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EFFECTS OF AN INTRODUCED BACTERIUM ON BACTERIAL COMMUNITIES ON ROOTS[1]

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Abstract. The objective of this work was to determine whether introduction of a bacterium altered microbial communities associated with roots. We conducted experiments in the field and in a growth chamber to determine whether coating soybean seeds with marked strains derived from the biological control agent *Bacillus cereus* UW85 affected the bacterial community in the rhizosphere of soybeans. We characterized 2651 individual isolates of bacteria from root-free soil and from soybean seedlings based on 43–50 physiological attributes. Discriminant analysis of the bacterial communities according to these attributes showed that the communities of rhizosphere bacteria that developed on non-treated plants and on plants grown from seeds coated with a single strain of bacteria were sometimes dramatically different. This occurred even when the introduced strain did not persist as a common member of the community. In two of four experiments we could as easily differentiate between bacterial communities on roots of UW85-treated and non-treated seedlings as between the communities in the rhizosphere and root-free soil. In the other two experiments we could differentiate only between communities in root-free soil and on roots. In the comparison of bacteria from root-free soil and from the soybean rhizosphere, we found that bacteria from root-free soil were more likely to degrade complex carbon sources than were rhizosphere bacteria, whereas bacteria from roots were resistant to more antimicrobial substances and were able to grow on a wider range of simple carbon sources than were bacteria isolated from root-free soil.

Key words: Bacillus cereus *UW85; biological control; damping-off; discriminant analysis; environmental release of microorganisms;* Glycine max; *microbial communities; microbial ecology; plant roots; rhizosphere; soil microbiology; soybean.*

INTRODUCTION

The community of bacteria associated with plant roots (the rhizosphere community) directly and indirectly affects the health of the plant. Pathogens in the community may inhibit plant growth or even kill the plant, deleterious rhizobacteria may reduce growth or make the host susceptible to pathogens, and beneficial bacteria may supply nutrients or protect the plant from disease-causing organisms (Kloepper et al. 1980, Chanway et al. 1988, Astrom and Gerhardson 1989, Lalande et al. 1989, Griffin 1990, Schippers et al. 1990). There have been many attempts to manipulate the development of microbial communities in the rhizospheres of crop plants in order to protect the plants from disease or to promote plant growth (Weller and Cook 1983, Weller 1988, Cook et al. 1990, Gorodecki and Hadar 1990, Smith et al. 1990, Parke et al. 1991). Usually this involves the introduction of a large population of one type of microorganism on seeds or in furrow during planting (Weller and Cook 1983, Handelsman et al. 1990, Mcquilken et al. 1990, Smith et al. 1990, Halverson and Handelsman 1991, Parke et al. 1991), with the intention of either promoting or interfering with the colonization of the rhizosphere by a specific microorganism. The term "introduction," as used in the literature on biocontrol of plant pathogens, can refer to either the introduction of an exotic organism or the application (often on seeds) of a large population of an organism that might or might not already be present at much smaller densities (Cook 1990). Throughout this paper, "introduction" will be used primarily in the latter sense.

The population dynamics of introduced organisms and their effects on plant health or on populations of

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pathogens have often been carefully investigated (Kloepper et al. 1980, Suslow and Schroth 1982, Loper et al. 1985, Okon and Kapulnik 1986, Bashan et al. 1989, Juhnke et al. 1989, Reddy and Rahe 1989, Parke 1990, Bull et al. 1991), but there have been few studies on the effects of an introduced microbe on indigenous members of microbial communities other than pathogens. Weller (1983) found an increase in total fluorescent pseudomonads but not in total bacterial populations after the introduction of *P. fluorescens* 2-79 on wheat. Yuen and Schroth (1986) found no effects on the population densities of Gram-negative bacteria, fluorescent pseudomonads, or actinomycetes on zinnia roots after inoculation with *Pseudomonas fluorescens* E6. In contrast, Kloepper and Schroth (1981) found that inoculation of potato seed pieces with *P. fluorescens* strains A1, E6, or B10 resulted in reduced populations of Gram-positive bacteria in the rhizosphere. An improved understanding of the influence of the introduced organism on microbial communities is required for biocontrol or plant growth promotion to be accomplished through the introduction of microorganisms. Moreover, improved information about the behavior of introduced organisms in microbial communities is needed to evaluate the possible risks and benefits of introducing genetically engineered or non-engineered microbes into the environment for biological control or other purposes (Tiedje et al. 1989).

We used the introduction of two antibiotic-resistant mutants of the biological control agent *Bacillus cereus* UW85 (Handelsman et al. 1990), which controls damping-off of seedlings (caused by *Pythium* spp. and *Phytophthora* spp.) and Phytophthora root-rot diseases of some legumes, to investigate the effects of an introduced bacterium on the complex microbial environment in the rhizosphere. Our work addresses how the communities of bacteria that develop in the rhizosphere differ from bacterial communities in root-free soil, and how introducing a large population of a single strain of bacterium can affect the bacterial community.

Methods

We examined the heterotrophic bacterial communities in root-free soil, in the rhizosphere of nontreated soybean plants, and in the rhizosphere of plants grown from seeds coated with *Bacillus cereus* UW85n1 (a spontaneous neomycin-resistant mutant of *B. cereus* UW85 that retains the biocontrol activity of its parent strain). Field experiments were conducted at one site in 1989 (Arlington) and at two sites in 1990 (Arlington and Hancock). UW85 was originally isolated from a healthy alfalfa root at the Arlington Experimental Farm, Columbia County, Wisconsin, in 1985. In 1990, to compare the effects on rhizosphere communities of the introduction of bacteria that have or lack biological control activity against *Phytophthora,* we included a second strain, *B. cereus* UW831, which is a mitomycin-C-induced mutant derived from UW85n1 that has lost the ability to protect alfalfa seedlings against *Phytophthora megasperma* in a growth-chamber bioassay. UW831 is physiologically indistinguishable from UW85n1 except that it lacks biocontrol activity and produces significantly less antifungal antibiotic than does UW85n1 (J. Handelsman, *unpublished data*). In 1989 we conducted a growth-chamber experiment (Growth chamber 1989) using soil from one of the field sites, to determine whether results from a growth-chamber experiment would reflect results from the field. We monitored the emergence of seedlings in the field experiments to determine severity of damping-off and efficacy of biocontrol, and determined the population densities and physiological attributes of heterotrophic bacteria in root-free soil and in the rhizosphere. The experimental protocols are described in detail below.

Arlington 1989

The first experiment was conducted at the Arlington Experimental Farm, on a Joy silt-loam (fine-silty, mixed, mesic aquic Hapludolls). Soybean seeds (*Glycine max* L. (Merr.) cultivar AP200, Agripro Seeds, Ames, Iowa) were coated with cultures of UW85n1 harvested from Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Maryland, USA) amended with polymyxin B (12.5 μg/mL), ampicillin (25 μg/mL), cycloheximide (50 μg/mL), and neomycin sulfate (5 μg/mL). Cultures were incubated for 4 d at 28°C, and examined microscopically for complete sporulation and purity of cultures before harvesting. The entire culture was coated onto seeds and allowed to air-dry; no sticker (adhesion promoter) was used. The density of spores on seeds was determined by plating dilutions of seed-washings onto this same medium on the day of planting. UW85n1-treated seeds were coated with 10^8 to 10^9 spores per seed; nontreated seeds did not show detectable quantities of UW85n1-like bacteria. Treated and nontreated seeds were planted at 2 cm depth in a randomized block design on 24 May 1989. Ten days after planting (2 d after most of the seedlings emerged, the earliest time when there was adequate certainty that roots were sufficiently large to be harvested), root samples from treated and nontreated seedlings and root-free soil samples were collected; five samples were taken from each treatment from each of 10 blocks. Roots were gently shaken free of loosely adhering soil prior to processing. Root samples were 1 cm in length, and taken from 2–3 cm below the soil line. Soil samples (1 cm^3) were collected from a corresponding depth, 20 cm from each nontreated plant. Samples were placed in sterile water, and sonicated for 30 s in a bath sonicator (model B220, Bransonic, Shelton, Connecticut, USA) to dislodge closely adhering soil and disperse rhizosphere bacteria. Ten additional samples of each plant treatment were sonicated for 15 s using a 250-W Vibra-cell probe sonicator (Sonics and Materials, Inc., Danbury, Connecticut, USA), which operates at 20 kHz and was set at 20% output. Ten-fold dilutions were

