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Genetic and physiological evidence for the production of *N*-acyl homoserine lactones by *Pseudomonas syringae pv. syringae* and other fluorescent plant pathogenic *Pseudomonas* species

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Abstract

N-acyl homoserine lactones (AHLs) function as cell density (quorum) sensing signals and regulate diverse metabolic processes in several gram negative bacteria. We report that strains of *Pseudomonas syringae* pvs. *syringae* (Pss), *tabaci* and *tomato* as well as *P. corrugata* and *P. savastanoi* produce difussible AHLs that activate the *lux* operons of *Vibrio fischeri* or the *tra*::*lacZ* fusion of *Agrobacterium tumefaciens*. In Pss strain B3A, AHL production occurs in cell density dependent manner. Nucleotide sequence and genetic complementation data revealed the presence of $ahlI_{Pss}$, a *luxI* homolog within the Ahl$^+$ DNA of Pss strain B3A. The $ahlI_{Pss}{}^+$ DNA expresses in AHL-deficient strains of *P. fluorescens* and *E. carotovora* subsp. *carotovora* (Ecc), and restores extracellular enzyme production and pathogenicity in the Ecc strain. The derivatives of Pss strains B3A and 301D carrying chromosomal *ahlI::lacZ* do not produce AHL, but like their wild type parents, produce extracellular protease and the phytotoxin syringomycin as well as elicit the hypersensitive reaction in tobacco leaves. While these strains also produce a basal level of β-galactosidase activity, the expression of *ahlI::lacZ* is substantially stimulated in the presence of multiple copies of the $ahlI_{Pss}{}^+$ DNA or by the addition of cell-free spent cultures containing AHL. The activation of β-galactosidase production occurs with spent cultures of some, but not all *Pseudomonas* strains which produce AHL as indicated by the Lux and *tra::lacZ* assays. Pss strains deficient in the global regulatory genes, *gacA* or *lemA*, produce very low levels of AHL. Since inactivation of $ahlI_{Pss}$ eliminates AHL production and since Ahl$^+$ *Pseudomonas* strains carry the homolog of $ahlI_{Pss}$, we conclude that $ahlI_{Pss}$ specifies a key step in AHL biosynthesis and it has been conserved in many plant pathogenic pseudomonads.

Introduction

Bacteria live in constantly changing environments. In order to thrive under these conditions, they need to adjust their metabolic processes in response to the changing conditions in the milieu. Because such adaptations must be quick and efficient, bacteria have developed means of communication via signaling mechanisms that allow them to sense the environment and respond accordingly (Kaiser and Losick, 1993; Ninfa, 1996; Salmond et al., 1995).

Studies over the past several years have established essential roles for extracellular factors in the regulation of gene expression in bacteria. For example, *N*-acyl homoserine lactones (AHLs) act as cell density (quorum) sensing signals controlling gene expression in several bacteria (Fuqua et al., 1996; Greenberg, 1997; Pierson et al., 1998; Salmond et al., 1995; Swift et al., 1996). Typical of these is *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), which controls bioluminescence in the marine bacterium, *Vibrio fischeri* (Meighen, 1991) as well as various phenotypes in other bacteria (see below). OHHL is the

catalytic product of OHHL synthase, an enzyme encoded by the *luxI* gene (Engebrecht and Silverman, 1984). Together with *luxR*, *luxI* forms a regulatory pair in which the LuxR-OHHL complex serves as the transcriptional activator of the genes specifying the functions of bioluminescence including *luxI* (Sitnikov et al., 1995). Examples of phenotypes controlled by *N*-acyl homoserine lactones in other gram negative bacteria include conjugation in *Agrobacterium tumefaciens* (Piper et al., 1993; Zhang et al., 1993); extracellular virulence factor production in *P. aeruginosa* (Passador et al., 1993; Ochsner and Reiser, 1995; Brint and Ohman, 1995; Latifi et al., 1995); pathogenicity factors, virulence and antibiotic production in *E. carotovora* (Bainton et al., 1992; Chatterjee et al., 1995; Jones et al., 1993; Pirhonen et al., 1993); antibiotic production in *P. aureofaciens* (Pierson et al., 1998); and pathogenicity and extracellular polysaccharide production in *E. stewartii* (von Bodman and Farrand, 1995).

In *Xanthomonas campestris* pv. *campestris*, a diffusible factor is required for the production of extracellular polysaccharide slime and the yellow pigments, xanthomonadins (Poplawsky and Chun, 1997). Another report documents that an unrelated diffusible compound also controls extracellular enzyme production and pathogenicity in this species (Barber et al., 1997). Recently, Flavier et al. (1997) have identified 3-hydroxypalmitic acid methyl ester as a novel autoregulating extracellular factor controlling virulence in *Ralstonia (Pseudomonas) solanacearum*.

The genus *Pseudomonas* contains bacteria that inhabit diverse ecosystems. A significant number of these species are pathogenic on many plant species where they cause severe diseases (Rudolph, 1995). *Pseudomonas* species utilize various factors during colonization of their plant hosts and symptom production. Examples of such factors are extracellular polysaccharides and enzymes; elicitors of the hypersensitive reaction, called harpins; avirulence gene products; phytotoxins such as syringotoxin, syringopeptin, syringomycin, coronatine and tabtoxin; and plant growth regulating hormones including auxins, cytokinins and ethylene. Many of these extracellular proteins and metabolites are produced in cultures in a growth phase dependent manner since the levels are generally higher as the bacteria enter the post-exponential growth stage. A number of fluorescent *Pseudomonas* species also transfer plasmids by conjugation (Rudolph, 1995). As stated above, expression of these traits in some other bacteria is controlled by *N*-acyl homoserine lactones (AHLs), raising the possibility that such diffusible metabolites are produced by plant pathogenic pseudomonads and that these metabolites may also function as quorum sensing signals. To test this possibility we examined the production of AHLs by several plant pathogenic *Pseudomonas* strains. While this work was in progress, Farrand and associates (Farrand et al., 1996; Shaw et al., 1997) reported the production of AHL analogs by several *P. syringae* strains. Our data confirm and extend these observations by documenting AHL production by *P. syringae* pv. *syringae* (Pss) and other strains of *Pseudomonas* not included in that study. In addition, we show that the *ahl* locus of Pss is expressed in AHL-deficient *E. carotovora* subsp. *carotovora* and *P. fluorescens*. Although we did not detect an effect of AHL on the production of various secondary metabolites or extracellular protease by Pss, our data establish that AHL of Pss does indeed function as a quorum sensing signal, that it behaves as an autoinducer and that its production is cell density dependent. Additionally, we have shown that the production of AHL requires functions of the global regulators, GacA and LemA, and that the $ahlI_{Pss}$ sequences required for the synthesis of AHL have been conserved in many fluorescent *Pseudomonas* species.

