Genetic regulation of phenazine-1-carboxamide synthesis by Pseudomonas chlororaphis strain PCL1391

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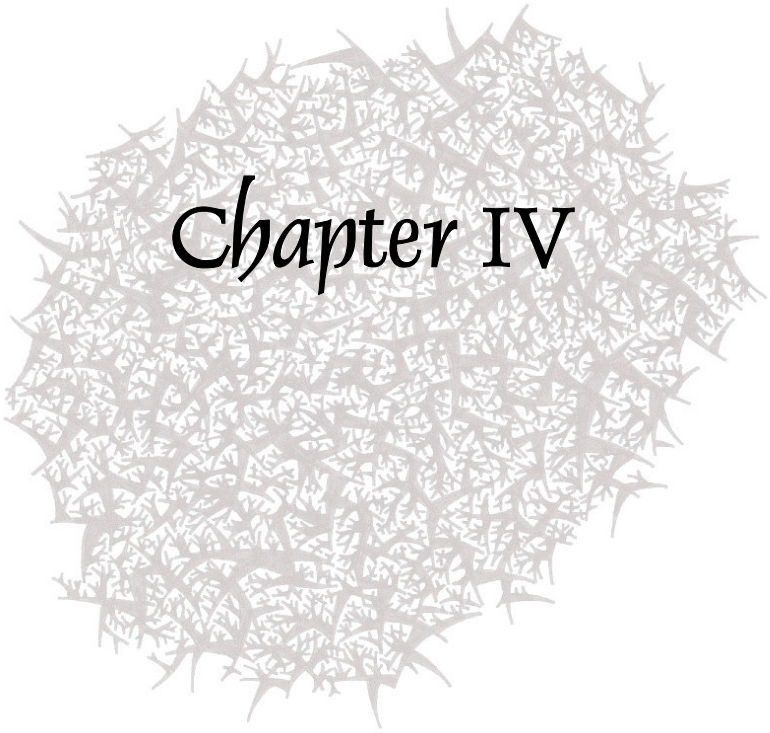

Chapter IV

The novel regulator Pip acts between PsrA/RpoS and quorum-sensing in the regulatory cascade of phenazine synthesis in *Pseudomonas chlororaphis* PCL1391

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Submitted

ABSTRACT

Secondary metabolites are important factors for interactions of bacteria with other microorganisms in their natural environment. *Pseudomonas chlororaphis* PCL1391 produces the antifungal metabolite phenazine-1-carboxamide (PCN) and is used as a model organism to study regulation of secondary metabolism in *Pseudomonas* species. Our previous work showed that a cascade of regulators controls PCN synthesis, starting with the GacA/GacS two-component signaling system, *via* the regulator PsrA, the stationary phase sigma factor RpoS and the quorum-sensing system PhzI/PhzR down to the phenazine biosynthetic (*phz*) operon. Here we describe the identification of a novel regulator, named Pip (phenazine inducing protein), which shows similarity to members of the TetR/AcrR family of regulators. Highly conserved homologues of *pip* were found in a large diversity of bacterial species, of which the functions are unknown. Expression studies and quantification of *N*-AHL and PCN in various mutant strains identified Pip as acting downstream of PsrA and RpoS and upstream of PhzI/PhzR.

INTRODUCTION

Understanding interactions between organisms is critical for the study of bacterial processes such as pathogenicity and competition, which take place in their natural habitat, such as the rhizosphere. Interactions of Gram-negative bacteria with other microorganisms often involve the production of secondary metabolites with various functions. For example, some Gram-negative bacteria produce *N*-acyl-homoserine lactones (*N*-AHLs), which are able to traffic across membranes. The *N*-AHL extracellular concentration reflects the number of bacteria present in a (semi)closed environment. *N*-AHLs enable bacteria to sense the density of their population and to induce specific (sets of) genes beyond a threshold concentration (quorum). This phenomenon, called quorum-sensing (Bassler, 1999; Fuqua *et al.,* 2001), controls the production of other secondary metabolites, for example in pseudomonads the production of toxins, exoenzymes, (Winson *et al.,* 1995) rhamnolipids (Ochsner & Reiser, 1995; Pearson *et al.,* 1997), hydrogen cyanide (Pessi & Haas, 2000) and phenazines (Chin-A-Woeng *et al.,* 2001). Many of these metabolites contribute to the competition with other microorganisms and to the protection of plants against phytopathogens.

Phenazine-1-carboxamide (PCN) is produced by *Pseudomonas chlororaphis* PCL1391, which suppresses tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis lycopersici* (Chin-A-Woeng *et al.,* 1998). PCN production, combined with efficient root colonization to deliver PCN in the rhizosphere, are crucial traits for the biocontrol ability of strain PCL1391 (Chin-A-Woeng *et al.,* 2000). Understanding the components regulating the synthesis of PCN is likely to contribute to insights in regulation of bacterial secondary metabolism in general.

Production of PCN was shown to be regulated by different factors, including environmental biotic and abiotic factors (van Rij *et al.,* 2004; van Rij *et al.,* 2005), and intrinsic regulatory systems. The GacS/GacA system is a classical two-component system (Rodrigue *et al.,* 2000) including a sensor (GacS) that recognizes an unknown environmental signal and a transcriptional regulator (GacA) that activates a cascade of regulators upstream of the *phz* operon (Chin-A-Woeng *et al.,* 2001; Girard *et al.,* 2006). PsrA was shown to be part of this regulatory cascade, and controls the expression of the stationary phase sigma factor encoded by *rpoS* (Girard *et al.,* 2006). Downstream of RpoS, PhzI synthesizes C_6-HSL that is supposed to bind to the transcriptional regulator PhzR. Activated PhzR binds in turn to the *lux* box upstream of the *phz* operon, which is responsible for the synthesis of PCN at the onset of the stationary phase. Our previous results showed

that a constitutively activated quorum-sensing system PhzI/PhzR is sufficient for synthesis of PCN when other regulators are mutated (Girard *et al.,* 2006).

Here we describe the identification of *pip* (phenazine inducing protein), a novel gene that is involved in controlling PCN synthesis. Our results show that Pip, a putative transcriptional regulator, acts downstream of PsrA and RpoS and stimulates the expression of the *phz* operon via the quorum-sensing system.

RESULTS

Isolation of mutant strain PCL1114

A transposon library of *P. chlororaphis* PCL1391 consisting of 18,000 transposants and established using pRL1063a (Chin-A-Woeng *et al.,* 2001) was screened for mutants exhibiting reduced PCN production on LC agar. On LC agar, PCN production is visually detectable by the yellow color of the colonies. Among 21 transposant colonies isolated for altered coloration, one was selected and tested for PCN production after growth in liquid complex LC medium. Quantitative HPLC analysis shows that this mutant, named PCL1114, is severely affected in PCN production (0.03% compared to the parental strain PCL1391). When strain PCL1114 was grown in King's B medium, another complex medium, similar results were obtained: 97.5% of decrease in PCN production. PCN production by PCL1114 was not detected during growth in poorer MVB1 medium (Fig. 1, panel A), which was used as a standard in the next experiments.

Genetic analysis of PCL1114

Plasmid rescue from chromosomal DNA of PCL1114 showed that the Tn5*luxAB* transposon is inserted in a small ORF of 669 bp in between positions 71 and 72. The gene corresponding to this ORF was named *pip* (phenazine inducing protein).

