Quorum Sensing Regulates Exopolysaccharide Production, Motility, and Virulence in *Pseudomonas syringae*

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The *N*-acyl homoserine lactone (AHL)-mediated quorum-sensing system in the phytopathogen *Pseudomonas syringae* pv. *syringae* requires the AHL synthase *AhlI* and the regulator *AhlR*, and is additionally subject to regulation by *AefR*. The contribution of quorum sensing to the expression of a variety of traits expected to be involved in epiphytic fitness and virulence of *P. syringae* were examined. Both an *aefR*[−] mutant and an *ahlI*[−] *ahlR*[−] double mutant, deficient in AHL production, were significantly impaired in alginate production and had an increased susceptibility to hydrogen peroxide compared with the wild-type strain. These mutants were hypermotile in culture, invaded leaves more rapidly, and caused an increased incidence of brown spot lesions on bean leaves after a 48-h moist incubation. Interestingly, an *aefR*[−] mutant was both the most motile and virulent. Like the wild-type strain, the AHL-deficient mutant strains incited water-soaked lesions on bean pods. However, lesions caused by an *ahlI*[−] *ahlR*[−] double mutant were larger, whereas those incited by an *aefR*[−] mutant were smaller. In contrast, tissue maceration of pods, which occurs at a later stage of infection, was completely abolished in the AHL-deficient mutants. Both the incidence of disease and in planta growth of *P. syringae* pv. *tabaci* were greatly reduced in transgenic tobacco plants that produced AHL compared with wild-type plants. These results demonstrate that quorum sensing in *P. syringae* regulates traits that contribute to epiphytic fitness as well as to distinct stages of disease development during plant infection.

Additional keywords: cell-to-cell signaling, GacA, swarming.

Pseudomonas syringae pv. *syringae* strains are the causal agent of brown spot disease of bean and incite frost injury to plants (Hirano and Upper 2000). Before initiating disease, *P. syringae* strains achieve large epiphytic population sizes on healthy leaves (Rouse et al. 1985). Understanding the factors that enable this epiphytic bacterium to successfully colonize leaf surfaces should lead to better strategies for the control of plant disease or frost injury. Recently, it has been demonstrated that the majority of cells of *P. syringae* strain B728a

occur in aggregates on leaves rather than as solitary cells (Monier and Lindow 2004). Cells in aggregates exhibited a much greater ability to survive periodic desiccation stress on leaves (Monier and Lindow 2003). Interestingly, increased tolerance to desiccation was related directly to the size of the cell aggregate (Monier and Lindow 2003). These findings suggest that some aspects of the epiphytic fitness of *P. syringae* are cell-density-dependent and that the preferential expression of certain traits within aggregated cells contributes to plant colonization.

Bacterial populations are capable of coordinating the expression of some traits in a cell-density-dependent manner by a process known as quorum sensing (Loh et al. 2002; Von Bodman et al. 2003). Bacteria that use quorum sensing can assess their local population density by detecting the production of a diffusible signal molecule which increases with local population density, resulting in coordinated gene expression. *P. syringae* and many other gram-negative bacteria use *N*-acyl homoserine lactone (AHL) as the quorum-sensing signal molecule. In many pathogenic bacteria, elevated levels of AHL, due to higher population densities, result in the expression of virulence factors and secondary metabolites that mediate successful colonization of the host.

The quorum-sensing system in *P. syringae* has many similarities to that of other gram-negative bacteria. The production of *P. syringae*'s AHL signal, 3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL), occurs in a cell-density-dependent fashion and requires the expression of the AHL synthase gene, *ahlI*, and the AHL regulator gene, *ahlR* (Dumenyo et al. 1998; Quiñones et al. 2004). In the presence of cellular precursors, *AhlI* catalyzes the production of 3-oxo-C6-HSL. *AhlR* forms a stable complex with the AHL signal and activates transcription of *ahlI* via a positive-feedback mechanism that leads to higher amounts of AHL to be produced with increasing cell concentrations. This *AhlI*-*AhlR* quorum-sensing system also is subject to modulation by additional regulatory proteins (Dumenyo et al. 1998; Kitten et al. 1998; Quiñones et al. 2004). Our previous work identified *AefR* as an important and novel regulator of quorum sensing in *P. syringae* (Quiñones et al. 2004). *AefR* was required for transcription of *ahlI* and, consequently, for maximal levels of AHL production. Another regulator, *GacA*, had a similar effect on the autoinduction process (Dumenyo et al. 1998; Kitten et al. 1998; Quiñones et al. 2004). Both *AefR* and *GacA* are thought to act as activators of the *AhlI*-*AhlR* quorum-sensing system via independent pathways (Quiñones et al. 2004).

AHL-mediated quorum sensing plays a major role in the interaction between plants and microbes (Loh et al. 2002; Von Bodman et al. 2003). Quorum sensing is required for the ex-

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pression of virulence factors and secondary metabolites in plant-pathogenic bacteria that contribute to colonization of plants and subsequent disease. For example, quorum sensing in *Erwinia carotovora* is required for the expression of several exoenzymes that are responsible for the maceration of plant tissues (Jones et al. 1993; Pirhonen et al. 1993). In *Pantoea stewartii*, AHL-mediated gene expression is involved in the production of the exopolysaccharide (EPS) virulence factor, stewartan (Beck von Bodman and Farrand 1995; Beck von Bodman et al. 1998). Other cell-density-dependent traits in plant-pathogenic bacteria recently have been reviewed (Von Bodman et al. 2003).

The role of AHL production in the expression of density-dependent traits that contribute to epiphytic fitness and virulence of *Pseudomonas syringae* has not yet been characterized fully. Although recent findings showed that disruption of *ahlI* results in an altered morphology of *P. syringae* colonies on solid agar (Kinscherf and Willis 1999), quorum sensing was reported not to have an effect on the production of virulence factors such as extracellular proteases or the phytotoxin syringomycin (Dumenyo et al. 1998; Kinscherf and Willis 1999). Furthermore, an *ahlI*⁻ mutant was reported to retain swarming motility and virulence on bean leaves (Kinscherf and Willis 1999), traits that are positively regulated in an AHL-dependent manner in other bacteria. To further understand the contribution of cell-to-cell signaling in *P. syringae* on epiphytic fitness and virulence, the present study examined the dependence of diverse behaviors on quorum sensing. We provide evidence that both the AhlI-AhlR quorum-sensing system and AefR are positive regulators of EPS production, oxidative stress tolerance, and plant tissue maceration, as well as negative regulators of swarming motility. In addition, this complex regulation of behaviors results in either increased or decreased early water-soaked lesions on bean pods and disease symptoms on leaves, depending on the environmental conditions. We also demonstrate that pathogen growth and disease incidence are altered in transgenic plants capable of producing 3-oxo-C6-HSL, the AHL that is recognized by *P. syringae*.

RESULTS

EPS production and resistance to oxidative stress is impaired in AHL-deficient mutants of *P. syringae*.

