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Both Leaf Properties and Microbe-Microbe Interactions Influence Within-Species Variation in Bacterial Population Diversity and Structure in the Lettuce (*Lactuca* Species) Phyllosphere[▽]

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Morphological and chemical differences between plant genera influence phyllosphere microbial populations, but the factors driving within-species variation in phyllosphere populations are poorly understood. Twenty-six lettuce accessions were used to investigate factors controlling within-species variation in phyllosphere bacterial populations. Morphological and physiochemical characteristics of the plants were compared, and bacterial community structure and diversity were investigated using terminal restriction fragment length polymorphism (T-RFLP) profiling and 16S rRNA gene clone libraries. Plant morphology and levels of soluble carbohydrates, calcium, and phenolic compounds (which have long been associated with plant responses to biotic stress) were found to significantly influence bacterial community structure. Clone libraries from three representative accessions were found to be significantly different in terms of both sequence differences and the bacterial genera represented. All three libraries were dominated by *Pseudomonas* species and the *Enterobacteriaceae* family. Significant differences in the relative proportions of genera in the *Enterobacteriaceae* were detected between lettuce accessions. Two such genera (*Erwinia* and *Enterobacter*) showed significant variation between the accessions and revealed microbe-microbe interactions. We conclude that both leaf surface properties and microbial interactions are important in determining the structure and diversity of the phyllosphere bacterial community.

The phyllosphere (the aerial parts of plants) is known to support large and diverse naturally occurring microbial communities (19, 51), of which bacteria, living both epiphytically and endophytically, are the most numerous and diverse (2, 6, 44). A range of environmental factors such as temperature, rainfall, wind, and solar radiation have been shown to play an important role in determining patterns of bacterial phyllosphere colonization (19). Much less is known of the role of plant factors in determining the diversity and dynamics of phyllosphere microbial communities. Plant species (20), gross plant morphology, the position and height of leaves (45), and leaf age (16) have all been associated with variation in the size of phyllosphere bacterial populations. Furthermore, various leaf surface features such epidermal cell wall junctions (12) and grooves along the veins, stomata, and the base of trichomes (29) and near hydathodes (32) have all been identified as preferential bacterial attachment sites, resulting in uneven bacterial distribution on leaf surfaces. The microbial population already resident in the phyllosphere is also likely to impact significantly on the ability of incoming organisms to successfully colonize the leaf surface. Variation in such resident populations between different plant species has been attributed to differences in a range of plant physiochemical characteristics, including the water and phosphorus contents of the leaves, levels of phenolic compounds (which may be inhibitory toward many bacteria), and leaf and mesophyll thickness (53). All of these plant characteristics have a genetic basis.

The majority of current knowledge of phyllosphere microbiology has been gathered using culture-dependent methods (19). Since microbial cells are usually attached to surfaces and may be integrated into biofilms in environmental samples, isolation methods will remove only a portion of these cells (49). This could potentially lead to an underrepresentation of the diversity of the sample, and this has been clearly demonstrated for phyllosphere populations in instances where comparisons with culture-independent techniques have been made (54). Furthermore, for the most part, studies have focused on variations in microbial populations between different plant species. A limited number of studies of microbial population differences at the cultivar level (i.e., within a species) have been conducted, for example, in field pea (15), sweet pepper (38), and potato (36, 37, 40). These studies, however, have been small scale (generally 2 to 4 cultivars) and have focused either on purely endophytic populations or on the effects of specific differences between plant lines (e.g., genetic modifications) on phyllosphere microbiology. The cultivar-level relationship between plant characteristics (and their underlying genotypes) and developing phyllosphere microbial populations has not been addressed (51).

In this study, differences in 16S (small-subunit) rRNA gene profiles of total phyllosphere bacterial populations (i.e., both epiphytic and endophytic bacteria) that developed on a set of 26 lettuce accessions representing a wide range of genetic diversity were investigated under field conditions. The influences of physical, chemical, and physiological plant parameters on the bacterial population profiles were investigated for all

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TABLE 1. Mean scores of plant characteristics of lettuce (*Lactuca sativa*) and *Lactuca serriola* accessions used in this study, showing associated significance groups

