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The *Pseudomonas chlororaphis* PCL1391 Sigma Regulator *psrA* Represses the Production of the Antifungal Metabolite Phenazine-1-Carboxamide

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The rhizobacterium *Pseudomonas chlororaphis* PCL1391 produces the antifungal metabolite phenazine-1-carboxamide (PCN), which is a crucial trait in its competition with the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* in the rhizosphere. The expression of the PCN biosynthetic gene cluster in PCL1391 is population density–dependent and is regulated by the quorum-sensing genes *phzI* and *phzR* via synthesis of the autoinducer *N*-hexanoyl-L-homoserine lactone (C_6-HSL). Here, we describe the identification of an additional regulatory gene of PCN biosynthesis in PCL1391. A mutation in the *psrA* gene (*Pseudomonas* sigma regulator), the gene product of which is a member of the TetR/AcrR family of transcriptional regulators, resulted in increased production of autoinducer molecules and PCN. Expression studies showed that inactivation of *psrA* resulted in increased expression of the *phzI* and *phzR* genes and the *phz* biosynthetic operon and that introduction of functional copies of *psrA* represses the expression of these genes, resulting in reduced production of autoinducer signal and PCN. Surprisingly, inactivation of *psrA* in the *phzI* or *phzR* quorum-sensing mutants, which do not produce detectable amounts of PCN and autoinducers by themselves, restored PCN biosynthesis. This phenomenon was accompanied by the appearance of compounds with autoinducer activities migrating at the positions of C_4-HSL and C_6-HSL on C18 reverse phase–thin-layer chromatography. These observations indicate that PsrA also represses at least one silent, yet unidentified, quorum-sensing system or autoinducer biosynthetic pathway in PCL1391. The expression of *psrA* declines at the onset of the stationary phase at the same moment at which quorum-sensing (-regulated) genes are activated. In addition, expression studies in a *psrA*$^-$ and a multicopy *psrA* background showed that *psrA* is autoregulated. Multiple copies of *psrA* repress its own expression. Mutation of *gacS*, encoding the sensor kinase member of a two-component global regulatory system significantly reduced production of autoinducers and PCN. We show a novel link between global regulation and quorum sensing via the PsrA regulator.

Additional keywords: biocontrol, tomato foot and root rot.

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Pseudomonas chlororaphis PCL1391 exhibits biocontrol activity of tomato foot and root rot caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *radicis-lycopersici* (Jarvis and Shoemaker 1978). The production of the antifungal metabolite phenazine-1-carboxamide (PCN) is crucial for this beneficial activity (Chin-A-Woeng et al. 1998). In addition, PCL1391 produces hydrogen cyanide (HCN), chitinase, and protease activity. PCN belongs to the class of phenazines, a class of heterocyclic antifungal compounds with broad spectrum activity, to which pyocyanin and phenazine-1-carboxylic acid also belong (Turner and Messenger 1986). The PCN biosynthetic operon was identified previously (Chin-A-Woeng et al. 2001a), and we have shown that expression of the biosynthetic operon is under regulation of quorum sensing (Chin-A-Woeng et al. 2001b). In gram-negative bacteria, this cell-cell communication system relies on diffusible *N*-acylhomoserine lactone (*N*-AHL) signal molecules to monitor the size of its population (Bassler 2002; Bauer and Coplin 2003; Loh 2002; Newton and Fray 2004; Swift et al. 2001; Von Bodman et al. 2003; Winzer et al. 2002). Quorum sensing is usually based on the action of two proteins that belong to the LuxI and LuxR family of two-component regulatory systems (Latifi et al. 1995; Throup et al. 1995). LuxI homologs synthesize an *N*-acyl-L-homoserine lactone signal that can diffuse through the cell envelope (Hanzelka and Greenberg 1996). A transcriptional regulator, a LuxR homolog, is activated by its cognate population density–dependent *N*-AHL signal molecule (Zhu and Winans 1999). In addition, the signal is often amplified by an auto-regulatory loop through which the autoinducer synthase is positively regulated (Salmond et al. 1995). The *luxI* and *luxR* homologs *phzI* and *phzR* are essential components in regulating phenazine production and are conserved in three genetically characterized phenazine-producing Pseudomonas biocontrol strains (Chin-A-Woeng et al. 2001b; Mavrodi et al. 1997; Wood et al. 1997). The genes are needed for the production of the autoinducer signal *N*-hexanoyl-L-homoserine lactone (C_6-HSL) and, consequently, for the production of PCN in PCL1391 (Chin-A-Woeng et al. 2001b).

In this paper, we describe the identification and characterization of *psrA*, which is shown to be involved in the repression of PCN production in *P. chlororaphis* PCL1391. Subsequently, the relation of *psrA* to the expression of the quorum-sensing genes *phzI* and *phzR*, *N*-AHL production, and its own regulation was studied. In addition, the effect of the *gacS*/*gacA* global regulatory system on *psrA* gene expression and related downstream effects in PCL1391 was determined, showing that both PsrA and the GacS/GacA regulatory system, either together or alone, are important regulators of phenazine production.

RESULTS

Isolation and genetic characterization of *P. chlororaphis* PCL1391 mutants affected in PCN biosynthesis.

In a screening on agar plates and in liquid cultures of 18,000 PCL1391 Tn*5luxAB* transconjugants, mutant PCL1111 was selected for its stronger blue-green pigmentation, which is indicative for an increased PCN production. The chromosomal regions flanking the Tn*5luxAB* insertion of PCL1111 were recovered in plasmid pMP6005 (Table 1). Analysis of 3.6 kb of the nucleotide sequence flanking the transposon (Fig. 1) revealed the Tn*5* transposon insertion in a 708-bp gene encoding a deduced 236-amino acid protein with 89% identity to PsrA (*Pseudomonas* sigma regulator) (Table 2) of *P. putida* WCS358 (Kojic and Venturi 2001). The predicted PsrA protein from strain PCL1391 has a conserved helix-turn-helix motif in its N-terminus. Based on the localization and similarity of the helix-turn-helix motif, PsrA is grouped together with the TetR/AcrR family of bacterial transcriptional regulators (Prosite accession number PS01081), which also includes EnvR from *Escherichia coli*, MtrR from *Neisseria gonorrhoeae*, and TcmR from *Streptomyces glaucescens*. A PsrA-binding site CAAACAAGTGTT TG matching the palindromic consensus C/GAAACN$_{2-4}$GTT TG/C (Kojic et al. 2002) was identified in the promoter region of the *psrA* gene of strain PCL1391 at nucleotide positions –16 to –30 bp, relative to the ATG codon. A very similar sequence

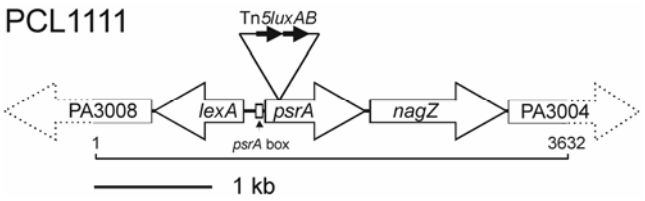

Fig. 1. Schematic representation of the Tn*5* insertion in the *Pseudomonas chlororaphis* PCL1391 mutant derivative PCL1111, as revealed by nucleotide sequencing of the Tn*5* flanking regions. Location of the Tn*5*-transposon insertion, direction of the *luxAB* reporter genes on the transposon, and putative *psrA* binding site are indicated. *lexA*, gene encoding LexA repressor protein; *psrA*, *Pseudomonas* sigma regulator; *nagZ*, β-*N*-acetyl-D-glucosaminidase; other gene names refer to the gene symbols as annotated in the *Pseudomonas aeruginosa* PAO1 genome (Stover et al. 2000). Black arrows indicate the *luxAB* genes of the Tn*5* transposon.

