Distribution and Biocontrol Potential of *phlD*$^+$ Pseudomonads in Corn and Soybean Fields

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ABSTRACT

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The abundance and diversity of *phlD*$^+$ *Pseudomonas* spp. colonizing the rhizospheres of young, field-grown corn and soybean plants were assayed over a 3-year period. Populations of these bacteria were detected on the large majority of plants sampled in the state of Ohio, but colonization was greater on corn. Although significant variation in the incidence of rhizosphere colonization was observed from site to site and year to year on both crops, the magnitude of the variation was greatest for soybean. The D genotype was detected on plants collected from all 15 counties examined, and it represented the most abundant subpopulation on both crops. Additionally, six other genotypes (A, C, F, I, R, and S) were found to predominate in the rhizosphere of some plants. The most frequently observed of these were the A genotype and a newly discovered S genotype, both of which were found on corn and soybean roots obtained from multiple locations. Multiple isolates of the most abundant genotypes were recovered and characterized. The S genotype was found to be phylogenetically and phenotypically similar to the D genotype. In addition, the novel R genotype was found to be most similar to the A genotype. All of the isolates displayed significant capacities to inhibit the growth of an oomycete pathogen in vitro, but such phenotypes were highly dependent on media used. When tested against multiple oomycete pathogens isolated from soybean, the A genotype was significantly more inhibitory than the D genotype when incubated on 1/10× tryptic soy agar and 1/5× corn meal agar. Seed inoculation with different isolates of the A, D, and S genotypes indicated that significant root colonization, generally in excess of log 5 cells per gram of root, could be attained on both crops. Field trials of the A genotype isolate Wayne1R indicated the capacity of inoculant populations to supplement the activities of native populations so as to increase soybean stands and yields. The relevance of these findings to natural and augmentative biocontrol of root pathogens by these bacteria is discussed.

Additional keywords: biogeography, DAPG.

Antibiotic-producing pseudomonads can colonize plant roots and promote plant health by suppressing the activities of plant pathogens. Howell and Stipanovic first established the importance of antibiotic production for the suppression of seedling pathogens using *Pseudomonas fluorescens* Pf-5 (9), a strain now known to produce at least three different antibiotics, including 2,4-diacetylphloroglucinol (DAPG) (25). Subsequently, a number of other DAPG-producing strains have been identified as effective biological controls of diverse root pathogens (30). The genes responsible for the biosynthesis of DAPG in *Pseudomonas* spp. have been characterized (1). One of these, *phlD*, has been used widely as a genetic marker for studying DAPG-producing pseudomonads. Populations of *phlD*$^+$ *Pseudomonas* spp. have been detected on roots grown in different soils (22,28,33,44), and root colonization by native populations of these bacteria have been correlated with root disease suppressiveness (4,14,29,31,35). Their widespread occurrence and their general capacity to inhibit the growth of root pathogens have led to the suggestion that *phlD*$^+$ pseudomonads may play a substantial role in root disease suppression in different cropping systems (45).

To further investigate the microbial ecology of these functionally important bacteria, we developed a rapid and inexpensive method for assessing the abundance and diversity of the most prolific *phlD*$^+$ *Pseudomonas* spp. present in the rhizosphere (21). This method provides quantitative estimates of population size of native and inoculated *phlD*$^+$ bacteria that are similar to those generated by other methods (13,21). However, when compared with the colony hybridization approach first described by Raaijmakers et al. (33), the amount of genotypic diversity revealed is more limited because subdominant populations in a sample generally are not identified and only 629 bp of sequence are available for analyses (12,13). Studies of the genotypic and phenotypic diversity of DAPG-producing *Pseudomonas* spp. have revealed a strong, though not absolute, correlation between restriction fragment length polymorphisms (RFLP) of the *phlD* gene and genomic structure assayed using arbitrarily primed polymerase chain reactions (PCRs) with various primers (18,34). Because of this, RFLP analyses of the *phlD* gene using *Hae*III have been considered a useful starting point for analyzing the ecology and population genetics of this beneficial group of soil bacteria (12,14,21,23,34,35).

Although a number of studies have indicated the ecological significance of DAPG producers in wheat cropping systems (45), little has been reported about the ability of *phlD*$^+$ pseudomonads to colonize the roots of corn and soybean. One well-studied *phlD*$^+$ strain, CHA0, colonized corn and soybean roots when inoculated under gnotobiotic conditions and suppressed the activities of root pathogens (16,17,41). Survival of this strain on corn roots also was observed when incubated in large outdoor lysimeters (43). Additionally, Picard and colleagues published a pair of studies that described the diversity of native populations of *phlD*$^+$ bacteria that colonized the roots of corn plants grown in a single soil in a greenhouse (27,28). In those studies, the abundance of *phlD*$^+$ bacteria increased significantly as the plants matured (28), and the large majority of those bacteria were genotypically identical based on RFLP analysis of the *phlD* gene using *Hae*III (27).

One goal of our laboratory is to develop bacterial seed treatments that suppress pathogens of field crops. To that end, we are

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evaluating the contributions of native and inoculant populations of *phlD*+ pseudomonads to the biological control of root pathogens in corn and soybean cropping systems. In this article, we describe the distribution, abundance, diversity, and biocontrol potential of the *phlD*+ pseudomonads that colonize the roots of young corn and soybean plants grown throughout the state of Ohio.

MATERIALS AND METHODS

Initial samplings. Between November 2000 and March 2001, several soil and crop residue samples were recovered from corn and soybean fields in Wood and Wayne Counties. Residue samples ($n = 9$) were processed directly for enumeration of *phlD*+ pseudomonads (described below), and the sampled soil was transferred to pots into which corn and soybean were planted. These pots were incubated in a greenhouse until the crops had reached the V1 or V2 stages of growth. Multiple independent root and soil samples were taken and processed as described below ($N = 24$).

Crop and site descriptions. Corn hybrid SC1091 (Seed Consultants, Inc., Washington Courthouse, OH) and soybean cv. Kottman (Grier Seed Farms, Fremont, OH) were used throughout this study. These two elite cultivars were selected because of their excellent yield potential and high degree of resistance to soilborne root diseases. The large majority of sampling sites were located on commercial farms as part of the Ohio State University annual crop performance trials in 2001, 2002, and 2003. Three additional study sites were established on research farms in Wayne and Wood Counties that are operated by the Ohio Agricultural Research and Development Center (OARDC). In Wayne County, two adjoining fields (sites 1 and 2), 0.3 ha in size, were established on a Wooster silt loam (pH 6.9, 15% clay). Both crops were planted with and without seed treatments in a randomized complete block design with four replicates each. Site 1 was left untilled, whereas site 2 was clean-tilled each spring prior to planting. A similar field, 0.5 ha in size, was established in Wood County (site 3) on a Hoytville silt loam (pH 6.7, 37% clay) with the same randomized complete block design as the other two sites, and the soil was disked only prior to planting each spring. All fields were rotated prior to the start of these experiments on a standard 3-year cycle (i.e., corn-soybean-wheat) with either corn (Wayne County) or wheat (Wood County) being planted in 2000. All subsequent plantings followed a simple corn-soybean rotation. Planting of these additional sites occurred during the first 3 weeks of May each year. Fertility and weed pressure were managed by farmer cooperators and OARDC farm managers according to standard practice for each location. Plots were harvested in October of each year.