Line no.	Accession	Plant characteristics[e]				Leaf characteristics[e]					
		Type	Size	Height	Head	Green color	Red color	Shape	Margin	Blistering	Thickness
1	Cobham Green	Butterhead	3.0[a]	2.0[b]	3.0[a]	2.0[b]	0.0[d]	3.0[a]	1.0[c]	1.0[c]	3.0[a]
2	*L. serriola*	Wild relative	3.0[a]	3.0[a]	1.0[c]	3.0[a]	1.0[c]	1.0[c]	1.0[c]	1.0[c]	1.0[c]
3	Stoke	Cos	1.0[c]	3.0[a]	1.0[c]	3.0[a]	0.0[d]	1.0[c]	1.0[c]	2.0[b]	2.0[b]
4	Batavia Blonde de Paris	Batavian	2.0[b]	2.0[b]	3.0[a]	1.0[c]	0.0[d]	2.0[b]	2.0[b]	2.0[b]	1.7[b]
5	Lobjoits Green Cos	Cos	3.0[a]	3.0[a]	1.7[b]	3.0[a]	0.0[d]	1.0[c]	1.0[c]	1.3[b]	2.3[a]
6	Ambassador	Butterhead	1.0[c]	2.0[b]	2.0[b]	2.0[b]	0.0[d]	3.0[a]	1.0[c]	1.3[b]	2.3[a]
7	Little Gem	Cos	1.0[c]	3.0[b]	2.0[b]	2.0[b]	0.0[d]	2.0[b]	1.0[c]	2.0[b]	2.7[a]
8[f]	*L. serriola* US96UC23	Wild relative	3.0[a]	3.0[a]	1.0[c]	3.0[a]	0.0[d]	1.0[c]	2.3[a]	1.0[c]	1.0[c]
9	Bloody Warrior	Cos	3.0[a]	1.0[c]	1.0[c]	3.0[a]	2.0[b]	1.0[c]	1.0[c]	2.0[b]	1.7[b]
10	New Chicken	Stem lettuce	3.0[a]	2.0[b]	1.0[c]	2.0[b]	0.0[d]	1.0[c]	1.0[c]	2.0[b]	1.3[b]
11	Romanie de Benicardo	Cos	3.0[a]	3.0[a]	1.0[c]	3.0[a]	0.0[d]	1.0[c]	1.0[c]	2.0[b]	3.0[a]
12	Lollo Rossa	Curly leaved	2.0[b]	2.0[b]	1.0[c]	1.0[c]	3.0[a]	3.0[a]	3.0[a]	2.0[b]	1.0[c]
13	Lollo Biondo	Curly leaved	1.7[b]	2.0[b]	1.0[c]	1.0[c]	0.0[d]	3.0[a]	3.0[a]	2.0[b]	1.0[c]
14	Lillian	Butterhead	3.0[a]	2.0[b]	3.0[a]	2.0[b]	0.0[d]	3.0[a]	1.0[c]	1.0[c]	3.0[a]
15	Batavia Tezier	Batavian	2.3[a]	3.0[a]	1.0[c]	1.0[c]	0.0[d]	1.0[c]	2.0[b]	1.0[c]	3.0[a]
16	Wunder von Stuttgart	Butterhead	2.0[b]	2.0[b]	3.0[a]	1.0[c]	0.0[d]	2.0[b]	1.0[c]	2.0[b]	2.0[b]
17	Adriatica 2	Butterhead	2.7[a]	1.7[b]	1.0[c]	2.0[b]	0.0[d]	2.0[b]	1.0[c]	2.0[b]	2.0[b]
18	Webb's Wonderful	Batavian	3.0[a]	2.0[b]	3.0[a]	2.0[b]	0.0[d]	3.0[a]	2.0[b]	2.7[a]	2.7[a]
19	Waldmann's Dark Green	Curly leaved	3.0[a]	2.0[b]	1.7[b]	2.0[b]	0.0[d]	1.7[b]	3.0[a]	3.0[a]	1.7[b]
20	Chinese Stem Lettuce	Stem lettuce	3.0[a]	1.0[c]	1.0[c]	1.0[c]	0.0[d]	1.0[c]	1.0[c]	1.0[c]	1.3[b]
21	Platinas	Iceberg	1.7[b]	2.0[b]	2.3[a]	2.7[a]	0.0[d]	3.0[a]	2.0[b]	3.0[a]	3.0[a]
22	Madrass	Iceberg	3.0[a]	2.0[b]	3.0[a]	2.0[b]	0.0[d]	2.3[a]	2.0[b]	3.0[a]	3.0[a]
23	Jazzie	Batavian	2.0[b]	2.0[b]	1.0[c]	1.0[c]	0.0[d]	3.0[a]	3.0[a]	1.0[c]	2.7[a]
24	Imagination	Batavian	2.0[b]	2.0[b]	1.5[b]	1.0[c]	0.0[d]	3.0[a]	3.0[a]	1.0[c]	2.3[a]
25[f]	Saladin	Iceberg	2.0[b]	2.0[b]	3.0[a]	3.0[a]	0.0[d]	2.7[a]	2.0[b]	3.0[a]	3.0[a]
26[f]	Iceberg	Batavian	2.3[a]	2.0[b]	3.0[a]	1.0[c]	1.0[c]	3.0[a]	2.0[b]	3.0[a]	2.3[a]

[a] Belongs to the significance group with the highest value for each characteristic.
[b] Belongs to the significance group with the second-highest value for each characteristic.
[c] Belongs to the significance group with the third-highest value for each characteristic.
[d] Belongs to the significance group with the fourth-highest value for each characteristic (where present).
[e] Characteristics were scored on a scale of 1 to 3, as follows: for size, head size, and height, small (head absent) (1), medium (2), and large (3); for green color, light (1), medium (2), and dark (3); for red color, pinking (1), speckled (2), red (3), and not present (0); for leaf shape, long (1), round (2), and broad (3); for leaf margin, smooth (1), mildly crenulated (2), and heavily crenulated (3); for leaf blistering: smooth surface (1), slight blistering (2), and heavy blistering (3); for leaf thickness, thin (1), medium (2), and thick (3).
[f] Mapping population parental line.

lettuce accessions using terminal restriction fragment length polymorphism (T-RFLP) profiling; populations developing on the three parental lines of three lettuce mapping populations were investigated in more detail through comparison of 16S rRNA gene clone libraries. This approach will facilitate further investigation of plant factors associated with differences in the bacterial populations by identifying genetic mapping populations in which they segregate for future genetic analysis. This offers the real possibility of identifying the genes that influence developing microbial populations and the mechanisms by which such influence occurs at a molecular level. An understanding of these processes will have important implications for the control of plant pathogens, spoilage organisms, and zoonoses, with zoonoses being particularly important in respect of minimally processed crops such as lettuce (52).

MATERIALS AND METHODS

Plant material. A set of 26 accessions (Table 1) representing a wide variety of crop types of lettuce (*Lactuca sativa*) and its wild relative (*L. serriola*) and including the parental lines of three lettuce genetic mapping populations (*L. serriola* US96UC23 [line 8] × cv. Salinas [syn. Saladin] [line 25], *L. serriola* US96UC23 cv. Saldin × cv. Iceberg [line 26], and *L. serriola* US96UC23 × cv. Iceberg) (46; P. Hand, unpublished data) were used. Seedlings were raised under glass in peat plugs in P150 modular trays (10 by 15 cells; Plantpak, Maldon, United Kingdom) until approximately 3 weeks postemergence, before they were hardened off outside for 48 h prior to planting out. Three replicate blocks, each of 32 plots (1.05 m^2), were planted in sandy loam soil in late spring (May). Plants were arranged in four rows of eight plots with 1-m gaps between the rows. Each plot contained a three-by-three array of plants from a single accession. Each accession was represented by a single plot in each block, with the exception of line 25 and line 26 (two of the mapping population parents). Four plots of these lines were planted in each replicate block. The positions of the lines were separately randomized for each block. The experimental area was irrigated by overhead spray. Mean daily temperatures ranged from 1.5°C to 25.4°C (mean average, 14.7°C) and the rainfall amount was between 0.0 and 29.7 mm day^{-1} (mean average, 3.4 mm day^{-1}) during the growing period.