Materials and methods

Bacterial strains and media

Bacterial strains are described in Table 1. Minimal salts, salt-yeast extract-glycerol supplemented with celery extract (SYG+CE) and nutrient-gelatin (NG) media were prepared according to Murata et al. (1991). Minimal glucose medium was supplemented with yeast extract (MM+YE) at 250 μg/ml. The AB minimal medium was prepared according to Chilton et al. (1974). Media were solidified by adding 1.5% agar. *Pseudomonas* strains were grown in King's B (KB) medium, *E. coli* strains in Luria-Bertani (LB) medium, and *Geotrichum candidum* on potato dextrose agar (PDA, pH 6.0). *Erwinia* strains were grown in SYG + CE or MM+YE media. Bacterial strains were grown at 28 °C except for *E. coli* which was grown at 37 °C. Where required, antibiotics were added at the indicated concentrations in μg/ml: tetracycline (Tc), 10 for *Erwinia*, *E. coli* and *Pseudomonas* strains and 100 for *P. fluorescens*; carbenicillin (Cb), 100; ampicillin (Ap), 100 and kanamycin (Km), 50.

Table 1. Bacterial strains and plasmids

Bacteria	Relevant characteristics	Reference or source
Erwinia carotovora subsp. *carotovora*		
AC5006	Lac$^-$ derivative of Ecc71	Murata et al., 1991
AC5092	*ahl::MudI* mutant of AC5006, Km^r	Cui et al., 1996
AC5070	RsmA$^-$, Km^r	Chatterjee et al., 1995
Ecc193	Wild type	Zink et al., 1984
SCRI193	Wild type	Jones et al., 1993
SCC3193	Wild type	Pirhonen et al., 1993
E. carotovora subsp. *atroseptica*		
Eca12	Wild type	Zink et al., 1984
E. carotovora subsp. *betavasculorum*		
JL11129	Wild type	Chatterjee et al., 1995
E. chrysanthemi		
EC16	Wild type	Chatterjee et al., 1979
P. syringae		
BR2R	Wild type, Rif^r	K. Willis
P. syringae pv. *syringae*		
B3A	Wild type	D. Gross
301D	Wild type	D. Gross
B15	Wild type	Lab collection
B452	Wild type	Morgan and Chatterjee, 1988
B453	Wild type	Lab collection
B454	Wild type	Lab collection
B455	Wild type	Lab collection
B456	Wild type	Lab collection
B728a	Wild type, Rif^r	Willis et al., 1990
NPS3136	LemA$^-$ derivative of B728a	Willis et al., 1990
BGACX	GacA$^-$ derivative of B728a	Rich et al., 1994
4B10	Wild type	K. Willis
AC800	*ahlI::lacZ* derivative of B3A, Km^r	This study
AC801	*ahlI::lacZ* derivative of 301D, Km^r	This study
P. syringae pv. glycinia		
race O	Wild type	Lab collection
Race 1	Wild type	Lab collection
Race 4	Wild type	Lab collection
Race 5	Wild type	Lab collection
Race 6	Wild type	Lab collection
P. syringae pv. tabaci		
Rif #2	Wild type	Lab collection
Str #5	Wild type	Lab collection
11528R	Wild type, Rif^r	K. Willis
P. syringae pv. tomato		
DC3000	Wild type	A. Collmer
P. corrugata		
0782-6	Wild type	Schroeder and Chun, 1995
1090-11	Wild type	Schroeder and Chun, 1995
0683-32	Wild type	Lab collection
P. savastanoi		
2009	Wild type	T.C. Currier

Table 1. Continued

Bacteria	Relevant characteristics	Reference or source
P. fluorescens		
Pf7-14	Wild type	Chatterjee et al., 1996
Agrobacterium tumefaciens		
NT1	Host strain for *tra::lacZ* indicator constructs	Piper et al., 1993
P. viridiflava		
SF312	Wild type	Lab collection
MI-4	Wild type	Lab collection
P. syringae pv. phaseolicola		
PDDC3019	Wild type	Lab collection
PM132	Wild type	Lab collection
PM141	Wild type	Lab collection
PM142	Wild type	Lab collection
Escherichia coli		
DH5α	ϕ80*lacZ* M15 Δ(*lacZYA-argF*) U169 *hsdR17 recA1 endA1 thi-1*	BRL
HB101	*proA1 lacY hsdS20*($r_B^-m_B^-$) *recA56 rpsL20*	Zink et al., 1984
M8820	*(proAB-argF-lacPOZYA) recA*$^+$	Castilho et al., 1984
POI1734	MudI1734*::ara(Mu cts),(proAB -argF-lacIPOZYA*), Km^r	Castilho et al., 1984
VJS533	a*ra (lac-proAB) rpsL 80lacZ M15 recA56*	Gray and Greenberg, 1992
Geotrichum candidum		
F-260	SR indicator strain	D. Gross
Plasmids		
pRK415	Mob$^+$, Tc^r	Keen et al., 1988
pRK2013	Mob$^+$, *tra*$^+$, Km^r	Figurski and Helinski, 1979
pLAFR5	Mob$^+$, Tc^r	Keen et al., 1988
pBluescript II SK+	Ap^r	Stratagene, La Jolla, CA
pSVB33	*traR*$^+$, Km^r	Piper et al., 1993
pJM749	Cb^r, *tra::lacZ*	Piper et al., 1993
pAKC951	Tc^r, *ahl*$^+$ cosmid	This study
pAKC953	7.0 kb *Bam*HI *ahl* fragment in pRK415	This study
pAKC954	*ahlI::lacZ* derivative of pAKC953	This study
pAKC955	4.0 kb MudI flanking *ahl* sequence cloned into pBluescript II SK+, Ap^r	This study
pAKC956	3.0 kb Mu*d*I flanking *ahl* sequence cloned in pBluescript II SK+, Ap^r	This study
pAKC957	4.3 kb *Bam*HI-*Eco*RI *ahl*$^+$ DNA in pBluescript II SK+. Ap^r.	This study
pHV200	8.8 kb *lux* DNA in pBR322, Ap^r	Gray and Greenberg, 1992
pHV200I	Frameshift mutation of *luxI* in pHV200, Ap^r	Pearson et al., 1994

Recombinant DNA techniques

Standard procedures (Sambrook et al., 1989) were used for restriction of DNA, isolation of plasmid DNA, electroporation of bacteria, gel electrophoresis, ligation of DNA, and Southern blot analyses. Chromosomal DNAs from bacteria were isolated using a kit obtained from Gentra Systems, Inc. (Research Triangle Park, NC, USA).

