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

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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. Bacterial 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 coordinately regulate 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 syntheses 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 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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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
P. aureofaciens		
30-84	Wild type, Phz$^+$	30
30-84Ice	*phzB-inaZ* genomic fusion, Phz$^-$ HHL$^+$	This study
30-84I	*phzI:kan* Phz$^-$ HHL$^-$ Kan^r	39
30-84Ice/I	*phzB-inaZ* genomic fusion, Phz$^-$ *phzI:kan* HHL$^-$ Kan^r	This study
30-84Z	*phzB-lacZ* genomic fusion, Phz$^-$ HHL$^+$	This study
30-84Z/I	*phzB-lacZ* genomic fusion, Phz$^-$ *phzI:kan* HHL$^-$ Kan^r	This study
C. violaceum CV026	Double mini-Tn*5* mutant derived from *C. violaceum* ATCC 31532, AHL biosensor	15
E. coli DH5α	F$^-$ *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argF-lacZYA*)*169* ϕ80*lacZ* ΔM15	Gibco-BRL
Plasmids		
pLAFR3	IncP1, Tet^r *cos rlx*$^+$	26
pUC18	ColE1, Amp^r	40
pIC20H	ColE1, Amp^r	19
pTn3Spice	Tn*3inaZ*	17
pLSP18-6	pUC18 carrying a 9.2-kb *Eco*RI fragment which contains the *phzFABCD* phenazine operon	30
pLSP18-6H3Δ3	pUC18 carrying the 5.6-kb *phzFABCD* phenazine operon	30
pLAFR3:*phzI*::*Km*	pLAFR3 carrying a ca. 7.0-kb *Hin*dIII fragment which contains a disrupted *phzI:kan* allele	39
pIC20H-*phzI*	pIC20H carrying a 2.2-kb *Pst*I-*Eco*RV fragment which contains *phzI*	39
pLSP18Ice	pUC18 carrying 3.8-kb *Bam*HI-*Eco*RI *inaZ* gene	This study
pLSP18-6H3Δ3InaZ	pLSP18-6H3Δ3 containing the 3.8-kb *inaZ* gene in the *Pst*I site of *phzB*	This study
pLAFR3-*FABZ*	pLAFR3 containing the 7.2-kb *phzFAB-inaZ* region from pLSP18-6H3Δ3InaZ	This study
pIC20H18-6	pIC20H carrying the 2.25-kb *Pst*I fragment from pLSP18-6 containing *phzCD*	This study
pLSP*phzB-inaZ*	pLAFR3-*FABZ* containing the 2.25-kb *Hin*dIII fragment from pIC20H18-6	This study

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids are listed in Table 1. All strains were grown at 28°C in Luria-Bertani (LB) medium containing 5 g of NaCl per liter (18), M9 minimal medium (18), or pigment production medium (PPMD). PPMD is a modified form of PPM medium in which Proteose Peptone no. 3 (Difco) is substituted at the same concentration for Bacto Peptone (Difco) (modified from reference 16). *p*-Aminobenzoic acid was used at a concentration of 1.6 μg/ml to suppress phenazine production prior to *phzB* expression analysis (29). When required, LB agar was supplemented with 4% (wt/vol) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 2 μl/ml; Sigma) dissolved in *N*,*N*-dimethyl formamide. Antibiotics were used at the following concentrations where appropriate: kanamycin sulfate, 50 μg/ml; and tetracycline, 50 μg/ml.

The *phzB-inaZ* reporter strain 30-84Ice was constructed as follows. The 3.8-kb *Bam*HI-*Eco*RI promoterless *inaZ* gene from transposon pTn*3*Spice (17) was cloned into pIC20H (19). This region was subcloned by using the flanking *Hin*dIII sites of pIC20H into the *Hin*dIII site of pUC18, resulting in pLSP18Ice. The *Pst*I fragment of pLSP18Ice containing the *inaZ* gene was cloned into the unique *Pst*I site within *phzB* in pLSP18-6H3Δ3, which contains the 5.6-kb phenazine biosynthetic operon *phzFABCD* (30), resulting in plasmid pLSP18-6H3Δ3InaZ. Digestion of pLSP18-6H3Δ3InaZ with *Eco*RI followed by ligation into pLAFR3 (26) resulted in pLAFR3-*FABZ*. To facilitate marker exchange, the *phzCD* region was inserted behind the *phzB-inaZ* fusion of pLAFR3-*FABZ*. This was accomplished by ligating the 2.25-kb *Pst*I fragment from pLSP18-6 (30) containing *phzCD* into pIC20H, resulting in pIC20H18-6, and then using the flanking *Hin*dIII sites to introduce it into the unique *Hin*dIII site in pLAFR3*phzB-inaZ*. The resulting plasmid, pLSP*phzB-inaZ*, was transformed into *Escherichia coli* DH5α and introduced into *P. aureofaciens* 30-84 by triparental mating as described previously (29). Double recombinants in which the *phzB-inaZ* fusion had replaced the wild-type *phzB* allele were identified by the loss of phenazine production and tetracycline sensitivity. One double recombinant was selected, tested for ice nucleation activity, verified by Southern analysis (data not shown), and designated 30-84Ice.

