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Pip, a Novel Activator of Phenazine Biosynthesis in *Pseudomonas chlororaphis* PCL1391▽†

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Secondary metabolites are important factors for interactions between bacteria and other organisms. *Pseudomonas chlororaphis* PCL1391 produces the antifungal secondary metabolite phenazine-1-carboxamide (PCN) that inhibits growth of *Fusarium oxysporum* f. sp. *radius lycopersici* the causative agent of tomato foot and root rot. Our previous work unraveled a cascade of genes regulating the PCN biosynthesis operon, *phzABCDEFGH*. Via a genetic screen, we identify in this study a novel TetR/AcrR regulator, named Pip (*p*henazine *i*nducing *p*rotein), which is essential for PCN biosynthesis. A combination of a phenotypical characterization of a *pip* mutant, in *trans* complementation assays of various mutant strains, and electrophoretic mobility shift assays identified Pip as the fifth DNA-binding protein so far involved in regulation of PCN biosynthesis. In this regulatory pathway, Pip is positioned downstream of PsrA (*Pseudomonas* sigma factor regulator) and the stationary-phase sigma factor RpoS, while it is upstream of the quorum-sensing system PhzI/PhzR. These findings provide further evidence that the path leading to the expression of secondary metabolism gene clusters in *Pseudomonas* species is highly complex.

Among gram-negative bacteria, pseudomonads are known to produce a wide variety of secondary metabolites, such as toxins (35), rhamnolipids (25, 27), hydrogen cyanide (HCN) (28) and phenazines (6). In contrast to primary metabolites, secondary metabolites are not essential for growth and reproduction. However, many of them play an important role in interactions between *Pseudomonas* species and other organisms, particularly during pathogenesis and biocontrol. For example pyocyanin produced by *Pseudomonas aeruginosa* is suggested to be involved in lung infection of cystic fibrosis patients (19), whereas Phl (2,4-diacylphloroglucinol) and HCN produced by *Pseudomonas fluorescens* protect tobacco plants from black root rot (20). The elucidation of how secondary metabolism is regulated is therefore relevant for medicine, agriculture, and industry.

In most species, the GacS/GacA two-component system is a global regulator of secondary metabolism, for example, for the production of HCN and Phl (20), the production of exoprotease and phospholipase C (30), and the production of phenazine (29). After binding of an unknown signal, the membrane-associated sensor GacS activates the GacA transcriptional regulator by phosphorylation (13, 38). Direct targets of GacA are so far unknown. In addition to GacA/GacS, quorum-sensing also regulates secondary metabolism in many species. Quorum-sensing involves a LuxI homologue synthesizing *N*-acylhomoserine lactone signal molecules (*N*-AHLs), which are able to traffic across membranes. Their extracellular concentration reflects the number of bacteria present in a (semi-) closed environment. *N*-AHLs bind to a LuxR homologue, thereby activating it. Activated LuxR homologues function as transcriptional regulators. Thus, *N*-AHLs enable bacteria to sense the density of their population and to induce specific (sets of) genes (9, 23).

Phenazine-1-carboxamide (PCN) is a secondary metabolite produced by *Pseudomonas chlororaphis* PCL1391, which suppresses tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis lycopersici* (5). PCN production and efficient root colonization for delivering PCN in the rhizosphere are crucial traits for the biocontrol ability of strain PCL1391 (4). Understanding the components regulating the synthesis of PCN is likely to give new insights in regulation of bacterial secondary metabolism in general.

Production of PCN was shown to be regulated by an intrinsic regulatory network, according to the following observations. (i) The GacS/GacA system activates a cascade of regulators upstream of the *phz* biosynthetic operon (6, 10). (ii) PsrA (*Pseudomonas* sigma factor regulator) was shown to be part of the PCN regulatory cascade. (iii) PsrA controls the production of the stationary-phase sigma factor RpoS (10). (iv) Downstream of RpoS, the LuxI homologue PhzI synthesizes *N*-hexanoyl-homoserine lactone (C_6-HSL), the *N*-AHL that is supposed to bind to the LuxR homologue 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 (10).

