Global Regulatory Networks Control the Hrp Regulon of the Gall-Forming Bacterium *Pantoea agglomerans* pv. *gypsophilae*

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Gall formation by *Pantoea agglomerans* pv. *gypsophilae* is dependent on the hypersensitive response and pathogenicity (*hrp*) system. Previous studies demonstrated that PagR and PagI, regulators of the quorum-sensing system, induce expression of the *hrp* regulatory cascade (i.e., *hrpXY*, *hrpS*, and *hrpL*) that activates the HrpL regulon. Here, we isolated the genes of the Gac/Rsm global regulatory pathway (i.e., *gacS*, *gacA*, *rsmB*, and *csrD*) and of the post-transcriptional regulator *rsmA*. Our results demonstrate that PagR and PagI also upregulate expression of the Gac/Rsm pathway. PagR acts as a transcriptional activator of each of the *hrp* regulatory genes and *gacA* in a *N*-butanoyl-L-homoserine lactone-dependent manner as shown by gel shift experiments. Mutants of the Gac/Rsm genes or overexpression of *rsmA* significantly reduced *Pantoea agglomerans* virulence and colonization of gypsophila. Overexpression of *rsmB* sRNA abolished gall formation, colonization, and hypersensitive reaction on nonhost plants and prevented transcription of the *hrp* regulatory cascade, indicating a lack of functional type III secretion system. Expression of *rsmB* sRNA in the background of the *csrD* null mutant suggests that CsrD may act as a safeguard for preventing excessive production of *rsmB* sRNA. Results presented indicate that the *hrp* regulatory cascade is controlled directly by PagR and indirectly by RsmA, whereas deficiency in RsmA activity is epistatic to PagR induction.

Pantoea agglomerans, a widespread epiphytic and endophytic bacterium on many plants, has evolved into a host-specific tumorigenic pathogen by acquiring a plasmid-borne pathogenicity island (PAI) (Barash and Manulis-Sasson 2009). *P. agglomerans* pv. *gypsophilae* elicits galls on gypsophila and hypersensitive reaction (HR) on beet, whereas *P. agglomerans* pv. *betae* causes galls on beet and gypsophila (Burr et al. 1991; Ezra et al. 2000). The pathogenicity of the gypsophila and beet pathovars is dependent on the presence of indigenous plasmids designated as pPATH_{Pag} and pPATH_{Pab}, respectively (Barash and Manulis-Sasson 2007). The pPATH_{Pag} (135 kb) of *P. agglomerans* pv. *gypsophilae* (strain Pag824-1) harbors a PAI of approximately 75 kb which carries a functional *hrp/hrc*

gene cluster containing a type III secretion system (T3SS) and genes of the *hrp* regulatory cascade. Additionally, it carries genes that encode for type III effectors (T3E) as well as a cluster of genes responsible for biosynthesis of indole-3-acetic acid (IAA) and cytokinins (CK) (Barash and Manulis-Sasson 2009).

In *Pantoea*, *Erwinia*, and *Pseudomonas* spp., the T3SS and T3E are regulated by HrpL—an alternative σ factor (Xiao et al. 1994). HrpL proteins are highly conserved and recognize a consensus sequence designated as “*hrp* box” in the promoters of the T3SS and T3E genes which, together, form the HrpL regulon (Nissan et al. 2005; Tang et al. 2006). In *Pantoea* spp., HrpL is sequentially activated by HrpXY, a two-component system, and HrpS, a transcription factor that, together with HrpL, forms the *hrp* regulatory cascade (Nizan-Koren et al. 2003).

Gall formation caused by phytopathogenic bacteria has been attributed primarily to IAA and CK secreted by the pathogen (Jameson 2000; Morris 1986; Spaepen et al. 2007). However, simultaneous disruption of the pathways for biosynthesis of CK and IAA, including the plasmid-borne indole-3-acetamide and the chromosomal-located indole-3-pyruvate pathways for IAA biosynthesis, reduced gall size by approximately 50% but did not abolish gall initiation (Manulis et al. 1998). In contrast, any mutation in the *hrp* gene cluster abolished gall initiation (Mor et al. 2001; Nizan-Koren et al. 2003). These findings suggest that gall formation relies on T3E. Additionally, we have recently demonstrated that disruption of the basipetal polar auxin transport (PAT) of gypsophila with specific inhibitors completely abolished gall initiation (Chalupowicz et al. 2013). PAT is known to be mediated by auxin efflux carriers encoded by the PIN gene family (Kleine-Vehn and Friml 2008). Our results suggest that PIN induction might serve as direct or indirect targets for T3E (Chalupowicz et al. 2013). Current hypotheses propose that cellular hormone transporters of the PIN family would create local auxin maxima at precise positions which, in turn, would lead to initiation of meristematic tissue and organ differentiation (Kramer 2004). We hypothesize that local auxin maxima might also be involved in gall initiation.

Two global regulatory networks that are known to play a major role in bacterial virulence are the quorum sensing (QS) system, also designated as cell-to-cell communication system (Barnard et al. 2007), and the Gac/Rsm signal transduction pathway (Lapouge et al. 2008). Expression of the RsmA-*rsmB* system is controlled by the two-signal transduction-component system GacS/A (Brencic and Winans 2005; Cui et al. 1995, 2001; Lapouge et al. 2008). RsmA is a post-transcriptional global regulator that binds to selected mRNA transcripts in a

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region that overlaps the ribosome-binding site, blocking ribosome binding and promoting transcript degradation (Barnard et al. 2007; Brencic and Winans 2005). *rsmB* is a small RNA (sRNA) that sequesters multiple molecules of RsmA, thereby neutralizing its effect (Lapouge et al. 2008). CsrD is an additional novel protein that acts as an antagonist of *rsmB* sRNA (referred to hereafter as *rsmB*) (Suzuki et al. 2006). GacS/A positively controls the expression of genes specifying sRNAs such as *rsmB* (Chatterjee et al. 2003; Lapouge et al. 2008).

The cell-to-cell regulatory system of *P. agglomerans* pv. *gypsophillae* strain Pag824-1 has been previously characterized (Chalupowicz et al. 2008). It produces *N*-butanoyl-L-homoserine lactone (C_4 -HSL) as a major QS signal and includes homologs of the *luxI* and *luxR* regulatory genes (Whitehead et al. 2001); namely, *pagI* (encoding C_4 -HSL synthase) and *pagR* (encoding the transcriptional regulator). Disruption of either *pagI*, *pagR*, or both genes simultaneously reduced by 50 to 55% the gall size in gypsophila cuttings inoculated with 10^6 CFU/ml (Chalupowicz et al. 2008). Higher reduction in gall size (70 to 90%) was achieved by overexpression of *pagI* or addition of exogenous C_4 -HSL. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in planta showed that the effect of the QS system on gall formation is mediated by expression of the *hrp* regulatory cascade (Chalupowicz et al. 2009). Furthermore, IAA and CK produced by the bacterium significantly upregulated the expression of the QS and, consequently, the *hrp* regulatory genes, presumably by a signaling effect (Chalupowicz et al. 2009).

The interrelationship between the QS system, Gac/Rsm signal transduction, and virulence has been investigated in various phytopathogenic bacteria (Mole et al. 2007). However, little is known about the precise regulatory interactions between these two global regulatory systems and activation of the *hrp* regulatory cascade genes apart from *Pectobacterium carotovorum* subsp. *carotovorum*. In this pathogen, QS-dependent regulation releases the suppressive effect of RsmA on the *hrp* regulatory genes and allows the expression of T3SS and T3E (Chatterjee et al. 2002b; Cui et al. 2005; Sjoblom et al. 2006).

The present study was undertaken to unravel the interactions between the plasmid-borne HrpL regulon of *Pantoea agglomerans* pv. *gypsophillae* and the chromosomal global regulatory

systems QS and the Gac/Rsm pathway. Our results demonstrate two main routes for controlling the Hrp regulon by the QS system: i) direct transcriptional activation of the *hrp* regulatory cascade and the *gacA* of the Gac/Rsm pathway by PagR and ii) control of the *hrp* regulatory cascade via RsmA.

RESULTS

Isolation and characterization of the Gac/Rsm pathway of *P. agglomerans* pv. *gypsophillae*.

