The Nature and Application of Biocontrol Microbes III: *Pseudomonas* spp.

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Diversity and Ecology of Biocontrol *Pseudomonas* spp. in Agricultural Systems

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ABSTRACT

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Diverse *Pseudomonas* spp. may act as biological controls of plant pathogens, but the ecology of those natural populations is not well understood. And, while biocontrol potential has been identified in multiple pseudomonad strains, the linkages between genotype and phenotype have yet to be fully delineated. However, intensive studies of one class of biocontrol strains, i.e., those that can produce 2,4-diacetylphloroglucinol (DAPG), have provided new insights into the diversity, distribution, and interactions of biocontrol pseudomonads. Those studies also laid the foundation for future research and development of pseudomonad-based biocontrol strategies. Over the past several years, numerous studies have also revealed that biocontrol pseudomonads are widely distributed in agricultural soils, and that multiple crop and soil factors can affect their abundance and activities. Recent work has shown that a variety of farm management practices that reduce soilborne disease pressure can also

alter the rhizosphere abundance of DAPG producers in complex ways. Such studies provide support for the hypothesis of an ecological feedback mechanism whereby a native biocontrol population increase and subsequently reduce root disease severity following infection. It is well established that complex biological interactions can take place among biocontrol pseudomonads, plant pathogens, their hosts, and other members of the microbial community. The net result of such interactions likely dilutes biocontrol efficacy at the field scale. Nonetheless, inoculation can be effective, and several successful applications of biocontrol pseudomonads have been developed. Future applications of microbial ecology research will hopefully improve the consistency and efficacy of biocontrol mediated by *Pseudomonas* spp. Current applications and future opportunities for improving pseudomonad-based biological control are discussed.

Additional keywords: *phlD*, phyllosphere.

Diversity of biocontrol pseudomonads. Because the search for biocontrol agents typically relies on functional screens, only the most active isolates are typically identified. However, the biocontrol potential of strains that do not perform well in the bioassays used for selection is rarely assessed. Thus, it is unclear what the distribution of biocontrol activities is within different species of pseudomonads. Among the 94 *Pseudomonas* spp. named in the Approved Lists of Bacterial Names published in 1980 (65), those most commonly reported to harbor strains that can suppress plant diseases include *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, and *P. putida*. Additionally, nonpathogenic isolates of *P. syringae* have been identified as useful biocontrol agents (29,30). Strains of two newly named pseudomonad species, *P. brassicacearum* and *P. thevervalensis*, also have been shown to be inhibitory to plant pathogen growth (60). Thus, it seems likely that additional lineages with biocontrol activity have yet to be discovered. However, it seems premature to suggest that most strains of these novel species are functionally inactive in terms of biological control, given the limited scope of the functional screens applied.

Biocontrol activities have been reported in a number of pseudomonad strains; however, there does not appear to be a strict relationship between biocontrol activity and phylogeny. This may be due, in part, to the occurrence of biocontrol-associated genes on plasmids or other mobile elements. However, most genes currently associated with biocontrol activities in pseudomonads, such as the siderophore and antibiotic biosynthesis genes and the regulators thereof, appear to be fixed to the chromosome (37). Nonetheless, the lack of comprehensive investigations into the functional diversity and phylogeny of the various *Pseudomonas* species currently obscures the evolutionary origins and distribution of biocontrol genes within the genus. If there is a general lack of association between biocontrol functions and chromosomal phylogeny at the species level, it might be more useful to consider the question of whether biocontrol can be associated directly with phylogenetically distinct subspecies. Subspecies diversity can be delineated readily by the use of several different genotypic and/or phenotypic traits, but such studies sometimes lead to ambiguous and overlapping classifications even at the species level. For example, discrepancies between 16S-based phylogenies and biochemical assays have led to a call for the reclassification of *P. fluorescens* and *P. putida* (7); but, to date, no comprehensive revision of these species has been completed. Such a revision might provide the firm taxonomic framework that is required to understand the evolution and ecological significance of biocontrol activities within the genus.

