Interpopulation Signaling via *N*-Acyl-Homoserine Lactones among Bacteria in the Wheat Rhizosphere

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The biological control bacterium *Pseudomonas aureofaciens* 30-84 utilizes an *N*-acyl-homoserine lactone (AHL) signal molecule to control phenazine antibiotic production in the wheat rhizosphere (D. W. Wood and L. S. Pierson III, *Gene* 168:49-53, 1996). In this study, we demonstrate that naturally co-existing, non-isogenic bacterial populations interact with strain 30-84 at the level of gene expression via the exchange of diffusible signals on wheat roots. Wheat plants from three geographic locations were used to generate a random library of 700 rhizobacterial isolates. Roughly 8% of the isolates from each location restored phenazine gene expression to an AHL-deficient strain of 30-84 in vitro. Five of these isolates were further tested for their ability to influence gene expression of an AHL-deficient reporter of strain 30-84 on wheat roots. All five, isolated from different geographic locations, restored phenazine gene expression by the reporter to wild-type levels. This suggests that in vitro assays can identify bacterial isolates with the potential to influence phenazine expression in strain 30-84 via AHLs on wheat roots. The occurrence of such strains in all fields sampled suggests that AHL-mediated communication is a common occurrence in the wheat rhizosphere.

Additional keywords: phenazine antibiotics, quorum sensing.

Plant-associated bacteria must compete with a diverse community of microorganisms in order to colonize and persist on plants. The expression of traits necessary for competitive survival is likely to be influenced by the ability of the organism to sense and respond to co-occurring organisms. Many bacteria have evolved sensory transduction systems that utilize diffusible signals or “pheromones” to sense and respond to their biotic environment, including their own population density (reviewed in Gray 1997; Kaiser and Losick 1993; Salmond et al. 1995; Swift et al. 1996; Wirth et al. 1996). These signals trigger many different responses including multicellular differentiation, fruiting body development, and sporulation, as well as physiological processes such as bioluminescence,

plasmid conjugal transfer, and secondary metabolism. Inter-cellular communication enables homogeneous bacterial populations to coordinate gene expression and behave as a multicellular organism (Dworkin 1997). However, because microorganisms rarely exist in pure cultures under natural conditions, and because signal transduction pathways involving diffusible signals appear to be common among microbes, it seems likely that signals produced by one population would be utilized by non-isogenic, co-existing populations. We use the term “cross-communication” to describe signal exchange between co-existing microbial populations. In this paper, we specifically test the hypothesis that diffusible signals produced by co-occurring rhizobacteria can affect gene expression in the biological control bacterium *Pseudomonas aureofaciens* strain 30-84 in its natural environment, the wheat rhizosphere.

One well-characterized class of diffusible signal molecules are the *N*-acyl-homoserine lactone (AHL) molecules (reviewed in Dunlap 1997; Fuqua et al. 1996; Salmond et al. 1995; Swift et al. 1996). These signals are used by a diverse range of gram-negative bacteria to regulate the expression of genes involved in biotic interactions (i.e., microbial competition and host interactions) in a coordinated manner. Each AHL-mediated regulatory system consists of at least two conserved regulatory proteins, an AHL synthase and a transcriptional regulator that responds to the AHL(s) it recognizes. According to models of AHL-mediated gene regulation (reviewed in Dunlap 1997; Fuqua et al. 1996; Salmond et al. 1995; Swift et al. 1996), the AHL signal diffuses through the cellular membrane and accumulates in the local environment in a population-dependent manner. Once an intracellular concentration threshold is attained, the AHLs interact with their cognate regulator to activate or repress the transcription of target genes.

