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RESEARCH ARTICLE

Phyllosphere Microbiota Composition and Microbial Community Transplantation on Lettuce Plants Grown Indoors

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ABSTRACT **The aerial surfaces of plants, or phyllosphere, are microbial habitats important to plant and human health. In order to accurately investigate microbial interactions in the phyllosphere under laboratory conditions, the composition of the phyllosphere microbiota should be representative of the diversity of microorganisms residing on plants in nature. We found that Romaine lettuce grown in the laboratory contained 10- to 100-fold lower numbers of bacteria than age-matched, field-grown lettuce. The bacterial diversity on laboratory-grown plants was also significantly lower and contained relatively higher proportions of *Betaproteobacteria* as opposed to the *Gammaproteobacteria*-enriched communities on field lettuce. Incubation of field-grown Romaine lettuce plants in environmental growth chambers for 2 weeks resulted in bacterial cell densities and taxa similar to those on plants in the field but with less diverse bacterial populations overall. In comparison, the inoculation of laboratory-grown Romaine lettuce plants with either freshly collected or cryopreserved microorganisms recovered from field lettuce resulted in the development of a field-like microbiota on the lettuce within 2 days of application. The survival of an inoculated strain of *Escherichia coli* O157:H7 was unchanged by microbial community transfer; however, the inoculation of *E. coli* O157:H7 onto those plants resulted in significant shifts in the abundance of certain taxa. This finding was strictly dependent on the presence of a field-associated as opposed to a laboratory-associated microbiota on the plants. Phyllosphere microbiota transplantation in the laboratory will be useful for elucidating microbial interactions on plants that are important to agriculture and microbial food safety.**

IMPORTANCE **The phyllosphere is a habitat for a variety of microorganisms, including bacteria with significant relevance to plant and human health. Some indigenous epiphytic bacteria might affect the persistence of human food-borne pathogens in the phyllosphere. However, studies on human pathogens are typically performed on plants grown indoors. This study compares the phyllosphere microbiota on Romaine lettuce plants grown in a Salinas Valley, CA, field to that on lettuce plants grown in environmental chambers. We show that phyllosphere microbiota from laboratory-grown plants is distinct from that colonizing plants grown in the field and that the field microbiota can be successfully transferred to plants grown indoors. The microbiota transplantation method was used to examine alterations to the phyllosphere microbiota after *Escherichia coli* O157:H7 inoculation on lettuce plants in a controlled environment. Our findings show the importance and validity of phyllosphere microbiota transplantation for future phyllosphere microbiology research.**

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The phyllosphere, or the total above-ground surfaces of plants, is a habitat for a variety of microorganisms (1). At 10^5 to 10^7 cells/g plant material, bacteria are typically the most abundant colonizers of the phyllosphere and constitute approximately 10^{26} cells globally (1). Members of the *Proteobacteria*, *Firmicutes*, and *Actinobacteria* phyla dominate the phyllosphere of agricultural and native plants, although the proportions of individual taxa can vary depending on plant species and phenotype, geographical location, time of year, and human intervention (2–13). Many of these epiphytic bacteria appear to be adapted for growth and survival in the phyllosphere, as shown by genome-wide and gene-targeted analysis approaches (14–23).

Most bacterial epiphytes confer no known beneficial or detrimental effects on plant health and productivity. However, some plant pathogens can colonize the phyllosphere prior to or in the apparent absence of infection (17). Other phyllosphere bacteria, such as certain of strains of *Erwinia*, *Pseudomonas*, and *Sphingomonas*, provide protection from plant pathogens through competition for limited nutrients and other mechanisms (24–26). Plants, and leafy green vegetables in particular, are also significant vectors of human pathogens, leading to food-borne illness (27). Although human pathogens are not adapted for growth in the phyllosphere, *Escherichia coli* O157:H7 and *Salmonella* survive at low levels over extended periods of time on plants in the field (28).

Investigations on plant-microbe and microbe-microbe interactions have largely been performed under controlled conditions in the laboratory. We hypothesized that the phyllosphere of lettuce plants grown in the laboratory contains different bacterial

inhabitants than plants grown outdoors and that it is possible to modify those bacterial communities to result in a field-relevant microbiota. We examined this by comparing the bacterial composition on Romaine lettuce plants grown in the laboratory and in an agricultural field. The effects of whole-plant relocation and microbiota transplantation were investigated in relation to bacterial community stability in the phyllosphere.

RESULTS

Comparison of phyllosphere bacteria on field- and laboratory-grown lettuce. We compared the bacterial amounts and diversity on 6-week-old Romaine lettuce plants grown in a field in the Salinas Valley, CA, USA, to the bacterial populations on the same cultivar at the same developmental stage in the same soil at the same time but grown in an environmental chamber in the laboratory (see Fig. S1 in the supplemental material). Culture-dependent and -independent methods were in agreement that the field-grown plants contained significantly higher numbers of bacteria (Fig. 1A). Field-grown lettuce contained 10- to 100-fold more CFU than lettuce plants grown in the laboratory, according to colony enumerations on full-strength tryptic soy agar (TSA) medium. The bacterial abundance measured on the field-grown plants was comparable to that in a similar study using dilute TSA medium concentrations (10). Total cell number estimates according to real-time PCR exhibited similar differences, as well as a higher number of total bacteria on all the plants than estimated by culturing alone (Fig. 1A).

High-throughput DNA sequencing was used to identify the bacteria on the laboratory- and field-grown plants. Weighted and unweighted UniFrac community distance metrics of the microbiota revealed that the phyllosphere bacterial communities were different depending on the location where the plants were grown (Fig. 1B). Specifically, field-grown plants contained significantly higher proportions of *Gammaproteobacteria*, represented primarily by *Enterobacteriaceae* and *Moraxellaceae* families. Conversely, laboratory-grown plants were enriched with *Betaproteobacteria*, represented by the *Comamonadaceae* and *Burkholderiaceae* families (Fig. 1C). These differences were consistent at the genus level (see Fig. S2C in the supplemental material). The relative quantities of *Erwinia*, *Acinetobacter*, and *Alkanindiges* bacteria were significantly higher on field plants, whereas laboratory-grown plants carried significantly more representatives of *Comamonas*, *Limnobacter*, and *Pelomonas* (Fig. S2C). Notably, such distinctions were not observed at the phylum level, and the two dominant phyla, *Proteobacteria* and *Firmicutes*, were present in similar proportions on plants at either locale. Those phyla comprised, on average, 84.5% and 12.6% of the total amounts on the field plants and 78.5% and 17.2% on laboratory-grown plants, respectively (Fig. S2A and B).