Detection of AHL using Agrobacterium tra::lacZ *and Pss* ahlI::lacZ *bioassays*

The activation of *tra::lacZ* expression was determined by the procedures described by Piper et al. (1993). Briefly, the indicator *A. tumefaciens*, NT1 carrying the plasmids, pSVB33 and pJM749 (Piper et al., 1993) was grown in AB minimal medium with mannitol as the carbon source and supplemented with Km and Cb. The cells were mixed with soft agar and 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 40 μg/ml) and overlaid on AB minimal agar medium containing mannitol in divided Petri plates. Cell suspensions or spent cultures of the bacterial strains to be tested were then spotted on the overlay and incubated at 28 °C. Appearance of blue color around the spots indicates the production of an AHL analog capable of activating the *tra::lacZ* gene fusion. A slight modification of this procedure involved seeding the test bacterium in the agar medium and incubating before overlaying with the indicator *Agrobacterium.* For the quantitative *tra::lacZ* assay, *Pseudomonas* strains were grown in KB medium and *Erwinia* strains in MM+YE medium to stationary phase. Spent cultures were collected, filtered and added (20%, v/v) to a culture of the *Agrobacterium* indicator strain grown in AB minimal medium to a final A_{600} of about 0.4. The cultures were incubated at 28 °C for 6 h and samples were taken for β-galactosidase assays (Miller, 1972).

To assay for the activation of *ahlI::lacZ,* spent cultures were added to fresh KB broth at a concentration of 20% (v/v) as described above. Cells of the *ahlI::lacZ* strain, AC800, harvested from mid-log culture were inoculated into the reconstituted medium to A_{600} of about 1.0. The cultures were incubated for two hours on a rotary shaker at 28 °C and samples were collected for β-galactosidase assay (Miller, 1972).

Bioluminescence assay for AHL

The plate bioluminescence assay for AHL has been described (Chatterjee et al., 1995). For quantitative assays, we used a modification of the procedure described by Chatterjee et al., (1995). A log phase (A_{600} = 0.2) culture of the Lux indicator strain, *E. coli* VJS533 carrying pHV200I grown in LB broth supplemented with Ap was divided into 10 ml aliquots. Spent cultures (50 μl samples) of each test bacterium were added to each aliquot. The cultures were incubated for an additional 2 h and samples were taken for bioluminescence measurements.

Cloning and sequence analysis of P. syringae *pv.* syringae *(Pss)* ahl *locus*

The genomic library of Pss strain B3A was constructed in the vector, pLAFR5 and the lambda particles were used to transduce plasmids into *E. coli* HB101. The library was mated into *P. fluorescens* Pf7-14 using the helper plasmid, pRK2013. Transconjugants were screened for AHL production using an agar plate assay (Chatterjee et al., 1995). Out of 1800 colonies, two Ahl^+ plasmids were obtained of which pAKC951 was used for further analysis. For nucleotide sequence analysis, the *ahl* fragments flanking the phage Mu sequence in pAKC954 (Table 1) were cloned into the *Hind*III-*Bam*HI site of pBluescript SK^+. The resulting plasmids, pAKC955 and pAKC956 were used for unidirectional deletions into the *ahl* sequence with the Erase-A-Base system (Promega Corp., Madison, WI, USA). The deletions were used for sequence analysis using the Sequenase Version 2.0 (USB, Cleveland, OH, USA). Oligonucleotide primers were also used in nucleotide sequence determinations. DNA and protein sequence analyses were performed using the PCgene software. The nucleotide sequence of $ahlI_{Pss}$ gene has been submitted to GenBank.

Assays for extracellular enzymes

Culture samples were prepared as previously described by Murata et al. (1991), and pectate lyase activity was assayed according to Chatterjee et al. (1995). For the detection of protease activity of Pss, cells were patched on nutrient-gelatin agar medium and incubated at 28 °C overnight.

Inactivation of $ahlI_{Pss}$ *by Mu*d*I mutagenesis*

The plasmid, pAKC953 (Table 1) was mutagenized with Mu*d*I1734 following the procedure of Castilho et al. (1984). Briefly, pAKC953 was transformed into a lysogenic *E. coli* strain POI1734. The strain carrying

the Ahl$^+$ plasmid was grown in LB broth at 28 °C to 80 Klett Units and heat-induced to lyse. The lysate was used for transduction of *E. coli* M8820, and the transductants were selected for Tc^r and Km^r. The plasmids carrying Mud*I* insertions were mated into *P. fluorescens* Pf7-14 using the helper, pRK2013. The Tc^r and Km^r transconjugants were screened for the loss of AHL production using the Lux plate bioassay procedure (Chatterjee et al., 1995). One of the plasmids (pAKC954) with inactivated *ahlI*$_{Pss}$ was used for further studies.

Construction of bacterial strains by marker exchange

To isolate Ahl$^-$ mutants of Pss, the *ahlI::lacZ* plasmid, pAKC954, was electroporated into strains B3A and 301D and the transformants were selected on KB medium containing Km. Single colonies were screened for antibiotic resistance, and the Km^r and Tc^s colonies were tested for their Ahl phenotype. Marker exchange was confirmed by Southern hybridization.

Assay for syringomycin

Fungal growth inhibition assay was used for the detection of syringomycin activity on PDA at pH 6.0. Briefly, bacteria were inoculated on to PDA medium and incubated at 28 °C for four days. *Geotrichum candidum* grown on a PDA plate was washed with sterile distilled water and this suspension was sprayed on to the seeded PDA plates. The plates were incubated overnight at 28 °C and syringomycin activity was indicated by clearing around the bacterial colonies due to the inhibition of fungal growth.

Assays for pathogenicity and hypersensitive reaction (HR)

The assays for pathogenicity of Ecc constructs on celery petioles, and the elicitation of HR by Pss on tobacco (*Nicotiana tabacum* L. cv. Samsun) leaves were done according to the procedures of Murata et al. (1991) and Cui et al. (1996), respectively. Bacterial cells were suspended in sterile water to a concentration of 2 x 10^8 cfu/ml for HR assays. For pathogenicity assays, Ecc cells were suspended in Tc solution and each inoculation site was injected with 2×10^8 cfu.

Table 2. Production of *N*-acyl homoserine lactone analogs by *Pseudomonas* species and occurrence of *ahlI* sequences

Bacterial species	Lux assay[1] AHL$^+$/ Total	*tra::lacZ* assay[2] AHL$^+$/ Total	*ahlI*$_{Pss}$ *homology*[3] Present/ Total
P syringae pv. *syringae*-SR^{+4}	6/6	6/6	6/6
P. syringae pv. *syringae*-ST^{+5}	1/5	1/5	1/5
P. corrugata	3/3	3/3	3/3
P. syrinage pv. *glycinia*	0/5	0/5	5/5
P. syringae pv. *tabaci*	3/3	3/3	3/3
P. syringae pv. *tomato*	1/1	1/1	1/1
P. syringae pv. *phaseolicola*	0/4	0/4	4/4
P. savastanoi	1/1	1/1	1/1
P. fluorescens	0/1	0/1	0/1
P. viridiflava	0/2	0/2	0/2

[1] Cell suspensions or spent cultures of bacteria grown in KB medium were spotted on LB plates with the *E.coli* Lux indicator strain.
[2] Cell suspensions or spent cultures of bacteria grown in KB medium were spotted on AB minimal medium overlaid with soft agar suspension of the *Agrobacterium* indicator strain.
[3] 232-bp segment of *ahlI* was used as the probe in Southern hybridization, representative data are shown in Figure 6.
[4] SR= syringomycin,
[5] ST= syringotoxin.