AHL regulatory systems have been demonstrated in a number of plant-associated bacteria (reviewed in Fuqua et al. 1996; Pierson et al. 1997, 1998a) including *Pseudomonas aureofaciens* strain 30-84. This root-colonizing strain is an effective biological control agent for take-all disease of wheat caused by the ascomycete *Gaeumannomyces graminis* var. *tritici*. Disease suppression is due primarily to the production of phenazine antibiotics (Pierson and Thomashow 1992). In addition to their role in disease suppression, phenazines enhance the survival of *P. aureofaciens* in the rhizosphere in competition with indigenous microorganisms (Mazzola et al. 1992). The expression of the phenazine biosynthetic operon (*phzFABCD*) is controlled by PhzI (Wood and Pierson 1996)

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and PhzR (Pierson et al. 1994). Recent work (Wood et al. 1997) demonstrated that AHL signals produced by an isogenic donor population restored phenazine gene expression in a *phzI* mutant population on wheat and confirmed that AHL signals are shared between populations in the rhizosphere. This work suggested that the local microbial community directly affects phenazine gene expression in strain 30-84 via AHL signals. The possibility of cross-communication among bacteria in their natural habitat is supported by in vitro studies that show that a large number of soilborne bacteria produce AHLs (Fuqua et al. 1996) and that strain 30-84 (Pierson and Pierson 1996) and other AHL-mediated regulatory systems respond to signals produced by different bacterial species (Bassler et al. 1997; Greenberg et al. 1979).

In order to test the hypothesis that cross-communication is a common phenomenon in the wheat rhizosphere, we utilized a collection of 700 wheat root-associated bacterial isolates from seven different fields in three separate geographic locations. Approximately 8% of these isolates from each location produced diffusible signals that affected gene expression in strain 30-84. Several of these strains restored phenazine gene expression in a *phzI* mutant to wild-type levels both in vitro and in situ on wheat roots. This suggests that rhizobacteria that can affect the expression of genes involved in biological control in strain 30-84 via AHL signals may be common in wheat rhizosphere communities.

RESULTS

Identification of test isolates with potential for interpopulation signaling.

Approximately 8% (59/700) of the bacterial isolates assayed in our primary in vitro plate assay restored the ability of the AHL-deficient strain 30-84I (Table 1) to produce phenazines (data not shown). This percentage was roughly consistent among areas sampled: percentages were 8% (45/565) for Pullman, Washington; 9% (4/44) for Ottawa, Canada; and 11% (10/91) south-central Arizona. Isolates shown to restore phenazine production to strain 30-84I were all gram negative but differed in a number of phenotypes including colony color and morphology; their ability to produce fluorescent siderophores (68%), hydrogen cyanide (9%) and extracellular protease (61%); and their ability to inhibit strain 30-84I (38%) and the fungal pathogen *G. graminis* var. *tritici* (59%) in vitro.

Isolates identified in the in vitro plate assay that potentially cross-communicated were further tested to determine the level to which they could induce *phzB::lacZ* expression in the

AHL-specific reporter strain 30-84Z/I. Induction of *lacZ* expression in strain 30-84Z/I by the addition of cell-free supernatants from different test isolates ranged from zero to more than two times that of the positive control (strain 30-84Ice) (data not shown). Interestingly, one third of the Washington State isolates tested in this assay were toxic to the 30-84Z/I reporter. To avoid the toxicity problem, we extracted diffusible signal from the test bacterial supernatants of all potentially cross-communicating strains with ethyl acetate. The 30-84Z/I reporter was able to grow in the ethyl acetate extracts of all test isolates with toxic cell-free supernatants. Nearly all of the extracts, whether from isolates with toxic or nontoxic cell-free supernatants, induced measurable to high β -galactosidase activity by the 30-84Z/I reporter. In contrast, extracts of the AHL-deficient strain 30-84Ice/I did not induce β -galactosidase activity by the reporter. Ethyl acetate extracts of all test isolates that induced measurable activity by strain 30-84Z/I were also tested to determine whether they could induce β -galactosidase activity by the AHL-specific reporter *Agrobacterium tumefaciens* A136. All extracts tested induced activity by the A136 reporter (data not shown). Induction of β -galactosidase activity in two distinct AHL-specific reporters confirms that diffusible AHL signals are extracted by this protocol.