Field plant acclimation to laboratory conditions. We tested whether plants relocated from the field to the growth chamber would maintain a field-associated phyllosphere microbiota. Whole Romaine lettuce plants germinated and grown in an agricultural field for 2 weeks were collected along with the surrounding soil and relocated to an environmental chamber for acclimation under relative humidity (RH) and temperature conditions resembling those in the field of origin (see Fig. S1 in the supplemental material). Two weeks later, on the same day that those lettuce plants were harvested for microbiota analysis, age-matched Romaine lettuce plants from the agricultural field were also collected. The average culturable bacterial population sizes on the laboratory-acclimated lettuce were similar to the quantities on the field plants and approximately 100-fold higher than on the laboratory-grown Romaine plant controls (Fig. S3A). The bacterial composition on laboratory-acclimated plants was also more similar to that on plants collected directly from the field than on plants germinated in an environmental chamber (Fig. 1D). However, the microbiota were not identical, and higher proportions of *Proteobacteria* (92.8%) were found on the laboratory-acclimated Romaine lettuce (Fig. S3C). The relative amounts of *Gammaproteobacteria* class bacteria were particularly increased (Fig. S3D), and the overall bacterial diversity on the acclimated lettuce was significantly reduced compared to that on lettuce plants harvested directly from the field (Fig. S3B).

Transplantation of field microbiota onto laboratory-grown lettuce. We next investigated whether it was possible to establish and maintain a field microbiota on plants germinated in the laboratory. Bacteria were dislodged from two representative field-grown lettuce plants in 2011 (T1_2011 and T2_2011) and 2012 (T1_2012 and T2_2012) and then applied onto laboratory-grown lettuce housed in an environmental chamber (see Fig. S1 in the supplemental material). Immediately after transplantation, the numbers of culturable bacteria were 8- to 34-fold higher than on the uninoculated laboratory-grown lettuce controls. Higher cell numbers were maintained on the transplanted plants for the duration of the experiment (Fig. 2A and E).

The microbial diversity on the field plants used for transplantation was similar to the microbiota found on other field plants collected at the same time in prior years (see Fig. S4 in the supplemental material) (13). The 2011 plants were grown early in the summer (July) and contained greater proportions of *Firmicutes*, whereas the 2012 plants were grown later in the summer (August) and were enriched in *Proteobacteria* (Fig. S4). Application of either the 2011 or 2012 field microbiota onto the laboratory-grown lettuce resulted in an immediate increase in bacterial diversity but no significant changes among the dominant bacterial taxa present (Fig. 2B and E; also see Fig. S5). Two days later, the bacterial taxa on transplanted lettuce were more similar to those in the field plant inoculum than to those on nontransplanted lettuce plant controls (Fig. 2B and F). The bacterial communities were stable for at least another 5 days (Fig. 2F).

Similar to field-grown plants, the phyllosphere microbiota on the transplanted lettuce was enriched in *Gammaproteobacteria* and typically contained reduced proportions of *Betaproteobacteria* (see Fig. S6A and C). Lettuce plants inoculated with the microbiota from lettuce grown in the early summer (2011) were also enriched for *Exiguobacterium* and *Bacillus* species, a result that is in agreement with the heightened proportions of *Firmicutes* on field lettuce at that time of year (Fig. S6A). Unlike the field-grown plants, transplanted lettuce contained different proportions of *Exiguobacterium*, *Pantoea*, *Pseudomonas*, and *Enterobacter* species (Fig. S6).

Establishment of a field microbiota using cryopreserved cells. A fraction of the cells washed from T1_2011 (field-grown lettuce) were preserved in glycerol at −80°C until being applied onto laboratory-grown Romaine lettuce. Similar to the results for plants containing the freshly transferred microbiota, transplantation using the cryopreserved microbiota resulted in a significant increase in bacterial populations on the lettuce (Fig. 2C). Two days after inoculation, the bacterial composition on the