The genes of the Gac/Rsm regulatory pathway (namely, *gacS*, *gacA*, *csrD*, *rsmB*, and *rsmA*) have not been previously identified in *P. agglomerans* pv. *gypsophillae* strain Pag824-1. Isolation of these genes was carried out by employing primers based on corresponding genes in taxonomically related bacteria (described below). The sequences of *gacS*_{Pag824-1}, *gacA*_{Pag824-1}, *csrD*_{Pag824-1}, and *rsmB*_{Pag824-1} were submitted to the GenBank under accession numbers JN203497, JN203496, JN392466, and FJ465143, respectively. A single copy of *rsmA* was previously isolated from strain Pag824-1 and submitted to the GenBank as FJ465141 (Chalupowicz et al. 2009).

The deduced GacS_{Pag824-1} (101-kDa) and GacA_{Pag824-1} (19-kDa) proteins possessed all the classical domains of two-component systems. Blast analysis revealed that GacS_{Pag824-1} shares a high identity of 70% in predicted amino acids with the GacS homolog BarA of *Erwinia amylovora* (accession number CBX79579) and 64% with BarA of *Klebsiella pneumonia* (accession number YP_001336759). The deduced GacA_{Pag824-1} protein possessed classical domains of a response regulator, including the phosphoryl receiver domain with a conserved aspartate at position 57 (Nizan-Koren et al. 2003). Outside the *Pantoea* genus, it shares the highest identity with the GacA homolog UvrY (90%) of *E. amylovora* (accession number CBX80276). The predicted CsrD_{Pag824-1} (72-kDa) protein possessed the signaling domains of diguanylate-cyclase GGDEF, and EAL which, in various bacterial species, affect production of exopolysaccharides and surface proteins involved in adhesion, motility, biofilm formation, and host–pathogen interactions (Hisert et al. 2005). CsrD_{Pag824-1} exhibits 77% identity with YhdA, a homolog of CsrD in *P. ananatis* (accession number YP_003521862.1), and 66% identity with YhdA of *E. amylovora* (accession number YP_003529640).

The *rsmB* and *rsmA* genes were previously characterized in *P. agglomerans* pv. *gypsophillae* strain PD713 (formerly *E. herbicola* pv. *gypsophillae*) (Ma et al. 2001) and were now identified in strain Pag824-1. *rsmB*_{Pag824-1}, an untranslated sRNA, has a size of 315 bp. Outside the *Pantoea* spp. it has a high identity of 87% with *csrB* (*rsmB* homolog) of *Salmonella enterica* serovar *Typhimurium* (accession number AFO76153) and 80% identity with *csrB* of *Escherichia coli* (accession number AFO31251). The deduced RsmA_{Pag824-1} is highly conserved and displays 97% identity to CsrA (RsmA homolog) of *E. coli*, *S. enterica*, *P. carotovorum* subsp. *carotovorum*, and *P. aeruginosa*.

The Gac/Rsm pathway regulates virulence and colonization of *P. agglomerans* in gypsophila.

The effect of the Gac/Rsm pathway on virulence of Pag824-1 was examined by generating marker exchange mutants of *gacS*, *gacA*, *rsmB*, and *csrD* using the suicidal vector pVIK165 (Kalogeraki and Winans 1997). Numerous attempts to generate marker exchange or deletion mutant of *rsmA* were unsuccessful. The inability to isolate an *rsmA* null mutant was also reported in other bacteria and could be attributed to strong cell-to-cell aggregation, slow growth, or critical role in bacterial metabolic processes required for multiplication (Lapouge et al. 2008; Lawhon et al. 2003; Molofsky and Swanson 2003;

Fig. 1. Effect of the Gac/Rsm pathway on gall development. Gypsophila cuttings were inoculated with 10^6 CFU/ml of Pag824-1 (wild type); mutants in *gacA* (MxgacA); *gacS* (MxgacS); *rsmB* (MxrsmB); *csrD* (MxcsrD); *rsmB* overexpression (*rsmBOE*) and *rsmA* overexpression (*rsmAOE*). Pathogenicity tests were performed on gypsophila cuttings. Mean gall size was obtained by measuring fresh weight of galls from eight cuttings in each treatment after 14 days. Results are presented as percentage of the mean gall size of the wild type (254 mg). The experiment was repeated three times. Bars indicate standard deviations.

Romeo et al. 1993). Because *rsmB* is known to sequester RsmA (Lapouge et al. 2008), strains overexpressing *rsmB* (*rsmBOE*) and *rsmA* (*rsmAOE*) were also generated. *rsmAOE* and mutants in *rsmB*, *gacA*, *gacS*, and *csrD* caused reduced gall formation by 49 to 61% (Fig. 1). The decrease in the size of galls induced by *rsmB*, *gacA*, and *gacS* mutants could be complemented to wild-type levels (results not shown). However, no gall development could be detected in the *rsmBOE* strain (Fig. 1). Moreover, the GacS/GacA two-component system is generally known to switch on the transcription of sRNA and, consequently, to regulate *rsmB* levels (Lapouge et al. 2008). The observation that marker exchange mutants of *gacS*, *gacA*, and *rsmB* significantly reduced gall size suggests that optimal RsmA activity is necessary for generating maximal gall size in *P. agglomerans* pv. *gypsophila*. This notion gains support from the observation that overexpression of *rsmA* also reduced gall size by approximately 53%. The effects of the various Gac/Rsm mutants on gall size correlated with gypsophila colonization (Fig. 2). Colonization of the various mutants was reduced by two orders after 7 days whereas growth of *rsmBOE* was hardly observed.

The QS system induces gene expression of the Gac/Rsm pathway.

In order to determine whether the Gac/Rsm signal transduction pathway is regulated by the QS system, the effect of *pagR* and *pagI* mutants on expression of *gacA* and *rsmB*, two key genes of the Gac/Rsm pathway, was investigated using qRT-PCR in planta. Inactivation of either *pagR*, *pagI*, or the two genes simultaneously (i.e., *pagΔRI*) reduced the transcript levels of *gacA* by 3.1-, 2.2-, and 2.9-fold, respectively, and those of *rsmB* by 2.3-, 2.8-, and 3.1-fold, respectively (Fig. 3). These results suggest that *gacA* and, subsequently, *rsmB* are under positive control of the QS system.

Fig. 2. Effect of the Gac/Rsm pathway on colonization of gypsophila by *Pantoea agglomerans* pv. *gypsophila*. Gypsophila cuttings were dipped in suspensions of various bacterial mutants for 45 min and colonization was determined. *Pag824-1*, the wild type strain; *rsmBOE*, overexpression of *rsmB*; *MxgacS*, mutant in *gacS*; *MxgacA* mutant in *gacA*; *rsmAOE*, *rsmA* overexpression; *MxrsmB*, mutant in *rsmB*; *MxcsrD*, mutant in *csrD*. Results presented are average of three independent experiments with five replicates for each time point. Bars indicate standard deviations.

PagR binds to the promoters of *hrp* regulatory genes and *gacA* in a C₄-HSL-dependent manner.

It has been previously reported that disruption of either *pagR*, *pagI*, or the two genes simultaneously substantially reduced the expression of *hrpXY*, *hrpS*, and *hrpL* in *P. agglomerans* pv. *gypsophila* (Chalupowicz et al. 2009). Studies presented in Figure 3 demonstrated a similar effect of the QS system on *gacA* and *rsmB*. These results suggest that PagR acts as a transcription factor of both the *hrp* regulatory genes and the Gac/Rsm pathway. To further support this premise, the promoters (i.e., 50 bp upstream of the transcription initiation) of the QS-controlled genes were analyzed for putative *lux* boxes containing the minimal consensus sequence [NNCT-(N₁₂)-AGNN] (Whiteley and Greenberg 2001). Results indicate that all three *hrp* regulatory genes and *gacA* harbor putative *lux* boxes (Table 1). The promoter of *rsmA* was also examined because, in *Pectobacterium carotovorum* subsp. *carotovorum*, the QS transcription factor ExpR binds *rsmA* and activates its transcription (Cui et al. 2005). However, although a palindrome could be detected in the promoter of *rsmA*_{Pag824-1}, it did not include the *lux* box minimal consensus sequence.