prepared from each sample. To obtain isolates from the general bacterial community, aliquots (0.1 mL) were plated onto trypticase soy agar (TSA) prepared at $\frac{1}{10}$ of the recommended strength (10% TSA; contains 1.5% agar [mass/volume]), which supports the growth of a wide range of heterotrophic bacteria (Martin 1975). After 3 d of incubation at ambient temperature (≈24°C), five isolates from each sample were randomly chosen from 10% TSA, from the plates containing the highest number of physically separate colonies (generally 50–200 colonies per plate), using a 50-point template and a random number table. These isolates were subcultured on 10% TSA until they appeared pure, and then transferred to 10% TSA plates in a six × eight pattern, suitable for replica plating. Bacteria were stored frozen in microtitre plates in tryptic soy broth + 10% glycerin at −20°C. Some isolates (22% for all four experiments) were not recovered after the purification or freezing processes.

Samples of root-free soil will be referred to as "soil samples," segments of roots that emerged from nontreated seeds will be referred to as "nontreated root samples," and samples of roots from UW85n1-treated seeds will be referred to as "UW85n1-treated root samples."

Growth chamber 1989

We collected soil on 11 May 1989 from the same field used in the Arlington 1989 experiment. This soil was air-dried in a greenhouse and then sieved (2-mm mesh opening). Plastic cones for nursery production of seedlings (50 cm^3, Ray Leach Conetainer Nursery, Canby, Oregon, USA) were plugged at the bottom with cosmetic puffs (synthetic equivalents of cotton balls) under 20 cm^3 of fine sterile vermiculite, and were then filled with sieved soil (20 cm^3). Seeds for this experiment were coated with UW85n1 at the same time as those used in the Arlington 1989 experiment. One hundred nontreated and 100 UW85n1-treated seeds were planted in the cones (one seed per cone), covered with 2 cm of sieved soil, and the cones placed in racks along with 60 cones not planted with seeds, in a completely randomized design. The racks were placed in a growth chamber at 24°C (which allows rapid growth of soybean seedlings) with a 12/12 light/dark cycle (photon flux density 244 $\mu mol \cdot m^{-2} \cdot s^{-1}$), and each cone was watered to saturation from the top at planting and on day 3 with sterile deionized water. Root and soil samples (50 samples per treatment, randomly selected from among cones with emerged seedlings or soil only) were harvested 4 d after planting (2 d after most of the plants emerged), and processed as described for the Arlington 1989 experiment.

Arlington 1990

To address the variability across years of the effects on UW85n1 on bacterial communities, on 3 July 1990 we planted an experiment in a different location in the same field as that used in the Arlington 1989 experiment. This planting date is somewhat later than that of 1989 because severe weather led to complete loss of an early June 1990 planting, necessitating a second planting when fields were no longer water-logged. Since the soybean cultivar AP200 was no longer commercially available, seed remaining from the 1989 experiments was used. Seeds were coated with *B. cereus* 2 d prior to the experiment using the same methods as in 1989, but for some unknown reason this resulted in somewhat lower densities of spores (3.3×10^6 spores/seed UW85n1 or 8.8×10^7 spores/seed UW831). Forty positions from which to collect soil cores for root-free soil samples, and 80 each of nontreated seeds, UW85n1-treated seeds, and UW831-treated seeds were included in a completely randomized design. Ten days after planting (2 d after most of the seedlings emerged), 40 seedlings were randomly selected from among those that emerged, and the seedlings and soil samples were processed as described for the Arlington 1989 experiment.

Hancock 1990

On 2 July 1990 we planted an experiment at the Hancock Experiment Station, Washara County, Wisconsin, on a Plainfield loamy sand (sandy, mixed, mesic typic Udipsamment) under center-pivot irrigation (three times per week; 5.32 cm water added in total). This experiment enabled us to compare the effects of UW85n1 and UW831 on rhizosphere communities under growing conditions very different from those at the Arlington Experiment Station, which would likely support different bacterial communities in the soil. Experimental design and procedures were as described for the Arlington 1990 experiment. Seeds were coated at the same time as were seeds for Arlington 1990. Soil samples and seedlings were harvested 8 d after planting (2 d after the majority emerged), and were processed as described for the Arlington 1989 experiment.

Characterization of bacterial isolates

Each bacterial isolate was tested for 50 (Arlington 1989), 49 (Growth chamber 1989), or 43 (1990 experiments) physiological attributes by replicate plating onto appropriate media, and positive or negative responses were recorded (tests are listed in Table 2). Growth on single, simple carbon sources was scored after 1 wk at room temperature (≈22°C) on ammonium basal salts minimal medium amended with 0.2% (mass/volume) of the carbon source (Proom and Knight 1955). Growth was compared to that on medium without an added carbon source (Minimal Medium [MM]–water) and only those colonies exhibiting substantially greater growth on the carbon source were scored as positive. Resistance to antimicrobial compounds (antibiotics

salts, etc.) was determined by scoring growth after 1 wk on 10% TSA amended with the appropriate compound. Anaerobic growth was determined by scoring for growth after 1 wk in an anaerobic jar on 10% TSA amended with 0.75% (mass/volume) glucose (anaerobic fermentation) or 0.1% (mass/volume) KNO_3 (anaerobic respiration). Blood haemolysis was determined by scoring (after 2 d) clearing of medium containing full-strength TSA + 1% (by volume) rabbit red blood cell suspension. The rabbit red blood cell suspension was prepared by centrifuging rabbit blood to pellet the red blood cells, and resuspending the red blood cells in saline to a final volume equal to the original blood volume. Motility was determined by stab-inoculating bacteria into 10% TSA with 0.3% agar, and scoring for diffusion through the agar. Gram determination was by the KOH method (Suslow et al. 1982). Apatite solubilization was determined according to Sperber (1958). Hydrophobicity was determined using the method of Rosenberg et al. (1980); isolates that formed a thick emulsion in the hexadecane layer were scored as hydrophobic. Pectin hydrolysis was assayed according to Andro et al. (1984). The rest of the tests were performed as described in Gerhardt (1981). *B. cereus* UW85n1 and UW831 are Gram-positive and grow anaerobically, show positive responses to BLO, GEL, CAS, LEC, LIP, STA, MOT, ZNC, NEO, AMP, and CVI (all acronyms defined in Table 2), and do not grow on minimal medium.

Statistical analyses

If bacteria isolated from within a sample are statistically independent, the mean square error (MSE) for arcsine square-root transformed proportions should be $\approx 821/n$, where n is the number of isolates per sample (Snedecor and Cochran 1980). The MSE was calculated for each attribute individually for both the Arlington and Growth chamber 1989 experiments, including only those samples with all five isolates. A blocking factor was included for the Arlington data. A 95% confidence interval was calculated for each MSE based on a χ^2 distribution (Snedecor and Cochran 1980), and if $(821/5) = 164.2$ was not included in the interval, the variance for that attribute was considered to be significantly different from the expected variance. Eighty-five percent (85%) of the attributes from Arlington 1989, and 87% of those from the 1989 Growth chamber experiment, had an MS significantly larger than 164.2, indicating a large amount of overdispersion caused by dependence of isolates within samples. Consequently, in subsequent analyses, isolates within samples were considered to be subsamples. Thus the attribute scores were averaged among isolates within each sample, and the sample scores (the proportion of positive responses to a given test) were used in further analyses. Arcsine square-root transformation did not affect the analytical results, so nontransformed proportions were used throughout.