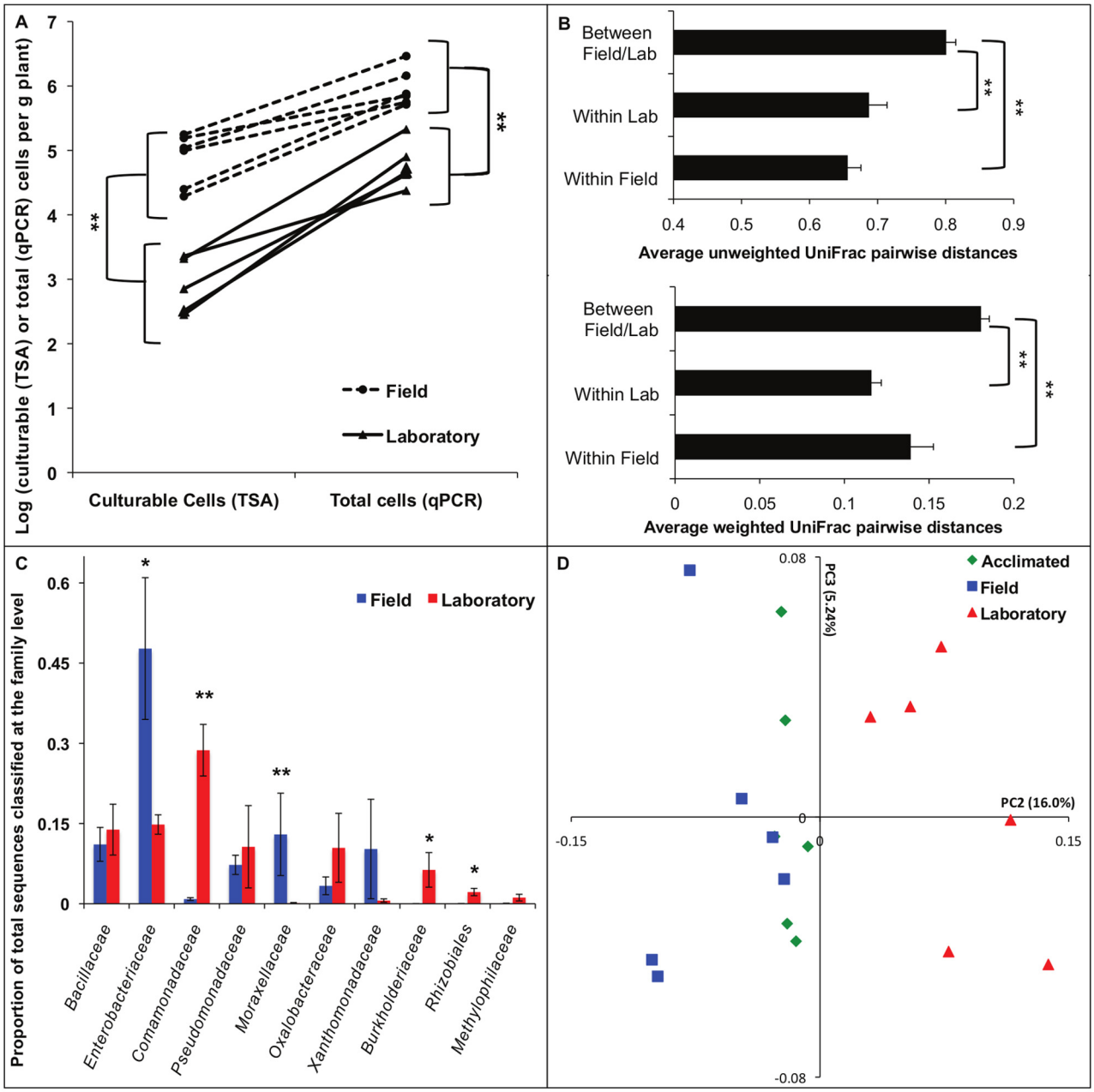

FIG 1 Characterization of the phyllosphere microbiota on laboratory-grown, field-grown, and laboratory-acclimated lettuce. (A, B, and C) Romaine lettuce cultivar Braveheart germinated in Salinas Valley soil in a controlled environment with field-like humidity and temperature conditions was used to compare the phyllosphere communities on 6-week-old plants to those on field-grown lettuce at the same developmental stage. (D) Whole lettuce plants collected from the field 2 weeks postgermination were acclimated to laboratory conditions for 2 weeks, and the bacteria on those plants were compared to the bacteria on laboratory-grown plants and untreated field plants. (A) Bacteria on laboratory- and field-grown lettuce were enumerated on TSA and by real-time PCR quantification of 16S rRNA gene numbers. (B) Averages ± standard errors of weighted and unweighted UniFrac community distances of the bacterial communities on field and laboratory lettuce as identified by 454 pyrosequencing. (C) Averages ± standard errors of the relative proportions of the top 10 families identified from 454 pyrosequencing of the bacterial 16S rRNA genes. (D) Principal coordinate analysis using weighted UniFrac community distances. An asterisk indicates significance by Student's *t* test (**, $P \leq 0.01$; *, $P \leq 0.05$).

transplanted plants was more similar to the field microbiota inoculum than to the bacteria on the laboratory-grown Romaine lettuce controls (Fig. 2D). The transplanted lettuce contained higher proportions of *Gammaproteobacteria* and *Bacillus* species. The populations of *Buttiauxella*, *Enterobacter*, *Pantoea*, *Bacillus*, *Exiguobacterium*, and *Arthrobacter* species were also enriched compared with the microbiota of the controls (Fig. S6B). Conversely, control (uninoculated) lettuce contained higher proportions of *Acinetobacter*, *Pseudomonas*, *Rhodanobacter*, *Acidovorax*, *Pelomonas*, *Lactococcus*, *Leuconostoc*, *Weissella*, *Rhizobium*, *Flavobacterium*, and *Chryseobacterium* species (Fig. S6B). Overall, the bacterial compositions of plants inoculated with the fresh or previously frozen bacterial cells were more similar to field plant microbiota than to bacterial communities on laboratory-grown lettuce (Fig. 3).

