Quorum-Sensing System Affects Gall Development Incited by *Pantoea agglomerans* pv. *gypsophilae*

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The quorum-sensing (QS) regulatory system of the gall-forming *Pantoea agglomerans* pv. *gypsophilae* was identified. Mass spectral analysis, together with signal-specific biosensors, demonstrated that *P. agglomerans* pv. *gypsophilae* produced *N*-butanoyl-L-homoserine lactone (C₄-HSL) as a major and *N*-hexanoyl-L-homoserine lactone (C₆-HSL) as a minor QS signal. Homologs of *luxI* and *luxR* regulatory genes, *pagI* and *pagR*, were characterized in strain *P. agglomerans* pv. *gypsophilae* Pag824-1 and shown to be convergently transcribed and separated by 14 bp. The deduced PagI (23.8 kDa) and PagR (26.9 kDa) show high similarity with SmaI (41% identity) and SmaR (43% identity), respectively, of *Serratia* sp. American Type Culture Collection 39006. PagR possesses characteristic autoinducer binding and a helix-turn-helix DNA-binding domain. Gall formation by *P. agglomerans* pv. *gypsophilae* depends on a plasmid-borne *hrp/hrc* gene cluster, type III effectors, and phytohormones. Disruption of *pagI*, *pagR*, or both genes simultaneously in Pag824-1 reduced gall size in gypsophila cuttings by 50 to 55% when plants were inoculated with 10⁶ CFU/ml. Higher reductions in gall size (70 to 90%) were achieved by overexpression of *pagI* or addition of exogenous C₄-HSL. Expression of the *hrp/hrc* regulatory gene *hrpL* and the type III effector *pthG* in the *pagI* mutant, as measured with quantitative reverse-transcriptase polymerase chain reaction, was reduced by 5.8 and 6.6, respectively, compared with the wild type, suggesting an effect of the QS system on the Hrp regulon.

Additional keywords: pPATH_{Pab}, pPATH_{Pag}, pathogenicity island.

Quorum sensing (QS) is the ability of a microorganism to perceive and respond to microbial population density, usually relying on the production and subsequent response to diffusible signal molecules (Fuqua et al. 2001). Regulation by QS has been identified in numerous gram-negative bacteria and can be associated with the expression of various genes that control diverse physiological functions, including epiphytic growth and virulence (von Bodman et al. 2003). The QS systems of many gram-negative bacteria are characterized by the presence of LuxR proteins, which function as transcriptional

regulators, and LuxI proteins, which function as synthases for *N*-acyl-homoserine lactone (acyl-HSL) signal molecules. Most LuxR proteins share relatively low identity but possess conserved amino acid residues (Whitehead et al. 2001). The LuxI family shares four groups of conserved sequences and several essential residues, which are mandatory for full activity of acyl-HSL synthase (Hanzelka et al. 1997; Parsek et al. 1997). The structures of acyl-HSL differ with regard to acyl side-chain length, hydroxyl or oxo substitutions at the third carbon, and the presence of double bonds (Fuqua and Eberhard 1999). Once critical levels of cell density and, consequently, of acyl-HSL accumulation have been achieved, the acyl-HSL binds to its cognate LuxR, causing it to activate the QS-controlled genes (Whitehead et al. 2001). The activated LuxR protein binds to a DNA element upstream of the transcription start site known as the *lux* box. QS regulation could be strain specific, with different strains making substantially different sets of acyl-HSL or no detectable acyl-HSL (Cha et al. 1998).

Expression of pathogenicity factors in a variety of plant-pathogenic bacteria might be critically dependent on QS (von Bodman et al. 2003). These include pathogens with differing virulence mechanisms and host ranges, such as *Pantoea stewartii* subsp. *stewartii*, a host-specific pathogen of corn that causes wilt and leaf blight, or *Erwinia carotovora* subsp. *carotovora* (synonym: *Pectobacterium*), which causes soft rots on a wide host range of plants. In the former pathogen, the QS controls production of EPS, which causes vascular occlusion (von Bodman and Farrand 1995); in the latter, it regulates production of extracellular enzymes required for cell maceration (Cui et al. 2005). The findings that virulence of *P. stewartii* subsp. *stewartii* and *E. carotovora* subsp. *carotovora* of the *Enterobacteriaceae* family is QS dependent stimulated us to investigate whether gall formation by the pathogens *P. agglomerans* pvs. *gypsophilae* and *betae*, which belong to the same family, is also controlled by the QS regulatory system. *P. agglomerans* pvs. *gypsophilae* and *betae* are two related pathovars that have been transformed into host-specific gall-forming pathogens by acquiring indigenous pathogenicity plasmids, designated as pPATH_{Pag} and pPATH_{Pab}, respectively (Barash and Manulis-Sasson 2007; Manulis and Barash 2003). pPATH_{Pag}, which has been more extensively studied, has a size of 135 kb and harbors a pathogenicity island (PAI) of approximately 75 kb. This PAI carries a *hrp/hrc*-gene cluster, genes encoding for type III effector proteins, biosynthetic genes of indole-3-acetic acid (IAA) and cytokinins (CK), multiple and diverse insertion sequences, and residual sequences of known genes from various bacteria

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(Barash and Manulis-Sasson 2007; Guo et al. 2002). *P. agglomerans* pv. *gypsophila* incites galls in gypsophila and a hypersensitive reaction (HR) in beet (Cooksey 1986; Ezra et al. 2000) whereas *P. agglomerans* pv. *betae* incites galls in both beet and gypsophila (Burr et al. 1991). Curing the pPATH_{pag} or pPATH_{pab} rendered the respective pathovar non-pathogenic (Weinthal et al. 2007), indicating that pathogenicity is entirely dependent on the presence of the plasmid.

Some strains of *E. herbicola*, which was merged into *P. agglomerans* (Gavini et al. 1989), have been previously reported to synthesize acyl-HSL (Cha et al. 1998). However, the nature of the signals, the genes responsible for QS regulation, and their involvement in pathogenicity has not previously been characterized. The present study was undertaken to elucidate the QS regulatory system in *P. agglomerans* pvs. *gypsophila* and *betae* and to determine whether it can control gall formation by *P. agglomerans* pv. *gypsophila*.

RESULTS

Detection and characterization of acyl-HSL compounds produced by *P. agglomerans*.

Initial indications that pathogenic (6-2, 420, 300, 350-1, 615, 102, 3-1a, and PD713) and nonpathogenic (1-10, 135, 163-6, 24-3, and 3-1) strains of *P. agglomerans* (Table 1) produced acyl-HSL compounds were obtained by using the reporter strain *Chromobacterium violaceum* CV026. Further characterization of the acyl-HSL compounds was achieved by using the luminescence reporters pSB536, pSB401, and pSB1075 in *Escherichia coli* JM109, whose cognate signals are *N*-butanoyl-L-homoserine lactone (C₄-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C₆-HSL), and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C₁₂-HSL), respectively (Winson et al. 1998). Culture filtrates of the wild-type pathogenic strains Pag824-1, Pag420, and Pab4188 and the nonpatho-