Five isolates (OT-113, OT-21, PU-4, PU-276, and PI-34) that were not toxic to strain 30-84I on plates and were capable of activating both AHL-specific reporters were chosen for further testing on roots (Table 2). These isolates originated from five different fields, each located in one of the three geographic locations sampled. All of the isolates were *Pseudomonas* spp. and identified as either *Pseudomonas marginalis* (OT-113, PU-276), *P. putida* biotype A (OT-21, PI-34), or *P. putida* biotype B (PU-4) by gas chromatography–fatty acid methyl esterase (GC-FAME) analysis (Microbe Inotech Laboratories, St. Louis, MO). Thin-layer chromatography (TLC) analysis of ethyl acetate extracts with an overlay of the 30-84I reporter showed that only two isolates, PU-276 and PI-34, produced a diffusible signal with an R_f value similar to that of the signal produced by strain 30-84 (Table 3). Interestingly, isolate PI-34 also produced a second diffusible signal and both signals had R_f values similar to those produced by another phenazine-producing strain, *Pseudomonas fluorescens* 2-79 (Thomashow and Weller 1988) (Table 3). Isolates OT-113, OT-21, and PU-4 each produced a single diffusible signal with an R_f value similar to that of the second signal produced by PI-34 and *P. fluorescens* strain 2-79 (Table 3). The AHL-specific reporter strain *Chromobacterium violaceum* CV026 also identified at least two distinct signals in ethyl acetate ex-

Table 1. Bacterial reporter derivatives used in this study

Strain	Relevant characteristics	Reference or source
<i>Pseudomonas aureofaciens</i>		
30-84	Phz ⁺ , Rif ^r , PhzI ⁺ , wild type	W. W. Bockus
30-84Z	Phz ⁺ , Rif ^r , PhzI ⁺ , <i>phzB::lacZ</i> genomic fusion	Pierson et al. 1994
30-84Ice	Phz ⁺ , Rif ^r , PhzI ⁺ , <i>phzB::inaZ</i> genomic fusion	Wood and Pierson 1996
30-84I	Phz ⁺ , Rif ^r , PhzI ⁺ , <i>phzI::Km</i> genomic fusion	Wood and Pierson 1996
30-84Z/I	Phz ⁺ , Rif ^r , PhzI ⁺ , <i>phzB::lacZ</i> , <i>phzI::Km</i> genomic fusions	Wood and Pierson 1996
30-84Ice/I	Phz ⁺ , Rif ^r , PhzI ⁺ , <i>phzB::inaZ</i> , <i>phzI::Km</i> genomic fusions	Wood and Pierson 1996
<i>Agrobacterium tumefaciens</i> A136	TraI ⁺ , Tc ^r , Km ^r , Cb ^r , Sp ^r , <i>traA113::lacZ</i> genomic fusion	Fuqua and Winans 1994
<i>Chromobacterium violaceum</i> CV026	Double mini-Tn5 mutant derived from <i>C. violaceum</i> ATCC31532, Km ^r	McClean et al. 1997

^a If an AHL donor is present.

tracts of strain PI-34 and one signal each in extracts of the other four strains on TLC plates (data not shown).

Interpopulation signaling on roots.

We tested the interpopulation signaling potential of each of the five isolates OT-113, OT-21, PU-4, PU-276, and PI-34 on wheat roots in separate experiments with two different cultivars (Fig. 1). In each experiment, total root population sizes ranged from 10^6 to 10^7 CFU. In all experiments, ice nucleation activity of the two positive controls (30-84Ice and 30-84Ice/I+30-84Z) was significantly greater (10 to 100×) than that of the negative control (30-84Ice/I). None of the five test isolates by themselves had measurable ice nucleation activity on roots (data not shown). However, when mixed in combination with the AHL-specific reporter 30-84Ice/I, all induced significantly greater ice nucleation activity by the reporter, compared with the reporter alone (Fig. 1). In most cases, the level of ice nucleation activity by the reporter in combination with the test isolate was not significantly different from either of the two positive controls. Control experiments demonstrated that cell-free culture supernatants of the test isolates in combination with the reporter on roots did not induce ice nucleation activity (data not shown). These data demonstrated that the test isolates were able to induce wild-type levels of gene expression in the 30-84Ice/I reporter on wheat roots.

