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ORIGINAL ARTICLE

Acyl-homoserine lactone-mediated cross talk among epiphytic bacteria modulates behavior of *Pseudomonas syringae* on leaves

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The leaf surface harbors a host of bacterial epiphytes that are capable of influencing the quorum sensing (QS) system of the plant pathogen *Pseudomonas syringae* pv. syringae (*Pss*). *Pss* uses QS to regulate expression of genes conferring extracellular polysaccharide production, motility and factors contributing to virulence to plants. About 7% of bacterial epiphytes isolated in this study produce the *Pss* cognate signal, 3-oxohexanoyl-homoserine lactone (3OC6HSL), often in amounts more than 10-fold higher than *Pss*. Premature induction of QS in *Pss* by these 3OC6HSL-producing epiphytes suppressed swarming motility and subsequent disease of the leaf. Co-inoculation of 3OC6HSL-producing strains with *Pss* reduced the number of lesions when inoculated together onto leaves compared with that of plants inoculated with *Pss* alone. Strains in which 3OC6HSL accumulation was quenched by expression of an *N*-acyl-homoserine lactonase did not decrease disease when co-inoculated with *Pss.* Disease incidence caused by a nonmotile mutant of *Pss* was not affected by 3OC6HSL-producing bacteria, suggesting that exogenous 3OC6HSL signal that altered the motility of *Pss* was responsible for reducing the apparent virulence of this pathogen. Thus, considerable cross talk involving exogenous 3OC6HSL occurs on leaves and this process can be exploited for disease control.
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Introduction

Many microbial traits are expressed in a cell-density-dependent manner under the control of a regulatory system known as quorum sensing (QS) (Miller and Bassler, 2001; Whitehead *et al.*, 2001). In this system, specific gene expression is regulated by a small, diffusible signal molecule that increases in concentration as cell density increases. Such signal molecules are normally produced at low, constitutive levels, and do not allow accumulation of high local concentrations unless cell numbers increase or the diffusion of the signal molecule is constrained. The best characterized of the bacterial QS systems uses *N*-acyl-homoserine lactones (AHL) as signal molecules. These AHLs consist of a homoserine lactone (HSL) ring and acyl chains of various lengths and modifications. Typically, this system requires an AHL synthase similar to LuxI and a response regulator similar to LuxR of *Vibrio fischerii* (Waters and Bassler, 2005). At low cell densities, *luxI* homologues are expressed at low levels, thus leading to low and constitutive levels of AHL production. As the population size increases, the local AHL concentration increases, resulting in increased AHL activation of the LuxR-like transcriptional activator, which then increases expression of the AHL synthase as well as downstream genes. Such cells constitute a 'quorum' in which traits such as motility, bioluminescence, genetic competence, production of extracellular polysaccharides and virulence factors important to the cell density or diffusion-limited phases of life of such microbes are expressed.

Several plant pathogenic bacteria exhibit QS-dependent behaviors (Loh *et al.*, 2002; von Bodman *et al.*, 2003). In the plant pathogenic bacterium *Erwinia carotovora*, 3-oxohexanoyl-homoserine lactone (3OC6HSL), which is produced by CarI and under the control of CarR, is required for the production of the carbapenem antibiotics and several exoenzymes involved in the maceration of plant tissues. *Pantoea stewartii* modulates the production of the extracellular polysaccharide

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stewartan, a virulence factor, through its production of 3OC6HSL under the control of EsaR. The beneficial rhizobacterium *Pseudomonas aureofaciens* has two separate, nonhierarchical QS systems. Both the PhzI/R and the CsaI/R systems are required for the production of phenazine antibiotics and exoproteases that aid in successful colonization of the wheat rhizosphere and antagonism of root-infecting fungi (Wood and Pierson, 1996; Wood *et al.*, 1997; Zhang and Pierson, 2001). AHL-mediated quorum induction is important in rhizosphere growth, nodule development and plasmid conjugation in *Rhizobium leguminosarum*, a symbiont of various legumes (Gonzalez and Marketon, 2003).

Quorum sensing controls several traits important to both the epiphytic fitness and virulence of the plant pathogenic bacterium *Pseudomonas syringae* pv. syringae (*Pss*), the causal agent of brown spot disease of bean. This pathogen can establish epiphytic populations on various plant species, and large populations of the pathogen precede and are predictive of disease on susceptible plants (Rouse *et al.*, 1985; Hirano and Upper, 2000). Epiphytic communities of *Pss* are spatially aggregated on leaves, and cells in large aggregates are more resistant to desiccation stress than solitary cells (Monier and Lindow, 2003). Such density-dependent behavior is apparently controlled by QS. AhlI confers production of 3OC6HSL under the control of *ahlR* and other regulatory genes in *Pss* (Quinones *et al.*, 2004). A variety of traits including extracellular polysaccharide production, oxidative stress tolerance, swarming motility, promotion of water-soaked lesions and extent of internal tissue maceration in bean pods are regulated by QS in *Pss* (Dulla *et al.*, 2005; Quinones *et al.*, 2005). As swarming motility of *Pss* strongly contributes to its ability to invade leaves and incite disease, the control of this trait by QS has a particularly large effect on the disease process (Haefele and Lindow, 1983, 1987).