The chromosomal organization around *pip* was analyzed by further sequencing, which showed the presence of an ORF of 1908 nucleotides upstream of *pip* (Fig. 2). This OFR (accession number DQ311664) has the same transcription orientation as *pip* and the protein shows 92% homology to a putative 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) of *P. fluorescens* PfO1 (accession number ZP_00262624). Computer analysis shows the presence of a putative *rho*-independent transcription terminator for the 4- hydroxyphenylpyruvate dioxygenase gene, seven nucleotides downstream of its stop codon (GTAACGGCGGCGGCAAAGGGCCGCCGTCCTGC), followed by a putative promoter

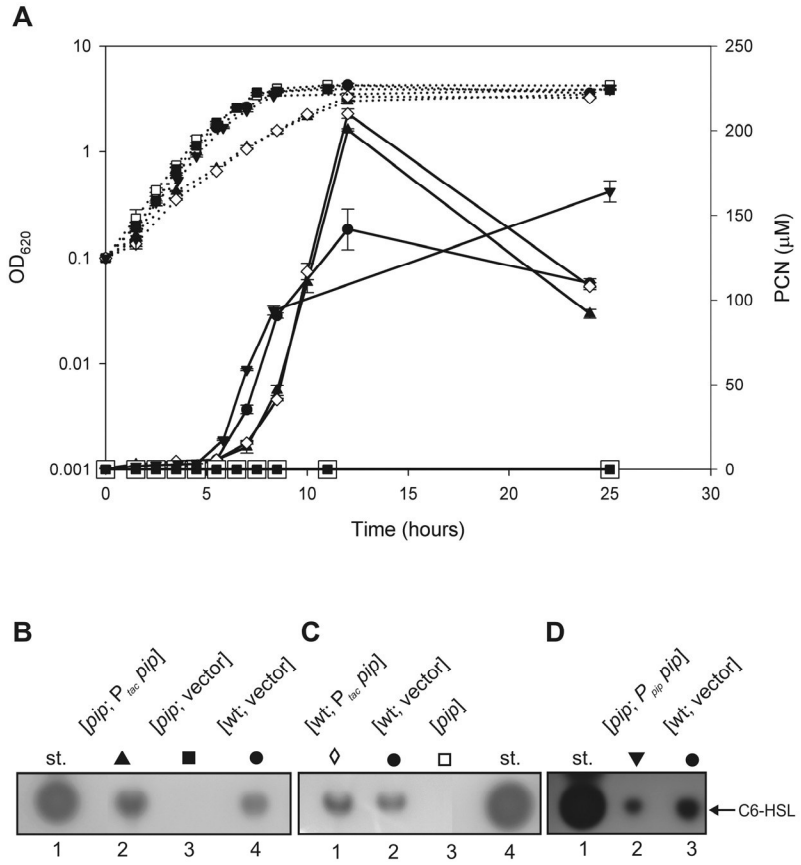

Figure 1. Analysis of PCN and *N*-AHL production by *P. chlororaphis* PCL1391 and PCL1114 derivatives. Panel A. Extractions were made from at least three independent cultures in 10 ml MVB1 in a time course and the PCN production level was determined by HPLC. The error bars indicate the standard deviations. On each graph, the OD at 620 nm (see left axis; dotted lines) and the PCN concentration (see right axis; solid lines) are plotted. Meaning of the symbols is indicated in panels B-D. The symbol for [*pip*] (□) was magnified for better visualization.
Panels B-D. C18-reverse phase TLC analysis of *N*-AHL production by various PCL1391 derivatives at OD_{620} = 3.0. Symbols are the same as in panel A and st indicates a standard of 2.5 nmol of synthetic C_6-HSL.

sequence upstream of the *pip* ORF. The start of transcription is predicted to be 35 nucleotides upstream of the *pip* start codon, downstream of a putative -10 box (GCCCATAAT) and a putative -35 box (TTTCCTT). No *rho*-independent terminator could be detected downstream of *pip*, however a putative gene is located there in the opposite direction of transcription. Its predicted protein product shows 58% homology with a putative transcriptional regulator of *Chromobacterium violaceum* ATCC 12472 (accession number AAQ59192).

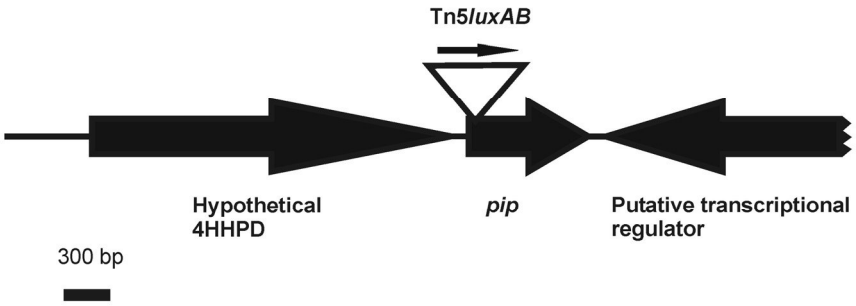

Figure 2. Genomic organization of the chromosomal region of *P. chlororaphis* PCL1391 surrounding *pip*. Each ORF is represented by an arrow which indicates the direction of transcription. The interrupted part of the arrow for the probable transcriptional regulator indicates that this ORF was not completely sequenced. The position of the transposon insertion is shown as an arrow head at the beginning of *pip* and the transcriptional directions are indicated. 4-HHPD stands for 4-hydroxyphenylpyruvate dioxygenase

Homologues of Pip were found in many pseudomonads (94% homology in *P. fluorescens* Pf-5; 83% homology in *P. putida* KT2440 and 79% homology in *P. aeruginosa* PAO1), as well as in a large variety of other Gram-negative species, such as *Azotobacter vinelandii*, *Burkholderia* sp., *Sinorhizobium meliloti*, *Agrobacterium tumefaciens*, *Ralstonia* sp. To our knowledge, no function has been published for any of these homologues. Domain homology search on the Pip sequence showed that Pip is homologous to members of the TetR/AcrR family, which are transcriptional regulators. In *E. coli*, TetR regulates a pump involved in tetracyclin resistance (Beck *et al.,* 1982) and AcrR regulates a pump involved in multidrug resistance (Olliver *et al.,* 2004). This search retrieved a TetR N-terminal domain (Pfam00440) for the stretch of amino-acids from position 31 to 77 (53 bits, E value of $3e^{-8}$). Alignments between Pip homologues of various species and the TetR N-terminal domain (Fig. 3) show that many amino acids are conserved in the N-terminal region, which is similar to the TetR N-terminus. In addition, towards the C-terminal two small regions corresponding to amino acids 107-130 and 172-191 from Pip are conserved. No homology with the TetR C-terminal domain was found. The Pip protein has an overall similarity of 42% with the AcrR transcriptional regulator of *E. coli* (accession number AAC73566).

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PCL1391      ---------MTTELSVVPEQSVNEPRKSRKNNPEKTRENILQEAIVEFVQQGLSGARVDA 51
DC3000       ---------MKKIDSAVAVEPAATVPKGRKNNPEKTREDILKAAVAEFVAHGLTGARVDA
KT2440       ---------MSDSVVGLGQPETEGGRKTRKNNPEKTREDILQAAINEFVQQGLAGARVDA
PfO1         ---------MTSELPAASALPAVEPRKSRKNNPEKTRENILQEAIVEFVQQGLSGARVDA
PAO1         ---------MSKPDPAVVEESAPRG-KGRKNNPEKTRQDILRAAIDEFVAQGLSGARVDA
Azotobacter  ---------MPDTKPVA--DPAARPRQPRKNNPEKTRQDILLAAIREFAEQGLSGARVDA
K96243       MPPSDHAKMKQGSKAAAPDAEARRDEARRKYDPEQTKRNILDVATQEFSAMGLSGARVDA
1021         --------------------MAERQANGRKNDPQRTQDDILEVATEEFSTHGLAGARVDQ
TetR_N       ----------------------------------------ILDAALELFAERGYDATTVRE
                                                     **  *   *   *     *

PCL1391      IAERIHTSKRMIYYYFGSKEQLYVEVLEKLYGDIRSTESRLHLAELAPRDAIRRLVEFTF 111
DC3000       IAERTKTSKRMIYYYFGSKEQLYVEVLEKLYGAIRNTESELNLGELEPVEAIHRVVEFTF
KT2440       IAERTATSKRMIYYYFGSKEQLYCECLVKLYGDIRKTEQSLDLESLPAEQAIRRLVEFTF
PfO1         IAERIHTSKRMIYYYFGSKEQLYVEVLEKLYGDIRNTENRLHLAELPPVEAIRRLVEFTF
PAO1         IAERTHTSKRMIYYYFGSKEQLYQAVLEKLYSDIRGIEGTLRLGALPPAKAMEKLVEFSF
Azotobacter  IAERTSTSKRMIYYYFGSKEQLYLAVLEKLYGDIRATEATLRLDELEPEAAIRRLVEFTF
K96243       IAERTNTTKRMLYYYFDSKEGLYEAVLEKVYGDIRTLERELNVGELEPREGLSRLVEFTF
1021         IAERTRTSKRMIYYYFGSKEALYLAVLERSYRKIRTLEADLELANLPPEEALRTLVATTF
TetR_N       IAKEAGVSKGALYRHFPSKEELLLAL-----------------------------------
             **      *   *  * *** *