Sample scores for each attribute test were compared individually for each experiment by a univariate F test among habitats (soil, nontreated roots, UW85n1-treated roots, UW831-treated roots), and least significant differences ($P = .05$) were calculated for attributes with significant F statistics ($P \leq .05$) (SAS Institute 1988). A blocking factor was included for the 1989 Arlington data, and was significant ($P \leq .05$) for five tests (GRA, ANF, CTB, LAC, WAT).

Multivariate analysis of variance (MANOVA) of the sample scores was used to determine whether there were significant differences among bacterial communities from the various habitats (SAS Institute 1988). MANOVA was performed separately for each experiment using (1) all habitats in an experiment, (2) nontreated and treated root habitats only, and (3) soil and nontreated root habitats only. Communities were considered significantly different if $P \leq .05$ based on Wilks' lambda F statistic (SAS Institute 1988). A blocking factor was not included in any multivariate analyses presented below; however, there was no change in the statistical significance when a blocking factor was included in the MANOVA for the 1989 Arlington experiment.

When the results of a MANOVA indicated that there were significant differences among communities, canonical (also known as "descriptive") and predictive discriminant analyses (Williams 1983, SAS Institute 1988) were used to explore the differences among communities from the various habitats. The two forms of discriminant analysis provide complementary approaches to evaluating multivariate data (Williams 1983). The coefficients in the discriminant functions are those that facilitate the best separation of samples according to habitat of origin (Williams 1983).

To avoid biased estimates of re-classification rates for the discriminant analyses, data from each experiment were first randomly split into two equal groups called "training" samples and "testing" samples (Press 1972). Training samples were used to define the discriminant model, which can then be applied to the testing samples to explore differences among communities without the bias of applying a model to the same data from which it was derived. Those attributes most useful in discriminating among communities were determined using stepwise discriminant analysis from the STEPDISC procedure on the training samples (SAS Institute 1988). We used a permissive P value of .15 for attributes to enter the function, followed by a more restrictive P value (.10) to remain in the function, which permitted the testing of many combinations of attributes while still requiring a fairly large contribution toward the discrimination for an attribute to remain in the function. The attributes selected with STEPDISC were then used to find both the canonical and predic-

TABLE 1. Populations of heterotrophic bacteria recovered on 10% trypticase soy agar (TSA).

Experiment	Source	log CFU* Mean	log CFU* SD†	n
Arlington 1989	Root-free soil	6.796	0.182	45
	Nontreated root	5.626	0.338	50
	UW85n1-treated root	5.699	0.383	50
Growth chamber 1989	Root-free soil	7.191	0.274	50
	Nontreated root	5.771	0.410	49
	UW85n1-treated root	5.639	0.458	46
Arlington 1990	Root-free soil	7.607	0.422	40
	Nontreated root	6.324	0.529	36
	UW85n1-treated root	6.481	0.387	40
	UW831-treated root	6.328	0.460	40
Hancock 1990	Root-free soil	6.959	0.182	38
	Nontreated root	6.346	0.307	39
	UW85n1-treated root	6.181	0.313	40
	UW831-treated root	6.263	0.390	42

* Log_{10}no. of colony-forming units (CFU) per centimetre of root segment or per cubic centimetre of root-free soil.
† SD = standard deviation.

tive linear discriminant functions, using only the training samples. The functions were then used to calculate discriminant scores for each of the testing samples.

Each testing sample was classified, based on its discriminant scores, into the training group from which it had the smallest squared distance (SAS Institute 1988). The proportions of testing samples test-classified into each habitat (training group) are presented in tabular form. We used a χ^2 test (Snedecor and Cochran 1980) to determine whether the number of samples test-classified into each habitat was significantly different from random.

To assess the stability of the stepwise and predictive discriminant analyses to different random sets of training and testing samples, the entire discriminant analysis procedure was repeated 20 times for each experiment, beginning with the random division of samples into training and testing sets. There was some variability in which attributes were selected using stepwise discriminant analysis, and in the precise classification rates; however, this variability did not affect the overall interpretation of the results, and the first analysis performed for each experiment is presented as representative results.

RESULTS

Emergence of seedlings

Low disease severity in both 1989 and 1990 prevented the assessment of the effectiveness of biological control by UW85n1 or UW831 against pre-emergence damping-off. The Arlington 1989 experiment was part of a larger experiment (Halverson and Handelsman 1991), and no effect on emergence was found. At Arlington in 1990, 83% of nontreated, 87% of UW85n1-treated, and 71% of UW831-treated seedlings emerged. At Hancock in 1990, 79% of nontreated, 84% of UW85n1-treated, and 79% of UW831-treated seedlings emerged. There were no significant differences in emergence among treatments for either 1990 experiment (χ^2 test of independence, $P > .05$). Emergence for the Growth chamber 1989 experiment was not determined.

Bacterial population densities

There were no significant differences in any experiment (Table 1) in the population densities of heterotrophic bacteria between nontreated and UW85n1-treated roots (t test, $P = .05$, 1989 experiments) or among nontreated, UW85n1-treated, and UW831-treated root samples (Fisher's protected least significant difference, $P = .05$, 1990 experiments).

Halverson et al. (*in press*) measured the density of neomycin-resistant populations of *Bacillus cereus*-like bacteria on 10 nontreated and 10 UW85n1-treated root samples from Arlington 1989. The population densities of UW85n1-like bacteria were 2.1 log of the number of colony-forming units (CFU) per centimetre of root for nontreated roots and 4.4 log CFU/cm root for UW85n1-treated roots (detection limit = 2.0 log CFU). Based on the population densities of heterotrophic bacteria for those same samples, we calculated that UW85n1-like bacteria comprised $\approx$0.02% of the total heterotrophic bacteria on nontreated roots and 3.98% on UW85n1-treated roots. However, the proportion of the total population comprised of UW85n1-like bacteria was extremely variable among the UW85n1-treated samples (0.1% to 20.0%), and this proportion may have been overestimated because of the combined use of a selective medium to estimate the populations of UW85n1 and a much less selective medium to estimate the total populations of heterotrophic bacteria. Although numerous *B. cereus*-like bacteria were isolated on 10% TSA (trypticase soy agar) from roots and soil in all four experiments, none of those isolates were resistant to neomycin. Based on our results and those of Halverson et al. (*in press*), it appears that UW85n1,

and probably UW831, were present on treated plants but not common members of the community at the time of sampling, for any of the four experiments.

Physiological attributes of bacterial isolates

The percentage of positive responses to each attribute test for isolates from the various habitats is presented in Table 2 for each experiment. A much higher proportion of attributes differed significantly among the three habitats at Arlington in 1989 (71%) than in 1990 (37%), although 63% of the attributes differed significantly among the three habitats at Hancock 1990. When Arlington 1990 data were analyzed using soil, nontreated, and UW85n1-treated habitats only (those treatments included in Arlington 1989 analysis), there were significant differences among habitats for each of those attributes indicated for all four treatments (Table 2), with the exception of CTB. Additionally, means for attribute tests OXI and TET were significantly different among habitats ($P \leq .05$).