PCR-based characterization of *phlD*+ populations. For microbiological analyses, plants were sampled between the V2 and V4 growth stages (36,37) to ensure homogenous sampling with regards to plant ontogeny regardless of site or year sampled. For the biogeography study, a single plot (4 rows by 13 m) was marked for sampling at each location. Eight plants were obtained from the middle two rows in a zigzag pattern across the central 10 m of each plot. For the other studies conducted at the OARDC research stations, two plants were sampled at random from each plot (i.e., $n = 16$ per crop per site). Plants were carefully dug, placed into plastic bags, and transported to the laboratory within 3 h of sampling. Samples were stored in a cold room (10°C) for no more than 24 h prior to processing.

The abundance and diversity of *phlD*-containing *Pseudomonas* spp. in the rhizosphere were determined using the PCR-based assay described previously (21), with slight modifications. Briefly, roughly equal-sized portions of individual seedling root systems (1 to 2 g fresh weight) were recovered individually from the soil, separated from the shoot, and placed in 15 ml of sterile distilled water. Bacteria were dislodged from the roots by vortexing and sonication (1 min each) prior to serial dilution (1:3.5) in a 96-well plate prefilled with sterile deionized water. From these "rhizosphere-wash" plates, 50 µl of each dilution was transferred into other 96-well plates containing 200 µl of 1/3× King's medium B supplemented with ampicillin (40 µg/ml), chloramphenicol (13 µg/ml), and cycloheximide (100 µg/ml) (KMB+++). Culture plates were incubated for 2 days at room temperature and bacterial growth was assayed spectrophotometrically (ELx800 microplate spectrophotometer; Bio-Tek Instruments, Winooski, VT). The terminal dilution displaying bacterial growth (optical density at 595 nm ≥ 0.1) was used to calculate abundance of the culturable pseudomonad population. Replica plates containing 18% glycerol were prepared to allow for strain recovery. Cultures were frozen for storage prior to use as whole-cell DNA templates. Portions of the *phlD* gene were amplified using the gene-specific primers B2BF and BPR4 (21) in a PTC-200 thermal cycler (MJ Research, Watertown, MA). Reaction products were loaded onto 1.5% agarose gels for separation and visualization. To determine the genotype of *phlD*+ populations, amplification products were digested with *Hae*III or *Taq*I. Reactions were incubated at either 37°C (*Hae*III) or 60°C (*Taq*I) for 2 to 4 h and then stored at –20°C. Digestion products were separated on agarose gels in 0.5× Tris-borate-EDTA (TBE) buffer and visualized by ethidium bromide staining. Gel images were processed using a Kodak EDAS 290 digital imaging system (Kodak, Rochester, NY).

Isolation and characterization of representative strains. Pseudomonads containing the *phlD* gene were recovered from the replica plates described above. An aliquot of a dilution found to contain *phlD*+ bacteria was streaked onto 1/3× KMB+++ agar (15 g/liter) plates and incubated at room temperature for up to 4 days. Multiple isolates of each colony type present were transferred to fresh media and allowed to grow for a minimum of 24 h prior to testing. Isolates were tested for the presence and structure of the *phlD* gene using the PCR-based assays described previously. Additional genotypic information was generated using amplified ribosomal DNA restriction analysis (ARDRA) of 16S sequences using *Msp*I or *Alu*I as described previously (22). Carbon source utilization profiles were generated using Biolog SF-N2 plates (Biolog, Inc., Hayward, CA) and analyzed as described previously (22). Several previously described, DAPG-producing pseudomonads were used as positive controls in these assays, including *Pseudomonas fluorescens* strains CHA0 (42), Pf-5 (25), Q8r1-96 (31), W2-6 (22), and MtV1 (23). Stock cultures of all bacterial strains were stored at –80°C in 1/3× KMB liquid media supplemented with 18% glycerol.

***Pythium* spp. isolation and growth inhibition assays.** Soybean roots (cv. Kottman) grown at the OARDC research farm located in Wayne County were used to isolate a small collection of *Pythium* spp. using standard methods (15). Briefly, *Pythium* spp. were isolated from diseased root pieces that had been washed and incubated on water agar. Hyphal tips from growing mycelia were transferred to water agar containing streptomycin (200 µg/ml) and ampicillin (50 µg/ml) (Sigma-Aldrich, St. Louis), and cultures were maintained on that medium for up to 6 months between transfers. For identification, plugs of isolated mycelia were transferred to water agar, allowed to infect autoclaved wheat leaves, and examined for distinctive morphological characteristics. The majority of isolates were keyed out to be *Pythium irregulare*, with isolate SF2 selected as the type strain for our collection.

Growth inhibition assays were performed as described previously (23). Briefly, subcultures of *Pythium* isolates were incubated on water agar for up to 1 week prior to use. Agar plugs (0.5 to 1.0 cm^2) were transferred to the center of agar (15 g/liter) assay plates formulated as 1/10× tryptic soy agar (TSA; tryptic soy broth at 3 g/liter; Difco Laboratories, Detroit), 1/5× corn meal agar (CMA; corn infusion at 10 g/liter; Difco), or 1/5× potato dextrose agar (PDA; dextrose at 4 g/liter, infusion from 40 g of

freshly boiled potato, pH 6.3). Fresh cultures of bacterial isolates were washed one time and resuspended in sterile distilled water. For each assay, 10-μl aliquots containing approximately 10^6 cells were spotted along the perimeter of the agar plate. The experiment was designed as a randomized block design with each isolate being assayed four times on each medium. The assay plates were incubated at room temperature. Oomycete growth inhibition was determined when the growth on the uninoculated control plates extended the full radius of the plate. For each replicate, an inhibition index (I) was calculated as $I = Y/(X + Y)$; where X represents the distance from the edge of the plug to the growing edge of the fungus and Y represents the distance from the edge of the bacterial growth to the growing edge of the fungus.

Evaluation of bacterial seed treatments. A rifampicin-resistant variant of each *phlD*$^+$ isolate was recovered by plating on media containing rifampicin at 50 μg/ml (i.e., 1/3× KMBRif$^+$ [21]). Fresh colonies of these isolates were washed and cells were resuspended in 0.5% methylcellulose. Corresponding untreated controls were treated with an equal volume of 0.5% methylcellulose solution (5 ml/kg of seed). Treated seed was stored at 10°C for up to 48 h prior to planting.