DISCUSSION

The results presented here are the first to show that signals produced by naturally co-occurring, non-isogenic, wheat-associated bacteria can affect the expression of genes by strain 30-84 in its natural habitat, the wheat root. Approximately 8% of the 700 isolates in our collection of wheat root-associated bacteria were able to activate the 30-84 reporters in vitro and this percentage was roughly consistent among isolates from the three different geographic locations. This collection is composed of a random sample of culturable bacteria found in the wheat rhizosphere, does not include viable but nonculturable microbes, and may include siblings of the same isolates. Since it is estimated that only 1 to 10% of the viable rhizobacterial population can be cultured in vitro, this percentage does not necessarily reflect the proportion of isolates capable of cross-communication in the rhizosphere. However, it does

indicate that a small, phenotypically diverse population of organisms that naturally occur in the rhizosphere of wheat, isolated from widely separated geographic locations, produce signals that can alter phenazine gene expression by strain 30-84 in vitro. All five isolates selected by in vitro analysis were able to activate the AHL-specific reporter, strain 30-84Ice/I, on the roots of more than one wheat cultivar, suggesting that in vitro assays can be used to predict the ability of bacterial strains to communicate via diffusible signals in the wheat rhizosphere. The occurrence of strains capable of intercellular communication with strain 30-84 via AHLs in all fields sampled suggests that AHL-mediated cross-communication is common in the wheat rhizosphere.

This study confirms earlier in vitro work that showed that a large number of soilborne bacteria produce AHLs and that strain 30-84 and other AHL-mediated regulatory systems respond to signals produced by different bacteria (Bassler et al. 1997; Fuqua et al. 1996; Greenberg et al. 1979; Pierson and Pierson 1996). We found that ethyl acetate extracts of test isolates that activated the *P. aureofaciens* 30-84Z/I reporter also activated the *A. tumefaciens* A136 and *C. violaceum* CV026 reporters in vitro. The fact that these extracts activated three separate AHL-specific reporter systems strongly suggests that they contain AHL derivatives. Based on the R_f values of signal extracts on TLC plates, the test isolates capable of cross-communication with strain 30-84 in the rhizosphere, as a group, produce at least two distinct signal molecules rec-

Table 3. Migration indices (R_f values) of *N*-acyl-homoserine lactone (AHL) signals produced by bacterial strains

Strain extract	R_f value ^a < 0.5	R_f value > 0.5
30-84Ice	...	0.58 ^b
OT-113	0.33	...
OT-21	0.35	...
PU-4	0.32	...
PU-276	...	0.58
PI-34	0.37	0.57
2-79	0.30	0.55

^a R_f values determined from thin-layer chromatography analysis of AHLs present in ethyl acetate extracts from bacterial cultures. AHL signals were detected by an overlay of the AHL-specific reporter 30-84I. All extracts produced either single spots with either a low index (R_f value < 0.5) or a high index (R_f value > 0.5), or two spots (one with a high and one with a low index).

^b Standard deviations in mean R_f values were less than 8% for all strains.

Table 2. Characteristics of select test isolates

Isolate	Strain identity ^a	Colony color ^b	HCN / protease ^c	Phenazine ^d	Inhibits 30-84I ^e	Inhibits <i>Ggt</i> ^f	30-84Z/I+ extract ^g	A136+ extract ^g
OT-113	<i>P. marginalis</i>	Yellow, +	± / ±	No	No	++	93 ± 5%	79 ± 12%
OT-21	<i>P. putida</i> bt A	Yellow, +	± / ±	No	No	++	129 ± 13%	85 ± 12%
PU-4	<i>P. putida</i> bt B	White, -	- / +	No	No	-	78 ± 5%	98 ± 5%
PU-276	<i>P. marginalis</i>	Orange, +	+ / -	PCA	Slight	+++	111 ± 14%	62 ± 8%
PI-34	<i>P. putida</i> bt A	Yellow, +	+ / -	No	No	-	74 ± 15%	86 ± 15%

^a Strain identity determined by gas chromatography-fatty acid methyl esterase (GC-FAME).

^b Colony color and presence/absence (+/-) of fluorescent siderophores on KMB after 48 h.

^c Ability to produce hydrogen cyanide and extracellular protease (± indicates inconclusive result).

^d Ability to produce phenazines (PCA indicates production of phenazine-1-carboxylic acid).