Because QS coordinates many traits important to the environmental success and virulence of bacteria, many studies have addressed processes that might disrupt QS-dependent gene expression. Many small molecules such as noncognate AHLs, AHL intermediates and dicyclic peptides produced by bacteria and compounds such as furanones produced by eukaryotes have the ability to interfere with AHL-mediated QS (Givskov *et al.*, 1996; Bauer and Robinson, 2002). Likewise, bacterial enzymes such as the *Bacillus* sp. AiiA-encoded AHL lactonase (Dong *et al.*, 2000), the *Ralstonia* sp. AiiD-encoded AHL acylase (Lin *et al.*, 2003) and the *Pseudomonas* sp. PvdQ-encoded AHL acylase (Huang *et al.*, 2003) are able to degrade AHLs and quench QS.

As the variety of AHL signal molecules produced by bacteria is limited, there is considerable opportunity for cross talk among bacteria in mixed communities. The most common AHLs made by QS bacteria have acyl chains of 4–12 carbons with and without a carbonyl oxygen on the third carbon of the chain. Thus, many bacterial taxa will share the same AHL signal, increasing the possibility of cross talk among bacterial species. Pierson *et al.* (1998) has found that as many as 8% of bacteria cultured from the wheat rhizosphere could modulate phenazine production and presumably QS in *P. aureofaciens* 30-84. They also found that expression of an AHL-dependent *phzI* gene in this species could be at least partially rescued when an AHL-deficient PhzI mutant strain was introduced into soil, suggesting that AHL signals from the soil bacterial community were sensed by the introduced strain (Pierson *et al.*, 1998). Likewise, 3OC6HSL produced by a *P. putida* strain could be detected by a nonproducing strain of this species on roots up to 68 μm away, but was most commonly detected only a few micrometers away from the producing strain (Gantner *et al.*, 2006). Similarly, AHLs produced by *Serratia* sp. could be detected by nearby cells of this *P. putida* AHL sensor on roots (Steidle *et al.*, 2001). Although these studies show that cross talk by sharing of AHL signal molecules can occur on roots, it remains unclear if such interactions are biologically significant or occur in other natural habitats. Given that leaves harboring *Pss* also accommodate a variety of other epiphytic bacteria, it seemed likely that many of its phyllosphere cohorts could potentially modulate its behavior by the production of exogenous 3OC6HSL.

In this study, we address the frequency with which epiphytic bacteria produce a signal molecule recognized by the QS system of *Pss* and whether such strains influence its behavior on plants. We show that a relatively high proportion of bacterial epiphytes produce the cognate AHL of *Pss*, and often at much higher levels. Furthermore, such strains alter QS-mediated traits of *Pss* such as swarming motility in an AHL-dependent manner, altering invasion and thus infection of susceptible plants.

Materials and methods

Bacterial strains, media and culture conditions

P. syringae pv. syringae B728a (Loper and Lindow, 1987), strain BHSL, an AHL-deficient derivative of strain B728a (Kinscherf and Willis, 1999), other *Pss* strains and *Agrobacterium tumefaciens* NT1 (pSVB33, pJM749) (Piper *et al.*, 1993) were maintained on King's medium B (KB) (King *et al.*, 1954) and grown at 28 °C. *Escherichia coli* strains DH5α, TOP10 (Invitrogen, Carlsbad, CA, USA), and 1480 (Williams, 1979) were maintained on Luria Agar and cultured at 37 °C. Concentrations of the antibiotics used were kanamycin 25 or 50 μg ml^{-1}, rifampicin 100 μg ml^{-1} and spectinomycin 20 μg ml^{-1}.

Isolation and screening of epiphytes

Leaves were collected from various unidentified plant species (other than bean) from California,

Wisconsin and Minnesota. Individual leaves were placed in sterile plastic bags with 10 ml of 10 mM potassium phosphate buffer (KPO_4 buffer; pH 7.0) and sonicated for 7 min to remove bacteria from leaves as in other studies (Quinones *et al.*, 2004, 2005). Appropriate dilutions of leaf washings were plated on KB and the plates were incubated at 28 °C for 3 days. Bacterial colonies differing in appearance were spotted on KB and LA plates for assay of AHL production. The plates were then immediately sprayed with a dense suspension ($>10^9$ cells per ml) of BHSL (pBQ9) (Quinones *et al.*, 2004) with an artists' airbrush and incubated at 28 °C overnight. Induction of QS was visualized by illuminating plates with a handheld UV lamp and observing the green fluorescent halos in the lawn of BHSL (pBQ9).

AHL extraction and identification
Isolates were grown overnight in Luria broth and cells were separated from the culture medium by centrifugation. Equal volumes of water-saturated ethyl acetate were used to extract AHLs from the cell-free culture supernatant. Extracts were concentrated to near dryness under vacuum and resuspended with a minimal amount of ethyl acetate. Samples and known amounts of authentic AHLs were applied to silica C18 reverse-phase TLC plates (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA). Development of the TLC plates and use of the AHL bioindicator strain *A. tumefaciens* NT1 (pSVB33, pJM749) (Piper *et al.*, 1993) were done as described by Shaw *et al.* (1997). In addition, extracts and AHL standards were also spotted onto TLC plates, air dried and overlaid with KB agar (approximately 3 mm thickness). A dense suspension ($>10^9$ cells per ml) of BHSL (pBQ9) was sprayed with an artists' airbrush onto the agar overlay and the plates were incubated at 28 °C overnight. The diameter of green fluorescent spots indicative of QS induction was measured.