Table 1. Bacterial strains, fosmids, and plasmids used in this study

Strains, fosmids, plasmids ^a	Relevant characteristics ^b	Reference or source
Strains		
<i>Escherichia coli</i>		
DH5α	<i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>gyrA</i> 96	Invitrogen, San Diego, CA, U.S.A.
JM 109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1</i> Δ(<i>lac-proAB</i>) <i>mcrA</i> [F' <i>traD36 proAB lacI^q lacZ</i> ΔM15], host for pSB536, pSB401, and pSB1075	Winson et al. 1998
S17-1/λpir	RK2 <i>tra</i> regulon, <i>pir</i> , host for <i>pir</i> -dependent plasmid (pVIK165)	Simon et al. 1983
EPI 300	[F' <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) <i>ebdA1 araD139</i>], host for fosmid vector	Episcentre, Madison, WI, U.S.A.
<i>Pa</i> strains 1-10, 135, 163-6, 24-3, 3-1	Nonpathogenic strains isolated from gypsophila and other sites	Brandl et al. 1996 Manulis et al. 1991
<i>Pag</i> strains 6-2, 420, 300, 350-1, 615, 102, 3-1a, PD713	Pathogenic strains on gypsophila	Manulis et al. 1991
Pag824-1	Pathogenic strain with spontaneous Rif ^r , gypsophila pathovar	Manulis et al. 1991
Pab4188	Pathogenic strain with spontaneous Rif ^r , beet pathovar	Burr et al. 1991
PagMx1	Rif ^r , Spec ^r , Pag824-1 marker exchange mutant on <i>pagI</i> (AHL ⁻)	This study
PagMxR	Rif ^r , Km ^r , Pag824-1 marker exchange mutant on <i>pagR</i>	This study
PagMxΔIR	Rif ^r , Km ^r , Pag824-1 marker exchange with double deletion in <i>pagI</i> and <i>pagR</i> .	This study
Pag824-1/pVSP4.3	Rif ^r , Km ^r , pathogenic strain carried in <i>trans</i> pVSP4.3	This study
PagMx1/pVSP4.3	Rif ^r , Km ^r , Spec ^r , marker-exchange mutant on <i>pagI</i> carried in <i>trans</i> pVSP4.3	This study
<i>Chromobacterium violaceum</i> CV026	Km ^r , mini Tn5 ⁺ <i>cvlI</i> :: Tn5xylE	McClean et al. 1997
Fosmids		
pCC1FOS	Cm ^r , vector for fosmid library, <i>oviV cos</i> site P1 <i>loxP</i>	Episcentre
pF48/6	Cm ^r , a 30-kb fosmid clone of Pag824-1 containing the QS regulatory genes	This study
Fly4188-735	Cm ^r , a 30-kb fosmid clone of Pab4188 containing the QS regulatory genes	This study
Plasmids		
pVSP61	Km ^r , pVS1 replicon	Loper and Lindow 1994
pBluescript-II KS+	Amp ^r , cloning vector, ColE1 ori	Stratagene, La Jolla, CA, U.S.A.
pRK2013	Km ^r , helper plasmid for triparental matings	Ditta et al. 1980
pLAFR3	Tet ^r , broad-host-range vector <i>IncP-1 rixRK2+ lacZ Tra⁻ Mob⁺ cos</i>	Staskawicz et al. 1987
pGEM	Amp ^r , polymerase chain reaction product cloning vector	Promega
pSB536	Amp ^r , <i>ahyRI</i> :: <i>luxCDABE</i> , reporter plasmid for C ₄ -HSL	Winson et al. 1998
pSB401	Tet ^r , <i>luxRI</i> :: <i>luxCDABE</i> , reporter plasmid for (C ₆ -C ₁₂)-HSLs	Winson et al. 1998
pSB1075	Amp ^r , <i>lasRI</i> :: <i>luxCDABE</i> , reporter plasmid for (C ₈ -C ₁₂)-HSLs	Winson et al. 1998
pVIK165	Km ^r , suicide vector for site-directed mutagenesis	Kalogeraki and Winans 1997
pKS4.3	Amp ^r , a 4.3-kb <i>HindIII</i> fragment containing <i>pagI</i> (under <i>lac</i> promoter) and <i>pagR</i> cloned into pKS ⁺	This study
pVSP4.3	Km ^r , a 4.3-kb <i>HindIII</i> fragment containing <i>pagI</i> (under <i>lac</i> promoter) and <i>pagR</i> cloned into pVSP61	This study
pKS1.85	Amp ^r , a 1.85-kb <i>EcoRI</i> fragment containing <i>pagI</i> (under <i>lac</i> promoter) and <i>pagR</i> cloned into pKS ⁺	This study
pLAFR4.3	Tet ^r , a 4.3-kb <i>HindIII</i> fragment containing <i>pagI</i> and <i>pagR</i> cloned into pLAFR3	This study
pLAFR1.4	Tet ^r , a 1.4-kb <i>PstI</i> fragment containing <i>pagR</i> cloned into pLAFR3	This study
pLPAGI	Tet ^r , Spec ^r , a 2.9-kb <i>HindIII</i> fragment of <i>pagI</i> containing the Spec resistance cassette as an <i>BamHI</i> fragment cloned into pLAFR3	This study
pVIK-R5	Km ^r , pVIK165 carrying internal fragment of <i>pagR</i>	This study
pGEM-I/RB	Amp ^r , Km ^r , pGEM carrying truncated <i>pagI/pagR</i> with insertion of Km resistance cassette as <i>BamHI</i> fragment	This study
pKS4188-7	Amp ^r , a 5-kb <i>BamHI</i> fragment containing <i>pabI</i> and <i>pagR</i> cloned into pKS ⁺	This study

^a *Pa* = *Pantoea agglomerans*, *Pag* = *P. agglomerans* pv. *gypsophila*, and *Pab* = *P. agglomerans* pv. *betae*.

^b Rif^r, Spec^r, Km^r, Cm^r, Amp^r, and Tet^r = resistant to rifampicin, spectinomycin, kanamycin, chloramphenicol, ampicillin, and tetracycline, respectively; AHL = acyl-homoserine lactone QS = quorum sensing; C₄-HSL = *N*-butanoyl-L-homoserine lactone; and C₆-HSL = *N*-hexanoyl-L-homoserine lactone.

genic strain *Pa3-1* showed strong luminescence responses with pSB536, weak responses with plasmid pSB401, and no luminescence with pSB1075 (Fig. 1). Measurements made with the microtiter plate reader indicated that the relative luminescence intensity with pSB401 was between 5 and 15% of that produced by pSB536. Thin-layer chromatography (TLC) of dichloromethane extracts from culture supernatants suggested that *Pag824-1* produced C_4 -HSL as a major compound and N -hexanoyl-D-homoserine lactone (C_6 -HSL) as a minor signal (results not shown). The active substances from the culture supernatant of *Pag824-1* were purified from the dichloromethane extract with a C18 preparative reverse-phase chromatography column (described below). Fractions that showed luminescence with the bioreporters pSB536 or pSB401 were analyzed by mass spectrometry. The electron spray ion (ESI) mass spectrum of the semipurified sample detected with pSB536 gave a strong molecular ion peak at 172.0917 m/z $[M+H]^+$, which is characteristic of C_4 -HSL (Fig. 2A). The ESI mass spectrum of the semipurified sample detected with pSB401 yielded a strong molecular ion peak at 200.1243 m/z $[M+H]^+$, which is characteristic of C_6 -HSL (Fig. 2B).

Isolation and characterization of the genes involved in QS regulation.

A fosmid library constructed from genomic DNA of *Pag824-1* was screened for acyl-HSL production with the reporter *C. violaceum* CV026. Clone pF48/6 (Fig. 3), which responded positively and was confirmed to have C_4 -HSL present in its culture supernatant with *E. coli* JM109 pSB536, was selected for further work. This clone, which contains an insert of approximately 30 kb, was digested with *Hind*III and subcloned into pBluescript-II KS+ to yield pKS4.3. Further subcloning of pKS1.85 was obtained by digesting pKS4.3 with *Eco*RI. DNA sequence analysis of pKS1.85 revealed two open reading frames (ORF) (Fig. 3). The first ORF, with ATG as a start codon, consisted of a 614-bp region with a putative protein of 204 amino acids (aa) and molecular mass of 23.8 kDa. The second ORF, with GTG as a start codon, spanned a 692-bp region that encoded a predicted protein of 230 aa and mo-

lecular mass of 26.9 kDa. The two ORF were convergently transcribed and separated by 14 bp (Fig. 3). The ORF sequences were subjected to BLAST analysis, which indicated that the first ORF was homologous to *luxI*, which encodes an acyl-HSL synthase, whereas the second ORF was a *luxR* homolog, which acts as a transcription regulator in QS systems. Consequently, the two ORF were designated as *pagI* (acyl-HSL induction gene of *P. agglomerans* pv. *gypsophilae*) and *pagR* (regulator gene of *P. agglomerans* pv. *gypsophilae*). The complete pKS4.3 subclone was sequenced and a 2.1-kb *Eco*RI-*Hind*III fragment, harboring pKS1.85 plus a 0.25-kb *Eco*RI-*Hind*III region upstream of the ORF of *pagI*, was submitted to the GenBank as accession number EF494651. The promoter regions of *pagI* and *pagR* contain a 20-nucleotide imperfect inverted repeat [CACT-N12-AGAA] that might represent a potential *lux* box motif. The underlined letters indicate a minimal consensus of the *lux* box (Whiteley and Greenberg 2001). The two putative *lux* boxes were centered at 47 and 15 bp from the transcription starts of *pagI* and *pagR*, respectively.