Morphological scoring, harvesting, and sample processing. Plants were harvested 6 weeks after they were transplanted (10 weeks after emergence), when those plant accessions that formed heads were of commercially harvestable size. Immediately prior to harvest, plants were scored for the morphological traits plant size (spread), head size, plant growth habit, leaf shape, level of leaf margin crenulations, level of leaf surface blistering, leaf lamina thickness, and intensity of red and green colors on a scale from 1 to 3 (Table 1). The central plant in each plot was harvested by cutting either at the base of the head or, for nonheading lines, approximately 5 cm above soil level. Harvested heads were separated into individual leaves. For nonheading lines, all leaves were stripped from the stems. Leaves from individual plants were thoroughly mixed to ensure representative subsampling. Separate subsamples of leaf material from individual heads were used for total microbial counts, DNA extraction, and leaf wax analysis. The remaining material was weighed and dried to constant weight at 80°C in drying

ovens (Birmingham and Blackburn Construction Company Ltd., Birmingham, United Kingdom). The water content was estimated, and the dried material was milled for chemical analyses.

Chemical analyses. Dried, milled leaf material was extracted in 100 ml g^{-1} distilled water at 100°C for 2 h, the debris was pelleted by centrifugation, and the supernatant was decanted and diluted to 50 ml with distilled water. Amounts of phenolic compounds were determined using the procedure of Vidhyasekaran et al. (48). Soluble carbohydrate content was measured according to the method of DuBois et al. (14). Mineral content was analyzed using the Kjeldahl method, followed by induced coupled plasma mass spectrometry (P, K, Mg, Mn, Na, and Ca) or flow injection (N [as NH_4]) assays. Cuticular wax levels were determined from leaf samples of known weight using a modification of the process of Pilon et al. (34).

Microbial load and population diversity and structure. Total bacterial load was estimated from between 2 g and 5 g of fresh leaf material of each sample, which had been macerated in 2 ml g^{-1} of sterile 18.1-g-liter^{-1} maximum recovery diluent (MRD; Oxoid, Hampshire, United Kingdom). Preliminary experiments showed that a 2-min exposure to 10,000 ppm available chlorine (Presept; Johnson & Johnson Medical Ltd., Gargrave, United Kingdom) was the minimum level of treatment that consistently surface sterilized leaves of all varieties from the field (determined by pressing treated leaves into contact with R2A agar plates, followed by incubation for 48 h at 18°C). For estimation of endophytic counts, leaf material was surface sterilized as noted above and washed twice in sterile distilled water prior to maceration. Ten-fold and 100-fold serial dilutions in MRD were used to enumerate bacterial colonies using spiral plating (spiral plater model D; Spiral Systems Inc., Cincinnati, OH) onto R2A agar (Oxoid), a low-nutrient medium that is more representative of the nutrient status of the leaf surface than other media with higher nutrient contents. Plates were incubated at 18°C for 48 h before enumeration.

Total DNA was extracted from 1 ml of undiluted macerate using a Fast DNA Spin for Soil kit (MP Biomedicals Europe, Illkirch, France) following homogenization in a bead beater (Biospec Products Inc., OK) and quantified spectrophotometrically by determination of the absorbance at 260 nm. Bacterial 16S rRNA genes were amplified from the DNA using primers 63f (27) and 1087r (17) in 25-μl reaction mixtures with an annealing temperature of 53°C for 38 rounds and with a final 10-min extension step. The amplification products were purified using a QIAquick PCR purification kit (Qiagen Ltd., West Sussex, United Kingdom).

For T-RFLP, forward and reverse primers fluorescently labeled with different tags (NED and VIC, respectively) (Applied Biosystems [United Kingdom], Cheshire, United Kingdom) were used, resulting in an amplification product with two differently labeled termini. Amplification products were digested with HhaI and HaeIII (New England Biolabs, MA) for 2 h at 37°C with appropriate buffers. Digests were then separated using an ABI 3700 DNA sequencer to produce T-RFLP profiles for each fluorescent tag (corresponding to the terminal restriction fragments at either end of the amplification product).

For clone library construction, total DNA extracts from lettuce lines 25 and 26 (*Lactuca sativa*) and *L. serriola* accession US96UC23 (the parents of the three lettuce mapping populations) were amplified using bacterial 16S rRNA primers without fluorescent labels. To ensure representative libraries, DNA from all replicate samples was pooled for each of the lines in equal proportions (12 replicates from lines 25 and 26 and 3 replicates from US96UC23). Purified amplification products were cloned using a Qiagen PCR cloning kit. Two hundred eighty-eight clones (3× 96) were randomly selected from each transformation, and plasmid DNA was amplified directly from transformants using a TempliPhi plasmid amplification and extraction kit (GE Healthcare Ltd., Buckinghamshire, United Kingdom) following the manufacturer's protocols. Forward and reverse sequences from each clone were determined from SP6 and T7 primer binding sites in the vector using an ABI 3700 automated sequencer and were combined to produce full-length sequences of the inserts. Chimeric inserts were identified using the Bellerophon program at http://foo.maths.uq.edu.au/~huber/bellerophon.pl and removed from the analyses before sequences were compared to the sequences in bacterial databases using the MegaBLAST search facility at http://www.ncbi.nlm.nih.gov/blast/blast.cgi. Sequences representing chloroplast or mitochondrial DNA were eliminated from further analyses.

Data analyses. Mean values for microbial counts and morphological and chemical characteristics were calculated across all replicates. Initial groupings were based on 95% confidence limits (±1.96 standard deviations [SDs]). Mean values were considered significantly different if the difference was greater than the sum of the associated 95% confidence limits. The significance of these groupings was then confirmed by analysis of variance.