Bacterial identification
Bacterial strains were grown on KB, scrapped from plates after 24 h of growth at 28 °C and washed once in sterile water to generate a dense cell suspension for use as a template for amplification of 16S rDNA genes. A 1.5 kb region of the 16S rRNA gene was amplified using the universal 16S sequencing primers 1510R (5′-GTGCTGCAGGGTTACCTTGTTACGACT-3′) and 6F (5′-GGAGAGTTAGATCTTGGCTCAG-3′) (van der Meer *et al.*, 1998). The amplified PCR products were cloned into pTOPO2.1 (Invitrogen) and the partial DNA sequences obtained by cycle sequencing by standard methods were compared with other sequences in GenBank using BLAST (Altschul *et al.*, 1997).

Swarming motility
Swarming motility of *Pss* was assessed on semisolid KB plates containing 0.4% agar as in previous studies (Kinscherf and Willis, 1999; Quinones *et al.*, 2005). Cells were grown for 2 days on KB and then harvested and washed in 10 mM KPO_4 buffer. Disks of filter paper (Fisherbrand; grade P8-creped 6 mm (Fisher Scientific, Pittsburgh, PA, USA)) were placed on the plates and inoculated with 10^7 cells of the appropriate bacterial strain and the plates were incubated overnight at 28 °C.

flgK *mutant construction*
A DNA fragment of approximately 1 kb in length harboring the internal portion of *flgK* was amplified from *Pss* B728a genomic DNA using the primers flgK-KO-fow (5′-CACCGTCGCTGATTTCAATTGGGCTCTCAGGTATC-3′) and flgK-KO-rev (5′-CACTGTTGGTGGTTTTGCCGATGCTGCGCTG-3′). This fragment was placed in the Gateway (Invitrogen) vector pENTR/D-Topo, and then transferred to pLVC-D as described by Marco *et al.* (2005). The resulting construct was transformed into *Pss* B728a and kanamycin was used to select for single crossover events as in other studies (Marco *et al.*, 2005). Resulting interruptions in *flgK* were verified to block swarming as described above.

Manipulation of AHL production in tested strains
Production of 3OC6HSL in *E. coli* 1480 was achieved through the following steps. *ahlI* was amplified with the primers ahlI-Fow-Nde1 (5′-GCACATATGCTGATCCTGGTGCGTGTGGG-3′) and *ahlI*-Rev-Nde1 (5′-CCTCATATGGACTCAGGCTGCGCTGTC-3′). The resulting fragment was digested with *Nde*I and ligated into the *Nde*I site of pKLN42 (Dulla and Lindow, 2008) containing P_{trp}:*gfp*, to yielded pGFD4 in which the green fluorescent protein (*gfp*) encoding region was replaced by *ahlI*. Gene orientation was determined by sequencing. Plasmids pGFD4 and the AiiA AHL lactonase-encoding pME6368 (Reimmann *et al.*, 2002) were introduced into appropriate strains by electroporation as in other studies (Joyner and Lindow, 2000). Alteration of AHL production in such strains was confirmed using indicator strains and assays described above.

Plant assays
Cells of all bacterial strains were grown on KB with appropriate antibiotic selection, harvested by scraping of the plate surface, washed once in sterile distilled water and resuspended to a final cell concentration of 10^6 cells per ml in 1 mM KPO_4 buffer either individually or in equal mixtures of two strains. The cell suspension was then spray inoculated onto fully expanded primary and trifoliate leaves of bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274; approximately 2 weeks old). Plants were then placed in a humid chamber at room temperature (approximately 24 °C) and periodically exposed to a fine mist of water to maintain high relative humidity and to maintain a thin film of

water on the leaf surface. Artificial light was maintained for 16 h periods within the 24 h cycle. After 24 h, plants were taken out of the chamber and placed on a greenhouse bench for 7 days to allow for disease development. The number of visible brown spot lesions was then counted on individual trifoliate leaves. Disease incidence was assessed on at least 14 individual leaves from each of five or more replicate pots. Epiphytic population sizes were determined by plating appropriate dilutions of washings of individual trifoliate leaves 24 h after inoculation as previously described (Quinones *et al.*, 2004, 2005).

Statistical analysis
A one-way analysis of variance was used to test if treatments affected virulence, measured as lesions per leaf. When necessary, data were $\log(X+1)$-transformed for normality. Multiple comparisons among treatment effects were made using a least significant difference (LSD) test. All analyses were performed using Statistica (StatSoft Inc., Tulsa, OK, USA).

Results

Identification of epiphytes inducing QS in Pss
Approximately 7% of the 1117 bacterial strains tested were able to elicit a fluorescent green halo around colonies of the *Pss* AHL indicator strain, demonstrating their production of a diffusible molecule capable of inducing expression of the AHL-inducible *ahlI* promoter linked to a *gfp* reporter gene in BHSL (pBQ9), which is incapable of producing AHLs (Figure 1). We did not include in this survey the small percentage of bacterial strains that produced a zone of growth inhibition to the *Pss* indicator strain. The identities of 10 randomly chosen isolates from the 77 putative AHL-producing strains found in this study were determined by partial sequencing of their 16S ribosomal DNA locus. Sequence analyses revealed that all of the strains were members of the *Pseudomonas*, *Erwinia* or *Pantoea* genera (Table 1).