Table 1. Microorganisms and plasmids

Strains and plasmids	Relevant characteristics	Reference or source
Bacterial strains		
PCL1391	Wild-type *Pseudomonas chlororaphis*, producing phenazine-1-carboxamide and biocontrol strain of tomato foot and root rot caused by *Fusarium. oxysporum* f. sp. *radicis-lycopersici*	Chin-A-Woeng et al. 1998
PCL1392	*lacZ*-tagged derivative of PCL1391 with the same root-colonising ability as the wild type	Chin-A-Woeng et al. 1998
PCL1103	PCL1391 *phzI*::Tn5luxAB	Chin-A-Woeng et al. 2001b
PCL1104	PCL1391 *phzR*::Tn*5luxAB*	Chin-A-Woeng et al. 2001b
PCL1111	PCL1391 *psrA*::Tn*5luxAB*	This study
PCL1119	PCL1391 *phzB*::Tn*5luxAB*	Chin-A-Woeng et al. 1998
PCL1123	PCL1391 *gacS*:: Tn*5luxAB*	This study
PCL1138	PCL1123 derivative, *gacS*::Tn*5luxAB*, *psrA*$^-$	This study
PCL1139	PCL1111 derivative, *psrA*::Tn*5luxAB*, *gacS*$^-$	This study
PCL1140	PCL1103 derivative, *phzI*::Tn*5luxAB*, *psrA*$^-$	This study
PCL1142	PCL1104 derivative, *phzR*::Tn*5luxAB*, *psrA*$^-$	This study
PCL1144	PCL1119 derivative, *phzB*::Tn*5luxAB*, *psrA*$^-$	This study
PCL1146	PCL1103 derivative, *phzI*::Tn*5luxAB*, *gacS*$^-$	This study
PCL1148	PCL1104 derivative, *phzR*::Tn*5luxAB*, *gacS*$^-$	This study
PCL1150	PCL1119 derivative, *phzB*::Tn*5luxAB*, *gacS*$^-$	This study
PCL1186	PCL1391, *psrA*$^-$	This study
PCL1187	PCL1391, *nagZ*$^-$	This study
PCL1196	PCL1111 harboring pMP6579	This study
PCL1197	PCL1119 harboring pBBR1MCS-5	This study
PCL1198	PCL1119 harboring pMP6579	This study
DH5α	*endA1 gyrA96 hrdR17*(*rK*$^-$- *mK*$^-$) *supE44 recA1*; general purpose *Escherichia coli* host strain used for transformation and propagation of plasmids	Hanahan 1983
CV026	*Chromobacterium violaceum N*-acylhomoserine lactone reporter strain	McClean et al. 1997
Plasmids		
pRL1063a	Plasmid harboring Tn*5* transposon containing promoterless *luxAB* and a kanamycin resistance marker	Wolk et al. 1991
pIC20H	General-purpose cloning vector with a carbenicillin resistance (Cb^r) marker	Marsh et al. 1984
pIC20R	General-purpose cloning vector with a Cb^r marker	Marsh et al. 1984
pBBR1MCS5	General-purpose cloning vector with a gentamycin resistance marker	Kovach et al. 1995
pMP5000	pIC20H with the tetracycline resistance (Tc^r) cassette from pWTT2081 in the multicloning site, Tc^r, Cb^r	Van der Bij et al. 1996
pME6010	*E. coli*/*Pseudomonas* shuttle vector, stably maintained in *Pseudomonas* species without antibiotic pressure, Km^r	Heeb et al. 2000
pMP6005	pRL1063a-based plasmid recovered from chromosomal DNA of PCL1111 after digestion with *Eco*RI	This study
pMP6015	pIC20R with a *Kpn*I/*Eco*RI 0.5-kb internal PCR fragment of *psrA* and a tetracycline marker from pMP5000 inserted in the multicloning site used to construct strains PCL1138, PCL1140, PCL1142, PCL1144, and PCL1186.	This study
pMP6016	pIC20R with a *Kpn*I/*Eco*RI 0.3-kb internal PCR fragment of *gacS* and a tetracycline marker from pMP5000 inserted in the multicloning site used to construct strains PCL1139, PCL1146, PCL1148, and PCL1150.	This study
pMP6049	pIC20H-derived plasmid harboring an internal PCR fragment of the *nagZ* gene of PCL1391 and a tetracycline cassette from pMP5000, used as a *Pseudomonas* suicide construct for homologous recombination to construct PCL1187.	This study
pMP6579	pBBR1MCS5-derived plasmid harboring the *psrA* gene and promoter region of PCL1391.	This study

GAAACTGCACTTTG was also identified in the promoter region of *rpoS* in strain PCL1391 (G. Girard, *unpublished data*). Analysis of the *phzI* and *phzR* promoter regions did not reveal an indication for the presence of a *psrA* binding site.

The *psrA* gene is the first gene of an operon consisting of at least three genes (Fig. 1). The order and identity of genes thus far analyzed is the same as in *P. aeruginosa* PAO1 (Stover et al. 2000), *P. putida* KT2440 (Nelson et al. 2002), and *P. syringae* DC3000 (Buell et al. 2003). Downstream of *psrA*, spaced by a 214-bp intergenic region, a 999-bp gene with 82% identity to the *nagZ* gene of *P. aeruginosa* PAO1 is located. *nagZ* encodes a β-*N*-acetylglucosaminidase with a role in peptidoglycan synthesis and recycling. This gene is followed by a partially analyzed open reading frame with homology to a sequence encoding a putative nucleoside phosphorylase (homologous to gene PA3004 of *P. aeruginosa* PAO1; Fig. 1). Introduction of a mutation in the *nagZ* gene by single homologous recombination with plasmid pMP6049 (Table 1), containing a 798-bp *nagZ* internal fragment, resulted in mutant PCL1187, which appeared to display the same phenotype with respect to the production of *N*-AHLs and PCN as the wild type (data not shown). Therefore, the phenotype of the *psrA*::Tn*5* mutant cannot be explained by polar effects of the transposon insertion on genes downstream of *psrA*. The divergently transcribed gene upstream of *psrA* (Fig. 1) is homologous with the *lexA* genes of *P. aeruginosa* (90%), *P. putida* (87%) (Garriga et al. 1992), and *E. coli* (41%) (Brent and Ptashne 1981; Calero et al. 1993; Little et al. 1981).

Characterization of the PCN-overproducing *psrA* mutant.