T-RFLP signal baselines were calculated according to the principles described by Abdo et al. (1), and the relative peak height data for each profile were extracted. The T-RFLP profiles generated for each sample were aligned to produce a single data set comprising either a relative proportion (based on normalized peak height) or 0 (absence of the peak) for each T-RF detected. Mean numbers of T-RFs for each lettuce accession were determined. Shannon diversity indices and the associated 95% confidence limits were calculated using the relative proportion of each T-RF (41). Two-dimensional representations of the T-RFLP profile data were produced from Bray-Curtis dissimilarity matrices following a nonmetric multidimensional scaling (NMDS) approach (Genstat statistical analysis software; VSN International Ltd.). Interactions between the T-RFLP profiles and chemical or morphological characteristics were investigated by applying the significance groups determined for these characteristics as factors in a multiresponse permutation procedure (MRPP) analysis of the NMDS output (Blossom statistical package; U.S. Geological Survey, Fort Collins Science Center). Bacterial populations from clone library data were compared nonparametrically by comparing the Cramér von Mises statistics between libraries according to the method of Singleton et al. (43) using the online analysis tool webLIBSHUFF (version 0.96) at http://libshuff.mib.uga.edu and by using the Mann-Whitney U test with correction for tied ranks.

Nucleotide sequence accession numbers. Bacterial sequences were submitted to the GenBank/EMBL/DDBJ databases under accession numbers FN813825 to FN813941 (Saladin line 25), FN813942 to FN814137 (Iceberg line 26), and FN814138 to FN814273 (*L. serriola* US96UC23).

RESULTS

Morphology and chemistry of lettuce genotypes. Significant differences between plant accessions ($P < 0.05$) were detected in all morphological characters examined (Table 1). Levels of leaf wax, nitrogen, potassium, calcium, magnesium, phenolic compounds, soluble carbohydrate, and water content were also found to differ significantly ($P < 0.05$) between accessions (Table 2). No significant variations in phosphorus, sodium, or manganese content were observed (data not shown). The *L. serriola* mapping parent, US96UC23 (line 8), was significantly taller and had thinner, narrower leaves with more crenulations, less surface blistering, lower levels of soluble carbohydrate, and a lower water content than either of the *L. sativa* parents (lines 25 and 26). These two lines differed significantly only in plant size and leaf coloration, with accession Iceberg (line 26) producing larger plants with paler green leaves and significantly higher levels of red pigmentation. No individual morphological or chemical component was significantly different between all three mapping parent accessions.

Microbial population size and structure. Total bacterial loads (normalized for sample weight) were between 4×10^5 and 5×10^6 CFU g $(FW)^{-1}$ (where FW is fresh weight) for all samples and were not significantly different between accessions. The bacterial population sizes of the culturable endophytic populations (surface-sterilized and macerated samples) ranged from 3×10^1 to 9×10^4 CFU g $(FW)^{-1}$ where they were detected. No culturable endophytes were detected from accessions 2, 3, 11, and 16 at a theoretical detection limit of 5×10^0 CFU g $(FW)^{-1}$. The numbers of T-RFs (i.e., individual peaks in a T-RFLP profile) detected were not significantly different between lettuce accessions. Comparison of Shannon diversity indices for the T-RFLP profiles (Fig. 1), which takes into account both number and relative proportion of the T-RFs, however, showed significantly different levels of diversity between the T-RFLP profiles from different accessions ($P < 0.05$).

Relationship between T-RFLP profiles and lettuce genotypes. MRPP analyses of the T-RFLP profile data indicated significant differences between profiles from different plant accessions ($P < 0.001$) according to plant morphotype (i.e., batavian type, iceberg type, etc.) (Fig. 2). The T-RFLP profiles

TABLE 2. Mean values for physiological and chemical characteristics of the lettuce (*Lactuca sativa*) and *Lactuca serriola* accessions used in this study, showing associated significance groups[e]