Two different experiments were performed. The first was a greenhouse experiment assessing colonization by the different genotypes. Bacteria were applied to seed at a rate of log 6 cells per seed. The corn and soybean seed were planted in pots 15 cm in diameter containing Metromix 360 (Scotts, Marysville, OH). Five seed were planted per pot. Pots were arranged in a factorial design consisting of six replicates of seven experimental treatments (i.e., two isolates each of three different genotypes and an untreated control) for each crop. These pots were incubated in a greenhouse and watered weekly with 1× Miracle-Gro fertilizer (Scotts). Plants (one seedling per pot) were harvested at V2 and assayed for rhizosphere colonization as described above. This experiment was repeated. The second experiment consisted of a series of field trials of an A genotype (strain Wayne1R) conducted to assess the effects of inoculation on crop health and productivity. Bacteria were applied to seed at a rate of log 5 cells per seed. Corn and soybean were planted on the same day at each of the three OARDC research stations described above in a randomized complete block design with four replicates of the two treatments (i.e., Wayn1R-treated and untreated seed) planted for each crop at each location. At all three sites, data on inoculant colonization, crop stand, and yield were collected. For stand counts, multiple measurements (n = 16 and 8 for corn and soybean, respectively) of plants per meter were made for each replicated plot at each site. At harvest, grain mass and percent moisture data were taken, and yields were calculated for each plot at 15 and 13 percent moisture for corn and soybean, respectively. In both experiments, estimates of inoculant colonization were determined by incubating dilutions of rhizosphere washes in 1/3× KMBRif$^+$ media for 3 days at room temperature. Bacterial growth was assayed spectrophotometrically and the *phlD* genotypes appearing in terminal dilution cultures were determined as described above.

Statistical analyses. All statistical analyses were performed with Minitab software (release 13.1; Minitab, Inc., State College, PA). Abundance and incidence data were analyzed using the dilutions in which positive detection events (i.e., the appearance of turbid growth or *phlD* amplification products) were obtained. When positive detection events did not occur, the limit of detection of the assay was used as the maximum possible abundance level for comparisons. Analyses of variance and multiple comparisons were performed using standard parametric and nonparametric procedures as appropriate (40). Classification of strains based on carbon source utilization was conducted using the hierarchical clustering and factor analyses options. Distinct groups of strains were defined by the 95th percentile (near-minimum) similarity coefficient of replicate assays of identical strains.

RESULTS

Initial detection of *phlD*$^+$ pseudomonads in Ohio soils and on crop residues. The *phlD* gene was detected in pseudomonads recovered from root residues of different corn and soybean plants (n = 9) recovered between November 2000 and March 2001. Other *phlD*$^+$ pseudomonads from those same soils colonized the roots of corn and soybean seedlings when grown in the greenhouse during the winter of 2001. All of the corn seedlings (n = 11) and just over half (i.e., 8 of 13) of the soybean seedlings examined were colonized by *phlD*$^+$ pseudomonads.

Rhizosphere colonization of field-grown corn and soybean plants. In order to characterize the biogeography of *phlD*$^+$ *Pseudomonas* spp., we examined the roots of plants sampled from farms located throughout Ohio. The incidence and genotypic diversity of *phlD*$^+$ pseudomonads colonizing the rhizosphere of corn and soybean during early growth stages were determined. Across the entire experiment, neither root mass nor cultured pseudomonad abundance was significantly correlated with the abundance of the *phlD*$^+$ populations on either crop. However, differences in rhizosphere colonization were observed between crops and between sites.

Across Ohio, the incidence of *phlD*$^+$ pseudomonads on corn roots was significantly greater than that on soybean roots in all 3 years of this study ($P < 0.01$, 0.04, and 0.02 for 2001, 2002, and 2003, respectively). Populations of *phlD*$^+$ pseudomonads were detected on 89% of the corn samples, and 75% harbored populations in excess of log 4.5 cells/g of root (Table 1). Two notable

TABLE 1. Occurrence of *phlD*$^+$ pseudomonads colonizing the rhizosphere of corn hybrid SC1091

Year	Site[x]	Incidence[y]		Genotype frequency[z]	
		≥Log 3.4	≥Log 4.5	D	Other
2001	Van Wert	1.0	1.0	1.0	0.25 I,S
2002	Van Wert	1.0	0.88	1.0	0.0
2003	Van Wert	1.0	0.50	1.0	0.67 S
2001	Wood	1.0	1.0	0.50	0.50 S
2002	Wood	0.37	0.37	0.67	0.33 S
2003	Wood	1.0	0.86	1.0	0.14 S
2001	Wyandot	1.0	1.0	1.0	0.0
2002	Wyandot	0.88	0.75	0.80	0.20 A
2003	Wyandot	n.d.	…	…	…
2001	Crawford	1.0	0.75	1.0	0.14 A
2002	Crawford	0.75	0.63	1.0	0.0
2003	Crawford	1.0	0.86	1.0	0.0
2001	Wayne	1.0	0.88	0.0	1.0 I
2002	Wayne	1.0	1.0	1.0	0.0
2003	Wayne	0.13	0.00	1.0	0.0
2001	Coshocton	1.0	1.0	0.88	0.25 A
2002	Coshocton	1.0	0.63	0.83	0.17 S
2003	Coshocton	1.0	0.63	0.88	0.25 A,S
2001	Mahoning	1.0	1.0	1.0	0.0
2002	Mahoning	1.0	0.88	1.0	0.0
2003	Mahoning	n.d.	…	…	…
2001	Darke	n.d.	…	…	…
2002	Darke	1.0	0.75	0.75	0.25 A
2003	Darke	1.0	0.88	1.0	0.0
2001	Clark	n.d.	…	…	…
2002	Clark	0.88	0.75	0.29	0.71 A
2003	Clark	0.50	0.37	0.0	1.0 S

[x] Samples were taken from commercial farms in each of the specified counties in Ohio. Sites in different counties were separated by >40 km, while those located in the same county, but sampled in different years, were separated by <5 km.

[y] Proportion of root samples harboring populations of *phlD*$^+$ pseudomonads at the abundance levels indicated. In several instances, incidence was not determined (n.d.) because samples were not taken for that location.

[z] Proportion of samples for which different *phlD*-restriction fragment length polymorphism-defined genotypes were determined. The identity of less frequently observed genotypes is noted. Infrequently, two genotypes were detected among the *phlD* sequences amplified from an individual plant sample causing the sum of the proportions to exceed 1.0.

exceptions to this pattern were observed. First, we observed that fewer than half of the drought-stricken corn plants sampled from the Wood County site in 2002 harbored detectable populations of *phlD*$^+$ pseudomonads. Additionally, only 1 of the 8 plants from the Wayne County transect sampled in 2003 was colonized. Significant site-to-site variation in abundance was not clearly present on corn in 2001 ($P < 0.17$) because of the remarkably high frequency of rhizosphere colonization on that crop grown throughout the state. However, significant differences in abundance among sites were observed for the 2002 and 2003 samplings ($P < 0.02$ both years) because of the two anomalous samples mentioned above. Across the entire data set, the median incidence of colonization on corn did not vary by county ($P = 0.97$) and varied only marginally by year ($P = 0.11$). In contrast to most of the corn samples, only 70% of the soybean samples harbored measurable populations of *phlD*$^+$ pseudomonads, and <40% harbored populations in excess of log 4.5 cells/g of root (Table 2). Significant variation in the ranked abundance of *phlD*$^+$ populations colonizing roots of plants grown in different counties was observed on soybean in 2001 and 2002 ($P < 0.02$ both years), but not in 2003 ($P = 0.20$) when colonization was highest. However, across the entire data set, the median incidence of colonization did not vary by county ($P = 0.53$), but it did vary by year ($P = 0.01$), with the highest level of colonization detected during the 2003 growing season (Table 2).