Fig. 1. Detection of acyl-HSLs production by *Escherichia coli* lux reporters. Acyl-homoserine lactones of the pathogenic strains *Pag824-1*, *Pab4188*, and *Pag420* and of the nonpathogenic strain *Pa3-1* were extracted from culture supernatants and assayed by *E. coli* reporters. *E. coli* JM109 (80 μ l) harboring the plasmid reporters pSB536, pSB401, or pSB1075 were added to black/clear 96-well microtiter plates containing dried culture extracts (10 μ l). Luminescence was captured on Kodak X film.

Fig. 2. A, Electron spray ion (ESI) mass spectrum of N -butanoyl-L-homoserine lactone (C_4 -HSL) gave a molecular ion peak at 172.0917 m/z $[M+H]^+$, achieving a mass accuracy of -29.7 ppm. B, ESI mass spectrum of N -hexanoyl-L-homoserine lactone (C_6 -HSL) gave a molecular ion peak at 200.1243 m/z $[M+H]^+$, achieving a mass accuracy of -19.0 ppm.

The homologs of *pagI* and *pagR* of the beet pathovar, namely *pabI* and *pabR*, were isolated from the fosmid library of *Pab4188* as follows. The fosmid clone Fly4188-735, which activated the *C. violaceum* CV026 biosensor, was digested with *Bam*HI and cloned into pBluescript II (KS⁺). The resulting subclones were screened for acyl-HSL production with *C. violaceum* CV026 reporter. A representative subclone, designated pKS4188-7 and containing the homologs of *pagI* and *pagR*, was used for isolation of these genes. The final sequence of the genes was determined after polymerase chain reaction (PCR) with primers PabIFwd and PabIRev for *pabI* and PabRFwd and PabRRev for *pabR*, which were based on a cor-

responding *P. agglomerans* pv. *gypsophillae* sequence (Table 2). The sequence of these two genes, including their promoter region, was submitted to the GenBank as accession number EU314940. Sequence comparison showed 99% identity to the QS genes of *Pag824-1* and showed identical genomic structure.

Analysis of the deduced PagI protein revealed the auto-inducer synthase domain (11 to 171 aa) and showed that this protein exhibited highest identity with SmaI of *Serratia marcescens* (41%) and significant identity with other LuxI proteins (Fig. 4A). All 10 amino acid residues conserved throughout the LuxI family (Arg 27, Phe 31, Trp 37, Glu 46, Asp 48, Asp 51, Arg 71, Phe 85, Glu 103, and Arg 106) (Parsek et al. 1997)

Fig. 3. Physical map of the fosmid clone pF48/6 and its derivatives containing the *pagR/pagI* locus. A 4.3-kb *Hind*III fragment from pF48/6 was subcloned into pBluescript II (KS⁺) to yield pKS-4.3, and a 1.85-kb *Eco*RI DNA fragment from pKS4.3 was further subcloned to obtain pKS-1.85. The locations of *pagR* and *pagI* genes are indicated by open arrows. The approximate insertion sites for generating the marker exchange mutants *PagMxR* (kanamycin resistant) and *PagMxI* (spectinomycin and streptomycin resistant) are marked as MxR and MxI, respectively. The 1-kb *Bam*HI deletion fragment for obtaining the double mutant *PagMxΔIR* is marked by ΔIR. The procedures for obtaining the mutants are described in Materials and Methods. The *Bam*HI sites were artificially introduced to pKS1.85. Relevant restriction sites are shown as H = *Hind*III and E = *Eco*RI.

Table 2. Relevant primers used in this study

Name	Sequence (5'-3')	Used for ^a
ProI-Hind5	CAACAGGGAAAGCTTGCCCC	Site-directed mutagenesis of <i>pagI</i>
IBa3	GAAATGGATCCTAATCACGC	...
IBa5	GCGTGATTAGGATCCATTTC	...
R-ProHind5	CCTTCAAAGCTTGCGCCAG	...
ProINde	GAGCCTGCCAGGAACCGTCTCATATGTCTGTC	Site-directed mutagenesis and deletion of <i>pagI/R</i>
PagIBa	CATCGTCGGATCCGTCGAATC	...
RBa3	ATAATACGGATCCGTGAAGGG	...
4kb-3	GCTGGGTCGGTGGAGGATTAAGGG	...
R-Fow SacI	GTAAATCCAGAATGAGCTCAATGC	Site-directed mutagenesis in <i>pagR</i>
R-Rev Xba	GTCCCTAGTTCTAGGCATGTGTG	...
Pag-1	CCTGACCGCTAAGCATGGTGA	<i>PagMxI</i> confirmation
Pag-2	GTGAATAACGCCACACATGC	...
PabRFwd	GCTGTCCCCGAGATTTTGTG	<i>pabR</i> sequencing
PabRRev	GAGATGTCCCGGCATTTACG	...
PabIFwd	CATCAAAAACGTCGGCGGTACTC	<i>pabI</i> sequencing
PabIRev	GCTCCGGCTGTCTGGACAAC	...
RT pthGFow	AGTCCGCTGAGTCGATCAGG	qRT-PCR of <i>pthG</i>
RT pthGRev	GGGATTGAAAAGCTCTGAGCCT	...
hrpLRTFow	GGTACAGATGACCTGGCTGGAA	qRT-PCR of <i>hrpL</i>
hrpLRTRev	CCAAAAACCCACGTCTCTGG	...
gyrBRTFow	GGTGAAAGTGCCGGATCCTAA	qRT-PCR of <i>gyrB</i>
gyrBRTRev	TGCTGCTCAACCGCTGTTT	...

^a qRT-PCR = quantitative reverse-transcriptase polymerase chain reaction.

were identified in PagI (Fig. 4A). Analysis of the deduced PagR protein exhibited the classic autoinducer-binding domain of the LuxR family (Stevens and Greenberg 1999) spanning a region comprising two-thirds of the N-terminus (11 to 155 aa) (Fig. 4B). The C-terminus contained a conserved helix-turn-helix DNA-binding domain (165 to 222 aa), through which PagR presumably binds to the *lux* box motif. This domain shares greatest identity with SmaR of *Serratia* sp. American Type Culture Collection (ATCC) 39006 (43%), and significant identities with other LuxR proteins (Fig. 4B). PagR contains all the five amino acid residues (Tyr 57, Asp 66, Gly 108, Glu 172, and Gly 182) previously reported to be absolutely conserved among known functional members of the LuxR family (Whitehead et al. 2001). The former and latter results confirm that PagI and PagR belong to the LuxI and LuxR protein families, respectively.

Disruption of *pagI* or *pagR* reduces gall development.

Marker-exchanged mutants in *pagI* (*PagMxI*) and *pagR* (*PagMxR*), and the double mutant *PagMxΔIR* were generated as described below. The resulting *PagMxI* and *PagMxΔIR* did not produce detectable levels of either C₄-HSL or C₆-HSL when tested with *C. violaceum* CV026 or the *E. coli* *lux* reporters. This result indicates that the same homoserine lactone synthase is responsible for biosynthesis of both signals. Acyl-HSL production was restored by complementation with pVSP4.3 (Table 1). *PagMxR* produced detectable amounts of Acyl-HSL, as shown by *C. violaceum* CV026.

The mutants were tested for pathogenicity on gypsophila cuttings, by comparison with the wild type (*Pag824-1*), using an inoculum concentration of 10⁶ cells/ml. Mutations in *pagI* or *pagR* or simultaneous mutations in both genes caused 50 to 55% reduction in gall size (Fig. 5). The colonization of the pathogen was determined at 7 days after inoculation, when initial galls were observed. The population of *Pag824-1* was 3 ± 1.44 × 10⁹ CFU/g compared with a range of 7 ± 1.14 × 10⁷ to 9 ± 1.29 × 10⁷ CFU/g with the three mutants. Thus, disruption of *pagI* or *pagR* was associated with a lower growth rate in planta. The reduced galls caused by *PagMxI* or *PagMxΔIR* could be complemented almost to the size of the wild-type galls with the pLAFR4.3 (Table 1) containing the intact *pagI* and *pagR* in trans. The mutant *PagMxR* was complemented with pLAFR1.4 containing the intact *pagR* in trans (results not shown). These findings suggest that gall development is under QS regulation. It should be pointed out that the adverse effect of these mutants on gall size could be overcome by applying an excess inoculum concentration (e.g., 10⁹ cells/ml).

