**N-Acyl-Homoserine Lactone-Mediated Regulation of Phenazine Gene Expression by *Pseudomonas aureofaciens* 30-84 in the Wheat Rhizosphere**

**DEREK W. WOOD,**† **FANGCHENG GONG,**† **MAVIS M. DAYKIN,**‡ **PAUL WILLIAMS,**‡ **AND LELAND S. PIERSON III***†*

*Department of Plant Pathology, University of Arizona, Tucson, Arizona 85721,*† **and Department of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom**‡

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*Pseudomonas aureofaciens* 30-84 is a soilborne bacterium that colonizes the wheat rhizosphere. This strain produces three phenazine antibiotics which suppress take-all disease of wheat by inhibition of the causative agent *Gaeumannomyces graminis* var. tritici. Phenazines also enhance survival of 30-84 within the wheat rhizosphere in competition with other organisms. Expression of the phenazine biosynthetic operon is controlled by the *phzR/phzI* N-acyl-homoserine lactone (AHL) response system (*L. S. Pierson III et al., J. Bacteriol. 176:3966–3974, 1994; D. W. Wood and L. S. Pierson III, Gene 168:49–53, 1996). By using high-pressure liquid chromatography coupled with high-resolution mass spectrometry, the AHL produced by PhzI has now been identified as N-hexanoyl-homoserine lactone (HHL). In addition, the ability of HHL to serve as an interpopulation signal molecule in the wheat rhizosphere has been examined by using isogenic reporter strains. Disruption of *phzI* reduced expression of the phenazine biosynthetic operon 1,000-fold in the wheat rhizosphere. Coinoculation of an isogenic strain which produced the endogenous HHL signal restored phenazine gene expression in the *phzI* mutant to wild-type levels in situ. These results demonstrate that HHL is required for phenazine expression in situ and is an effective interpopulation signal molecule in the wheat rhizosphere.

Bacterial populations mediate numerous physiological traits through the production of diffusible signal molecules (38). One class of signal molecules found commonly among diverse gram-negative bacteria are the N-acyl-homoserine lactones (AHLs). This family of signal molecules allows bacterial populations to coordinate gene expression (9). AHL-mediated regulation is commonly referred to as quorum sensing, autoinduction, or population density-responsive gene regulation.

AHL-mediated gene regulation has been best characterized in *Vibrio fischeri*, where it controls the bioluminescent phenotype of these organisms (5, 8, 9, 25). This type of regulatory circuit is also found in a wide range of gram-negative bacteria, where it mediates phenotypes related to microbe-microbe and host-microbe interactions (reviewed in references 9 and 35). These systems are comprised of two proteins which belong to the LuxI and LuxR families. LuxI homologs are AHL synthetases which utilize S-adenosylmethionine and specific acylated-acyl carrier proteins to synthesize specific AHL signal molecules (11, 24, 31, 34). These AHLs diffuse across the cellular membrane and accumulate in the local environment (13). Once a specific intracellular concentration is attained, each AHL interacts with its cognate LuxR homolog, presumably modifying it in some way to allow it to bind to target promoters and mediate transcription.

*Pseudomonas aureofaciens* 30-84 is a fluorescent pseudomonad originally isolated from wheat roots, which can be used as a seed treatment to protect wheat from take-all disease caused by *Gaeumannomyces graminis* var. tritici (30). The ability of this strain to reduce the severity of take-all disease is due to the production of the phenazine antibiotics phenazine-1-carboxylic acid, 2-hydroxyphenazine-1-carboxylic acid, and 2-hydroxyphenazine (30). In addition to their role in disease suppression, phenazines enhance the rhizosphere survival of *P. aureofaciens* in competition with indigenous microorganisms (20, 30). The expression of the phenazine biosynthetic operon (phzFABCD) in vitro is controlled by PhzI and PhzR, which are members of the LuxI/LuxR family of quorum sensing regulators (28, 29, 39).