Here we describe the identification of *pip* (*p*henazine *i*nducing *p*rotein), 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

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Characteristics and description[a]	Reference or source
Strains		
P. chlororaphis		
PCL1391	wt; producing phenazine-1-carboxamide; biocontrol strain of tomato foot and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici*	5
PCL1103	*phzI*; derivative of PCL1391 in which a promoterless Tn*5luxAB* has been inserted in *phzI*; Km^r	6
PCL1104	*phzR*; derivative of PCL1391 in which a promoterless Tn*5luxAB* has been inserted in *phzR*; Km^r	6
PCL1111	*psrA*; derivative of PCL1391 in which a promoterless Tn*5luxAB* has been inserted in *psrA*; Km^r	7
PCL1114	*pip*; derivative of PCL1391 in which a promoterless Tn*5luxAB* has been inserted in *pip*; Km^r	This study
PCL1123	*gacS*; derivative of PCL1391 in which a promoterless Tn*5luxAB* has been inserted in *gacS*; Km^r	7
PCL1954	$rpoS_{SHR}$; derivative of PCL1391; *rpoS*::pMP7418; Km^r	10
PCL1955	$rpoS_{SHR}$; P_{tac} *rpoS*; derivative of PCL1955 containing pMP7420; Km^r,Gm^r	10
PCL1962	*psrA* (empty vector); derivative of PCL1111 containing pBBR1-MCS5; Km^r Gm^r	10
PCL2001	*phzR* (empty vector); derivative of PCL1104 containing pBBR1-MCS5; Km^r Gm^r	10
PCL2008	pip_{SHR}; derivative of PCL1391; *pip*::pMP7451; Km^r	This study
PCL2011	*pip* (empty vector); derivative of PCL1114 containing pBBR1-MCS5; Km^r Gm^r	This study
PCL2012	*pip* P_{tac} *pip*; derivative of PCL1114 containing pMP7455; Km^r Gm^r	This study
PCL2013	*pip* P_{tac} *phzR*; derivative of PCL1114 containing pMP7447; Km^r Gm^r	This study
PCL2019	wt; P_{tac} *pip*; derivative of PCL1391 containing pMP7455; Gm^r	This study
PCL2036	*pip* P_{tac} *rpoS*; derivative of PCL1114 containing pMP7420; Km^r Gm^r	This study
PCL2038	*psrA* P_{tac} *pip*; derivative of PCL1111 containing pMP7455; Km^r Gm^r	This study
PCL2040	$rpoS_{SHR}$ P_{tac} *pip*; derivative of PCL1954 containing pMP7455; Km^r Gm^r	This study
PCL2082	*phzI* (empty vector); derivative of PCL1103 containing pBBR1MCS-5; Km^r Gm^r	This study
PCL2083	*phzI* P_{tac} *phzR*; derivative of PCL1103 containing pMP7447; Km^r Gm^r	This study
PCL2085	*pip* P_{pip} *pip*; derivative of PCL1114 containing pMP7487; Km^r Gm^r	This study
PCL2086	*phzR* P_{pip} *pip*; derivative of PCL1104 containing pMP7487; Km^r Gm^r	This study
PCL2087	*psrA* P_{pip} *pip*; derivative of PCL1111 containing pMP7487; Km^r Gm^r	This study
PCL2089	wt; P_{pip} *pip*; derivative of PCL1391 containing pMP7487; Gm^r	This study
C. violaceum CV026	Double mini-Tn*5* mutant from *C. violaceum* ATCC 31532; AHL biosensor	24
Escherichia coli DH5α	λ^- ϕ80d*lacZ*Δ*M15*Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	12
Plasmids		
pRL1063a	Harboring promoterless Tn*5luxAB* transposon; Km^r	36
pRK2013	Helper plasmid for triparental mating	8
pIC20H	General purpose cloning vector; Cb^r	22
pGEM-T easy	Plasmid designed for direct ligation of PCR fragments	Promega
pBBR1MCS-5	Empty vector; general purpose cloning vector; Gm^r	17
pMP5285	Suicide vector for *Pseudomonas* spp.; used for homologous recombination; Km^r Cb^r	18
pMP7420	pBBR1MCS-5 containing the *rpoS* gene of PCL1391 downstream of the P_{tac} promoter, obtained by EcoRI digestion of pMP7424	10
pMP7444	pRL1063a containing *pip* and flanking regions; Km^r	This study
pMP7447	P_{tac} *phzR*; pBBR1MCS-5 containing the *phzR* gene of PCL1391 under control of the P_{tac} promoter, inserted between the XhoI and EcoRI sites	10
pMP7451	pMP5285 containing an internal 350-bp PCR fragment of *pip*	This study
pMP7455	P_{tac} *pip*; pBBR1MCS-5 containing the *pip* gene of PCL1391 under control of the P_{tac} promoter	This study
pMP7465	P_{tac} *psrA*; pBBR1MCS-5 containing the *psrA* gene of PCL1391 under control of the P_{tac} promoter	10
pMP7487	P_{pip} *pip*; pBBR1MCS-5 containing the *pip* gene of PCL1381 under control of its own promoter	This study