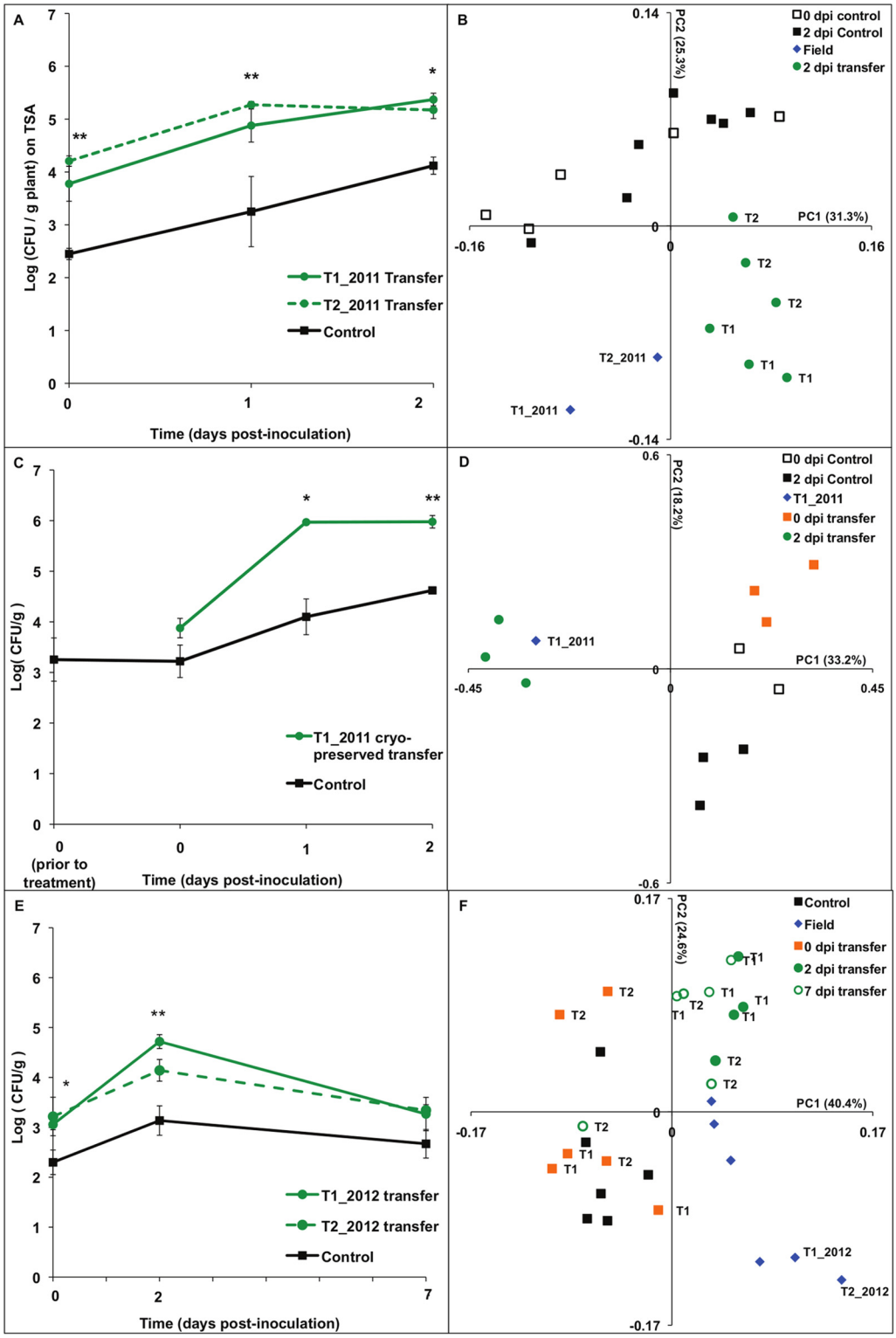

FIG 2 Phyllosphere microbiota transplantation with fresh and cryopreserved microbial communities. The microbiota associated with field-grown plants was inoculated directly or after cryopreservation into the phyllosphere of laboratory-grown plants, and the resulting communities were quantified on TSA and identified by pyrosequencing. (A, C and E) Culturable cell amounts were enumerated on TSA agar on either control plants or plants inoculated with freshly collected field microbiota from 2011 (A) or 2012 (E) or cryopreserved cells from the same field plants used in 2011 (C). Each time point shows the average value ± standard error for 3 plants. (B, D, and F) The bacterial communities on field plants in 2011 (B and D) and 2012 (F) and on laboratory-grown transplanted plants were compared to those on the laboratory-grown control plants by principal coordinate analysis using the weighted UniFrac metric. Freshly collected microbiota from 2011 (B) or 2012 (F) or cryopreserved cells from 2011 (D) were applied. (B and D) Open symbols represent plants sampled before any treatment was applied. (F) Other field plants collected at the same time are included. An asterisk indicates significance by Student's *t* test (**, $P \leq 0.01$; *, $P \leq 0.05$).