The production of EPS is subject to regulation by quorum sensing in several phytopathogenic bacteria (Von Bodman et al. 2003); therefore, we examined the production of alginate in various mutants of *P. syringae* deficient in AHL production. Strains were grown on mannitol-glutamate solidified medium supplemented with yeast extract at 0.25 g/liter (MGY) in the presence of 0.6 M sorbitol, a medium previously demonstrated to promote a mucoid phenotype and a maximal expression of the alginate biosynthetic gene cluster in *P. syringae* (Peñaloza-

Vázquez et al. 1997; Willis et al. 2001). Wild-type *P. syringae* strain B728a exhibited a mucoid, slimy colony type after growth for 3 days under these conditions, whereas an *ahlI*⁻*ahlR*⁻ double mutant and an *aefR*⁻ mutant both exhibited a reduced mucoid phenotype (data not shown). As expected, a *gacA*⁻ mutant had a dry, nonmucoid colony type, as previously reported (Willis et al. 2001). We then performed a modified carbazol assay to quantify the amounts of alginate produced by the wild-type and AHL-deficient mutant strains. The amount of alginate produced by an *ahlI*⁻*ahlR*⁻ double mutant was threefold less than that produced by wild-type cells (Table 1). Furthermore, the amount of alginate recovered from an *aefR*⁻ mutant was approximately fourfold less than that from wild-type cells (Table 1). No alginate was isolated from a *gacA*⁻ mutant, confirming that the GacS/GacA two-component regulator is required for alginate biosynthesis (Willis et al. 2001). The fact that both the *ahlI*⁻*ahlR*⁻ double mutant and an *aefR*⁻ mutant had an impaired but not abolished alginate production suggests that the AhlR and AefR regulators are not required for, but contribute to, maximal levels of alginate production in *P. syringae*.

To further characterize the defect in alginate production observed in AHL-deficient mutants of *P. syringae*, we assessed whether an *aefR*⁻ mutant or an *ahlI*⁻*ahlR*⁻ double mutant showed increased susceptibility to hydrogen peroxide, a compound known to generate reactive oxygen intermediates (ROIs) (Farr and Kogoma 1991). Previous studies demonstrated that a *P. syringae* mutant deficient in alginate biosynthesis was hypersensitive to environmental stresses, such as heat and compounds that generate ROIs (Keith and Bender 1999). An increased susceptibility to ROIs was observed in the AHL-deficient mutants, as evidenced by a larger zone of growth inhibition around discs that were impregnated with a hydrogen peroxide solution (Table 1). The zones of growth inhibition of both an *aefR*⁻ and an *ahlI*⁻*ahlR*⁻ double mutant were similar to each other, but significantly greater than that exhibited by wild-type cells (Table 1). Interestingly, a *gacA*⁻ mutant was even more susceptible to ROIs than either of the AHL-deficient mutants (Table 1).

Swarming motility is enhanced in AHL-deficient mutants.

When inoculated onto filters at a cell concentration of 1×10^7 cells/filter, wild-type cells of *P. syringae* showed limited swarming motility on a low-agar medium (Fig. 1). Wild-type cells displayed only an irregular blebbing that appeared at the margin of the colony. An *ahlI*⁻*ahlR*⁻ double mutant exhibited much more motility and an irregular dendritic colony pattern that spread rapidly away from the point of inoculation when compared with the wild-type strain (Fig. 1). Interestingly, an *aefR*⁻ mutant swarmed the greatest distance from the point of inoculation and displayed more extended tendrils that occurred in a very regular and defined dendritic pattern (Fig. 1). The

Table 1. Alginate production and susceptibility to reactive oxygen intermediates in *Pseudomonas syringae* strains differing in *N*-acyl homoserine lactone (AHL) production

Strain	Genotype	Alginate production ($\mu\text{g}/\text{mg}$ of total protein $\pm$ SD) ^y	Hydrogen peroxide susceptibility (growth inhibition zone [mm] $\pm$ SD) ^z
B728a	Wild type	913.84 $\pm$ 108.83 a	11.38 $\pm$ 0.51 a
A9	<i>ahlI</i> ⁻ <i>ahlR</i> ⁻	302.94 $\pm$ 58.82 b	13.69 $\pm$ 0.48 b
538	<i>aefR</i> ⁻	225.65 $\pm$ 63.71 b	13.15 $\pm$ 0.68 b
BGACX	<i>gacA</i> ⁻	17.89 $\pm$ 4.98 c	18.23 $\pm$ 0.44 c

^y Alginate production was quantified after growth for 3 days on mannitol-glutamate solidified medium supplemented with yeast extract at 0.25 g/liter containing 0.6 M sorbitol. Results are the mean and the standard deviation (SD) from a single experiment consisting of three replicates. Values followed by the same letter were not significantly different at $P = 0.01$ according to Bonferroni's means comparison test.

^z Susceptibility to reactive oxygen intermediates was determined by measuring the diameter of the growth inhibition zone surrounding filters containing 3% hydrogen peroxide. Results are the mean and standard deviation from a single experiment consisting of five replicates. Values followed by the same letter were not significantly different at $P = 0.01$ according to Bonferroni's means comparison test.

aeiR⁻ mutant swarmed approximately 2.5-fold farther than an *ahlI*⁻ *ahlR*⁻ double mutant and 5.6-fold farther than the wild-type strain (Table 2).

When the wild-type and AHL-deficient mutants were spotted at a higher cell concentration (1×10^9 cells/filter), the differences in swarming motility among the strains were less pronounced (Fig. 1, bottom panels). Wild-type cells exhibited greater swarming motility after an overnight incubation than when a smaller number of cells were spotted. However, the *ahlI*⁻ *ahlR*⁻ double mutant strain was still more motile than the wild type (Fig. 1; Table 2). Moreover, an *aeiR*⁻ mutant was the most hypermotile even when inoculated at these higher inoculum concentrations (Fig. 1), moving approximately twofold farther than the wild-type strain (Table 2). As expected from previous studies (Kinscherf and Willis 1999), no swarming motility was observed for a *gacA*⁻ mutant at any cell concentration tested in this assay (Fig. 1). Because a more pronounced effect was observed at relatively lower cell densities, these results demonstrate that the AhlI-AhlR quorum-sensing system and the regulator AeiR both apparently repress swarming motility, especially during the early stages of quorum sensing.

To further dissect the differences in swarming motility observed between the wild-type strain and AHL-deficient mutants of *P. syringae*, their speed of movement was examined on a low-agar medium. At a given time, each of the strains exhibited substantial differences in the distance to which they moved. An *aeiR*⁻ mutant was hypermotile and moved the farthest. This strain was the first to begin movement, approximately 5 h after inoculation (Fig. 2). An *ahlI*⁻ *ahlR*⁻ double mutant moved less distance than an *aeiR*⁻ mutant, but substantially more than the wild-type strain, and initiated swarming motility approximately 8 to 9 h after inoculation. No motility was observed in a *gacA*⁻ mutant (Fig. 2). To further compare swarming motility among the various strains, the rates of movement were obtained by calculating the slope of the best-fit line relating the swarmed distance as a function of time

(Fig. 2). The rates of movement for both an *aeiR*⁻ mutant and an *ahlI*⁻ *ahlR*⁻ double mutant were constant with time and similar to each other (0.13 cm/h). In contrast, the wild-type strain had a slower rate of movement (0.07 cm/h).