Characterization of QS-activating compounds
All QS-activating epiphytes were found to produce 3OC6HSL and many of these strains produced over 10-fold AHL than *Pss* (Table 1). These strains also produced variable numbers of other AHL species that could be detected with the *A. tumefaciens* NT1 (pSVB33, pJM749) biosensor when overlayed on TLC plates on which the AHLs in ethyl acetate extracts of culture media had been separated. *Pss* B728a primarily produces 3OC6HSL to which *ahlI* is most responsive. This strain also produces very small amounts of 3-oxo-octanoyl-HSL, and is responsive to this and other AHLs only at much higher concentrations than 3OC6HSL (Quinones

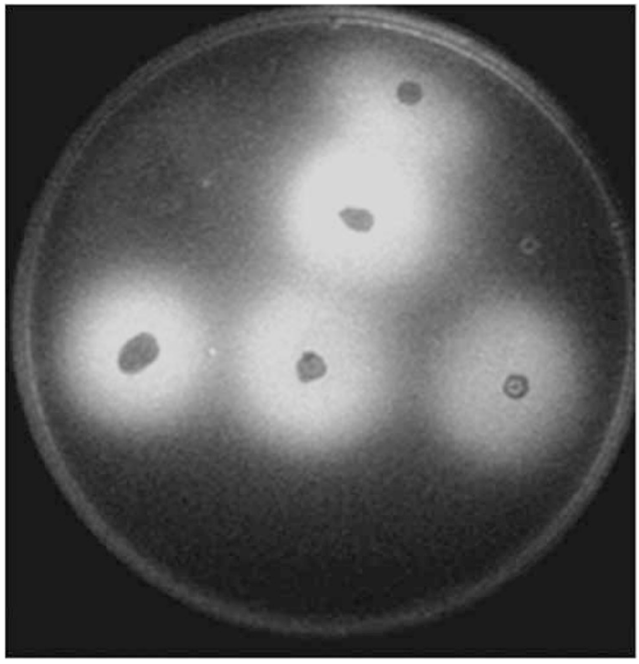

Figure 1 Colonies of bacterial epiphytic isolates stimulate the production of *N*-acyl-homoserine lactones (AHL)-induced green fluorescent protein (GFP) in a lawn of the *Pseudomonas syringae* pv. syringae (*Pss*) bioindicator strain BHSL (pBQ9). A full color version of this figure can be found at the *ISME Journal* online.

et al., 2004). All epiphytic bacterial isolates that produced 3OC6HSL also produced 3-oxo-octanoyl-HSL. In addition, some strains also produced 3-oxo-dodecanoyl-HSL, 3-oxo-decanoyl-HSL, dodecanoyl-HSL and decanoyl-HSL (Table 1). No induction of the *ahlI:gfp*-containing *Pss* BHSL (pBQ9) AHL biosensor strain was observed in culture extracts of any AHL-producing epiphytic bacteria into which plasmid pME6863 (Reimmann *et al.*, 2002) harboring the AiiA-encoded AHL lactonase was introduced, indicating that their ability to modulate QS in *Pss* was due to one or more AHLs that they had produced.

Modulation of QS-controlled swarming by Pss
Because swarming motility in *Pss* is suppressed by the activation of QS (Quinones *et al.*, 2005), we assessed whether epiphytic bacterial strains capable of AHL production such as *Erwinia* sp. FT1, a potent producer of 3OC6HSL, could modulate swarming motility of *Pss* in culture. Strain BHSL (pBQ9) does not produce AHLs and thus exhibits a hyperswarming phenotype on semisolid agar (Figure 2c). When BHSL (pBQ9) and strain FT1 were spotted near each other, BHSL (pBQ9) moved away from the point of inoculation only on the side of the spot opposite that of strain FT1 and remained stationary on the side proximal to it (Figure 2a). Furthermore, quorum induction of BHSL (pBQ9), evident as the production of GFP fluorescence, was apparent only on the side of the BHSL (pBQ9) colony nearest strain FT1

Table 1 AHL production by a collection of epiphytic bacteria

Strain	*Taxa*[a]	*AHLs produced*[b]	*Relative QS induction*[c]
B728a	*Pseudomonas syringae*	OC6	1
FT1	*Erwinia*	OC12, OC10, OC8, OC6, C12	15.1
FT3	*Erwinia*	OC12, OC10, OC8, OC6, C12	19.2
FT4	*Pseudomonas*	OC8, OC6, C10	1.3
FT5	*Pseudomonas*	OC8, OC6	8.7
FT6	*Pseudomonas*	OC8, OC6	9.3
FT7	*Pseudomonas*	OC8, OC6	1.5
PW1	*Enterobacter*	OC8, OC6	1.9
PW2	ND	OC8, OC6	0.5
WI2b	ND	OC8, OC6	10.9
WI3b	*Pantoea*	OC8, OC6	11.4
WI4a	*Erwinia*	OC10, OC8, OC6	11.3
WI6e	*Pseudomonas*	OC8, OC6	9.4
WI7a	*Erwinia*	OC8, OC6	9.9
WI9a	*Erwinia*	OC8, OC6	9.0
WI9b	*Pantoea*	OC8, OC6	11.8
WI11c	*Pantoea*	OC8, OC6	9.3
WI12a	*Pseudomonas*	OC8, OC6, C10	1.7
WI13a	ND	OC8, OC6	8.2
WI14b	*Pantoea*	OC8, OC6	12.4