Although quorum sensing systems have been identified in several diverse bacteria, direct genetic evidence that AHLs function in the habitats occupied by these bacteria has been lacking. Chin-a-Woeng et al. used scanning electron microscopy to examine the spatial relationships of microcolonies formed by fluorescent pseudomonads in the tomato rhizosphere (4). They speculated that the high localized cell densities attained by these microcolonies on roots may facilitate effective AHL-mediated signaling. Direct biochemical evidence was provided by Boettcher and Ruby, who showed that N-(3-oxohexanoyl) homoserine lactone (OHHL), the cognate *V. fischeri* autoinducer, could be extracted directly from the light organs of *Euprymna* species at concentrations sufficient to induce bioluminescence in vitro (2).

The present work demonstrates that PhzI is responsible for the production of N-hexanoyl-homoserine lactone (HHL) and that HHL is required for phenazine gene expression by *P. aureofaciens* 30-84 in the wheat rhizosphere. In addition, an isogenic HHL-producing strain restored phenazine gene expression in a *phzI* mutant to wild-type levels in situ, demonstrating that HHL functions as an effective intercellular signal molecule in the wheat rhizosphere. This study provides the first genetic evidence that AHL-mediated quorum sensing occurs in situ on plant roots.

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† Present address: Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.

‡ Corresponding author. Department of Plant Pathology, University of Arizona, Tucson, AZ 85721. Phone: (520) 621-9419. Fax: (520) 621-9290. E-mail: lsp@u.arizona.edu.
AHL restore the ability of 30-84I to produce phenazine, resulting in an orange halo around the test inoculum. A recombinant unable to produce the endogenous AHL was selected, verified by Southern analysis (data not shown), and designated 30-84Ice/I.

Strain 30-84Z/I was constructed analogously to 30-84Ice/I, using strain 30-84Z. Strain 30-84Z carries a phzB-lacZ chromosomal fusion and produces β-galactosidase in lieu of phenazines (29). A recombinant which was white on LB-X-Gal and unable to produce endogenous AHL was selected. This construct was verified by Southern analysis (data not shown) and designated 30-84Z/I.

Identification of the AHLs produced by P. aureofaciens. Supernatant extracts of P. aureofaciens 30-84 or 30-84I were prepared from cultures grown for 3 to 4 days at 25°C (optical density at 620 nm of 1.4) in 2 liters of PPM medium or M9 minimal medium. Supernatant extracts of E. coli DH5α (pIC20H) and E. coli DH5α(pIC20H-pH2) were prepared from cultures grown for 18 h at 30°C in 2 liters of M9 minimal medium (18). Cells were removed by centrifugation, and cultures were extracted with 1 liter of acidified ethyl acetate (0.1 ml of glacial acetic acid per liter) or dichloromethane. After 1 h, the organic solvent was separated from the supernatant and 5 g of Na2SO4 was added to remove any aqueous residue. The organic solvent was then removed by rotary evaporation at 30°C, and the residue was dissolved in 1 ml of acetonitrile prior to thin-layer chromatography (TLC) or high-pressure liquid chromatography (HPLC) analysis. TLC was performed essentially as described by Shaw et al. (32) except that Chromobacterium violaceum CV026 rather than Agrobacterium tumefaciens was used as the AHL indicator organism (21, 23, 33, 37). CV026 can be used as a biosensor for exogenous AHLs via the induction, or inhibition of production, of the purple pigment violacein. The standards N-butanoyl-L-homoserine lactone (BHL), HHL, and N-octanoyl-L-homoserine lactone (OHL) were synthesized as described previously (3). Culture supernatant extracts and synthetic AHL standards (as 10 mM solutions) were spotted (2 to 3 μl) onto glass-backed RP18 reverse-phase TLC plates (BDH, Poole, Dorset, England) and dried in a stream of cold air. Samples were separated by using 60% (vol/vol) methanol in water as the solvent. Once the solvent front had migrated to within 2 cm of the top of the chromatogram, the plate was removed from the chromatography tank, dried in air, and overlaid with a thin film of C. violaceum CV026 seeded in 0.3% (wt/vol) LB agar. After overnight incubation at 30°C, AHLs were located as purple spots against a white background (21, 33).