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Despite the lack of association of phylogenetic variation with biocontrol phenotypes, the genetic diversity of pseudomonads has been defined at multiple levels using a variety of methods. Yamamoto et al. (71) showed that the sequences of just two conserved single copy genes can be used to classify isolates to the species level, but this approach has yet to be taken up by biocontrol researchers. This approach would be particularly useful in placing newly cultured strains within a standardized phylogenetic framework. To date, comprehensive genetic studies of pseudomonad biocontrol agents have only been conducted on one class of biocontrol pseudomonads, i.e., those that produce 2,4-diacetylphloroglucinol (DAPG). Researchers studying this group of pseudomonads have used a variety of methods including genomic fingerprinting, restriction fragment length polymorphism (RFLP) analyses of amplified genes, and gene sequences (16,40,46,58, 60,67). Such studies revealed multiple subspecies with varying levels of resolution. Despite differences in the strain collections analyzed in each study, certain subspecies were consistently identified. For example, genomic fingerprinting using random amplified polymorphism DNA and repetitive sequence based-polymerase chain reaction (PCR) led to nearly identical results as RFLP analyses of the *phlD* gene (40,46,47). These types of fine-scale taxonomic studies also have proven useful at identifying subspecies genotypes that differ in their host preference (5,34,45), a trait likely to influence biocontrol efficacy. Extremely fine degrees of genotypic variation have also been found using genomic subtraction of closely related isolates (39). However, the full significance of this and other forms of subspecies genetic variation in these biocontrol pseudomonads has yet to be established.

One goal of conducting genetic diversity studies is to use genotypic information to make predictions about complex and more difficult to assay phenotypes, such as biological control potential. Within *Pseudomonas* spp., clonal growth appears to be the dominant mode of propagation, and genotypes have been linked strongly with various phenotypes. For example, studies of carbon-source utilization profiles have shown that there is a high degree of concordance between genetic classifications of pseudomonads at the species level (13) and at the level of genotypic subspecies with known variations in biocontrol activities (33,35,46, 57). To more directly link differences in genotype with differences in biocontrol phenotype, one can analyze variants in genes closely related to biocontrol function, as has been done for several different antibiotic biosynthesis genes (14,40,57). However, the linkage between readily assayable genotype and biocontrol phenotype will not always be absolute. This is because small and unmeasured changes in genome sequence may confound the associations. For example, phase variation in some pseudomonad populations can regulate secretion of secondary metabolites that can mediate plant pathogen suppression (8,66). And, spontaneous mutations in the *gacS* and *gacA* genes of different biocontrol strains can arise in the rhizosphere, resulting in substantial subpopulations that are deficient in secondary metabolite biosynthesis and altered colonization potential (9,61). The significance of genetic variation within functionally distinct subspecies of biocontrol pseudomonads (e.g., those that produce DAPG) has yet to be fully exploited for applied biological control. For example, now that multiple distinct genotypes of DAPG-producing pseudomonads have been identified, standardized collections of these known variants need to be systematically assayed for efficacy in controlling diseases in different cropping systems.

Factors affecting the distribution of biocontrol pseudomonads. Comprehensive culture-based studies of the rhizosphere have indicated that individual plants harbor mixed populations of pseudomonads, many of which can suppress plant pathogens *in vitro* (3,4,48,60). However, given that complex interactions between rhizosphere bacteria can significantly affect the expression of biocontrol activities (53), it is generally unclear to what extent these different populations actually contribute to pathogen sup-

pression under typical growing conditions. Clearly, biasing population structure through inoculation with biocontrol bacteria can lead to significant disease suppression in the field, especially when significant populations colonize the target tissues. However, it seems reasonable to assume that multiple abiotic and biotic factors will affect the abundance and activities of native biocontrol pseudomonads. Thus, a key area of future research will focus on defining the environmental conditions that significantly alter the distribution of biocontrol pseudomonads.

To date, a number of studies have characterized the environmental factors that can affect the abundance of different pseudomonad populations on plant surfaces. Above ground, colonization of newly emerged tissues is rapid and promoted by rainfall, high humidity, ice nucleation activity, virulence, and host susceptibility (26,27,70). Below ground, the relative abundance and diversity of plant-associated *Pseudomonas* spp. are known to vary with host species, cultivar, and plant tissue (3,4,41,48) as well as soil type, structure, and fertility (5,36,52). Soil temperature and moisture also can affect root colonization and activities of biocontrol pseudomonads in significant ways (20,62). The influence of plant species on root colonization appears to be particularly important in determining colonization of specific bacterial populations that might lead to biocontrol. For example, Mazzola and Gu (42) showed that wheat plants, which can harbor significant populations of biocontrol pseudomonads, grown in orchard soils suffering from apple replant disease can increase the abundance of *Pseudomonas* spp. and reduce disease pressure. Large-scale surveys of corn and soybean fields in Ohio have revealed that soil type and crop host play key roles in determining the degree of root colonization by native populations of biocontrol pseudomonads (45). In total, these studies have revealed several key factors that influence the abundance of biocontrol pseudomonads in agricultural systems.