Abbreviations: AHL, *N*-acyl-homoserine lactone; ND, not determined; QS, quorum sensing.
[a]Strain identity determined by partial 16 s rDNA sequencing.
[b]AHLs produced was determined by ethyl acetate extraction, separation by TLC and visualization by the bioindicator *A. tumefaciens* NT1 (pSVB33, pJM749).
[c]Relative QS induction of the bioindicator BHSL (pBQ9) estimated from 1 or 5 ml ethyl acetate culture extracts.

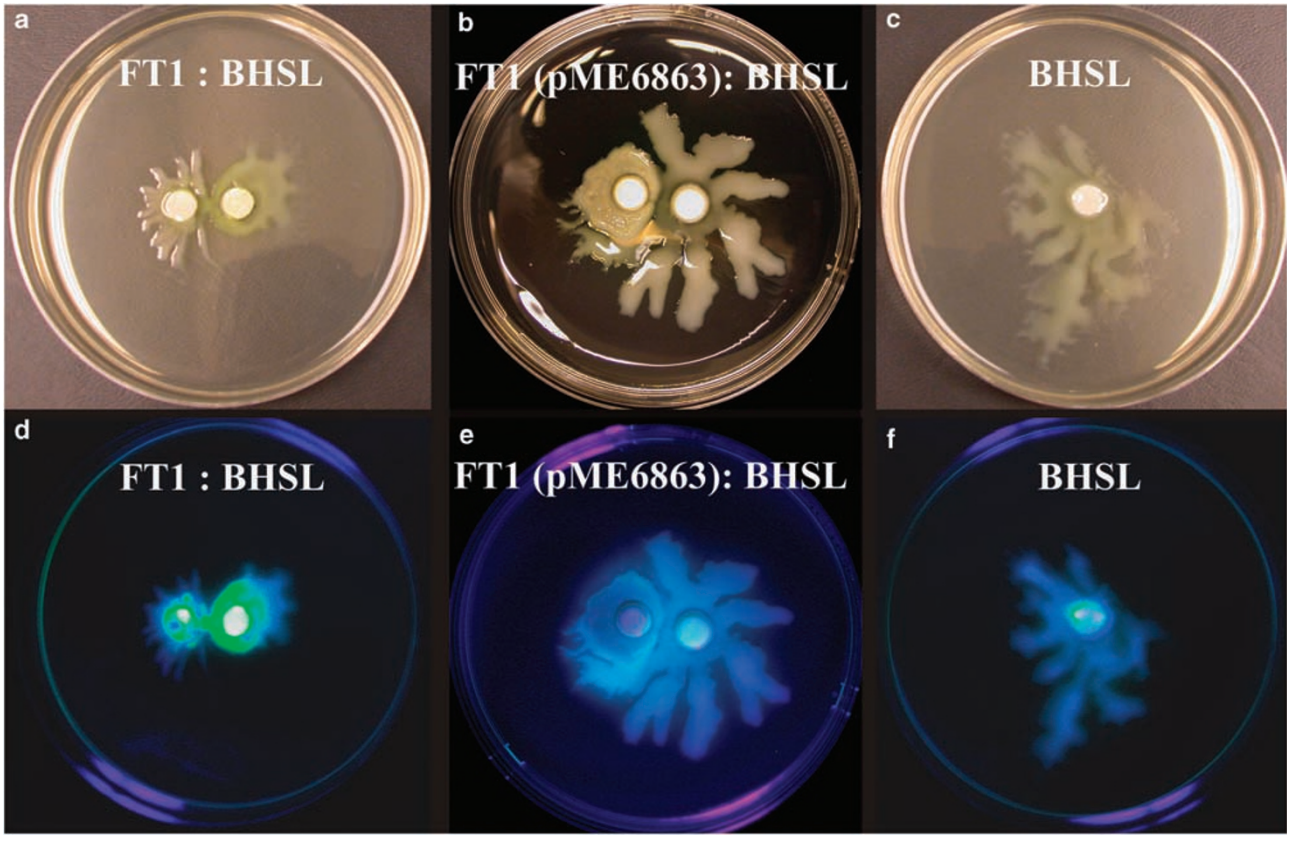

Figure 2 Swarming on soft agar plates of the *N*-acyl-homoserine lactones (AHL)-deficient, hyper motile *Pseudomonas syringae* pv. syringae (*Pss*) strain BHSL (pBQ9) (**c**) is suppressed when grown near AHL-producing *Erwinia* sp. strain FT1 (**a**, **d**) when visualized with white light (**a**) or UV light (**d**). No such suppression is seen when grown near strain FT1 (pME6863) in which AHL accumulation is suppressed with an AHL lactonase (**b**). AHL-induced green fluorescent protein (GFP) production in BHSL (pBQ9) is seen in the area proximal to strain FT1 and not in the finger-like projections moving away from the site of coincidence (**d**) but not in the vicinity of FT1 (pME6863) (**e**) or in cultures of BHSL alone (**f**).