Strain 30-84Ice/I was constructed by introducing pLAFR3:*phzI*::*Km* (39) into 30-84Ice via triparental mating and selecting for kanamycin-resistant, tetracycline-sensitive recombinants in which the wild-type *phzI* allele had been replaced by the disrupted *phzI* allele. Loss of endogenous AHL production in the recombinants was verified using an in vitro plate assay that determines the ability of test strains to restore phenazine production in the *phzI* mutant 30-84I (39). Briefly, *P. aureofaciens* 30-84I and other strains to be tested for AHL production are grown at 28°C overnight in PPMD medium. Fifty microliters of 30-84I is spread evenly across the surface of PPMD agar, and each test strain (5 μl) is spotted onto this lawn and incubated at 28°C. Those strains which produce recognizable 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 PPMD or M9 minimal medium. Supernatant extracts of *E. coli* DH5α(pIC20H) and *E. coli* DH5α(pIC20H-*phzI*) 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 liters 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 ca. 5 g of Na_2SO_4 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*-oxanoyl-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 30 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 PhzI. 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 C_8 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 C_8 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 rechromatographing on an analytical HPLC attached to a photodiode array (PDA) system (Waters 996 PDA system operating with a Millenium 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 couples the resolving power of C_8 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. aureofaciens* 30-84Z essentially as described previously (39). 30-84Z was grown in PPMD 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 PPMD medium supplemented (10 μg/ml) with the following synthetic AHLs: BHL, HHL, OHHL, *N*-(3-oxooctanoyl)-L-homoserine lactone (OOHL), *N*-(3-oxohexanoyl)-L-homocysteine thiolactone (OHTL), *N*-(3-oxodecanoyl)-L-homoserine lactone (ODHL), and *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL). Synthetic AHLs were synthesized as described by Chhabra et al. (3). β-Galactosidase activity 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-inaZ* reporter fusion in *P. aureofaciens* 30-84Ice and 30-84Ice/I was compared to β-galactosidase activity from the *phzB-lacZ* fusion of strain 30-84Z. Strains were grown in PPMD medium supplemented with *p*-aminobenzoic acid (1.6 μg/ml) at 28°C for 18 h. The cultures were centrifuged, resuspended in fresh PPMD medium (1:100), and grown with shaking at 28°C. After 1.5 h, 3 μl of HHL (1 μg/μl in dimethyl sulfoxide) or dimethyl sulfoxide was added to the 30-84Ice/I cultures (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 10-μl droplets from appropriate dilutions were spotted to aluminum foil trays coated with paraffin and set on a −11°C ethanol (EtOH) bath. The number of frozen droplets was scored after 7 min. Ice nucleation frequency was calculated on a per-cell basis, using the equation described by Lindgren et al. (17). The 30-84Z culture was assayed for β-galactosidase activity hourly as described by Miller (22).