Results

Production of N*-acyl homoserine lactone (AHL) analogs by plant pathogenic pseudomonads*

Although the production of AHL analogs has been reported in many gram negative bacteria (Fuqua et al., 1996), similar data were not available until recently for fluorescent plant pathogenic pseudomonads (Farrand et al., 1996; Shaw et al., 1997). We screened strains of *Pseudomonas* species (Table 2) for the production of AHL analogs by assaying for the activation of *Agrobacterium tra::lacZ* fusion (Piper et al., 1993) and bioluminescence in *E. coli* which requires activation of the *Vibrio fischeri lux* operons (Chatterjee et al., 1995). Many *Pseudomonas* strains produced analogs detectable by both Lux and *tra::lacZ* assays; for example, *P. syringae* pv. *syringae* (Pss) B3A, 301D, B15, B728a, B456, 4B10; *P. syringae* BR2R; *P. syringae* pv. *tabaci* Rif #2, Str #5, 11528R; *P. syringae* pv. *tomato* DC3000; *P. corrugata* 0782-6, 0683-32, 1090-11 and *P. savastanoi* 2009. By contrast, Pss B452, B453, B454, B455; *P. syringae* pv. *glycinia* strains belonging to races 0, 1, 4, 5, and 6; *P. syringae* pv. *phaseolicola* PDDC3019, PM132, PM141, PM142; *P.*

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AHLI        MSSGFEFQVASYSKVPVTLLETLYALRKKIFSDRLEWKVRVSQAFEFDDYDSAAA    55
PSYI        MSSGFEFQLASYTTMPVTLLETLYSMRKKIFSDRLEWKVRVSHAFEFDEYDNAAT    55
HSLI        MLEIFDVN---HTLLSETKSEELFTLRKETFKDRLNWAVQCTDGMEFDQYDNNNT    52
SWRI        MIELFDVD---YNLLPDNRSKELFSLRKKTFKDRLDWLVNCENNMEFDEYDNRHA    52
YENI        MLKLFNVN---FNNMPERKLDEIFSLREITFKDRLDWKVTCIDGKESDQYDDENT    52
            * . *. .    . ..    ......*. .*.***.* *   .. * *.**.  .

AHLI        TYLIGSWNGVPLAGLRLINTCDPYMLDGP-FRSFFDYPAPRNAGMAESSRFFVDT   109
PSYI        TYLVGSWNGVPLAGLRLINTCDPYMLEGP-FRSFFDCPAPKNAAMAESSRFFVDT   109
HSLI        TYLFGIKDNTVICSLRFIETKYPNMITGTFF-PYFKEINIPEGNYLESSRFFVDK   106
SWRI        TYIFGTYQNHVICSLRFIETKYPNMISDGVFDTYFNDIKLPDGNYVEASRLFIDK   107
YENI        NYILGTIDDTIVCSVRFIDMKYPTMITGP-FAPYFSDVSLPIDGFIESSRFFVEK   106
            .*. *  ..  . ..*.*.   * *...  * ..*.      ..  *.**.*...

AHLI        ERARSLGILHAPLTEMLLFSMHNHAA AGLESIITVVSKAMARIVRKSGWEHRVL   164
PSYI        ARARSLGILHAPLTEMLLFSMHNHAALSGLQSIITVVSKAMARIVRKSGWEHHVL   164
HSLI        SRAKDILGNEYPISSMLFLSMINYSRDKGYDGIYTIVSHPMLTILKRSGWGIRVV   161
SWRI        ARIQALQLHQAPISAMLFLSMINYARNCGYEGIYAIISHPMRIIFQRSGWHISVV   162
YENI        ALARDMVGNNSSLSTILFLAMVNYARDRGHKGILTVVSRGMFILLKRSGWNITVL   161
            .  ...   . .....*...* *..   * ..* ...*. *  . ..***   *.

AHLI        ATGEASPGETVLLLDMPVNADNHQRLLGSIALRQPV-TDDLLRWPIPLDASASLQ   218
PSYI        STGEASPGETVLLLEMPVTADNHQRLLGNIALRQPV-TDDLLRWPIALGVSGSAP   218
HSLI        EQGLSEKEERVYLVFLPVDDENQEALARRINRSGTFMSNELKQWPL--KGPAAIA   214
SWRI        KTGCSEKNKNIYLIYMPIDDANRNRLLARINQHATKMG-----------------   200
YENI        NQGESEKNEVIYLLHLGIDNDSQQQLINKILRVHQVEPKTLETWPI--IVPGIIK   214
              * .. .. . *. . ..... *   *

AHLI        RARMDSAA   226
PSYI        QACMHSAA   226
HSLI        QA------   216
SWRI        --------   200
YENI        --------   214
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Figure 1. Multiple alignment of deduced amino acid sequences of AhlI$_{Pss}$ of *Pseudomonas syringae* pv. *syringae* B3A, PsyI of *P. s.* pv. *tabaci* (Chen and Shaw, 1995), HslI of *Erwinia carotovora* strain Ecc71 (Chatterjee et al., 1995), SwrI of *Serratia liquefaciens* (Eberl et al., 1996) and YenI of *Yersinia enterocolitica* (Throup et al., 1995). Identical amino acid is indicated by an asterik (∗) and conserved residue is indicated by a dot(.). Alignment was performed with clustal program of Pcgene software.

fluorescens Pf7-14; *P. viridiflava* SF312 and MI-4 did not produce AHL analogs detectable by either of the two assay procedures.

Cloning and sequence analysis of the ahl *locus*

To determine the genetic basis for the production of AHL by *Pseudomonas syringae*, we cloned a DNA segment of Pss B3A specifying AHL synthesis into pLAFR5, producing the plasmid, pAKC951 (see Materials and methods for the details). Pf7-14 carrying pAKC951 activated light production in the Lux assay and produced a distinct blue zone around the patched colony in the *tra::lacZ* assay. By contrast, Pf7-14 carrying the vector (pLAFR5) remained negative in these assays. The DNA locus specifying the synthesis of AHL was designated as *ahl*$_{Pss}$ (**a**cyl-**h**omoserine **l**actone synthesis gene of *Pseudomonas syringae* pv. *syringae*).