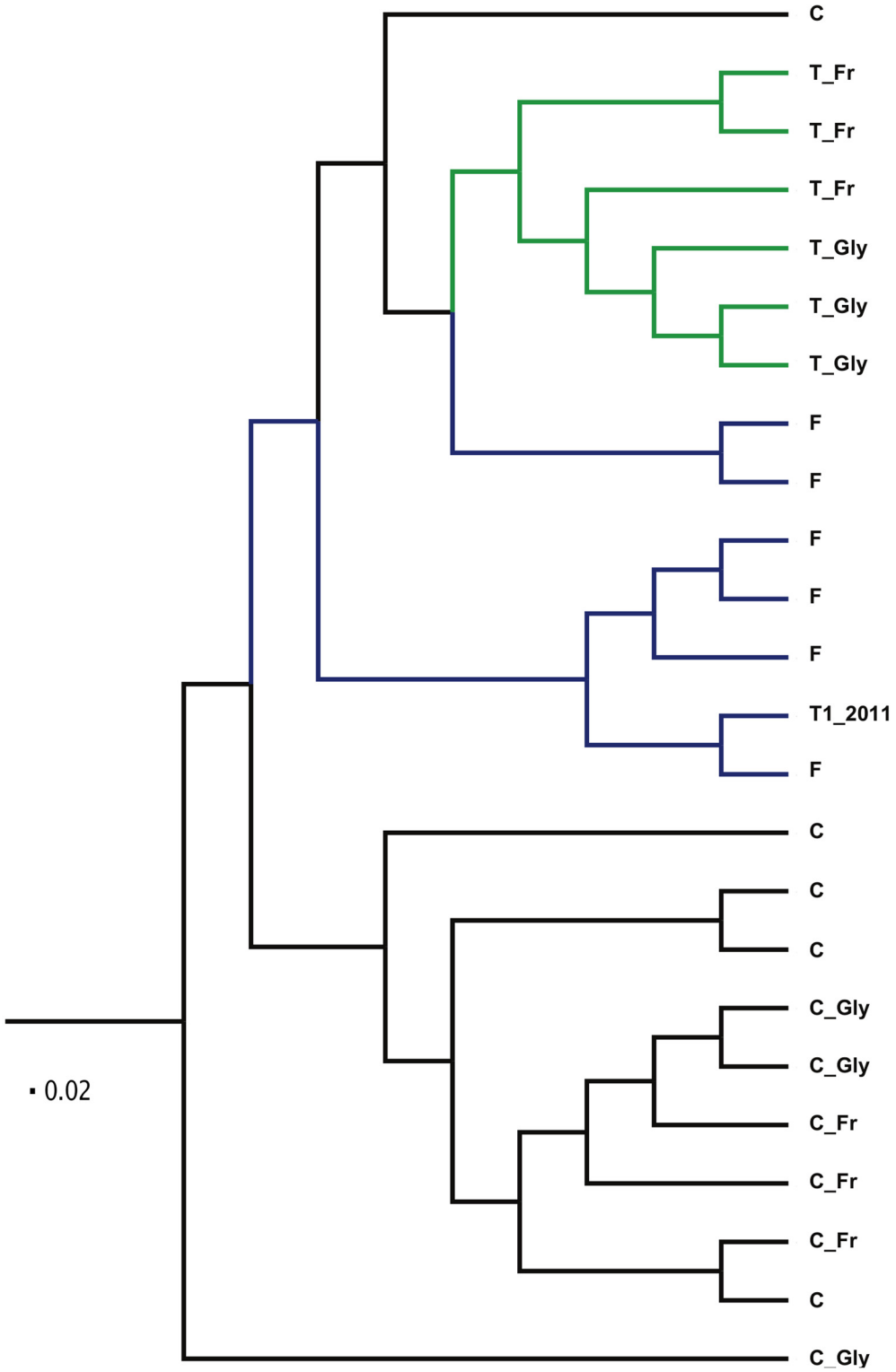

FIG 3 UPGMA comparison of transplantation techniques. A phylogenetic tree using the weighted UniFrac distances was created in QIIME using sequences belonging to untreated laboratory-grown lettuce (C), laboratory-grown plants dipped in buffer (C_Fr) or buffer plus glycerol (C_Gly), untreated field-grown lettuce not used for microbiota transplantation (F), and laboratory-grown plants sampled 48 h after being transplanted with a fresh (direct) T1_2011 field community (T_Fr) or a glycerol-preserved T1_2011 field community (T_Gly). One of the communities from the "C" treatment was designated the outgroup. The phylogenetic tree was visualized and modified (to display as a cladogram and to add color to the branches) in FigTree version 1.4.1 (http://tree.bio.ed.ac.uk/).

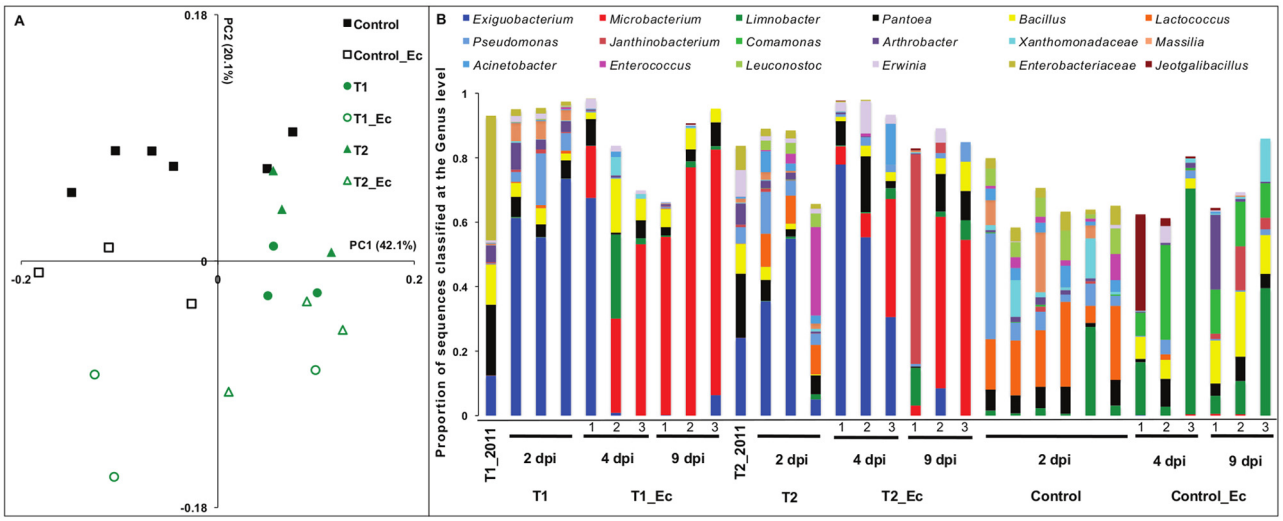

FIG 4 The effect of *E. coli* O157:H7 on phyllosphere bacteria. Cryopreserved microbiota from the T1_2011 or T2_2011 field plants collected from the field trial in 2011 was applied to laboratory-grown lettuce prior to *E. coli* O157:H7 inoculation; microbiota from these plants are referred to as T1_Ec and T2_Ec and compared to microbiota of laboratory-grown plant controls also inoculated with *E. coli* O157:H7 (Control_Ec). All DNA sequences classified as *Escherichia* were removed prior to analysis to show the changes in the diversity of the other bacteria present. (A) Principal coordinate analysis using the weighted UniFrac metric comparing the phyllosphere communities on T1_Ec, T2_Ec, and Control_Ec plants 4 days posttransplantation (open symbols) to the corresponding transplanted plants or untreated controls 2 days posttransplantation without any exposure to *E. coli* O157:H7, which are referred to as T1, T2, and Control (closed symbols). (B) The relative abundances of bacterial genera in the phyllosphere of the field plants and the laboratory-grown plants with the applied field communities with and without the *E. coli* O157:H7 inoculant. The numbers directly above the time point information for the *E. coli*-inoculated plants correspond to the plant numbers in Table S1 in the supplemental material.

***Escherichia coli* O157:H7 population dynamics on laboratory-grown plants with transplanted communities.** To examine the effect of an exogenous organism on the indigenous bacterial communities in the phyllosphere, *Escherichia coli* O157:H7 strain ATCC 700728 was applied at a level of approximately 10^6 CFU/g plant onto laboratory-grown Romaine lettuce plants containing or lacking either a T1_2011 or T2_2011 field plant microbiota. Within 2 days, the numbers of culturable *E. coli* O157:H7 bacteria declined to approximately 10^3 CFU/g plant, and they decreased further by 7 days postinoculation (d.p.i.), to an average of 2.1 CFU/g plant (see Fig. S7 and Table S1 in the supplemental material). Reductions in *E. coli* O157:H7 viability occurred equally on both uninoculated and transplanted lettuce (Fig. S7).

Lettuce plants inoculated with *E. coli* O157:H7 were highly enriched in sequences classified as *E. coli*, representing averages of 87%, 62%, and 52% of the total 16S rRNA gene sequences identified in the lettuce phyllosphere at 0, 2, and 7 d.p.i., respectively (Table S1). This finding supports previous studies concluding that *E. coli* dies shortly after inoculation onto plants in the field or in environmental chambers maintained at a low RH, although the organism's DNA can remain detectable by PCR over longer periods of time (29). Examination of the other bacteria on those plants showed that there were also significant and consistent changes in composition among those organisms following *E. coli* O157:H7 inoculation (Fig. 4A). The outcomes of *E. coli* O157:H7 inoculation on the microbiota differed between the control and transplanted lettuce (Fig. 4B). Specifically, *Microbacterium* (a member of the *Actinobacteria* phylum) was significantly enriched on *E. coli* O157:H7-containing plants with either T1_2011- or T2_2012-phyllosphere microbiota (Fig. 4B). Notably, this genus was found on field-grown and not laboratory-grown lettuce (Fig. 4B).

