Effect of Population Density of *Pseudomonas fluorescens* on Production of 2,4-Diacetylphloroglucinol in the Rhizosphere of Wheat

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ABSTRACT

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The role of antibiotics in biological control of soilborne pathogens, and more generally in microbial antagonism in natural disease-suppressive soils, often has been questioned because of the indirect nature of the supporting evidence. In this study, a protocol for high pressure liquid chromatography/mass spectrometry is described that allowed specific identification and quantitation of the antibiotic 2,4-diacetylphloroglucinol (Phl) produced by naturally occurring fluorescent *Pseudomonas* spp. on roots

of wheat grown in a soil suppressive to take-all of wheat. These results provide, for the first time, biochemical support for the conclusion of previous work that Phl-producing fluorescent *Pseudomonas* spp. are key components of the natural biological control that operates in take-all-suppressive soils in Washington State. This study also demonstrates that the total amount of Phl produced on roots of wheat by *P. fluorescens* strain Q2-87, at densities ranging from approximately 10^5 to 10^7 CFU/g of root, is proportional to its rhizosphere population density and that Phl production per population unit is a constant (0.62 ng/ 10^5 CFU). Thus, Phl production in the rhizosphere of wheat is strongly related to the ability of the introduced strain to colonize the roots.

Various mechanisms account for the ability of fluorescent *Pseudomonas* strains to control plant pathogens, including competition for iron and other nutrients, niche exclusion (35), induction of systemic resistance (23,24), and production of antimicrobial metabolites (32). Our studies have focused on the role of the antibiotic 2,4-diacetylphloroglucinol (Phl) in biological control of soilborne pathogens by fluorescent *Pseudomonas* spp. (4,36). Phl is a phenolic metabolite with antibacterial, antifungal, anthelmintic, and phytotoxic properties (32). Genetic studies, modeled after Koch's postulates, demonstrated that Phl produced by fluorescent *Pseudomonas* strains plays a major role in the suppression of various soilborne plant pathogens (7,11,28,34). Numerous strains of Phl-producing *Pseudomonas* spp. were isolated from soils from diverse geographical regions and were readily isolated from roots of plants grown in soils that are naturally suppressive to diseases such as black root rot of tobacco and Fusarium wilt of tomato (12). Moreover, Phl-producing fluorescent *Pseudomonas* spp. were highly enriched in soils that are suppressive to take-all of wheat and are key components of the natural biological control that operates in these soils (25,26).

The significance of antibiotics in biological control, and more generally in microbial antagonism in natural disease-suppressive soils, often has been questioned in the past because of the indirect nature of the supporting evidence and the perceived constraints to antibiotic production in soil environments (9,37). Because of the biotic and abiotic complexity of soils, there are several inherent

difficulties in detecting antibiotics produced by microorganisms in soil and rhizosphere environments. Depending on the nature of the metabolite, recovery and detection may be hampered by chemical instability of the compound, irreversible binding to soil colloids or organic matter, or microbial decomposition (31). Sensitive methods have been developed by which the expression of specific traits of biocontrol agents, such as antibiotic production, can be measured in natural environments. One approach involves the use of reporter gene systems (17,18). One of the disadvantages of this approach in studying in situ antibiotic production is that it does not provide an accurate measure of the amount of the antibiotic produced. Bioanalytical techniques like thin layer chromatography and high pressure liquid chromatography (HPLC) are now being used to detect and quantify antibiotics produced by microorganisms in situ (31). The versatility, resolving capability, and quantitative accuracy of HPLC make it one of the best direct methods to study the production of antibiotics in situ. HPLC has been successfully used for the in situ detection of a variety of antibiotics including phenazine-1-carboxylic acid (33), herbicolin A (13), pyrrolnitrin (14), gliotoxin (19), and Phl (2,11).