Gel shift assays were employed to verify that PagR can bind to the putative *lux* box promoters of the genes shown in Table 1. DNA fragments of 50 bp containing the promoter regions of *hrpL*, *hrpXY*, *hrpS*, and *gacA* were end-labeled with digoxigenin (DIG) and used for gel shift experiments with PagR fused to the maltose-binding protein (MBP). The DNA fragments and the MBP-PagR fusion protein were incubated in the binding buffer with and without C₄-HSL. Resolution of the binding mixture on nondenatured polyacrylamide gel revealed that MBP-PagR binds to *hrpL*, *hrpS*, *hrpXY*, and *gacA* DNA fragments in a C₄-HSL-dependent manner (Fig. 4A). Addition of identical excessive unlabeled DNA or a synthetic segment

Fig. 3. Effects of the quorum-sensing mutants in *pagR* (*PagMxR*), *pagI* (*PagMxI*), and the double mutant (*PagMxΔIR*) on *gacA* and *rsmB* transcript levels. Template cDNA for quantitative reverse-transcription polymerase chain reaction were generated from total RNA extracts of gypsophila cuttings 16 h after inoculation with bacterial cell suspension (10⁶ CFU/ml). Gene expression was normalized with *gyrB* and *mdh* that were used as endogenous reference and *Pag824-1* as the control. Results represent the mean of three different experiments with standard deviation.

Table 1. Promoter regions of the hypersensitive response and pathogenicity (*hrp*) regulatory genes *gacA* and *rsmA* containing putative *lux* boxes

Gene	Putative <i>lux</i> boxes in regulatory genes 5'→3'
<i>hrpL</i>	AGAATTTTCCAGCTGACAGTATCAGGAGTGAATTTTGCAAAGAGTTGCAG
<i>hrpXY</i>	CTCAATATCATCTCTGGAACTCTGTAGATCTGCAGCCAGACCGGGTAAACG
<i>hrpS</i>	TAGCCTTACCCCTATCTCAGTGAATTCCTTACTCGTGGTTTCAGCATAAGA
<i>gacA</i>	GTCGCATCCTGGAGGATGTCAAAGGTCTGGCCGTGGTTCGGAGAGGTCAAC
<i>rsmA</i>	GCATCGATGTGCATTGGTGTATTCTCATACATTGTGCAGCGTTAAAT
Random DNA	ACCACGTTTTTAATTTAAATGACTTTAATCTGTGGCATCTGGGATGGATTTC

^a The minimal consensus sequence for *lux* box is marked by bold letters. The underlined sequences in *rsmA* indicate a non-*lux* box palindrome.

of random DNA deficient of the minimal *lux* box consensus sequence abolished the retarded band, indicating that the binding is sequence specific. Purified MBP obtained from isopropyl-thio-galactopyranoside (IPTG)-induced cells of JM109 carrying pMAL-c4X empty vector did not bind the promoters. Contrary to its homolog from *P. carotovorum* subsp. *carotovorum*, the QS transcription factor PagR of *Pag824-1* did not bind to the *rsmA* promoter in any of the conditions used.

The ability of PagR to bind to its target gene *hrpL* has been examined in the presence of different signal concentrations. PagR bound to *hrpL* in vitro with increasing efficiency at C₄-HSL concentrations between 5 and 200 µM (Fig. 4B).

RsmA is required for expression of the *hrp* regulon and operates via the *hrp* regulatory cascade.

It has been previously reported that *Pag824-1* elicits HR on beet due to the presence of *pthG* (Ezra et al. 2000). It is now well established that a functional T3SS is required for HR induction caused by T3E (Alfano and Collmer 2004). To investigate whether genes of the Gac/Rsm system are involved in HR elicitation by *Pag824-1*, beet leaves were inoculated with the different Gac/Rsm mutants. *rsmB*, *gacA*, or *gacS* mutants or a strain overexpressing *rsmA* (*rsmAOE*) elicited HR on beet leaves (Fig. 5). In contrast, inoculation with a strain overexpressing *rsmB* (*rsmBOE*) did not elicit HR. These results indicate that RsmA activity is required for expression of a functional T3SS.

The possibility that the effect of RsmA activity on the expression of the T3SS is mediated by the *hrp* regulatory cascade was further examined by qRT-PCR in *gypsophila* (Fig. 6). In the overexpressing *rsmB* mutant, the relative expression of *hrpL*, *hrpS*, and *hrpY* was significantly lower than in the wild type. At 16 h postinoculation (hpi), transcript level of *hrpL* and *hrpS* could not be detected, whereas a very low expression of *hrpY* was measured. At 24 hpi, the transcription of *hrpL* and *hrpS* was three and four times lower, respectively, than the wild type (Fig. 6). These results suggest that RsmA activity is required for effective expression of the *hrp* regulatory cascade.

The effect of CsrD on *rsmB* regulation.

The regulatory protein CsrD has been shown to decay *csrB* (homolog of *rsmB*) through an RNAaseE-mediated pathway in *E. coli* (Suzuki et al. 2006). The ability of CsrD to reduce transcript levels of *rsmB* in *Pag824-1* was examined (Fig. 7). Results obtained illustrate that the expression of *rsmB* in the *csrD* mutant was only slightly increased by approximately 1.75-fold whereas the overexpression of *rsmB* was 5-fold higher than in the wild type (Fig. 7). However, overexpression of *rsmB* in a *csrD* mutant increased its transcripts levels by 11-fold compared with the wild-type strain. These results suggest that CsrD may act as a safeguard for excessive expression of *rsmB* that binds RsmA, which adversely affects the optimal level of RsmA activity. However, its ability to effectively prevent the expression of high levels of *rsmB* might be limited to physiological conditions. When *rsmB* is artificially overexpressed, the antagonistic effect of CsrD is substantially reduced.

DISCUSSION

Gall formation by *Pantoea agglomerans* pv. *gypsophila* is absolutely dependent on a plasmid-borne functional *hrp* gene cluster and T3E (Barash and Manulis-Sasson 2009). The linear cascade of the *hrp* regulatory genes (i.e., *hrpXY*, *hrpS*, and *hrpL*) which activates the HrpL regulon is crucial for pathogenicity of this pathogen (Nizan-Koren et al. 2003). Global regulation networks, notably the QS system and the Gac/Rsm pathway, have been shown to be involved in expression of various virulence factors, including the T3SS system (Mole et al. 2007; Tang et al. 2006). However, the interrelationship between these systems and the precise mechanisms by which they interact are only partially understood and might vary among different bacterial plant pathogens. We have previously shown that QS genes elevate the expression of the *hrp* regulatory cascade in *P. agglomerans* pv. *gypsophila* (*Pag824-1*) (Chalupowicz et al. 2009). The present study illustrates that PagR and PagI also upregulate the transcription of *gacA* and

Fig. 4. Gel-shift assay of purified maltose-binding protein (MBP)-PagR fusion protein with digoxigenin-labeled promoter DNAs. **A**, MBP-PagR binding to *rsmA*, *hrpL*, *hrpS*, *hrpXY*, and *gacA*. A DNA promoter (2 to 3 ng) was added to each reaction mixture. Commercially available *N*-butanoyl-L-homoserine lactone (C₄-HSL) (100 µg) and unlabeled DNA were added to the indicated lanes. PagR-free MBP vector served as the control. **B**, Effect of increasing C₄-HSL concentrations on binding of PagR to *hrpL* promoter. MBP-PagR DNA (2 to 3 ng) was added to each reaction mixture followed by addition of C₄-HSL at various concentrations. Arrow points to DNA-bound protein.

rsmB (Fig. 3), two key genes of the Gac/Rsm pathway, implying that QS controls both the genes of the *hrp* cascade and the Gac/Rsm route. Additionally, by employing gel-shift assays, we demonstrated that PagR directly binds to the putative *lux*-box-containing promoters of individual genes of the *hrp* regulatory cascade and the *gacA* in a C₄-HSL-dependent manner (Fig. 4A). Together, these results suggest that the cell-to-cell communication system positively and directly upregulates the two pathways simultaneously through PagR, which acts as a common transcriptional activator.