Line no.	Accession	Physiological characteristics				Mineral content			
		Wax	Water	Soluble carbohydrate	Phenolics	Mg	Ca	N	K
1	Cobham Green	0.97[a]	94.8[a]	156.3[b]	74.7[b]	0.17[a]	0.84[a]	2.69[a]	4.05[a]
2	*L. serriola*	0.97[a]	90.0[d]	124.4[a]	89.9[a]	0.25[a]	0.71[a]	4.05[a]	3.71[a]
3	Stoke	0.41[a]	94.0[a]	108.7[a]	89.6[a]	0.22[a]	1.05[a]	3.11[a]	4.41[b]
4	Batavia Blonde de Paris	0.46[b]	95.3[a]	164.3[a]	76.7[b]	0.20[a]	0.58[a]	3.43[a]	4.98[a]
5	Lobjoits Green Cos	0.64[a]	94.2[b]	193.8[a]	77.1[b]	0.23[a]	0.78[a]	3.59[a]	4.92[a]
6	Ambassador	0.61[a]	96.5[a]	96.8[b]	76.5[b]	0.15[b]	0.48[a]	3.34[a]	4.56[a]
7	Little Gem	0.35[b]	95.1[a]	154.6[b]	58.3[c]	0.17[a]	0.77[a]	2.80[a]	4.67[a]
8[f]	*L. serriola* US96UC23	2.28[a]	89.9[d]	62.5[e]	136.9[a]	0.30[a]	0.90[a]	3.66[a]	4.66[a]
9	Bloody Warrior	0.69[a]	93.8[b]	154.2[a]	96.2[a]	0.19[a]	0.53[a]	3.28[a]	5.01[a]
10	New Chicken	0.75[a]	94.0[b]	68.0[d]	93.2[a]	0.17[a]	1.13[a]	3.05[a]	4.45[b]
11	Romanie de Benicardo	2.90[a]	94.2[a]	135.2[c]	142.8[a]	0.20[a]	0.42[b]	3.60[a]	3.67[b]
12	Lollo Rossa	0.74[a]	94.7[b]	126.3[b]	150.8[a]	0.17[a]	0.58[a]	3.35[a]	5.26[a]
13	Lollo Biondo	0.33[a]	95.5[a]	101.6[d]	86.2[b]	0.24[a]	0.85[a]	3.29[a]	5.36[a]
14	Lillian	0.66[a]	94.6[b]	185.4[a]	90.3[b]	0.15[a]	0.64[a]	2.64[a]	3.91[a]
15	Batavia Tezier	0.72[a]	94.5[b]	161.4[a]	101.1[a]	0.18[a]	0.64[a]	2.76[b]	4.57[a]
16	Wunder von Stuttgart	0.57[a]	94.8[a]	109.8[c]	85.5[b]	0.21[a]	0.86[a]	2.89[a]	4.10[a]
17	Adriatica 2	0.73[a]	93.7[b]	94.6[d]	98.4[a]	0.27[a]	1.03[a]	4.19[a]	6.05[a]
18	Webb's Wonderful	0.53[a]	95.3[a]	182.2[a]	96.1[a]	0.17[a]	0.65[a]	3.10[a]	5.19[a]
19	Waldmann's Dark Green	0.99[a]	93.9[b]	127.3[b]	112.8[a]	0.14[b]	0.75[a]	3.09[a]	4.74[a]
20	Chinese Stem Lettuce	0.85[a]	93.0[c]	214.8[a]	100.0[a]	0.15[b]	0.52[b]	2.64[b]	4.55[a]
21	Platinas	1.07[a]	94.7[a]	186.4[a]	107.0[a]	0.14[b]	0.53[a]	2.99[a]	4.98[a]
22	Madrass	0.47[b]	95.1[a]	232.5[a]	96.5[a]	0.16[a]	0.51[a]	3.36[a]	4.79[a]
23	Jazzie	0.54[a]	95.3[b]	161.9[a]	67.0[c]	0.18[a]	0.75[a]	2.91[a]	5.48[a]
24	Imagination	0.58[a]	95.1[b]	236.6[a]	94.6[b]	0.15[a]	0.60[a]	2.35[b]	4.07[b]
25[f]	Saladin	0.73[a]	94.8[a]	202.9[a]	101.0[a]	0.19[a]	0.58[a]	3.29[a]	4.48[a]
26[f]	Iceberg	0.60[a]	95.2[a]	181.2[a]	97.2[a]	0.17[a]	0.55[a]	3.20[a]	4.79[a]

[a] Belongs to the significance group with the highest value for each characteristic.
[b] Belongs to the significance group with the second-highest value for each characteristic.
[c] Belongs to the significance group with the third-highest value for each characteristic (where present).
[d] Belongs to the significance group with the fourth-highest value for each characteristic (where present).
[e] The units for the various characteristics are as follows: wax, mg g fresh weight^{-1}; water, % fresh weight; soluble carbohydrate and phenolics, mg g dry weight^{-1}; Mg, Ca, N, and K, % dry weight.
[f] Mapping population parental lines.

of the bacterial population on the iceberg types were significantly different from all the other profiles. The profiles from the curly leaved, Cos, and butterhead morphotypes clustered together, with the profiles from the batavian, stem lettuce, and *L. serriola* morphotypes differing significantly from the major-

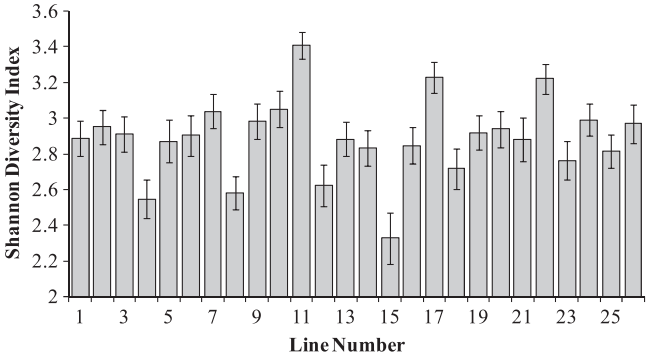

FIG. 1. Comparison of Shannon diversity indices for T-RFLP data (calculated from both number and relative intensity of T-RFs) for each of the *Lactuca* accessions. Error bars show 95% confidence limits. Lines 8, 25, and 26 (*L. serriola* US96UC23, cv. Iceberg, and cv. Saladin, respectively) are the parental lines of the three mapping populations.

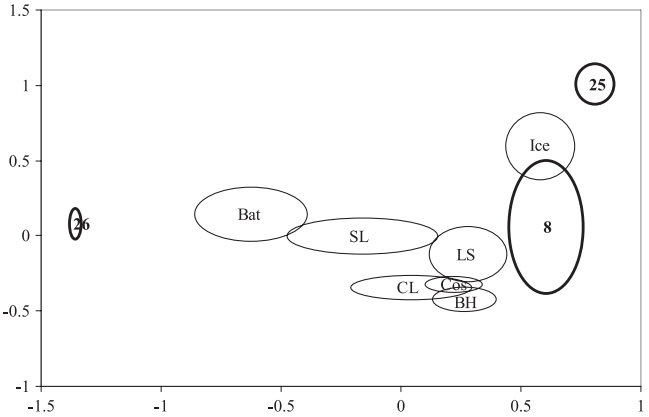

FIG. 2. Two-dimensional plots of nonparametric multidimensional scaling analyses of the T-RFLP profile data showing significant groupings on the basis of plant morphotype. The axes are scalar values representing multiple parameters. The markers indicate mean values, and ellipses indicate least significant difference ($P < 0.05$) in each case. Regular-face labels and light ellipses indicate data for lettuce morphotypes: Bat, batavian type; BH, butterhead type; Cos, Cos type; CL, curly leaved type; Ice, iceberg type; SL, stem lettuce type; LS, *Lactuca serriola* wild-type relatives. Boldface labels and heavy ellipses indicate data for the three parental accessions for the mapping populations (line 8 [*L. serriola* US96UC23], line 25 [batavian type cv. Iceberg], and line 26 [iceberg type cv. Saladin]).

ity of other profiles but forming a spectrum of profiles, from the *L. serriola* morphotypes (most similar to the curly leaved, Cos, and butterhead profiles) to the batavian accessions (least similar). Applying groupings of accessions identified by the previous analysis of individual plant characteristics as factors in further MRPP analyses showed that characteristics that affected whole-plant architecture (plant size and head size) and those that affected leaf topology (shape, crenulations, and blistering) were significant factors ($P < 0.001$) in differentiating the T-RFLP profiles, as were levels of soluble carbohydrate, Ca, leaf wax, water content, and phenolic compounds ($P < 0.05$). Leaf thickness, leaf color characteristics, and levels of N, Mg, and K were not found to significantly influence T-RFLP profiles.