^e Ability to inhibit strain 30-84I in vitro (slight indicates zone of inhibition less than 5 mm).

^f Ability to inhibit *Gaeumannomyces graminis* var. *tritici* (*Ggt*) in vitro (++ indicates zones of inhibition greater than 7 mm, +++ indicates fungicidal activity).

^g Amount of β-galactosidase activity produced by a combination of an ethyl acetate extract of the test isolate plus either the *P. aureofaciens* 30-84Z/I reporter or the *A. tumefaciens* A136 reporter. β-galactosidase activity of test isolates was expressed as a percentage of the activity of the positive control (strain 30-84Ice) in each experiment. Values are the average of three replicates; standard errors are given.

ognizable by strain 30-84. Interestingly, only two of the strains produced a signal detected by 30-84I with an R_f value similar to that of the signal produced by strain 30-84. Thus, at least two distinct signal molecules are capable of influencing phenazine gene expression by *P. aureofaciens* strain 30-84 in situ on wheat roots.

AHL-mediated gene regulation, also known as "quorum sensing," has been described as a mechanism by which bacteria sense and respond to their own population densities. The discovery that gene expression can be affected by AHL signals produced by co-occurring, non-isogenic bacteria suggests that the overall level of signal in the immediate environment

Fig. 1. Ice nucleation activity of strain 30-84Ice (ICE) or strain 30-84Ice/I (ICE/I) alone or in combination with test isolates on wheat roots after 10 days. Test isolates include strains 30-84Z (Z), OT-113, OT-21, PI-34, PU-4, and PU-276. All of the isolates were tested on two wheat cultivars: **A**, Penawawa or **B**, Fielder. Standard errors are indicated by error bars and means with the same letter are not significantly different.

determines patterns of gene expression. Thus, the “quorum” sensed by the responding population is likely to be composed of both isogenic and non-isogenic signal donors. The reality of cross-communication via diffusible signals has important implications for microbial ecology. Theoretically, non-isogenic populations of microorganisms may interact at the level of gene expression by altering the level of signal in the environment by either contributing toward the total amount of recognizable signal, acting as sinks, or producing antagonistic signals that interfere with gene expression (Pierson et al. 1998b). It is unknown whether cross-communication confers a selective advantage to one or all of the interacting populations. However, in strain 30-84, these signals regulate genes involved in competition and rhizosphere competence (Mazzola et al. 1992), suggesting that cross-communication may directly affect the outcome of competitive interactions among microorganisms in the rhizosphere. Thus, the production and exchange of AHL signals may play an important role in determining the structure and function of the rhizosphere community. Future research will explicitly test the ecological significance of AHLs produced by co-existing bacteria for competition among bacterial populations in the rhizosphere with the *P. aureofaciens* strain 30-84 system.

MATERIALS AND METHODS

Bacteria.

Characteristics of reporter derivatives of *P. aureofaciens*, *A. tumefaciens*, and *C. violaceum* are listed in Table 1.

Wheat root-associated bacteria were isolated from plants grown in seven different fields located in either Ottawa, Canada (prefix OT); Pullman, Washington (prefix PU); or south-central Arizona (prefix MA or PI for Maricopa or Pima counties, respectively). Isolates were sonicated from wheat roots and adhering soil in 0.1 M MgSO₄ solution and cultured on pigment-producing medium D (PPMD; Canada isolates only) or Luria-Bertani (LB) medium as described previously (Wood et al. 1997). For long-term storage, isolates were maintained in a 1:1 mixture of PPMD culture and 80% glycerol at -80°C. Unless otherwise stated, all reporters and test isolates were grown at 28°C in PPMD medium.

Screening isolates for the production of AHLs recognized by strain 30-84.