Quorum-sensing mutants invade leaves more rapidly.

Because AHL-deficient strains of *P. syringae* were hypermotile in culture, we investigated whether they also had an increased ability to invade leaves. To address this question, the surface of bean leaves was spray inoculated with each strain to establish similar initial epiphytic bacterial populations. At various time points after inoculation, we assessed the invasion into the leaf tissue by measuring the internal populations that were protected from hydrogen peroxide killing, as in previous studies (Wilson et al. 1999). The *aeiR*⁻ mutant and the *ahlI*⁻ *ahlR*⁻ double mutant strain both had an increased ability to invade the leaf tissue (Fig. 3). When examined 3 or 6 h after inoculation, the hydrogen peroxide-insensitive population sizes for both of these mutant strains were more than 10^3 CFU/leaf,

Table 2. Swarming motility of *Pseudomonas syringae* strains differing in *N*-acyl homoserine lactone (AHL) production

Strain	Genotype	Distance swarmed (mm) $\pm$ SD ^y	
		1×10^7 cells/filter ^z	1×10^9 cells/filter ^z
B728a	Wild type	2.50 $\pm$ 0.78 a	15.83 $\pm$ 3.85 a
A9	<i>ahlI</i> ⁻ <i>ahlR</i> ⁻	5.67 $\pm$ 1.45 b	19.56 $\pm$ 3.22 b
538	<i>aeiR</i> ⁻	14.11 $\pm$ 4.02 c	29.17 $\pm$ 3.49 c
BGACX	<i>gacA</i> ⁻	ND	ND

^y Swarming motility was assessed after growth on King's B agar containing 0.4% agar for 16 h at 26°C. Results are the mean and standard deviation from a single experiment consisting of three replicates. Values followed by the same letter within a given inoculum were not significantly different at $P = 0.01$ according to Bonferroni's means comparison test. ND = Not detected.

^z Inoculum concentration applied to filter disc.

Fig. 1. Swarming motility is enhanced in *N*-acyl homoserine lactone (AHL)-deficient mutants of *Pseudomonas syringae*. Sterile filter discs were inoculated with either 1×10^7 cells (top panels) or with 1×10^9 cells (bottom panels) of *P. syringae* parental strain B728a, *ahlI*⁻ *ahlR*⁻ double mutant strain A9, *aeiR*⁻ mutant strain 538, or *gacA*⁻ mutant strain BGACX and placed on King's B agar containing 0.4% agar. Plates were incubated at 26°C for 16 h. Results are from a single representative experiment with three replicates.

1,000-fold higher than that of wild-type cells. Only very small internal populations of the wild-type strain were detected by 6 h after inoculation (Fig. 3). Similarly, no cells of a *gacA*⁻ mutant were detected in sites on the leaves protected from hydrogen peroxide within the first 6 h of colonization (Fig. 3). After 18 h, the internal population sizes for each strain were relatively large; however, the population sizes of an *aeiR*⁻ mutant often were higher than the other strains (Fig. 3). The epiphytic population sizes of both wild-type and AHL-deficient mutant strains were similar for each strain at a given sampling time, ranging from 1×10^5 CFU/leaf to more than 10^6 CFU/leaf (data not shown). These observations suggest that the low internal population of the wild-type and *gacA*⁻ mutant strain was due to their inability to actively enter leaves and was not due to a lack of viable cells on the leaf surface. Thus, the hypermotile *aeiR*⁻ and *ahII*⁻ *ahIR*⁻ mutants have an enhanced ability to explore and invade protected sites on leaves during the early stages of colonization.

Quorum sensing in *P. syringae* influences virulence toward bean.

We examined the contribution of the AhII-AhIR quorum-sensing system and of AeiR in the formation of brown spot lesions on bean leaves. *P. syringae* strain B728a produces small brown necrotic lesions surrounded by a yellow halo of chlorotic tissue that are easily enumerated (Hirano and Upper 2000); therefore, we compared the virulence of various AHL-deficient mutants after spray inoculation of leaves. Plants were incubated under moist conditions for either 24 or 48 h (short or long treatment, respectively) to achieve high (10^7 CFU/leaf) and stable epiphytic population sizes before being transferred to a greenhouse to allow lesions to form for 7 days. Visible brown spot lesions were quantified only on trifoliolate leaves that were fully expanded at the time of inoculation. The results obtained demonstrated that the number of brown spot lesions on leaves treated with the wild-type, *aeiR*⁻, or *ahII*⁻ *ahIR*⁻ mutant strains did not significantly differ when exposed to moist conditions for only 1 day (Fig. 4). However, when plants were

Fig. 2. Rate of swarming motility in wild-type and *N*-acyl homoserine lactone (AHL)-deficient mutants of *Pseudomonas syringae*. Swarming motility was assessed on King's B agar containing 0.4% agar after inoculating sterile filter discs with 10^7 cells of *P. syringae* parental strain B728a (●), *ahII*⁻ *ahIR*⁻ double mutant strain A9 (■), *aeiR*⁻ mutant strain 538 (▲), and *gacA*⁻ mutant strain BGACX (◆). The distance that cells moved from the point of inoculation was measured every hour. Results are from a single representative experiment with three replicates. Vertical bars represent the standard deviation from the mean.

incubated for 2 days under moist conditions, both an *aeiR*⁻ mutant and an *ahII*⁻ *ahIR*⁻ double mutant incited more lesions than the wild-type strain (Fig. 4). Lesions incited by the *ahII*⁻ *ahIR*⁻ double mutant strain were threefold higher, whereas the *aeiR*⁻ mutant strain incited nearly sevenfold more lesions than the wild type after an extended moist period (Fig. 4). As previously reported (Rich et al. 1994), a *gacA*⁻ mutant was avirulent under all conditions tested in this study (Fig. 4).

To further investigate whether the AhII-AhIR quorum-sensing system and AeiR regulate factors required for early stages of symptom development, bean pods with superficial incisions were inoculated with the wild-type and AHL-deficient mutant strains. When injected into bean pods, *P. syringae* causes sunken water-soaked lesions that are indicative of tissue necrosis and fluid release within 2 days of infection (Willis et al. 1990). After incubation under humid conditions, the diameters of the water-soaked lesions were measured at the site of inoculation. Although all strains incited water-soaked lesions, the sizes of the lesions were different for each strain. When compared with the wild-type strain, the water-soaked lesions on bean pods inoculated with an *ahII*⁻ *ahIR*⁻ double mutant were 20% larger; in contrast, the lesions incited by an *aeiR*⁻ mutant were 41% smaller (Fig. 5). A *gacA*⁻ mutant did not cause water-soaked lesions on bean pods (Fig. 5). These findings demonstrate that the AhII-AhIR quorum-sensing system and AeiR have distinct roles in the initiation of early disease symptoms on bean.