The differences in the physiological attributes of bacteria from each of the communities can best be illustrated by defining three composite attributes, using only those tests that were applied to isolates from all four experiments. The three composite attributes are extracellular activities (8 tests), growth on simple carbon source (14 tests), and resistance to antimicrobial compounds (12 tests). The number of extracellular activities observed, carbon sources utilized, and resistances expressed by each isolate were calculated, and sample means for each of these three composite attributes were analyzed for significant differences across habitats within each experiment (Table 3). For each of the three field experiments there were significantly more carbon sources utilized, more resistances to antimicrobial compounds, and fewer extracellular activities for bacteria from nontreated roots than from root-free soil. In the growth chamber experiment this was true only for extracellular activities, which may indicate that the development of microbial communities in the growth chamber was not representative of the development in field situations. In most cases, nontreated and *Bacillus*-treated root samples were not significantly different for these three composite attributes, and where significant differences were found, the results differed among the experiments.

Multivariate analysis of variance

In each experiment there were significant differences among the soil, nontreated root, and UW85n1-treated root habitats ($P = .0042$ for Arlington 1990, and $P \leq .0001$ in the other three experiments). Significant differences were also found for MANOVAs including only nontreated and UW85n1-treated roots (i.e., root-free soil was excluded) for Arlington 1989 ($P = .0006$) and Growth chamber 1989 ($P \leq .0001$), but not for Arlington 1990 or Hancock 1990 ($P > .10$ for each). For all four experiments there were significant differences between communities in soil and on nontreated roots ($P = .013$ for Arlington 1990, $P \leq .0001$ for other experiments).

For only one attribute (CVI, Hancock 1990) was a significant difference observed between UW85n1-treated and UW831-treated root communities, based on Fisher's protected LSD (Table 2). Multivariate analysis of variance of root samples alone (soil samples excluded) showed no significant difference among samples from nontreated, UW85n1-treated, and UW831-treated roots ($P = .763$ for Arlington 1990, and $P = .182$ for Hancock 1990).

Collectively the results of the MANOVAs suggest that in every experiment there were significant differences between the communities of bacteria in root-free soil and on plant roots, but significant effects of coating seeds with UW85n1 occurred only in the Arlington 1989 and Growth chamber 1989 experiments.

Discriminant analyses

Soil vs. nontreated roots.—Averaged across 20 repetitions of predictive discriminant analysis, soil- and nontreated-root testing samples were correctly classified 78.7 ± 4.9% (mean ± SD) for Arlington 1989, 88.1 ± 5.9% for Growth chamber 1989, 65.5 ± 5.3% for Arlington 1990, and 74.5 ± 5.2% for Hancock 1990. Based on a χ^2 test for equal proportions, the test classifications were always non-random for Arlington 1989 and Growth chamber 1989 ($P \leq .01$; 1 of 20 from Arlington at $.01 < P \leq .05$), 19 of 20 repetitions were non-random for Hancock 1990 (16 of 20 at $P \leq .01$; 3 of 20 at $.01 < P \leq .05$), and 12 of 20 were significantly non-random ($.01 < P \leq .05$) for Arlington 1990. The inconsistent discrimination for Arlington 1990 reflects the less significant differences by MANOVA for that experiment, while the consistent, significant test classifications for the other three experiments indicate large differences between the communities that can be described by discriminant functions.

The linear discriminant functions for the soil/nontreated root analyses contained 3 to 16 attributes, selected with stepwise discriminant analysis. The attributes selected were quite variable across repetitions of the analysis; in each experiment, at least 88% of the attributes appeared in at least one repetition. For each experiment, no more than five attributes were selected in more than 50% of the repetitions.

Soil, nontreated roots, and UW85n1-treated roots.—Averaged over the 20 repetitions (1989 experiments only), testing samples were correctly classified 67.1 ± 3.3% (mean ± SD) for Arlington 1989, and 75.4 ± 5.1% of the time for Growth chamber 1989. Test classifications were significantly different from random ($P \leq .01$) for all repetitions in both experiments. As found for the soil vs. nontreated root analyses, the attributes chosen with the stepwise procedure were variable across repetitions within each experiment. For Arlington 1989 there was an average of 10.9 ± 1.9 attributes included

TABLE 2. Percentage of bacterial isolates showing positive responses to tests for physiological attributes in each of the habitats for four different experiments.

		Percentage of positive responses to attribute tests							
		Arlington 1989				Growth chamber 1989			
Attribute test	Code	Root-free soil	Non-treated roots	UW85n1-treated roots		Root-free soil	Non-treated roots	UW85n1-treated roots	
Gram positive	GRA	83.4[a]	62.1[b]	56.4[b]	*	68.0[b]	83.3[a]	70.1[b]	*
Anaerobic respiration	ANR	28.6[a]	15.1[b]	23.4[ab]	*	49.8[a]	38.4[a]	12.2[b]	*
Anaerobic fermentation	ANF	5.3	1.6	7.1		9.7	5.4	2.5	
Blood haemolysis	BLO	38.9[a]	31.5[ab]	20.8[b]	*	51.4[a]	42.9[a]	29.1[b]	*
Gelatin hydrolysis	GEL	76.1[a]	40.9[c]	54.4[b]	*	79.0	74.1	74.2	
Casein hydrolysis	CAS	67.8[a]	31.8[c]	45.4[b]	*	69.6[a]	39.4[b]	35.3[b]	*
Lecithinase activity	LEC	38.3[a]	18.9[b]	11.2[b]	*	49.1[a]	37.7[a]	16.5[b]	*
Lipase activity	LIP	16.1	10.5	10.6		45.8[a]	25.9[b]	15.1[b]	*
Starch hydrolysis	STA	74.8[a]	35.0[c]	51.5[b]	*	43.0[b]	58.9[a]	37.4[b]	*
Apatite solubilization	APA	18.7	13.5	11.6		30.7[a]	4.6[b]	20.5[a]	*
Motility	MOT	35.6	33.1	30.1		60.3	49.6	56.9	
Oxidase positive	OXI	48.1[a]	28.9[b]	42.2[a]	*	32.6	42.0	28.1	
Hydrophobic	HYD	72.8	67.7	76.4		ND	ND	ND	
Pectin hydrolysis, pH7.8	PGA	7.9[b]	6.4[b]	32.0[a]	*	23.5	15.9	26.2	
Pectin hydrolysis, pH5.5	PLY	14.3[b]	8.6[b]	40.6[a]	*	6.5[b]	9.0[ab]	18.0[a]	*
Growth at 12°C	CLO	45.2[b]	92.1[a]	85.1[a]	*	98.6[ab]	99.6[a]	95.8[b]	*
Growth at 37°C	CHI	88.2[a]	80.6[ab]	73.4[b]	*	81.1[b]	95.6[a]	95.1[a]	*
Growth at pH4	PHL	1.0	2.6	2.8		0.0	0.8	0.0	
Growth at pH10	PHH	99.6	99.6	99.6		99.6	100.0	98.8	
Pigmentation on 10% TSA	PIG	5.8[c]	25.0[b]	42.8[a]	*	3.8[b]	7.7[b]	15.8[a]	*
Fluorescence on King's B	FLU	6.0	7.7	2.8		0.4	0.0	0.0	
NaCl (2%, mass/volume)	NAL	99.6	98.5	99.5		99.1[a]	99.5[a]	95.3[b]	*
NaCl (5%, mass/volume)	NAH	52.4[b]	76.0[a]	57.4[b]	*	60.4	69.3	62.6	
$CaCl_2$ (5 mmol/L)	CAL	30.8	39.4	33.1		40.2	45.1	42.6	
$ZnSO_4$ (1 mmol/L)	ZNC	32.5	45.3	38.3		45.5[a]	36.4[a]	16.2[b]	*
$CuSO_4$ (1 mmol/L)	COP	3.2	8.8	13.2		0.0	0.8	2.2	
Neomycin (10 μg/mL)	NEO	26.8[b]	84.5[a]	86.9[a]	*	43.2[b]	67.0[a]	69.9[a]	*
Ampicillin (25 μg/mL)	AMP	50.9	58.6	53.6		69.3[a]	51.9[b]	37.0[c]	*
Tetracycline (10 μg/mL)	TET	6.2[b]	14.4[b]	25.6[a]	*	12.4[b]	27.9[a]	12.6[b]	*
Crystal violet (0.125 μg/mL)	CVI	28.9[b]	76.9[a]	87.1[a]	*	86.3[b]	96.1[a]	90.7[ab]	*
Rifampicin (10 μg/mL)	RIF	14.9[b]	35.0[a]	42.9[a]	*	6.9[b]	15.6[b]	29.7[a]	*
Vancomycin (20 μg/mL)	VAN	23.5[b]	52.8[a]	57.1[a]	*	24.2	13.7	22.6	
Streptomycin (20 μg/mL)	STR	19.7[b]	52.1[a]	48.9[a]	*	13.7	12.2	22.4	
CTAB† (26 μg/mL)	CTB	23.7[c]	45.5[b]	62.4[a]	*	26.9[a]	4.4[b]	21.6[a]	*
Spectinomycin (25 μg/mL)	SPC	44.0[b]	83.6[a]	89.4[a]	*	43.0[b]	41.2[b]	59.6[a]	*
MM–glucose‡	GLU	69.4[b]	87.4[a]	72.0[b]	*	64.5[a]	49.6[b]	77.9[a]	*
MM–mannose	MAN	34.7[b]	84.0[a]	73.1[a]	*	39.7[b]	40.8[b]	67.7[a]	*
MM–lactose	LAC	16.8[b]	42.7[a]	39.3[a]	*	5.8[b]	2.0[b]	24.3[a]	*
MM–xylose	XYL	60.5[b]	85.1[a]	60.9[b]	*	17.7[b]	15.6[b]	50.2[a]	*
MM–mannitol	MNL	68.1	79.2	68.9		59.9[ab]	47.7[b]	69.3[a]	*
MM–myo-inositol	INO	47.3[b]	66.9[a]	41.4[b]	*	40.5	45.7	43.1	
MM–rhamnose	RHA	23.3[b]	41.0[a]	13.8[b]	*	6.9[b]	2.9[b]	15.3[a]	*
MM–sucrose	SUC	78.9[b]	94.8[a]	84.4[b]	*	69.5[a]	55.9[b]	78.6[a]	*
MM–galactose	GAL	58.7[b]	89.4[a]	84.6[a]	*	43.5[ab]	37.8[b]	55.8[a]	*
MM–azelaic acid	AZE	10.6	23.2	16.4		12.5[a]	4.2[b]	0.0[b]	*
MM–succinic acid	SCN	56.4[b]	84.2[a]	75.1[a]	*	49.4[b]	61.8[ab]	69.9[a]	*
MM–malonic acid	MAL	32.6[b]	73.1[a]	62.7[a]	*	31.1[c]	46.6[b]	60.3[a]	*
MM–sodium tartrate	TAR	9.2[c]	31.5[a]	21.4[b]	*	27.0[ab]	17.1[b]	36.2[a]	*
MM–citric acid	CIT	58.0[b]	78.5[a]	68.7[ab]	*	65.8	75.7	79.2	
MM–water	WAT	1.0	0.0	0.0		12.5[a]	0.0[b]	0.0[b]	*
MM–casamino acids	CAA	ND§	ND	ND		ND	ND	ND	
Number of isolates‖		173	189	182		232	217	184	
Number of samples‖		45	50	50		50	49	46	