Quantification of PCN by high-performance liquid chromatography (HPLC) analysis showed that PCL1111 exhibited an up to tenfold overproduction of PCN (0.2 and 1.7 g of PCN per liter of King's medium B (KB) growth medium after 16 and 72 h of growth, reaching an optical density at 620 nm (OD_{620}) of 3 and 12, respectively; Table 3) as compared with the wild type (0.06 and 0.15 g of PCN per liter of growth medium at an OD_{620} of 3 and 12, respectively). Introduction of a *psrA* mutation in the *lux* reporter strain PCL1119 (*phzB*::Tn*5luxAB*) using pMP6015 resulted in strain PCL1144, which exhibited an earlier and a fivefold higher expression of the *phz* operon than its parental strain PCL1119 (Fig. 2A). In addition, a 2.5-fold increased production of C_6-HSL in *psrA* mutant PCL1111 (17.5 nM) in comparison with wild-type PCL1391 (7.0 nM) was detected in the growth medium (retardation factor (R_f) = 0.36) (Fig. 3A, lanes 1 and 5). The activity detected at the R_f value of 0.57 observed for PCL1111 (Fig. 3A, lane 5) was only detected when larger amounts of culture supernatant extract of the wild type were analyzed and has the same R_f value as synthetic C_4-HSL (Chin-A-Woeng et al. 2001b).

Strain PCL1111 (*psrA*::Tn*5luxAB*) was not altered in the production of HCN, protease, and chitinase, as compared with its wild type (Table 2). Motility and its ability to colonize the tomato root system [4.1 ± 0.3 $\log_{10}$ (PCL1111 CFU + 1)/cm of root tip] in competition with the reference strain PCL1392, a *lacZ*-tagged derivative of the wild-type strain PCL1391, which is not impaired in colonization [3.9 ± 0.4 $\log_{10}$ (PCL1392 CFU + 1)/cm of root tip], were not affected after seedling inoculation. Since PCN production was elevated in strain PCL1111, this strain was tested for its efficiency to control tomato foot and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici*. Although PCL1111 was found to suppress tomato foot and root rot better than the wild type (e.g., 41 vs. 49% diseased plants, respectively) in three bioassays performed, the differences were not statistically significant (data not shown).

An independently constructed *psrA* mutant, strain PCL1186, made by homologous recombination, using a pIC20R-derived suicide construct containing a tetracycline resistance cassette and a 600-bp internal fragment of the *psrA* gene (pMP6015), displayed the same phenotypic characteristics as did PCL1111 (*psrA*::Tn*5luxAB*) (Table 3).

Influence of a *psrA* mutation on autoinducer and PCN production.

Transcriptional fusions of the *luxAB* genes with *phzI* in strain PCL1103 (*phzI*::Tn*5luxAB*) and with *phzR* in PCL1104 (*phzR*::Tn*5*l*uxAB*) allowed us to monitor the expression of these genes. Due to the insertional inactivation, these reporter strains do not produce detectable amounts of autoinducers (Fig. 3A, lanes 2 and 3; Table 3) and PCN (Chin-A-Woeng et al. 2001b). To characterize the influence of a *psrA* mutation on

Table 2. Characteristics of *Pseudomonas chlororaphis* PCL1391 and transposon derivatives

	Bacterial strains[a]		
Traits	**PCL1391**	**PCL1111**	**PCL1123**
Tn*5luxAB* inserted gene	none	*psrA*	*gacS*
PCN production[b]	+	++++	–
Antifungal activity[c]	+	+	–
Autoinducer production	+	++	±
HCN production	+	+	+
Protease production	+	+	–
Lipase production	+	+	+
Chitinase production	+	+	–
Motility	+	+	+
Tomato root tip colonization[d]	+	+	+

[a] + = wild-type level; ++ = twofold increase; ++++ = tenfold increase; – = absent, ± = decreased to <0.1 nM.
[b] Phenazine-1-carboxamide (PCN) production was determined after 72 h of growth
[c] Activity was tested in a petri dish assay for antifungal activity (Geels and Schippers 1983) against *F. oxysporum* f. sp. *radicis-lycopersici.*
[d] Colonizing ability was tested after seedling inoculation in competition with PCL1392, a Tn*5lacZ*-tagged derivative of PCL1391 not impaired in root colonization, in a gnotobiotic sand system (Simons et al. 1996).

Table 3. Production of *N*-hexanoyl homoserine lactone (C_6-HSL) and phenazine-1-carboxamide (PCN) by *Pseudomonas chlororaphis* PCL1391 and mutant derivatives

Strain	**C_6-HSL[a] (nM)**	**PCN[b] (g/liter)**
PCL1391 (wild type)	7	0.15
PCL1111 (*psrA*::Tn*5luxAB*)	17.5	1.7
PCL1103 (*phzI*::Tn*5luxAB*)	n.d.[c]	n.d.
PCL1104 (*phzR*::Tn*5luxAB*)	n.d.	n.d.
PCL1123 (*gacS*::Tn*5luxAB*)	< 0.1	n.d.
PCL1186 (*psrA*$^-$)	17	1.8
PCL1140 (*phzI*::Tn*5luxAB*, *psrA*$^-$)	5	0.17
PCL1142 (*phzR*::Tn*5luxAB*, *psrA*$^-$)	27.5	0.09
PCL1196 (PCL1111 pMP6579)	6.3	0.1
PCL1198 (PCL1119 pMP6579)	5	n.d.
PCL1146 (*phzI*::Tn*5luxAB*, *gacS*$^-$)	n.d.	n.d.
PCL1148 (*phzR*::Tn*5luxAB*, *gacS*$^-$)	n.d.	n.d.
PCL1150 (*phzB*::Tn*5luxAB, gacS*$^-$)	< 0.1	n.d.
PCL1138 (*gacS*::Tn*5luxAB, psrA*$^-$)	n.d.	n.d.
PCL1139 (*psrA*::Tn*5luxAB*, *gacS*$^-$)	n.d.	n.d.

[a] A 100-ml volume of a 72-h King's medium B culture supernatant of the strains was extracted with dichloromethane, and samples equivalent to 20 ml of culture supernatant were tested in the *Chromobacterium* thin-layer chromatography overlay assay. Concentration of C_6-HSL was calculated from the intensities of spots and a calibration curve of synthetic C_6-HSL.
[b] The cell-free supernatants of culture samples were extracted twice with an equal volume of toluene. PCN was separated by high-performane liquid chromatography, and production in the growth medium was calculated from peak areas using a calibration curve of PCN.
[c] n.d. = not detected