Some bacterial plant pathogens, such as *Erwinia* spp., express enzymes involved in plant tissue maceration that contribute to disease symptoms under quorum-sensing control (Von Bodman et al. 2003; Whitehead et al. 2001). To determine whether this mechanism also occurs in *P. syringae*, internal disease symptoms were examined in bean pods that were inoculated as above. After incubation for 3 to 4 days at 28°C, the pods were cross-sectioned longitudinally to examine the integrity of internal tissues. The wild-type strain B728a caused extensive internal tissue maceration and partial dehydration (Fig. 6). In contrast, both an *ahII*⁻ *ahIR*⁻ double mutant and an *aeiR*⁻ mutant did not cause any internal tissue maceration (Fig. 6). Pods inoculated with these strains remained green, fleshy, and indistinguishable from those inoculated with the *gacA*⁻ mutant

Fig. 3. Analysis of movement by wild-type and *N*-acyl homoserine lactone (AHL)-deficient mutants of *Pseudomonas syringae* into hydrogen peroxide-protected sites after inoculation onto bean leaves. The population sizes of *P. syringae* parental strain B728a (●), *ahII*⁻ *ahIR*⁻ double mutant strain A9 (■), *aeiR*⁻ mutant strain 538 (▲), and *gacA*⁻ mutant strain BGACX (◆) from macerated bean leaves that were surface sterilized with hydrogen peroxide at various times are shown. Results are from a single representative experiment with three replicates. Vertical bars represent the standard error of the mean of $\log_{10}$ -transformed bacterial population sizes.

strain or with buffer alone (Fig. 6). A lack of tissue maceration also was observed when bean pods were inoculated with an *ahlI*⁻ mutant (data not shown). Based on these observations, quorum sensing in *P. syringae* appears to be required for the expression of virulence determinants that are responsible for internal tissue maceration.

Exogenous AHL confers resistance to *P. syringae* pv. *tabaci* in tobacco.

In *P. syringae* strain B728a, quorum sensing appears to regulate virulence toward bean; therefore, we explored whether altering the abundance of AHL within the plant could modulate disease. To investigate this, we inoculated transgenic tobacco plants that produce 3-oxo-C6-HSL (Fray 2001). Because tobacco is not a host for *P. syringae* B728a, we tested virulence using *P. syringae* pv. *tabaci* strain 11528R, a causal agent of wild fire disease in tobacco (Dye et al. 1980). 3-oxo-C6-HSL has been shown to regulate quorum sensing in *P. syringae* pv. *tabaci* (Shaw et al. 1997), and the genes required for 3-oxo-C6-HSL production show high sequence identity to those in strain B728a (Quiñones et al. 2004). Various cell concentrations of this strain were infiltrated into the leaf apoplast of transgenic and wild-type tobacco, and disease symptoms were evaluated daily. Symptoms were first observed 2 days after inoculation only in plants that were inoculated with the highest cell concentrations (10^7 and 10^9 CFU/ml) and were confined to the areas infiltrated with the pathogen (data not shown). Although necrotic leaf tissue was observed in both wild-type and AHL-producing transgenic plants, lesions in wild-type tobacco plants were much more chlorotic. By 3 days after inoculation, disease symptoms in wild-type tobacco were much more pronounced than in AHL-producing plants when inoculated with 10^5 CFU/ml (Fig. 7). A similar, but subtler, difference in disease symptoms was observed between the transgenic and the wild-type plants when inoculated with an even higher cell concentration (10^7 and 10^9 CFU/ml) (Fig. 7). Interestingly, leaves of wild-type plants that were inoculated with 10^3 CFU/ml had

some chlorotic areas with discrete tissue necrosis, whereas AHL-producing transgenic plants exhibited no disease symptoms (Fig. 7). However, by 5 days after inoculation, symptoms on AHL-producing and wild-type plants were similar at any given inoculum concentration. Thus, exogenous AHL conferred a decreased susceptibility to *P. syringae* pv. *tabaci* in tobacco, particularly at early stages of infection with low inoculum concentrations.

To address how exogenous AHL suppressed disease, growth of *P. syringae* pv. *tabaci* strain 11528R was compared within wild-type and AHL-producing tobacco. No differences in pathogen growth in AHL-producing and wild-type plants was observed at any sampling time when plants were inoculated with a cell concentration of 1×10^5 CFU/ml, resulting in an initial population of approximately 10^3 CFU/leaf disc (Fig. 8). A rapid 1,000-fold increase in pathogen abundance was observed within 1 day after inoculation and a further 10-fold increase occurred by 2 days. Interestingly, when a low population (approximately 10^2 CFU/leaf disc) was established, a difference in pathogen growth was observed between transgenic and wild-type plants (Fig. 8). By 1 day after inoculation, the population sizes of strain 11528R were found to be slightly lower in tobacco plants producing 3-oxo-C6-HSL than in wild-type plants. The population sizes of 11528R remained as much as 10-fold lower in AHL-producing transgenic plants when compared with wild-type plants for two or more days (Fig. 8).

The effect of exogenous AHL on the disease process was further assessed after establishing an epiphytic population of the pathogen on wild-type and AHL-producing tobacco. Strain 11528 was spray inoculated on transgenic and control tobacco plants, and the incidence of leaf spot lesions was recorded after incubating plants under moist conditions. The number of leaf lesions on AHL-producing tobacco was 3.6-fold lower than on wild-type plants (Fig. 9). Based on these observations, production of AHL on tobacco leaves effectively reduces spot lesion formation from epiphytic populations of *P. syringae* pv. *tabaci* strain 11528.

Fig. 4. Lesion formation on bean leaves inoculated with an *aefR*⁻ mutant and an *ahlI*⁻ *ahlR*⁻ double mutant of *Pseudomonas syringae*. Bean leaves were spray inoculated with a cell suspension containing 10^6 CFU/ml of *P. syringae* parental strain B728a, *ahlI*⁻ *ahlR*⁻ double mutant strain A9, *aefR*⁻ mutant strain 538, or *gacA*⁻ mutant strain BGACX. Plants were incubated under high relative humidity for either 24 h (black bars) or 48 h (gray bars) at 28°C and brown spot lesions were counted after 7 days. Bars with the same letter were not significantly different ($P < 0.01$) according to Duncan's multiple range test.

Fig. 5. Sizes of water-soaked lesions on bean pods produced by wild-type and N-acyl homoserine lactone (AHL)-deficient mutants of *Pseudomonas syringae*. Wounded bean pods were inoculated with a drop containing 10^8 cells of *P. syringae* parental strain B728a, *ahlI*⁻ *ahlR*⁻ double mutant strain A9, *aefR*⁻ mutant strain 538, or *gacA*⁻ mutant strain BGACX. The diameter of the water-soaked lesions was measured after 3 days of incubation under moist conditions at 28°C. Bars with the same lowercase letter were not significantly different ($P < 0.01$) according to Bonferroni's means comparison test.