* Indicates that sample means for that attribute test are significantly different among habitats ($P \leq .05$). Means followed by the same superscript letter within an experiment and within an attribute are not significantly different by Fisher's protected least significant difference ($P = .05$).

† CTAB is hexadecyltrimethylammonium bromide (Sigma Chemical Company, Saint Louis, Missouri, USA).

‡ MM–carbon source denotes 0.2% (mass/volume) carbon source in minimal medium.

§ ND = not determined.

‖ Up to 6 bacterial isolates from each root sample (1 cm) or soil sample (1 cm^3).

TABLE 2. Continued.

Percentage of positive responses to attribute tests									
Arlington 1990					Hancock 1990				
Root-free soil	Nontreated roots	UW85n1-treated roots	UW831-treated roots		Root-free soil	Nontreated roots	UW85n1-treated roots	UW831-treated roots	
90.8[a]	78.8[b]	77.0[b]	70.7[b]	*	86.8[a]	54.0[b]	32.9[c]	41.3[c]	*
ND	ND	ND	ND		ND	ND	ND	ND	
ND	ND	ND	ND		ND	ND	ND	ND	
22.2	13.4	17.8	17.3		36.3[a]	21.8[b]	12.5[b]	15.1[b]	*
68.9[a]	42.1[b]	50.1[b]	47.2[b]	*	69.3[a]	32.7[b]	21.1[c]	18.3[c]	*
47.2[a]	15.0[c]	25.2[bc]	32.2[b]	*	54.1[a]	26.3[b]	15.1[c]	14.4[c]	*
3.8	2.5	2.9	1.3		6.8	7.8	1.7	3.6	
15.8	13.3	9.1	9.4		22.9[a]	19.6[ab]	12.0[bc]	8.6[c]	*
61.2	60.3	55.5	54.8		71.6[a]	36.9[b]	24.6[c]	22.9[c]	*
ND	ND	ND	ND		ND	ND	ND	ND	
42.3	30.3	41.2	38.8		52.3	50.0	43.6	60.3	
33.2	20.6	18.9	31.3		17.2[b]	35.6[a]	34.5[a]	29.6[a]	*
ND	ND	ND	ND		ND	ND	ND	ND	
10.0	9.6	9.2	9.7		21.8[a]	8.5[b]	7.6[b]	7.5[b]	*
0.0	0.0	1.3	0.0		1.4	1.3	3.1	3.2	
47.8[c]	81.6[a]	67.7[b]	72.2[ab]	*	56.8[ab]	58.0[a]	43.7[c]	44.6[bc]	*
84.7	82.4	85.6	75.4		77.1	80.2	69.3	73.7	
ND	ND	ND	ND		ND	ND	ND	ND	
ND	ND	ND	ND		ND	ND	ND	ND	
5.2[b]	19.0[a]	19.0[a]	11.0[ab]	*	7.6	9.6	9.4	6.0	
2.9	2.4	2.8	3.2		1.0	5.3	7.2	3.7	
ND	ND	ND	ND		ND	ND	ND	ND	
72.1[a]	53.0[b]	47.5[b]	57.5[b]	*	73.5[a]	40.9[b]	24.3[c]	28.7[c]	*
ND	ND	ND	ND		ND	ND	ND	ND	
28.8	32.4	38.4	32.2		40.0[b]	61.2[a]	66.1[a]	61.1[a]	*
3.3	10.8	8.5	7.2		5.2	5.6	5.6	3.3	
51.2[b]	75.3[a]	68.3[a]	71.7[a]	*	47.1[b]	67.4[a]	75.3[a]	73.1[a]	*
73.7	74.3	76.9	72.0		62.4[b]	80.2[a]	82.7[a]	82.1[a]	*
9.8	18.9	23.5	17.6		19.4	29.7	22.3	22.9	
90.5	92.7	90.8	93.9		83.0[a]	78.6[ab]	68.5[b]	79.2[a]	*
10.9[b]	34.7[a]	32.3[a]	28.0[a]	*	21.4[b]	47.9[a]	57.1[a]	55.7[a]	*
13.1[b]	28.1[a]	36.8[a]	39.5[a]	*	15.7[b]	52.2[a]	53.6[a]	49.5[a]	*
15.4	25.2	18.3	22.6		23.6[b]	44.5[a]	53.0[a]	52.7[a]	*
13.9[b]	21.3[ab]	27.3[a]	32.8[a]	*	17.7[c]	42.8[b]	49.9[ab]	55.1[a]	*
34.0[c]	59.1[a]	44.0[bc]	50.9[ab]	*	33.7	37.0	33.3	29.2	
85.9	86.7	85.7	82.1		77.4[b]	88.7[a]	85.1[ab]	88.4[a]	*
41.7[c]	73.8[a]	58.9[b]	62.0[ab]	*	52.6	50.0	39.6	42.9	
23.5	37.7	32.1	35.8		35.3[a]	26.5[ab]	19.3[b]	27.6[ab]	*
62.6	67.0	62.4	66.3		56.6	61.0	53.4	57.1	
92.0	88.0	84.2	80.7		86.6[a]	73.6[b]	70.7[bc]	60.4[c]	*
68.6	62.2	52.8	58.4		47.1[a]	34.4[b]	32.7[b]	30.6[b]	*
26.9[b]	44.3[a]	39.0[ab]	47.6[b]	*	22.1	24.3	20.2	27.4	
84.4	85.8	82.4	82.6		70.6	71.5	67.3	71.0	
52.3	66.2	57.3	62.8		45.7[b]	64.7[a]	61.2[a]	57.3[ab]	*
5.5	5.4	13.6	8.9		5.7[b]	31.0[a]	39.6[a]	42.9[a]	*
24.6[b]	48.3[a]	44.8[a]	49.4[a]	*	21.7[b]	52.4[a]	40.8[a]	44.9[a]	*
30.0[b]	56.7[a]	46.8[a]	45.7[a]	*	24.5[b]	52.3[a]	50.3[a]	50.8[a]	*
28.1[b]	52.5[a]	34.0[b]	35.0[b]	*	22.6	37.6	34.0	32.0	
48.3	62.1	58.6	59.7		39.9[b]	57.6[a]	39.5[b]	46.7[ab]	*
2.1	1.7	2.9	0.8		1.7	2.1	0.5	2.1	
92.5	87.2	84.0	83.3		88.7	90.5	92.8	90.7	
232	134	158	161		210	181	186	212	
40	36	40	40		38	40	40	42	