expression of *phzI* and *phzR*, *psrA* was mutated in PCL1103 (*phzI*::Tn*5luxAB*) and PCL1104 (*phzR*::Tn*5luxAB*) by homologous recombination using pMP6015, resulting in strains PCL1140 and PCL1142, respectively. Surprisingly, after mutation of psrA, PCN production was restored to wild-type level in the *phzR* mutant PCL1104 and the *phzI* mutant PCL1103, as was judged by restoration of blue/green pigment production on agar plates (data not shown) and quantification of PCN by C18-reverse phase-HPLC analysis of culture supernatant extracts. Strain PCL1140 (*phzI*::Tn*5luxAB*, *psrA*$^-$) produced 0.17 g of PCN per liter of growth medium, and PCL1142 (*phzR*::Tn*5luxAB*, *psrA*$^-$) produced 0.09 g of PCN per liter of growth medium (Table 3). Moreover, analysis of cell-free spent culture medium of these double mutants using the *Chromobacterium N*-AHL reporter assay showed the production of two compounds with inducer activity. The activities produced by both strains PCL1140 (*phzI*::Tn*5luxAB*, *psrA*$^-$) and PCL1142 (*phzR*::Tn*5luxAB*, *psrA*$^-$) were present at the same positions as C_6-HSL and C_4-HSL (Fig. 3B, lanes 3 and 4). For PCL1140 (*phzI*::Tn*5luxAB*, *psrA*$^-$) and PCL1142 (*phzR*::Tn*5luxAB*, *psrA*$^-$), the strains appeared to produce 5.0 and 27.5 nM C_6-HSL, respectively (Table 3). Furthermore, in contrast to strain PCL1103 (*phzI*::Tn*5luxAB*), *phzI* in PCL1140 (*phzI*::Tn*5luxAB*, *psrA*$^-$) was induced in the absence of exogenously added synthetic C_6-HSL (Fig. 2B). Expression of *phzI* in PCL1140 (*phzI*::Tn*5luxAB*, *psrA*$^-$) i) was induced at the start of the stationary phase (Fig. 2B), ii) reached a basic level higher than that of PCL1103 (*phzI*::Tn*5luxAB*) (Fig. 2B), and iii) reached a

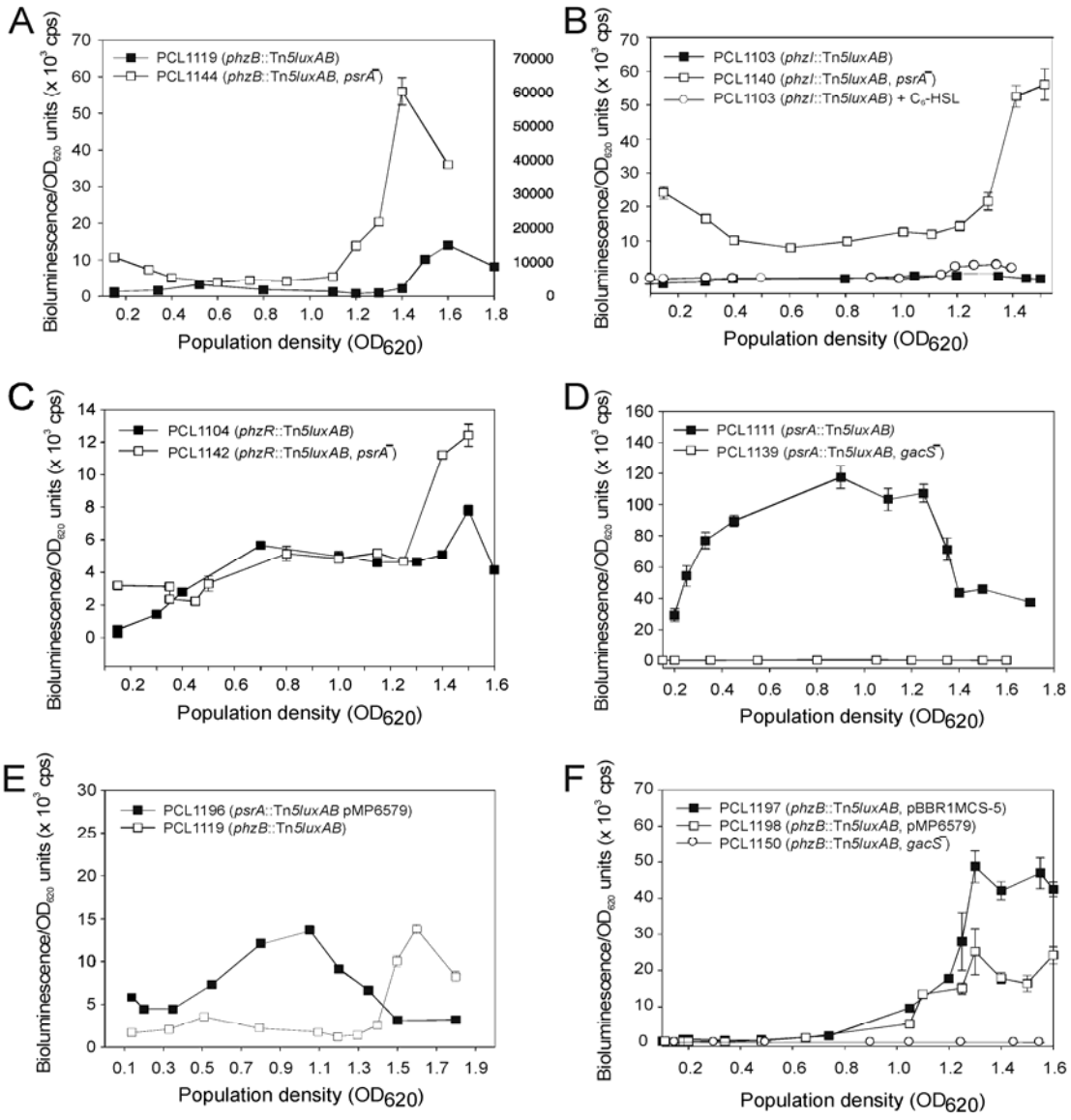

Fig. 2. Expression of the regulatory genes *phzI*, *phzR*, and *psrA*, and of the phenazine-1-carboxamide (PCN) biosynthetic operon of *Pseudomonas chlororaphis* PCL1391 in *psrA* and *gacS* mutant strains. Strains were grown in KB medium. Where indicated, synthetic *N*-hexanoyl-L-homoserine lactone was added to the culture to a concentration of 5 μM at the initial optical density at 620 nm of 0.1. Values depicted in the panels are values for the luminescence measured in counts per seconds (cps) per optical density unit during growth in time. **A** through **C,** Comparison of the expression of the biosynthetic *phzB* (PCL1144, panel A) and the regulatory *phzI* (PCL1140, panel B) and *phzR* genes (PCL1142, panel C) in *psrA*-deficient backgrounds versus in their parental backgrounds (PCL1119, PCL1103, PCL1104, respectively). **D,** Expression of *psrA*::Tn*5luxAB* in a *gacS*-deficient background (PCL1139) versus its *psrA* mutant background (PCL1111). **E,** Expression of *psrA*::Tn*5luxAB* in PCL1196 (PCL1111 harboring a multicopy plasmid pMP6579 with the *psrA* gene). For comparison, the expression of *phzB*::Tn*5luxAB* in PCL1119 is also included. **F,** Expression of the PCN biosynthetic genes in a *psrA* multicopy background (PCL1198) and in a *gacS*-deficient background (PCL1150) versus in its parental background PCL1197 (*phzB*::Tn*5luxAB*, pBBRMCS-5). Data points were sampled in triplicate. If error bars are not indicated, standard errors were too small to visualize in the graph. Expression studies were performed at least twice in independent experiments with similar results.

higher level in the stationary phase than when synthetic C_6-HSL (5 μM) was added to PCL1103 (Fig. 2B). A small positive effect of the *psrA* mutation on the expression of *phzR* was also observed at the onset of the stationary phase in PCL1142 (Fig. 2C) and was consistent with the 1.2-fold increased production of C_6-HSL (Fig. 3B, lane 4; Table 3).

Analysis of *psrA* expression during growth and autoregulation.