DISCUSSION

The production of EPS by plant-pathogenic bacteria is considered to be an important strategy that contributes significantly to their growth, survival, and virulence (Lindow and Brandl 2003; Loh et al. 2002; Von Bodman et al. 2003). EPS production in *Pantoea stewartii*, *Ralstonia solanacearum*, and *Xanthomonas campestris* is subject to regulation by quorum sensing (Von Bodman et al. 2003). Based on these observations, the present study examined whether quorum sensing contributes to the production of alginate, a major component

of EPS in *Pseudomonas syringae* strain B728a. Various AHL-deficient mutants of *P. syringae* B728a were significantly impaired in alginate production (Table 1). Both an *aeiR*⁻ mutant and an *ahlI*⁻ *ahlR*⁻ double mutant produced 30% less alginate than the parental strain. Although an *ahlI*⁻ mutant previously was reported to be unaffected in alginate production (Willis et al. 2001), our study clearly demonstrated that, although AHL-deficient mutants are not devoid of alginate, AeiR and the AhlI-AhlR quorum-sensing system contribute to maximal levels of alginate production. Our finding that alginate production in *P. syringae* strain B728a is regulated in an AHL-dependent

Fig. 6. Bean tissue maceration is abolished in *N*-acyl homoserine lactone (AHL)-deficient mutants of *Pseudomonas syringae*. Bean pods were inoculated with a drop containing 5×10^7 cells of *P. syringae* parental strain B728a, *ahlI*⁻ *ahlR*⁻ double mutant strain A9, *aeiR*⁻ mutant strain 538, or *gacA*⁻ mutant strain BGACX and incubated at 28°C for 3 days. Internal tissue maceration was examined after performing a longitudinal cross section of the pods.

Fig. 7. Leaf symptoms on *N*-acyl homoserine lactone (AHL)-producing and wild-type tobacco plants by *Pseudomonas syringae* pv. *tabaci*. Regions between lateral veins on tobacco leaves were infiltrated with *P. syringae* pv. *tabaci* 11528R at various cell concentrations: 10^3 , 10^5 , 10^7 , or 10^9 CFU/ml. Disease symptoms were assessed 3 days after inoculation. Results shown are from a single representative experiment with 15 replicates for each inoculum concentration tested.

manner helps explain the cell-density-dependent, epiphytic fitness that is exhibited by this strain (Quiñones et al. 2004). An association between alginate synthesis and susceptibility to hydrogen peroxide also was observed (Table 1). The increased susceptibility to hydrogen peroxide of an *aeiR*⁻ and an *ahlI*⁻*ahlR*⁻ double mutant was similar to that observed in an *algT*⁻ mutant, a regulatory gene of the alginate biosynthetic cluster in *P. syringae* (Keith and Bender 1999). Alginate has been postulated to facilitate the colonization or dissemination in planta of *P. syringae* because its production is associated with increased epiphytic fitness (Yu et al. 1999) and resistance to desiccation and toxic molecules (Keith and Bender 1999). Therefore, the limited survival of an *aeiR*⁻ and an *ahlI*⁻*ahlR*⁻ double mutant on dry leaves (Quiñones et al. 2004), may be due, in part, to the reduced production of alginate in these mutant strains.

Motility also is considered an important epiphytic fitness trait in *P. syringae* B728a, enabling cells to locate resources and to access sites that allow them to avoid environmental stresses (Haelele and Lindow 1987; Lindow et al. 1993). Swarming is thought to be a ubiquitous form of motility among eubacteria (Harshey 1994) and is an intrinsically surface-linked and cell-density-dependent phenomenon (Eberl et al. 1999). Bacteria displaying swarming motility propagate on semisolid surfaces in a coordinated and social manner (Eberl et al. 1999). AHL-dependent gene regulation is required for swarming motility in *P. aeruginosa* and *Serratia liquefaciens* by promoting the synthesis of biosurfactants that facilitate the spreading of bacteria on the surface (Köhler et al. 2000; Lindum et al. 1998). Although a previous study reported that swarming motility in *P. syringae* B728a was not abolished in an *ahlI*⁻ mutant (Kinscherf and Willis 1999), the present study provides compelling evidence for the role of both the AhlI-AhlR quorum-sensing system and AeiR in the regulation of swarming motility. Both an *aeiR*⁻ mutant and an *ahlI*⁻*ahlR*⁻ double mutant were hypermotile under conditions when the wild-type cells displayed little movement (Fig. 1). Under such conditions, an *aeiR*⁻ mutant moved 5.6-fold more and an *ahlI*⁻*ahlR*⁻ double mutant moved 2.3-fold more than the wild-type strain (Table 2). Therefore, quorum sensing

downregulates swarming motility when the population is at relatively lower cell densities.

The enhanced swarming motility of the quorum-sensing mutants was observed in two separate scenarios. First, although both mutants moved faster than the parental strain, the *aeiR*⁻ mutant strain appeared to be the most hypermotile because it also started to swarm at an earlier time (Fig. 2). These findings indicate that AeiR, a regulator of autoinduction that is expressed even during low cell densities (Quiñones et al. 2004), plays a more important role in suppressing swarming motility at early stages of quorum sensing. It is noteworthy that the quorum-sensing system in *Yersinia pseudotuberculosis* has a negative effect in swarming motility (Atkinson et al. 1999); mutants in either the AHL synthase gene, *yspI*, or in the AHL regulator gene, *yspR*, were hypermotile. It is tempting to speculate that a common feature in the lifestyle in both of these bacterial pathogens may have selected for suppression of swarming motility. One possible explanation for this type of negative regulation in *P. syringae* would apply if nutrients are relatively localized on leaves and epiphytes prosper primarily at the sites of nutrient release (Leveau and Lindow 2001, 2002; Lindow and Brandl 2003). In that case, it would be beneficial for immigrant bacteria to a leaf to be motile, enabling them to explore the leaf surface for sites of nutrient release. Upon reaching such sites, multiplication to large population sizes in cell aggregates would occur, and further motility on the leaf would seem to be detrimental to such cells. Sites away from cell aggregates would be expected to be lower in resource abundance as well as AHL signals. Therefore, AHL-mediated suppression of swarming would tend to inhibit bacterial movement away from high-nutrient sites while promoting resource acquisition at earlier stages of leaf colonization. Presumably, *Y. pseudotuberculosis* also exploits localized sites of infection (Logsdon and Meccas 2003). In the second scenario, when swarming motility was examined at higher inoculum concentrations, the differences in swarming motility between the quorum-sensing mutants and the wild-type strain were subtle, with the wild-type strain being more motile than at lower cell concentrations.

Fig. 8. Population sizes of *Pseudomonas syringae* pv. *tabaci* within *N*-acyl homoserine lactone (AHL)-producing and wild-type tobacco plants. AHL-producing transgenic (open symbols) or wild-type (closed symbols) tobacco plants were infiltrated with a *P. syringae* pv. *tabaci* 11528R cell suspension that contained either 1×10^5 CFU/ml (triangles) or 1×10^3 CFU/ml (squares). Results presented are from a single representative experiment with 10 replicates. Vertical bars represent the standard error of the mean of $\log_{10}$ -transformed bacterial population sizes.

Fig. 9. Incidence of disease symptoms on leaves of *N*-acyl homoserine lactone (AHL)-producing and wild-type tobacco incited by *Pseudomonas syringae* pv. *tabaci* after surface inoculation. Tobacco leaves were spray inoculated with a *P. syringae* pv. *tabaci* 11528R cell suspension containing 1×10^6 CFU/ml and incubated under moist conditions at 28°C for 14 days. Results presented are from a single representative experiment that consisted of 10 replicates per treatment, and the mean difference ($P = 0.003$) was determined by a Student's *t* test. Vertical bars represent the standard error of the mean of leaves infected per plant.