Gall development is inhibited by higher concentrations of C₄-HSL.

Because production of the acyl-HSL signal is strictly coordinated with bacterial multiplication, it was of interest to determine whether excess QS signal would affect virulence. This hypothesis was examined by two procedures. In the first of these, the coding region of *pagI* and *pagR* was cloned into

pVSP61, in which *pagI* is under *lac* promoter, to generate pVSP4.3. This was then electroporated into the wild-type strain *Pag824-1* and the *PagMxI* mutant to yield the transconjugants *Pag824-1/pVSP4.3* and *PagMxI/pVSP4.3*, respectively. In the second procedure, gypsophila cuttings were inoculated with *Pag824-1* and *PagMxI* in the presence of 0, 5, 10, 25, or 50 μM C₄-HSL. The results indicate that overexpression of *pagI* in the wild-type strain *Pag824-1* or the *PagMxI* mutant reduced gall size by 90 or 70%, respectively (Fig. 6A). Moreover, reduction in gall size could also be achieved by the presence of C₄-HSL in the inoculum of *Pag824-1* and *PagMxI* (Fig. 6A, numbers 5 and 6). When 5 to 50 μM of C₄-HSL was added, the gall size reduction was proportional to the amount added, with approximately 90% inhibition by 50 μM (Fig. 6B).

Effect of mutation in *pagI* on expression *hrpL* and *pthG*.

It has been previously shown that the type III secretion system (T3SS) and T3SS effectors are essential for gall formation (Barash and Manulis-Sasson 2007). Therefore, it was suspected that the reduction in gall size by the QS regulatory system might be mediated by the T3SS and effectors. To confirm this hypothesis, we selected *hrpL* and *pthG* as target genes. *HrpL* is a key component of the Hrp regulon (Nizan-Koren et al. 2003) whereas *PthG* is a T3SS effector, which acts as a virulence factor in gypsophila and as an avirulence factor in beet (Ezra et al. 2000). Results obtained by quantitative reverse-transcriptase (qRT)-PCR described in Figure 7 demonstrate that the transcript levels of *hrpL* and *pthG* in a *PagMxI* background are reduced by 5.8 and 6.6, respectively, compared with that of the wild-type *Pag824-1*.

DISCUSSION

Autoinduction mediated by acyl-HSL signal molecules is a common form of gene regulation among plant-associated gram-negative bacteria (Barnard et al. 2007; von Bodman et al. 2003; Whitehead et al. 2001). In this article, we present genetic and biochemical evidence for the presence of a functional QS regulatory system in *P. agglomerans*, which includes acyl-HSL signals and gene members of the *luxI* and *luxR* families; namely, *pagI* and *pagR*. Moreover, we demonstrate that the QS regulation plays a significant role in virulence of the gall-forming *P. agglomerans*, possibly by affecting the expression of the Hrp regulon. It is noteworthy that the QS system described for *P. agglomerans* pv. *gypsophilae* shows a striking similarity to that reported for *Serratia* sp. ATCC 39006. Both bacteria produce C₄-HSL as major and C₆-HSL as minor QS signals, the deduced PagI and PagR protein exhibits highest identity to SmaI and SmaR of *Serratia* sp. ATCC 39006 (Fig. 4), and the homologous genes are found in the adjacent genome and convergently transcribed (Thomson et al. 2000). In contrast, the QS system of the taxonomically related *P. stewartii* subsp. *stewartii* might be more evolutionarily distant in terms of the cognate QS signal (i.e., 3-oxo-C₆-HSL) and identities of the proteins involved in QS regulation (Fig. 4); and, although the

Fig. 4. Alignment of the deduced protein sequence of *pagI* and *pagR* with selected members of LuxI and LuxR. **A**, PagI showed amino acid identity to SmaI (41%) from *Serratia* sp. ATCC 39006 (accession number CAB92553.1), SwrI (42%) from *Serratia liquefaciens* (accession number AAO38762.1), ExpI (37%) from *Erwinia carotovora* subsp. *atroseptica* (accession number YP_048233.1), CarI (38%) from *Pectobacterium carotovorum* (CAA52352.1), YenI (41%) from *Yersinia enterocolitica* (accession number CAA53693.1), EsaI (38%) from *Pantoea stewartii* subsp. *stewartii* P54656), and AhII (30%) from *Pseudomonas syringae* pv. *syringae* (accession number CAD98865.1). **B**, PagI showed amino acid identity to SmaR (40%) from *Serratia* sp. ATCC 39006 (accession number CAB92554.1), SwrR (40%) from *S. liquefaciens* (accession number AAO38761.1), ExpR (42%) from *Dickeya dadantii* (accession number ABV57378.1), YenR (42%) from *Y. enterocolitica* (accession number CAA53694.1), EsaR (37%) from *Pantoea stewartii* subsp. *stewartii* (accession number P54293), CarR (34%) from *Pectobacterium carotovorum* subsp. *carotovorum* (accession number AAC38403.1), and AhIR (44%) from *E. chrysanthemi* (accession number AF448800_2). The alignment was generated by using Clustalx and Gendoc programs. Black shading indicates conserved residues. Numbers on the right-hand side indicate position of the amino acid residues.

A

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      *           20           *           40           *           60
PagI : MNSAIEGELRDYDEFFPELATETLYRLRRTTERERLDWKVESCIDGMEKQDFDNENTTYLLG : 60
SmaI : ---MIEEDVDYNNLLPDNRSKELFSLRKKTEKDRLDWLVNCCENNMEDEYDNRHATYIFG : 57
SwrI : ---MIEEDVDYNNLLPDNRSKELFSLRKKTEKDRLDWLVNCCENNMEDEYDNRHATYIFG : 57
ExpI : ---MLEEDVDNHTLLSETKSEELFTLRKETEKDRLNWAVQCTDGMERDQYDNNTTYLLG : 57
CarI : ---MLEEDVDNHTLLSETKSEELFTLRKETEKDRLNWAVQCTDGMERDQYDNNTTYLLG : 57
YenI : ---MLKIDNVNFNMMPERKLDEHSLREITEKDRLDWKVTCIDGKESDQYDNDENTTYLLG : 57
EsaI : ---MLEEDVSYEELQTTRESEELYKLKKTESDRLGWEVICSQGMESDEFDGPGTRVILG : 57
AhlI : MSSGFEPQVASYSKVPVTTLETTYALRKKIESDRLEKRVRSQAFEDDYSAANNTYLLG : 60

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      *           80           *           100          *           120
PagI : MHNGKLLCGAREFDSTQPMMS-EIEHEYFEGISGLPTDIPCCISRLFLDKDRRDSAGL : 119
SmaI : TYQNHVICSLREFIETKYPNMISDGVNNYFNDIK--LPDGNVVEASRLFDKARIQALQL : 115
SwrI : TYQNHVICSLREFIETKYPNMISDGVNDFYFNDIK--LPDGNVVEASRLFDKARIQALQL : 115
ExpI : IKDNTVICSLREFIETKYPNMIT-GTEFFYFKEIN--IPDGNVLESSRFEVDKSRADILG : 114
CarI : IKN-TVICSLREFIETKYPNMIT-GTEFFYFKEIN--IPEGNYLESSRFEVDKSRADILG : 113
YenI : TIDDTIVCSVREFIDMKYPMIT-GTFAPYFSDVS--LPIDGFISSRFEVEKALARDMVG : 114
EsaI : ICEGQLVCSVRETSLDRNMIT-HTFQHCPSDVT--LPAYG-TESSRFEVDKARARALLG : 113
AhlI : SWNGVPLAGLRLLINTCDPYMLD-GPPRSFFDYPA--PRNAGMAESSRFEVTERARSLGI : 117