PCL1391      DHHDRNVDFVRIICTENIHYGEYVKQSPAIREMSSLVLEALGNTLRRGVQEGVFRAGIEV 171
DC300        DHHDRNVDFVRIVSIENIHNAEYVKRSEAIKAMNNTILHALGEILQRGVDEGVFRAGLDP
KT2440       DHHDRNVDFVRIVSIENIHNAEYVKRSDAIKAMNNTILDSLGEILRRGAEEGVFRAGLDA
PfO1         DYHDRHVDFVRIISIENIHKGEHIASSELVQSVNSSIVQSIAEILRRGEAEGVFRAGLEA
PAO1         DHHDSNVDFVRIVCIENIHNGENVKQSDTIQAKSQNIIRALDGILRRGEASGLFRDGVHP
Azotobacter  DHHAANVDFIRLVSIENIHHGEHIARSPSIRSLSKSIVSQIAAILARGADAGVFRAGIDP
K96243       DYHDRHRDFVRLVTIENIHGAKYIEQLKSFKNRNVSIIKTLEELLARGVESGVFRDDVDP
1021         DHDEANPDFVRLVSIENIHRAAHMLRSDAIRDLNVSVIQMIEAIIERGLGDGTFRRKADP
TetR_N       ------------------------------------------------------------

PCL1391      LDVHLLISSFCFYRVSNRHTFGEIFQIDLPDEAIKQRHREMICESVLRYLQA-------- 223
DC3000       VDLHLMISSFCFYRISNRHTFSEIFQIELWSEEVKQRHKAMICDAVLRYLKR--------
KT2440       IDLHMLMSSFCFYRVSNRHTFGEIFQIDLSDEQIKLRHKAMICDAVLRYIQLEPRT----
PfO1         LDVHLLISSFCFYRVSNRHTFGEIFQIDLPDESIKQRHREMICESVLRYLQA--------
PAO1         LDVHMLISSFCFYRVSNRYTVSRIFRTDLHDAEVRQRHRRMIGEAVLRYIRA--------
Azotobacter  LDLHLLISSFCFFRVSNRYTFSTIHQIDLGGAEARARHREMICEAVIRHVRA--------
K96243       FDLHLLISSFCFHRVANRYTFGTAFGRDPSSPRLRARHRAMITDTVLRYV-------AR-
1021         IDVHMLISAFCFFRVSNRYTFGTIFRRDLSEPKTIARHRTMIADAVVSYLKSDEPRAARR
TetR_N       ------------------------------------------------------------
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Figure 3. Alignment of Pip homologues from various bacterial species with the TetR N-terminal domain of *E. coli*.
Homologues of Pip from *P. chlororaphis* PCL1391 were found in *P. syringae* pv. *tomato* str. DC3000 (NP_792164), *P. putida* KT2440 (NP_745664), *P. fluorescens* PfO-1 (ZP_00262623), *P. aeruginosa* PAO1 (AAG03632), *Azotobacter vinelandii* (ZP_00091468), *Burkholderia pseudomallei* K96243 (YP_111478) and *Sinorhizobium meliloti* 1021 (NP_436576). The aminoacids that are conserved in all the Pip homologues are indicated in bold. The aminoacids that are conserved in all the Pip homologues and in the TetR N-terminal domain are indicated by an asterisk. The numbers on the right margin indicate the aminoacid counting of Pip in strain PCL1391.

Phenotypic characterization of *pip* mutant PCL1114

pip mutant PCL1114 was characterized for motility and production of HCN, siderophore, exoprotease and chitinase. The wild-type strain PCL1391 and the *gacS* mutant PCL1123 (Chin-A-Woeng *et al.,* 2005) were used as control strains. Measurements showed no difference between the wild type and the *pip* mutant in motility. After overnight incubation, a blue coloration was observed for both

PCL1391 and PCL1114 on filter paper used for HCN detection. Similarly, a halo of comparable size for PCL1391 and PCL1114 was measured in the chitinase, exoprotease and siderophore tests (Table 1).

Table 1. Phenotypic tests

	Strains		
Property	PCL1391 wt	PCL1114 *pip* mutant	PCL1123 *gacS* mutant
PCN production	+	-	-
C_6-HSL production	+	-	-
Swarming diameter (mm)	17.3 (±3.0)	18.0 (±1.0)	1.3 (±0.2)
Swimming diameter (mm)	20.3 (±5.5)	21.7 (±1.5)	1.3 (±0.2)
Chitinase production (mm)	9.3 (±0.6)	9.3 (±0.3)	0.7 (±0.3)
Exoprotease activity	+	+	-
Siderophore production	+	+	++
HCN production	+	+	+
MIC* for gentamicin (µg/ml)	15	15	n. d. **
MIC for tetracyclin (µg/ml)	40	40	n. d.
MIC for rifampicin (µg/ml)	16	16	n. d.
MIC for carbenicillin (µg/ml)	>1,600	>1,600	n. d.

*MIC, minimum inhibitory concentration
**n. d., not determined

Since Pip shows homology with TetR, which is involved in tetracycline resistance (Beck *et al.,* 1982) and with AcrR, which regulates multidrug resistance (Olliver *et al.,* 2004), we tested if the *pip* mutant had a modified resistance to various antibiotics as compared to its wild-type strain. No difference was found between PCL1114 and PCL1391 in their sensitivity towards gentamicin, tetracycline, rifampicin and carbenicillin (Table 1).

Effect of a *pip* mutation on production of PCN and *N*-AHL

Mutant strain PCL1114 was selected for its impaired PCN production (see above). In addition, analysis of *N*-AHL production showed that C_6-HSL could not be detected in the supernatant of PCL1114 (Fig. 1, panel C, lane 3).

In order to test if the inhibition of PCN production was indeed due to the defect in *pip*, several new mutant strains were constructed. PCL2008 contains a suicide vector, which recombined into *pip* by homologous recombination (see Experimental Procedures), and is impaired in PCN and C_6-HSL production, like PCL1114 (data not shown). A second strain, PCL2012, is derived from PCL1114 and contains the vector pMP7455, which harbors *pip* under control of the *tac* promoter, resulting in constitutive expression of *pip*. This strain produced 1.4-fold more PCN (Fig. 1, panel A) than strain PCL1960 (wt PCL1391 containing the empty cloning

vector as a control) as analyzed after 12 hours of growth. Strain PCL2012 (*pip*; P_{tac} *pip*) also produces high amounts of C_6-HSL (Fig. 1, panel B, lane 2). The *pip* gene was also expressed under its own promoter *in trans* in the *pip* mutant (PCL2085). This new strain showed restored production of PCN and *N*-AHL (Fig. 1, panels A and D). Over-expression of *pip* in a wild-type background (transformation of pMP7455 into PCL1391 resulting in PCL2019) gave the same phenotype as in the *pip* mutated background (Fig. 1, panel A and panel C, lane 1). These results clearly show that the impaired production of PCN and *N*-AHL in PCL1114 is indeed caused by the disruption or *pip*.