It is possible that, at this growth stage, a separate regulatory mechanism with a more pronounced effect, one mediated by GacA, then would control swarming motility (Kinscherf and Willis 1999).

Previous studies have demonstrated that swarming motility may facilitate the entry of *P. syringae* into leaves. The movement of *P. syringae* on the surface of bean leaves has been shown to be assisted by flagellar motility and to require high relative humidity or moisture (Leben et al. 1970; Panopoulos and Schroth 1974). Flagellar mutants of *P. syringae* were remarkably impaired in their infection potential, presumably due to their inability to enter into the leaf tissue through stomates (Hattermann and Ries 1989; Panopoulos and Schroth 1974). Furthermore, flagellar motility contributed to the epiphytic fitness of *P. syringae* by allowing it to establish large, stable epiphytic populations and to survive environmental stress (Haefele and Lindow 1987; Lindow et al. 1993). In the present study, we investigated whether epiphytic populations of the hypermotile AHL-deficient mutants also would show an enhanced ability to access areas that were protected from hydrogen peroxide killing and to infect leaves. Intriguingly, both an *aeiR*⁻ mutant and an *ahII*⁻ *ahIR*⁻ double mutant achieved higher internal population sizes when compared with wild-type cells when measured soon after inoculation (Fig. 3). The internal population sizes of all strains increased with time, but the AHL-deficient mutants were always more numerous within the leaves, especially at earlier time points. A direct correlation between hypermotility and an increased incidence of disease also was observed. When incubated on moist bean leaves for 48 h, both an *aeiR*⁻ mutant and an *ahII*⁻ *ahIR*⁻ double mutant incited a much higher number of lesions on bean leaves than the wild type (Fig. 4). Interestingly, an *aeiR*⁻ mutant, which displayed hypermotility in culture, was also the most virulent. In contrast, when strains were provided only a short and humid incubation period (24 h), there were no significant differences in the number of lesions caused by the wild-type and AHL-deficient mutants (Fig. 4). It is possible that, under the conditions used in this study, at least two different infection processes may be occurring on a leaf after inoculation with a relatively few cells of *P. syringae*. Under moist conditions, a rapid multiplication of *P. syringae* occurs on leaves for 24 h. However, when the leaf surfaces are subsequently allowed to dry, further epiphytic growth ceases and some of epiphytic cells succumb to desiccation stress (Beattie and Lindow 1994). As epiphytic populations increase in size, some cells gain entrance to the intercellular spaces on the leaf, where the infection process can be initiated (Beattie and Lindow 1999; Wilson et al. 1999). In the present study, when cells were provided ample time (48 h) for both multiplication and motility on leaves, the hypermotile cells of the AHL-deficient mutants presumably entered the leaf in larger numbers than the wild-type strain and, therefore, had a higher likelihood of initiating a successful infection. Our previous findings showed that the epiphytic populations of the AHL-deficient mutants are less tolerant to desiccation stress (Quiñones et al. 2004). Therefore, when the plants were allowed to dry after only a short (24-h) moist period, the epiphytic population of the AHL-deficient mutants may have preferentially succumbed relative to that of the wild-type strain before invasion of the leaf could occur. The increased mobility of the AHL-deficient mutants may have been offset by their lower tolerance to desiccation stress, resulting in similar numbers of infections when provided a limited time for invasion of leaves before stressful conditions were imposed.

AHL-mediated quorum sensing affected various stages of symptom development during plant infection. *P. syringae* B728a causes lesions on both pods and leaves (Hirano and Upper 2000) and, during the early stages of lesion formation

on pods, water-soaked lesions result from the release of fluid from the plant tissue (Hirano and Upper 2000; Willis et al. 1990). AefR and the AhII-AhIR quorum-sensing system appear to differentially regulate traits involved in the formation of water-soaked lesions on pods. An *aeiR*⁻ mutant produced water-soaked lesions that were 41% smaller, while an *ahII*⁻ *ahIR*⁻ double mutant produced lesions that were 20% larger than those incited by the wild type (Fig. 5). Therefore, *P. syringae* seems to employ different regulatory pathways leading to the formation of water-soaked lesions. In contrast to the wild type, none of the AHL-deficient mutants incited any internal tissue maceration of bean pods (Fig. 6). AHL-mediated regulation of traits responsible for the maceration of plant tissue has been implicated in the virulence of other plant-pathogenic bacteria. For example, quorum sensing in *E. carotovora* is required for the production of exoenzymes such as cellulases, pectin lyase, pectate lyase, polygalacturonase, and proteases that lead to tissue maceration (Jones et al. 1993; Pirhonen et al. 1993). Tissue maceration caused by the wild-type strain is evident only 4 days or more after inoculation; therefore, tissue maceration is a later stage of infection than water soaking. Thus, an AhII-AhIR quorum-sensing system and AefR in *P. syringae* are involved in both early and late stages of disease development. This quorum-sensing regulation in *P. syringae* during various stages of plant infection appears to be a different strategy than that used by another plant pathogen, *E. carotovora* subsp. *atroseptica*. The AHL-mediated quorum sensing in *E. carotovora* subsp. *atroseptica* regulates only traits required for later stages of host plant infection and not initial plant colonization (Smadja et al. 2004).

Several regulatory genes affect quorum sensing as well as other traits in *P. syringae*; therefore, the different observed phenotypes in an *aeiR*⁻ mutant, a *gacA*⁻ mutant, and an *ahII*⁻ *ahIR*⁻ double mutant can be explained. Our previous findings suggested that AefR and GacA play an indirect role in the regulation of quorum sensing because the defect in *ahII* transcription in either an *aeiR*⁻ or a *gacA*⁻ mutant can be overcome by exogenous AHL (Quiñones et al. 2004). In addition to regulating AHL production, the regulators AefR and GacA may directly control other traits via separate regulatory cascades. For example, the regulation of AHL production by GacA occurs via a different pathway from the one involved in syringomycin and protease production and lesion formation on bean leaves (Kinscherf and Willis 1999; Kitten et al. 1998). Given that AefR and GacA are separate regulators that independently modulate quorum sensing (Quiñones et al. 2004), one would expect a difference in the expression of other traits such as EPS production, motility, and virulence between an *aeiR*⁻ and a *gacA*⁻ mutant. Distinct phenotypes also were observed between an *aeiR*⁻ mutant and an *ahII*⁻ *ahIR*⁻ double mutant when examined for water-soaking lesion development. Our previous findings demonstrated that *aeiR* is expressed even at low cell densities and that the defect in AHL production in an *aeiR*⁻ mutant was more pronounced at earlier stages of growth (Quiñones et al. 2004). Therefore, it is possible that AefR may play an important role in the expression of early disease symptoms, such as water soaking, during the infection process and may account for the reduced lesions that were observed in an *aeiR*⁻ mutant. Clearly, additional studies are needed to further characterize the interaction between the AhII-AhIR quorum-sensing system and the regulators AefR and GacA.