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      *           140          *           160          *           180
PagI : HGLPASKVLFLAMIDYCVKRNRYGIYAVAS-----FGMKIEVIQKGLSEKGEVIYY : 171
SmaI : HQAPISAMFLSLMINVARNCGYEGIYATISHPMRIIFORSGWHISVVKTCSEKKNKIYL : 175
SwrI : HQAPISAMFLSLMINVARNCGYEGIYATISHPMRIIFORSGWHISVVKTCSEKKNKIYL : 175
ExpI : NEYPISAMFLSLMINVSRDKGYDGIYITVSHFMLTILKRSWGIGISVVEQGLSEKPEKVYL : 174
CarI : NEYPISAMFLSLMINVSKDKGYDGIYITVSHFMLTILKRSWGIGIRVVEQGLSEKPEKVYL : 173
YenI : NNSLSLTILFLAMVNNYARDRGHGILTVVSRGMFILLKRSGWNIIVLNQGESERKEVIYL : 174
EsaI : EHYPISCVLFLAMVNWQNNAYGNIYITVSRAMLKILTRSGWQIKVKEAFLTEKBRILY : 173
AhlI : LHAPLTEMLLFSMHNHAASAGLESIIIVSKAMARIVRKSQWEHRVLTATGEASPGCTVLL : 177

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      *           200          *           220
PagI : IFMPASMSIVEDIIITRDKASGWLREMSRHLRHL----- : 204
SmaI : IYMPIDDANRRRLARINQHATKWDNPLDSWPLSFRICEKRPDELQLHT : 224
SwrI : IYMPIDDANRRRLARINQHATKMG----- : 200
ExpI : VFLEPVDDENQEAALARRINRSGTFMSNELKQWPLRLPAIAQA----- : 216
CarI : VFLEPVDDENQEAALARRINRSGTFMSNELKQWPLRVPAAIAQA----- : 215
YenI : LHLGIDNDSQQQLINKILRVHQVEPKTLETWPIIVPGIIK----- : 214
EsaI : LTLPAQDDKQQLGGDVVSRGTGCEPFAVTTWPLTLFV----- : 210
AhlI : LDFVNADNHQRLIGSIALRQPVTDLLRWPIPLDASASLQRARMSAA- : 225

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B

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      *           20           *           40           *           60
PagR : MKDITYNDTRINLLIQNELNAFFEDYKGIIMAYAIMNKKDPSQMRIINN-PEWFDIYLE : 59
SmaR : MSNFFNNTSINISIKNYLEKNLKVFNNIKYAYAIMNKKNPENDEFAIISNR-MEWFDEYTK : 59
SwrR : MSNFFFNNETINASKEELERGLSKYNNIKYAYAIMNKRNPASFISIISNR-TWFFEFYIK : 59
ExpR : MSVSFSNVDFINSTIQNYLNRLKLSYGDLYAYLIMNKKKPTDVVITISNYPSEWVEIYRS : 60
YenR : MIDYFDNESINEDIKNYIQRRIKTYGDLCYSYLVNMKKTPHPTIISNYPLDWVKYKK : 60
EsaR : MFSEFLENQTTITDTLQTYIQKRLSPLGSPDMAYTVVSKKNPSNVLIISYSPDEWIRLYRA : 60
CarR : -----MDHEHSFIKRLKGVGDVWFSYFMMSKTSTSHPYIISNYPEAMNEYLK : 50
AhlR : --MKINGAPAPLRQLIEDFENDLHHIGDFTYAFSTPKTRNVKPVILSNYPDSWLKSYVA : 58

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      *           80           *           100          *           120
PagR : RKYQFIDPVLIQSLRCVEDFEWENNVLSSGGYNLTRIFNESFQYNTFQGTFFLHDYINN : 119
SmaR : NNLQFIDPVLITASCCTFPLWDENIMISSGLKMPKIFNMAKNYDVINGYTEVLHDHNNH : 119
SwrR : NNYQFIDPVLITASHRITPSTWQKDLIGAGLKLPKIFMAKNYNTINGYTEVLHDHNNH : 119
ExpR : NNYQHIDPVLITAINKISPSWDDNLVSSKLKFSRIFNLKSEYDIVNGYTEVLHDHNNH : 120
YenR : NSYHLIDPVLITAKDKVAPSAWDDNSVINKKSTDSAVFKLAREYNTVNGYTEVLHDNSNN : 120
EsaR : NNFQLTDPVILTAFKRTSPAWDENITLMSDLRFTKIFSLSKQYNTVNGYTEVLHDHNNH : 120
CarR : KEMFLSDIIVASLARIPTBSWDDNDLVTLRAKNQDVFISSVQHDISSGYTEVLHDHNNH : 110
AhlR : SNYHLIDIITKHAWHSITPFWREARNVQGVETDDFLKRSQYQLSSGATEVLHDASGL : 118

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      *           140          *           160          *           180
PagR : LATPSIISQKDSGIDMEK----NRSKIVSFLIKLHQKTLNLY-----SQHHQKKNVF : 167
SmaR : LVVPSIIMDKSCDDDEIKIIVDKKNDLQMLLTTHEKLIITLYQEIN-DTHQFNKKNQKEI : 178
SwrR : LVVPSIMLDKHCDAADVEQQIDNNKAEIQMLLITMHGKMTTLYQEMS-TPADFEKMNQREF : 178
ExpR : LATPSFMFEENRSGELEEVHNNKEKLQMLLISAHEKLTSLYREMS-KNKNNSKSEQEFL : 179
YenR : MATPNISNGSDDSISEFDERIEINKEIQMLLIITHEKMLGLYQNSDKNENRNTQIERDI : 180
EsaR : LATPSVIKGNQTALEQRLAAEQGTQMQLLIDFNEQMYRLAGTEGERAPALNQSADKTI : 180
CarR : VATPSIAN-HLEDADFEKCMKSHENDLQMLLVNVHEKVMAYQRAIK-DQAKPPDSSRNAL : 168
AhlR : FAATPSLCNARQQN-DFDQRIREKAADIQMSLIRFHDRLIKTR----APHELFPPQAQCK : 172

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      *           200          *           220          *           240
PagR : LSPRERCILKWVGAGKTYNEVAVILSIAERTVVKFLGNVMMKLGVNNAHAVKLGTELRL : 227
SmaR : LSKRENEIYWASMGKSYOEIALILGILKLTIVKYHVGNAVKKLGVTNAKHAIKRLGVELKL : 238
SwrR : FSKRENEIYWASLGKSYOEIALILGILKLTIVKYHIGNAVKKLGVTNAKHAIKRLGVELKL : 238
ExpR : FSKRENEIYWASMGKTYOEIALILGILKLTIVKFIHGNVVRKLGVLNAKHAIKRLGVELKL : 239
YenR : FSPRENEIYWASVGKTYNEISILGILKRSIVKFIHGNVVRKLGVLNAKHAIKRLGVELKL : 240
EsaR : FSSRENEVLYWASMGKTYNEIAATGHSVSTVKFIKNNVVKLGVSNAKQAIKRLGVELDL : 240
CarR : LSPRETEVLFLVSSGRITYKEVSRILGISEVTVKFIHNNSVRKLDVINSRHAITKALELNL : 228
AhlR : LSPRETEVLKWVAMGKSYSEIAETFSISERTVVKFHMNNVSSKLDVRTAKQAVYKATNIGM : 232

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      *
PagR : LDN----- : 230
SmaR : IRPILPDAE----- : 247
SwrR : IRPLLADTEG----- : 248
ExpR : IKPVEPVKARS----- : 250
YenR : IKPI----- : 244
EsaR : IRPAASAAR----- : 249
CarR : FNSPCEPVVMKHESR : 244
AhlR : V----- : 233

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lux homologs are convergently transcribed, they overlap with each other (von Bodman and Farrand 1995).