Rhizosphere assay. Wheat seeds (cultivar Fielder) or pea seeds (cultivar Little Marvel; Royal Seed Co.) were surface sterilized by suspending ca. 50 g of seed in 100 ml of EtOH and gently shaking for 1 min. EtOH was removed by aspiration, and 100 ml of bleach (5% HOCl) was added with gentle shaking for 1 min. Bleach was removed by aspiration, and seeds were rinsed five times, 1 min each, with 100 ml of sterile distilled H_2O and allowed to dry for 5 h under a laminar flow hood. Surface sterilized seeds were pregerminated on LB agar plates (with moistened sterile germination paper attached to the lids) for 3 days to ensure sterility before planting. The seedlings were suspended for 1 min in bacterial cultures grown with 24 to 30 h of shaking at 28°C in PPMD medium. Bacterial strains were grown individually and used singly or mixed in specific ratios immediately prior to seedling treatment. The treated seedlings were sown in 24- by 100-mm glass tubes which contained 7 cm of sterile sand and 5 ml of sterile 1/3 Hoagland's solution (macronutrients only) (12). The seedlings were covered with 1 cm of sterile sand and placed in Conviron growth chambers (22°C/18°C, 25% relative humidity with a 12-h light/dark cycle). Ten replicates of each treatment were prepared, and all experiments were repeated at least twice. At the end of 10 days, plants were aseptically removed from tubes, and all loosely adhering sand was removed by shaking. Bacteria were isolated via sonication of total excised root mass in 2 ml of PBS, (pH 7.0). These bacteria were serially diluted in PBS and plated on LB agar supplemented with X-Gal to determine total bacterial numbers. The use of the chromogenic substrate X-Gal allowed a distinction between colonies which carried the *phzB-lacZ* fusion (30-84Z and 30-84Z/I) and those with the *phzB-inaZ* fusion (30-84Ice and 30-84Ice/I) which contributed to ice nucleation activity. Although total bacterial populations were reduced compared to the initial inoculum, the final ratio of each bacterial strain closely matched the initial inoculation ratios, indicating that population relationships were maintained over the course of 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.

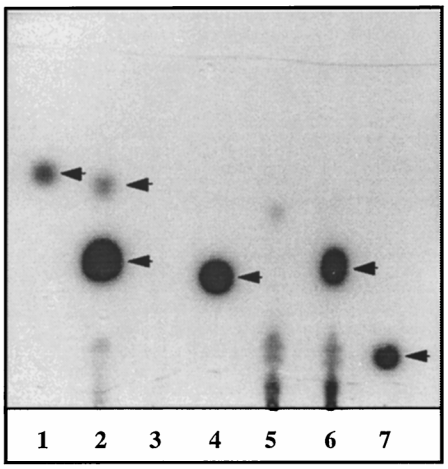

FIG. 1. Thin-layer chromatogram of the AHLs present in cell-free supernatants of *P. aureofaciens* 30-84, 30-84I, *E. coli* DH5α(pIC20H), and *E. coli* DH5α(pIC20H-*phzI*) detected by using a *C. violaceum* CV026 overlay. Cell-free culture supernatants from each organism were extracted with dichloromethane and chromatographed as described in Materials and Methods alongside synthetic AHL standards. Lane 1, BHL; lane 2, *P. aureofaciens* 30-84; lane 3, *P. aureofaciens* 30-84I; lane 4, HHL; lane 5, *E. coli* DH5α(pIC20H); lane 6, *E. coli* DH5α(pIC20H-*phzI*); 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 ($\alpha = 0.05$).

RESULTS

Identification of the *N*-acyl-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. aureofaciens* 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. aureofaciens* 30-84 a major purple spot which was absent from the *phzI* mutant 30-84I. This spot migrated with an R_f 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_f 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. aureofaciens*, we subjected the solvent extracts of various culture supernatants to HPLC. In these studies, a derivative of *P. aureofaciens* 30-84 unable to produce phenazines (30-84ICE) was used to avoid problems associated with the phenazines (strong UV absorbance and interference with the *C. violaceum* CV026 bioassay). After elution using a linear acetonitrile gradient followed by isocratic acetonitrile mobile phases on a preparative C_8 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