Purification and chemical characterization of the P. aureofaciens AHL synthesized via PhzH. Crude supernatant extracts were prepared as described above and subjected to sequential fractionation using both gradient and isocratic elution methods. Samples were applied to a C18 reverse-phase preparative HPLC column (Kromasil KR100-5C8-SP [250 by 8 mm]; Hichrom, Reading, England) and initially eluted with a linear gradient of acetonitrile in water (20 to 95%) over a 30-min period at a flow rate of 2 ml/min and monitored at 210 nm. Six fractions (F1 to F6) covering each 5-min interval were collected and assayed for activity using the C. violaceum AHL reporter assay as described by Milton et al. (23). To
locate the AHL as a single peak, active fractions were rechromatographed on a preparative C18 HPLC column, using an isocratic phase of acetonitrile in water (35:65% [vol/vol]) followed by a third subfractionation using a mobile phase of acetonitrile in water (25:75% [vol/vol]). The final active subfraction was collected, pooled and analyzed first by rechromatography on an analytical HPLC column attached to a photodiode array (PDA) system (Waters 996 PDA system operating with a Millennium 2010 Chromatography Manager; Waters, Watford, Herts, England), and both the retention time and spectral properties were compared with those of a series of synthetic AHL standards. The major active fraction was then subjected to HPLC-mass spectrometry (LC-MS) (Micromass Instruments, Manchester, England), using the same isocratic mobile phase (acetonitrile in water, 25:75% [vol/vol]). This technique coupled the resolving power of C18 reverse-phase HPLC directly with mass spectrometry such that the mass of the molecular ion (M + H) and its major component fragments can be determined for a compound with a given retention time. Samples eluting from the HPLC column (mobile phase, acetonitrile in water, 25 to 75% [vol/vol]) were ionized by positive-ion electrospray mass spectrometry (ES-MS), and the spectra obtained were compared with those of the synthetic material subjected to the same LC-MS conditions.

In vitro expression of phzB reporter strains. Various synthetic AHLs were tested to determine if they could induce phzB-lacZ expression in P. aeruginosa 30-84Z, essentially as described previously (39). 30-84Z was grown in PPM medium at 28°C to a low cell density at which phzB expression had not yet been initiated (optical density at 620 of 0.1). These cultures were centrifuged and resuspended in an equal volume of fresh PPM medium supplemented (10 μg/ml) with the following synthetic AHLs: BHL, HHL, OHL, N-(3-oxoocanoyl)-L-homoserine lactone (OHL), N-(3-oxohexanoyl)-L-homoserine lactone (OHL), N-(3-oxodecanoyl)-L-homoserine lactone (ODHL), synthetic AHLs were treated with Chibhara et al. (33), E. Galatosidas (33), phzB-DH5DH5 strain and ODHL. Synthetic AHLs were then spotted to aluminum foil trays coated with paraffin and set on a (17). Tenfold serial dilutions from each culture were made in phosphate-buffered (11°C ethanol (EOH) 0% FBS). PhzI expression was measured hourly as described by Miller (22) and compared to that of control treatments without AHL. A more complete range of synthetic HHL concentrations (1 nM to 4.7 μM) was also tested to determine the activity of this specific AHL on the induction of phzB-lacZ expression in 30-84Z.

Ice nucleation activity of the phzB-inA reporter fusion in P. aeruginosa 30-84Ce and 30-84Ie-1 was compared to β-galactosidase activity from the phzB-lacZ fusion of strain 30-84Z. Strains were grown in PPM medium supplemented with p-aminobenzoic acid (1.6 μg/ml) at 28°C for 18 h. The cultures were centrifuged, resuspended in fresh PPM medium (1:100), and grown with shaking at 28°C. After 1.5 h, 3 μl of HHL (1 μg/ml in dimethyl sulfoxide) or dimethyl sulfoxide was added to the 30-84Ce/I (0.5 μM, final HHL concentration). Ice nucleation activity was measured hourly as described by Lindgren et al. (17). Tenfold serial dilutions from each culture were made in phosphate-buffered saline (PBS; pH 7.2). Appropriate dilutions were plated to LB medium to determine viable counts. Fifty μl droplets from appropriate dilutions were spotted to an aluminum foil tray coated with paraflin and set on a −11°C ethanol (EOH) (0%). The final concentration was maintained over the course of the experiment. Axenic conditions were maintained throughout the experiment. Axenic conditions were verified by plating total root sonicates from uninoculated controls to LB agar. Serial dilutions were tested for ice nucleation activity at −11°C as described above.