In contrast to *P. agglomerans* pv. *gypsophillae*, in *Pectobacterium carotovorum* subsp. *carotovorum*, the cell-to-cell communication system exerts an indirect control of *hrpL*, *hrpN*, and genes of the plant cell-wall-degrading enzymes (PCWDEs) (Chatterjee et al. 2002a; Cui et al. 2005). In this soft-rot-inducing bacterium, the effect of the QS transcription factor ExpR specifically binds to the *rsmA* promoter in the absence of the cognate signal 3-oxo-C₆-HSL and activates its transcription. The RsmA destabilizes the mRNA transcripts of *hrpL*, *hrpN*, and PCWDEs and prevents their expression. Binding of the cognate signal inactivates the ExpR and thereby inhibits *rsmA* expression and stabilizes the mRNA transcripts encoded by the virulence genes (Cui et al. 2005; Sjoblom et al. 2006). Thus, the mode of action of ExpR on the virulence genes *hrpL* and *hrpN* that are related to the T3SS is fundamen-

tally different from PagR; its control is mediated by RsmA rather than by acting directly as a transcriptional activator of the *hrp* cascade, and it performs in the absence of the cognate signal.

Present and previous studies (Chalupowicz et al. 2008, 2009) indicate that the QS system of *Pantoea agglomerans* pv. *gypsophillae* is the first step in the regulatory hierarchy that subsequently triggers other virulence responses. Instead, in *Pseudomonas syringae* pv. *syringae* or *P. aureofaciens*, the GacS/A two-component system plays a pivotal role in activating *ahhI* encoding the synthesis of its cognate *N*-acyl homoserine lactones (AHL) signal (Quinones et al. 2004, 2005). The AhhI/R (LuxI/R-homologs) of the *Pseudomonas* QS system, in combination with the TetR family transcription activator AefR, is essential for intercellular maceration and epiphytic fitness as well as for the production of other virulence factors such as the phytotoxin syringomycin (Chancey et al. 1999; Kitten et al. 1998). However, it is, noteworthy that, similarly to *Pantoea agglomerans* pv. *gypsophillae*, in most other major bacterial plant pathogens such as *Pectobacterium carotovorum* subsp. *carotovorum* or *Ralstonia solanacearum*, the cell-to-cell system operates upstream to the Gac/Rsm pathway (Mole et al. 2007). Also, in *Xanthomonas campestris* pv. *campestris*, the diffusible signal factor of the cell-to-cell communication system is upstream of the two-component system RpfC/RpfG, which

Fig. 5. Hypersensitive response (HR) induced by mutants of the Gac/Rsm pathway on beet leaves. Approximately 100 μ l of bacterial suspension containing of each bacterial mutant at 10^7 CFU/ml and water as control was infiltrated into beet leaves (*Beta vulgaris* 'Red Egyptian'). The point of bacterial entry is indicated by an arrow. HR response was detected within 48 h.

leads to the degradation of the global regulator cyclic di-GMP that further affects the global transcription activator Clp (Mole et al. 2007; Tao et al. 2010).

It has been previously shown that addition of 50 μ M C₄-HSL during inoculation of gypsophila cuttings by *Pantoea agglomerans* pv. *gypsophila* reduced gall size by up to 90% and the degree of reduction appeared to be proportional to the concentration of the added signal (Chalupowicz et al. 2008). It was generally proposed that the addition of exogenous QS signal results in premature production of virulence factors that elicit host defenses prior to reaching the bacterial quorum required for infection (Mae et al. 2001). However, the explanation for the “signal concentration effect” might be more complex. For example, in some pathogens such as *Pectobacterium carotovorum* subsp. *carotovorum* or *P. stewartii* subsp. *stewartii*,

the unliganded apo form of the LuxR homolog (i.e., ExpR and EsaR), binds to the DNA and acts as transcription factor without the AHL signal. Binding of the AHL results in dissociation of the transcription factor from its target promoter (Barnard et al. 2007; von Bodman et al. 2008). The possibility that an excessive AHL signal might also lead to the dissociation of the LuxR homologs in bacteria that require the signal for binding cannot be excluded. However, results obtained here might suggest that this is not the case in *Pantoea agglomerans* pv. *gypsophila* because the binding of PagR to the *hrpL* promoter in vitro was still increasing at a high concentration of C₄-HSL.

As earlier described, RsmA acts as a global regulator that controls numerous bacterial functions, including pathogenesis (Barnard et al. 2007; Lapouge et al. 2008). RsmA activity is tightly controlled through one or more sRNAs whose transcription is regulated by the GacS/A two-component system (Lapouge et al. 2008). In the present study, only one sRNA molecule (namely, *rsmB*) could be detected and upregulated by the GacS/A, which appears to play a critical role in modulation of RsmA activity. This conclusion was supported by analyzing the effects of various Gac/Rsm marker exchange mutants on gall formation and pathogen colonization (Figs. 1 and 2). The observation that mutants in *gacS*, *gacA*, *rsmB*, and *csrD* significantly reduced gall size and colonization of gypsophila plants could be attributed to the control of the Gac/Rsm on RsmA activity via its binding to *rsmB*. These mutants are expected to reduce *rsmB* transcription and enhance RsmA activity (Lapouge et al. 2008). The finding that overexpression of *rsmA* reduced gall size and colonization to the levels of the marker-exchanged mutants (Figs. 1 and 2) further supports the view that optimal rather than maximal RsmA activity is required to attain full virulence. It is possible to hypothesize that the optimal concentration for gall formation results from the effect of RsmA on multiple factors that indirectly also influence virulence.

The reduction of gall size by an excessive activity of RsmA could result from decreasing transcripts of genes involved in T3SS expression, such as *hrpL* or additional virulence genes of *P. agglomerans* pv. *gypsophila*, as reported for *Dickeya dadantii* (Yang et al. 2008). In the latter pathogen, a *gacA* deletion mutant was shown to reduce pathogenicity through lower transcription of T3SS-related genes such as *dspE*, *hrpN*, or *hrpA* and additional virulence genes (Yang et al. 2008).

Fig. 6. Transcript levels of *hrpL*, *hrpS*, and *hrpY* in the wild-type *Pantoea agglomerans* pv. *gypsophila* 824-1 (Pag824-1) and its *rsmB* overexpression mutant (*rsmBOE*) in planta. Template cDNAs were generated from total RNA extracts of infected gypsophila cuttings 8, 16, 24, and 48 h after inoculation. Gene expression was normalized with *gyrB* used as an endogenous reference. Results represent three different experiments with standard deviation.

Fig. 7. Effect of mutation in CsrD on transcript levels of *rsmB*. Transcript levels were determined in Pag824-1 (wild type), *csrD* mutant (*MxcsrD*), *rsmB* overexpression mutant (*rsmBOE*), and *rsmBOE* in the background of *csrD* mutant (*rsmBOE/MxcsrD*). Template cDNAs were generated from total RNA extracts of infected gypsophila cuttings. Results represent three different experiments with standard deviation.

In contrast to mutants of the Gac/Rsm pathway and overexpression of RsmA, the overexpression of *rsmB* completely eliminated gall formation and colonization, presumably due to a lack of RsmA activity (Figs. 1 and 2). Because deficiency in RsmA activity was obtained through *rsmB* sequestration rather than mutagenesis, we cannot unequivocally claim a complete absence of RsmA activity, and the possibility that some residual activity of RsmA was still retained cannot be entirely excluded. However, even if some activity remains, it was insufficient to fulfill the functions of RsmA in virulence. Because pathogenicity of *P. agglomerans* pv. *gypsophila* is *hrp* dependent, it was hypothesized that the phenotype observed by RsmA deficiency operates via T3SS expression. To confirm this premise, the various mutants were injected into beet leaves, which serve as nonhost for the *gypsophila* pathovar (Ezra et al. 2000). In these experiments, with the exception of *rsmBOE*, all other Gac/Rsm mutants induced HR response when inoculated into beet leaves (Fig. 5). Because HR elicitation by plant-pathogenic bacteria requires the functionality of T3SS (Alfano and Collmer 2004), it can be concluded that the RsmA activity is essential for T3SS expression.

We further explored whether the dramatic effect of RsmA deficiency on T3SS expression is performed through the *hrp* regulatory cascade or other genes of the Hrp regulon. To this aim, the effect of *rsmBOE* on transcription of the *hrp* regulatory genes was investigated by qRT-PCR (Fig. 6). Results clearly illustrate that RsmA activity is crucial for expression of *hrpY*, *hrpS*, and *hrpL*, each of which is essential for activation of the Hrp regulon of *P. agglomerans* pv. *gypsophila* (Nizan-Koren et al. 2003). Because RsmA is known to operate by promoting degradation of selected mRNA transcripts (Lapouge et al. 2008), its apparent positive effect on the *hrp* regulatory genes can be

best explained by hypothesizing the presence of an intermediate regulator which negatively and effectively prevents the transcription of the *hrp* regulatory cascade. This as-yet-unknown regulator is negatively controlled by RsmA. Thus, elimination of RsmA activity releases the inhibition of the hypothetical regulator which, in turn, inactivates the transcription of the *hrp* regulatory cascade (Fig. 8). Similarly to our results, inactivation of the *rsmA* genes of *X. oryzae* pv. *oryzae* or *X. campestris* pv. *campestris* caused a complete loss of virulence and failure to elicit HR in a nonhost, indicating that RsmA is essential for T3SS expression as well as other virulence factors of these pathogens (Chao et al. 2008; Zhu et al. 2011).