Expression of *psrA* during growth, starting at OD_{620} = 0.1, was analyzed, using PCL1111 (*psrA*::Tn*5luxAB*). Expression of *psrA* reached a maximum in the late exponential phase of growth (OD_{620} = 1.2), followed by a rapid decrease (Fig. 2D). The moment of downregulation coincided with the moment at which *phzB* expression was induced (Fig. 2A).

To study the expression of *psrA*::Tn*5luxAB* in a *psrA*-positive background, the complete *psrA* gene, including 199 bp of its putative promoter region, was amplified with PCR and was cloned into pBBR1MCS5, resulting in plasmid pMP6579. Introduction of *psrA* (pMP6579) into PCL1111 (*psrA*::Tn*5luxAB*) reduced PCN production to approximately wild-type levels (Table 3, PCL1196). Similarly, C_6-HSL production was restored to approximate wild-type levels, from 25 to 6.3 nM, in PCL1196 (Fig. 3C, lane 2; Table 3). When the expression of *psrA* was assessed in a *psrA*-positive background using PCL1196, maximal expression (1.4×10^4 counts per second [cps]) (Fig. 2E) was tenfold lower than in the absence of *psrA* (1.2×10^5 cps) (Fig. 2D).

To assess the repressing role of *psrA* on *phz* expression, pMP6579 was also transferred to PCL1119 (*phzB*::Tn*5luxAB*). The characteristic population density–dependent expression of the *phzB* gene was observed, but the overall expression was lower in a *psrA*-multicopy background in PCL1198 as compared with PCL1119 harboring the empty vector (PCL1197) (Fig. 2F). Multiple copies of *psrA* in strain PCL1198 or wild type caused a slightly repressing effect on production of C_6-HSL (5.0 nM) as compared with that in the parental strains carrying the empty control vector (7.0 nM) (Table 3).

***psrA* expression in a *gacS*-deficient background.**

Strain PCL1123 (*gacS*::Tn*5luxAB*) was identified as being impaired in production of PCN, protease, and chitinase (but not HCN and lipase) and, consequently, in the inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* (Table 2). Similarly, *gacS* mutants of *P. aeruginosa* PAO1 (Reimmann et al. 1997) and *P. aureofaciens* 30-84 (Chancey et al. 1999) are disturbed in quorum sensing and phenazine production. The production of autoinducers in PCL1123 (*gacS*::Tn*5luxAB*) is severely reduced (to <0.1 nM) as compared with that in the wild type (Table 3; Fig. 3A, lanes 1 and 4), and expression of the *phz* genes is abolished in the *gacS*-deficient *phzB* reporter strain PCL1150 (Fig. 2F), which is also reflected in *gacS* mutants of the quorum-sensing reporters, since *phzI* expression in the double mutant PCL1146 (*phzI*::Tn*5luxAB*, *gacS*$^-$) cannot be induced by the addition of exogenous C_6-HSL and *phzR* expression is repressed in the *gacS*-deficient *phzR* reporter PCL1148 (data not shown).

To analyze the effect of *gacS* on the expression of *psrA*, the reporter strain PCL1111 (*psrA*::Tn*5luxAB*) was mutated in *gacS* by homologous recombination using plasmid pMP6016, resulting in strain PCL1139. Expression of *psrA* (Fig. 2D) was abolished in PCL1139, showing that the expression of *psrA* is dependent upon the presence of *gacS*. PCN production by PCL1139 was not detected (as judged from HPLC analysis of spent culture medium extracts) (Table 3); neither were C_6-HSL and C_4-HSL detected in extracts of PCL1139 spent culture medium (Fig. 3B, lane 5). The reciprocal mutant PCL1138 (*gacS*::Tn*5luxAB, psrA*$^-$) displays a phenotype identical to PCL1139 (data not shown).

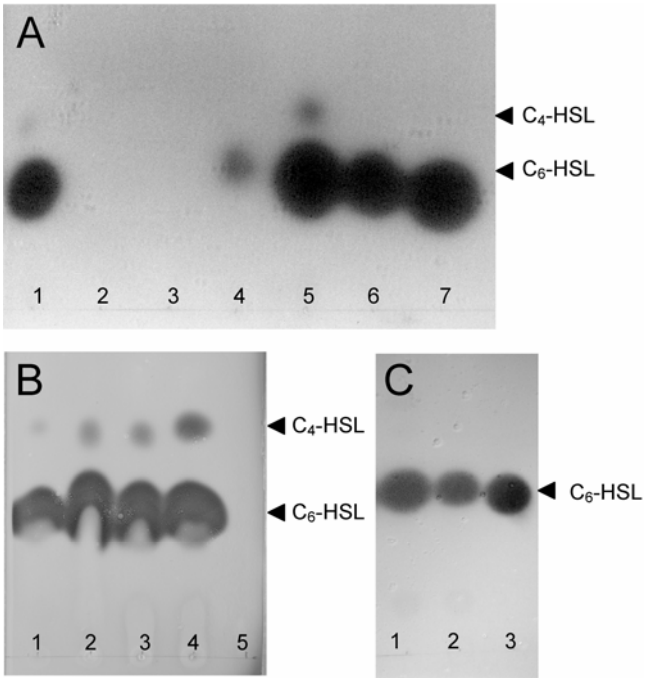

Fig. 3. C18-reverse phase thin-layer chromatography (TLC) analysis of *N*-acyl-L-homoserine lactones produced by *Pseudomonas chlororaphis* PCL1391 and mutant derivatives. A volume of 100 ml of a 72-h King's medium B culture supernatant of the strains was extracted with dichloromethane, and samples equivalent to 20 ml of culture supernatant were tested in the *Chromobacterium* TLC overlay assay. **A,** Autoinducer profiles of regulatory and biosynthetic mutants of *P. chlororaphis* PCL1391. Lane 1, PCL1391 wild type, lane 2, PCL1103 (*phzI*::Tn*5luxAB*); lane 3, PCL1104 (*phzR*::Tn*5luxAB*); lane 4, PCL1123 (*gacS*::Tn*5luxAB*); lane 5, PCL1111 (*psrA*::Tn*5luxAB*); lane 6, PCL1119 (*phzB*::Tn*5luxAB*); lane 7, synthetic *N*-hexanoyl-L-homoserine lactone (C_6-HSL) standard (1×10^{-10} mol). **B,** C_6-HSL production by *Pseudomonas chlororaphis* PCL1391 and *psrA* mutant derivatives. Lane 1, PCL1391 wild type; lane 2, PCL1111 (*psrA*::Tn*5luxAB*); lane 3, PCL1140 (*phzI*::Tn*5luxAB*, *psrA*$^-$); lane 4, PCL1142 (*phzR*::Tn*5luxAB*, *psrA*$^-$); lane 5, PCL1139 (*psrA*::Tn*5luxAB*, *gacS*$^-$). **C,** C_6-HSL production of the complemented *P. ororaphis* PCL1111 (*psrA*::Tn*5luxAB*). Lane 1, PCL1111 (*psrA*::Tn*5luxAB*); lane 2, PCL1111 harboring pMP6579 (*psrA*); lane 3, synthetic C_6-HSL standard (1×10^{-10} mol). Experiments were performed at least twice with similar results.