in the discriminant function (NEO, PIG, SPC, and XYL were included in >50% of the repetitions). For Growth chamber 1989, there was an average of 16.5 ± 2.5 attributes per function (ANR, APA, OXI, NAL, PIG, NEO, AMP, TET, CTB, XYL, INO, TAR, and WAT were selected in 50% or more of the repetitions). At least 90% of the attributes were included in at least one function for each experiment.

The linear discriminant function used in predictive discriminant analysis of Arlington 1989 is shown in Table 4a. Test classification of the testing samples using this linear discriminant function shows that root sam-

TABLE 3. Physiological attributes of bacteria from different soil habitats. Acronyms of the physiological tests listed in the footnotes are explained in Table 2.

Experiment	Habitat	Attribute group*		
		Carbon sources†	Extracellular-activities‡	Resistances§
Arlington 1989	Root-free soil	6.25[a]	3.34[c]	3.27[a]
	Nontreated root	9.61[c]	1.84[a]	6.34[b]
	UW85n1-treated root	7.82[b]	2.67[b]	6.63[b]
Growth chamber 1989	Root-free soil	5.34[a]	3.68[b]	4.32[a]
	Nontreated root	5.03[a]	3.04[a]	4.36[a]
	UW85n1-treated root	7.28[b]	2.52[a]	4.47[a]
Arlington 1990	Root-free soil	6.74[a]	2.29[b]	4.17[a]
	Nontreated root	8.37[b]	1.56[a]	5.26[b]
	UW85n1-treated root	7.56[ab]	1.71[a]	5.13[b]
	UW831-treated root	7.77[b]	1.72[a]	5.26[b]
Hancock 1990	Root-free soil	6.08[a]	2.84[c]	4.43[a]
	Nontreated root	7.26[b]	1.55[b]	5.88[b]
	UW85n1-treated root	6.54[ab]	0.98[a]	5.92[b]
	UW831-treated root	6.80[ab]	0.94[a]	5.93[b]

* Means for an attribute group followed by the same superscript letter within an experiment are not significantly different (MANOVAs followed by Fisher's protected least significant differences, $P = .05$).

† Mean number of simple carbon sources utilized, from tests GLU, MAN, LAC, XYL, MNL, INO, RHA, SUC, GAL, AZE, SCN, MAL, TAR, and CIT (maximum no. = 14).

‡ Mean number of extracellular activities, from tests BLO, GEL, CAS, LEC, LIP, STA, PGA, and PLY (maximum no. = 8).

§ Mean number of resistances to antimicrobial compounds, from tests NAH, ZNC, COP, NEO, AMP, TET, CVI, RIF, VAN, STR, CTB, and SPC (maximum no. = 12).

TABLE 4. Predictive discriminant analysis for Arlington 1989 experiment.

a) Linear discriminant function.*

Attribute test‡	Coefficient vectors†		
	Root-free soil	Nontreated root	UW85n1-treated root
Neomycin (10 μg/mL)	−79.40	−60.86	−64.06
Pectin hydrolysis, pH7.8	29.85	19.12	27.37
MM–rhamnose	39.85	42.56	33.11
Crystal violet (0.125 μg/mL)	−2.78	−7.82	2.12
MM–xylose	−1.17	4.12	−2.39
Fluorescence on King's B	225.99	202.29	185.74
CTAB§ (26 μg/mL)	−73.24	−61.41	−54.12
$ZnSO_4$ (1 mmol/L)	85.71	75.75	70.50
Growth at pH10	2419.00	2355.00	2298.00
Anaerobic respiration	−56.62	−51.05	−48.27
MM–mannitol	−40.53	−42.22	−33.13
Gelatin hydrolysis	38.72	28.10	31.48
MM–citric acid	68.39	57.63	56.98
MM–malonic acid	−46.33	−37.40	−41.31
Constant	−1225.00	−1162.00	−1113.00

b) Test-classification of samples.‖

Habitat of origin	% of samples classified into habitat			% error¶	Number of samples
	Root-free soil	Nontreated root	UW85n1-treated root		
Root-free soil	77.3	13.6	9.1	22.7	22
Nontreated root	24.0	52.0	24.0	48.0	25
UW85n1-treated root	4.0	20.0	76.0	24.0	25

* The linear discriminant function was trained on half of the samples from each habitat.

† Coefficient vector = $\mathrm{COV}^{-1}\,\bar{x}_j$ and constant = $-0.5\,\bar{x}_j'\mathrm{COV}^{-1}\bar{x}_j$, where COV = pooled covariance matrix and $\bar{x}_j$ = mean vector of sample scores for habitat j.

‡ MM–carbon source denotes 0.2% (mass/volume) carbon source in minimal medium.

§ CTAB is hexadecyltrimethylammonium bromide (Sigma Chemical Company, Saint Louis, Missouri, USA).

‖ Test-classified samples were those not used in developing the linear discriminant function.

¶ Total error = 31.6%.

TABLE 5. Predictive discriminant analysis for growth chamber 1989 experiment.

a) Linear discriminant function.*

Attribute test‡	Coefficient vectors†		
	Root-free soil	Nontreated root	UW85n1-treated root
MM–xylose	−25.42	−36.87	−19.98
Neomycin (10 μg/mL)	0.95	16.92	11.44
Growth at 37°C	51.37	62.82	73.51
NaCl (2%, mass/volume)	155.08	175.62	121.00
MM–water	−26.32	−53.30	−53.87
Oxidase positive	18.93	42.35	24.36
CTAB§ (26 μg/mL)	−11.08	−36.67	−11.02
MM–myo-inositol	13.68	17.17	−7.69
MM–sodium tartrate	−23.81	−35.37	−9.29
Anaerobic respiration	46.12	46.41	18.95
Lecithinase activity	−31.87	−42.62	−15.28
MM–glucose	−17.77	−30.70	−19.75
MM–succinic acid	39.51	63.79	51.16
Starch hydrolysis	5.96	13.21	14.78
MM–rhamnose	0.79	17.82	18.65
MM–citric acid	−8.34	−14.74	−2.68
Constant	−101.16	−142.20	−104.81

b) Test-classification of samples.