DISCUSSION

Numerous fundamental questions remain in phyllosphere community ecology. Because the phyllosphere is essentially a heterogeneous surface environment, epiphytic bacteria are constrained in mobility and access to resources needed for growth and survival as well as contact with other microorganisms. While it is well established that there is significant variation in bacterial composition between plants, it is unclear how biotic and abiotic factors influence pre- and postcolonization events and the resiliency of the microbiota against colonization by exogenous microorganisms. In order to examine phyllosphere community ecology in detail, particularly as it relates to human and plant pathogens, it is important to be able to construct field-relevant phyllosphere communities under controlled conditions in the laboratory. Our results clearly show that this is possible using fresh and cryopreserved field microbiota transplantation onto laboratory-grown plants.

By investigating Romaine lettuce grown in the laboratory and field, we identified core and variable features of lettuce phyllosphere bacterial populations. The field plants analyzed in this study followed the same seasonal-based trends among the dominant phyla identified in our previous work (13). However, consistent among all plants, regardless of growth conditions, was the predominance of the *Proteobacteria* and *Firmicutes* phyla. These phyla comprise the majority of bacteria in the phyllosphere of other agricultural and native plant species (8, 11, 13, 14, 30). This result is notable, given the likelihood that the plants in the agricultural field were likely exposed to very different bacterial species than those in the environmental chamber. By comparison, the rhizosphere is colonized by a broader diversity of bacteria encompassing a variety of phyla

that appears to be largely determined by soil and host genotype (31–33).

The similarity between the laboratory and field lettuce plants was not maintained at lower taxonomic levels. *Gammaproteobacteria*, and specifically members of the *Enterobacteriaceae* family, were enriched on the field-grown Romaine lettuce. These bacteria are also significant colonizers of other plants grown outdoors (8, 10, 13, 34). In contrast, members of the *Betaproteobacteria*, and specifically the *Comamonadaceae* and *Burkholderiaceae* families, were highly abundant on laboratory-grown Romaine lettuce plants. These families were also significant colonizers of the phyllosphere of other plants indoors (9, 30, 35). Notably, *Betaproteobacteria* were typically found in the phyllosphere of field-grown lettuce but in much lower proportions than the *Gammaproteobacteria*. It is not clear why different classes of *Proteobacteria*, comprised of distinct lineages with functionally diverse members, were enriched on either the laboratory- or field-grown lettuce. Such differences might be the result of the presence (or absence) of certain bacterial species within the built environment as opposed to natural settings.

The total and culturable bacterial quantities were lower on laboratory-grown than on field-grown lettuce. Other studies on plants grown indoors have also reported similarly low cell numbers (9, 30), but to the best of our knowledge, our work represents the first direct comparison of phyllosphere bacterial microbiota diversity and amounts between plants grown in the laboratory and in an agricultural setting. The lower cell quantities on plants grown indoors might be due to lower numbers of bacteria that can be deposited onto the plants or, alternatively, the result of changes in plant physiology outdoors increasing the carrying capacity for bacteria in the phyllosphere. The latter seems less likely because laboratory-grown lettuce plants transplanted to contain a field microbiota in this study contained higher numbers of cells than the laboratory plant controls. Potential sources of bacteria in the field include plant seeds, irrigation water, soil, human contact, dust, and air. The impact of irrigation water appears to be relatively minor, because only limited differences in bacterial abundance and composition were found for overhead-sprinkler- and surface drip-irrigated lettuce (13). To minimize other factors, we used the same soil and batch of lettuce seed and limited human contact with the plants at both locations. In comparison, aerosols and dust appear to be good sources of phyllosphere colonists (9, 36) and might have accounted for the increased numbers of bacteria on lettuce grown outdoors. Airborne bacteria were recently shown to be important in forming the initial phyllosphere communities that are then subsequently modified based on selective pressures and spatial associations among plants (9). Additional studies are needed to identify the precise biotic and abiotic factors that regulate the differences in bacterial composition on plants grown in- and outdoors.

Relocation of Romaine lettuce to the laboratory did not result in global rearrangements to the phyllosphere microbiota. The bacteria on field-grown Romaine lettuce plants incubated in the laboratory for 2 weeks were similar to those found on equivalently developed plants harvested directly from the field. For example, bacteria on the acclimated plants were overwhelmingly *Gammaproteobacteria* and not *Betaproteobacteria*. The significant enrichment of *Gammaproteobacteria* on those plants might indicate that certain bacteria in that family are well adapted for growth on lettuce in general and not only in the field. To that regard, the best characterized bacterial colonists of plants include strains of *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Pantoea* and these genera are members of the *Gammaproteobacteria* family.

We also investigated whether it was possible to recreate a field-like microbiota on laboratory-grown lettuce by using whole-community transplantation. The application of fresh and cryopreserved leaf washes resulted in increased bacterial population sizes and phylogenetic diversity, indicating that the phyllosphere of laboratory-grown plants is able to accept new colonists. Those plants then contained a field-like bacterial composition within 2 days after inoculation, and the microbiota were stable for at least 7 days. The results were representative of the bacteria in the field plant inoculum and consistent between replicate plants. Moreover, the cryopreserved cells were equally as effective at creating a field-like microbiota as the fresh leaf washes. In general, *Gammaproteobacteria* populations were enriched on laboratory plants posttransplantation, while *Betaproteobacteria* populations decreased. With few exceptions, bacterial genera associated with field plants increased, whereas those from laboratory-grown lettuce declined.