The nucleotide sequence of about 950 bp of the *ahl* DNA disclosed an open reading frame with a deduced protein product of 226 amino acids. GenBank search for sequences homologous with the *ahl*$_{Pss}$ DNA revealed 82% homology with *psyI* of *P. syringae* pv. *tabaci*, a gene predicted to encode a homoserine lactone synthase (Chen and Shaw, 1995). The predicted product of the *ahl*$_{Pss}$ ORF has high homology with members of the LuxI family of AHL synthases (Figure 1). Among these, the highest homology (i.e., 85% identity and 91% similarity) was with PsyI. The predicted amino acid sequence of the ORF also has 42% identity and 55% similarity with SwrI of *Serratia liquefaciens* (Eberl et al., 1996), 40% identity and 51% similarity with CarI and HslI of *Erwinia carotovora* (Swift et al., 1993; Chatterjee et al., 1995), 36% identity and 50% similarity with YenI of *Yersinia enterocolitica* (Throup et al., 1995). An unexpected observation was that, other than the near identity with PsyI, the putative product of the ORF has higher sequence homology with LuxI homologs from the members of *Enterobacteriaceae* such as *Erwinia species*, *Yersinia enterocolitica* and *Enterobacter agglomerans* than LuxI homologs from other pseudomonads. The gene was designated as *ahlI*$_{Pss}$ because of the homology of its putative product with PsyI and other LuxI homologs as well as its ability to complement the AhlI$^-$ mutant of Ecc strain 71 (see below).

Characterization of AHL-deficient Pss strains

To determine the role played by the *ahl*$_{Pss}$ locus in Pss, we constructed by marker exchange AhlI$^-$ mu-

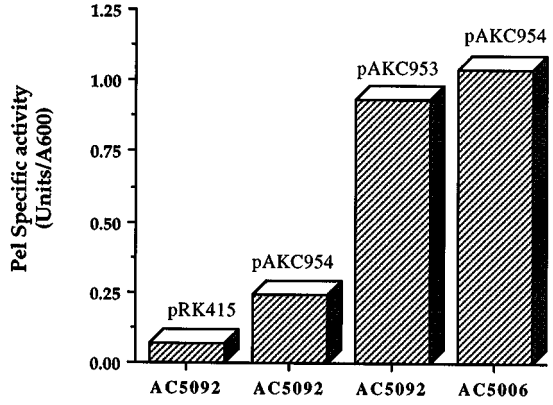

Figure 2. The Ahl$^+$ DNA of Pss B3A restores pectate lyase production by *E. carotovora subsp. carotovora* AhlI$^-$ strain AC5092. AC5092 and its parent, AC5006, carrying the indicated plasmids were grown in SYG+CE medium supplemented with Tc. Culture supernatants were assayed for pectate lyase activity as described in Murata et al. (1991). pRK415, vector control; pAKC953, *ahlI*$_{Pss}$$^+$; pAKC954, *ahlI::lacZ*.

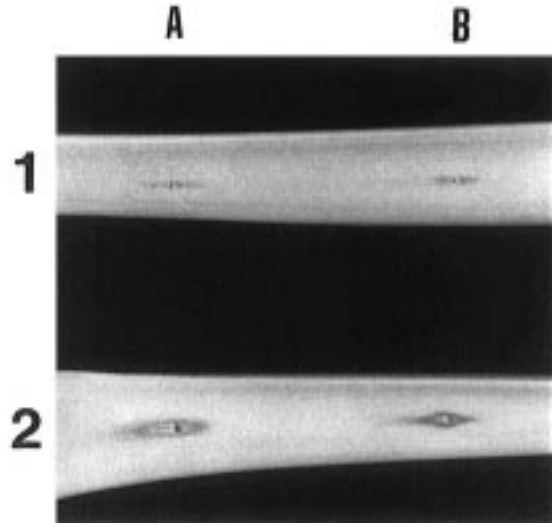

Figure 3. Celery tissue maceration by *E. carotovora* subsp. *carotovora* AC5092 (AhlI$^-$) carrying Ahl$^+$ DNA and its parent, AC5006. Bacterial cells were suspended in sterile Tc solution and tested for maceration of celery petioles. About 2 x 10^8 cells were injected into each inoculation site. The inoculated petioles were incubated in a moist chamber at 25 °C for 24 h. 1A, AC5092 carrying pRK415; 1B, AC5092 carrying pAKC954 (*ahlI::lacZ*); 2A, AC5092 carrying pAKC953 (*ahl*$^+$); 2B, AC5006 carrying pAKC954 (*ahlI::lacZ*).

tants, AC800 and AC801 of Pss B3A and 301D, respectively. The presence of Mu*d*I in *ahlI*$_{Pss}$ gene in the chromosomes of AC800 and AC801 was confirmed by Southern hybridization. The mutants, unlike their parent strains, did not produce AHL as determined by the *tra::lacZ* assay of *A. tumefaciens* (data not shown) and by the Lux assay. In the latter assay, spent cultures of AhlI$^-$ mutants, AC800 and AC801 did not activate bioluminescence to detectable levels. By contrast, spent cultures of the parent strains, Pss B3A and 301D produced 3×10^6 quanta/min/ml and 4×10^6 quanta/min/ml, respectively. To test for the restoration of AHL production in the mutants, the *ahl*$_{Pss}$$^+$ plasmid, pAKC951 and the vector, pLAFR5 were transformed into AC800 and AC801 and spent cultures were tested for the presence of AHL by the Lux assay. While the spent cultures of AC800 and AC801 carrying the vector did not activate bioluminescence, the spent cultures of the same strains carrying the *ahl*$_{Pss}$$^+$ plasmid generated about 4×10^7 quanta/min/ml and about 7×10^7 quanta/min/ml, respectively.

The strains B3A and 301D and their AhlI$^-$ derivatives were tested for various characteristics. Like their Ahl$^+$ parents, the AhlI$^-$ mutants produced the phytotoxin, syringomycin (SR) and extracellular protease (Prt) activity and elicited the hypersensitive reaction (HR) in tobacco leaves (data not shown). These observations demonstrate that AHL is not required for the production of SR, Prt or the elicitation of the HR by Pss.

Pss ahl *locus restores AHL production in AHL-deficient* E. carotovora *subsp.* carotovora *(Ecc) and functions as a quorum sensing signal*

To further test the regulatory function of Pss AHL, the plasmid, pAKC953 and its *ahlI::MudI* derivative, pAKC954 were transformed into AC5092, an AhlI$^-$ derivative of Ecc strain 71. The bacterial constructs were grown in salts, yeast extract and glycerol medium supplemented with celery extract and the spent cultures were assayed for AHL. Strain AC5092 carrying pAKC953 activated high level of light production (about 10^7 quanta/min/ml) in the *E. coli* Lux bioassay system. By contrast, the same strain carrying pAKC954 did not activate light production. These data demonstrate that *ahl*$_{Pss}$ was functional in Ecc and that Ecc produced the precursors required for the synthesis of Pss AHL.