(Figure 2d). This suppression of swarming motility was abolished in strain FT1 (pME6863) in which AHL accumulation was quenched by an AHL lactonase (Figure 2b). Likewise, no induction of *ahlI* was apparent in BHSL (pBQ9) in the presence of strain FT1 (pME6863) (Figure 2e).

All AHL-producing epiphytic bacteria greatly affected the incidence of disease when co-inoculated with *Pss.* The process of infection of bean leaves by *Pss* when inoculated alone and when co-inoculated with AHL-producing epiphytic bacteria was monitored by quantifying the number of lesions that occurred 7 days after inoculation. Before being placed on a greenhouse bench, plants were kept moist for 24 h after inoculation to enable population sizes to increase and for movement of cells on and into the leaf to occur. Compared to the number of lesions formed by *Pss* when inoculated alone onto plants, the incidence of infections was reduced as much as 50% on plants co-inoculated with the AHL-producing strains FT1 and *Pseudomonas* sp. FT5 (Table 2). Importantly, the incidence of disease on plants inoculated with *Pss* alone and co-inoculated with FT1 (pME6863) in which AHL accumulation had been quenched was similar (Table 2), indicating that AHL production by strain FT1 had contributed to the suppression of disease. Further support for AHL-dependent suppression of disease caused by *Pss* was obtained by co-inoculating it with 3OC6HSL-producing *E. coli* strain 1480 (pGFD4). The parental *E. coli* strain 1480 had no significant effect on disease when co-inoculated with *Pss*, whereas disease was reduced over 50% when *E. coli* strain 1480 (pGFD4) was co-inoculated (Table 2). A similar reduction in infection events was seen when plants were treated with a mixture of *Pss* B728a and 10 μM 3OC6HSL. In contrast, treatment of plants with a mixture of *Pss* and butanoyl- and decanoyl-HSL, AHLs to which *Pss* is nonresponsive, resulted in similar numbers of lesions as treatment with *Pss* B728a alone (Table 2).

Reductions in infections of plants by *Pss* caused by co-colonization of AHL-producing bacteria were not observed when a nonmotile, *flgK* mutant of *Pss* was used. Although the *flgK* mutant incited only about 50% as many infections as the wild-type strain B728a, co-inoculation of this nonmotile mutant with either strain FT1 or *E. coli* 1480 (pGFD4) resulted in similar numbers of lesions as that caused by the *flgK* mutant alone (Table 3). Likewise, the number of lesions on plants co-inoculated with the *flgK* mutant and FT1 (pME6863) quenched for AHL accumulation was similar to that on plants co-inoculated with the *flgK* mutant alone or with strain FT1 (Table 3).

The reduced disease incidence conferred by co-inoculation of plants by AHL-producing bacteria was not associated with a reduction in epiphytic populations of *Pss.* The epiphytic population size of *Pss* B728a was similar at a given sampling time on plants inoculated only with this strain as that on plants co-inoculated with strain FT1, FT1 (pME6863) or *Pseudomonas* sp. FT5 (data not shown). Although population sizes increased from about 10^5 cells per leaf shortly after inoculation to about 10^7 cells per leaf after 24 h incubation, they had all decreased to about 10^6 cells per leaf after 7 days incubation on a greenhouse bench.

Discussion

Given that surveys of Gram-negative, plant-associated bacteria have found that AHL production is a common trait (Cha *et al.*, 1998), it was important to

Table 2 Numbers of brown spot lesions on bean leaves inoculated only with *Pss* B728a, on plants co-inoculated with various AHL-producing bacteria, or on plants treated with AHLs themselves

Treatment		*Disease trial*[a]			
	Exogenous 3OC6HSL	*Experiment 1*	*Experiment 2*	*Experiment 3*	*Experiment 4*
Pss only	−	52 ± 4.3 A	35 ± 1.8 A	27 ± 1.6 A	49 ± 6.4 A
Pss+*Erwinia* sp. FT1	+	28 ± 6.1 B	16 ± 1.0 B		
Pss+*Erwinia* sp. FT1 (pME6863)	−		32 ± 1.5 A		
Pss+*Pseudomonas* sp. FT5	+	31 ± 5.2 B			
Pss+*E. coli* 1480[b]	−			27 ± 1.8 A	
Pss+*E. coli* 1480 (pGFD4)[b]	+			12 ± 0.9 B	
Pss+3OC6HSL[c]	+				33 ± 2.8 B
Pss+butanoyl-HSL[c]	−				55 ± 7.9 A
Pss+decanoyl-HSL[c]	−				46 ± 7.2 A

Abbreviation: 3OC6HSL, 3-oxohexanoyl-homoserine lactone.
[a]Average number of lesions per leaf was determined by counting brown spot lesions present on trifoliate leaves approximately 7 days after inoculation with *Pss* B728a . The standard error of the mean is shown. Within individual experiments, treatments with the same letter designation do not differ significantly ($P > 0.05$) as determined by an LSD test. Representative experiments are shown; similar results were seen in repeat experiments.
[b]*Pss* B728a was co-inoculated with *E. coli* strain 1480 or with *E. coli* 1480 carrying plasmid pGFD4 harboring the *ahlI* gene of *Pss* B728a under the control of the P_{trp} promoter and thus producing 3-oxo-hexanoyl-HSL.
[c]Inoculum of *Pss* B728a was applied to plants immediately after spraying them with solutions (10 μM) of the designated AHLs.