To further elucidate the role of introduced and naturally occurring Phl-producing *Pseudomonas* spp. in biological control of soilborne plant pathogens and microbial antagonism in natural disease-suppressive soils, we developed a protocol for HPLC/mass spectrometry (MS) to identify and quantify Phl produced on roots of wheat plants grown in raw soil. We then describe the effect of population density on production of Phl in situ by *P. fluorescens* Q2-87. Finally, we demonstrate, for the first time, that Phl is produced by naturally occurring fluorescent *Pseudomonas* spp. on roots of wheat grown in a soil that is suppressive to take-all of wheat.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. fluorescens* strains Q2-87 and Q8r1-96 both produce the antibiotic Phl. Strains Q2-87 and Q8r1-96 were isolated in 1987 and 1996, respectively, from wheat grown in soil collected from an agricultural field near

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Quincy, WA (22,25). Spontaneous rifampin-resistant derivatives of strains Q2-87 and Q8r1-96 were used in the experiments. Both strains were cultured on King's medium B (KMB) agar (15) supplemented with rifampin (100 $\mu\text{g ml}^{-1}$). Naturally occurring fluorescent *Pseudomonas* spp. were isolated from wheat roots on KMB agar supplemented with cycloheximide (100 $\mu\text{g ml}^{-1}$), chloramphenicol (13 $\mu\text{g ml}^{-1}$), and ampicillin (40 $\mu\text{g ml}^{-1}$) (KMB⁺) (29).

Soils. Soils were collected from two sites near Quincy, WA. Soil from an agricultural field, designated Quincy take-all decline (TAD), is suppressive to take-all of wheat. In 1980, the Quincy TAD field had been cropped continuously to wheat for 22 years; between 1980 and 1995, other crops besides wheat also were grown. A noncropped virgin soil, designated Quincy virgin, was covered by native vegetation such as sagebrush and bunchgrass and was located near the Quincy TAD field. The Quincy virgin soil is conducive to take-all of wheat. The soils were collected in March 1995 from the upper 30 cm of the soil profile, air-dried for 1 week, and passed through a 0.5-cm-mesh screen prior to use. Their physical and chemical properties were determined by the Analytical Sciences Laboratory, University of Idaho, and described previously (26,36).

Seed treatment. Wheat seeds (cv. Penewawa) were treated with an aqueous solution of 1% methyl cellulose (Sigma Chemical Co., St. Louis) or with suspensions of strains Q2-87 or Q8r1-96 in 1% methyl cellulose. The coated seeds were air-dried for 5 h in a laminar flow cabinet. Final densities of Q2-87 were approximately 10^2 , 10^3 , 10^4 , 10^5 , or 10^6 CFU per seed, as determined by dilution plating on KMB⁺ supplemented with rifampin (100 $\mu\text{g ml}^{-1}$). The final density of Q8r1-96 was approximately 10^5 CFU per seed.

Plant cultivation. Approximately 25 wheat seeds were sown in square PVC pots (8 cm high, 7.5 cm wide) containing 200 g of raw soil after 50 ml of water supplemented with metalaxyl (Novartis Crop Protection, Greensboro, NC) was added at 2.5 mg active ingredient per ml to control *Pythium* root rot. A 1-cm layer of soil was spread on top of the seeds. Plants were grown in a controlled-environment chamber at 16°C with a 12-h photoperiod. Pots received 50 ml of dilute (2:3, vol/vol) Hoaglund's solution (macroelements only) twice a week. After 3 weeks of growth, the plants were harvested and loosely adhering soil was removed from the roots by gently shaking the plants. The root systems were excised and stored overnight in petri dishes at 4°C prior to the isolation of bacteria and the extraction of Phl. Each treatment was replicated two to four times, and five pots were used for each replicate.

Isolation of Phl-producing *Pseudomonas* spp. from roots of wheat. One gram of roots and associated rhizosphere soil harvested from each pot was suspended in 5.0 ml of sterile water and shaken vigorously for 1 min on a Vortex mixer. The samples were subsequently sonicated in an ultrasonic cleaner (Branson 521; Branson Cleaning Equipment Co., Shelton, CT) for 1 min, and then serial dilutions of the root wash were plated onto KMB⁺ plus rifampin to determine the population density of Q2-87 or Q8r1-96. To determine the population density of naturally occurring Phl-producing fluorescent *Pseudomonas* spp., root washes obtained from wheat plants grown in Quincy virgin and Quincy TAD soils were plated onto KMB⁺. Plates were incubated at 25°C and colonies were enumerated after 48 h. Colonies of fluorescent *Pseudomonas* spp. were differentiated from nonfluorescent colonies under UV light (wavelength 366 nm). The number of fluorescent *Pseudomonas* spp. that harbor the genes for Phl was determined by colony hybridization followed by polymerase chain reaction (PCR) analysis (26).