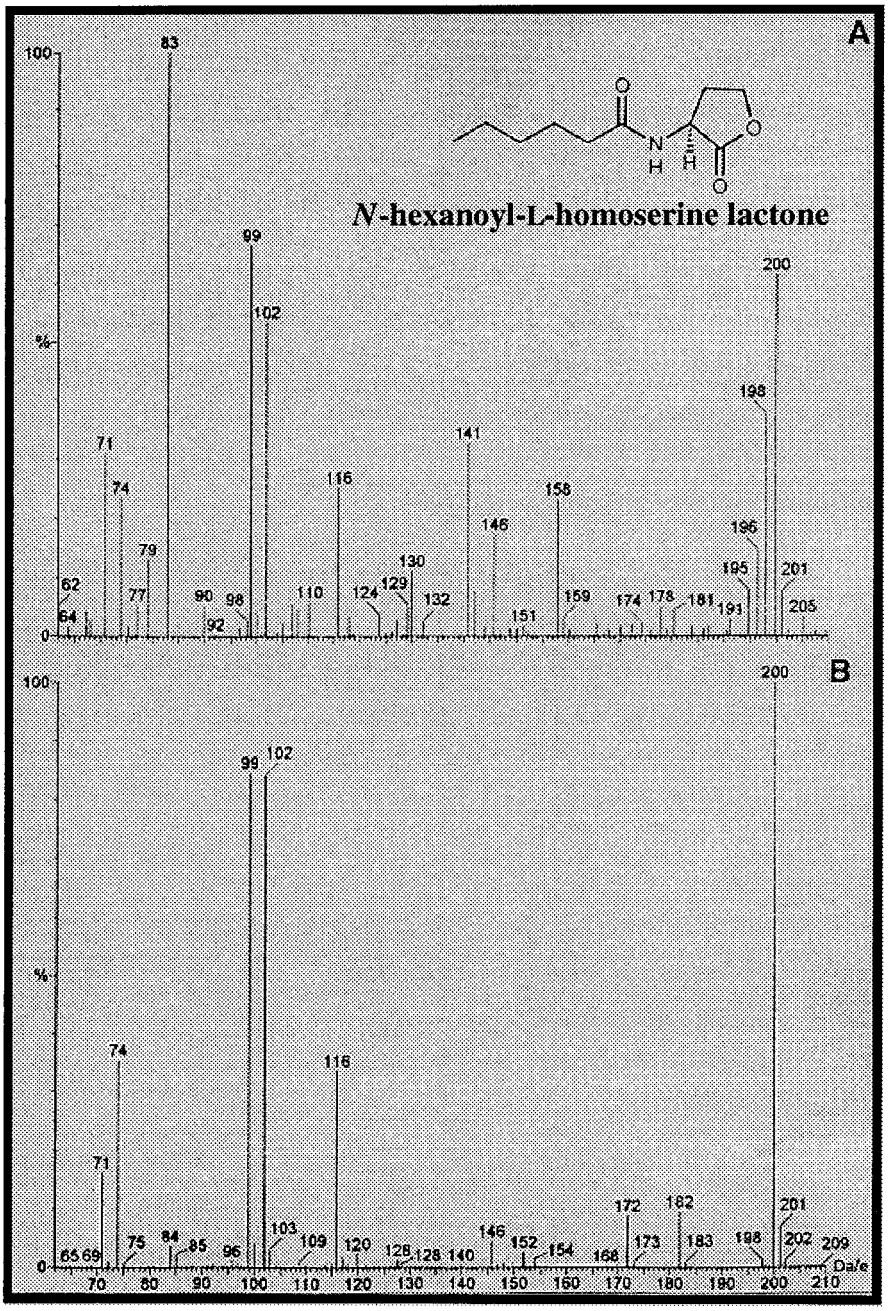

FIG. 2. LC-MS spectra comparing AHL purified from spent culture supernatants of *P. aureofaciens* 30-84Ice (A) and synthetic HHL (B). The inset shows the chemical structure of HHL.

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 C_6 acyl side chain [$CH_3(CH_2)_4C{\equiv}O^+$], 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 R_f 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

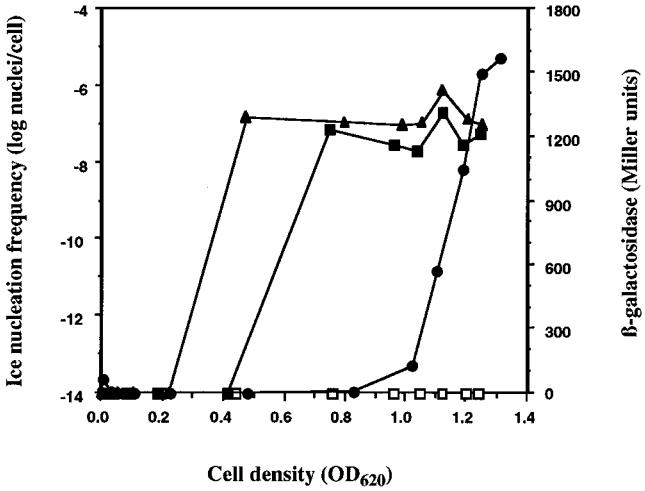

FIG. 3. Induction of *phzB* expression in liquid cultures of various reporter strains. Induction of the *phzB-inaZ* fusion in strain 30-84Ice (▲), strain 30-84Ice/I (□), strain 30-84Ice/I supplemented with 0.5 μM synthetic HHL (■), and the *phzB-lacZ* fusion in strain 30-84Z (●) was determined in PPMD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

extracts, with an R_f value closer to that of synthetic BHL, was not apparent in the sample prepared from *E. coli* DH5α(pIC20H-*phzI*). 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 may indirectly require *phzI*.