The stability, or resilience, of a community is defined as its resistance to change against an applied disturbance. We evaluated the resilience of Romaine lettuce phyllosphere microbiota against exposure to *E. coli* O157:H7. Following inoculation, *E. coli* O157:H7 ATCC 700728 did not survive in high quantities in the lettuce phyllosphere, and culturable cells were no longer detectable on most plants 7 days after inoculation. This result is agreement with field and laboratory studies on *E. coli* O157:H7 epiphytic fitness (28, 29, 37–41). Despite an inability to survive on lettuce, *E. coli* O157:H7 altered the composition of the other bacteria present on the plants on replicate plants in two independent trials. Microbe-mediated disturbances of indigenous microbial communities have also been reported for other habitats, such as the lettuce rhizosphere upon exposure to the plant pathogen *Rhizoctonia solani* (42).

Altered bacterial communities were found for both control and field microbiota-transplanted plants within 2 days after *E. coli* O157:H7 inoculation. Contrary to previous reports suggesting that increased biological diversity results in a more stable habitat (43), the increase in bacterial diversity of the field microbiota-containing laboratory-grown lettuce in this study did not confer community stability against the *E. coli* O157:H7 cell amounts used in this study. Our results are consistent with the general lack of resilience of bacterial phyllosphere populations against disturbance on Romaine lettuce plants in the field (13). However, it is possible that the phyllosphere microbiota might be more resilient against lower total numbers of *E. coli* O157:H7 or different strains of inoculated bacteria. Although the reasons for the change in microbiota in response to *E. coli* O157:H7 are not presently known, it is possible that certain bacteria on the lettuce are able to use the cellular components from dead *E. coli* cells as nutrients for growth.

Notably, inoculation of *E. coli* O157:H7 onto lettuce containing the field plant microbiota resulted in reproducible increases in the proportions of *Microbacterium*. *Microbacterium* was previously identified in the spinach and lettuce phyllosphere (13, 44). Although it remains to be shown why the relative amounts of *Microbacterium* increased, it was previously shown that strains of this genus exhibit antagonistic activity against *E. coli* O157:H7 (44). This genus has also been investigated as a biocontrol agent

against fungal plant pathogens (45, 46). A relatively high abundance of *Microbacterium* might therefore serve as an indicator of *E. coli* O157:H7 exposure on field-grown lettuce or provide novel applications to ensure plant and human health. Because *Microbacterium* was not natively found on laboratory-grown plants, these findings show the value of using field plant microbiota for bacterial community analysis of the phyllosphere.

We established that it is possible to reconstruct a field microbiota even on Romaine lettuce grown under standard conditions in an environmental chamber. This approach, largely benefiting from the vulnerability of the phyllosphere microbiota to modification, prevents the need to cultivate plants axenically for assessments of microbe-microbe interactions. The approach presented here will also be useful for elucidating the factors that determine phyllosphere microbial community structure and the roles of those communities in plant and human health.

MATERIALS AND METHODS

Plant growth conditions. For a complete description of the different lettuce cultivars used and other variables regarding the plants and plant inoculations throughout this study, see Fig. S1. In 2011 and 2012, Romaine lettuce (*Lactuca sativa*) cultivars Green Towers (2011) and Braveheart (2012) were grown from seed at different times in the year at one location in the Salinas Valley, CA, USA. The field plot design and growth conditions were previously described (13, 40). Permits and approvals for use of U.S.-owned land were granted by the United States Department of Agriculture. In 2011, lettuce plants were harvested in June (early season), whereas plants in 2012 were collected in September (late season). Lettuce plants in the field were watered by sprinkler (overhead) irrigation and were otherwise untreated prior to harvest.

In the laboratory, Romaine lettuce cultivars Green Towers and Braveheart from the same lot of seed that was used in the field were germinated in soil and grown in an environmental chamber (PGR15; Conviron, Pembina, ND, USA). The chamber was maintained at a constant 60% relative humidity (RH), a light intensity of 230 μmol m^{-2} s^{-2}, and daily temperatures of 18°C (12 h without light) and 23°C (12 h with light). In 2011, laboratory-grown plants were seeded in Sunshine mix potting soil no. 1 (Sun Gro Horticulture Distribution, Bellevue, WA). In 2012, plants were seeded in soil collected from the field in Salinas Valley. Beginning 1 week after germination, the soil was fertilized weekly with Hoagland nutrient water. Approximately 2 days prior to each experiment, the lettuce pots were transferred to an environmental chamber (Percival; Geneva Scientific LLC, Fontana, WI). The environmental chamber was maintained at a 12-h photoperiod (light intensity of 230 μmol m^{-2} 114 s^{-2}), constant 60% RH, and temperatures of 18°C (12 h) and 23°C (12 h).

Plant sampling and phyllosphere microbiota collection, enumeration, and inoculation. Lettuce plants grown in the field or laboratory were harvested and processed in the same manner. A sterile scalpel was used to cut the lettuce approximately 3 cm above the soil surface. The lettuce leaves were then submerged in 0.1% peptone buffer (50 to 250 ml) in sterile 1.62-liter (55 oz) Whirl-Pak bags (Nasco, Ft. Atkinson, WI), and sonicated for 7 min in a Branson 8510 ultrasonic cleaner water bath (Branson Ultrasonics Corporation, Danbury, CT). An automated spiral plater (Autoplate 4000; Spiral Biotech, Inc., Boston, MA) was used to plate serial dilutions of the leaf washes onto tryptic soy agar (TSA) containing 25 μg/ml natamycin (47). With the exception of the fraction of leaf washes used for cryopreservation or direct lettuce inoculation, leaf washes from field-grown and laboratory-acclimated plants were transferred to 50-ml tubes and centrifuged at 3,220 × *g* for 15 min, and the resulting cell pellet stored at −80°C prior to DNA extraction. Because no observable bacterial pellet was formed upon centrifugation of the leaf washes from laboratory-grown plants, the leaf washes from those plants were concentrated onto 0.22-μm nitrocellulose membrane filters (EMD Millipore Corp., Billerica, MA, USA). The membranes were rinsed with 1 ml of phosphate-buffered saline (8.01 g/liter NaCl, 0.2 g/liter KCl, 1.78 g/liter Na_2HPO_4, 0.27 g/liter KH_2PO_4, pH 7.4) and stored at −80°C prior to DNA extraction.

To measure the phyllosphere microbiota on field-grown plants after acclimation to laboratory conditions, entire Romaine lettuce plants were uprooted 2 weeks after germination during the field study in 2012 (see Fig. S1 in the supplemental material). Those plants and the surrounding soil were placed into pots and subsequently incubated for 2 weeks in the environmental growth chamber. The acclimated lettuce plants and those collected from the field at the same time were sampled as described above.