Colony hybridization and PCR analysis. Transfer of bacterial colonies to Hybond-N⁺ nylon membranes (Amersham International, Little Chalfont, United Kingdom) was performed by standard methods (27). After air-drying, the membranes were baked for 1 h at 80°C in a vacuum oven. To remove bacterial cell debris, the membranes were washed for 1.5 h at 42°C in a solution containing 2× SSPE (20 mM NaH₂PO₄ [pH 7.4], 0.36 M NaCl, and 2 mM EDTA), 0.1% sodium dodecyl sulfate (SDS), and pronase (100 $\mu\text{g ml}^{-1}$)

and washed again for 1 h at 56°C in 2× SSPE-0.1% SDS. Hybridizations were performed under high stringent conditions by standard methods (27). The Phl probe consisted of a 745-base pair (bp) DNA fragment amplified by PCR from Phl-producing strain *P. fluorescens* Q2-87 (26) and was generated by random-primed labeling using the nonradioactive digoxigenin system (Boehringer GmbH, Mannheim, Germany). The hybridized probe was immunodetected with anti-digoxigenin-AP-Fab fragments, and Phl-positive colonies were visualized with the calorimetric substrates nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate, according to protocols provided by the supplier. The lower limit of detection for indigenous Phl-producing *Pseudomonas* spp. was 10^4 CFU g⁻¹ fresh weight of root (26). Positive colonies of fluorescent *Pseudomonas* strains were isolated and purified on KMB agar plates. PCR analysis of the isolated colonies was performed as a quality check of the colony hybridization. The oligonucleotide primers Phl2a and Phl2b were developed from sequences within *PhlD*, one of the six genes in the Phl biosynthetic locus of *P. fluorescens* Q2-87 (GenBank accession no. U41818). Primers Phl2a and Phl2b were synthesized by Operon Technologies Inc. (Alameda, CA) and yielded a 745-bp fragment. DNA isolation by cell lysis and PCR amplification was carried out according to the methods described by Raaijmakers et al. (26).

Antibiotic extraction from roots of wheat grown in natural soil. Phl was isolated from roots of wheat according to the method described by Bonsall et al. (2). Thirty grams of wheat roots with adhering rhizosphere soil, without remnants of seeds and obtained from approximately 80 to 100 plants, was mixed in a 250-ml flask with 40 ml of 80% acetone acidified to pH 2.0 with 10% trifluoroacetic acid (TFA) and then shaken (200 rpm) for 2 h at room temperature. Samples were subsequently filtered (Whatman no. 1; Whatman International, Ltd., Maidstone, England) through a Buchner funnel (Coors, Golden, CO), and the filtrate was centrifuged at $12,400 \times g$ for 30 min at 4°C to remove small soil particles. The supernatant was evaporated to a volume of 8 ml, acidified to pH 2.0 with 10% TFA, extracted twice with 10 ml of ethylacetate, and evaporated to dryness. Extracts were suspended in 1 ml of 35% acetonitrile (ACN) and 0.1% TFA and then centrifuged in an Eppendorf 5415 centrifuge (Brinkmann Instruments, Inc., Westbury, NY) at $16,000 \times g$ for 20 min at 4°C prior to separation and identification by HPLC and MS. The extraction efficiency of Phl was approximately 60% (2).