Certain trends in the clustering of plant accessions could be identified in these analyses; The Cos, stem lettuce, and *L. serriola* morphotypes were grouped together with respect to all morphological characteristics that differentiated the T-RFLP profiles, while the other morphotypes showed more variable groupings. The majority of iceberg type accessions grouped differently from the majority of other morphotypes with respect to leaf topographical characteristics. The majority of curly leaved and butterhead morphotypes also grouped together. Considering whole-plant characteristics (plant size and head size) and the chemical factors that differentiated the T-RFLP profiles produced patterns of clustering with no discernible correlation with plant morphotype.

The MRPP analyses showed that the T-RFLP profiles associated with the morphotypes to which the three parental lines belonged differed significantly from one another. Further analysis also showed that the profiles of the bacterial populations from the individual parental lines of the mapping populations were also significantly different (Fig. 2). To obtain a more detailed breakdown of the structure of the microbial populations from these three parental lines, 16S rRNA gene clone libraries were constructed. A total of 24 bacterial genera were identified from the lettuce phyllosphere, based on analysis of 449 useful clones. Chao1 estimates of population richness (9) indicated that more than 85% of estimated genera were present in the clone libraries in each case (Table 3). These estimates were significantly lower ($P < 0.05$) for the population from *L. serriola* US96UC23 than the populations from *L. sativa* accessions Saladin (line 25) and Iceberg (line 26), indicating lower diversity in the population from the *L. serriola* accession. This was in agreement with the comparison of the Shannon diversity indices for the T-RFLP profiles of these populations. The Chao1 estimates also, however, showed significantly greater richness in the population from cv. Iceberg compared to that from cv. Saladin ($P < 0.01$), which the Shannon diversity index measurements of T-RFLP profiles had not resolved.

Comparison to sequence databases showed that the overwhelming majority of detected sequences (>95% in all three lines) were from the gammaproteobacterial division, with *Pseudomonadaceae* and *Enterobacteriaceae* being the predominant families. Comparisons of the Cramér von Mises test statistics based on sequence data and Mann-Whitney U tests (with appropriate correction for tied ranks) based on the identified genera both showed that the three clone libraries were all significantly different from each other ($P > 0.01$ and $P > 0.001$, respectively). Isolates of the *Pseudomonadaceae* were represented exclusively by the genus *Pseudomonas* and were detected at similar levels in all three libraries, making this the major genus in all three populations. In contrast, considerable variation was observed in both the bacterial genera detected within the *Enterobacteriaceae* and their relative proportions (Table 3). Sequences from 5 enterobacterial genera were identified from *L. serriola* US96UC23, sequences from 9 genera were detected from cv. Saladin, and sequences from 13 genera were detected from cv. Iceberg. Furthermore, the proportions of some of the genera common to all populations differed considerably between the lines, most notably, the proportions of *Enterobacter*, *Erwinia*, and *Pantoea* species. The proportion of sequences representing the genus *Enterobacter* was nearly four times greater in cv. Saladin than in cv. Iceberg and nearly 20-fold greater than in US96UC23. Conversely, the proportion of *Erwinia* species in the cv. Saladin population was approximately 3-fold lower than that in the US96UC23 population and 4-fold lower than that in the cv. Iceberg population. Sequences

TABLE 3. Sequence identification from clone library screens for *L. serriola* US96UC23 (line 8) cv. Saladin (line 25) and cv. Iceberg (line 26) amplified with bacterial 16S rRNA primers 63f and 1087r, including Chao1 estimates of population size based on numbers of identified bacterial genera

Bacterial taxon	% representation[a]		
	L. serriola US96UC23	Saladin (line 25)	Iceberg (line 26)
Gammaproteobacteria			
Enterobacteriaceae			
Averyella	—	—	0.5
Buttiauxella	—	1.7	2.6
Citrobacter	—	—	0.5
Enterobacter	2.2	30.2	7.6
Erwinia	16.1	3.4	14.3
Hafnia	—	—	0.5
Klebsiella	—	2.6	0.5
Leclercia	—	0.9	—
Pantoea	24.6	5.2	8.7
Photorhabdus	—	1.7	—
Rahnella	—	0.9	4.1
Raoultella	—	—	0.5
Serratia	2.2	0.9	7.6
Tiedjeia	—	—	0.5
Yersinia	—	—	0.5
Xenorhabdus	1.5	—	—
Unidentified	2.2	1.7	5.6
Total *Enterobacteriaceae*	**48.8**	**49.2**	**54.0**
Pseudomonadaceae (*Pseudomonas*)	**48.2**	**45.7**	**43.4**
Aeromonadaceae (*Aeromonas*)	—	**1.7**	—
Chromatiaceae (*Rheinheimera*)	—	—	**0.5**
Moraxellaceae			
Acinetobacter	1.5	0.9	—
Alkanindiges	—	—	0.5
Total *Moraxellaceae*	**1.5**	**0.9**	**0.5**
Total gammaproteobacteria	**98.5**	**97.5**	**98.4**
Firmicutes (*Carnobacterium*)	—	—	0.5
Unidentified bacteria	1.5	2.6	1.0
No. of clones	137	116	196
No. of genera detected	10	15	30
Chao1 estimate of population size (genera) ± 1.96 SDs	11 ± 3	17 ± 2	36 ± 9
% coverage	90.9	88.2	83.3

[a] —, division, family, or genus not detected. Boldface indicates total percentages for each category.

representing the genus *Pantoea* were also a greater proportion of the population from US96UC23 than from either of the other two lines.