RsmA appears to fulfill two different functions in virulence of *P. agglomerans* pv. *gypsophila*: under surplus activity, which results from disruption of the Gac/Rsm pathway, it partially suppresses the Hrp regulon and reduces virulence (Fig. 1) whereas, under very low or deficient activity, it abolishes virulence. In both cases, *rsmB* appears as the central regulator, which is under rigorous control by GacS/A and CsrD of the Gac/Rsm pathway. In *E. coli*, the CsrD was proposed to function by binding to sRNA molecules such as *rsmB* and convert them into substrates for RNaseE degradation (Suzuki et al. 2006). The *csrD* mutant in the present study increased the expression of *rsmB* by 1.75 times while overexpression of *rsmB* in the wild type and in the *csrD* mutant substantially elevated its transcription 5 and 11 times, respectively (Fig. 7). These results could imply a dose effect; namely, the expression of *csrD* in the wild-type strain is limited to bind and neutralize the *rsmB* present at physiological concentration but not when artificially overexpressed.

A linkage between IAA, global regulatory circuits, and the Hrp regulon has been previously demonstrated (Chalupowicz et al. 2008, 2009, 2013). The IAA originating from the pathogen

Fig. 8. Schematic model describing the global virulence regulation of *Pantoea agglomerans* pv. *gypsophila*. The transcription factor PagR of the quorum-sensing (QS) system is the first step in the regulatory hierarchy that simultaneously activates the hypersensitive response and pathogenicity (*hrp*) regulatory cascade and the Gac/Rsm pathway via activation of *gacA*. Transcriptional activation by PagR is signal dependent and requires active PagI. The Gac/Rsm pathway also manipulates the transcription of the *hrp* cascade by controlling RsmA activity via rigorous regulation of *rsmB*. RsmA activity is essential for expression of the *hrp* cascade genes, presumably due to its negative regulation of a hypothetical regulator that can effectively inhibit the transcription of the *hrp* regulatory genes cascade upon its release from RsmA inhibition. Pathogen-derived indole-3-acetic acid (IAA) upregulates expression of the QS genes, whereas type III effectors encoded by the Hrp regulon induce plant-derived IAA, which is essential for virulence.

upregulated the expression of the QS genes but was not essential for gall formation (Chalupowicz et al. 2009). In contrast, plant-derived IAA, as expressed by PAT, was crucial for virulence and substantially increased upon infection (Chalupowicz et al. 2013). This increase was assumed to be either directly or indirectly mediated by PthG expression and possibly other

T3E (Chalupowicz et al. 2013). Further studies are necessary to unravel these interactions.

A schematic illustration describing the interactions between the QS system, Gac/Rsm pathway, and *hrp* regulatory cascade in relation to IAA produced by the pathogen or the host plant is given in Figure 8. The two major players that control the *hrp*

Table 2. Bacterial strains and plasmids used in this study

Strains, plasmids	Relevant characteristics ^a	Reference or source ^b
Strain		
<i>Escherichia coli</i>		
DH5 α	<i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>gyrA</i> 96	Invitrogen
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1</i> Δ (<i>lac-proAB</i>) <i>mcrA</i> [F' <i>traD36 proAB lacIq lacZ</i> Δ M15]	Winson et al. 1998
S17-1/ λ pir	RK2 <i>tra</i> regulon, <i>pir</i> , host for <i>pir</i> -dependent plasmid (pVIK165)	Simon et al. 1983
<i>Pantoea agglomerans</i> pv. <i>gypsophila</i> 824-1		
Pag 824-1	Pathogenic strain, <i>gypsophila</i> pathovar with spontaneous rifampicin resistance	Manulis et al. 1991
PagMx <i>rsmB</i>	Rif ^r , Cm ^r , marker exchange mutant with chloramphenicol cassette in <i>rsmB</i>	This study
PagMx <i>rsmB/C</i>	Rif ^r , Cm ^r , Tet ^r , <i>rsmB</i> mutant harboring pLAFR3:: <i>rsmB</i> for complementation	This study
Pag <i>rsmAOE</i>	Rif ^r , Km ^r , overexpression of <i>rsmA</i> in Pag824-1	This study
Pag <i>rsmBOE</i>	Rif ^r , Km ^r , overexpression of <i>rsmB</i> in Pag824-1	This study
PagMx <i>gacA</i>	Rif ^r , Cm ^r , marker exchange mutant with chloramphenicol cassette in <i>gacA</i>	This study
PagMx <i>gacA/C</i>	Rif ^r , Tet ^r , Cm ^r , <i>gacA</i> mutant harboring pLAFR3:: <i>gacA</i> for complementation	This study
PagMx <i>gacS</i>	Rif ^r , Km ^r , site directed mutagenesis in <i>gacS</i>	This study
PagMx <i>gacS/C</i>	Rif ^r , Km ^r , Tet ^r , <i>gacS</i> mutant harboring pLAFR3:: <i>gacS</i> for complementation	This study
PagMx <i>csrD</i>	Rif ^r , Km ^r , site directed mutagenesis in <i>csrD</i>	This study
PagMx <i>csr/rsmBOE</i>	Rif ^r , Km ^r , Cm ^r , overexpression of <i>rsmB</i> in <i>csrD</i> mutant	This study
PagMxI	Rif ^r , Spec ^r marker exchange mutant in <i>pagI</i>	Chalupowicz et al. 2008
PagMxR	Rif ^r , Km ^r , marker exchange mutant in <i>pagR</i>	Chalupowicz et al. 2008
Pag Δ IR	Rif ^r , Km ^r , double deletion mutant of <i>pagI</i> and <i>pagR</i>	Chalupowicz et al. 2008
Plasmids		
pBluescript-II KS+	Amp ^r , cloning vector, <i>ColE1</i> ori	Stratagene
pGEM-T-Easy	Amp ^r , cloning vector for PCR products	Promega
pLAFR3	Tet ^r , broad host range cloning vector, <i>IncP-1</i> rixRK2 ⁺ <i>lacZa</i> <i>Tra</i> ⁻ <i>mob</i> ⁺ <i>cos</i>	Staskawicz et al. 1987
pVSP61	Km ^r , pVS1 replicon	Loper and Lindow 1994
pCL1920	Spec ^r , low-copy-number cloning vector	Lerner and Inouye 1990
pAKC1042	Spec ^r , 1.7-kb <i>EcoRI/BamHI</i> fragment containing <i>rsmB</i> _{Pag824-1} in pCL1920	Ma et al. 2001
pMAL-c4X	Amp ^r , MBP fusion protein expression vector	New England Biolabs
pVIK165	Km ^r , suicide plasmid	Kalogeraki and Winans 1997
pBS-1B	Amp ^r , a 1-kb <i>EcoRV</i> fragment containing part of <i>rsmB</i> _{Pag824-1} gene from Fos763	This study
pBS-2.1B	Amp ^r , a 2.1-kb <i>EcoRV</i> fragment containing part of <i>rsmB</i> _{Pag824-1} gene from Fos763	This study
pCL- <i>rsmA</i>	Spec ^r , a 3-kb <i>SacI</i> fragment containing the <i>rsmA</i> _{Pag824-1} gene from Fos768	This study
pBS-1.5- <i>gacA</i>	Amp ^r , a 1.5-kb <i>EcoRV</i> fragment containing part of <i>gacA</i> _{ag824-1} gene	This study
pBS-0.5- <i>gacA</i>	Amp ^r , a 0.5-kb <i>EcoRV</i> fragment containing part of <i>gacA</i> _{Pag824-1} gene	This study
pBS-2- <i>gacS</i>	Amp ^r , a 2-kb <i>PstI</i> fragment containing part of <i>gacS</i> _{Pag824-1} gene	This study
pCL- <i>gacS</i>	Spec ^r , a 10-kb <i>BamHI</i> fragment containing the entire <i>gacS</i> _{Pag824-1} gene	This study
pCL- <i>csrD</i>	Spec ^r , a 2.5-kb <i>HindIII</i> fragment containing the <i>csrD</i> _{Pag824-1} gene	This study
pVIK- <i>rsmB</i> -Cm	Km ^r , Cm ^r , a 2.3-kb <i>XbaI/KpnI</i> fragment of <i>rsmB</i> containing the Cm resistance cassette as a <i>BamHI</i> fragment cloned into pVIK165 for marker exchange mutagenesis	This study
pVIK- <i>csrD</i>	Km ^r , a 0.86-kb <i>SacI/XbaI</i> internal fragment of <i>csrD</i> cloned into pVIK165 for site directed mutagenesis	This study
pVIK- <i>gacS</i>	Km ^r , a 1.39-kb <i>SacI/KpnI</i> internal fragment of <i>gacS</i> cloned into pVIK165 for site directed mutagenesis	This study
pVIK- <i>gacA</i> -Cm	Km ^r , Cm ^r , a 2.2-kb <i>SacI/KpnI</i> fragment of <i>gacA</i> containing the Cm resistance cassette as a <i>BamHI</i> fragment cloned into pVIK165 for marker exchange mutagenesis	This study
pLAFR3- <i>rsmB</i>	Tet ^r , a 1.8-kb <i>BamHI/EcoRI</i> fragment containing <i>rsmB</i> ORF cloned into pLAFR3 for complementation	This study
pLAFR3- <i>gacA</i>	Tet ^r , a 0.9-kb <i>HindIII/EcoRI</i> fragment containing <i>gacA</i> ORF cloned into pLAFR3 for complementation	This study
pLAFR3- <i>gacS</i>	Tet ^r , a 10-kb <i>BamHI</i> fragment containing <i>gacS</i> ORF cloned into pLAFR3 for complementation	This study
pLAFR3- <i>csrD</i>	Tet ^r , a 2.5-kb <i>BamHI</i> fragment containing <i>csrD</i> ORF cloned into pLAFR3 for complementation	This study
pVSP- <i>rsmB</i> OE	Km ^r , a 0.47-kb <i>HindIII/EcoRI</i> fragment containing <i>rsmB</i> ORF cloned into pVSP61 under <i>lac</i> promoter for overexpression	This study
pVSP- <i>rsmB</i> OE-Cm	Km ^r , Cm ^r , a 0.47-kb <i>HindIII/EcoRI</i> fragment containing <i>rsmB</i> ORF cloned into pVSP61 under <i>lac</i> promoter and <i>EcoRI</i> Cm resistance cassette for overexpression in <i>csrD</i> mutant strain	This study
pVSP- <i>rsmA</i> OE	Km ^r , a 0.34-kb <i>HindIII/EcoRI</i> fragment containing <i>rsmA</i> ORF cloned into pVSP61 under <i>lac</i> promoter for overexpression	This study
pMAL- <i>pagR</i>	Amp ^r , ORF of <i>pagR</i> cloned into pMAL-c4X for expression and purification	This study