HPLC photodiode array (PDA) spectroscopy. The Waters HPLC system (Waters Corporation, Milford, MA) consisted of a 717 Plus autosampler, 600E solvent delivery system, 600 controller, and 996 PDA detector. The HPLC protocol described by Bonsall et al. (2) was modified as follows. Root extracts were fractionated by C₁₈ reverse-phase HPLC (Waters symmetry column, 3.9×150 mm; Waters Corporation) with 50- to 200- μl sample injections. Solvent conditions included a flow rate of 0.5 ml/min with a 2-min initialization at 10% ACN-0.1% TFA, followed by a 20 min gradient to 100% ACN-0.1% TFA using Waters curve profile #5 (Waters Corporation). HPLC gradient profiles were monitored at 270 and 330 nm, which represent the peak maxima of Phl in the designated solvent system. A seven-point standard curve used for quantification was generated by spiking known concentrations of pure Phl into root samples (30 g) collected from wheat grown in Quincy virgin soil prior to the extraction procedure described above. A highly significant linear relationship was found for the standard curve ($\text{Phl} = 0.00156 \times A$, $r^2 = 0.99$, $P < 0.0001$), in which Phl represents the total amount of Phl (ng) and A represents the area of the Phl peak. The lowest amount of Phl extractable from the rhizosphere was 200 ng. For detection of Phl produced by relatively low population densities of introduced or resident Phl-producing *Pseudomonas* spp. (approximately 10^5 CFU/g of root), two replicate samples were pooled.

MS. In a mass spectrometer, molecules of a compound are ionized to give the parent molecular weight ion (the actual molecular

weight of the compound) and a series of fragment ions. Consequently, mass spectra show a series of peaks corresponding to the mass (m) to charge (e) ratio values (m/e values) of the positive ions produced from the compound. The structure of the compound can be deduced from this fragmentation pattern either by library matching (e.g., Wiley library, John Wiley & Sons, Inc., New York) or by spectral interpretation. The Waters Integrity LC-MS system (Waters Corporation) used in this study consisted of an Alliance 2690 separation module with a 996 PDA detector and an electron ionization Thermabeam mass detector (TMD) (Waters Corporation, Milford, MA). Root extracts were fractionated by C_{18} reverse-phase HPLC with 100- μ l sample injections as described above. Solvent conditions included a flow rate of 0.35 ml/min with a 2-min initialization at 10% ACN-2.0% acetic acid, followed by a 20-min linear gradient to 100% ACN-2.0% acetic acid. The TMD conditions included a helium flow rate of 15 psi with the temperatures of the ion source, expansion region, and nebulizer set

at 220, 80, and 84°C, respectively. TMD chromatograms were monitored at a total ion current of 170-355 m/e . The detection limit of Phl for electron ionization involving MS was 200 ng.

Statistical analysis. The experiments described in this study were performed at least twice. Regression analyses (SAS release 6.11; SAS Institute Inc., Cary, NC) were performed to determine the relationship between (i) rhizosphere population densities of Q2-87 and in situ production of Phl, and (ii) rhizosphere population densities of Q2-87 and the amount of Phl produced per population unit (10^5 CFU). In all regression analyses, variables and intercepts were tested for differences from zero by performing t tests ($\alpha = 0.05$). When the intercepts were not significantly different from zero, regressions were forced through the origin. The probability based match (PBM) algorithm was used in MS to determine the percent match between the submitted spectrum and the library spectrum (e.g., Wiley library, John Wiley & Sons, Inc.). Differences between treatments in population densities and

Fig. 1. High pressure liquid chromatography (HPLC) chromatograms and electron ionization mass spectrum of 2,4-diacetylphloroglucinol (Phl). **I**, Pure Phl; **II**, pure Phl spiked into extracts of root samples collected from nontreated wheat grown in raw Quincy virgin soil; **III**, extracts from root samples collected from wheat treated with strain Q8r1-96; and **IV**, extracts from root samples collected from wheat treated with strain Q2-87. **A**, HPLC chromatograms, dotted lines, and arrowheads indicate the area of the Phl peak; **B**, photodiode array UV spectra of Phl for chromatogram peak shown in **A** (indicated with an arrow); and **C**, electron ionization mass spectra of Phl for chromatogram peak shown in **A** (indicated with an arrow).

amounts of Phl produced in situ were determined by analysis of variance followed by Tukey's studentized range test (SAS release 6.11; SAS Institute Inc.). When data were not normally distributed, differences between treatments were analyzed by Wilcoxon rank-sum test ($\alpha = 0.05$).