Diversity and distribution of phlD$^+$ genotypes. The genotypes of the most abundant *phlD*$^+$ populations were determined by RFLP analyses of the amplified *phlD* gene fragments. Genotypic assignments were based on comparison with previously published profiles and were named accordingly (14,21). In total, seven distinct *phlD*-RFLP-defined genotypes (A, C, D, F, I, R, and S) were detected in our studies.

The most frequently observed population corresponded to the D genotype. In our preliminary investigations conducted prior to the 2001 field season, the most abundant *phlD*$^+$ populations detected in cold and saturated field soils and on root residues were of the D genotype (data not shown). On commercial farms, the roots of young corn and soybean plants both were colonized frequently by native populations of this genotype (Tables 1 and 2). Overall, the large majority of field-grown corn plants harboring detectable populations of *phlD*$^+$ bacteria were colonized by the D genotype (77, 84, and 81% sampled in 2001, 2002, and 2003, respectively). Frequent root colonization by the D genotype also was observed on field-grown soybean plants (78, 67, and 52% of plants in 2001, 2002, and 2003, respectively). This general pattern of ubiquity and dominance was observed in 41 of the 46 sampled locations. Interestingly, the few exceptions to this rule were noted among the three most southern sample sites. For example, <20% of the corn samples from Clark County (Table 1) and <30% of the soybean samples from Preble and Clinton Counties (Table 2) contained detectable populations of the D genotype. Interestingly, the D genotype was not observed on corn samples taken from the 13-m transect at the Wayne County site in 2001 (Table 1), even though it was detected on corn sampled at a different time from nearby sites (i.e., 10 to 100 m away) in 2001 (data not shown), as well as in 2002 and 2003.

Populations of five other genotypes (A, C, I, R, and S) were detected repeatedly on corn and soybean roots, though much less frequently than the D genotype. In general, the detection of these other genotypes was more variable from year to year (Tables 1 and 2). Most prominent among these were populations of the A genotype, which was detected in 10 of the 17 counties sampled. The S genotype was the next most frequently observed on both crops. This newly discovered genotype was identified in rhizosphere samples taken from four of nine counties planted to corn and two of eight counties planted to soybean. In contrast, the I and C genotypes were found at approximately half of the soybean sites, but only rarely or not at all on corn. The newly discovered R genotype was detected on just one plant in Clinton County. It was also found on two plants grown in Wayne County during the subsequent evaluation of seed treatments (described below). Even more rare, the F genotype was detected on just one plant sampled from Delaware County in 2003 (Table 2).

Rhizosphere colonization by native *phlD*$^+$ populations was further investigated on two OARDC research farms located in Wayne and Wood Counties (Table 3). In those fields, neither root mass, total cultured pseudomonad population, nor plant growth stage was significantly correlated with population size on either crop. Populations of native *phlD*$^+$ pseudomonads were detected in rhizosphere of young corn and soybean plants with frequencies similar to those observed for the crops grown at different locations in the same county. The number of *phlD*$^+$ pseudomonads ranged from below the limit of detection (i.e., log 2.7) to log 6.5 on individual rhizosphere samples and incidence ranged from 13 to 100% for each replicate sample ($n = 8$). The *phlD*$^+$ populations generally were more abundant on colonized soybean roots than on colonized corn roots (Table 3). However, the incidence of root colonization was significantly greater on corn than on soybean across the entire experiment ($P < 0.02$). In these fields, the average incidence of *phlD*$^+$ bacteria observed in the rhizosphere of corn was double that of soybean (70 and 35 percent, respectively). The percentage of samples harboring populations >log 4.5 also was higher on corn (36%) than on soybean (29%). Highly significant differences among sites were detected in 2001 ($P <$ 0.006), with the highest populations observed on plants grown at site 3, located in Wood County. The *phlD*$^+$ populations also were

TABLE 2. Occurrence of *phlD*$^+$ pseudomonads colonizing the rhizosphere of soybean cv. Kottman

		Incidence[y]		Genotype frequency[z]	
Year	Site[x]	≥Log 3.4	≥Log 4.5	D	Other
2001	Henry	1.0	0.75	0.88	0.12 A
2002	Henry	1.0	0.37	0.88	0.12 A
2003	Henry	1.0	0.50	1.0	0.0
2001	Huron	0.37	0.12	1.0	0.0
2002	Huron	0.75	0.50	0.83	0.17 C
2003	Huron	1.0	0.75	0.71	0.29 C,I
2001	Mercer	0.63	0.25	n.d.	…
2002	Mercer	0.50	0.37	0.75	0.25 C
2003	Mercer	n.d.	…	…	…
2001	Delaware	0.63	0.44	1.0	0.0
2002	Delaware	0.75	0.25	1.0	0.0
2003	Delaware	1.0	0.63	0.4	0.80 C,F,I
2001	Preble	0.00	0.00	…	…
2002	Preble	0.63	0.50	0.0	1.0 A,C
2003	Preble	1.0	1.0	0.13	0.88 C
2001	Clinton	0.75	0.25	0.33	0.67 S
2002	Clinton	0.00	0.00	…	…
2003	Clinton	1.0	0.38	0.20	0.80 A,C,R
2001	Wood	1.0	0.50	0.71	0.57 S
2002	Wood	0.63	0.25	0.50	0.50 I
2003	Wood	1.0	0.75	0.57	0.50 A,S
2001	Wayne	0.25	0.12	n.d.	…
2002	Wayne	0.50	0.00	0.75	0.25 A
2003	Wayne	1.0	0.63	0.63	0.38 A,I

[x] Samples were taken from commercial farms in each of the specified counties in Ohio. Sites in different counties were separated by >40 km, while those located in the same county, but sampled in different years, were separated by <5 km.

[y] Proportion of root samples harboring populations of *phlD*$^+$ pseudomonads at the abundance levels indicated. In one instance, incidence was not determined (n.d.), because samples were not taken from that location.

[z] Proportion of samples for which different *phlD*-restriction fragment length polymorphism-defined genotypes were determined. The identity of less frequently observed genotypes is noted. Infrequently, two genotypes were detected among the *phlD* sequences amplified from an individual plant sample causing the sum of the proportions to exceed 1.0. In two instances, genotype data was not determined (n.d.), because restriction digests of amplified DNA products were too degraded to be interpreted.

most abundant at this same site in 2002, but the differences in the mean population sizes among sites were not significant for either crop. The proportion of plants colonized by *phlD*+ pseudomonads also was greatest at site 3 in both years.

Isolation and baseline characterization of dominant genotypes. Attempts were made to isolate strains representative of the predominant *phlD*+ genotypes observed to colonize the rhizosphere of corn and soybean in 2001 and 2002. In winter and spring 2001, several isolates corresponding to the A and R genotypes were recovered from the roots of corn and soybean plants grown in soil taken from the OARDC research farm located in Wayne County. Additionally, a D genotype strain was isolated from corn root residues obtained from the research farm located in Wood County. The soil and plant samples used in those preliminary studies were taken from fields located within 100 m of the field plots from which subsequent samples were taken. Additional isolates of the A, R, and S genotypes were obtained from root samples processed in 2001. Despite a systematic approach to obtaining representative isolates, only 3 of the 24 attempts to recover a D genotype strain were successful. In contrast, nearly half of the attempts to recover A and S genotypes were successful (6 of 11 and 3 of 6, respectively). Attempts to isolate the C and I genotypes (present in three or fewer samples each year) were unsuccessful. It was noted that, when recovered, the D genotypes initially formed relatively small colonies on 1/3× KMB+++ agar compared with the A, R, and S genotypes, an indication that the relatively low recovery rates of that genotype reflected poor culturability on the solid form of that medium.