Wheat root isolates were grown overnight (18 to 24 h) and 5 µl of each culture was spotted onto (i) PPMD plates freshly spread with a lawn of an overnight culture of strain 30-84I and (ii) PPMD plates that, after 24 h, were sprayed with an overnight culture of strain 30-84I. Strain 30-84I is a derivative of strain 30-84 that is defective in *phzI* and therefore lacks the ability to synthesize the AHL signal. The plates were scored after 24 h for the ability of each test isolate to restore phenazine production to strain 30-84I (resulting in orange halos around the test isolates). Those isolates that restored the ability of strain 30-84I to produce phenazines were presumed to produce an AHL signal recognized by strain 30-84I. Strains 30-84Ice and 30-84Z, which produce the endogenous AHL signal but do not produce phenazines, were included on each plate as positive controls. Test isolates were also scored for their ability to inhibit strain 30-84I by measuring zones of inhibition around each test isolate. Each isolate was tested on three replicate plates.

Isolates shown to complement strain 30-84I for phenazine production were further characterized to determine whether they were phenotypically different from strain 30-84 and each other. The ability to produce metabolites known to play a role in disease suppression including fluorescent siderophores (pyoverdins), hydrogen cyanide, and phenazines was determined as described previously (Pierson and Thomashow 1992). Extracellular protease production was assayed by the formation of a zone of clearing around the test isolate on skim milk agar (10% [vol/vol] Difco Skim Milk, 3% [wt/vol] agar). Isolates were also tested for their ability to inhibit the fungal pathogen *G. graminis* var. *tritici* in vitro, as described previously (Pierson and Weller 1994). All strains were also Gram stained.

Isolates selected from the in vitro plate assay were tested to determine if they could induce *phzB::lacZ* expression in the AHL-specific reporter, strain 30-84Z/I, as described previously (Wood et al. 1997). Briefly, 5-ml cultures of each test isolate were grown 18 to 24 h to a high cell density ($OD_{620} \geq 1.0$). Cell-free supernatants of each test culture were obtained by centrifugation ($3,000 \times g$, 5 min) and filter sterilization (a combination 0.8 µm/0.2 µm filter). Cells of strain 30-84Z/I from 10 ml of a low cell density culture ($OD_{620} = 0.12$) were also collected by centrifugation. The cells of the reporter were resuspended in the cell-free supernatants of the test isolates (with 10% fresh media) and allowed to grow 18 to 24 h. The level of β-galactosidase activity was measured according to the method of Miller (1972). Strains 30-84Ice and 30-84Ice/I (Table 1) were included in each assay as positive and negative controls, respectively. Because the absolute level of β-galactosidase activity (expressed in Miller units) varied from experiment to experiment, β-galactosidase activity of test isolates was expressed as a percentage of the activity of the positive control (strain 30-84Ice) in each experiment (i.e., [Miller units of the test isolate/Miller units of the 30-84Ice control] × 100).

Rapid ethyl acetate extraction and biological assay.

Crude AHL preparations were isolated from cell-free supernatants of 5-ml overnight cultures by a procedure modified from Wood et al. (1997). Briefly, cell-free supernatants were extracted with an equal volume of ethyl acetate (acidified with 0.1 ml of glacial acetic acid per liter) for 1 to 2 h on a rotating mixer. The preparations were centrifuged ($3,000 \times g$, 5 min) and the ethyl acetate phase collected. The ethyl acetate was removed by rotary evaporation and residual ethyl acetate evaporated under nitrogen gas. The extract was resuspended in 2 ml of PPMD broth and inoculated with 20 µl of a low density culture ($OD_{620} = 0.12$) of an AHL-specific reporter, either *P. aureofaciens* 30-84Z/I or *A. tumefaciens* A136. After incubation for 16 to 18 h, the cultures were assayed for β-galactosidase activity. Each test isolate was assayed in three separate experiments with each of the two reporters.

Test for interpopulation signaling on roots with the InaZ assay.

Several test isolates were selected for further testing based on their ability to activate both the *P. aureofaciens* 30-84Z/I and the *A. tumefaciens* A136 reporter strains in vitro and their lack of inhibition of strain 30-84I on plates. These isolates were tested to determine if they also could activate the AHL-

specific reporter of strain 30-84, 30-84Ice/I, on wheat roots. Each InaZ root assay was performed as described previously (Wood et al. 1997) and consisted of the following bacterial treatments: (i) strain 30-84Ice (a positive InaZ control); (ii) strain 30-84Ice/I (a negative InaZ control); (iii) a 50:50 ratio of strains 30-84Ice/I (reporter) and 30-84Z (an AHL donor; an additional positive control); (iv) a 50:50 ratio of strain 30-84Ice/I (reporter) and a test isolate; and (v) the test isolate alone. Each isolate was tested on two different wheat cultivars (Penawawa or Fielder) in separate experiments with a minimum of 10 replicate plants per bacterial treatment.