FIG. 1. Thin-layer chromatogram of the AHLs present in cell-free supernatants of P. aeruginosa 30-84, 30-84E, E. coli DH5α (pIC20H), and E. coli DH5α (pIC20H-plz) detected by using a C. violaceum CV026 overlay. Cell-free supernatants from each organism were extracted with dichloromethane and chromatographed as described in Materials and Methods alongside synthetic AHL standards. Lane 1, BHL; lane 2, P. aeruginosa 30-84; lane 3, P. aeruginosa 30-84; lane 4, HHL; lane 5, E. coli DH5α (pIC20H); lane 6, E. coli DH5α (pIC20H-plz); lane 7, OHL. The arrowheads mark the positions of the purple spots indicative of AHL-mediated AHL induction. Note that all compounds not marked by arrows are yellow-brown-colored products arising from contaminants within the extracts and were not due to activation of the CV026 reporter.

Statistical analysis. Treatment effects were determined by analysis of variance, using SAS (version 6.11 for UNIX, 1993; SAS Institute Inc., Cary, N.C.). Mean comparisons were made after analysis of variance using the Duncan-Waller K-ratio test (α = 0.05).

RESULTS

Identification of the N-acetyl-homoserine lactone produced by phzI. Earlier work indicating that PhzI was responsible for the production of an AHL signal molecule (39) was confirmed by the following analyses. Cell-free supernatants from cultures of P. aeruginosa 30-84 and 30-84I (phzI) grown to stationary phase in M9 medium were extracted with dichloromethane, and samples were spotted onto RP18 reverse-phase TLC plates. After chromatography, TLC plates were overlaid with a thin film of C. violaceum CV026 seeded in LB agar as described in Materials and Methods. The results obtained are shown in Fig. 1. We identified in spent culture supernatants from P. aeruginosa 30-84 a major purple spot which was absent from the phzI mutant 30-84I. This spot migrated with an R value similar to that of synthetic HHL (Fig. 1; compare lanes 2, 3, and 4). A second, faster-migrating but weaker spot with an R value similar to that of BHL was also noted in the wild type but was absent from the mutant (Fig. 1; compare lanes 1, 2, and 3). To identify the AHL(s) produced by P. aeruginosa, we subjected the solvent extracts of various culture supernatants to HPLC. In these studies, a derivative of C. violaceum CV026 bioassay). After elution using a linear acetonitrile gradient followed by isocratic acetonitrile mobile phases on a preparative C18 reverse-phase HPLC column, the active fractions (as determined by their ability to induce violacein production in C. violaceum CV026 [21, 37]) were again collected. When chromatographed on an analytical HPLC using an isocratic mobile phase of 25% (vol/vol) acetonitrile in water, the active compound eluted with
the same retention time (18 min) and PDA spectrum (data not shown) as the synthetic HHL standard. When mixed, the active compound and synthetic HHL coeluted within the same fraction. To confirm the identity of the *P. aureofaciens* AHL as HHL, the active fraction was subjected to LC-MS, which couples the resolving power of HPLC with mass spectroscopy. The ES-MS spectrum obtained (Fig. 2) reveals the presence of a molecular ion [M + H] of 200 together with the characteristic fragmentation products at 102 and 99 which correspond to the homoserine lactone moiety and the C₆ acyl side chain [CH₃(CH₂)₄C=O⁺], respectively. We were unable to attain sufficient material to identify the second putative AHL revealed in the CV026 TLC assay. (Fig. 1).