Representative isolates of the A, D, R, and S genotypes were recovered in these studies (Fig. 1). The A and D genotypes were confirmed to belong to the two different ARDRA groups to which similar strains had been assigned previously (10,23). The *Msp*I digests of the 16S sequences amplified from the newly defined R genotypes gave rise to the same RFLP pattern as the A type strains (e.g., CHA0 and Pf-5). In contrast, the newly defined S genotypes had the same pattern as strains belonging to the more diverse ARDRA B group, which is known to contain strains with different *phlD*-RFLP genotypes. Use of *Alu*I revealed identical banding patterns for all of the strains tested (data not shown).

The abilities of the recovered isolates to grow in vitro on different carbon sources were determined. After 7 days of incubation, each isolate had developed turbid growth on 56 to 74 substrates, and, of these, 48 substrates supported growth of all of the tested strains. As a rule, A and R genotypes grew on significantly fewer substrates (56 to 61) than the D and S genotypes (62 to 70). However, the two assays performed with Pf-5 indicated that this strain could grow on ≈70 substrates presented on the SF-N2 MicroPlates. Multivariate statistical analyses (i.e., cluster and ordination analyses using different algorithms) of the carbon source utilization data after 3 and 7 days of incubation revealed two major groupings (Fig. 2). The D and S genotype strains were classified as clearly distinct from the A and R genotypes. This result correlated perfectly with the ARDRA-defined groupings and was determined largely by differences in utilization of six substrates (D-galactose, D-sorbitol, D-galactonic acid lactone, D-galacturonate, D-glucoronate, and D-saccharic acid) on which only the D and S genotype strains could grow. The D and S genotypes also appeared to differ phenotypically from one another after 7 days of growth. This tentative distinction between these two groups was due largely to the ability of D genotypes to grow on *N*-acetyl-D-glucosamine, L-threonine, and D-cellobiose, capacities not shared by the two S genotypes assayed. The utilization of carbon substrates by isolates of the A and R genotypes was very similar, but Pf-5 appeared distinct in some multivariate analyses because it was able to grow on several additional substrates.

In vitro inhibition studies. The capacity of the various *phlD*+ isolates to inhibit the growth of oomycete pathogens was evaluated in vitro. Significant variation in inhibition potential was observed among *phlD*+ genotypes against *Pythium irregulare* SF2 on

TABLE 3. Abundance of *phlD*+ pseudomonad populations that colonized the rhizosphere of corn and soybean plants grown on two Ohio Agricultural Research and Development Center research farms

Year, site[x]	Crop	Abundance[y]	Incidence[z]	
			≥Log 3.4	≥Log 4.5
2001				
1	Corn	3.9	0.37	0.25
	Soybean	5.5	0.25	0.25
2	Corn	3.9	0.50	0.25
	Soybean	5.6	0.12	0.12
3	Corn	5.0	1.0	0.88
	Soybean	5.0	1.0	0.88
2002				
1	Corn	4.3	0.50	0.37
	Soybean	5.1	0.25	0.25
2	Corn	3.8	0.88	0.00
	Soybean	3.9	0.25	0.00
3	Corn	4.8	0.86	0.43
	Soybean	5.2	0.25	0.25

[x] The two crops were planted at three different locations. Site 1 (no-till) was planted adjacent to site 2 (conventional till) in Wayne County, OH and site 3 (minimum-till) was located in Wood County, OH.

[y] Mean population size on colonized plants expressed as log CFU per gram of fresh weight ($n \geq 7$).

[z] Proportion of root samples harboring populations of *phlD*+ pseudomonads at the abundance levels indicated.

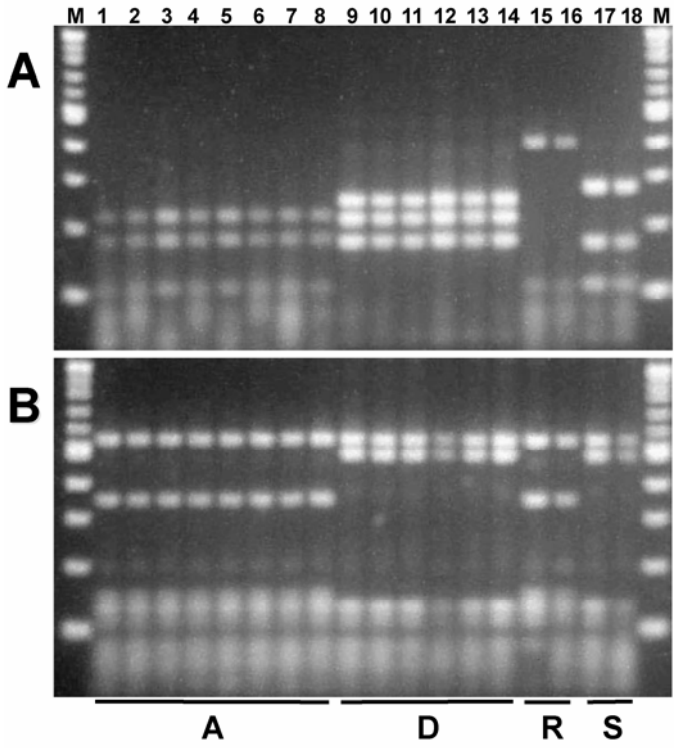

Fig. 1. Genotypic characterization of representative *phlD*+ pseudomonads isolated from corn and soybean rhizospheres. **A,** Restriction fragment length polymorphism analysis of amplified *phlD* sequences using *Hae*III **B,** Amplified ribosomal DNA restriction analysis using *Msp*I. The 100-bp ladder (New England Biolabs, Beverly, MA) (M) was used to estimate the size of restriction fragments. Numbers at the top of the figure correspond to analyses for strains CHA0 (1), Pf-5 (2), Crawf1 (3), Cosho1 (4), Wood2 (5), Wayne1 (6), Wayne1R (7), Wayne3 (8), Q8r1-96 (9), W2-6 (10), MtV1 (11), Wood1 (12), Delaw1 (13), Huron1 (14), Wayne2 (15), Wayne4 (16), Wood3 (17), and Clinto1 (18). Four distinct *phlD*+ genotypes were identified that corresponded to the previously characterized A and D genotypes of 2,4-diacetylphloroglucinol producers as well as two new genotypes designated R and S.

different media (Fig. 3). On 1/10× TSA, the A and R genotypes were strongly inhibitory to the growth of the pathogen, but the D and S genotypes were not. The observed differences between genotypes were significant ($P < 0.05$), with the exception that Crawf1 (an A genotype) and Wood3 (an S genotype) were only weakly inhibitory to growth ($I < 0.2$). The A and R genotypes also were significantly more inhibitory than the D and S genotypes when cultured on 1/5× CMA ($P < 0.05$). However, the magnitude of the difference was much less on this corn-based medium because the inhibition indexes for even the most effective A genotypes were still <0.1. In contrast, the D and S genotypes, but not the A and R genotypes, were effective at inhibiting *Pythium* spp. growth when cultured on 1/5× PDA. In addition, on 1/5× PDA, significant variation was observed among the D genotype isolates ($P > 0.05$) in their capacities to inhibit the pathogen.