In silico restriction digests of all sequences corresponding to the major genera detected (*Pseudomonas*, *Erwinia*, *Enterobacter*, and *Pantoea*) identified predicted T-RFs for *Pseudomonas*, *Erwinia*, and *Enterobacter* species that both represented the majority of sequences from these genera in the clone libraries and either were unique to the target genus or were predicted to be represented at very low levels in sequences from other genera. No suitably specific T-RFs could be identified for the genus *Pantoea* with the primer and restriction enzyme combinations used. The sizes of the predicted T-RFs were then compared to the experimentally generated profiles and representative peaks were identified. Additional MRPP analysis using these partially genus-specific T-RFs individually showed significant ($P < 0.001$) grouping of the profiles from the lettuce accessions with respect to the genera *Erwinia* and *Enterobacter*. The *Erwinia* profiles clustered into four groups (accessions 1, 8, and 21; accessions 4, 10, 16, 18, and 20; accessions 14, 17, 19, and 26; and all other accessions). In contrast, the *Enterobacter* profiles fell into six clusters. Similarly to the *Erwinia* profiles, accessions 1, 8, and 21 grouped together. Accessions 20, 25, and 26 all grouped singly and independently, while accessions 2, 6, and 11 also grouped together, with the other accessions falling into a single group. The analysis also showed the profiles from the parental mapping accessions grouping into separate clusters for both genera, confirming the differences seen in the clone libraries. No significant grouping of lettuce accessions was identified with respect to *Pseudomonas*. By grouping the lettuce accessions according to significant differences in the *Enterobacter* subprofiles and applying these groupings as factors to a further MRPP analysis of the *Erwinia*-specific T-RFLP data, however, the *Enterobacter* subpopulation was found to be a significant ($P < 0.001$) factor in the variation of the *Erwinia* T-RFLP subprofiles. The converse analysis (using *Erwinia*-based groupings of accessions as factors in the *Enterobacter* analysis) did not show significant clustering ($P = 0.22$). Consequently, the *Erwinia* subpopulation was shown not to be a significant factor in the variation of the *Enterobacter* T-RFLP subprofiles.

DISCUSSION

Levels of bacterial epiphytic phyllosphere colonization have been reported to range from 10^1 to 10^8 CFU g^{-1} between different plant genera (18, 20, 53). Total bacterial colonization of the lettuce phyllosphere in this study ranged from 4×10^5 to 5×10^6 CFU g $(FW)^{-1}$, which is comparable to the levels of epiphytic lettuce colonization (18). No significant differences in levels of bacterial colonization were detected between the closely related lettuce accessions used in this study. Endophytic bacterial load has been shown to vary between plants of the same species (38, 40); however, the culturable bacterial counts from surface-sterilized lettuce tissue in this study (i.e., endophytic counts) accounted for less than 5% of the total culturable population. Although leaf decolorization due to exposure to active chlorine penetrated only a few mm beyond the leaf margins, it is possible that the sterilization procedure may have resulted in an underestimate of endophytic counts. Despite this, however, this result still indicated that the majority of bacteria detected were epiphytic and showed that the detected variation in the compositions of the bacterial populations was not influenced by population size. Environmental effects are also well-known to influence microbial phyllosphere populations; however, the experimental design used (replicate plantings at a single site and in a single geographical location grown over a single time frame) was intended to minimize the variation due to environmental effects by exposing all replicates of all accessions to the same environmental conditions.

The emergence of plant morphotype (a composite of numerous leaf and whole-plant morphological traits) as a significant factor in differentiating the bacterial T-RFLP profiles (Fig. 2) suggested an important role for leaf morphology in determining the differences detected between the developing microbial communities. The more detailed analysis, applying groupings of accessions identified by the previous analysis of individual plant characteristics as factors in further MRPP analyses, revealed that all the factors that influenced the T-RFLP profiles had the potential to affect the leaf surface microenvironment. On one scale, differences in plant and head sizes could alter the flow of air and deposition of water and soluble nutrients on the plant surface, while at a smaller scale, leaf shape, margin crenulations, and surface blistering all impact leaf topography. Differences in topographical features may reflect even smaller-scale differences, such as the distribution of veins and hydathodes and the density of epidermal cell wall junctions, all of which have been reported as being preferred bacterial attachment sites (12, 29, 32). Leaf wax levels have also been shown to directly influence adherence of microorganisms (5) and have been associated with UV protection (26), presumably by altering the UV-absorptive or -reflective properties of leaf surfaces. Topography and water repellency (as influenced by surface wax deposits) have also been shown to have localized effects on leaf surface water distribution, potentially influencing the distribution of available water and water-soluble nutrients (31, 33). Since the level of soluble carbohydrate was also found to be a factor influencing T-RFLP profiles, the impact of these distributions may be of particular importance. Sugar levels have also been reported to vary in the apoplastic fluid of sugarcane cultivars. In this case, differential responses of some associated endophytic bacteria to some of these sugars were also noted (3). Since differences in apoplastic fluid content could affect concentrations of surface-deposited nutrients, epiphytic populations may also be influenced in this manner.

It should be noted, however, that the influence of certain of these characters may vary depending on the growth stage of the leaf. For example, surface blistering is likely to have more impact on the microbial populations developing on more open, outer leaves than in compact lettuce heads, where the leaves are pressed closely together. Leaf chemistry and the distribution of nutrients are also known to vary between different leaf growth stages. This study (where leaf samples were pooled from different growth stages and positions in the plant architecture) can therefore provide only a general snapshot of differences in the microbial populations, not an exhaustive analysis of the variations at each growth stage and location within the plant architecture. It also remains to be seen if the factors identified in this study to be influencing phyllosphere popula-

tions interact with environmental variables in other environments.