To demonstrate that *phzI* directs HHL synthesis, culture supernatants of *E. coli* DH5α(pIC20H) and *E. coli* DH5α(pIC20H-*phzI*) were extracted with dichloromethane and subjected to TLC analysis. Figure 1 (lanes 5 and 6) shows a single spot in the sample prepared from *E. coli* DH5α(pIC20H-*phzI*) which is lacking in the *E. coli* DH5α containing the vector alone. The *Rf* value of this spot corresponds with synthetic HHL. Final confirmation that this product was HHL was obtained by LC-MS (data not shown). The second spot observed in 30-84
AHL-mediated gene expression in the rhizosphere

FIG. 3. Induction of phzB expression in liquid cultures of various reporter strains. Induction of the phzB-lacZ fusion in strain 30-84Ice (A), strain 30-84Ice/I (C), strain 30-84Ice/I supplemented with 0.5 μM synthetic HHL (B), and the phzB-lacZ fusion in strain 30-84Z (D) was determined in PPMD medium as described in the text.

FIG. 4. Ice nucleation activity of the phzB-inaZ reporter fusion of strains 30-84Ice and 30-84Ice/I in the rhizosphere. (A) Ice nucleation activity of the phzB-inaZ reporter in bacteria isolated from wheat roots inoculated with 30-84Ice alone (column 1), a 10:90 ratio of 30-84Ice/I and the phzI+ strain 30-84Z (column 2), a 50:50 ratio of 30-84Ice/I and the phzI strain 30-84I/Z (column 3), or 30-84Ice/I alone (column 4). (B) Ice nucleation activity of the phzB-inaZ reporter in bacteria isolated from wheat roots inoculated with 30-84Ice alone (column 1), a 10:90 ratio of 30-84Ice/I and the phzI+ strain 30-84Z (column 2), a 50:50 ratio of 30-84Ice/I and 30-84Z (column 3), or 30-84Ice/I alone (column 5). The relative inoculation ratios of bacterial mixtures were maintained throughout the experiment, as determined by total viable counts at harvest. Ice nucleation frequency was determined on a per-cell basis as described in the text. Treatments with the same letter are not statistically different as determined by analysis of variance and the Duncan-Waller K-ratio test (P < 0.5).

PhzR system. Strain 30-84Ice/I is an isogenic derivative of 30-84Ice in which the phzI gene has been disrupted by insertion of a kanamycin resistance cartridge. This strain is unable to produce HHL, as determined by in vitro plate complementation and qualitative analysis of ethyl acetate culture extracts using 30-84I as an indicator (data not shown). 30-84Ice/I did not produce any ice nucleation activity in liquid culture, consistent with its inability to activate phenazine gene expression in the absence of HHL (Fig. 3). The addition of synthetic HHL (0.5 μM) to 30-84Ice/I cultures restored phzB-inaZ expression to wild-type levels. Therefore, the phzB-inaZ fusion is extremely sensitive, is cell density responsive, responds to the cognate AHL, and is not expressed in a phzI mutant, indicating that it accurately reflects expression from the phenazine promoter.

Strains 30-84Ice or 30-84Ice/I were used to inoculate wheat seedlings in the rhizosphere assay. After 10 days, total bacteria were isolated from roots, and their ice nucleation activity was determined (Fig. 4A). The activity of the phzB-inaZ fusion in strain 30-84Ice represents the normal level of phenazine gene expression, with an Rₜ value closer to that of synthetic BHL, than was apparent in the sample prepared from E. coli DH5α(pIC20H-lacZ). These data indicate that HHL is produced by P. aureofaciens 30-84, that phzI is required for its synthesis, and that P. aureofaciens produces a second putative AHL whose synthesis is not apparent in the sample prepared from E. coli DH5α(pIC20H-lacZ). In addition, ODHL and OdDHL had no effect on phenazine gene expression, followed by OHHL, BHL, and OOHL, respectively. ODHL and OdDHL had no effect on phenazine gene expression, indicating that it was under the control of the PhzI/PhzR system. Strain 30-84Ice/I is an isogenic derivative of 30-84Ice in which the phzI gene has been disrupted by insertion of a kanamycin resistance cartridge. This strain is unable to produce HHL, as determined by in vitro plate complementation and qualitative analysis of ethyl acetate culture extracts using 30-84I as an indicator (data not shown). 30-84Ice/I did not produce any ice nucleation activity in liquid culture, consistent with its inability to activate phenazine gene expression in the absence of HHL (Fig. 3). The addition of synthetic HHL (0.5 μM) to 30-84Ice/I cultures restored phzB-inaZ expression to wild-type levels. Therefore, the phzB-inaZ fusion is extremely sensitive, is cell density responsive, responds to the cognate AHL, and is not expressed in a phzI mutant, indicating that it accurately reflects expression from the phenazine promoter.