The AhlI$^-$ mutant, AC5092 does not produce extracellular enzymes and is consequently nonpathogenic (Cui et al., 1995). Therefore, we wished to determine if the AHL specified by the *ahl* locus of Pss could substitute for that of Ecc and correct the pleiotropic phenotype of AC5092. The bacteria were grown in salts, yeast extract and glycerol medium supplemented with celery extract and the culture supernatants were

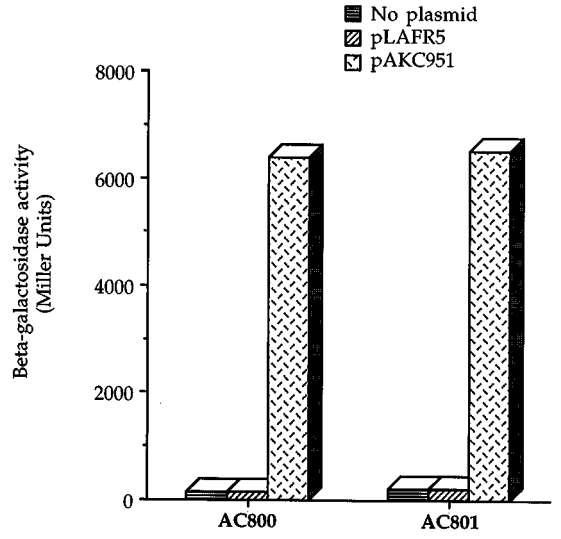

Figure 4. Activation of the expression of *ahlI::lacZ* in *P. syringae* pv. *syringae* AC800 and AC801 by *ahl*$^+$ DNA. AC800 and AC801 were grown in King's B medium. The strains carrying the *ahl*$^+$ plasmid, pAKC951 or the vector, pLAFR5 were grown in King's B medium supplemented with Tc. Cultures were grown to saturation and used for β-galactosidase assay.

assayed for pectate lyase (Pel) activity. Figure 2 shows that AC5092 carrying pAKC953 produced substantial Pel activity, comparable to the level in the parental strain AC5006. By contrast, the mutant strain carrying the AhlI$^-$ construct (pAKC954) produced a low level of this enzyme. Similarly, Peh, Prt and Cel activities, as indicated by agarose plate assays (Chatterjee et al., 1995), were higher in the Ahl$^+$ than in the Ahl$^-$ strains (data not shown). The bacterial constructs were also tested for pathogenicity on celery petioles, and as shown in Figure 3, pAKC953 restored pathogenicity in AC5092. Taken together, these data demonstrate that the AHL specified by pAKC953 is functionally interchangeable with the quorum sensing signal, OHHL produced by Ecc71.

Autoinduction of the ahl *locus in Pss strains B3A and 301D*

Acyl homoserine lactones are usually referred to as autoinducers because they activate the expression of the genes specifying their synthesis (Fuqua et al., 1996; Greenberg, 1997). We were interested in determining if the expression of the *ahlI*$_{Pss}$ gene in Pss also is AHL dependent. For this, we used the strains AC800 and AC801 which carry chromosomal copies of *ahlI::lacZ*. The *ahl*$_{Pss}$$^+$ plasmid, pAKC951, was transformed into AC800 and AC801. The constructs were grown in KB medium and assayed for β-galactosidase activity. As shown in Figure 4, strains AC800 and AC801 carrying extrachromosomal copies of *ahlI*$_{Pss}$$^+$ produced 30- to 40-fold higher levels of β-galactosidase activity than the same strains carrying the plasmid vector, pLAFR5. The addition of spent cultures of the Ahl$^+$ parent strains, but not those of AC800 and AC801, also activated *ahlI::lacZ* expression (Table 3). These observations are similar to the findings with *P. aeruginosa, A. tumefaciens* and *V. fischeri* where AHLs were shown to behave as autoinducers of the *luxI, traI and lasI* genes, respectively (Eberhard et al., 1991; Piper et al., 1993; Passador et al., 1993).

Table 3. Activation of *ahlI::lacZ* expression in AC800 by spent cultures of *Pseudomonas* and *Erwinia* strains

Source of spent culture[1]	Strain	β-galactosidase activity (Miller Units[2])
P. syringae pv. *syringae*	B3A	1047 ± 16
	301D	946 ± 17
	B15	680 ± 11
	B728a	583 ± 11
	NPS3136	15 ± 4
	BGACX	21 ± 3
	B456	650 ± 18
P. syringae	BR2R	976 ± 40
P. syringae pv. *tabaci*	11528R	566 ± 40
P. syringae pv. *tomato*	DC3000	ND[3]
P. corrugata	0782-6	151 ± 26
E. carotovora subsp.	AC5006	567 ± 12
carotovora	Ecc193	48 ± 29
	SCC3193	15 ± 11
E. carotovora subsp. *atroseptica*	Eca12	29 ± 17
E. stewartii	DC283	1365 ± 24

[1] *Pseudomonas* strains were grown in KB medium and *Erwinia* strains were grown in minimal medium supplemented with yeast extract.
[2] Values are corrected for the control; mean values are followed by standard deviation.
[3] ND = not detectable.

AHL production in Pss B3A is cell density dependent

The synthesis of AHLs and the expression of AHL-controlled traits in many bacteria are cell density-dependent (Fuqua et al., 1996). Thus, significant expression of AHL biosynthetic genes does not begin until the bacterial culture has reached a certain threshold cell density. To determine if AHL produc-

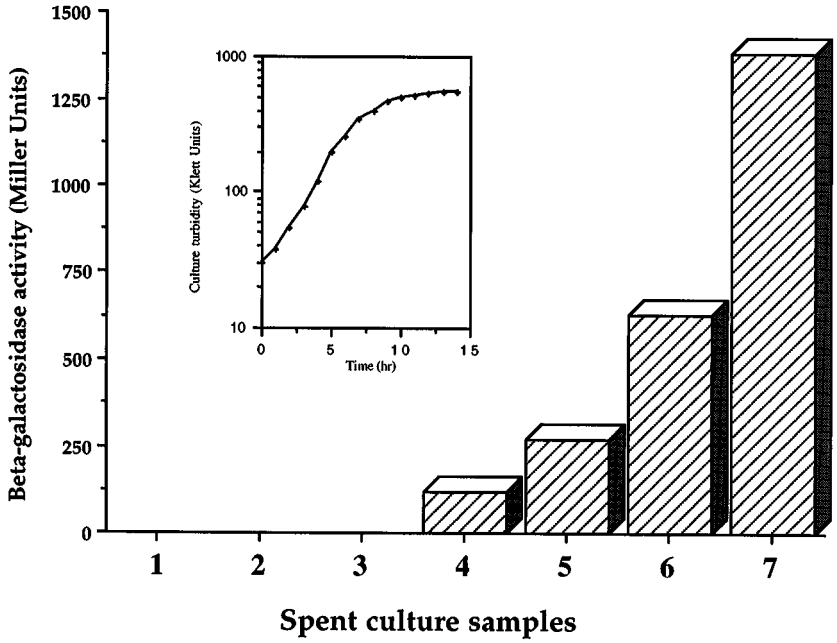

Figure 5. Cell density dependent AHL production in Pss strain B3A. The bacterium was grown in KB medium and samples were collected when the culture had reached different turbidities. Spent cultures, obtained by removing cells by centrifugation, were added at a final concentration of 20% (v/v) to samples of an exponentially growing culture of AC800 (*ahlI::lacZ*). The cultures of AC800 were incubated at 28 °C for two hours and samples were assayed for β-galactosidase activity. Under these conditions the level of activation of *ahlI::lacZ* expression is directly proportional to the amount of AHL. Pss B3A culture samples were collected at the following Klett Units: 1, 50; 2, 100; 3, 200; 4, 300; 5, 400; 6, 500 and 7, 600.