[a] See Materials and Methods for an explanation of the notation.

the expression of the *phz* operon via the quorum-sensing system PhzI/PhzR.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* strains were cultured at 28°C in liquid MVB1 (34), LC (10), or King's medium B (16) and shaken at 195 rpm on a Janke and 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 medium was supplemented with kanamycin (50 μg/ml), carbenicillin (200 μg/ml), gentamicin (30 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (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 diluted 10-fold 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 (6). The Tn*5*

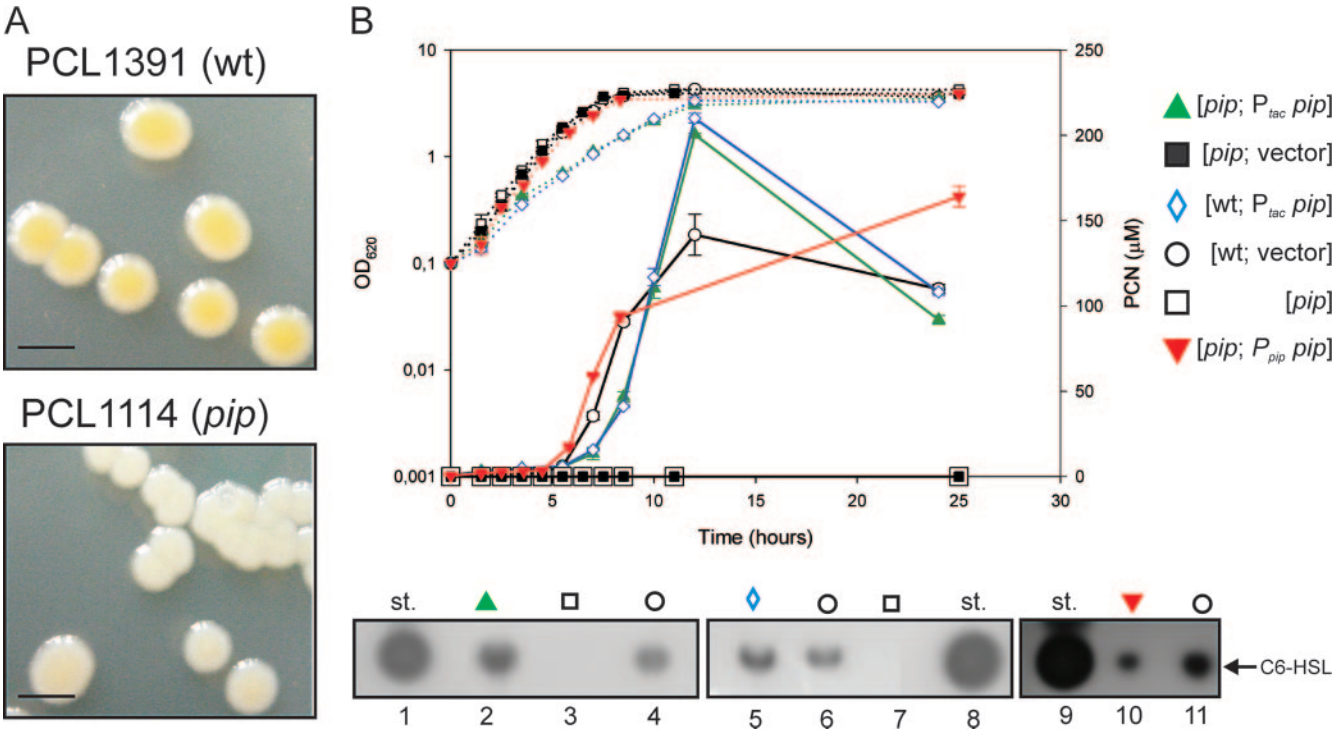


FIG. 1. Analysis of PCN and *N*-AHL production by *P. chlororaphis* PCL1391 and PCL1114 derivatives. (A) Phenotypic aspect of PCL1391 and PCL1114 colonies. Bar, 5 mm. (B) Extractions were made from at least three independent cultures in 10 ml of MVB1 medium in a time course, and the PCN production level was determined by HPLC. The error bars indicate the standard deviations. On each graph, the OD_{620} (left axis; dotted lines) and the PCN concentration (right axis; solid lines) are plotted. The symbol for the *pip* strain (□) was magnified for better visualization. Below the graph is the result of C_8 reverse-phase TLC analysis of *N*-AHL production by various PCL1391 derivatives at an OD_{620} of 3.0. st, standard of 2.5 nmol of synthetic C_6-HSL. For details, see Materials and Methods.