Strains 30-84Ice or 30-84Ice/I were used to inoculate wheat seedlings in the rhizosphere assay. After 10 days, total bacteria were isolated from roots, and their ice nucleation activity was determined (Fig. 4A). The activity of the phzB-inaZ fusion in strain 30-84Ice
cells. Induction of the phzB-lacZ fusion in strain 30-84Ice/I, strain 30-84Ice/I supplemented with 0.5 μM synthetic HHL, and the phzB-lacZ fusion in strain 30-84Z was determined in PPMD medium as described in the text.

PhzI is required for phenazine gene expression in the rhizosphere. To determine whether phzI was required for phenazine gene expression by P. aureofaciens 30-84 in the wheat rhizosphere, the ice nucleation reporter strains 30-84Ice and 30-84Ice/I were constructed. Strain 30-84Ice contains a chromosomal phzB-inaZ transcriptional fusion designed to express ice nucleation protein in lieu of phenazines. Ice nucleation protein is reported to be approximately 100,000 times more sensitive than β-galactosidase as a reporter of gene expression. Ice nucleation of the phzB-inaZ reporter in bacteria isolated from wheat roots inoculated with 30-84Ice alone (column 1), a 50:50 ratio of 30-84Ice/I and the phzI+ strain 30-84Z (column 2), a 50:50 ratio of 30-84Ice/I and the phzI strain 30-84I/Z (column 3), or 30-84Ice/I alone (column 4). (B) Ice nucleation activity of the phzB-inaZ reporter in bacteria isolated from wheat roots inoculated with 30-84Ice alone (column 1), a 10:90 ratio of 30-84Ice/I and the phzI+ strain 30-84Z (column 2), a 50:50 ratio of 30-84Ice/I and 30-84Z (column 3), or 30-84Ice/I alone (column 5). The relative inoculation ratios of bacterial mixtures were maintained throughout the experiment, as determined by total viable counts at harvest. Ice nucleation frequency was determined on a per-cell basis as described in the text. Treatments with the same letter are not statistically different as determined by analysis of variance and the Duncan-Waller K-ratio test (P < 0.5).
expression in situ. Expression of this fusion in the phzI mutant 30-84Ice/I was 1,000-fold lower than in strain 30-84Z. These data demonstrate that phzI is essential for high-level expression of the phenazine operon in situ in the wheat rhizosphere.

Rhizosphere complementation of phzI mutants by isogenic strains. To determine if AHL could function as an effective signal molecule between distinct genetic populations in the rhizosphere, we designed the following in situ complementation assay. Strain 30-84Ice/I was used as a reporter to detect the presence of exogenous HHL produced by isogenic populations in the rhizosphere. Strain 30-84Ice/I was used to inoculate wheat seedlings alone or in a 50:50 ratio with isogenic strains either able (30-84Z) or unable (30-84Z/I) to produce HHL. After 10 days, total bacteria were isolated from roots, and the ice nucleation activity derived from each reporter strain was determined (Fig. 4A). Expression from the 30-84Ice/I phzB-inaZ reporter cooincided with 30-84Z was restored to 100% of wild-type levels (Fig. 4A, columns 1, 2, and 4). In contrast, expression from the 30-84Ice/I phzB-inaZ reporter cooincided with 30-84Z/I reached only 35% of wild-type levels (Fig. 4A, columns 2 and 3). This finding demonstrates that HHL is an effective interpopulation signal molecule that can alter gene expression in distinct genetic populations in the rhizosphere.