Because different pathogens may vary in their sensitivity to the antagonistic activities of different biocontrol strains, we evaluated the inhibitory potential of two A genotype strains (Wayne1 and Crawf1) and one D genotype strain (Wood1) when co-cultured with eight different *Pythium* isolates. In those assays, the A genotypes generally were effective at suppressing the growth of *Pythium* spp., but the inhibition index varied significantly depending on the oomycete strain tested ($P < 0.01$) and the medium used for the assay plate ($P < 0.01$). In most instances, results were consistent with those reported above (Fig. 3). However, the growth of one of the eight *Pythium* isolates was not at all inhibited on 1/5× CMA by any of the three bacterial strains tested (data not shown). As noted above for *P. irregulare* strain SF2, the A genotype was more inhibitory than the D genotype on both media ($P < 0.05$ for all comparisons). Additionally, in five of the eight assays on 1/10× TSA, Wayne1 was significantly more effective than Crawf1 at inhibiting *Pythium* growth ($P < 0.05$); however, no such variation within genotype was observed on 1/5× CMA.

Evaluation of seed treatments. Although turbidity of rifampicin-containing media was used primarily to estimate population size, the presence of *phlD* was assayed in a subset of samples for each experiment. In all instances, the genotype of rifampicin-resistant bacteria present in the terminal dilution cultures matched that of the inoculant strains used (data not shown). Infrequently, the targeted gene also was detected in cultures of untreated samples, but these events always occurred in the lowest dilutions of our assay plates where soil residues also contributed to turbidity (data not shown).

Under greenhouse conditions, all of the *phlD*$^+$ isolates displayed the capacity to colonize roots grown from inoculated seed (Table 4). On individual seedlings, the abundance of the rifampicin-resistant inoculant strains ranged from log 4.0 to log 6.9 cells/g of root for corn and from log 3.6 to log 6.5 cells/g of root for soybean. As a group, the D and S genotypes typically developed larger populations than the A genotype on both crops (Table 4). However, no significant differences in rhizosphere colonization among the individual isolates were observed (data not shown). No significant differences in germination rate, shoot growth, or root biomass recovered at the time of sampling were observed in these assays (data not shown).

A single isolate of the A genotype, Wayne1R, was selected for field testing based on its capacity to inhibit pathogens in vitro on corn- and soybean-containing media. In the field, root colonization by the inoculant strain Wayn1R was evaluated between V2 and V4 on both crops at three different locations over 3 years. In soybean fields, significant root colonization by the inoculant strain was observed at all three sites ($P < 0.05$). Rifampicin-resistant pseudomonads were recovered from 64% of the samples developing from inoculated soybean seed. On those samples, the abundance of the inoculant strains ranged from log 4.4 to log 6.3 CFU/g of root. In contrast, colonization of corn roots was observed less frequently, with <25% of the rhizospheres developing from treated seed testing positive for the inoculant strain. In soybean, significant increases in stand and yield were noted in the large majority of trials (Table 5). Although not significant in any individual trial, due to the limited extent of sampling, significant increases in soybean stand ($P = 0.06$) and yield ($P = 0.04$) were noted across the entire study. However, no significant differences in corn stand or yield were noted ($P = 0.55$ and 0.26, respectively).

DISCUSSION

Different soils known to be suppressive to root diseases of wheat, pea, or tobacco have been shown to harbor significant

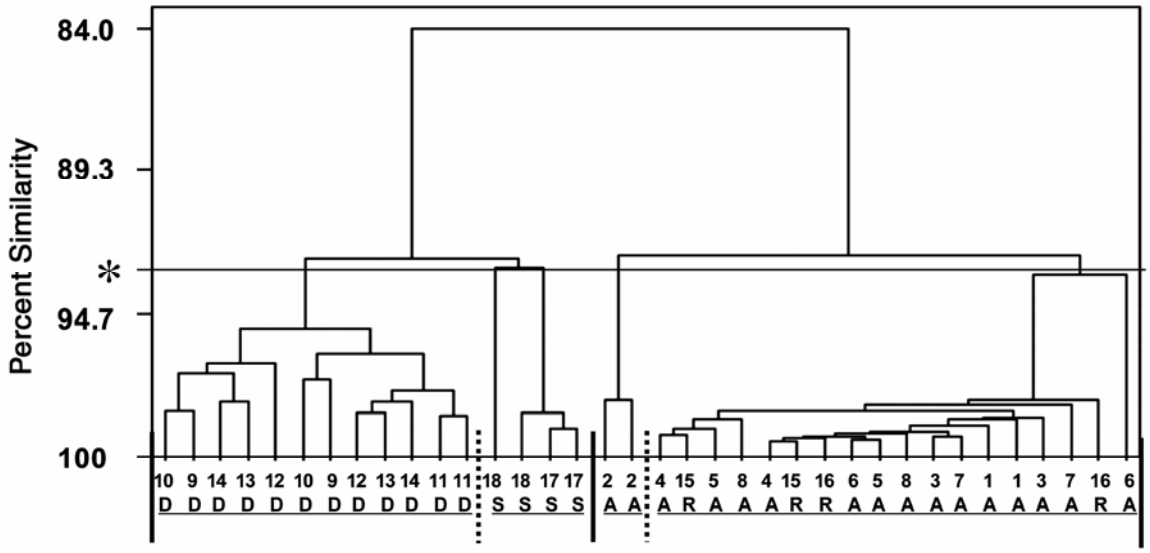

Fig. 2. Cluster analysis of carbon-source utilization patterns of *phlD*-containing *Pseudomonas* strains cultured for 7 days on SFN2 plates. The dendrogram was generated by Minitab using the Average linkage algorithm and the similarity matrix generated using Pearson's correlation coefficient. Multiple isolates representing the four different *phlD*-restriction fragment length polymorphism genotypes (A, D, R, and S) isolated from corn and soybean were assayed. Numbers at the bottom of the figure correspond to strains CHA0 (1), Pf-5 (2), Crawf1 (3), Cosho1 (4), Wood2 (5), Wayne1 (6), Wayne1R (7), Wayne3 (8), Q8r1-96 (9), W2-6 (10), MtV1 (11), Wood1 (12), Delaw1 (13), Huron1 (14), Wayne2 (15), Wayne4 (16), Wood3 (17), and Clinto1 (18). Two independent assays were performed on each strain to allow for a quantitative definition of group identity. The 95th percentile near-minimum similarity value of the paired replicates (*) was used to define distinct groups.