Table 3 Numbers of brown spot lesions on bean leaves inoculated with *Pss* B728a alone, a *flgK* mutant alone or on plants co-inoculated with the nonmotile mutant and AHL-producing bacteria

Treatment		*Disease trial*[a]	
	Exogenous 3OC6HSL	*Experiment 1*	*Experiment 2*
Pss B728a	−	23 ± 1.6 A	20 ± 1.9 A
flgK	−	10 ± 1.0 B	11 ± 1.3 B
flgK+*E. coli* 1480	−	9 ± 0.8 B	
flgK+*E. coli* 1480 (pGFD4)	+	10 ± 1.3 B	
flgK+*Erwinia* sp. FT1	+		9 ± 1.2 B
flgK+*Erwinia* sp. FT1 (pME6863)	−		9 ± 1.2 B

Abbreviation: 3OC6HSL, 3-oxohexanoyl-homoserine lactone.
[a]Average number of disease lesions per leaf was determined by counting brown spot lesions present on trifoliate leaves approximately 7 days after inoculation with *Pss* strains. The Standard Error of the mean is shown. Treatments within a given experiment with the same letter designation do not differ significantly ($P > 0.005$) as determined by an LSD test. Representative experiments are shown; similar results were seen in repeat experiments.

examine whether AHL-mediated interactions could occur between microbes in natural habitats. The function of QS in plant-associated bacteria has been addressed almost exclusively in the context of the infection process or of symbiosis with single strains despite the possibilities of behavioral changes due to cross talk that might be mediated by sharing of AHL signal molecules in mixed microbial communities (Loh *et al.*, 2002). Because QS in the plant pathogenic bacterium *Pss* B728a controls swarming motility and thus invasion into the leaf tissue that contributes to brown spot disease, the assessment of lesion formation was a sensitive indicator of alterations in behavior that might be conferred by AHL-producing neighbors on plants. Our study thus revealed that other residents on leaves commonly could produce an AHL to which *Pss* would respond and that these exogenous signal molecules strongly modulated the behavior of *Pss* on plants.

A large percentage (approximately 7%) of culturable epiphytes on leaves produces an AHL to which the QS system in *Pss* B728a is responsive. A similar abundance of AHL producers has been reported in the rhizosphere. Pierson *et al.* (1998) found that 8% of the culturable bacteria from the wheat rhizosphere were able to induce phenazine gene expression in the biocontrol strain *P. aureofaciens* 30-84, presumably due to AHL production. These two species are responsive only to 3OC6HSL, and 3-oxo-octanoyl-HSL, respectively. It is likely that other AHLs are made by other epiphytes, suggesting that plant surfaces often harbor a variety of AHLs. Not surprisingly, many of the QS-modulating strains that were characterized produced a variety of AHLs detectable with the AHL bioindicator *A. tumefaciens* NT1 (pSVB33, pJM749) (Piper *et al.*, 1993). Although all strains studied produced 3OC6HSL and 3-oxo-octanoyl-HSL, as would be expected if their only means of modulating QS in *Pss* was through production of AHLs because the *ahlIR* QS system of *Pss* B728a produces and is most responsive to 3OC6HSL, a variety of other AHLs are also apparently being made on leaves. As our study was designed only to identify culturable bacteria able to affect QS in *Pss*, it is possible that other nonculturable AHL-producing taxa could also modulate QS of *Pss*. Given the large fraction of the bacterial community that is able to produce AHLs on plants, the possibility of QS cross talk in this habitat is highly likely.

The tentative identification of AHL-producing bacterial epiphytes made by partial sequencing of the 16S rDNA genes revealed that only a limited number of different genera possessed this ability to interfere with QS in *Pss*. All of the AHL producers characterized were members of the genera *Pseudomonas*, *Pantoea* or *Erwinia*. Our survey of QS modulators attempted to address the diversity of culturable bacteria on leaves by selecting colonies of varying characteristics for evaluation. No other genera were found to contain members capable of AHL production in the phyllosphere, suggesting that this phenomenon is restricted to a few groups. This is perhaps not surprising given that these genera are among the most common epiphytes and have been shown to have AHL-mediated QS systems.

Surprisingly, some epiphytes produced dramatically more AHL than did *Pss* in culture, up to 19-fold more 3OC6HSL than *Pss* (Table 1). No obvious association with taxa was noted. Perhaps these epiphytic strains harbor QS systems that are simply less responsive to AHL, and hence have evolved to produce larger amounts of AHL than other species. We did not determine whether these strains have functional *luxR* homologues to detect the signal they produce, although we did note that *Pseudomonas* sp. FT5 produced antibiotics inhibitory to *Pss* only when AHL production was quenched through AHL lactonase expression. Alternatively, it is possible that these bacteria produce AHLs not to control their own traits in a QS-dependent manner, but to alter the behavior of nearby residents in a way that is beneficial to them.

Perhaps *Pss* exhibits traits under the control of QS that would be beneficial not only to its own epiphytic survival, but also to neighboring AHL-producing cells, such as by producing exopolysaccharide (EPS) that benefits epiphytic survival (Yu *et al.*, 1999; Quinones *et al.*, 2005).