When comparing the control treatments 30-84Ice/I cooincided with 30-84Z/I (Fig. 4A, column 3) and 30-84Ice/I alone (Fig. 4A, column 4), we noted a difference in phzB-inaZ expression. The reason for an increase in phzB-inaZ expression in 30-84Ice/I cooincided with 30-84Z/I is unclear since these two strains are isogenic and simply contain different reporter genes in phzB. Although we cannot explain this phenomenon at this time, it is important to note that phzB-inaZ expression in this mixture is still significantly less than that seen in 30-84Ie or 30-84Ice/I in combination with the HHL donor strain 30-84Z.

To examine how the relative proportion of AHL-producing bacteria could influence phzB-inaZ expression in 30-84Ice/I, additional rhizosphere assays were performed with various ratios (100:0, 90:10, 50:50, 10:90, and 0:100) of the 30-84Z HHL donor strain to 30-84Ice/I reporter (Fig. 4B). Expression of phzB-inaZ in 30-84Ice/I increased relative to the percentage of HHL donor present. These data indicate that the relative proportion of HHL-producing bacteria in the rhizosphere can have significant effects on phenazine gene expression in situ. Similar results were seen when this assay was repeated with pea as the host plant, indicating that this phenomenon is not restricted to the rhizosphere of wheat (data not shown).

DISCUSSION

Since the discovery of AHL-mediated signaling in luminescent bacteria in the early 1970s, many gram-negative bacteria have been shown to produce a variety of AHL signals which regulate the expression of genes essential for host-microbe interactions (9, 35). Although these regulatory systems have been well characterized in a number of bacteria, to our knowledge there has been no direct genetic evidence to date showing that AHL-mediated signaling operates in the natural habitat of these organisms. The present study was therefore designed to (i) identify the specific AHL produced by the PhzI protein of P. aureofaciens 30-84 and (ii) determine if this molecule could mediate interpopulation signaling within the confines of the rhizosphere.

The phzI gene of P. aureofaciens 30-84 was shown to be essential for the production of HHL, based on the following lines of evidence: (i) TLC analysis of crude ethyl acetate extracts from P. aureofaciens 30-84 and E. coli DH5a(pIC20H-phzI) culture supernatants, but not those from 30-84I (a phzI mutant) or E. coli DH5a(pIC20H), show Rf values similar to that of synthetic HHL, (ii) HPLC analysis demonstrates that the biologically active fraction elutes with the same retention time and PDA spectrum as synthetic HHL (this fraction was not present in 30-84I), and (iii) LC-MS analysis of these fractions gave molecular ion peaks consistent with the structure of HHL.

Synthetic HHL induced phzB-lacZ expression in P. aureofaciens 30-84Z at concentrations as low as 1 nM in vitro. When compared with a range of synthetic AHLs with acyl chain lengths of between 4 and 12 carbons, HHL was clearly the most active compound, with OdDHL being the least active. Thus, in common with observations made for AHL-responsive genes of other bacteria such as Erwinia carotovora (9), P. aeruginosa (37), and C. violaceum (21), the length of the N-linked acyl chain is a key structural feature in determining the activity of an AHL analog. Of the synthetic AHLs tested, OHHL (V. fischeri, E. carotovora, E. stewartii, Yersinia enteroxolitica, and Enterobacter agglomerans), OOH (A. tumefaciens), and BHL (Serratia liquefaciens) are naturally produced by other bacteria (9). In addition, HHL is produced by a number of bacteria, including A. tumefaciens (41), Aeromonas hydrophila (33), A. salmonicida (33), P. aeruginosa (37), S. liquefaciens (7), and V. fischeri (14). The ubiquitous nature of AHL response systems in gram-negative bacteria (9) and the range of AHLs recognized by 30-84 indicate their potential as mediators of interspecies communication in the rhizosphere.