In my laboratory, we are investigating the associations between native populations of pseudomonads that produce the antibiotic compound DAPG, plant health, and crop productivity in different cropping systems. Our data indicate that, in most instances, native populations of DAPG producers are positively correlated with corn stand and yields (D. Rotenberg and B. B. McSpadden Gardener, *unpublished data*). This is consistent with the hypothesis that native populations of DAPG producers contribute to maintaining plant health by suppressing pathogens (69). However, farm management practices can also significantly alter the relative abundance of biocontrol pseudomonads by simply altering the soil environment. For example, a comparison of continuously cropped versus rotated corn and soybean fields, planted under no-till conditions, has shown that DAPG producers are more abundant under conditions of elevated soilborne disease pressure (Table 1). In this particular instance, soil moisture was elevated and soil temperatures depressed in response to elevated levels of corn residue. This led to elevated levels of *Pythium* infections and lower stands of both corn and soybeans following previous corn crops. An earlier study also indicated that the abundance of DAPG producers was increased following root infection by the take-all fungus, *Gaeumannomyces graminis* var. *tritici* (47). That study also provided evidence that shifts in the abundance of several bacterial populations, including other fluorescent pseudomonads, may facilitate pathogen suppression by DAPG-producing *Pseudomonas* spp. Other recent work has shown that compost amendments can significantly increase soil fertility and the abundance of DAPG producers in the rhizosphere while having varying effects on crop stand and vigor on a per plot basis (M. S. Benitez, D. Rotenberg, A. Camp, and B. B. McSpadden Gardener, *unpublished data*).

While it is currently unclear whether changes in rhizosphere bacterial community structure associated with pathogen infection generally lead to subsequent pathogen suppression, we hypothesize that competitive saprophytes that use antibiotics to secure

access to consumable resources will increase as a result of the “collateral damage” caused by invading root pathogens. The net effect might be to slow pathogen ingress and/or reduce the viability of pathogen biomass located proximal to these competitive saprophytes. In some systems, this competition between pathogens and opportunistic saprophytes may lead to the development of long-lasting disease suppression (e.g., take-all decline) (15,56). However, it seems unlikely that continuous cropping will typically lead to pseudomonad-based disease suppressive soils. In the continuous no-till corn fields described above, net pathogen fitness exceeded that of their bacterial competitors, and various root diseases continued to increase in severity over the 5 years for which monoculture was maintained. Thus, in addition to crop and soil type, crop health and cropping system will also influence the abundance and activities of specific populations of biocontrol pseudomonads.

Interactions with plants, insects, and microbes. *Pseudomonas* strains with the capacity to promote plant health have been described as biocontrol agents and plant-growth promoting (rhizo)bacteria. Collectively, they can be considered opportunistic mutualists, offering their plant hosts additional fortification against pathogens albeit on uncertain terms. Their beneficial effects result from the expression of multiple activities that act to directly and indirectly inhibit pathogen activities. Direct antagonism is largely mediated by antibiotic production (25,55), which is hypothesized to be a primary determinant in specific suppression of soilborne diseases in various systems (15,59,69). Whereas most studies have focused on suppression of fungi and oomycetes, others have shown that plant-pathogenic nematodes may also be suppressed by the production of various inhibitory compounds (12,68). Most biocontrol strains are known to produce one or more antibiotic factors. And while genomic analyses are increasing the depth of our knowledge of the physiology of individual strains (37), the full spectrum of metabolites and the amounts of each secreted under typical cropping conditions remains a mystery. Competition for nutrients may also be important, but its quantitative contribution to biological control is difficult to measure. Indeed, predicting biocontrol efficacy from in vitro assays has not provided consistent results (17,28). Indirect antagonism is largely mediated by the stimulation of host defenses (1). Although little is known about the factors that influence the expression of these activities and the specific conditions under which they promote plant health in the field, a large number of investigations point to the complexity of ecological interactions that can alter the relationships between *Pseudomonas* spp., their plant hosts, and plant pathogens.