tion in Pss B3A also occurs in a cell density-dependent fashion, we measured the activation of *ahlI::lacZ* expression in strain AC800 by spent cultures of Pss B3A collected at different culture turbidities. Cultures of AC800 supplemented with the spent cultures were assayed for the activation of *ahlI::lacZ* expression as described in Materials and Methods. In this assay, the level of β-galactosidase reflects the amount of AHL present in spent cultures. The data (Figure 5) show that significant activation of *ahlI::lacZ* occurred with samples that were collected from cultures at 300 or higher Klett units. By extrapolating from the data on activation of *ahlI-lacZ* expression, we concluded that AHL levels remained low in cultures at low cell densities, but were progressively higher as the Pss strain attained higher cell density. These observations demonstrate that Pss produces an AHL in a cell density dependent manner.

Activation of ahlI::lacZ *expression in Pss by spent cultures of plant pathogenic* Pseudomonas *and* Erwinia

Because extrachromosomal copies of *ahl*$^+$ plasmid as well as spent cultures of Ahl$^+$ parent strains increased the expression of *ahlI::lacZ* in Pss strains AC800 and AC801, we wished to determine the activation of *ahlI::lacZ* fusion by spent cultures from other Ahl$^+$ plant pathogenic *Pseudomonas* and *Erwinia* strains. The representative data (Table 3) show that spent cultures of Ahl$^+$ strains of *Erwinia* and *Pseudomonas* did indeed activate *ahlI::lacZ* expression. Based on the level of activation, the strains fell into three classes: class I was represented by *E. carotovora* subsp. *atroseptica* Eca12*; E. carotovora* subsp. *carotovora* Ecc193 and SCC3193; and *P. corrugata* 0782-6: the spent cultures of these strains activated *ahlI::lacZ* expression to low levels. Class II consisted of *E. carotovora* subsp. *betavasculorum* strain JL11129; Ecc strains AC5006 and SCRI193; *E. chrysanthemi* strain EC16; *E. stewartii* strain DC283; Pss B3A, 301D, B15, B728a, B456; *P. syringae* BR2R and *P. syringae* pv. *tabaci* 11528R, whose spent cul-

tures caused high levels of β-galactosidase production. Class III was represented by *P. syringae* pv. *tomato* DC3000: the spent culture of this strain activated bioluminescence and the expression of *tra::lacZ* fusion, but did not activate *ahlI::lacZ* to detectable levels (Table 3) or purple pigment (violacein) production in the *Chromobaterium violaceum* bioassay (McClean et al., 1997) for OHHL. These differential responses could be due to differences in AHL structure, the quantity of the AHLs produced or both.

AHL production in Pss is controlled by the global regulators, gacA *and* lemA

The expression of cell density (quorum)-sensing functions, including the production of AHLs in *P. aeruginosa* and *P. aureofaciens*, is controlled by the global regulator genes, *gacA* and *lemA* (Reimmann et al., 1997; Pierson et al., 1996). To find out if these genes might also control the production of AHL in Pss, we used the well characterized Pss strains: BGACX, a GacA$^-$ strain (Rich et al., 1994), and NPS3136, a LemA$^-$ strain (Willis et al., 1990), both derived from Pss strain B728a. Spent cultures of these strains were assayed for AHL using the *E. coli* Lux bioassay and by determining the activation of *ahlI::lacZ* expression in Pss strain AC800. We found that the spent culture of the wild type parent, B728a activated high level of bioluminescence (about 3×10^7 quanta/min/ml). This value was about 80-fold and 100-fold higher than the levels of bioluminescence obtained with the spent cultures of the GacA$^-$ and LemA$^-$ mutants, respectively. Similarly, spent culture of the GacA$^+$ and LemA$^+$ parent strain was considerably more efficient in the activation of the *ahlI::lacZ* expression than those of the GacA$^-$ or the LemA$^-$ mutants (Table 3).

ahlI$_{Pss}$ *homologs exist in other plant pathogenic pseudomonads*

To determine the presence of *ahlI*$_{Pss}$ homologs in the plant pathogenic pseudomonads (Table 1), we conducted Southern hybridization using a 232 bp DNA fragment extending from *Bgl*II to *Sal*I sites in the predicted coding region of the *ahlI*$_{Pss}$ gene as the probe, and the data for the representative strains are shown in Figure 6. Based upon the pattern of hybridization of *Eco*RI-digested chromosomal DNAs, the *Pseudomonas* strains were grouped into the following categories: Pss strains 301D, B3A, B15, 4B10, B728a and B456 produced strong signals. By contrast, *P. syringae* strain BR2R, *P. syringae* pv. *tabaci* strains

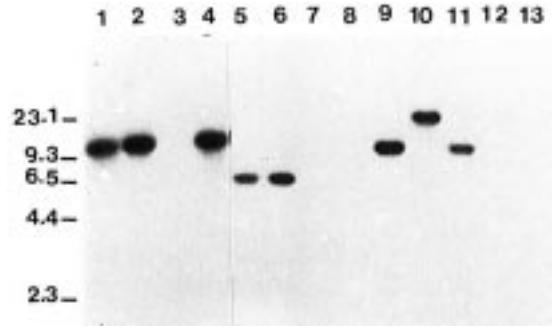

Figure 6. Southern hybridization of chromosomal DNA of *Pseudomonas* species with *ahlI*$_{Pss}$. Genomic DNAs were digested with *Eco*RI, electrophoresed, transferred to nylon membranes, and hybridized with ^{32}P-labeled 232bp *Bgl*II-*Sal*I fragment of *ahlI*$_{Pss}$. Washings were carried out sequentially at 65 °C as follows: 30 min in 2X SSC, 30 min in 2X SSC + 0.1% SDS, 30 min in 1X SSC + 0.1% SDS, 30 min in 0.5X SSC + 0.1% SDS and 30 min in 0.2X SSC. The X-ray films were developed after overnight exposure. The positions of λ-*Hind*III marker are shown. 1, Pss B3A; 2, Pss B728a; 3, Pss B452; 4, Pss B456; 5, *P. syringae* BR2R; 6, *P. syringae* pv. *tabaci* 11528R; 7, *P. syringae* pv. *tomato* DC3000; 8, *P. corrugata* 0782-6; 9, *P. syringae* pv. *glycinia* Race 1; 10, *P. savastanoi* 2009; 11, *P. syringae* pv. *phaseolicola* PM132; 12, *P. viridiflava* SF312 and 13, *P. fluorescens* Pf7-14. Weak signals, not seen in this figure, were detected with *P.s.* pv. *tomato* DC3000 and *P. corrugata* 0782-6 after three days exposure.