^a Rif^r, Cm^r, Tet^r, Km^r, Spec^r, and Amp^r indicate resistance to rifampicin, chloramphenicol, kanamycin, tetracycline, spectinomycin, and ampicillin, respectively; ORF = open reading frame.

^b Invitrogen, San Diego, CA, U.S.A.; Stratagene, La Jolla, CA, U.S.A.; Promega, Madison, WI, U.S.A.; New England Biolabs, Beverly, MA, U.S.A.

regulatory cascade are PagR and RsmA. PagR directly elevates the *hrp* regulatory genes and indirectly controls the activity of RsmA by enhancing the expression of the Gac/Rsm pathway through transcriptional activation of *gacA*. Although the QS system affects only the intensity of the *hrp* cascade expression, a minimal activity of RsmA is mandatory for its transcription. A hypothetical regulator is proposed to explain the demand for RsmA. RsmA negatively controls the hypothetical regulator, which completely suppresses the transcription of the *hrp* regulatory genes. Deficiency in RsmA releases the regulator from inhibiting the *hrp* cascade's expression and virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and plant inoculation.

Bacterial strains and plasmids used in this work are listed in Table 2. Wild-type and mutant strains of *P. agglomerans* pv. *gypsophila* were grown in Luria-Bertani (LB) broth or agar

at 28°C, whereas *E. coli* strains were cultured on the same medium at 37°C. Antibiotics were used in the following concentrations: kanamycin (Km), 50 µg/ml; rifampicin, 150 µg/ml; ampicillin (Amp), 150 µg/ml; spectinomycin (Spec), 50 µg/ml; tetracycline (Tet), 15 µg/ml; and chloramphenicol (Cm), 20 µg/ml. All strains were maintained as glycerol stocks at -80°C.

Plant inoculation was performed essentially according to (Lichter et al. 1995). Cuttings of *Gypsophila paniculata* var. *golan* (Danziger Ltd., Bet Dagan, Israel) were inoculated after removing a section of approximately 2 mm from the bottom of the stem and dipped into bacterial suspension of 10⁶ cells/ml. The cuttings were then placed in vermiculite-filled trays for symptoms visualization. The greenhouse temperature was maintained at 22 to 25°C and high humidity was generated by computer-controlled mist sprinklers every 20 min for 10 s. Bacterial colonization of gypsophila cuttings was performed according to Chalupowicz and associates (2009) and expressed as CFU per gram fresh weight.

Table 3. Sequence of primers used in this study

Primer	Sequence (5'→3')	Restriction site	Used for ^a
pcc-gacA1	ATGGACATGAGCATGCCAGGTAT	...	Creating <i>gacA</i> _{Pcc} probe
pcc-gacA2	ACTACTTAACAATGTCTCCGCGG	...	
ea-gacS1	TTTGCTTATCGTCTGATGCG	...	Creating <i>gacS</i> _{Ea} probe
ea-gacS2	TCCAGCACGTCATTAATAATG	...	
ec-csrD1	TTCGGCCTTTGTACGCTG	...	Creating <i>csrD</i> _{E.coli} probe
ec-csrD2	ATCAAGTGGCTGTGAGGA	...	
rsmB-sp	GG <u>ACTAGT</u> CATTGCGGTACCA	<i>SpeI</i>	Cloning an upstream arm for <i>rsmB</i> mutant
rsmB-b1	GGCGGATCCGACGATATCTT	<i>BamHI</i>	
rsmB-b2	CGCGGATCCGGGAGCACTGTTAT	<i>BamHI</i>	Cloning downstream arm for <i>rsmB</i> mutant
rsmB-r	GCGGAATTCATGGCGATCCA	<i>EcoRI</i>	
gacA-x	CGCTCTAGAGTTTAAGGATTATTC	<i>XbaI</i>	Cloning upstream arm for <i>gacA</i> mutant
gacA-b1	CACGGATCCGTGTGAATGGT	<i>BamHI</i>	
gacA-b2	GGCGGATCCGGCCATTTCGAC	<i>BamHI</i>	Cloning downstream arm for <i>gacA</i> mutant
gacA-r	GGCGAATTCATAAGCCACACC	<i>EcoRI</i>	
rsmB-OE-h	GGTAAGCTTTGAGTACCTTGTGC	<i>HindIII</i>	Cloning ORF of <i>rsmB</i> for overexpression
rsmB-OE-r	GCGGAATTCAGCCGTTGCGT	<i>EcoRI</i>	
rsmA-OE-h	GCCAAGCTTTTAATCTTTCAAGGA	<i>HindIII</i>	Cloning ORF of <i>rsmA</i> for overexpression
rsmA-OE-r	GCGGAATTCGAAATGAAAGAT	<i>EcoRI</i>	
gacS-s	AACGAGCTCAGTATTATCAATGAC	<i>SacI</i>	Cloning internal fragment of <i>gacS</i> for site directed mutagenesis
gacS-k	CCGGGTACCAGTCCAGCGTG	<i>KpnI</i>	
gacA-h-comp	CGCAAGCTTTTGAGGTAACAAACGG	<i>HindIII</i>	Cloning ORF of <i>gacA</i> for complementation
gacA-r-comp	CCGGAATTCGGTTTCGGTGTGGT	<i>EcoRI</i>	
csrD-s	AGA <u>GAGCTC</u> ACGCGTATGATG	<i>SacI</i>	Cloning internal fragment of <i>gacS</i> for site directed mutagenesis
csrD-x	CGCTCTAGATCATCACGATTAAC	<i>XbaI</i>	
PagR-R	GGTGAATTCATGAAGGATACATACTA	<i>EcoRI</i>	Cloning ORF of <i>pagR</i> for purification in MBP system
PagR-H	GTAAAGCTTCGTCAGTTGTCCAGCAG	<i>HindIII</i>	
mdhFow	ACTCTGGCGTAACCATTC	...	qRT-PCR of <i>mdh</i>
mdhRev	GGTCAGATCAGCCACTTC	...	
gyrBFow	GGTGAAAGTGCCGGATCCTAA	...	qRT-PCR of <i>gyrB</i>
gyrBRev	TGCTGCTCAACCGTGTGTT	...	
gacSFow	CTCCAATCACAAGCAGAAC	...	qRT-PCR of <i>gacS</i>
gacSRev	CGGCAATTCATCCACATC	...	
gacAFow	TTTGTCGGAAGGGAATTG	...	qRT-PCR of <i>gacA</i>
gacARev	TCTACATCGCCACTGATAT	...	
pthGFow	GGCAGAGCGAACAATTATG	...	qRT-PCR of <i>pthG</i>
pthGRev	AGCGACTATCAGCAATC	...	
rsmAFow	GTGAGTTGGTGAACCCCTCAT	...	qRT-PCR of <i>rsmA</i>
rsmARev	ATCTCTCGCGATGCACAGACACT	...	
rsmBFow	CCAGGATGCTAAGGATACA	...	qRT-PCR of <i>rsmB</i>
rsmBRev	TAATGCTCCCTGCTCTTC	...	
csrDFow	TCTCATCCATATCGGTATCAG	...	qRT-PCR of <i>csrD</i>
csrDRev	CCATTGGTCCAGTTGTTG	...	
hrpSFow	ATCCAGTTACAGCCTCTC	...	qRT-PCR of <i>hrpS</i>
hrpSRev	CTAATCCCAACACAAATCG	...	
hrpYFow	TGAGCGAAGCGGGAAATG	...	qRT-PCR of <i>hrpY</i>
hrpYRev	GGTATTGCGAGTGAATCTGG	...	
hrpLFow	GGTACAGATGACCTGGCTGGAA	...	qRT-PCR of <i>hrpL</i>
hrpLRev	CCAAAAACCCACGTCTCTGG	...	