RESULTS

Identification of Phl by HPLC/MS. HPLC/MS analysis was performed on four different samples: (i) pure Phl, (ii) pure Phl spiked into extracts of root samples collected from nontreated wheat grown in raw Quincy virgin soil, (iii) extracts of root samples collected from wheat treated with Phl-producing strain *P. fluorescens* Q8r1-96, and (iv) extracts of root samples collected from wheat treated with Phl-producing strain *P. fluorescens* Q2-87 (Fig. 1). The HPLC chromatograms showed that peaks from all four samples elute at retention times ranging from 25 min to 26.5 min, the same area where pure Phl elutes under the given conditions (Fig. 1 A.I to A.IV). Because the peak maxima of 270 and 330 nm are characteristic of Phl in the specified solvent mixture (2), spectral analysis of the peaks by PDA spectroscopy allowed a positive identification of the Phl peak in all four chromatograms including those of the root extracts collected from wheat treated with either Q8r1-96 or Q2-87 (Fig. 1 B.I to B.IV). Phl was not detected in root extracts collected from nontreated wheat grown in raw Quincy virgin soil (data not shown). Subsequent MS showed that the mass spectrum of pure Phl is characterized by three intense peaks with m/e values of 210, 195, and 177 (Fig. 1 C.I to C.IV). The highest mass peak of 210 is the parent molecular ion (M^+) and represents the molecular weight of Phl. The peak at m/e 195 is most likely due to a neutral fragment loss of $\cdot\text{CH}_3$ (M^+)-15 at $m/e = 210$), whereas the peak at m/e 177 corresponds to a neutral fragment loss of $\cdot\text{H}_2\text{O} + \cdot\text{CH}_3$ (M^+)-33 at $m/e = 210$) from the parent molecular ion. In addition to these three major peaks, the mass spectrum of Phl also is characterized by three minor peaks with m/e values of 153, 121, and 90. PBM of the derived mass spec-

trum of pure Phl with the Wiley library spectrum showed a 93% fit. Identical mass spectra were obtained from root extracts spiked with pure Phl (79% fit) and from root extracts collected from wheat treated with either Q8r1-96 (83% fit) or Q2-87 (95% fit). These results demonstrate that, in addition to HPLC, MS enables positive identification of Phl produced by fluorescent *Pseudomonas* strains introduced onto roots of wheat grown in a raw soil.

Effect of population density on Phl production by *P. fluorescens* Q2-87. Dose-response studies were performed to determine the relationship between rhizosphere population densities of Q2-87 and in situ production of Phl. Wheat seeds treated with different densities of Q2-87 were sown in raw Quincy virgin soil, and rhizosphere population densities of Q2-87 and Phl production were determined after 3 weeks of plant growth. Regression analysis showed a highly significant linear relationship between the total amount of Phl produced on roots of wheat and the rhizosphere population density of Q2-87 (Fig. 2). At population densities of Q2-87 smaller than 10^5 CFU/g fresh weight of root, Phl could not be detected. Logarithmic and quadratic relationships between the total amount of Phl and the rhizosphere population density of Q2-87 were considerably less significant ($r^2 = 0.45$ and 0.59 , respectively) than the linear relationship shown in Figure 2. Data analysis also demonstrated that the amount of Phl produced per population unit (10^5 CFU) was independent of the rhizosphere population density of Q2-87 ($r^2 = 0.07$, $P = 0.24$) and averaged 0.62 ng/ 10^5 CFU (Fig. 3). These results indicate that, for population densities of Q2-87 ranging from approximately 10^5 to 10^7 CFU/g fresh weight of root, the total amount of Phl produced on roots of wheat is proportional to the rhizosphere population density, and Phl production per population unit is a constant.