Analysis of autoregulation of *pip* expression

Both TetR and AcrR negatively auto-regulate their own expression (Hillen & Berens, 1994; Ma *et al.,* 1996). We tested whether Pip shows a similar autoregulatory activity. Analysis of the orientation of the Tn5*luxAB* in PCL1114 showed that the *luxAB* genes and *pip* have the same direction of transcription, which allows measurements of *pip* expression by quantifying the *luxAB* activity (Fig. 2). The expression of *pip* was measured in three derivatives, containing P_{tac} *pip*, P_{pip} *pip*, and the empty cloning vector, respectively (Fig. 4, panel A). PCL2085 (*pip*::Tn5*luxAB*; P_{pip} *pip*) showed a maximum of expression (2210±67 cps), which is intermediate between that of PCL2011 (*pip*::Tn5*luxAB*; empty vector) (3590±231 cps) and that of PCL2012 (*pip*::Tn5*luxAB*; P_{tac} *pip*) (262±1 cps). These results demonstrate that Pip regulates negatively its own transcription.

Regulatory relationships between Pip and other regulators of PCN production

Several genes, including *psrA*, *rpoS* and *phzI*/*phzR*, are known to play a role in the regulation of PCN synthesis (Chin-A-Woeng *et al.,* 2001; Girard *et al.,* 2006). Experiments were conducted to understand if and, if so, how *pip* interacts with these genes. For easier reading below, the insertion of Tn5*luxAB* is not always mentioned, but in the strains used in the studies, all *psrA* and *pip* mutants are actually *psrA*:: Tn5*luxAB* and *pip*:: Tn5*luxAB*, respectively.

In MVB1 medium the *psrA* and *rpoS* genes positively regulate PCN and *N*-AHL production (Girard *et al.,* 2006). To test whether *pip* could be regulating these two genes, the effect of a *pip* mutation on the production of RpoS in strain PCL1391 protein was tested by Western blot analysis. Samples were taken during the logarithmic growth phase (OD_{620} 1.0) and at the beginning of the stationary phase (OD_{620} 2.2) of cells grown in MVB1 medium. The amounts of RpoS appeared to be

similar at the two time points for all the tested strains. A blot of the results at OD_{620} 1.0 is shown (Fig. 5). PCL1954 (*rpoS* mutant), used as a negative control, showed no RpoS production (Fig. 5, lane 3), whereas PCL1955 (*rpoS* mutant with constitutive *rpoS* expression *in trans*) showed restored RpoS production (Fig. 5, lane 4). The amounts of RpoS were similar for PCL1391 and PCL1114 (Fig. 5, lanes 1 and 2). Subsequently, the ability of constitutively produced PsrA and RpoS to restore PCN production in *pip* mutant PCL1114 was tested. Measurements showed that PCL2036 (*pip*; P_{tac} *rpoS*) and PCL2046 (*pip*; P_{tac} *psrA*) do not produce detectable amounts PCN (results not shown).

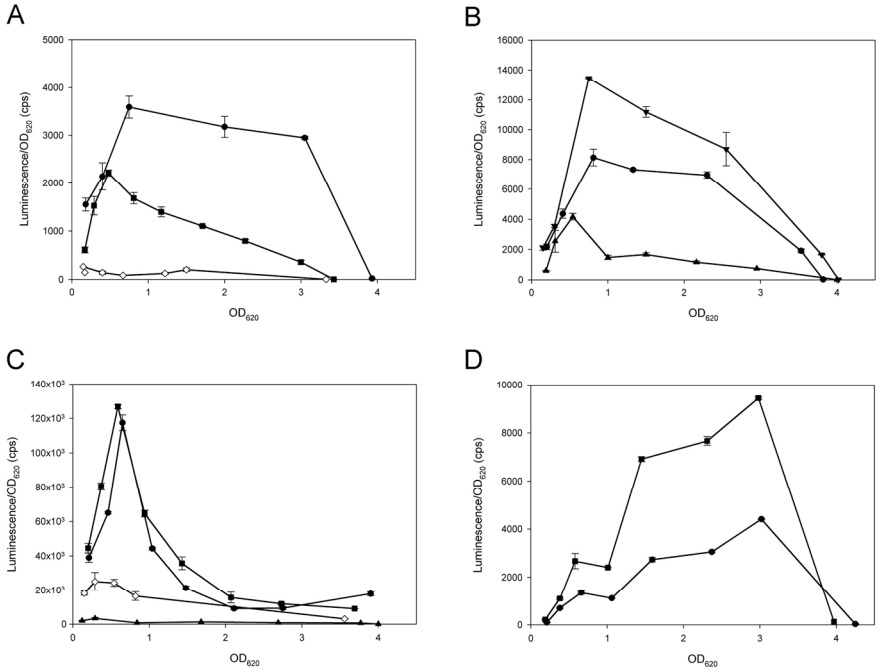

Figure 4. Expression analyses of *P. chlororaphis* PCL1391 *pip*, *psrA* and *phzR* derivative strains.
Each panel corresponds to a particular chromosomal background, and only the genes in the vector pBBR1MCS-5, added to the background, are indicated in the detailed legend. Cell cultures were grown in MVB1 medium and samples were taken at regular time intervals. Activity of the *luxAB* reporter was determined by quantifying bioluminescence. Measurements were performed in duplicate.
Panel A. Strains with a *pip*::Tn5*luxAB* background: PCL2011 (pBBR1-MCS5, ●), PCL2085 (P_{pip} *pip* ■), PCL2012 (P_{tac} *pip*, ◊).
Panel B. Strains with a *pip pip*::Tn5*luxAB* background: PCL2011 (pBBR1-MCS5, ●), PCL2036 (P_{tac} *rpoS* ▼) and PCL2046 (P_{tac} *psrA* ▲).
Panel C. Strains with a *psrA*::Tn5*luxAB* background: PCL1962 (pBBR1-MCS5 ●), PCL2087 (P_{pip} *pip* ■), PCL2038 (P_{tac} *pip*, ◊) and PCL2045 (P_{tac} *psrA* ▲).
Panel D. Strains with a *phzR*::Tn5*luxAB* background: PCL2001 (pBBR1-MCS5 ●) and PCL2086 (P_{pip} *pip* ■).

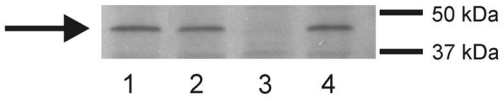

Figure 5. Western-blot analysis of RpoS production in *P. chlororaphis* PCL1391 and derivative strains. Cells were grown in MVB1 until OD_{620} 1.0. Lane 1: PCL1391. Lane 2: PCL1114 (*pip*::Tn5*luxAB*). Lane 3: PCL1954 (*rpoS*::pMP7418). Lane 4: PCL1955 (*rpoS*::pMP7418 + P_{tac} *rpoS*). On the right side of the blot two markers are shown. An arrow on the left indicates the position of RpoS.

Constitutive expression of *rpoS* in *pip* mutant (PCL2036) resulted in a 25% increase of P_{pip} activity compared to PCL2011. In contrast, constitutive expression of *psrA* in PCL2046 had a negative influence (45% reduction) on P_{pip} activity (Fig. 4, panel B). The reverse experiments were performed to evaluate if *pip* had an effect on *psrA* expression. Like for constitutive expression of *pip* in *pip*::Tn5*luxAB*, constitutive expression of *psrA* in *psrA*::Tn5*luxAB* resulted in complete inhibition of luminescence (Fig. 4, panel C, compare PCL1962 [*psrA* with the empty vector] and PCL2045 [*psrA*; P_{tac} *psrA*]). Constitutive expression of *pip* in the same *psrA*::Tn5*luxAB* background (PCL2038) resulted also in a severe decrease (60%) of *lux* activity, although the level remained higher than in PCL2045. The vector containing the *pip* gene under its own promoter was also used for transformation of the *psrA* mutant. Activity of P_{psrA} was comparable between the derivative strain containing P_{pip} *pip* and the derivative strain with the empty cloning vector (Fig. 4, panel C).

Previously it was shown that *psrA* and *rpoS* mutants produce only low amounts of PCN and *N*-AHL (Girard *et al.,* 2006). To test whether *pip* could regulate PCN and *N*-AHL production downstream of *psrA* and/or *rpoS*, *pip* was overexpressed in PCL1111 (*psrA* mutant) and PCL1954 (*rpoS* mutant) by transformation with pMP7455, resulting into PCL2038 and PCL2040, respectively. These two strains showed a restored production of PCN (Fig. 6, panel A) and C_6-HSL, as compared to PCL1962 (*psrA* mutant with empty vector) and PCL1957 (*rpoS* mutant with empty vector) (Fig. 6, panel C; compare lanes 3 and 2, 5 and 4).