The presence of the QS regulatory genes in pathogenic and nonpathogenic strains of *P. agglomerans*, which lack the

Fig. 5. Effect of gene disruption on gall size. Gypsophila cuttings were inoculated with 10^6 cells/ml of lane 1, *Pag824-1* (wild type); lane 2, *PagMxI*; lane 3, *PagMxR*; lane 4, *PagMxΔIR*; and lane 5, water control. Symptoms were observed at 18 days after inoculation and severity of disease was expressed as gall size. Mean gall size was obtained by measuring the fresh weight of galls from five cuttings in each treatment. The weight of *Pag824-1*-induced gall was 200 mg and a reduction of 50 to 55% was measured with each of the mutants. The experiment was repeated three times and significant difference in gall size between the wild type and the three mutants was determined by means of a χ^2 test (at $P \leq 0.05$).

pPATH_{pag} (Barash and Manulis-Sasson 2007), suggests that they do not reside on the pathogenicity plasmid and might be chromosomal. This assumption was further confirmed by hybridizing the genomic and plasmid DNA of pathogenic and nonpathogenic *P. agglomerans* strains with probes containing *pagI* and *pagR* compared with *hrpJ*. Whereas the probe containing *pagI-pagR* hybridized the genomic DNA of all the strains but not with the plasmid DNA, *hrpJ*, which resides on the pPATH (Manulis and Barash 2003), hybridized only with the plasmid DNA of pathogenic strains (results not shown). It has been claimed that bacteria have acquired *luxI-luxR* genes through horizontal gene transfer (Lerat and Moran 2004). For example, the *smal* and *smar* genes of *Serratia* sp. ATCC 39006 are flanked by insertion elements and remnants of genes that encode transposases (Thomson et al. 2000), suggesting an occurrence of a late integration into the chromosome. Although we could not detect any mobile elements in the vicinity of the *pagI-pagR* locus, we found that the (G + C) contents of *pagI* and *pagR* were 44.22 and 39.83%, respectively, compared with 55 and 56.8%, respectively, in *P. agglomerans* (Gavini et al. 1989). This observation could support the hypothesis of integration of the *pagI-pagR* locus into the chromosome of *P. agglomerans* as a genomic island (Hacker and Kaper 2000), and might explain the absence of QS regulation in some *P. agglomerans* strains (Cha et al. 1998), in which such a transfer presumably did not occur.

Plant-pathogenic bacteria use QS signals to regulate major pathogenicity factors, as exemplified by EPS in *P. stewartii*

Fig. 6. Overexpression of *pagI* or additions of *N*-butanoyl-L-homoserine lactone (C_4 -HSL) reduce gall development. **A**, Gypsophila cuttings were inoculated with 10^6 cells/ml and observed for gall development at 18 days after inoculation and severity of disease was expressed as gall size. Inoculations were performed with lane 1, *Pag824-1*; lane 2, *PagMxI*; lane 3, *Pag824-1/pVS1*; and lane 4, *PagMxI/pVS1* or addition of synthetic C_4 -HSL (50 μ M) during inoculation with lane 5, *Pag824-1* and lane 6, *PagMxI*. The experiments were repeated three times with five cuttings for each treatment. The mean gall size of treatments 1 to 6 in mg fresh weight were 230 (a), 110 (b), 20 (d), 60 (c), 16 (d), and 50 (c). Statistically significant differences (Student's t at $P < 0.05$) were indicated by letters in parentheses. **B**, Galls induced by *Pag824-1* following inoculation of gypsophila cuttings in the presence of various C_4 -HSL concentrations (0, 5, 10, 25, and 50 μ M). Gypsophila cuttings were inoculated with 10^6 cells/ml of *Pag824-1*. The mean gall size of C_4 -HSL treatments (0 to 50 μ M) in milligrams fresh weight were 250 (a), 200 (a), 130 (b), 75 (c), and 32 (d). Statistically significant differences (Student's t at $P < 0.05$) were indicated by letters in parentheses.

subsp. *stewartii*, *Xanthomonas campestris*, and *Ralstonia solanacearum*, T3SSs in *P. stewartii* subsp. *stewartii* and *Erwinia carotovora*, and exoenzyme production in *E. carotovora*, *X. campestris*, and *R. solanacearum* (Barnard et al. 2007; von Bodman et al. 2003). The symptoms controlled by the QS systems in these bacterial pathogens include leaf blight, wilt, and soft rots. In addition, the QS system controls HR and necrosis symptoms during *Agrobacterium vitis*–plant interactions (Hao and Burr 2006). However, a direct involvement of QS regulation in gall development has not been previously reported. In the crown gall pathogen, *A. tumefaciens*, both acyl-HSL and a subclass of opines, the conjugal opines, act simultaneously to induce the transfer of the resident Ti plasmid to plasmidless agrobacteria, often cohabiting the gall (Farrand 1998). Although the latter process might increase the proportion of pathogenic bacteria within the gall's population and indirectly affect the frequency of gall initiation, it does not modulate gall development as observed in *P. agglomerans* pv. *gypsophylae*.

Gall initiation and development by *P. agglomerans* pv. *gypsophylae* is primarily dependent on the presence of a functional *hrp/hrc* gene cluster harboring the T3SS system and T3SS effectors (Barash and Manulis-Sasson 2007). However, although inactivation of the pathways leading to biosynthesis of IAA and cytokinins in *P. agglomerans* pv. *gypsophylae* cannot prevent gall initiation, a reduction in gall size by up to 50 to 55% could be observed (Manulis et al. 1998). Therefore, the reduction of 50 to 55% in gall size caused by disrupting the QS system via mutations in *pagI* or *pagR* (Fig. 5) may be analyzed in light of two hypotheses: i) that the QS regulatory system controls the gall size by affecting the secretion of CK or IAA by *P. agglomerans* pv. *gypsophylae* and ii) that the effect of QS on gall formation is mediated by the Hrp regulon. Although the first hypothesis cannot yet be excluded, the finding that disruption of *PagI* significantly reduced expression of *hrpL* and *pthG* by approximately sixfold (Fig. 7) provides a strong support for the second hypothesis. The *hrp* regulatory cascade of *Pag824-1* consists of an *hrpXY* operon encoding a two-component system: *hrpS* encoding a transcriptional factor of the NtrC family and *hrpL* encoding an alternative sigma factor that activates “*hrp* box” promoters (Nizan-Koren et al. 2003). An *hrp* promoter is characteristic of the genes of the T3SS system and genes encoding for T3SS effectors (Alfano and Collmer 2004), which emphasizes the key function of HrpL in the Hrp regulon. PthG is a T3SS effector that exhibits a dual function: it encodes a protein that functions as both a virulence factor in *gypsophila* and an avirulence factor in multiple beet species (Ezra et al. 2004). It acts as a main factor in the divergent evolution of *P. agglomerans* into two pathovars, *P. agglomerans* pvs. *gypsophylae* and *betae* (Barash and Manulis-Sasson 2007). The information on the effect of QS on *hrp/hrc* genes is quite scanty. It has been previously shown that expression of HrpL in *E. carotovora* subsp. *carotovora* is controlled by the post-transcriptional regulator RsmA, which in turn is regulated by the QS regulatory system (Chatterjee et al. 2002). According to this model, RsmA suppresses expression of HrpL when activated by the QS transcription activators ExpR1 and ExpR2 of *E. carotovora* subsp. *carotovora* (Mole et al. 2007). The RsmA-rsmB and RsmC system has also been reported to regulate *hrpN* of *E. carotovora* subsp. *carotovora* (Cui et al. 1999). Whether the QS regulatory system of *P. agglomerans* pv. *gypsophylae* is able to activate the Hrp regulon directly or indirectly via the RsmA-rsmB system remains to be elucidated. An effect of mutation in *esal* (the *pagI* homolog of *P. stewartii* subsp. *stewartii*) on reduction of *hrp/hrc* gene expression has been previously reported as unpublished results by von Bodman and associates (2003). It is noteworthy that the significant reduction in the pathogen's population observed in *pagI* mu-

tant might arise from the suppression of the *hrp/hrc* gene cluster, which is required for growth in planta (Alfano and Collmer 2004; Lindgren 1997).