Phl production on roots of wheat grown in a take-all decline soil. Recently, we demonstrated that naturally occurring Phl-producing fluorescent *Pseudomonas* spp. were highly enriched in take-all-suppressive soils from Washington State and are key components of the specific suppression that operates in these soils (25, 26). To provide biochemical support for the role of Phl in take-all decline, Phl production and population densities of naturally occurring Phl-producing *Pseudomonas* spp. were determined on roots

Fig. 2. Relationship between the rhizosphere population density of *Pseudomonas fluorescens* Q2-87 and the total amount of the antibiotic Phl produced on roots of wheat grown in raw Quincy virgin soil. Wheat seeds were treated with Q2-87 at densities of approximately 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 CFU per seed in order to achieve a range of population densities in the rhizosphere. Results of four experiments are shown, representing a total of 22 replicates; five pots were used for each replicate.

Fig. 3. Relationship between the rhizosphere population density of *Pseudomonas fluorescens* Q2-87 and the amount of Phl produced per population unit (10^5 CFU). Wheat seeds were treated with Q2-87 at densities of approximately 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 CFU per seed in order to achieve a range of population densities in the rhizosphere. Results of four experiments are shown, representing a total of 22 replicates; five pots were used for each replicate. SE = standard error.

of wheat plants grown for 3 weeks in Quincy TAD soil and in Quincy virgin soil (Table 1). Neither Phl-producing *Pseudomonas* spp. nor Phl were detected on roots of wheat grown in raw Quincy virgin soil, which is conducive to take-all of wheat. In contrast, indigenous Phl-producing fluorescent *Pseudomonas* spp. were present on roots of wheat grown in the Quincy TAD soil at a density of 4.3×10^6 CFU/g fresh weight of root, and the total amount of Phl detected was 19.1 ng/g fresh weight of root. The amount of Phl detected on roots of wheat treated with strain Q8r1-96, which originally was isolated from the Quincy TAD soil, was 254.5 ng/g fresh weight of root. Given the differences in population densities, however, no significant difference was observed in Phl production per population unit between Quincy TAD and the Q8r1-96 treatment. Moreover, Phl production per population unit (10^5 CFU) in Quincy TAD and the Q8r1-96 treatment was also similar to the average value reported earlier for strain Q2-87 (Fig. 3).

DISCUSSION

To detect and quantify Phl and other antibiotics by HPLC, the extracted and separated compounds need to be positively identified. Identification of Phl and other compounds on the basis of their retention time is not sufficient. Spectral analysis by PDA detectors is required to provide the necessary information about peak homogeneity and identity. In most cases, spectral analysis over a range of wavelengths will provide sufficient information on the compound. However, spectral analysis may have its limits. Determining the identity of compounds in overlapping peaks may be difficult if the spectra of the compounds are similar and the peaks are poorly resolved (5). Also, deviations in the spectral characteristics of the compound can be induced by volatilization of the solvents and may occur after derivitization. This study demonstrates that, in addition to HPLC, MS provides a means to confirm the identity of antibiotics produced by fluorescent *Pseudomonas* spp. on roots of plants grown in natural soils.

Fluorescent *Pseudomonas* spp. have been implicated in the specific suppression that operates in soils that are naturally suppressive to certain soilborne pathogens (1,3,16). The results presented in this study demonstrate, for the first time, that the antibiotic Phl is produced by naturally occurring fluorescent *Pseudomonas* spp. on roots of wheat grown in a take-all-suppressive soil and not in a complementary take-all-conducive soil. The amount of Phl produced per population unit on roots of wheat grown in the Quincy TAD soil was very similar to the amounts produced by *Pseudo-*

monas strains Q2-87 and Q8r1-96, two strains that were originally isolated from Quincy TAD soil and that provide significant control of take-all of wheat (22,25,34). These biochemical data support the conclusion of previous work that Phl-producing fluorescent *Pseudomonas* spp. are key components of the natural biological control that operates in take-all-suppressive soils in Washington State (25).