The in vitro activity of HHL was determined by examining the influence of synthetic HHL on phenazine gene expression in the *phzB-lacZ* reporter strain 30-84Z. HHL induced *phzB-lacZ* expression in 30-84Z to high levels with the activity increasing in proportion to the concentration of HHL (1 nM to 4.7 μM) (data not shown).

Phenazine gene expression was induced in response to a range of AHLs in addition to HHL. Supplementing PPMD medium with 10 μg of synthetic BHL, HHL, OHHL, OOHL per ml resulted in earlier and higher-level induction of the *phzB-lacZ* fusion compared to unsupplemented medium (data not shown). In addition, *phzB* gene expression was induced by OHTL, an analog of the *V. fischeri* autoinducer OHHL in which the oxygen in the lactone ring has been substituted by a sulfur atom (3). HHL and OHTL showed the highest levels of induction, followed by OHHL, BHL, and OOHL, respectively. ODHL and OdDHL had no effect on phenazine gene expression in these experiments.

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 and therefore is a good candidate to study potentially low level gene expression in the rhizosphere (17). Consistent with this, expression of ice nucleation activity in 30-84Ice was detected much earlier in vitro than β-galactosidase activity in the analogous *phzB-lacZ* reporter 30-84Z (Fig. 3). The expression of the *phzB-inaZ* fusion was induced in a cell density-dependent manner, suggesting 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.

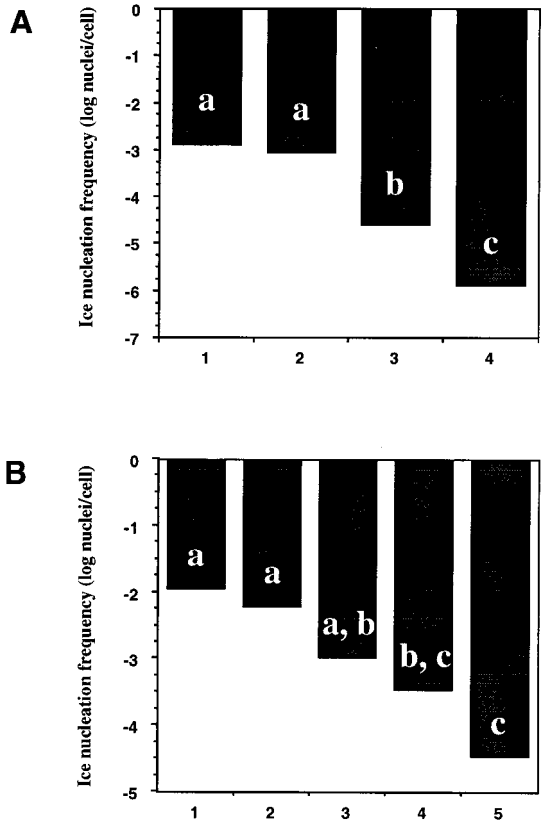

FIG. 4. Ice nucleation activity of the *phzB-inaZ* reporter fusion of strains 30-84Ice and 30-84Ice/I in the rhizosphere. (A) Ice nucleation activity of the *phzB-inaZ* reporter in bacteria isolated from wheat roots inoculated with 30-84Ice alone (column 1), a 50:50 ratio of 30-84Ice/I and the *phzI*$^+$ 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), a 90:10 ratio of 30-84Ice/I and 30-84Z (column 4), 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 ($P < 0.5$).

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 in situ. Expression of this fusion in the *phzI* mutant 30-84Ice/I was 1,000-fold lower than in strain 30-84Ice. 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 coinoculated with strain 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 coinoculated 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 coinoculated 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 coinoculated 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 30-84Ice 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* DH5α(pIC20H-*phzI*) culture supernatants, but not those from 30-84I (a *phzI* mutant) or *E. coli* DH5α(pIC20H), show R_f 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 enterocolitica*, and *Enterobacter agglomerans*), OOHL (*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 *P. aureofaciens* 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 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 *P. aureofaciens* 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 *P. aureofaciens* 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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