To test the relationship between Pip and quorum-sensing further, a *pip* mutant derivative was constructed that constitutively expresses *phzR*. The resulting strain, PCL2013, showed a restored production of both PCN and C_6-HSL (Fig. 6, panels A and B). Transformation with a plasmid containing *pip* under its own promoter showed a positive effect on P_{phzR} activity (Fig. 5, panel D). A summary of all these results is shown in Fig. 7.

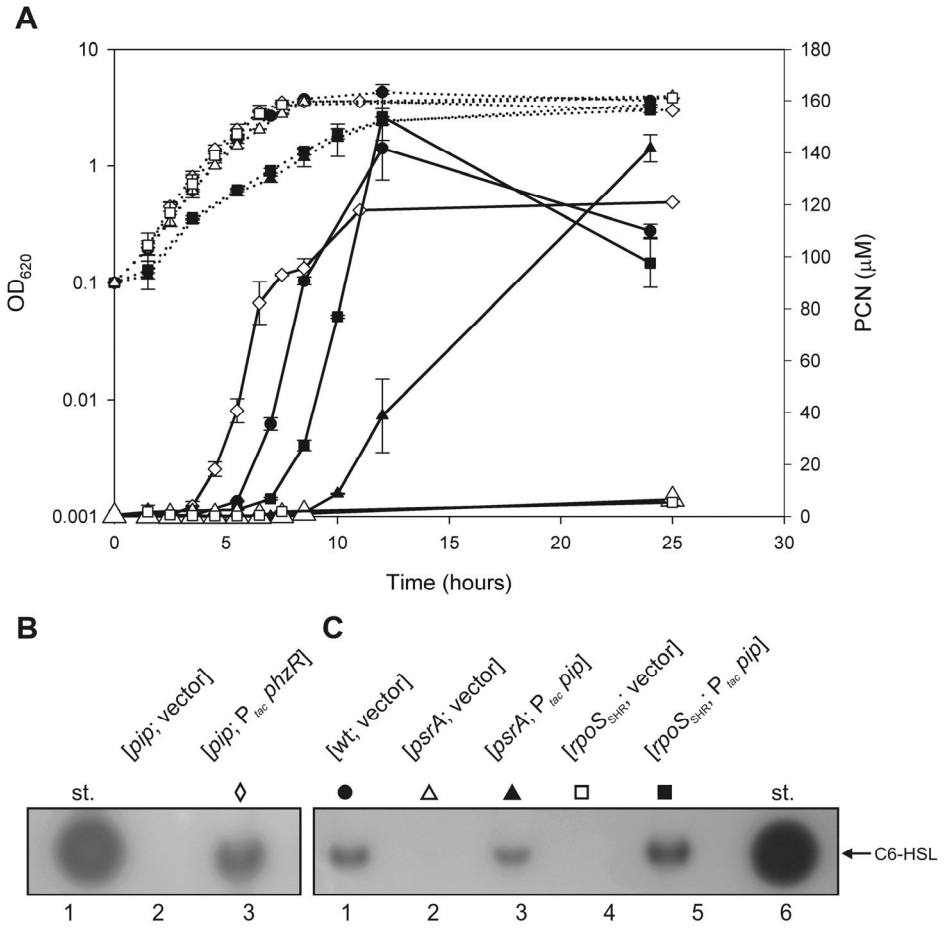

Figure 6. Analysis of PCN and *N*-AHL production in *P. chlororaphis* PCL1391 derivative strains.
Panel A. Extractions were carried out from at least three independent cultures in 10 ml MVB1 in a time course and the PCN concentration was determined by HPLC analysis. On each graph, the absorbance is plotted along the left axis (dotted lines) and the PCN concentration is plotted along the right axis (closed lines). Meaning of the symbols is indicated in panels B-C. Since measurements were made at the same time points for the cultures, the points indicating PCN amounts in PCL1962 (Δ) are hidden by the points indicating the amounts of PCN for PCL1957 (□).
Panels B and C. C18-reverse phase TLC analysis of *N*-AHL produced by PCL2011, *pip*::Tn*5luxAB* + pBBR1MCS-5 (lane 2) and other derivatives indicated by the symbols of panel A. st: 2.5 nmol of synthetic C_6-HSL.

DISCUSSION

In order to identify new genes involved in the regulation of PCN production, a mutant library was screened for decreased PCN production, which yielded mutant strain PCL1114 (Fig. 1). The analysis of the flanking regions of the Tn5 transposon showed an interrupted ORF, which we named *pip* (Fig. 2). Homologues of *pip* are found in a wide variety of bacterial species (Fig. 3). To our knowledge this is the first time that a phenotype is described as a result of mutating *pip*.

Location of *pip* in the genome

Sequence analysis showed upstream of *pip* the presence of a gene encoding a putative 4-HPPD, in the same direction of transcription as *pip* (Fig. 2). In principle this enzyme is not linked to the metabolism of *N*-AHL or PCN, since it catalyzes the second step in the pathway for the catabolism of the aminoacid tyrosin (Moran, 2005). Searches for reciprocical best BLAST hits in the sequenced genome database version 2 of *P. aeruginosa* PAO1 showed that the *pip* homologue is downstream of a putative 4-HPPD gene in at least *P. fluorescens* Pf-5, *P. aeruginosa* PAO1, *P. putida* KT2440, *P. syringae* B728a and *P. syringae* pv. *tomato* DC3000. This is also the case for *Azotobacter vinelandii*. This conserved genomic organization could indicate that *pip* is functionally coupled to the 4-HPPD. However, a terminator of transcription was found for the putative 4-HPPD and a putative promoter just upstream of *pip* supports the hypothesis that the two genes are independently transcribed. Downstream of *pip* an ORF was detected with an opposite direction of transcription (Fig. 2), indicating that no downstream effect of the Tn5 insertion in *pip* is expected that would influence PCN production. Complementation of the *pip* mutant confirmed this (Fig. 1). Interestingly, the ORF downstream of *pip* encodes a putative transcriptional regulator, whereas in all the other *Pseudomonas* species displayed in the sequenced genome database of *P. aeruginosa* PAO1, the *pip* homolog is located upstream of a putative shikimate 5-dehydrogenase. Similar observations were made concerning the flanking regions of *rpoS* in strain PCL1391 (Girard *et al.,* 2006), which seems to indicate that the chromosome of strain PCL1391 is organized in a different way as compared to closely related pseudomonads.

Pip belongs to the AcrR/TetR family of regulators

Alignments showed that Pip contains an N-terminal domain similar to that of the TetR transcriptional regulator (Fig. 3). The TetR N-terminal is a domain that

contains a helix-loop-helix (HLH) motif (Isackson & Bertrand, 1985), which indicates that Pip would be a transcriptional regulator. Pip also shows overall homology with the transcriptional regulator AcrR. AcrR belongs to the same family of regulatory proteins as TetR (Grkovic *et al.,* 2002). TetR and AcrR have similar functions. The TetR protein regulates the resistance mechanism against the antibiotic tetracycline in *E. coli* (Hillen & Berens, 1994). Located in the membrane, TetA exports the tetracycline-magnesium $[MgTc]^+$ complex. TetR recognizes two palindromic operator *(tetO*$_{1,2}$) sites in the promoter region of the divergently oriented genes *tetR* and *tetA*, thereby inhibiting their expression. Binding of $[MgTc]^+$ to TetR relieves this inhibition by reducing the affinity of TetR for *tetO*. (Hillen & Berens, 1994). AcrR regulates the production of the AcrAB pump (Ma *et al.,* 1996) that is responsible for multidrugs resistance (Ma *et al.,* 1995; Okusu *et al.,* 1996). Similarly to TetR, AcrR represses the *acrAB* operon and its own transcription (Ma *et al.,* 1996).