^a ORF = open reading frame, MBP = maltose-binding protein, and qRT-PCR = quantitative reverse-transcription polymerase chain reaction. Underlined and bold sequences describe restriction sites.

DNA manipulation.

Isolation of genomic DNA from *P. agglomerans* or plasmid DNA from *E. coli*, cloning, ligation, transformation, colony hybridization, Southern or Western blots, and other DNA manipulations were performed according to standard procedures (Ausubel et al. 1995) or as recommended by the supplier. The cloning vectors used in this study are listed in Table 2. DNA fragments were amplified by PCR with *Taq* (Super-Therm polymerase JMR-801; Roche, Mannheim, Germany) and synthetic oligonucleotides (Table 3) were synthesized according to manufacturer's specifications by Sigma-Aldrich (Rehovot, Israel). The PCR was performed directly from bacterial colonies. Amplification was carried out in Biometra thermo cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.) as previously described (Chalupowicz et al. 2008).

The *E. coli* and *P. agglomerans* strains were transformed by electroporation with Gene Pulser apparatus (Bio-Rad Laboratories) according to manufacturer's instructions. Triparental mating was performed with the *E. coli* helper plasmid pRK2073 (Spec^r) on LB agar plates as described elsewhere (Ditta et al. 1980; Manulis et al. 1998).

DNA sequencing was performed in the Instrumentation and Services Center at Tel-Aviv University.

Isolation of the Gac/Rsm genes.

A probe for isolating the *gacS* from *P. agglomerans* pv. *gypsophila* (strain Pag824-1) was obtained by PCR amplification of the *barA* gene (*gacS* homolog) of *Erwinia amylovora* CFBP1 (accession number FN434113) using primers ea-*gacS*1 and ea-*gacS*2 (Table 3). The obtained 0.47-kb fragment was DIG labeled to create the *gacS*_{Ea} probe. Total genomic DNA of Pag 824-1 was digested with several restriction enzymes following by Southern blot hybridization under low-stringency conditions with the *gacS*_{Ea} probe. The *gacS*_{Pag824-1} gene was located in a 2-kb DNA fragment of the *Pst*I digestion. Subsequently, the genomic DNA of Pag824-1 was digested with *Pst*I and the 2-kb fragment was extracted from the gel, ligated into pBluescript-II KS+ (Stratagene, La Jolla, CA, U.S.A.), transformed into *Escherichia coli* DH5 α , and screened for positive *gacS*_{Pag824-1} via colony hybridization. A positive subclone, designated as pBS-2-*gacS* (Table 2), was sequenced. The obtained 2-kb sequence was analyzed by open reading frame (ORF) finder but only a partial ORF was revealed. To complete the gene sequence, a 10-kb fragment of the *Bam*HI digestion was cloned into the low-copy-number plasmid pCL1920 to create pCL-*gacS* (Table 2) and the rest of the gene sequence was obtained (ORF of 2,730 bp) and characterized.

The strategy described above for isolation of *gacS*_{Pag824-1} was also employed for the isolation of *gacA*_{Pag824-1}, *csrD*_{Pag824-1}, *rsmB*_{Pag824-1}, and *rsmA*_{Pag824-1} and described in Table 2 as pBS-1B, pBS-2.1B, pCL-*rsmA*, pBS-1.5*gacA*, pBS-0.5*gacA*, pBS-2*gacS*, pCL-*gacS*, and pCL-*csrD*. The homologous gene from *Pectobacterium carotovorum* subsp. *carotovorum* (accession number X95564) and the PCR primers pcc-*gacA*1 and pcc-*gacA*2 (Table 3) were used for probe isolation of *gacA*_{Pag824-1} whereas the homologous gene from *E. coli* K12 (accession number ECK3240) and the PCR primers ec-*csrD*1 and ec-*csrD*2 were employed for probe isolation of *csrD*_{Pag824-1}. Final isolation and characterization of the ORF with promoter regions was performed from total DNA extract of Pag824-1. The isolation and characterization of the *rsmA* gene was previously described (Chalupowicz et al. 2009) and a similar procedure was employed for isolation of *rsmB*. A fosmid library of Pag 824-1 was screened by colony hybridization under high stringency with a 1.7-kb probe obtained from plasmid pAKC1042 digested with *Eco*RI/*Bam*HI. This plasmid contained the *rsmB* gene from *Pantoea agglomerans* pv. *gypsophila* PD713 (Ma et al. 2001).

Positive clones were then digested with *Eco*RV and analyzed by Southern blot hybridization using the same probe. Only a single band of sRNA could be observed in gel electrophoresis. The *rsmB* was localized in two positive fragments of 1 and 2 kb, which were cloned into *Eco*RV sites of pBluescript-II KS+ for sequencing and characterization (as described above).

Construction of mutants.

The strategy described by Kalogeraki and Winans (1997) was employed for generating marker exchange mutants of the genes that constitute the Gac/Rsm pathway. In order to generate a *gacS* mutant, a central fragment of *gacS*_{Pag824-1} was amplified with primers *gacS*-s and *gacS*-k (Table 3) followed by digestion with *Sac*I and *Kpn*I to create a 1.39-kb fragment. This fragment was cloned into pVIK165 (Km^r) predigested with the same enzymes to yield pVIK-*gacS*. The resultant plasmid, which carried the replication origin of the plasmid R6K without its *pir* gene (Kalogeraki and Winans 1997), was replicated in *E. coli* S17-1/ λ *pir*. Mutants designated as Mx*gacS* (Table 2) which were generated following electroporation of pVIK-*gacS* into Pag824-1 were selected on LB plates amended with Km. Mutations were verified by Southern blot analysis using *gacS*_{Pag824-1} as a probe. Complementation of the mutant was achieved by a 10-kb fragment harboring the entire ORF of *gacS*_{Pag824-1} that was cloned into the *Bam*HI site of pLAFR3 to create pLAFR3-*gacS* (Table 2). The latter plasmid was introduced by triparental mating into PagMx*gacS* and selected on plates containing Tet and Km.