11528R, Rif #2, Str#5, *P. syringae* pv. *phaseolicola* PM132, PM141, PM142, PDDC3019, *P. savastanoi* 2009 and *P. syringae* pv. *glycinia* races O, 1, 4, 5 and 6 produced relatively weak bands. Even weaker bands were obtained with *P. corrugata* 0782-6, 1090-11, 0683-32 and *P. syringae* pv. *tomato* DC3000 after prolonged exposure. No hybridizing signals were obtained with Pss B452, B453, B454, B455; *P. fluorescens* Pf7-14 and *P. viridiflava* SF312, MI-4. An unexpected finding was the occurrence of *ahlI* homologs in strains of *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *glycinia* which do not produce AHL analogs detectable by any of the three bioassay systems.

Discussion

We have presented evidence for the production of AHL analogs by *Pseudomonas syringae* pv. *syringae* and other fluorescent plant pathogenic pseudomonads and the existence of *ahlI* sequences in these bacteria. First, upon co-culturing the test *Pseudomonas* strains with the bioluminescence indicator strain, these bacteria induced bioluminescence presumably by producing AHLs. Second, the cloned DNA segment

allowed AHL-deficient Ecc and *P. fluorescens* to produce AHL(s), detectable by light assay. Third, mutants carrying an insertion in $ahlI_{Pss}$ did not elicit bioluminescence, but AHL production was restored in these mutants by the $ahl_{Pss}{}^{+}$ plasmid. Further, the amino acid sequence of the predicted product of $ahlI_{Pss}$ gene shows high homology with PsyI a predicted AHL synthase in *P. syringae* pv. *tabaci* (Chen and Shaw, 1995) and other members of LuxI family of AHL synthases. In addition, as in *P. aeruginosa* and *A. tumefaciens*, AHL synthesis in Pss B3A occurs in a cell density-dependent manner.

The cloned $ahlI_{Pss}$ was expressed in AHL-deficient Ecc restoring extracellular enzyme production and pathogenicity. There are two possible interpretations of this finding. A putative transcriptional factor of extracellular enzymes interacts with the Pss AHL to activate enzyme production in AC5092. Alternatively, pAKC953 also carries the gene for AhlR (a putative LasR = LuxR homolog), and this transcriptional factor then interacts with its cognate AHL to activate the expression of exoenzyme genes. However, since there is no evidence for an AhlR-like molecule activating exoenzyme production in Ecc, the putative product of $ahlR_{Pss}$ probably does not function as the activator of extracellular enzymes in AC5092.

Our survey of pseudomonads for AHL production using biological activity and for the presence of *ahlI* sequences by Southern hybridization revealed an unexpected pattern: strains of *P. syringae* pvs. *glycinia* and *phaseolicola* possess $ahlI_{Pss}$ homologs (Figure 6), but do not produce AHL analogs detectable by any of the three bioassays. Furthermore, these strains did not activate pigment production in the *Chromobacterium violaceum* bioassay for AHLs (McClean et al., 1997). There are several possible explanations for these observations. First, it is conceivable that the genes for AHL biosynthesis remain silent under the cultural conditions used in these experiments. Second, the occurrence of *ahlI* homologs in plant pathogenic *Pseudomonas* does not always result in the production of structurally and functionally similar AHL species. Another trivial and unlikely explanation could be that the *Pseudomonas* strains produce substances inhibitory to the indicator bacterial strains and this somehow prevents a positive response in the bioassay protocols. The hybridization data also reveal considerable haterogeneity among plant pathogenic pseudomonads with respect to the occurrence of homologs of $ahlI_{Pss}$. These findings are consistent with the observation that *luxI* homologs generally have low sequence homology with each other (Swift et al., 1993).

Other than the activation of *lux* and *tra* operons, none of the phenotypes we tested were affected in our AHL-deficient mutants. Both the mutants and their parents produced extracellular protease and the phytotoxin, syringomycin as well as elicited the hypersensitive reaction in tobacco leaves. A phenotype affected by AHL was also not detected in *Yersinia enterocolitica* where *yenI*, a homolog of *luxI*, specifies the synthesis of *N*-(3-oxohexanoyl)-L-homoserine lactone and *N*-(hexanoyl)-L-homoserine lactone (Throup et al., 1995). A $YenI^{-}$ mutant, deficient in both AHL species, produced Yop proteins, a major pathogenity factor in this bacterium. However, this mutant lacked a number of cellular proteins whose functions are not yet known (Throup et al., 1995). A lack of AHL effect also was noted in *Enterobacter agglomerans*: an AHL-deficient mutant due to an insertional inactivation of *eagI*, the *luxI* homolog, was phenotypically similar to the parent $EagI^{+}$ strain (Swift et al., 1993).

Pseudomonas syringae pv. *syringae* is an epiphytic bacterium and it survives on leaf surfaces where conditions markedly differ from those of the laboratory culture. It is conceivable that Pss uses AHL to recruit an associated organism(s) in the micro-environment to produce a factor(s) which it utilizes for its growth and survival. Under these conditions, AHL analogs do not need to have a physiological role in Pss. In fact, in another strain of Pss, Ahl^{-} mutants have been found to be less fit on plant surfaces, indicating that AHL production may be required for the survival of the bacterium in its natural environment (S. Lindow, pers. comm.). Therefore, although we have not succeeded in associating any phenotype with AHL production, it would be premature to conclude that AHL analogs do not play any role in the physiology and ecology of Pss.

The data presented in this report and the findings with *P. aeruginosa* (Reimmann et al., 1997) and *P. aureofaciens* (Pierson et al., 1996) now firmly establish a crucial role of *gacA* and *lemA* in AHL production in *Pseudomonas* species. The issue that now has to be resolved is the mechanism by which *gacA/lemA* activates the AHL biosynthetic genes. A most likely target of this regulation is AhlR (= PhzR, Wood and Pierson, 1996; LasR, Gambello and Iglewski, 1991; RhlR, Latifi et al., 1995). We now have conclusive evidence for the occurrence of AhlR in Pss B3A and have initiated studies to determine if it is the target of *gacA/lemA* regulation.

Acknowledgments

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