The differences in Ca content observed may reflect variation in uptake, transport, or utilization by the different lettuce accessions. Carbon-source availability rather than mineral nutrition is usually considered to be the major constraint to bacterial growth in the phyllosphere (25); consequently, Ca levels are unlikely to have a direct impact on the bacterial population. Calcium signaling pathways have, however, been implicated in a number of plant responses: regulation of stomatal guard cell closure has been linked with Ca cycles (30), and Ca signaling has also been shown to have implications for the structure of trichomes, which have been identified to be a preferred site of bacterial adhesion (13). Conversely, both stomatal density (35) and transpiration rate (4) have been shown to influence the Ca concentration in plant tissues. Thus, differences in Ca levels could reflect multiple factors capable of indirectly influencing phyllosphere population composition.

Both constitutive and induced expression levels (and the rate of induction) of numerous phenolic compounds have been associated with plant defense against pathogenic bacteria and fungi (22, 46). Since the majority of plants harbor both endophytic and epiphytic bacterial populations without invoking defense responses, it would seem unlikely that induced phenolic pathways would influence general phyllosphere populations; however, variations in levels of constitutively expressed phenolic compounds may have an effect. The question remains whether variation in the phyllosphere population has any reciprocal effect on the host plant.

The gammaproteobacteria have been shown to be the dominant group in phyllosphere populations from many, diverse plant species (9, 16, 23, 39, 45, 51). In the majority of these studies, the pseudomonads have been the dominant family detected (30 to 55% of the population), with *Enterobacteriaceae* family members also being strongly represented (10 to 27%) (9, 16, 45), although in one study on soybean, the *Enterobacteriaceae* were found to be dominant, at 57% of the population (23). Plant surfaces are considered relatively hostile environments for bacterial colonization due to temperature and humidity fluctuations as well as low nutrient availability (25), and significant seasonal changes in population composition have been noted (39). It may be that these (largely culturable) genera represent the organisms best adapted to survive in the niche environments that occur in the phyllosphere.

The fact that differences in bacterial profiles between lettuce accessions could be detected only when the relative proportions of the T-RFs were considered (i.e., by comparing Shannon diversity indices) suggested subtle variations in the relative proportions of certain bacterial ribotypes rather than gross differences in community composition. The clone library data indicated that differences in the proportions of *Erwinia*, *Pantoea*, and *Enterobacter* (*Enterobacteriaceae*) accounted for the majority of the variation in the mapping parent populations. There has been some synonymous use of the genus names *Erwinia* and *Pantoea* in the past. In order to confirm their identity, sequences identified as being from these genera were compared to type strain sequences at the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). All sequences aligned closer to sequences of their identified genus type strains than to any others. The significant differences noted between the T-RFLP subprofiles for *Erwinia* and *Enterobacter* confirmed that these genera contributed to the identified differences in the bacterial populations across all the accessions. From the MRPP analyses, the factors that were significantly associated with the differences between bacterial populations that developed on *L. serriola* US96UC23 and those which developed on lettuce cv. Iceberg and cv. Saladin were levels of soluble carbohydrate, water content, and leaf blistering, all of which were significantly lower in US96UC23 than in the other two lines. None of these characteristics, however, were found to be significant factors in the distribution of the three dominant genera for which marker T-RFs were determined. The fact that these T-RFs were not significantly influenced by any specific parameter but did show variation across the accessions suggests that a combination of factors (potentially with low-level influences) may be important.

The significant clustering of the T-RFLP subprofiles for *Erwinia* in respect of the differences observed for the *Enterobacter* T-RFLP subprofiles indicates that the relative proportion of *Enterobacter* species may influence colonization of the phyllosphere by *Erwinia*. A comparison of the relative proportions of these two genera from the clone library data suggests a negative effect of *Enterobacter* on *Erwinia* colonization. The lack of significance in the reciprocal analysis, however, shows that the *Enterobacter* population is unaffected by variations in the relative proportions of *Erwinia* species. This suggests that other factors may be involved in determining variation in the proportion of *Enterobacter*, although since the proportions of this genus were not found to be significantly influenced by the morphological and chemical characters identified, the source of this variation is not evident. From the clone library data, the proportions of *Pantoea* species follow a trend similar to that observed for *Erwinia* (i.e., opposite to the proportion of *Enterobacter* species); however, since no representative T-RFs could be identified for *Pantoea*, the relative proportions of these two genera in the other accessions could not be compared. It is possible, however, that other, more subtle effects may have roles in determining phyllosphere population structure. Temporal succession patterns are known to occur in aquatic biofilm systems (24, 27) and have been shown in the phyllosphere of the cottonwood (*Populus deltoides*) (39). Unlike the aquatic situations, where the biofilms were forming on abiotic surfaces, we have shown that phyllosphere populations are influenced by leaf characteristics. As a consequence, plant genotype may directly influence the early composition of the phyllosphere community (which may or may not persist) and consequently (indirectly) affect the later composition of the developing community. Such influence, however, will always be subject to variation in the organisms available to colonize the phyllosphere from the environment.

One area meriting further investigation would be the impact of population differences in the phyllosphere metabolome. It is unknown whether differences in population composition are reflected in different metabolic functionalities and what impact any such metabolic differences may have on further colonization. In addition, further investigation of the observed variation within the *Enterobacteriaceae* may be of particular benefit in terms of the risk of contamination with human pathogens. Three of the four most significant genera associated with food poisoning (*Salmonella*, *Yersinia*, and *Escherichia* [49]) are

members of this family, and both enterohemorrhagic *Escherichia coli* (42) and *Salmonella enterica* (7, 21) have been shown to bind to various salad leaves, including lettuce. Variations in the levels of other *Enterobacteriaceae* may also be important; for example, levels of *Salmonella* and *E. coli* O157:H7 contamination have been reported to be enhanced in the presence of *Erwinia* (8, 50) and reduced by the presence of *Enterobacter asburiae* (11, 55).

The underlying plant genetic component associated with the variation in plant characteristics that influence phyllosphere bacterial communities (and their individual components) allows more detailed investigations of the mechanisms by which these effects operate. The identification of differences between the parental lines of the lettuce mapping populations provides a route to carry out such investigations and will identify target traits for genetic investigation. In the longer term, this opens up the real possibility of managing the phyllosphere bacterial population through a plant breeding approach.

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