Habitat of origin‖	% of samples classified into habitat			% error¶	Number of samples
	Root-free soil	Nontreated root	UW85n1-treated root		
Root-free soil	80.0	8.0	12.0	20.0	25
Nontreated root	16.7	70.8	12.5	29.2	24
UW85n1-treated root	26.1	17.4	56.5	43.5	23

* The linear discriminant function was trained on half of the samples from each habitat.
† Coefficient vector = $COV^{-1}\,\bar{x}_j$ and constant = $-0.5\,\bar{x}_j' COV^{-1}\bar{x}_j$, where COV = pooled covariance matrix and $\bar{x}_j$ = mean vector of sample scores for habitat j.
‡ MM–carbon source denotes 0.2% (mass/volume) carbon source in minimal medium.
§ CTAB is hexadecyltrimethylammonium bromide (Sigma Chemical Company, Saint Louis, Missouri, USA).
‖ Test-classified samples were those not used in developing the linear discriminant function.
¶ Total error = 30.9%.

ples are easily discriminated from soil samples, and that coating soybean seeds with UW85n1 dramatically affected the communities of bacteria that developed on the roots (Table 4b). The linear discriminant function and test classification of testing samples for the Growth chamber 1989 experiment (Table 5) indicates that this is true for the growth chamber experiment as well.

The relationships among samples from the three habitats are more easily assessed visually, using canonical discriminant analysis. In both 1989 experiments (Fig. 1) the samples from a given habitat cluster together, and each habitat is somewhat distinct from the others. Training samples are not shown, but the placement of training samples in Figs. 1A and B is nearly identical to that of testing samples. These graphs show again that the bacterial communities in the three habitats are quite different from one another, and that samples from UW85n1-treated roots are nearly as easily distinguished from nontreated root samples as are root samples from root-free soil samples.

To test whether the results from the growth chamber experiment provide a good model for behavior in the field, the linear discriminant function for training samples from Growth chamber 1989 (Table 5a) was used to test-classify the testing samples from the Arlington 1989 experiment. The misclassification rate (69.0% error) was actually slightly higher than would be expected from test-classifying random samples, indicating that the development of bacterial communities in the growth chamber experiment was dramatically different from that in the field.

It is also useful to assess how well results from a field experiment in one growing season can be applied to results in another season. From the individual MANOVAs, we know that coating seeds with UW85n1 did not measurably affect the rhizosphere bacterial communities at Arlington in 1990, although it did in 1989. We created a linear discriminant function using the training samples from Arlington 1989, and then test-classified testing samples from Arlington 1990. Some attribute tests were performed in 1989 but not in 1990, so stepwise discriminant analysis was performed using only those attribute tests applied to both experiments. The attributes selected were those presented in Table 4a, excluding PHH and ANR. The linear discriminant function constructed using these 12 attributes was then

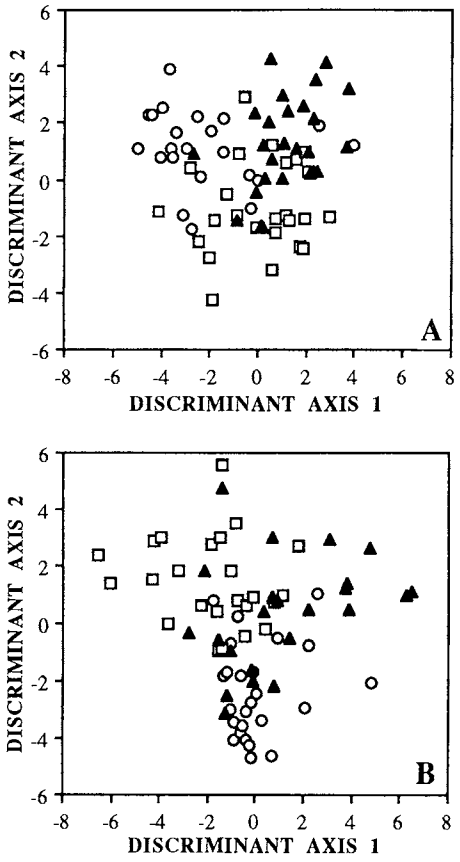

FIG. 1. Plots of canonical discriminant scores for testing samples from Arlington 1989 (A) and Growth chamber 1989 (B). ○ = root-free soil samples, □ = nontreated root samples, and ▲ = UW85n1-treated root samples.

used to test-classify both the Arlington 1989 testing samples and the Arlington 1990 testing samples. For the Arlington 1989 testing samples there was an overall misclassification of 27.6% of the samples (22.7% root-free soil, 36.0% nontreated root, and 24.0% UW85n1-treated root), which is actually slightly better than the classification rate shown in Table 4b. When the Arlington 1990 samples were test-classified, the overall misclassification rate was considerably higher, at 51.7%. However, 70% of the root-free soil samples were correctly classified, and only 22.2% of the nontreated root samples and 30.0% of the UW85n1-treated root samples were misclassified as soil samples. Root samples from 1990 were most likely to be classified as most similar to the nontreated root samples from 1989 (50.0% of nontreated and 45% of UW85n1-treated root samples). These results are expected if the composition of the soil and nontreated root communities are fairly consistent across the two years, and coating seeds with UW85n1 did not affect the development of the bacterial community in the rhizosphere in 1990.

DISCUSSION

Our results illustrate the potential impact of introducing large populations of a single strain of bacteria on the indigenous community of bacteria on roots. This highlights the need for research on bacterial communities for the successful implementation of biological control and for the development of policies concerning the release of large populations of wild-type or genetically modified microorganisms into the environment. In two of the experiments, coating seeds with a large population of *Bacillus cereus* UW85n1, a common method of introducing biocontrol agents, resulted in significantly different bacterial communities in the rhizosphere. In the two experiments in which such changes in the bacterial community were detected, the changes occurred even when UW85n1 itself was not a common member of the rhizosphere community. In the other two experiments no differences could be detected between communities on roots of treated and nontreated plants, despite inoculation with a large population of UW85n1 or UW831 on the seeds at the time of planting. In each of four experiments the bacterial communities that developed in the soybean rhizosphere were physiologically different from those in root-free soil.

Various physiological differences between bacteria cultured from the rhizosphere and those from nonrhizosphere soil have been observed for decades (for pioneering examples see Lochhead and Taylor 1938, Lochhead and Rouatt 1955, Rovira 1956). We found that bacteria isolated from roots were able to utilize a wider range of simple carbon sources, but they degraded fewer complex carbon sources than did bacteria isolated from soil (Tables 2 and 3), which is consistent with the findings of others (Sundin et al. 1990). The rhizosphere may enrich for bacteria that are best suited for rapidly utilizing simple carbon sources or it may select against organisms that produce extracellular enzymes.

At the very high density of bacteria in the rhizosphere compared with root-free soil, production of antimicrobial compounds by even a small proportion of the bacterial community in the rhizosphere, or by the plant itself, could create a strong selection for resistance to antimicrobial substances. This hypothesis is supported by our results that bacteria isolated from roots are more often resistant to a wide range of antimicrobial compounds than are bacteria from the root-free soil (Tables 2 and 3). We do not know whether it is the breadth of types of resistance or resistance to specific antimicrobial compounds that is important. However, if resistance to particular antimicrobial compounds provides an advantage in root colonization, caution is necessary in the use of antibiotic-resistance markers for studying the population dynamics of bacteria introduced into the rhizosphere. Moreover, understanding more about physiological attributes characteristic of rhizosphere bacteria may be useful in selecting or designing bacteria for successful rhizosphere colonization.