For direct (fresh) microbiota transfer onto laboratory-growth lettuce (see Fig. S1 in the supplemental material), leaf washes containing the phyllosphere microbiota from field-grown plants in either 2011 or 2012 were centrifuged for 15 min at 3,220 × *g* to collect the bacterial cells as described above and approximately 50% of the suspended pellet was immediately mixed into 1 liter of an autoclaved 0.1 mM potassium phosphate buffer (PPB) solution (0.01742 g/liter K_2HPO_4, 0.01361 g/liter KH_2PO_4). Whole laboratory-grown Romaine lettuce plants at the same stage of growth were then submerged for 3 s into the microbiota suspension. Control plants were submerged into 1 liter of the PPB solution. The plants were gently shaken to remove large droplets and then immediately incubated in the growth chamber at 60% RH as described above. One-third of the concentrated phyllosphere microbiota from individual plants was also stored in 20% (wt/vol) final concentration glycerol at −80°C. The cryopreserved cells were thawed approximately 6 months later and suspended in 1 liter of 0.1 mM PPB solution for microbiota transplantation onto laboratory-grown plants as described above (Fig. S1). Control plants were submerged in 0.1 mM PPB solution containing an equal amount (0.048%, wt/vol) of glycerol.

For investigating the interactions between *E. coli* O157:H7 and phyllosphere microbiota (see Fig. S1 in the supplemental material), cryopreserved microbiota stored in glycerol at −80°C were used to inoculate laboratory-grown Romaine lettuce as described above. After incubating the lettuce plants for 48 h, 10^7 CFU/ml of a rifampin-resistant strain of *E. coli* O157:H7 ATCC 700728 (*E. coli* O157:H7) was inoculated as previously described (13, 40). Briefly, *E. coli* O157:H7 was delivered by a single spray of 1 ml onto each lettuce plant, resulting in a final cell density of approximately 10^7 CFU/plant. *E. coli* O157:H7 cell numbers in the inoculum and phyllosphere were quantified by plating serial dilutions of leaf washes onto TSA with 50 μg/ml rifampin.

Quantitative PCR. All DNA extractions were performed according to bacterial DNA isolation protocols described previously (13). Total bacterial abundance was determined by quantitative real-time PCR (qPCR) using the universal 16S rRNA primers 534F (5′ CCAGCAGCCGCGGTA AT 3′) and 783R (5′ ACCMGGGTATCTAATCCKG 3′) (48) as described previously (13, 49). Each plant microbiota was quantified in triplicate and compared as performed previously (13). Briefly, bacterial cell numbers were quantified using comparisons of cycle threshold (C_T) values to a standard curve of DNA extracted from an overnight *E. coli* O157:H7 ATCC 700728 culture. The number of cells in the culture was estimated by plating for CFU enumeration on TSA agar, and total genomic DNA was extracted by applying the same method used for the phyllosphere samples.

Pyrosequencing and bioinformatic analysis. The 16S rRNA V5 to V9 regions were amplified from phyllosphere microbiota genomic DNA by PCR using bar-coded 799f (50) and 1492r (51) primers, and pyrosequencing was performed as previously described (13). Sequencing was performed on the GS-FLX 454 Titanium platform (454 Life Sciences, Branford, CT) at The Core for Applied Genomics and Ecology (CAGE) at the University of Nebraska, Lincoln, and at Selah Genomics (University of South Carolina, Columbia, SC, USA). Sequences were submitted to the NCBI BioProject database with the project identification number PRJNA237242 (sample accession numbers SAMN02640062 to SAMN02640169).

The Quantitative Insights Into Microbial Ecology (QIIME) (52) software package was used to analyze the 16S rRNA gene sequences. Prior to

taxonomic and phylogenetic analysis, the DNA sequences were subjected to the following preprocessing steps and quality controls: (i) sequences with incorrect bar codes or more than two base pair mismatches in the primer were removed; (ii) sequences containing windows of 50 consecutive base pairs with an average quality score of less than 20 were truncated at the start of the low-quality region; and (iii) sequences with less than 200 bp or more than 600 bp, not including bar codes, were also not considered. The data set was quality filtered using USEARCH version 5.2.236 (53) and resulted in an average of 5,624 sequences per sample. Operational taxonomic units (OTUs) were picked with a 97% identity cutoff using the default parameters. OTUs that were found only once in a given sample (singletons) were removed from the data set, yielding a total of 164 OTUs per sample remaining in the data set. Representative sequences for each OTU were then aligned in QIIME using the PyNast algorithm (54), and phylogenetic trees of the assigned OTUs were created using FastTree (55). Sequences in the original data set that were classified as chloroplasts were removed and not considered in the subsequent analyses.

The QIIME sequence analysis pipeline was employed for taxonomic assignment using the Ribosomal Database Project (RDP) classifier RDP10 database (training set 6) (56). Jackknifing sequence subsampling was performed for beta diversity analysis by calculating UniFrac distance matrices (57), using 100 subsampled sequences per sample averaged over 10 iterations. Assessments of beta diversity encompassed principal coordinate analyses (PCA) and unweighted pair-group method with arithmetic mean (UPGMA) phylogenetic trees.

Statistical analysis. Statistical comparisons of taxonomic data were performed in Excel with Student's *t* test. The taxonomic data came from the bioinformatic analysis of the sequenced 16S rRNA amplicons and consisted of the relative proportions of OTUs assigned to specific taxonomic groups at the phylum, class, family, and genus level. For statistical analysis of the phylogenetic diversity (alpha diversity) between treatment groups, the Mann-Whitney directional test was utilized. Significant differences between treatment groups were assumed, and it is noted in the figures if the tests yielded a *P* value of less than 0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01564-14/-/DCSupplemental.

Figure S1, TIF file, 12.6 MB.
Figure S2, TIF file, 12.9 MB.
Figure S3, TIF file, 16 MB.
Figure S4, TIF file, 8.9 MB.
Figure S5, TIF file, 11.3 MB.
Figure S6, TIF file, 12.4 MB.
Figure S7, TIF file, 9.6 MB.
Table S1, DOCX file, 0.1 MB.

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