populations of *phlD*+ pseudomonads that effectively colonized crop roots and reduced disease severity (4,14,31,35). Interestingly, the abundance and genotypes of root-colonizing *phlD*+ bacteria present in these disease-suppressive soils were similar in size to those observed in this work. The selection of elite cultivars of corn and soybean based on stand, vigor, and yield potential is standard practice for crop breeders (7). We think that this selection process may have resulted in the concomitant selection of traits that promote symbioses with biocontrol bacteria such as those described in this study. Because the suppression of root disease by DAPG-producing, *phlD*+ pseudomonads has been correlated with population size in the rhizosphere of wheat (31), it will be interesting to determine whether such a correlation exists in corn and soybean fields. Comparisons of hybrids and cultivars with varying degrees of susceptibility to root disease will be needed to test the hypothesis that native populations of *phlD*+ *Pseudomonas* spp. (or their unassayed co-colonists in the rhizosphere) play a significant role in disease suppression in corn and soybean fields. Recently, it has been shown that different cultivars of wheat support different levels of DAPG-producing inoculant strains (26); however, it is not clear if such differences are related to resistance to soilborne pathogens. Mazzola et al. (20) have shown that some cultivars of wheat can promote suppression of apple replant disease while others cannot, and that multiple populations of fluorescent pseudomonads might contribute to such suppression. We are currently investigating whether similar correlations between rhizosphere colonization by native populations of *phlD*+ pseudomonads and disease suppression occur in corn and soybean cropping systems. The high degree of genotypic and phenotypic similarity among the isolates obtained in this study and those with proven biocontrol activities (e.g.,

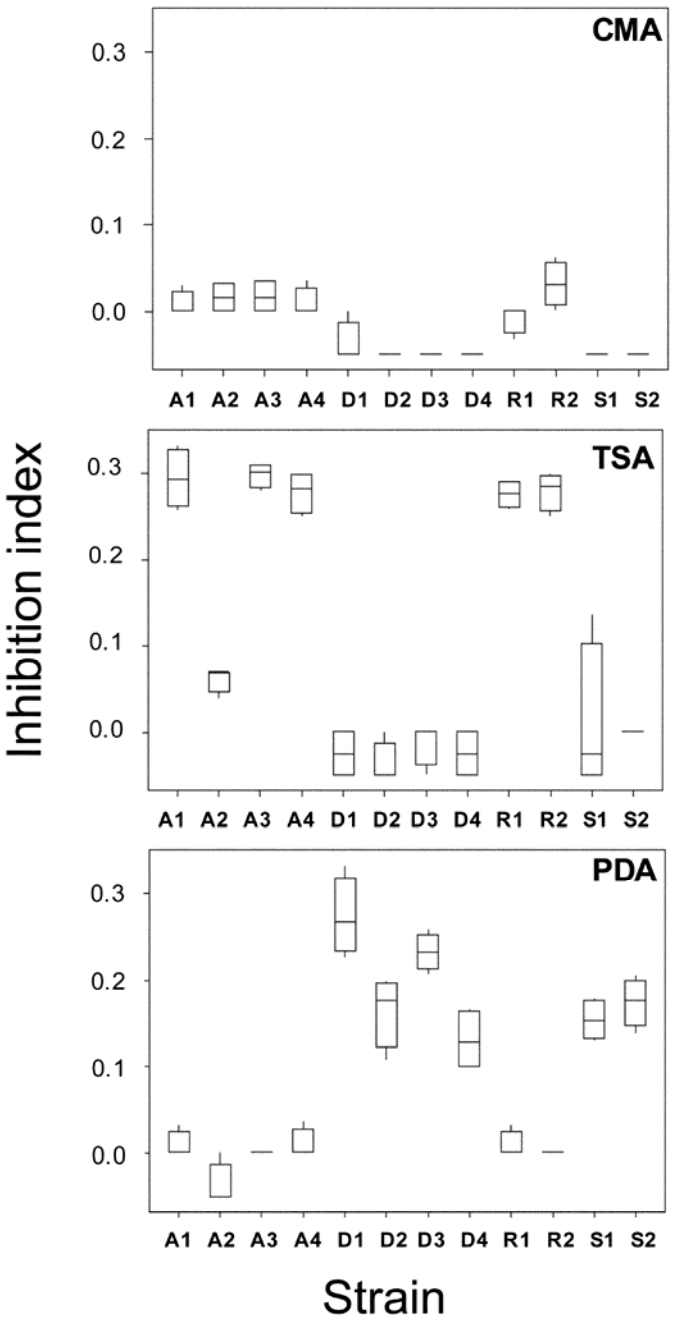

Fig. 3. Variation of pathogen growth inhibition activity dependent on genotype and environmental conditions. Inhibition of *Pseudomonas irregulare* SF2 growth was assayed in vitro on three different media: 1/5× corn meal agar (CMA), 1/10× tryptic soy agar (TSA), and 1/5× potato dextrose agar (PDA). Multiple isolates representing the four different *phlD*-RFLP genotypes (A, D, R, and S) isolated from corn and soybean were assayed. Numbers at the bottom of the figure correspond to strains Wayne1 (A1), Crawf1 (A2), CHA0 (A3), Cosho1 (A4), Delaw1 (D1), Huron1 (D2), Wood1 (D3), Q8r1-96 (D4), Wayne4 (R1), Wayne2 (R2), Wood3 (S1), and Clinto1 (S2). Data from multiple independent assays (n = 6) are presented as box and whisker plots with the range, interquartile range, and median inhibition indices noted for each isolate. Negative indices indicate overgrowth of the colony by the pathogen on the assay plate.

TABLE 4. Colonization of corn and soybean rhizospheres by rifampicin-resistant *phlD*+ bacteria inoculated onto seed[x]

	Log CFU/g of root[z]	
Crop, treatment[y]	Trial 1	Trial 2
Corn		
A	5.2 ab	4.4 b
D	5.0 b	5.1 ab
S	5.8 a	5.4 a
Uninoculated	3.0 c	2.9 c
Soybean		
A	5.0 b	4.3 b
D	5.6 a	4.6 ab
S	5.3 ab	5.1 a
Uninoculated	2.7 c	3.2 c

[x] Corn and soybean seed were treated with equal volumes (10 ml/kg) of either water or a cell suspension (log 6 cells/seed) and planted into Metromix 36 within 24 h of treatment. Plants were harvested during early vegetative growth (V2) and the population of rifampicin-resistant inoculant strains determined as described in the text.

[y] Letters refer to the *phlD*-restriction fragment length polymorphism-defined genotype of the bacteria used as seed treatments.

[z] Mean counts of rifampicin-resistant bacteria are presented. Values in the same column followed by different letters are significantly different (P < 0.05) by Tukey's multiple comparison test.

TABLE 5. Influence of Wayn1R seed treatments on crop stand and yield[z]

	Change in stand (%)			Change in yield (kg/ha)		
Crop, site	2001	2002	2003	2001	2002	2003
Corn						
1	–10	+2	–1	–354	+502	–389
2	–6	+3	+1	+458	–13	+157
3	–2	+2	+2	+490	n.d.	–207
Soybean						
1	+10	+4	–1	+87	+54	+256
2	+22	+6	–6	+162	+54	+128
3	–6	+11	+10	+47	–47	–101

[z] Corn and soybean seed were treated with equal volumes (5 ml/kg) of either water or cell suspension (log 5 cells/seed) and planted within 48 h of treatment. Plant stand was measured during early vegetative growth (V2–V4) and moisture adjusted yield was calculated at harvest. Numbers presented are relative to the uninoculated control treatment; n.d. indicates yield was not determined in this instance because the crop was not harvested following late-season frost damage.