Perhaps the most significant result from this study

is the observation that coating a single bacterial strain (UW85n1) on soybean seeds had a dramatic impact on the bacterial community of the rhizosphere in the 1989 experiments, even when the introduced strain itself was not a common member of the community. That the communities on UW85n1-treated roots in the 1989 experiments were as easily distinguished from the communities on nontreated roots as from communities in root-free soil is particularly striking (Tables 4 and 5), and highlights the potential importance of microbes early in plant growth in shaping the selective pressures in the rhizosphere. The changes in the rhizosphere communities associated with coating seeds with UW85n1 were qualitative shifts in the microbial community, since there were no significant differences in the heterotrophic bacterial population densities between nontreated and UW85n1-treated roots at the time of sampling (Table 1). Similar results from a concurrent study of fluorescent pseudomonads isolated in the Arlington 1989 experiment (Gilbert 1991) indicate that such shifts in the physiological attributes can occur both at the community level and within a more narrowly defined taxonomic level.

Nevertheless, in the 1990 experiments there were no discernible differences between the communities of bacteria on roots of treated and nontreated plants. This result is also striking, because the introduction of large quantities of UW85n1 or UW831 on the seeds with no detectable effect on rhizosphere communities suggests that the rhizosphere bacterial communities may be resilient to the introduction of large populations of bacteria. This may be through a selection pressure for particular types of bacteria from the soil strong enough to overcome the effects of the introduced organism on community development. It is also possible that the somewhat smaller inoculum in 1990 ($\approx 10^7$ spores per seed) compared with 1989 (10^8 to 10^9 spores per seed), may have been insufficient to induce the effects observed in 1989. Also, the 1990 planting date was more than one month later in the growing season than in 1989; differences in temperature, moisture, etc., may have had a significant influence on community development. Additionally, the same seed lot was used in both years, and the extra year of aging of the soybean seed for the 1990 experiment may have led to differences in seed exudation or other aspects of seed physiology that could have affected the colonization process. However, the proportion of seedlings that emerged and the rate of emergence were not lower in 1990 than in 1989.

The variable impact of UW85n1 on the development of the rhizosphere bacterial community suggests that further studies are required to determine whether the dramatic effect of UW85n1 in 1989 was a spurious result or whether it occurs frequently. More broadly, these results suggest the need for studying the impact of additions of large populations of microorganisms on microbial communities, particularly over longer time periods than in our study, for the development of a true "risk-based" policy for the release of microbes into the environment (Miller et al. 1990).

Results of experiments under controlled conditions (such as a growth chamber) are often used to predict ecological behavior in the field. In many cases, soil is collected from the field, sieved, and stored prior to use. Although communities from root-free soil, nontreated roots, and UW85n1-treated roots were as easily distinguished within the Growth chamber 1989 experiment as they were within the Arlington 1989 experiment, the communities across the two experiments were quite different. Indeed, the linear discriminant function from the growth chamber experiment did not classify samples better than randomly when applied to samples from the field experiment. This indicates that the growth chamber experiment was not a good model for biological effects in the field.

UW85n1 may have both direct and indirect effects on rhizosphere bacteria. UW85n1 could produce metabolites that are toxic to particular types of bacteria or other microbes but not to others. UW85 produces a low molecular weight, amino-containing, polyol antibiotic that inhibits the growth of many Gram-negative bacteria, as well as of germinating spores of the oomycetes *Phytophthora megasperma* and *Pythium ultimum* on solid media (L. Silo, B. Lethbridge, S. Raffel, G. Gilbert, and J. Handelsman, *unpublished manuscript*), but we do not yet know if this activity occurs in the rhizosphere.

Different bacterial communities early in the colonization of the root surface could significantly alter the selective pressures of the rhizosphere, both by changing patterns of nutrient utilization and nutrient production and through production of antimicrobial compounds. Describing changes in the rhizosphere community through several points in time would be an important next step in understanding the mechanistic basis for the changes caused by, or robustness to, UW85n1 in the bacterial community.

Low disease severity prevents us from determining whether there was a correlation between alterations in the rhizosphere bacterial community by UW85 and biocontrol activity. It is possible, although not investigated, that treatment with UW85n1 reduced the populations of pathogens that were present in the rhizosphere at subclinical levels. Further study on the effects of UW85 on particular members of the rhizosphere community, and their possible role in biocontrol of damping-off diseases is in progress.

In addition to the active mechanisms just described, the spore preparation coated on the seeds could be passively responsible for the observed changes in the rhizosphere community. These preparations contained large numbers of spores rich in calcium, DNA, and dipicolinic acid, cellular debris, and possibly nutrients or antibiotics leached from the growth medium, since the spores were not washed. The chemical composition

of the bacterial preparation itself could have enriched for or selected against particular types of bacteria, altering the rhizosphere bacterial communities. Further studies are needed to differentiate between the possible role of passive nutrient enrichment and that of actively growing cells of *B. cereus* UW85n1 in altering the microbial communities of roots. Other indirect mechanisms by which UW85n1 could alter the rhizosphere communities include alteration of the host physiology (Selvadurai et al. 1991, Wei et al. 1991) and selection for or against particular bacterivores such as protozoans or nematodes that would selectively graze on particular components of the rhizosphere community. These possibilities also deserve further consideration.

Our approach to the study of bacterial communities has both strengths and limitations. We described organisms based on their physiological attributes, which avoids the problem of classifying isolates into ecologically meaningful taxonomic groups. However, our approach also has several drawbacks. First, our technique relies on culturing bacteria on growth media under aerobic conditions, and it is likely that only a small portion of bacteria that inhabit the soil or the rhizosphere are represented in our collections (Lambert et al. 1987, Giovannoni et al. 1990, Ward et al. 1990). However, this technique likely selects for a consistent physiological subset of the total bacterial community and this subset is most likely to be of some ecological importance, if only as an indicator for factors that disturb the total community. In addition, because our analysis is based on the collective physiological attributes of groups of bacteria, it is possible that there were changes in the types of bacteria present that we could not detect, because there was sufficient redundancy of function within the bacterial communities. Lastly, but most importantly, the physiological attributes included here are simply markers for distinguishing among different groups of bacteria, and may or may not be causal to the appearance or absence of a bacterium in a habitat. In spite of these limitations, our results provide a basis for constructing testable hypotheses on the importance of particular physiological attributes for colonization of nontreated soybean roots or roots treated with *B. cereus* UW85n1.

Our study highlights the usefulness of multivariate statistical techniques in soil microbial ecology. Such techniques have a long history of use in macroecology, but have only rarely been used to study the ecology of soil-borne microorganisms (Feest and Campbell 1986, Pfender and Wootke 1988, Lambert et al. 1990). Based on the 20 repetitions of the analysis, many combinations of attributes can be used successfully to discriminate between root and soil communities. The attributes selected in each stepwise repetition are those that maximize the discrimination for that particular set of training samples. Where the differences between communities are large, the discriminant procedure shows relatively little variability in this regard; where the differences are smaller, as for Arlington 1990, the discriminant procedure is less reliable. The main advantage of multivariate analyses over univariate analysis of each attribute individually, is that the discriminant functions are optimized combinations of attributes, and may discern important interactions among attributes that are otherwise cryptic. Potential interactions among attributes, as well as the possibility that these attributes may be only biological markers for more ecologically important attributes, should be considered when designing experiments in rhizosphere ecology.

The results of our work show the importance of studies of soil microorganisms at the community level, in addition to research on population dynamics and metabolic activity of individual strains. By studying a cross section of the bacterial community, effects of the introduced bacteria and interactions among community members can be discerned that would have been overlooked by autecological methods.

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