The presence of AHL-producing bacteria on leaves very strongly affected the behavior of *Pss* on plants, causing a large reduction in the incidence of infections by the plant pathogen. Although we used AHL-producing *Erwinia* sp. strain FT1 predominantly in our study, all of the AHL-producing bacteria that we tested mediated the same changes in behavior of *Pss*. These changes in the behavior of *Pss* on plants were due primarily to the production of AHL by bacteria on leaves, thus providing strong evidence for widespread AHL-mediated cross talk in the phyllosphere. The changes in behavior of *Pss* in the presence of AHL-producing bacteria on plants were predictable based on our understanding of the contribution of QS to behavioral phenotypes of *Pss*. Alteration of QS-controlled swarming motility in *Pss* most easily explains the changes in behavior of *Pss* on leaves caused by AHL-producing neighbors. QS in *Pss* negatively regulates swarming motility and thus invasion into leaf tissue. Furthermore, loss of flagellar motility resulted in reduced virulence (Table 3). When excess 3OC6HSL was introduced onto the leaf surface by *Erwinia* sp. FT1, other AHL-producing strains such as *E. coli* 1480 (pGFD4), or topical treatment with purified 3OC6HSL (Table 2), *Pss* cells apparently became prematurely autoinduced, leading to decreased mobility on the leaf surface and thus less invasion into the tissue. The inability of the AHL-producing strains to suppress disease of a nonmotile *flgK* mutant indicates that suppression of motility, and not competition for nutrients or other interactions, is the major factor contributing to reduced lesion formation. Inappropriate induction of EPS production on activation of QS could also hinder movement of *Pss* on the leaf surface. The reduced invasiveness would result in accessibility of fewer internal plant sites in which *Pss* could multiply and thus initiate an infection.

Given that the occurrence of brown spot lesions on plants co-inoculated with *Pss* and AHL-producing bacteria was reduced over 50%, apparently many cells of *Pss* are located sufficiently near AHL producers that experienced a change in QS status. Although nonisogenic epiphytic bacteria strains appear to be highly spatially segregated on leaves, they often occur together in cellular aggregates (Monier and Lindow, 2004, 2005) and such cells should readily perceive the AHLs produced by their neighbors (Dulla and Lindow, 2008). If, however, many lesions are initiated by more solitary cells, then the dispersion of AHLs across the leaf must has been relatively unimpeded. Although AHLs have been shown to travel several micrometers in the tomato rhizosphere, most commonly it was perceived only a few micrometers from a producing cell (Steidle *et al.*, 2001; Gantner *et al.*, 2006). As the QS status of adjacent cellular aggregates of *Pss* on leaves was largely dictated by the size of the cellular aggregate itself and not by AHLs made by other aggregates (Dulla and Lindow, 2008), we believe that AHL transport on leaves is a relatively local phenomenon and that the spatial coincidence of epiphytes largely accounts for the high level of apparent cross talk seen here. It seems likely that a given cell will encounter AHL signals both of its own origin as well as from neighboring cells. It is also possible, however, that the host plants are responding to the abundant AHLs produced by some epiphytic bacteria to induce changes in the susceptibility to bacterial infection. Both *Medicago truncatula* and tomato have been shown to respond to exogenous AHLs by altering patterns of gene expression, including the activation of PR proteins that might contribute to disease resistance (Mathesius *et al.*, 2003; Schuhegger *et al.*, 2006). We were unable to induce a disease-suppressing response in bean by treating with the noncognate AHLs of *Pss* such as butanoyl- and decanoyl-HSL (Table 2), suggesting that the effect of the AHLs was on *Pss* and not the plant. The reduced disease seen here was similar in magnitude to that observed when *P. syringae* pv. tabaci was sprayed onto 3OC6HSL-producing tobacco plants compared to normal tobacco (Quinones *et al.*, 2004). Such an effect could have been due to either the premature induction of disease resistance as suggested by Dong *et al.* (2000) and Fray *et al.* (1999) or a suppression of swarming motility of this strain on such plants. As the effects of exogenous AHL on motility of *Pss* and modulation of host responses might be expected to have an additive effect on lesion formation, the lack of an effect on lesion formation by the *flgK* mutant of *Pss* by exogenous AHLs produced by neighboring bacteria (Table 3) suggests that suppression of motility most likely explains the effects of AHL on disease in this model system.

The size of local populations needed to exhibit quorum behavior may be strongly influenced by the composition of neighboring cells in natural habitats. Under wet conditions where AHLs do not accumulate due to diffusion, relatively large cell aggregates of *Pss* were needed to constitute a 'quorum' on plants (Dulla *et al.*, 2005; Dulla and Lindow, 2008). However, the quorum size is low under dry conditions, which apparently reduces diffusional losses of AHL, and with extended time during which AHLs can accumulate from *Pss* cells (Dulla and Lindow, 2008). This study indicates that the concept of a quorum of *Pss* cells in a mixed community of bacteria must consider not only its own cell aggregate size but also the likelihood that neighboring cells can produce cognate AHLs and the amount of such AHLs they produce. The existence of a high proportion of AHL-producing strains on plants indicates that there is great potential for cross talk among plant-associated bacteria.

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