CHA0, Pf-5, and Q8r1-96) indicates that native populations of *phlD*$^+$ pseudomonads may contribute substantially to root disease suppression in corn and soybean cropping systems. Furthermore, our field trials of Wayne1R indicate that colonization by native populations may be significantly supplemented so as to increase soybean stand and yield. However, further work will be required to quantitatively assess the contributions and relative value of native and inoculant populations to plant health in different soils.

Several components of soil chemistry have been shown to influence antibiotic production in vitro (6,38) and in situ (5,24) by different *phlD*$^+$ isolates, and crops may respond differently to the presence of inoculated DAPG producers (16,41). Our data on the effects of media composition on pathogen inhibition activities expressed by different *phlD*$^+$ genotypes indicate that the relative biocontrol activities of these pseudomonads likely varies on different crops. In this study, we showed that media derived from soybean and potato provided environments conducive to pathogen inhibition by different *phlD*$^+$ genotypes, but media derived from corn seed did not. These data indicated that the A genotype was more likely to express biocontrol activities in the rhizosphere of corn and, especially, soybean plants than the D and S genotypes, despite the observation that the latter two genotypes may more abundantly colonize the roots of both crops. Such in vitro inhibition assays commonly use 1/5× PDA to prove that compounds such as DAPG (3,19) or strains, such as the D genotype (23) can inhibit the growth of root pathogens. We think that such assays might be misleading if chemistry of the root environment differs substantially from that of the assay plate. Assessment of biocontrol potential of bacterial strains based on such in vitro assays is further complicated by the observation that sensitivity of the target pathogens can also vary (3,19, this work). Still, it is interesting to note that our data on the capacity of Wayne1R, an A genotype, to increase stand and yields of soybean, but not corn, in the field is consistent with our in vitro inhibition assays. However, comparisons of the effectiveness of different genotypes on both crops will be needed to further determine the predictive value of the in vitro assays used in this study.

Diverse pseudomonads containing the *phlD* gene, and all generally capable of producing DAPG, have been isolated from the roots of different plant species grown in different soils. Initially, Keel et al. (11) showed that there were two major, distinct groups of *Pseudomonas* spp. differing in multiple genotypic and phenotypic traits. In subsequent studies, these two groups were delineated further and substantial subspecies diversity was found to exist in each (18,22,34,39,44). The ecological significance of this diversity is not entirely clear, but it is known that variation exists in phenotypes directly related to biocontrol (e.g., root colonization ability [13,14,31] and the amount of DAPG produced in vitro [18,38]). Across the state of Ohio, we observed that the D genotype predominated in the rhizosphere of both crops. Strains with this genotype have been shown to be present in different soils, with known isolates obtained from several disease-suppressive soils located in the United States and Europe (14,22,34,44). Nonetheless, the omnipresence of this genotype across Ohio was unexpected. Our results are consistent with those of Picard et al. (28) who indicated that, in a single soil, the large majority (144 of 167) of *phlD*$^+$ pseudomonads were nearly identical. Further work indicated that those isolates corresponded to D genotype based *Hae*III digests of a 745-bp fragment of the *phlD* gene (27). Subgroup diversity in the *phlD*-RFLP group D strains has been observed using random amplified polymorphic DNA analyses (18, 27,32) and ERIC-PCR (24), but the full extent and relevance of this diversity is unclear. Significantly, multiple D genotype strains have been shown to effectively colonize crop roots when applied at low doses in soil or on seed, an ability that makes them excellent targets for development as biocontrol agents (14,31,32). The widespread occurrence of D genotypes on corn and soybean indicates a high-level of rhizosphere competence of this genotype on both of these crops. However, as noted above, it is uncertain as to whether the D genotype will express any significant biocontrol activities in the rhizosphere of corn or soybean given the results of our in vitro assays.

Several other distinct genotypes predominated in the rhizosphere of some corn and soybean plants, and their infrequent detection relative to the D genotype indicates that they are either less widely distributed or less prolific rhizosphere colonists. Although it has been reported that only one *phlD*-RFLP genotype will be numerically dominant in any given soil (22,34,44), more recent studies have indicated that multiple *phlD*-RFLP genotypes can be detected in a soil when large numbers of samples are examined (13,14,35, this work). The S genotype, which appears to be fairly common in Ohio, is genotypically distinct from all other previously identified *phlD*$^+$ strains, although it is likely to be phylogenetically similar to the D genotype strains because of its noted similarities in the ARDRA, carbon source utilization, and in vitro inhibition assays. Interestingly, we also found that the A genotype could predominate in the rhizosphere of both crops. Strains of this genotype are known to express multiple phenotypes related to biological control of plant pathogens and plant growth promotion (10). Although isolates of the A genotype have been obtained from several different soils (2,11,22,34,44), the precise distribution of these strains in most environments remains unexplored. The A genotype is known to be less rhizosphere competent than the D genotype on wheat and pea when grown under controlled conditions (13,14). Our results suggest that differences in colonization capacity also might exist on corn and soybean. Last, it is worth noting that the C genotype seems to express a host preference for soybean compared with corn. Isolates of this genotype were obtained previously from a take-all decline soil from Quincy, WA, but they have not been identified in other surveys of the genotypic diversity of *phlD*$^+$ pseudomonads present on both monocots and dicots (11,18,22,34,44). The frequent occurrence of the S, A, and C genotypes in Ohio may indicate a certain degree of geographic isolation or host preference; however, more intensive surveys of corn and soybean fields in other regions will be needed in order to determine which phenomenon is more significant.

This is the first study to systematically investigate the distribution of *phlD*$^+$ pseudomonads across multiple spatial scales. In each sampled plot (15 m^2), only one or two *Hae*III-defined *phlD* genotypes were predominated on the eight plants sampled, but six were found across the entire region (75,000 km^2). In most cases, the same genotypes were detected with similar frequencies in three consecutive years at locations within the same county (i.e., separated by <5 km). This indicates that similarities in soil type, crop management, prevailing climate, and the crop host may all be important factors selecting for similar populations of DAPG producers. Sampling conducted at the two OARDC research farms revealed that differences in rhizosphere colonization across Ohio were due to a mix of both crop- and site-specific factors. In Wayne County, soybean plants were less frequently colonized than corn when planted in adjacent plots (spanning 30 m^2) and across the entire field (approximately 2,500 m^2). At the Wood County sites, the frequency of rhizosphere colonization generally was higher than at the Wayne County site, and crop-to-crop variation was much less apparent because colonization was relatively high on both crops. In total, our data leads us to conclude that, although soybean roots will be less frequently colonized than those of corn, the predominant genotype present in either crop's rhizosphere will be selected from among those present in a soil by the prevailing environmental conditions. Additionally, the distribution of different genotypes seems to be patchily distributed at both the field and landscape scales. Multiple soil factors (e.g., pH, organic matter content, moisture, and temperature) likely affect the abundance, distribution, and activities of bacteria that suppress plant pathogens (8,46). Our laboratory is currently investi-

gating the relationships among different environmental variables, the abundance and diversity of *phlD*$^+$ pseudomonads, and their impact on plant health in a variety of soils.

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