Given that quorum sensing regulated virulence of *P. syringae* B728a on bean, we explored whether it was possible to alter *P. syringae*'s ability to cause disease by expressing AHL in plants. This question was addressed by employing transgenic tobacco plants that were engineered to express the *yenI* gene of *Y. enterocolitica*, resulting in the synthesis of 3-oxo-C6-HSL

and hexanoyl homoserine lactone (C6-HSL) (Fray et al. 1999). The AHLs are produced in both plant roots and leaves and are easily detected on the leaf surface by various AHL-biosensor strains (Fray et al. 1999). Tobacco is not a normal host for *P. syringae* pv. *syringae*; therefore, we assessed the virulence of *P. syringae* pv. *tabaci* in AHL-producing tobacco. *P. syringae* pv. *tabaci* responds to 3-oxo-C6-HSL (Shaw et al. 1997), and the genes encoding the AHL synthase and AHL regulator show 89% sequence identity to strain B728a (Quiñones et al. 2004), suggesting that these pathovars share a similar quorum-sensing system. The expression of AHL in transgenic tobacco altered the process of disease caused by *P. syringae* pv. *tabaci*. When *P. syringae* pv. *tabaci* was infiltrated directly into leaves, the severity of disease in the AHL-producing plants was decreased when compared with wild-type tobacco. Interestingly, the differences in disease severity between wild-type and AHL-producing tobacco were less pronounced with increasing numbers of cells of *P. syringae* pv. *tabaci* infiltrated into the plant. Obvious differences in disease were observed when 10^5 CFU/ml was infiltrated, and a more pathogen-resistant phenotype was observed when 10^3 CFU/ml were infiltrated. Less-apparent differences in symptom severity were observed at 10^7 and 10^9 CFU/ml (Fig. 7). Likewise, the multiplication of *P. syringae* pv. *tabaci* was suppressed significantly when inoculated into AHL-producing tobacco, but only when low inoculum concentrations were used (Fig. 8). The observation of reduced disease severity and bacterial growth following inoculation with low but not high levels of inoculum was similar to that observed when *E. carotovora* also was inoculated into AHL-producing tobacco (Mäe et al. 2001).

As proposed by Mäe and collaborators, AHL production by the transgenic plant could induce the expression of bacterial virulence traits even when the pathogen cells were at sufficiently low population sizes in the plant. This early expression of the bacterial virulence traits was hypothesized to enable recognition of the previously “stealth” pathogen by the plant and, hence, initiate a successful defense reaction in the plant (Alfano and Collmer 1996; Mäe et al. 2001). However, when relatively large numbers of bacterial cells are inoculated into plants, the pathogen would already be present in sufficient numbers to overwhelm plant defenses and trigger gene expression of virulence factors that would result in disease. In this situation, the AHL signal produced by the transgenic plant would not noticeably alter the normal pattern of virulence gene expression because the large number of bacteria would already be producing their own high levels of AHL. Although *P. syringae* and *E. carotovora* differ greatly in the types of symptoms that they induce in plants, it appears that they both share a similar population size-dependent regulation of virulence genes and, therefore, a similar “stealth-style” of pathogenesis. The production of AHL in tobacco clearly altered the normal pattern of disease and was effective in controlling bacterial multiplication, particu-

larly when the populations of the pathogen were small. However, a recent study reported that production of AHL in potato plants resulted in an increased disease incidence in *E. carotovora* subsp. *atroseptica* at low inoculum levels (Toth et al. 2004). It is possible that the induction of plant defenses in these transgenic potato plants may initially be retarded or in some way reduced by the pathogen, allowing a successful infection to occur (Toth et al. 2004).

The incidence of infection in AHL-producing tobacco also was greatly reduced when infection resulted from an epiphytic inoculum. In this more natural disease process, a variety of bacterial traits that contribute to infection would be operating, unlike when inoculum was infiltrated directly into leaves. When compared with the wild-type plants, AHL-producing tobacco had 3.6-fold fewer diseased leaves (Fig. 9). To our knowledge, this is the first evidence that foliar disease occurring by a natural infection process can be suppressed by expressing AHL in plants. There are two possible explanations for the reduction in lesion formation in AHL-producing plants. After surface inoculation on the transgenic plant, most of the *P. syringae* pv. *tabaci* cells probably occurred as solitary cells or in small groups that normally would not have led to AHL production. These cells probably encountered high concentrations of AHL produced on the leaf surface of the transgenic plant. Presumably, this would result in an early expression of traits that normally occur only during later stages of colonization. Therefore, the AHL might have caused repression in motility when the epiphytic population of *P. syringae* pv. *tabaci* was at a relatively low cell density, resulting in an inefficient invasion into the leaf. As an alternative explanation, premature expression of virulence traits by *P. syringae* pv. *tabaci* cells that had entered the plant may have allowed the plant cells to recognize and halt the pathogen in sites that it had invaded, while still at low population sizes. Thus, expression of AHL signals in plants appears to be an efficient strategy for preventing disease caused by foliar pathogens such as *P. syringae*. Further work will be necessary, involving a plant host that produces AHL and also is compatible with *P. syringae* pv. *syringae* to assess whether these observations apply to all pathovars of *P. syringae*.

Although we only described a few phenotypes, this study suggests that AHL-regulated traits in *P. syringae* are important in its interaction with the plant host. A genomic analysis of quorum-sensing-regulated traits should provide more insights into the processes of epiphytic growth, survival, and virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids that were used in this study are described in Table 3. The source and characteristics of *P. syringae* pv. *syringae* B728a, a pathogen of bean, have

Table 3. Bacterial strains and plasmids used in this study

Strain, plasmid	Relevant characteristics ^a	Reference or source
<i>Pseudomonas syringae</i> pv. <i>syringae</i>		
B728a	Rf ^r , wild type, bean pathogen	Loper and Lindow 1987
538	Rf ^r Km ^r , Δ aejR::Tn5, B728a derivative	Quiñones et al. 2004
A9	Rf ^r Km ^r , Δ ahlI-ahlR::Kan, B728a derivative	Quiñones et al. 2004
BGACX	Rf ^r Km ^r , Δ gacA::Kan, B728a derivative	Rich et al. 1994
NPS3136	Rf ^r Km ^r , Δ gacS::Kan, B728a derivative	Willis et al. 1990
<i>P. syringae</i> pv. <i>Tabaci</i> 11528R	Rf ^r , wild type, tobacco pathogen	B. Staskawicz, University of California–Berkeley
<i>Agrobacterium tumefaciens</i> NTI	AHL indicator strain harboring <i>traG::lacZ</i> construct	Piper et al. 1993
Plasmids		
pSVB33	Km ^r , <i>traR</i> +	Piper et al. 1993
pJM749	Cb ^r , contains a <i>traG::lacZ</i> gene fusion	Piper et al. 1993

^a AHL = *N*-acyl homoserine lactone, Rf = rifampicin, Km = kanamycin, Cb = carbenicillin, and r = resistance.

been described previously (Loper and Lindow 1987). *P. syringae* strains were grown at 28°C in King's B agar (KB) (King et al. 1954), King's B broth (KBB), or MGY (Keane et al. 1970). The following concentrations of antibiotics were used: carbenicillin, 100 µg/ml; kanamycin, 50 µg/ml; and rifampicin, 100 µg/ml.