transposon of pRL1063a contains an origin of replication that functions in *E. coli* (36). Chromosomal DNA was isolated from PCL1114, digested with EcoRI, 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 performed using BLAST (http://www.ncbi.nih.gov/BLAST). A search for bacterial promoters and terminators was 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).

Recombinant DNA techniques. General DNA techniques were performed as described previously (31). PCRs were carried out with Super *Taq* enzyme (Enzyme Technologies Ltd., United Kingdom). Only for the construction of *pip* under control of the P_{tac} or P_{pip} promoter were PCRs 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, Leusden, The Netherlands) and T4 DNA ligase was from Promega (Leiden, The Netherlands).

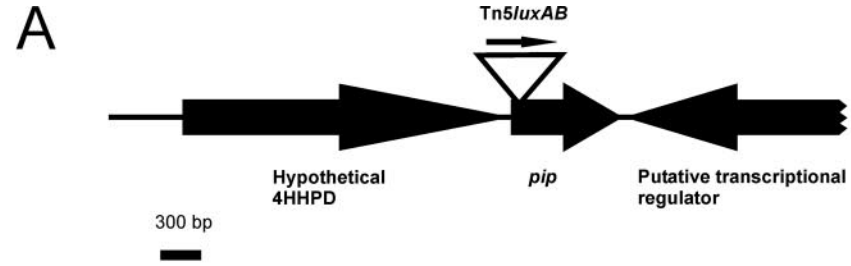
Construction of plasmids and PCL1391 mutant strains. In order to construct a suicide plasmid for disruption of *pip* by single homologous recombination, an internal *pip* fragment of 350 bp was obtained by PCR on PCL1391 chromosomal DNA with the primers oMP814 (5′-ATATATGAATTCCCGGCGCTCGGGTGGATGCC-3′) and oMP815 (5′-ATATATGAATTCTCTCGCCCAGGGCATGGAGG-3′). The PCR fragment was cloned in the EcoRI site of the vector pMP5285. The obtained suicide vector was named pMP7451 and introduced into PCL1391 by triparental mating using the helper plasmid pRK2013. The resulting mutant was named PCL2008. PCL2008 is impaired in PCN and C_6-HSL production, like PCL1114 (data not shown), confirming that the phenotype of PCL1114 is not due to a secondary mutation in the genome.