An interesting result of the present work is the pronounced effect of exogenous C₄-HSL on gall size. Thus, addition of 50 μ M C₄-HSL during inoculation of *gypsophila* cuttings by *P. agglomerans* pv. *gypsophylae* could reduce gall size up to 90% compared with 50 to 55% by *pagI* or *pagR* mutants (Fig. 6A). Moreover, the degree of reduction appeared to be proportional to the concentration of the added signal (Fig. 6B). The effect of exogenous acyl-HSL on reduction of virulence has been previously demonstrated by employing transgenic tobacco plants carrying acyl-HSL synthase genes from *E. carotovora* subsp. *carotovora* or *Pseudomonas syringae* pv. *tabaci* (Fray 2002; Mae et al. 2001; Quinones et al. 2005) or addition of exogenous cognate signal to wild-type tobacco inoculated with *E. carotovora* subsp. *carotovora* (Mae et al. 2001). QS regulation is designed to coordinate expression of virulence factors with bacterial population, allowing the pathogen to evade host defenses and proliferate to a critical level where the coordinated production of virulence determinants may overwhelm the host (Swift et al. 1996). Consequently, it was proposed that addition of exogenous QS signal results in a premature production of virulence factors that elicits host defenses prior to reaching the critical bacterial population required for infection (Mae et al. 2001; Quinone et al. 2005). However, the explanation for the effect of exogenous acyl-HSL could be more complex because potato plants genetically modified to produce N-acyl-HSL increased susceptibility to soft rot *erwiniae* (Toth et al. 2004). In contrast to the necrotrophic *E. carotovora* subsp. *carotovora*, *Pantoea agglomerans* pv. *gypsophylae* is a hemibiotrophic bacterium whose pathogenicity is totally dependent on a functional *hrp/hrc* gene cluster and T3SS effectors (Barash and Manulis-Sasson 2007). Although induction of basal defenses in *gypsophila* by *P. agglomerans* pv. *gypsophylae*'s infection has been previously claimed (Chalupowicz et al.

Fig. 7. Effect of mutation in *pagI* on transcript levels of *hrpL* and *pthG* as determined by quantitative reverse-transcriptase polymerase chain reaction. cDNAs samples were generated from total RNA extracts of *Pag824-1* and *PagMxI* grown to early exponential phase on minimal A medium. The Target transcripts were normalized using *gyrB* (DNA gyrase subunit B) as endogenous reference and the wild-type strain *Pag824-1* was used as the control. Fold changes in target genes were determined by the $2^{-\Delta\Delta CT}$ method. Experiments were repeated three times and error bars represent the standard deviation.

2006), it might be questioned whether they can account for 90% reduction in gall size. A possible explanation is that the presence of the cognate signal in an excessive concentration causes PagR to reduce virulence via the *hrp/hrc* regulatory system in an as yet unknown manner.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and pathogenicity test.

The bacterial strains employed in this study are presented in Table 1. The *P. agglomerans* and *C. violaceum* strains were generally grown in Luria-Bertani (LB) broth or agar at 28°C, whereas the *Escherichia coli* strains were grown on the same medium but at 37°C. Tryptic soy agar (TSA) (Difco Laboratories, Detroit) was used to culture *C. violaceum* CV026 reporter for acyl-HSL detection. Minimal A medium was prepared according to Ausubel and associates (1995). Where applicable, antibiotics were added at the following concentrations: ampicillin, 150 µg/ml; kanamycin (Km), 50 µg/ml; spectinomycin (Spec), 50 µg/ml; rifampicin (Rif), 150 µg/ml; tetracycline (Tet), 15 µg/ml; chloramphenicol, 20 µg/ml; and streptomycin (Sm), 50 µg/ml. All strains were maintained as glycerol stocks at -80°C (Sambrook et al. 1989).

Pathogenicity tests were performed on cuttings of *Gypsophila paniculata* var. Golan (Danziger Ltd, Bet Dagan, Israel), essentially according to Lichter and associates (1995). After removal of an approximately 2-mm section from the bottom of the stem, the cuttings (20 for each treatment) were inoculated by dipping into a bacterial suspension of 10⁶ cells/ml for 30 min and placed in trays filled with vermiculite. The greenhouse temperature was maintained at 22 to 25°C, and high humidity was generated by computer-controlled mist sprinklers that were activated every 20 min for 10 s. Pathogenicity was scored 15 to 20 days after inoculation. Bacterial colonization in gypsophila cuttings was carried out according to Valinsky and associates (2002). Five cuttings were used for each treatment and the results were averaged over three independent experiments. Experiments with external addition of C₄-HSL were performed by dipping the gypsophila cutting into a bacterial suspension of 10⁶ cells/ml in the presence of various concentrations of the signal.

Bioassay of acyl-HSL compounds with QS reporters.

Acyl-HSL compounds were initially detected with the acyl-HSL reporter strain *C. violaceum* CV026, which responds to a wide range of acyl-HSL compounds (McClellan et al. 1997). The tested *P. agglomerans* strain was cross-streaked with the reporter strain on TSA plates and incubated at 28°C. Appearance of the purple pigment violacein after 15 h indicated production of acyl-HSL. *C. violaceum* CV026 was also used to detect acyl-HSL on 20-by-20-cm silica gel 60 F₂₅₄ TLC plates (Merck, Darmstadt, Germany). The tested bacterial strain was grown overnight in 10 ml of LB broth and the cells were removed by centrifugation. The supernatant was extracted twice with equal volumes of acidified dichloromethane (0.1 ml of acetic acid per liter of dichloromethane), taken to dryness in a rotary vacuum evaporator at 40°C, dissolved in 10 µl of methanol, and applied to the TLC plate. Various acyl-HSL standards (Sigma-Aldrich, Rehovot, Israel), including C₄-HSL and C₆-HSL, were co-applied on the TLC plates with the authentic compounds. The amounts of the applied acyl-HSL standards ranged from 0.7 µg (e.g., C₄-HSL) to 10 ng (e.g., C₆-HSL). The plates were developed for 3 h with *N*-hexane-ethanol-chloroform (40:20:40 vol/vol) and dried in the hood at room temperature. For acyl-HSL detection, *C. violaceum* CV026 was grown in 3 ml of LB broth for 15 h and transferred into 50 ml of LB for an additional 2 h of growth. The cell suspension was then mixed with 100 ml of LB agar that had been pre-

cooled to 45°C and evenly poured onto the plate. The appearance of purple spots after 18 h of incubation at 28°C signified the presence of acyl-HSL compounds.

A set of highly sensitive chimeric luminescence reporters in *E. coli* JM109 (Table 1) (Swift et al. 1997; Winson et al. 1998) was employed for detecting acyl-HSL compounds, essentially according to Gao and associates (2003). These reporters included *E. coli* JM109 pSB536 (*ahyRI::luxCDABE amp^r*), which responds to short-chain alkanoyl HSL, most strongly to its cognate C₄-HSL; *E. coli* JM109 pSB401(*luxRI::luxCDABE Tet^r*) which is activated by C₆-C₁₂-HSL, most strongly with its cognate 3-oxo-C₆-HSL; and *E. coli* JM109 pSB1075 (*lasRI::luxCDABE amp^r*), which is activated by C₁₂-C₈-HSL, most strongly with its cognate 3-oxo-C₁₂-HSL. The three *E. coli* reporters were grown overnight as shake culture in 5 ml of LB broth with appropriate antibiotics at 37°C and then subcultured twice in fresh LB medium, each time with a 10-fold dilution. The second subcultures, which were allowed to grow to an optical density of 0.2 at 600 nm, were centrifuged and the pellets were resuspended in 10 times the original volume of LB. Each reporter suspension (80 µl) then was added to the wells of black microtiter plates containing dried tested acyl-HSL fractions and were incubated at 37°C for 3 to 4 h inside Ziploc bags to minimize evaporation. The luminescence was measured with a Wallace Victor-2 multimode microtiter plate reader (Perkin-Elmer, Gaithersburg, MD, U.S.A.). Relative luminescence was calculated as luminescence in the sample (counts per second) minus an average of the background luminescence of the reporter only. For routine, semiquantitative detection of lux-inducing activity, the microtiter plates containing the dried tested fractions and the reporter were placed on a piece of Kodak Biomax Light-1 X-ray film. Bioluminescence was detected after an exposure of 3 to 4 h.

Purification and structural determination of acyl-HSL.