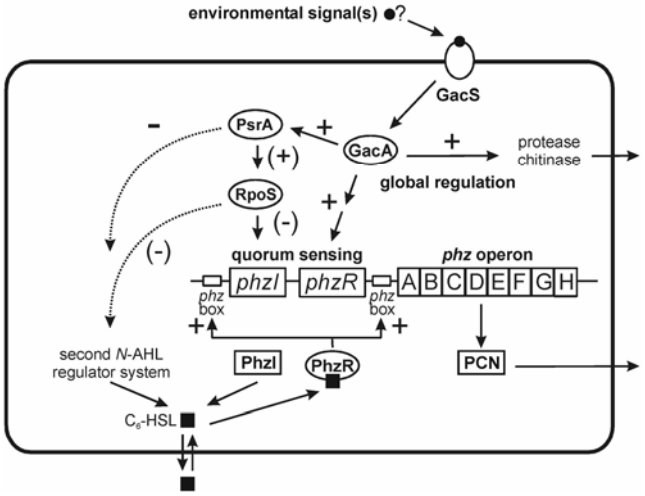

Fig. 4. Model for the regulation of the phenazine (*phz*) biosynthetic operon in *Pseudomonas chlororaphis* PCL1391. Abbreviations: A to H, *phzA* to *phzH* genes; C_6-HSL, *N*-hexanoyl- L -homoserine lactone; *N*-AHL, *N*-acyl-L-homoserine lactone; PCN, phenazine-1-carboxamide.

DISCUSSION

Production of secondary metabolites, including phenazine antibiotics, in *Pseudomonas* spp. appears to be controlled by complex regulation systems, including quorum sensing and a two-component global regulatory GacS/GacA system (Chancey et al. 1999; Reimmann et al. 1997). Other regulators of phenazine production in *P. aeruginosa* PAO1 include RsmA/RsmZ (regulator of secondary metabolites) (Chatterjee et al. 1995; Cui et al. 1995; Pessi et al. 2001), the stationary phase sigma factor RpoS (Suh et al. 1999; Venturi 2003), a LuxR-type repressor protein QscR (Chugani et al. 2001), the global repressor MvaT (Diggle et al. 2002), and the Sm-like protein Hfq (Sonnleitner et al. 2003). In addition, a negative regulator RpeA (repressor of phenazine expression) was identified in *Pseudomonas aureofaciens* 30-84 (Whistler and Pierson 2003).

In previous studies, we have identified genes involved in regulation of phenazine biosynthesis (Chin-A-Woeng et al. 2001b). In this paper, we describe additional genes contributing to the regulation of phenazine biosynthesis. Screening for PCL1391 transposon mutants for increased PCN production resulted in the identification of the *psrA* gene (Fig. 1). The transcriptional activity of *psrA* was profiled along with the quorum-sensing regulatory genes and *phz* biosynthetic genes during growth. Although our results do not indicate that one or more additional factors (for example a signal compound) are required for the activity of PsrA, this cannot be excluded. It is not known if PsrA requires additional factors for its activity. Our results show that, in addition to the already identified *phzI*/*phzR* and *gac* regulatory genes, the *psrA* gene also significantly contributes to the regulation of PCN biosynthesis by (at least partly) influencing production of C_6-HSL and C_4-HSL (Fig. 3). We show the repressing effect of *psrA* expression on the quorum-sensing genes, *phz* biosynthetic genes, and on itself (Fig. 2). Moreover, the expression of *psrA* appears to be dependent upon the presence of *gacS* (also called *lemA* and *apdA*; Hrabak and Willis, 1992), the sensor kinase part of a two-component global regulatory system that is also involved in regulation of quorum sensing and production of secondary metabolites (Reimmann et al. 1997; Sacherer et al. 1994). On the basis of our results, we present a model for the regulation of PCN production of PCL1391 by PsrA, which includes regulators involved in quorum sensing, global regulation, and stress signaling (Fig. 4; discussed below).

The deduced amino acid sequence shows that PsrA belongs to the TetR family of transcriptional regulators. Many of the gene products in this family function as repressors in regulating the level of susceptibility to hydrophobic antibiotics and detergents. Mutation of *psrA* resulted in a more than twofold and, after prolonged growth, up to tenfold increase in PCN production and in substantially increased autoinducer production in KB medium (Fig. 3A, lane 5). The significantly increased levels of PCN and *N*-AHLs produced in the *psrA* mutant can be explained by the increased expression of the *phzI*, *phzR*, and phenazine biosynthetic genes (Fig. 2A through C). Our data show that PsrA contributes to an additional level of regulation in PCL1391 by repressing quorum sensing and quorum sensing–regulated genes. In addition, *psrA* is also indicated to repress its own expression, as was indicated by the position of the PsrA binding site in the *psrA* promoter region (–16 to –30 bp relative to the ATG codon) and was shown by expression studies (Figs. 2D and E).

PsrA was identified as a positive regulator of *rpoS* expression in *P. putida* WCS358 and *P. aeruginosa* PAO1 (Bertani et al. 2003; Kojic and Venturi 2001). The stationary-phase sigma factor RpoS, or σ^s, is a stress response protein involved in the regulation of many stationary phase, stress-related, and other processes in *Pseudomonas* (Sarniguet et al. 1995; Schuster et al. 2004; Suh et al. 1999), including phenazine production in *P. aeruginosa*. Regulation of phenazine production in *P. aeruginosa* PAO1 involves two systems, the Rhl and Las quorum-sensing systems. There are no indications that Rhl- or Las-like systems are present in PCL1391, neither does *P. aeruginosa* PAO1 posses *phzI*/*R* genes. Recently, we have studied the role of RpoS in PCL1391 in more detail, showing a clear regulatory effect (direct or indirect) of RpoS on quorum sensing and PCN production (G. Girard, *unpublished data*), and therefore, we included a role of RpoS in our model of PCN regulation (Fig. 4).

The increased autoinducer production in a PCL1391 *psrA* mutant (Fig. 3A) is in contrast with *P. aeruginosa* PAO1 and *P. putida* WCS358, in which no difference in the amounts of autoinducer molecules in supernatants of *psrA* null mutants and its wild type were detected (Kojic and Venturi 2001). The distinct quorum-sensing systems in the three strains may have contributed to the different data. Since *psrA* was identified as an inducer of RpoS expression, we assume that in a *psrA* mutant of PCL1391, *rpoS* expression is reduced, which would lead to higher expression levels of the *phzI*/*phzR* genes and, consequently, of the *phz* biosynthetic operon. The *rpoS* promoter of PCL1391 (G. Girard, *unpublished data*) indeed contains a putative binding site for the PsrA regulator ($^{-177}$GAAACTGCA CTTTG^{-164}, consensus underlined). The sequence is nearly identical to the consensus recognition sequence in the promoter of *rpoS* of *P. putida* WCS358 in which binding of PsrA to the promoter was mapped (Kojic et al. 2002). Furthermore, recent data show a lower *rpoS* expression level in a PCL1391 *psrA* mutant (G. Girard, *personal communication*).