The negative feedback of Pip on its own expression was also clearly shown: increased expression of *pip in trans* in PCL1114 derivatives results in decreased expression of chromosomal *pip*, since P_{pip} *pip* inhibits *pip* expression less than P_{tac} *pip* and more than the presence of the empty cloning vector (Fig. 4, panel A). The homology with AcrR and TetR and the negative feedback of Pip on its own expression (Fig. 4) suggest that Pip might also regulate the production of an efflux pump. However, *tetR* is located next to *tetA* in the chromosome of *E. coli*, as is *acrR* located next to *acrA* (Hillen & Berens, 1994; Ma *et al.,* 1996). In the case of strain PCL1391, no gene encoding a putative pump could be found next to *pip* in the genome (Fig. 2). It also does not share its promoter region with the promoter region of another gene, as *tetR* and *acrR* do. Also unlike TetR and AcrR (Beck *et al.,* 1982; Okusu *et al.,* 1996), Pip does not seem to be involved in resistance to antibiotics (Table 1). We considered the idea that Pip might regulate an *N*-AHL or PCN pump (see also Chapter 5). It seems unlikely in the case of PCN, because then a *pip* mutation should not affect the amounts of *N*-AHL, but it does (Fig. 1). In the case of *N*-AHL, it would explain the effect of *pip* mutation on both *N*-AHL and PCN (Fig. 1). However, it is assumed that C_6-HSL, with such a short acyl chain, is able to diffuse through membranes (Fuqua *et al.,* 2001) and does not traffic *via* a pump: an efflux pump was found *in P. aeruginosa* that exports long chain *N*-AHLs, but not *N*-AHLs with short acyl chains (Evans *et al.,* 1998).

Pip is a new regulator of PCN synthesis

Since *pip* stimulates *N*-AHL and PCN synthesis (Fig. 1), we tried to clarify how Pip interacts with other proteins known to regulate PCN synthesis. Firstly, several experiments were conducted to test if Pip is positioned upstream of RpoS in the regulatory cascade of PCN production. Western blot analysis showed that Pip does not influence *rpoS* expression (Fig. 5). Neither constitutive expression of *psrA* nor constitutive expression of *rpoS* could restore PCN and *N*-AHL production in the *pip* mutant. All together, these results suggest that Pip does not regulate *psrA* and *rpoS*.

In contrast, the stimulation of P_{pip} activity by RpoS (Fig. 4, panel B) indicates that Pip is located downstream of RpoS in the PCN regulatory cascade. However, constitutively produced PsrA has a negative effect on P_{pip} activity (Fig. 4, panel B). Since PsrA positively regulates *rpoS* (Girard *et al.,* 2006), it seems contradictory that RpoS and PsrA have opposite effects on the expression of *pip*. An explanation could be given by the fact that PsrA and Pip show an inhibitory effect on their own expression (Fig. 4, panels A and C). Since PsrA and Pip are both TetR homologues, they are most likely to exert their negative autoregulation in similar ways. In a situation of constitutive expression, Pip or PsrA is present in excess and therefore might bind to other targets, although less specifically. This would result for example in PsrA inducing negative feedback on *pip*, although to a lower level than the negative feedback of Pip itself. This hypothesis was verified by the reverse experiment, that showed that Pip induces negative feedback on *psrA*, although to a lower level than the negative feedback of PsrA itself (Fig. 4, panel C). These results support the hypothesis that the negative effect of PsrA on *pip* expression would be the result of a non-physiological high concentration of PsrA in the cell.

If so, the previous results do not contradict the notion that PsrA and RpoS regulate *pip* expression. This hypothesis was confirmed by the fully restored production levels of PCN and *N*-AHL in *psrA* and *rpoS* mutants constitutively expressing *pip* (Fig. 6). In addition, PCN and *N*-AHL levels were correlated in all the *pip* derivatives and a *pip* mutation was suppressed by constitutive production of PhzR in strain PCL2013. Finally, increasing the copy number of *pip* results in an increased transcription of *phzR* (Fig. 4, panel D). Taken together, these results show that Pip acts in the cascade of PCN synthesis, downstream of PsrA/RpoS and upstream of PhzI/PhzR and the *phz* operon. Subsequent studies showed that Pip does not regulate exoprotease, chitinase, HCN and siderophore production or motility (Table 1), indicating that Pip is regulating PCN rather specifically. Negative

autoregulation of *pip* is the simplest explanation for the fact that constitutive production of Pip complements *psrA* and *rpoS* mutations better than constitutive production of RpoS (Girard *et al.,* 2006). Constitutive expression of *rpoS* is partially compensated by the negative feedback of *pip* on itself, which cannot occur when *pip* is constitutively expressed. This autoregulation of Pip adds complexity to the regulation scheme that can be drawn from these results (Fig. 7).

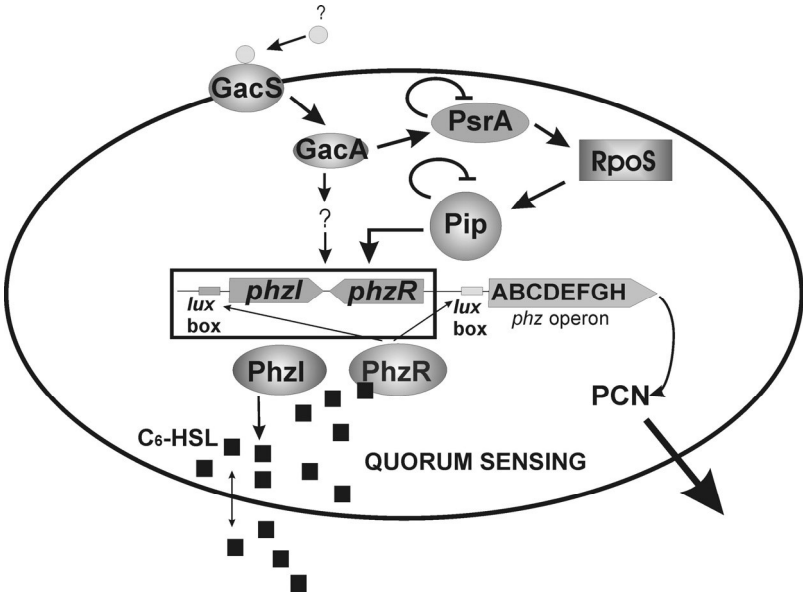

Figure 7. Schematic model showing the role of Pip in the genetic cascade regulating PCN synthesis in *P. chlororaphis* PCL1391. The regulatory cascade of PCN starts with the sensing of a so far unidentified environmental signal by GacS and subsequent activation of GacA. The TetR-homologue PsrA regulates *rpoS* probably by binding to its promoter. The alternative sigma factor RpoS is positively regulates *pip*, the product of which stimulates expression the quorum-sensing system *phzI/phzR*. Both *pip* and *psrA* exhibit negative autoregulation. PhzI is responsible for the production of C_6-HSL, which is supposed in turn to bind to PhzR. The PhzR-C_6-HSL complex binds to *lux* boxes in the promoter sequences of *phzI* and the *phz* operon. Subsequently *phzI* is upregulated and expression of the *phz* operon is switched on, which finally results in the synthesis of phenazine-1-carboxamide (PCN).

Concluding remarks

The discovery of *pip* is an important step in unraveling the regulatory mechanism of PCN regulation. A complex regulatory model for regulation of the *phz* operon arises from previous results and the results of this study, as depicted in Fig. 7. The autoregulation of Pip adds complexity to this model. This study also brings new insights into the role of homologues of the TetR/AcrR family, which are widely distributed in bacterial species, as far as in *Thermus thermophilus* and in *Streptomyces coelicolor*, two organisms with considerable biotechnological potential (Challis & Hopwood, 2003; Henne *et al.,* 2004).