Diverse rhizosphere microbes and their plant hosts can secrete multiple metabolites that can alter gene expression (53) and may, ultimately, alter the physiology of biocontrol pseudomonads, soilborne plant pathogens, and their plant hosts in significant ways. Different *Pseudomonas* spp. are capable of producing a variety of ecologically significant compounds, including antibiotics, plant hormones, nutrient chelators, exoenzymes, and signal molecules (54). Such secreted compounds can act to suppress pathogen activity via antibiosis and competition, and some may also stimulate plant host defenses (1). Interestingly, it has been suggested that such molecules are more frequently expressed by plant-associated pseudomonads as opposed to soilborne pseudomonads (21). Furthermore, gene expression required for the biosynthesis of such compounds has been shown to be up-regulated in the rhizosphere (23,51). Beyond the diversity of compounds that might mediate interspecies interactions, it is also apparent that some compounds can have multiple effects on the community. For example, DAPG has been shown to inhibit the growth of diverse pathogens, stimulate plant host defenses, and stimulate amino acid uptake in roots (54). In turn, the biosynthesis of DAPG can be affected by a variety of abiotic (19,52) and biotic (51) variables, indicating that, quantitatively, its production is context dependent. Indeed, the expression of DAPG-mediated biocontrol activities can be altered by pathogens (18,50,64), other biocontrol strains (34,38), and other members of soil and rhizosphere microbial communities that might otherwise be neutral or positive to plant health (2,31,32,47). Additionally, the biosynthesis of DAPG may be affected by other compounds thought to contribute to biological control. For example, pyoluteorin (a pseudomonad-derived antibiotic) and salicylate (a compound that can stimulate host defense pathways) can be synthesized by several DAPG-producing pseudomonads, but act to repress biosynthesis of DAPG in culture (63). Such studies indicate the variety and potential complexity of intra- and interspecies microbial interactions that might modulate the expression of any single biocontrol mechanism. However, the frequency and magnitude of such interspecies interactions and, thus, their phenomenological significance have not been assessed under field conditions. Such studies might lead to a more compelling description of rhizosphere ecology and reveal why soilborne diseases are not consistently controlled through inoculation with biocontrol agents.

Current applications of biocontrol pseudomonads and prospects for improvement. Strains of fluorescent pseudomonads have been found that suppress a wide variety of plant diseases. Thus, there is substantial interest in developing some of these into biopesticides, to be used alone or in combination with

TABLE 1. Effects of different corn and soybean rotations on the abundance of 2,4-diacetylphloroglucinol (DAPG)-producing (i.e., *phlD*⁺) pseudomonads, soil variables, and plant health¹

Rotation ¹	Log <i>phlD</i> ^{2v}	% <i>phlD</i> ^{2w}	Incidence ^{2x} <i>phlD</i> ⁺ >log 5/g	% Volumetric water content ^{2y}	Soil temperature (°C)	Root fresh weight (mg)	<i>Pythium</i> infection ^{2z}
CCCC	5.1 a	20 b	0.67	22.5 b	21.5 a	370 c	4.4 d
SCSC	4.9 a	6 a	0.43	19.5 a	22.3 b	531 d	3.9 c
CSCS	5.5 b	60 c	0.67	21.8 a	21.9 a	177 a	2.9 b
SSSS	5.1 a	18 b	0.50	19.4 a	23.0 b	245 b	2.4 a

¹ Mean values are tabulated, and for each column, values followed by different letters are significantly different based on Dunn's multiple range test ($P < 0.01$).

^{2v} Cropping sequence of corn (C) and soybeans (S) from 2001 through 2004. The crop assayed in 2004 is underlined.

^{2w} Population size of DAPG-producing *Pseudomonas* spp. estimated by the polymerase chain reaction-based assay described by McSpadden Gardener et al. (45).

^{2x} Relative abundance of DAPG-producing pseudomonads expressed as a percentage of all cultured pseudomonads.

^{2y} Incidence of plants harboring more than 10^5 *phlD*⁺ pseudomonads per gram of root (fresh weight).

^{2z} Volumetric water content measured at the time of sampling using radio interferometry.

² Mean root infection levels by oomycetes assayed using *Pythium*-specific primers targeting the actin gene. Amplifications were scored across three fivefold dilutions on a scale from 0 (no amplification) to 6 (maximum amplification in most dilute samples).

^v Population size of DAPG-producing *Pseudomonas* spp. estimated by the polymerase chain reaction-based assay described by McSpadden Gardener et al. (45).

^w Relative abundance of DAPG-producing pseudomonads expressed as a percentage of all cultured pseudomonads.

^x Incidence of plants harboring more than 10^5 *phlD*⁺ pseudomonads per gram of root (fresh weight).