Surprisingly, a *psrA* mutation can compensate for mutations in *phzI* and *phzR* (Fig. 2B and C) in that it enables the mutants to produce PCN and autoinducer compounds (Fig. 3B, lanes 2 and 3). Studies with reporter strains in which *psrA* is mutated show that the expression of *phzI*, *phzR*, and of the *phz* biosynthetic genes is increased (Fig. 2A through C). Single mutation of *phzI* or *phzR* abolishes autoinducer production (Fig. 3A; lanes 2 and 3) as well as PCN production. However, we observe that in a *phzI*/*psrA* double mutant the *phzI* promoter is induced in a population density–dependent manner (Fig. 2B) and that PCN production is restored to wild-type levels. Moreover, two compounds with autoinducer activity are detectable in the *phzI*/*psrA* and *phzR*/*psrA* double mutants (Fig. 3B, lanes 3 and 4). Taking into account the structural requirements of molecules inducing the *Chromobacterium N*-AHL reporter (McClean et al. 1997) and their migration characteristics, these two compounds are most likely C_4-HSL and C_6-HSL. Since the inactivation of *psrA* can restore the synthesis of autoinducer activity and PCN in a previously defective strain (Chin-A-Woeng et al. 2001), we hypothesize that PsrA also acts either as a repressor of a second system capable of producing *N*-AHLs or acts indirectly, via induction of *rpoS* expression, leading to repression of the second *N*-AHL-producing system (Fig. 4). The observed PCN production by the *psrA*/*phzR* double mutant PCL1142 indicates that there is another regulator for the biosynthetic operon, which could be another LuxR homolog. Alternatively, we speculate that PsrA might act on a quorum sensing–independent regulator of the phenazine biosynthethic operon, especially since the relatively high AHL production in PCL1142 does not result in PCN levels higher than wild-type levels (Table 3). An interesting candidate for such a regulator is the recently identified RpeA (repressor of phenazine expression) protein identified in *P. aureofaciens* 30-84 (Whistler and Pierson 2003). In the absence of this negative regulator, *phzR* was not required and phenazine production in a *phzR*/*rpeA* double mutant did not differ from the wild-type *P. aureofaciens* 30-84 (Whistler and Pierson 2003).

In several *Pseudomonas* strains, more than one quorum-sensing system has been identified. The CsaI-CsaR system has been identified as a second quorum-sensing system in the highly related phenazine-producing *P. aureofaciens* 30-84 (Zhang and Pierson 2001). However, this system did not regulate phenazine production but was, rather, involved in protease production and biosynthesis of cell surface components and produces signals different from those produced by PhzI.

In many gram-negative bacteria, the GacS/GacA regulatory system plays a role in the control of extracellular products and virulence (Ahmer et al. 1999; Laville et al. 1992; Mahajan et al. 1999; Sacherer et al. 1994). The histidine sensor kinase GacS relays environmental information to GacA (Hrabak and Willis 1992; Rich et al. 1994) and is part of a two-component regulatory system together with GacA (Appleby et al. 1996; Heeb and Haas 2001; Pernestig et al. 2001). The nature of the environmental stimulus for GacS is not known. A *gacS* mutant of PCL1391 does not produce sufficient amounts of C_6-HSL to mount PCN production. In addition, secreted protease and chitinase activities are lacking (Table 2). The global activator GacA is a member of the FixJ family of response regulators (Laville et al. 1992) and is a well-conserved response regulator identified in many gram-negative bacteria, including *Pseudomonas* spp. (de Souza et al. 2003; Heeb and Haas 2001). GacA i) positively regulates production of secondary metabolites in *P. fluorescens* CHA0 (Sacherer et al. 1994), ii) regulates pyocyanin production in *P. aeruginosa* PAO1 by controlling the production of C_4-HSL (Reimmann et al. 1997), and iii) mediates the synthesis of autoinducers in *P. aureofaciens* 30-84 (Chancey et al. 1999). Transcription of *rpoS*, assessed with an *rpoS-lacZ* transcriptional fusion, was positively influenced by GacS and GacA in *P. fluorescens* Pf-5 (Whistler et al. 1998). The GacA/GacS two-component system has a clear positive effect on transcription of *rpoS* and quorum-sensing genes, but whether this is directly or through another regulator was not known to date. Our new data now show that *gacS* expression has a positive influence on the expression of *psrA* (Fig. 2D). Since *gacS* expression is required for expression of *psrA*, and *psrA* appears to be necessary for induction of RpoS (Kojic and Venturi 2001; G. Girard, *unpublished data*), we propose that the GacS/GacA system is linked to RpoS via the PsrA regulator (Fig. 4).

Although the *psrA* mutation can suppress the effect of a mutation in one of the quorum-sensing genes in strain PCL1391 (Fig. 3B, lanes 3 and 4) by inducing a second, yet to be identified autoinducer synthase system, the presence of *gacS* is needed for PCN production, since *gacS*/*psrA* double mutants do not produce PCN. The observation that a *psrA* mutant is not affected in the production of other secondary metabolites and exoenzymes indicates that *psrA* acts downstream of GacS/GacA and confirms the role of GacS/GacA as a global regulatory system that not only regulates PCN biosynthesis but also that of chitinase and protease (Table 2).

MATERIALS AND METHODS

Microorganisms and media.

The bacterial strains and plasmids used are listed in Table 1. KB (King et al. 1954) was used for routinely culturing *Pseudomonas* strains. *E. coli* and the *Chromobacterium violaceum* were grown in LB medium (Sambrook and Russel 2001). Solid growth media contained 1.8% agar (Difco Laboratories, Detroit). Kanamycin (50 μg/ml), tetracycline (80 μg/ml), carbenicillin (50 μg/ml), and gentamycin (10 μg/ml) were added for antibiotic selection of *Pseudomonas* strains, *E. coli*, and *Chromobacterium violaceum*, as applicable. For selection of *E. coli* 20 μg of tetracycline per ml was used.

DNA modifications.

Digestion with restriction endonucleases, ligation, transformation of *E. coli* cells with plasmid DNA, and polymerase chain reaction using Pwo polymerase (Roche Molecular Biochemicals, Basel, Switzerland) were performed using standard molecular biological protocols (Sambrook and Russel 2001). Nucleotide sequencing was performed by Eurogentec B.V. (Herstal, Belgium) using AB1377-based fluorescent sequencing technology and Baseclear (Leiden, The Netherlands) using dye terminator chemistry or dye-primer chemistry. Computer analysis of protein and nucleotide sequences was performed with the European Molecular Biology Open Software Suite (EMBOSS) version 2.0.1 (Rice et al. 2000). The PCL1391 chromosomal sequences containing *gacS* and *psrA* were deposited in GenBank under accession numbers AF502252 and AF502251, respectively.

Isolation and characterization of mutants.

A mutant library of PCL1391 consisting of 18,000 transposon mutants was established using pRL1063a (Wolk et al. 1991), harboring a Tn*5* transposon carrying promoterless *luxAB* reporter genes. Mutants were selected for absence of or change in PCN-related pigment production either on LB agar plates or in 200-μl liquid KB cultures grown in 96-well microtiter plates for 3 days (Chin-A-Woeng et al. 1998). Phenotypic characterization of the mutants for production of PCN, hydrogen cyanide, chitinase, or protease for motility (Chin-A-Woeng et al. 1998) and for tomato root–colonizing ability (Simons et al. 1996) was performed as previously described. Tomato–*F. oxysporum* f. sp. *radicis-lycopersici* biocontrol assays were also performed as described previously (Chin-A-Woeng et al. 1998).