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Pseudomonas* strains were cultured at 28°C in liquid MVB1 (van Rij *et al.,* 2004) or LC (*Girard et al., 2006*) or King's Medium B (King *et al.,* 1954) and shaken at 195 rpm on a Janke und Kunkel shaker KS501D (IKA Labortechnik, Staufen, Germany). *Escherichia coli* strains were grown at 37°C in LC medium under vigorous aeration. Media were solidified with 1.8% Bacto agar (Difco, Detroit, MI). When appropriate, growth media were supplemented with kanamycin (50 µg/ml), carbenicillin (200 µg/ml), gentamicin (30 µg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 µg/ml) or hexanoyl-homoserine lactone (C_6-HSL) (5 µM) (Fluka, Sigma-Aldrich, Zwijndrecht, The Netherlands). To follow growth, the absorbance of liquid cultures was measured at 620 nm.

Isolation and sequence analysis of chromosomal regions flanking the transposon in the *pip* mutant PCL1114

A transposon library was obtained by transformation of strain PCL1391 with the plasmid pRL1063a (Chin-A-Woeng *et al.,* 2001). The Tn5 transposon of pRL1063A contains an origin of replication that functions in *E. coli* (Wolk *et al.,* 1991). Chromosomal DNA was isolated from PCL1114, digested with *Eco*RI, religated and transferred into *E. coli* by transformation. One clone was picked among the colonies obtained after kanamycin resistance selection. The plasmid containing the regions flanking the transposon was named pMP7444, and sequenced using primers oMP458 (5'-TACTAGATTCAATGCTATCAATGAG-3') and oMP459 (5'-AGGAGGTCACATGGAATATCAGAT-3'). Similarity and domain searches were done using BLAST (http://www.ncbi.nih.gov/BLAST). Search for bacterial promoters and terminators were done using Softberry (http://www.softberry.com). Alignments of amino-acid sequences were obtained using the ClustalW software (http://www.ch.embnet.org/software/ClustalW.html).

Phenotypic analyses

To test swimming and swarming ability, the method described previously (Deziel *et al.,* 2001) was used, in which 1/20 KB-0.3% agar plates were used for the analysis of swimming, and 1/20 KB-0.5% agar plates were used for analysis of swarming.

Table 2. Bacterial strains and plasmids used

Bacterial strains and plasmids	Description	Reference or source
Bacteria		
Pseudomonas chlororaphis		
PCL1391	Wild-type *Pseudomonas chlororaphis*, producing phenazine-1-carboxamide; biocontrol strain of tomato foot and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici*	(Chin-A-Woeng *et al.*, 1998)
PCL1104	Derivative of PCL1391 in which a promoterless Tn5*luxAB* has been inserted in *phzR*; Km^r	(Chin-A-Woeng *et al.*, 2001)
PCL1111	Derivative of PCL1391 in which a promoterless Tn5*luxAB* has been inserted in *psrA*; Km^r	(Chin-A-Woeng *et al.*, 2005)
PCL1114	Derivative of PCL1391 in which a promoterless Tn5*luxAB* has been inserted in *pip*; Km^r	This study
PCL1123	Derivative of PCL1391 in which a promoterless Tn5*luxAB* has been inserted in *gacS*; Km^r	(Chin-A-Woeng *et al.*, 2005)
PCL1954	Derivative of PCL1391; *rpoS*::pMP7418; Km^r	(Girard *et al.*, 2006)
PCL1962	Derivative of PCL1111 containing pBBR1-MCS5; Km^r, Gm^r	(Girard *et al.*, 2006)
PCL2001	Derivative of PCL1104 containing pBBR1-MCS5; Km^r, Gm^r	(Girard *et al.*, 2006)
PCL2008	Derivative of PCL1391; *pip*::pMP7451; Km^r	This study
PCL2011	Derivative of PCL1114 containing pBBR1-MCS5; Km^r, Gm^r	This study
PCL2012	Derivative of PCL1114 containing pMP7455; Km^r, Gm^r	This study
PCL2013	Derivative of PCL1114 containing pMP7447; Km^r, Gm^r	This study
PCL2019	Derivative of PCL1391 containing pMP7455;, Gm^r	This study
PCL2035	Derivative of PCL1104 containing pMP7455; Km^r, Gm^r	This study
PCL2036	Derivative of PCL1114 containing pMP7420; Km^r, Gm^r	This study
PCL2038	Derivative of PCL1111 containing pMP7455; Km^r, Gm^r	This study
PCL2040	Derivative of PCL1954 containing pMP7455; Km^r, Gm^r	This study
PCL2045	Derivative of PCL1111 containing pMP7465; Km^r, Gm^r	(Girard *et al.*, 2006)
PCL2046	Derivative of PCL1114 containing pMP7465; Km^r, Gm^r	This study
PCL2085	Derivative of PCL1114 containing pMP7487; Km^r, Gm^r	This study
PCL2086	Derivative of PCL1104 containing pMP7487; Km^r, Gm^r	This study
PCL2087	Derivative of PCL1111 containing pMP7487;	This study

	Km^r, Gm^r	
Chromobacterium violaceum		
CV026	Double mini-Tn5 mutant from *C. violaceum* ATCC 31532, AHL biosensor; Km^r	(Milton *et al.,* 1997)
Escherichia coli		
DH5α	*Escherichia coli*; *supE44* Δ*lac*U169(Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi1 relA1*	(Hanahan, 1983)
Plasmids		
pRL1063a	Plasmid harboring promoterless Tn5*luxAB* transposon; Km^r	(Wolk *et al.,* 1991)
pRK2013	Helper plasmid for tri-parental mating	(Ditta *et al.,* 1980)
pIC20H	General purpose cloning vector; Cb^r	(Marsh *et al.,* 1984)
pGEM-T easy	Plasmid designed for direct ligation of PCR fragments	Promega
pBBR1MCS-5	General purpose cloning vector; Gm^r	(Kovach *et al.,* 1995)
pMP5285	Suicide vector for *Pseudomonas* spp. Used for homologous recombination; Km^r, Cb^r	(Kuiper *et al.,* 2001)
pMP7420	pBBR1MCS-5 containing the *rpoS* gene of PCL1391 downstream of the P_{tac} promoter, obtained by *Eco*RI digestion of pMP7424; Gm^r	(Girard *et al.,* 2006)
pMP7444	pRL1063a containing *pip* and flanking regions, Km^r	This study
pMP7447	pBBR1MCS-5 containing the *phzR* gene of PCL1391 under control of the P_{tac} promoter, inserted between the *XhoI* and *Eco*RI sites; Gm^r	(Girard *et al.,* 2006)
pMP7451	pMP5285 containing an internal 350 bp PCR fragment of *pip*; Km^r	This study
pMP7455	pBBR1MCS-5 containing the *pip* gene of PCL1391 under control of the P_{tac} promoter; Gm^r	This study
pMP7465	pBBR1MCS-5 containing the *psrA* gene of PCL1391 under control of the P_{tac} promoter; Gm^r	(Girard *et al.,* 2006)
pMP7487	pBBR1MCS-5 containing the *pip* gene of PCL1381 under control of its own promoter; Gm^r	This study

For measuring the production of chitinase, plates were poured with 2% agar dissolved in 0.05 M sodium acetate and Cm-Chitin-RBV solution (Loewe Biochemica GmbH, Sauerlach, Germany) following recommendations of the manufacturer. A volume of 200 µl of supernatant of 3-day old LC cultures was applied in wells made in the agar plates. After overnight incubation at 28°C, the distance between the edge of the well and the edge of the halo was measured.

In order to test protease production, bacteria were tested as described previously (Chin-A-Woeng *et al.,* 1998), except that the concentration of milk was increased to 10% in MVB1 agar plates.

The test for siderophore production was performed on solid medium as described previously (Schwyn & Neilands, 1987).

The production of HCN was measured as described previously (Castric, 1975). Whatman 3MM paper was soaked into a chloroform solution containing copper(II) ethyl acetoacetate (5 mg/ml) and 4,4'-methylene-bis-(N,N-dimethylanilin) (5 mg/ml), and subsequently dried and stored in the dark. A piece of this paper was placed in the lid of a Petri dish in which bacteria had been plated on MVB1-agar (1%). The Petri dishes were sealed with parafilm and incubated overnight at 28°C. Production of HCN by the bacteria was shown by blue coloration of the paper.