Briefly, bacterial strains were grown individually 18 to 24 h to a high cell density ($OD_{620} \geq 1.0$). Surface-sterilized, pregerminated wheat seeds were placed in 10 ml of the appropriate bacterial culture (mixed treatments contained 5 ml of each strain), blotted briefly, and planted immediately in 25-ml culture tubes (20 cm long $\times$ 2.5 cm diameter) containing sterile sand (to a 6 cm depth) moistened with 5 ml of sterile 1/3 Hoagland's solution (macro nutrients only; Hoagland and Arnon 1950). Seeds were covered lightly with sterile sand and incubated in a growth chamber (25/20°C, 12/12 h light/dark cycle). After 10 days, plants were harvested aseptically, heights were measured, and the roots and lightly adhering sand (excluding the remaining caryopsis) were harvested into 15-ml sterile plastic tubes containing 10 ml of phosphate-buffered saline (pH 7.4). Bacteria were removed from roots by vortexing (10 s) and sonicating (30 s) the tubes twice. Bacterial populations were assayed by serial dilution plating on LB. For mixed treatments containing the 30-84Ice/I reporter and the test isolate, the total population was determined on LB and the population of the 30-84Z/I reporter was determined by plating on LB with kanamycin (50 μ g/ml). Populations of the 30-84Ice/I reporter and the 30-84Z donor were differentiated on LB with 2% (wt/vol) 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; Sigma, St. Louis, MO). Strain 30-84Z appears blue due to the expression of the structural *phzB::lacZ* fusion.

The ice nucleation assay was performed immediately after harvest by spotting 10- μ l drops of the appropriate dilution of each bacterial treatment on an aluminum foil tray (pretreated with 1% [wt/vol] paraffin) in an ethanol ice bath (-11°C) as described previously (Wood et al. 1997). For each sample, we selected the dilution at which between 2 and 8 of 10 drops froze. The proportion of a total of 40 drops frozen was determined after 7 min. The ice nucleation frequency is determined as $N(T) = \ln[1/(1 - f)]/[10^D/V][1/C]$, where D is the number of 10-fold serial dilutions, *f* is the fraction of frozen droplets, *V* is the volume of droplets (in milliliters), and *C* is the cell concentration (CFU/ml) of each sample (Lindgren et al. 1989). Ice nucleation frequencies in our experiments are reported on a scale that ranges from zero (e.g., ice nucleation frequency of 1) to about -6 (e.g. ice nucleation frequency of 1 in 10^6 cells). Mean ice nucleation activity was compared among treatments by analysis of variance (ANOVA) and least significant difference (LSD) multiple comparison tests.

Identification of strains and characterization of AHLs.

The five bacterial isolates used in the root experiment were identified by GC-FAME analysis (Microbe Inotech Laboratories, St. Louis, MO). AHLs produced by these isolates were characterized as described previously (Wood et al. 1997) by a

procedure modified from Shaw et al. (1997). Briefly, ethyl acetate extracts from culture supernatants of test isolates were spotted (2 to 10 μ l) onto reverse phase TLC plates. Samples were separated by TLC with 60% (vol/vol) methanol in water. Once the solvent front had migrated to within 2 cm of the top of the plate, the plate was hot air dried and overlaid with a thin film of 0.3% (wt/vol) PPMD agar inoculated from an overnight culture of an AHL-specific reporter, either strain 30-84I or *C. violaceum* CV026 (1/100 dilution). After 24 to 48 h, AHLs were located as either orange or purple spots resulting from the production of either phenazines by the 30-84I reporter or the pigment violacein by *C. violaceum* CV026, respectively. Relative migration indices (R_f values) were calculated as the distance to the center of the spot from the origin relative to the distance to the solvent front from the origin.

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