The above-described procedure was essentially employed for obtaining marker exchange mutants of *gacA*, *rsmB*, and *csrD* as follows. For generating a *gacA* mutant, an internal fragment of *gacA*_{Pag824-1} was constructed by amplifying a 453-bp fragment from the beginning of the ORF using primers *gacA*-x and *gacA*-b1 and a 828-bp fragment from the end of the gene using primers *gacA*-b2 and *gacA*-r (Table 3). The two fragments were cloned into *Xba*I and *Eco*R sites of pBS, respectively; and, subsequently, a chloramphenicol-resistant (Cm^r) cartridge was inserted in the *Bam*HI site located between the two fragments. The pBS was then digested with *Sac*I and *Kpn*I to yield a 2.2-kb construct, which was subcloned for mutagenesis into the *Kpn*I/*Sac*I sites of the suicidal vector pVIK165 to yield the plasmid pVIK-*gacA*-Cm (Table 2). To obtain the *rsmB* mutant, an 878-bp fragment was amplified from the beginning of *rsmB*_{Pag824-1} gene using the primers *rsmB*-sp and *rsmB*-b1, whereas a second fragment of 504 bp was amplified from the end of the gene using primers *rsmB*-2 and *rsmB*-r (Table 3). The two fragments were cloned into the *Spe*I and *Eco*RI sites of pBS and, subsequently, a Cm^r (925-bp) cartridge was inserted into the *Bam*HI site of *rsmB*. The entire 2.3-kb construct was subcloned into *Xba*I/*Kpn*I sites of pVIK165 to yield the plasmid pVIK-*rsmB*-Cm (Table 2) for mutagenesis. The *csrD* mutant was constructed by PCR amplification of an internal fragment of the gene (860 bp) using the primers *csrD*-S and *csrD*-X (Table 3) harboring the restriction sites *Sac*I and *Xba*I, respectively. After digestion with the appropriate restriction enzymes, the resultant fragment was cloned into pVIK165 to yield the plasmid pVIK-*csrD* (Table 2) for insertional mutagenesis. pVIK-*gacA*-Cm, pVIK-*rsmB*-Cm, and pVIK-*csrD* were mobilized into Pag824-1 by triparental mating. The generated mutants (namely, Mx*gacA*, Mx*rsmB*, and Mx*csrD*) were selected and confirmed by Southern blots. Complementation of each mutant was performed by introducing *in trans* the undisrupted gene cloned to pLAFR3 as described for *gacS* (pLAFR3-*rsmB*, pLAFR3-*gacA*, and pLAFR3-*csrD*).

Overexpression mutants of *rsmA*_{Pag824-1} (*rsmAOE*) and *rsmB*_{Pag824-1} (*rsmBOE*) were generated by cloning their respective ORFs into the plasmid pVSP61 under its *lac* promoter. A

340-bp fragment containing the entire ORF of *rsmA* was PCR amplified with primers *rsmA*-OE-h and *rsmA*-OE-r (Table 3), which contained the *Hind*III and *Eco*RI restriction sites, respectively. Following sequence confirmation, the fragment was cloned into the *Hind*III/*Eco*RI sites of pVSP61 to yield pVSP-*rsmA*OE. *rsmB* overexpression was created in a similar manner by PCR amplification of its 470 bp using the primers *rsmB*-OE-h and *rsmB*-OE-r (Table 3) containing the *Hind*III and *Eco*RI restriction sites, respectively. Following sequence confirmation, it was cloned into pVSP61 via the same restriction sites to obtain pVSP-*rsmB*OE. The two vectors were then mobilized separately into the wild-type strain by triparental mating. Southern blot was used for confirmation of overexpression of both genes. Overexpression of *rsmB* in the background of *MxcsrD* (*rsmB*OE/*MxcsrD*) was generated by introducing a *Cm^r* cartridge into an *Eco*RI site of pVSP-*rsmB*OE, to replace its *Km^r*. The *csrD* mutant was introduced into *rsmB*OE by triparental mating and verified by Southern blot analysis.

qRT-PCR analysis.

The procedure used for qRT-PCR analysis was essentially as described by Chalupowicz and associates (2008). Total RNA was extracted from mutant strains of *P. agglomerans* pv. *gypsophillae* from inoculated gypsophila cuttings after 8, 16, 24, and 48 h at 25 to 28°C by MasterPure RNA Purification Kit (Epicentre Biotechnologies Madison, WI, U.S.A.). RNA quantification was performed with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Epsom, Surrey, U.K.). To remove residual genomic DNA, the isolated RNA was treated with DNase (Turbo DNase-free; Ambion Inc., Austin, TX, U.S.A.). Reverse transcription was carried out from 1 µg of total RNA using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, U.S.A.). The resultant cDNAs were subjected to PCR amplification with Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, U.K.) in a final volume of 20 µl. The specific primers for qRT-PCR were designed by the Primer Express 3.0 program (Applied Biosystems) according to the sequence of the corresponding genes. The primer pairs as well as the internal normalization control genes, DNA gyrase subunit B (*gyrB*), and malate dehydrogenase (*mdh*) of *Pag824-1* are presented in Table 3. Real-time detection was performed in triplicates with a 7300 Real Time PCR System (Applied Biosystems Instrument, Foster City, CA, U.S.A.).

The values from inoculated plants at inoculation time were used as control for expression of the regulatory genes. Relative quantification and statistical analysis were performed with the real-time PCR data analysis software (Applied Biosystems Instrument). The results represent the mean of three different experiments with five replications per time point.

Gel shift assay.

Overexpression and purification of PagR for DNA binding studies were performed by the pMAL Protein Fusion and Purification System (New England Biolabs Inc., Ipswich, MA, U.S.A.). The coding region of *pagR* was PCR amplified from *Pag824-1* using the primers *PagR*-H and *PagR*-R (Table 3). The latter primer included the common ATG start codon for replacing the native GTG of *pagR* in order to facilitate translation. The obtained 680-bp fragment was digested with *Eco*RI and *Hind*III and cloned into a pMAL-c4X expression vector to yield pMAL-*pagR* (Table 2). *E. coli* (JM109) harboring pMAL-*pagR* was grown in LB medium supplemented with Amp and glucose (0.2%) to repress the maltose genes of the host. IPTG was added to the culture when growth reached an absorbance at 600 nm of approximately 0.5 and grown for an additional 3 h prior to harvesting by centrifugation. The resultant MBP-PagR was purified by a small-scale batch method

using amylose resin as described by the manufacturer. Crude extracts and purified MBP-PagR protein were analyzed on 10% (wt/vol) sodium dodecyl sulfate polyacrylamide gel electrophoresis gel stained with Coomassie blue. The MBP-β-gal-α protein produced by *E. coli* JM109 harboring the empty vector pMAL-c4X was used as a negative control.

A gel mobility shift assay was performed by the DIG Gel Shift Kit (second generation; Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. Synthetic DNA segments of 50 bp containing the promoter regions of *hrpL*, *hrpS*, *hrpXY*, and *gacA* (Table 1) were end-labeled with DIG with terminal transferase and were used as a probe for gel shift analysis. The interaction between the DNA fragments and MBP-PagR fusion protein was assayed using the binding buffer (100 mM Hepes [pH 7.6], 5 mM EDTA, 50 mM [NH₄]₂SO₄, 5 mM dithiothreitol, 1% [wt/vol] Tween 20, and 150 mM KCL) with or without addition of synthetic C₄-HSL purchased from Sigma-Aldrich. The reaction mixtures were incubated at room temperature for 15 min and resolved on a nondenaturing 5% polyacrylamide gel. Further steps—namely, transfer of DNA to a positively charged nylon membranes (Hybond-N⁺; Amersham Biosciences, Little Chalfont, U.K.) and development of the membrane—were carried out following the instructions of the manufacturer.

Bioinformatics procedures.

DNA and protein sequence homology searches (e.g., blastn, blastx, and PSI blast) were carried out with the National Center for Biotechnology Information (NCBI) Blast program. ORF determination and analysis against protein data base was done with the NCBI ORF Finder. The alignments of the amino acid sequences and DNA restriction sites were carried out by the CLC Sequence Version 6.1. Alignment of DNA sequences was performed with GCG-Genetic Computer Group (Program Manual for the Wisconsin package).

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