To test antibiotic resistance, various dilutions (10^4, 10^5, 10^6 and 10^7 fold) of overnight cultures were spotted in duplicate on 1.8% agar LC plates containing various concentrations of the following antibiotics: tetracyclin (20, 40, 80 and 160 µg/ml), carbenicillin (200, 400, 800 and 1,600 µg/ml), rifampicin (2, 4, 8 and 16 µg/ml) and gentamicin (7.5, 15, 30 and 60 µg/ml). Growth of bacteria was judged after two days of incubation at 28^0C.

Recombinant DNA techniques

General DNA techniques were performed as described previously (Sambrook & Russel, 2001). Polymerase chain reactions were in carried out with Super Taq enzyme (Enzyme Technologies Ltd, UK). Only for the production of *pip* under control of the P_{tac} or P_{pip} promoter, PCRs were performed using Phusion from Finnzymes (Bioké, Leiden, The Netherlands). Primers were synthesized by Isogen Life Science (Maarssen, The Netherlands). Restriction enzymes were purchased from New England BioLabs Inc. (Westburg) and ligase from Promega (Leiden, The Netherlands).

Construction of plasmids and PCL1391 mutant strains

In order to construct a suicide plasmid for disruption of *pip* by single homologous recombination, an internal *pip* fragment of 350 bp was obtained by PCR on PCL1391 chromosomal DNA with the primers oMP814 (5'-ATATATGAATTCCCGGCGCTCGGGTGGATGCC-3') and oMP815 (5'-ATATATGAATTCTCTCGCCCAGGGCATGGAGG-3'). The PCR fragment was cloned into the *Eco*RI site of the vector pMP5285. The obtained suicide vector was named pMP7451 and transferred to PCL1391 by triparental mating using the helper plasmid pRK2013. The resulting mutant was named PCL2008.

In order to constitutively express *pip*, a plasmid was constructed harboring *pip* under control of the constitutive P_{tac} promoter. Two primers were designed according to the *pip* sequence obtained from pMP7444: oMP816 (5'-ATATATGAATTCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGACAATGACCACAGAACTCTCCGTAGTGCCC-3'), which contained the P_{tac} promoter and oMP817 (5'-ATATATGAATTCAGGATGCGGTTGAAACCCTGTGCCGCG-3'). These primers were used for PCR on chromosomal DNA of PCL1391. The obtained fragment was cloned in the *Eco*RI site of pBBR1MCS-5. The resulting vector was named pMP7455 and was introduced into PCL1114 by triparental mating to obtain PCL2012. The cloning vector pBBR1MCS-5 was introduced into PCL1114 in order to obtain the control strain PCL2011.

For over-expression of *phzR*, pMP7447 (Girard *et al.,* 2006) was introduced into PCL1114 to obtain PCL2013. For over-expression of *rpoS*, pMP7420 (Girard *et al.,* 2006) was transferred to PCL1114 to obtain PCL2036. For over-expression of *psrA*, pMP7465 (*Girard et al., 2006*) was introduced into PCL1114 to obtain PCL2046. In order to study the effect of over-expression of *pip* on *phzR* expression, pMP7455 was introduced into PCL1104 (Chin-A-Woeng *et al.,* 2001), which resulted in PCL2035.

The *pip* gene was also constitutively expressed in wild-type, *psrA* and *rpoS* mutant backgrounds. Therefore the plasmid pMP7455 was introduced into PCL1391, PCL1111 (Chin-A-Woeng *et al.,* 2005) and PCL1954 (Girard *et al.,* 2006) by triparental mating and the resulting strains were named PCL2019, PCL2038 and PCL2040, respectively.

Primers oMP1045 (5'-ATATATGAATTCGAGGTCAGCCGGGCCAAGGAG-3') and oMP817 (5'-ATATATGAATTCCAGGATGCGGTTGAAACCCTGTGCCGCC-3') were used for PCR on chromosomal DNA of PCL1391 with Phusion enzyme (Finnzymes) to obtain *pip* with 424 nucleotides of the sequence upstream of its start codon. The 1.1 kb product was cloned in the *Eco*RI site of pBBR1MCS-5. The orientation of the insert was verified by PCR and a clone was selected in which *pip* and the β-galactosidase gene of pBBR1MCS-5 have opposite directions of transcription. This plasmid was named pMP7487 and verified by sequencing for the lack of possible mutations introduced by PCR. It was introduced into strains PCL1114, PCL1104 and PCL1111 (*pip*, *phzR* and *psrA* mutants, respectively) to obtain strains PCL2085, PCL2086 and PCL2087, respectively.

Extraction and analysis of phenazine and *N*-acyl-homoserine lactones

Phenazine extraction was carried out on supernatants of 10 ml liquid MVB1 cell cultures at regular time points during growth and/or after overnight growth as described previously (van Rij *et al.,* 2004). For *N*-AHL extraction, supernatants from 50 ml MVB1 cultures were harvested at OD_{620} 3.0 and mixed with 0.7 volume of dichloromethane and shaken for 45 min after which the organic phase was collected. The extract was dried using a rotary evaporator. The dried residue was redissolved in 25 µl of acetonitrile and spotted on RP-C18 TLC plates (Merck, Darmstadt, Germany). The TLC plates were developed in methanol-water (60:40, v:v). For detection of *N*-AHLs, the TLC was overlaid with 0.8% agar LC containing a 10-fold diluted overnight culture of the *Chromobacterium violaceum* indicator strain CV026 (Milton *et al.,* 1997) and kanamycin (50 µg/ml). After incubation for 48 h at 28°C, chromatograms were analyzed for the appearance of violet spots, indicating the presence of *N*-AHLs.

Expression analysis of bioluminescent Tn5*luxAB* reporter strains

Expression of *pip* was monitored in various derivatives making use of the *luxAB* reporter genes of the Tn5 derivative in PCL1114. Expression was determined by quantification of bioluminescence during culturing. Cells from overnight MVB1 cultures were washed with fresh medium and diluted to OD_{620} 0.1 in 10 ml fresh MVB1 medium. During growth, OD_{620} was measured at regular intervals, and 100 µl samples were taken in duplicate to quantify luminescence. A volume of 100 µl of *N*-decyl-aldehyde substrate solution [0.2% *N*-decyl-aldehyde (Sigma, St. Louis, MO, USA) in a 2% bovine serum albumin solution] was added. After 5 min of incubation at room temperature, bioluminescence was determined with a MicroBeta 1450 TriLux luminescence counter (Wallac, Turku, Finland) and normalized to the luminescence per OD_{620} unit.

Western blot analysis

Cells were grown after inoculation of 10 ml MVB1 with an overnight culture washed with fresh medium and subsequently diluted to OD_{620} of 0.1. Cells were harvested at OD_{620} 1.0 or 2.2 (cultures are diluted 10-fold before OD_{620} measurement) in volumes corrected for equal cell amounts. Cell pellets were resuspended in 200 µl of cracking buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 2 mM EDTA, 10% glycerol, 0.01% bromophenol blue, 1% β-mercaptoethanol) and boiled for 3 minutes. The samples were subsequently loaded on a 10% SDS-

polyacrylamide gel and proteins were separated and blotted following a standard Western blot procedure (Ausubel *et al.,* 1997). A dried aliquot of rabbit antibodies against RpoS was kindly provided by Prof. K. Tanaka (Tokyo, Japan). This sample was resuspended in 100 µl PBS, diluted 1000-fold and allowed to react with the blot. The blots were subsequently incubated with peroxidase-labeled goat anti-rabbit antiserum (Amersham Biosciences, Roosendaal, The Netherlands). Finally, blots were incubated in a luminal solution (250 µM sodium luminol (Sigma), 0.1 M Tris-HCl, pH 8.6, 0.01% H_2O_2) mixed with 60 µl enhancer solution [67 µM p-hydroxy coumaric acid (Sigma) in DMSO]. Hybridizing protein bands were detected using a Super R-X photographic film (Fujifilm, Düsseldorf, Germany).

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