A culture of the wild-type strain *Pag*824-1 was grown for 15 h in 1 liter of LB broth without antibiotic, and the cells were removed by centrifugation. The supernatant was extracted twice with equal volumes of acidified dichloromethane, and the organic phase was taken to dryness by rotary vacuum evaporation as described above. The resulting residue was re-dissolved in 1 ml of methanol and applied to a Vydac C₁₈ RP-high-performance liquid chromatography (HPLC) preparative column (25 by 2.2 cm, 10-nm particle size; Pharmacia, Uppsala, Sweden). The HPLC included high-pressure pump model 2249, fluorescence detector 474, PDA detector 996, 600s controller, vacuum degasses, and noncooled auto sampler 717. The instrument was fully controlled by the Millennium 32 software. The column was eluted at 6 ml min⁻¹ with a linear water-acetonitrile gradient of 20 to 48% (vol/vol) for 1 h. The fractions were collected at intervals of 15 s to 3 min, dried in the hood, dissolved in 1 ml of methanol, and tested for C₄-HSL and C₆-HSL with the *E. coli* biosensors pSB536 and pSB401, respectively, as described above. Fractions containing the same signal were pooled, dried by Speed Vac Plus, and used for mass spectrometry.

For mass spectrometry, samples were dissolved in a 50% (vol/vol) methanol/0.5% (vol/vol) formic acid mixture and diluted 1:1 with a calibration mixture consisting of 3 × 10⁻⁶ M sex pheromone inhibitor iPD1 (Bachem Bioscience Inc., King of Prussia, PA, U.S.A.), 6 × 10⁻⁶ M CsI (Sigma-Aldrich), 50% (vol/vol) methanol, and 1% (vol/vol) formic acid. Mass spectra were obtained with a QSTAR Pulsar mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.) using ESI in a Nanospray source (Protana Inc., Ontario, Canada). The sample was injected through a QSTAR capillary and measured in positive ion mode using a source potential of 900 V.

DNA manipulations.

The cloning vectors used in this study are listed in Table 1. DNA isolation, agarose gel electrophoresis, restriction analysis, and other DNA manipulation methods were performed according to standard procedures (Ausubel et al. 1995; Sambrook et al. 1989) or as recommended by the supplier. Small-scale plasmid isolation from *P. agglomerans* strains was carried out according to Kado and Liu (1981). Southern blots were prepared from approximately 400 ng of plasmid DNA and 5 µg of total DNA. Hybridization was performed by labeling a DNA probe with the enhanced chemiluminescence system (Amersham Biosciences, Uppsala, Sweden). DNA fragments were amplified by PCR with *Taq* polymerase (Super-Therm polymerase JMR-801; Roche, Mannheim, Germany), and synthetic oligonucleotides (Table 2) were synthesized according to the manufacturer's specifications (Sigma-Aldrich). The *E. coli* and *P. agglomerans* strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's instructions. PCR-amplified DNA and plasmid DNA were purified with the AccuPrep plasmid extraction kit (Bioneer, San Francisco) prior to sequencing or cloning into pGEM-T Easy Vector (Promega Corp., Madison, WI, U.S.A.). Automated sequencing with *Taq* DNA polymerase was carried out at the Biological Services Laboratories of Tel-Aviv University with an ABI Prism 3100 genetic analyzer (Applied Biosystems). When necessary, the sequence was completed with custom primers.

DNA sequence homology searches were performed with the National Center for Biotechnology Information BLAST programs blastn, blastx, and PSI blast. The alignment of the amino acid sequences was carried out with the programs ClustalX and Genedoc. Restriction maps, ORF searches, and additional molecular analyses were carried out with the Sci Ed Central for Window 95, Scientific and Educational Software (Cary, NC, U.S.A.).

Cloning procedures and construction of mutants.

The CopyControl Fosmid Library Production Kit (Epicentre Biotechnologies, Madison, WI, U.S.A.) was used to construct a genomic library of *Pag824-1* and *Pab4188* in *E. coli* EPI300 according to the manufacturer's instructions. The vectors pBluescript KS⁺, pGEM-T Easy vector, pVSP61, or pLAFR3 (Table 1) were used for gene subcloning. To obtain a marker-exchange mutant of *pagI* (*PagMxI*), a 1.35-kb *HindIII* DNA fragment harboring the C₄-HSL synthase was disrupted by insertion of a Ω cartridge conferring Sm/Spec resistance into the *Bam*HI site. The *Bam*HI site was artificially generated with the PCR primers ProI-Hind5, IBa3, IBa5, and R-ProHind5 (Table 2), 15 bp downstream of the start codon (Fig. 3). The construct obtained was cloned into pLAFR3 to yield pLPAGI (Table 1). The Sm/Spec insertion mutant was mobilized into *Pag824-1*(Rif^r) by triparental mating with the helper plasmids pRK2013 (Km^r) according to Nizan-Koren and associates (2003). The presence of the Ω insertion was confirmed by PCR amplification with primers Pag-1 and Pag-2 (Table 2) based on the two end-sides of *pagI*. The strategy described by Kalogeraki and Winans (1997) was employed for generating the marker-exchange mutant of *pagR* (*PagMxR*). A 622-bp central fragment of *pagR* was amplified with primers R-FowSac and R-RevXba (Table 2), digested with *SacI* and *XbaI*, and cloned into pVIK165 (Km^r) predigested with the same enzymes to obtain pVIK-R5. The resultant plasmid, which carried the vegetative replication origin of plasmid R6K without its *pir* gene (Kalogeraki and Winans 1997), was replicated in *E. coli* S17- λ *pir*. Mutants generated following electroporation of pVIK-R5 into *Pag824-1* were selected on LB plates amended with kanamycin. Mutations were verified by

Southern blot analysis with the *SacI/XbaI* fragment used as a probe.

A double mutation within *pagI* and *pagR* was created by deleting the 1-kb *Bam*HI fragment that spans the 3' coding region of both genes and introducing a Km^r cassette into the same site (Fig. 4). The *Bam*HI sites were artificially introduced into the two genes by PCR amplification with primers ProInde, PagIBa, RBa3, and 4kb-3 (Table 2). The mutated DNA Km^r fragment was cloned into the *Bam*HI site of pGEM to yield pGEM-I/RB, and the deleted *pagI/R* construct was subsequently introduced into *Pag824-1* by electroporation to generate the marker-exchange double mutant *PagMx Δ IR*. The generation of a double mutant (Km^r) was confirmed by digesting the total genomic DNA with *PstI*, followed by Southern hybridization with the deleted DNA fragment as probe. The mutant *Pag Δ IR* did not produce detectable amounts of acyl-HSL when tested with the *C. violaceum* CV026 biosensor.

Complementation of *PagMxI* and *PagMx Δ IR* for pathogenicity was achieved by triparental mating with pLAFR4.3. Complementation of *PagMxR* was achieved by digesting the pKS4.3 with *PstI*, and a 1.4-kb fragment containing the *pagR* was selected and cloned into pLAFR3 to obtain pLAFR1.4.

Gene expression analysis by qRT-PCR.

Total RNA was extracted from *Pag824-1* and *PagMxI* strains following growth on minimal A agar medium for 16 h at 28°C. The MasterPure RNA Purification Kit (Epicentre Biotechnologies) was employed for RNA extraction from 2×10^8 cells. Reverse transcription was carried out on 1 µg of total RNA pretreated with TURBO DNA-free (Ambion Inc., Austin, TX, U.S.A.) using the Verso cDNA Kit (Thermo Scientific, Epsom, U.K.) with random hexamer primers. The resultant cDNAs were subjected to PCR amplification with the ABsolute Blue QPCR SYBER[®] Green ROX Mix kit (Thermo Scientific). The specific primers used for *hrpL* and *pthG* amplification were designed according to the sequence of the corresponding genes and the degenerate primers for the housekeeping gene, *gyrB*, are listed in Table 2. The expected size of the amplicons was approximately 85 bp. Real-time detection was performed with a Rotor-Gene 3000 device (Corbett Research, Sydney, Australia) and results were analyzed with the rotor-gene 6 software. The endogenous control was the *gyrB* gene and the calibrator was the wild-type *Pag824-1* extracted sample. A mixture of all the used cDNAs in the various treatments was employed as a template for calibrating curves designed for each pair of primers. Relative quantification was calculated on the basis of $\Delta\Delta C_T$ (cycle threshold). The ΔC_T value was determined by subtracting the C_T results for the target gene from the endogenous control gene C_T and then normalizing as suggested by Rotor Gene ($\Delta\Delta C_T$). Each experiment was conducted three times and statistical analysis was performed using Microsoft Excel (Microsoft).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Plate-Frome Bio-Informatique de Strasbourg's ClustalX website: www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html

National Resource for Biomedical Supercomputing Genedoc software: www.nrbsc.org