Since the Tn*5* transposon in the transconjugants contains an origin of replication that functions in *E. coli* (Wolk et al. 1991), chromosomal DNA regions flanking the transposon were recovered from the genome by excision with *Eco*RI, followed by circularization. After transfer to *E. coli*, the recovered constructs were analyzed by nucleotide sequencing. Nucleotide sequencing of the flanking regions was performed with unique primers oMP458 (5′-TACTAGATTCAATGCTAT CAATGAG-3′) and oMP459 (5′-AGGAGGTCACATGGAA TATCAGAT-3′) directed to the left and right ends of the Tn*5* transposon, respectively.

Phenazine extractions and analyses.

Phenazines were extracted as described previously (Chin-A-Woeng et al. 1998). The cell-free supernatants of culture samples were adjusted to pH 2 with HCl and were extracted twice with an equal volume of toluene. The solvent phase was removed with a rotary evaporator and the residue was dissolved in 20% (vol/vol) acetonitrile. PCN was separated by HPLC, using an Econosphere C18 5μm, 259 × 4.6 mm column (Alltech Associates, Inc., Deerfield, IL, U.S.A.) at 30°C, with a linear gradient of 20 to 80% acetonitrile acidified with 0.1% trifluoroacetic acid and a flow rate of 1 ml/min. Quantification was performed using a calibration curve of purified PCN and HPLC analysis software (Chromeleon software package v6.2; Dionex Corporation, Sunnyvale, CA, U.S.A.).

Detection and quantification of autoinducer activities.

Autoinducer activity was extracted from 100 ml of supernatant of 16- or 72-h KB culture with dichloromethane. Dichloromethane was added to the supernatant in a ratio of 3:7 dichloromethane/supernatant (vol/vol) and was shaken for 1 h (120 rpm). The organic phase was removed and dried by evaporation in vacuo (McClean et al. 1997). Supernatant extracts were redissolved in 100 μl of 100% acetonitrile, and subse-

quently 20 µl was fractionated with C18 thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany). After development in a solvent mixture of methanol/water (60:40, vol/vol), the plates were dried. TLC plates were overlaid with 30 ml of 0.8% LB top agar mixed with 2 ml of a 16-h culture of the *Chromobacterium violaceum* CV026 indicator strain (200 µl/ml) and kanamycin (50 µg/ml) (McClean et al. 1997). The activity of samples was judged after 16 h of growth at 28°C by the appearance of violet spots caused by violacein production as a result of activation of the reporter genes in the *Chromobacterium* strain. Amounts of C_6-HSL were determined using densiometry calculations of the spots and a calibration curve of standard concentrations of C_6-HSL (5, 2, 1, and 0.1 pmol), using the Scion Image software package v4.0.2 (Scion Corporation, Frederick, MD, U.S.A.). Synthetic *N*-butanoyl-L-homoserine lactone (C_4-HSL), *N*-hexanoyl-L-homoserine lactone (C_6-HSL), and N-octanoyl-l-homoserine lactone (C_8-HSL) references were provided by P. Williams, University of Nottingham, U.K.

Expression studies using bioluminescent Tn*5luxAB* reporter strains.

Expression of Tn*5luxAB*-tagged genes was determined by quantification of *luxAB* activity during culturing. Cells from overnight KB cultures were washed with fresh medium and resuspended in KB medium to an optical density at 620 nm (OD_{620}) of 0.1. Cultures were grown in a volume of 10 ml under vigorous shaking. Growth was followed by measurement of OD_{620} at regular intervals, and 100-µl samples were taken in triplicate to quantify *luxAB* activity. A volume of 100 µl of an *n*-decyl-aldehyde substrate solution (0.2% *n*-decyl-aldehyde [Sigma, St. Louis] in a 2.0% bovine serum albumin solution) was added. After thorough mixing, bioluminescence was determined using a luminescence counter (MicroBeta 1450 TriLux, Wallac, Turku, Finland). Luminescence was determined after approximately every 0.1 unit increase of OD_{620}.

Construction of mutants by homologous recombination.

Disruption of the *gacS* homologous gene by homologous recombination was performed using pMP6016, a pIC20R-based *Pseudomonas* suicide plasmid containing a 0.3-kb internal PCR fragment of the *gacS*-homologous gene of strain PCL1391 and a tetracycline resistance cassette. The PCR primers contained *Kpn*I and *Eco*RI (underlined) restriction sites and annealed to nucleotide positions 1,084 to 1,103 (5′-CCGGAATTCGAGCCACGAAATCCGTACCC-3′) and 1,294 to 1,313 (5′-CGGGGTACCTCAGGGTGTCCTGCAACAGG-3′), having taken the adenine of the first ATG of the PCL1391 *gacS* gene as position one. Plasmid pMP6016 was transformed to strain PCL1391 Tn*5luxAB* derivatives by electroporation, and recombinants resulting from chromosomal integration were selected on KB agar medium supplemented with tetracycline (160 µg/ml) for PCL1391 or kanamycin (50 µg/ml) and tetracycline for Tn*5*-derivatives. Transformants were analyzed for proper recombination using Southern hybridization (Sambrook and Russell 2001). Double mutants were isolated by selection on medium with antibiotics for selecting both the initial Tn*5* insertion and the newly introduced mutation. The second mutation introduced was confirmed using Southern hybridization.

A mutagenesis strategy similar to that described above was used for the introduction of mutations in *psrA* and *nagZ*. The *psrA* fragment for constructing the suicide plasmid pMP6015 for the introduction of a mutation in the *psrA* homologous gene was obtained by PCR, using primers oMP657 (5′-CGGAATTCAACCGTTGAACGCATTCTC-3′), containing an *Eco*RI restriction site and annealing to positions 16 to 34, and oMP656 (5′-CGGAATTCAGGAACGGCACCATCAG-3′), containing a *Kpn*I restriction site and annealing to positions 590 to 607 of the *psrA* gene of strain PCL1391.

The *nagZ* PCR fragment for construction of suicide plasmid pMP6049 was produced by using primers oMP646 (5′-GAATTCGATCATCTTTGCCCGGAATA-3′), containing an *Eco*RI restriction site, and oMP647 (5′-GGGTACCGACGGTGTCACTTTCAGAC-3′), containing a *Kpn*I restriction site, annealed to positions 90 to 109 and 869 to 888 of the *nagZ* gene, respectively.

Cloning of the *psrA* gene and promoter.

The complete *psrA* gene and 199 bp of the promoter region of *psrA* was amplified with PCR using primers oMP691 (5′-GAATTCGCTTGGCAATCCTCCTTTTT-3′) and oMP692 (5′-GAATTCATCGGCGAAAGATCTGAAAG-3′), both containing an *Eco*RI restriction site annealing to positions –199 to –179 and 779 to 798 relative to the start of the *psrA* gene, respectively. The 1.0-kb *psrA* fragment obtained was cloned into the *Eco*RI site of pBBR1MCS5, resulting in pMP6579. Plasmids were transformed to *Pseudomonas* using electroporation (1.25 kV/cm, 2.5 µF, 200 Ω) (Gene Pulser, Bio-Rad Laboratories, Richmond, CA, U.S.A.).

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