The ice nucleation reporter strains P. aureofaciens 30-84Ice (phzB-inaZ) and 30-84Ice/I (phzB-inaZ phzI) were used to examine AHL-mediated communication within the rhizosphere. These strains contain a sensitive ice nucleation reporter fusion within the phenazine operon which allows gene expression to be monitored directly in the wheat rhizosphere. When these two strains were introduced into the wheat rhizosphere and tested for ice nucleation activity, expression of the phzB-inaZ fusion in strain 30-84Ice/I was 1,000-fold lower than in strain 30-84Ice. This finding indicates that phzI is required for normal phenazine gene expression in the rhizosphere and that the total amount of phenazines produced is significantly reduced in a phzI mutant.

To further examine the potential of AHLs as signal molecules in the rhizosphere, strain 30-84Ice/I was used as a biological sensor for the presence of exogenous HHL under rhizosphere conditions. Strain 30-84Ice/I contains an intact copy of phzR, the cognate HHL recognition protein required for expression of the phenazine operon. This strain is therefore able to induce the phzB-inaZ fusion in response to exogenous HHL (Fig. 3). Coinoculation of strain 30-84Ice/I in a 50:50 ratio with the HHL-producing strain 30-84Z on wheat roots resulted in ice nucleation activities comparable to those seen in the wild-type reporter 30-84Ice. However, coinoculation with 30-84Z/I, which does not produce HHL, did not restore expression to these same levels. These results indicate that HHL functions as an effective signal molecule between distinct genetic populations in the rhizosphere.

Similar experiments in which the HHL sensor strain 30-84Ice/I was coinoculated in various ratios with the HHL donor strain 30-84Z showed that increasing the relative proportion of HHL producing bacteria within the rhizosphere resulted in a concomitant increase in expression from the phzB-inaZ reporter. These results suggest that the relative proportion of rhizosphere bacteria producing AHLs recognized by P. aureofaciens may influence phenazine production in situ.

The ability of one population to influence gene expression in
a distinct population by the production of AHL signal molecules has potential to influence the efficiency of biological control. One of the main problems facing the practical use of biological control is the lack of consistent performance during field trials (36). The discovery that gene expression in \textit{P. aerofaciens} 30-84 is affected by exogenous AHL signals in the rhizosphere, and that this expression is modified by a range of AHL signals in vitro, suggests that the local microbial environment can directly alter phenazine gene expression in 30-84 via AHL signals in vitro, suggests that the local microbial environment can directly alter phenazine gene expression in 30-84 via AHLs. This hypothesis is supported by in vitro studies which show that a large number of soilborne bacteria produce AHLs (9) and that other AHL-mediated regulatory systems respond to signals produced by different bacterial species (1, 10). If other rhizosphere organisms altered phenazine gene expression in 30-84 by the production of AHLs, this would affect the success of 30-84 as a biological control agent. For example, if 30-84 is introduced into an environment that contains a high percentage of organisms that produce recognizable AHL, it may more effectively produce the protective phenazine antibiotics. However, if introduced into a microbial community that does not produce recognizable AHL, or produces antagonistic AHLs, the persistence of 30-84 and its ability to suppress disease may be compromised. In this context, synthetic AHLs which antagonize the activity of the cognate AHL have been identified (6). This hypothesis may provide some insight into experiments by Pierson and Weller (27). This work showed that combinations of various pseudomonads were more effective at controlling take-all than any of the strains individually. Since several of the strains used in these mixtures had no ability to suppress disease on their own, it is possible that these secondary strains produced compounds which altered the ability of the primary strains to produce antagonistic compounds responsible for control.

While it has often been proposed that bacterial populations use quorum sensing in situ to mediate the expression of genes involved in host-microbe and microbe-microbe interactions, genetic evidence in support of this hypothesis has been lacking. The data presented above provide direct genetic evidence that quorum sensing-mediated control of phenazine biosynthesis in \textit{P. aerofaciens} occurs within the wheat rhizosphere. We anticipate that quorum sensing systems in other bacteria will be found to function similarly in situ. Future work will be directed toward understanding the interrelationships between distinct quorum sensing systems of rhizosphere bacteria. Such studies will provide insight into the ecological relationships between bacterial populations in nature at the genetic level.

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