Alginate production.

P. syringae strains were grown for 2 days on KB amended with the appropriate antibiotics. Single colonies then were streaked on MGY containing 0.6 M sorbitol and grown for 3 days at 28°C to promote maximal levels of alginate production, as in previous studies (Keith and Bender 1999; Willis et al. 2001). Cells were removed from plates and resuspended in 0.9% NaCl as described previously (May and Chakrabarty 1994). The alginate was separated from the bacterial cells by centrifugation at $13,000 \times g$ for 20 min and further quantified by a modified carbazol assay (May and Chakrabarty 1994). Total cellular protein was determined by a Bradford protein assay (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, U.S.A.). The concentration of the isolated alginate from three replicates was determined from a standard curve of alginic acid from *Macrocystis pyrifera* (Sigma-Aldrich, St. Louis) and reported as micrograms of alginate per milligram of total cellular protein.

Susceptibility to ROIs.

To determine the susceptibility to ROIs, *P. syringae* strains were grown on KB for 2 days, removed from plates, and resuspended in 10 mM potassium phosphate buffer, pH 7.0 (PB). Cells were then mixed with KBB containing 0.6% melted agar to a final concentration of 5×10^8 CFU/ml, and 2 ml of this suspension was overlaid on a KB plate. Once the overlay had solidified, a filter disc (Fisherbrand, Grade P8-Creped, 6 mm in diameter) containing 3% hydrogen peroxide was placed in the center of each plate and the plates were incubated at 28°C for 18 h. The susceptibility to killing by hydrogen peroxide was determined by measuring the diameter of the zone of inhibited growth surrounding the filters, as in previous studies (Keith and Bender 1999). Each treatment consisted of five replicates and six measurements were taken for each replicate.

Motility assays.

Swarming motility was assessed on semisolid KB plates that contained 0.4% agar, as in previous studies (Kinscherf and Willis 1999). Cells first were grown on KB for 2 days, and then rinsed and resuspended in PB. Filter discs (Fisherbrand, Grade P8-Creped, 6 mm in diameter) were placed in the center of the plate and inoculated with a drop containing either 1×10^7 or 1×10^9 cells. Plates then were incubated at 26°C. The radius of swarming of the cells to the edge of the filter discs was measured at various times. Measurements of colony diameter were made, and the experiments were repeated several times, each with three replicates.

To determine the ability of *P. syringae* strains to enter the interior of leaves, 2-week-old bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274, containing 8 to 10 seedlings per pot) having fully expanded trifoliolate leaves were spray inoculated with a suspension containing 10^6 CFU/ml in PB. Three pots were inoculated for each strain. At various times, three trifoliolate leaves were removed randomly and surface sterilized with 15% hydrogen peroxide for 10 min, as in previous studies (Wilson et al. 1999), followed by a treatment with catalase at 7.5 µg/ml for 10 min. Leaves then were macerated in 5 ml of PB, and serial dilutions of each sample were plated on KB containing rifampicin plus 12.5 µg of benomyl

per ml, using a spiral diluter/plater (Spiral Systems, Inc. Cincinnati, OH, U.S.A.).

Inoculation of bean plants.

Cells of *P. syringae* strains were grown on KB for 2 days at 28°C, removed from plates, resuspended in PB, and diluted into 1 mM potassium phosphate buffer to yield a final concentration of 10^6 CFU/ml (Quiñones et al. 2004; Wilson et al. 1999). Bean plants with fully expanded primary and trifoliolate leaves were spray inoculated with the cell suspensions and then placed in a humid chamber maintained at nearly 100% relative humidity (RH) at 28°C. After either 24 or 48 h, plants were transferred to a greenhouse bench (<50% RH) and allowed to develop disease symptoms for 7 to 8 days. The numbers of visible brown spot lesions were counted on trifoliolate leaves (six leaves per plants; three pots per treatment) that were fully expanded at the time of inoculation. To determine the population sizes of epiphytic bacteria at various times during the inoculation process, four trifoliolate leaves were removed randomly from plants at a given time, and each leaf was immersed individually in 20 ml of washing buffer (100 mM potassium phosphate buffer containing 0.1% Bacto-peptone) (Difco Laboratories, Detroit) in 25-mm-diameter test tubes. The tubes then were sonicated in an ultrasonic bath for 7 min and vortexed briefly, as previously described (Quiñones et al. 2004). Serial dilutions of each sample were plated on KB containing rifampicin plus 12.5 µg of benomyl per ml as above.

Virulence on wounded bean pods was assessed by placing a droplet of cells (10^8 cells in PB) on the site of a 1-mm incision made with a razor blade to three sites on each 10-day-old, excised bean pod (*Phaseolus vulgaris* cv. Bush Blue Lake 274). Pods then were incubated for 3 to 4 days under humid conditions at 28°C. Disease symptoms were quantified by measuring the diameter of water-soaked lesions perpendicular to the razor wound. Longitudinal sections of the pods also were made to examine internal tissue maceration.

Inoculation of tobacco plants.

The growth and pathogenicity of *Pseudomonas syringae* pv. *tabaci* was investigated in wild-type tobacco (*Nicotiana tabacum* L. cv. Samsun) and transgenic plants expressing *yenI* from *Y. enterocolitica* and producing 3-oxo-C6-HSL and C6-HSL (Fray et al. 1999). The production of the AHL was confirmed by testing leaf discs (6 mm in diameter) in a bioassay similar to that previously described (Quiñones et al. 2004) by using *Agrobacterium tumefaciens* NT1 (pSVB33, pJM749) as the AHL indicator strain. To examine the growth of *P. syringae* pv. *tabaci* within plants, cells were grown on KB for 2 days, removed from plates, and resuspended in PB. Bacterial suspensions of various cell concentrations were infiltrated through a small incision into the apoplast of tobacco leaf tissue using a 1-ml needleless syringe. Immediately after infiltration, plants were incubated under moist conditions (100% RH) in a humid chamber at 28°C with 14 h of light/day. Plants were evaluated daily for disease symptoms. To determine the population sizes of bacteria within leaves, 1.5-cm-diameter leaf discs (10 leaf discs per treatment) were excised randomly from the inoculated areas of each leaf and macerated in PB. Serial dilutions of each sample were plated on KB containing rifampicin plus 21.6 µg of natamycin per ml as above. To examine the incidence of foliar infections caused by topically applied *P. syringae* pv. *tabaci*, leaves of AHL-producing and control tobacco plants were spray inoculated to wetness with a suspension containing 10^7 CFU/ml diluted in 1 mM potassium phosphate buffer. After inoculation, plants were placed in a humid chamber, and the number of leaves displaying disease symptoms was assessed after 14 days.

Statistical analysis.

Descriptive statistical analysis, Student's *t* test, Duncan's multiple test range, and Bonferroni's means comparison test were performed with Microsoft Excel 2000 (Microsoft Co., Redmond, WA), Systat (version 10.2; Systat Software, Inc., Point Richmond, CA), or SAS Software (version 6.03; SAS Institute Inc., Cary, NC, U.S.A.).

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