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^z Mean root infection levels by oomycetes assayed using *Pythium*-specific primers targeting the actin gene. Amplifications were scored across three fivefold dilutions on a scale from 0 (no amplification) to 6 (maximum amplification in most dilute samples).

chemical pesticides. To date, commercial interest in *Pseudomonas* biocontrol strains has been more limited than for spore-forming microbes such as *Bacillus* and *Trichoderma*. Nonetheless, several pseudomonad strains are currently registered with the U.S. Environmental Protection Agency as biopesticides and have been made commercially available to growers (22). These include *P. chlororaphis* strain 63-28 for control of wilt diseases and stem and root rot; *P. chlororaphis* TX-1 for control of turfgrass diseases; *P. syringae* strain ESC-10 for control of fungal post-harvest diseases; *P. fluorescens* strain A506 for control of fire blight and russetting of pome fruits; and combinations of *P. fluorescens* strains A506 and 1629RS and *P. syringae* 742RS for prevention of frost-induced damage on several crops. Additionally, the extensive field testing of additional strains by different academic and government laboratories (11) indicates that additional opportunities exist for commercial development of pseudomonads for the biological control of a variety of crop diseases. However, to do so, more comprehensive evaluations of the cost and benefits of experimental strains relative to other disease control strategies need to be done in order for their commercial potential to be properly evaluated.

The relative safety and environmental impact of natural and genetically engineered pseudomonad biocontrol strains has been thoroughly evaluated. As with any disease control measure, one can expect application of biocontrol pseudomonads to alter the structure of plant-associated microbial communities. Indeed, the goal of biological control is to reduce plant pathogen activity, often by suppressing pathogen abundance directly or indirectly. The magnitude of disturbances to nontarget organisms that may be caused by inoculation is generally considered to be small and transient (6,24,49). This is consistent with the lack of negative impacts associated with widespread rhizobial inoculation of legumes, and it is in stark contrast to the dramatic shifts in microbial community structure that results following the application of more conventional farm practices. Given the widespread distribution of pseudomonads with biocontrol activities in the environment, it may be possible to develop targeted biocontrol strategies. For example, strains of DAPG-producing pseudomonads have been recovered from most agricultural soils. We hypothesize that targeted isolation and reintroduction of individual strains on a site-specific basis would provide an approach that minimizes the potential disruption of microbial community structures by virtue of avoiding the introduction of nonnative strains. Recently, we implemented such an approach and found it to be effective at increasing productivity in conventional and organic soybean fields (43,44).

A multitude of possibilities still exists for the successful application and management of *Pseudomonas*-mediated biological control of plant pathogens; however, more research into the microbial ecology of the genus will be required to ensure success of future biocontrol applications. The path from functional screens to the characterization of genetic mechanisms has proven useful in the past, but more directed screens may point the way in the future. Intensive studies of DAPG producers have shown that subspecies diversity is extensive and may be tapped for applications to different plant disease problems. The use of biocontrol-related gene markers to identify novel but functionally related strains could rapidly accelerate strain discovery. However, currently, only genes involved in antibiotic biosynthesis have been revealed as useful candidates for such directed selection procedures. Characterizing the genetic basis for the pseudomonad-mediated induction of plant host defense pathways should provide additional useful markers, opening up the possibility of further expanding collections related to the most effective pseudomonad biocontrol strains. Biocontrol markers will also be useful for mapping the distribution of biocontrol populations in production environments. Further investigating the biogeography of various biocontrol populations can be useful for identifying novel genetic

variants especially from diverse environments, which then can be used for directed screening programs.

Distinct genotypes appear to be endemic to specific geographic locations (10), so systematic bioprospecting of soils and plants from different ecosystems should provide a wealth of novel and potentially useful biocontrol strains. New screening programs might focus on evaluating the utility of a core set of strains for application on a single crop and its most important pathogens. This could lead to commodity-specific applications where strain testing is tailored toward the salient features of a more comprehensive pest management program. A greater understanding of which biological control and pathogen populations are present and active in a given area also may be useful for identifying locations with the greatest probability of responding to inoculations, e.g., those where native populations of biocontrol pseudomonads are low.

The limited viability of *Pseudomonas* spp. has hindered practical applications, especially in those systems where on-farm applications are not considered economically feasible. However, the use of strains with superior colonization potential (e.g., Q9r1-96) (56) may allow for workable applications. For example, we have developed a two-step inoculation process that allows for convenient in-hopper seed treatments with proven yield increasing potential (43,44, *unpublished data*). The process uses partially hydrated and autoclaved soybean seeds as a matrix for keeping the DAPG-producing pseudomonads viable for 6 months or more. Two to seven days prior to planting, the farmer activates the inoculum by the addition of a specified amount of chlorine-free water, creating a condition allowing for the multiplication of the bacteria. At planting, the DAPG producers are rinsed from the matrix and diluted to a level that provides for seedling root colonization following spray treatment and mixing of the seed in the hopper. Further research on the ecology of biocontrol pseudomonads may reveal the underlying causes or contexts under which biological control fails or, alternatively, is most successful. Such knowledge will be useful for further developing biocontrol applications with increased efficacy and consistency.

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