The natural environments in which either introduced or naturally occurring antibiotic-producing *Pseudomonas* spp. function are subjected to changes in abiotic and biotic conditions. Several abiotic factors such as oxygen, temperature, specific carbon and nitrogen sources, and microelements have been identified or proposed to influence in situ antibiotic production by fluorescent *Pseudomonas* spp. (6,10,21,28,30). Among the biotic factors that may play a determinative role in the regulation of antibiotic production are the plant host (8,20), the pathogen (6), the indigenous microflora (39), and the cell density of the producing strain (38,40). This study demonstrates that the total amount of Phl produced on roots of wheat by *P. fluorescens* Q2-87, at densities ranging from approximately 10^5 to 10^7 CFU/g of root, is proportional to its rhizosphere population density (Fig. 2) and that Phl production per population unit is a constant (Fig. 3). These results indicate that Phl production in the rhizosphere of wheat is related strongly to the ability of the introduced strain to colonize the roots. The results also suggest that within the given range of population densities there is no abiotic or biotic limitation for *P. fluorescens* Q2-87 to produce the antibiotic or that certain limitations apply equally to the whole population range. It should be emphasized, however, that the amount of Phl reported for the different rhizosphere population densities of strain Q2-87 are average values for whole root systems, sampled at 0 to 8 cm from the stem base and harvested after 3 weeks of plant growth; therefore, it does not take into account spatial-temporal colonization and production patterns. The time and place of antibiotic production needs to be considered with respect to the efficacy of biological control of several plant pathogens. For example, an antibiotic may reach threshold concentrations for activity within certain microsites while remaining below this threshold level at other sites where pathogen infection also occurs. This may offer an explanation for the observation that population densities of *P. fluorescens* Q2-87 higher than the threshold density (approximately 2×10^5 CFU/g root) required to control take-all of wheat did not significantly improve the level of suppression (25).

The relationship between the population density of *P. fluorescens* Q2-87 and Phl production in the rhizosphere of wheat described in this study should not be confused with cell density-dependent regulation of antibiotic biosynthetic genes. Cell density-dependent regulation through the production of *N*-acyl-homoserine lactones (AHLs) has been demonstrated for a variety of microorganisms, including phenazine-producing *Pseudomonas* strain 30-84 (38), and is referred to as autoinduction or quorum sensing. Whether the expression of the Phl biosynthetic locus in strain Q2-87 and other fluorescent *Pseudomonas* strains is AHL-mediated is yet unclear and is currently under investigation.

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TABLE 1. Production of 2,4-diacetylphloroglucinol (Phl) and population densities of introduced and naturally occurring Phl-producing fluorescent *Pseudomonas* spp. in Quincy virgin and Quincy take-all decline (TAD) soils^a

Treatment	Phl (ng/g of root)	Population ^b (CFU/g of root)	Phl/population (ng/10 ⁵ CFU)
Quincy virgin	nd ^w	nd	nd
Quincy virgin + Q8r1-96 ^x	254.5 a ^y	4.2×10^7 a ^z	0.56 a ^z
Quincy TAD	19.1 b	4.3×10^6 b	0.48 a

^a Average values of two experiments are shown; in each experiment, treatments were replicated two times, and five pots were used for each replicate.

^b To isolate Phl-producing *Pseudomonas* spp., one gram of roots plus adhering rhizosphere soil was suspended in 5.0 ml of sterile water, shaken on a Vortex mixer, sonicated, and then dilution-plated. Strain Q8r1-96 was enumerated on KMB⁺ plus rifampin. Indigenous Phl-producing *Pseudomonas* spp. were isolated on KMB⁺ and enumerated by colony hybridization followed by polymerase chain reaction analysis as described in text.

^w nd = Neither Phl nor populations of Phl-producing *Pseudomonas* spp. were detected (detection limit was 10^4 CFU/g of root).

^x *P. fluorescens* strain Q8r1-96 was applied to wheat seeds at a density of approximately 10^5 CFU per seed.

^y Within the column, different letters indicate a statistically significant difference (Wilcoxon rank-sum test, $\alpha = 0.05$).

^z Within the column, different letters indicate a statistically significant difference (Tukey's studentized range test, $\alpha = 0.05$).

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