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 EcoRI site of pBBR1MCS-5. The resulting

TABLE 2. Overview of the results for PCN and *N*-AHL production by various derivative strains

Metabolite	Production with the indicated mutation in:[a]								
	psrA		*rpoS*		*pip*				
	Empty vector	P_{tac} *pip* strain	Empty vector	P_{tac} *pip* strain	Empty vector	P_{tac} *psrA* strain	P_{tac} *rpoS* strain	P_{tac} *pip* strain	P_{tac} *phzR* strain
PCN	−	+	−	+	−	−	−	+	+
N-AHL	−	+	−	+	−	−	−	+	+

[a] Cloned genes were added in *trans*. See Materials and Methods for an explanation of the notation.



B

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PCL1391          ---------MTTELSVVPEQSVNEPRKSRKNNPEKTRENILQEAIVEFVQQGLSGARVDA 51
DC3000           ---------MKKIDSAVAVEPAATVPKGRKNNPEKTREDILKAAVAEFVAHGLTGARVDA
KT2440           ---------MSDSVVGLGQPETEGGRKTRKNNPEKTREDILQAAINEFVQQGLAGARVDA
PfO1             ---------MTSELPAASALPAVEPRKSRKNNPEKTRENILQEAIVEFVQQGLSGARVDA
PAO1             ---------MSKPDPAVVEESAPRG-KGRKNNPEKTRQDILRAAIDEFVAQGLSGARVDA
Azotobacter      ---------MPDTKPVA--DPAARPRQPRKNNPEKTRQDILLAAIREFAEQGLSGARVDA
K96243           MPPSDHAKMKQGSKAAAPDAEARR DEARRKYDPEQTKRNILDVATQEFSAMGLSGARVDA
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-------------------------------
                 **      *   *  * *** *

KT2440           DHHDRNVDFVRIICTENIHYGEYVKQSPAIREMSSLVLEALGNTLRRGVQEGVFRAGIEV 171
PCL1391          DHHDRNVDFVRIVSIENIHNAEYVKRSEAIKAMNNTILHALGEILQRGVDEGVFRAGLDP
PfO1             DHHDRNVDFVRIVSIENIHNAEYVKRSDAIKAMNNTILDSLGEILRRGAEEGVFRAGLDA
PAO1             DYHDRHVDFVRIISIENIHKGEHIASSELVQSVNSSIVQSIAEILRRGEAEGVFRAGLEA
DC3000           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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C

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Pip   MTTELSVVPEQSVNEPRKSRKNNPEKTRENILQEAIVEFVQQGLSGARVDAIAERIHTSK 60
AcrR  ---------------MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTR
                     *     **  **  *   * *** *      **

Pip   RMIYYYFGSKEQLYVEVLEKLYGDIRSTESRLHLAELAPRDAIRRLVEF TFDHHDRNVDF 120
AcrR  GAIYWHFKDKSDLFSEIWELSESNIGELELEYQAKFPGDPLSVLREILIHVLESTVTEER
        **  *  *  *  *  *     *   *                  *

Pip   VRIVSIENIHNAEYVKRSEAIKAMN-NTILHALGEILQRGVDEGVFRAGLDPLDVHLLIS 179
AcrR  RRLLMEIIFHKCEFVGEMAVVQQAQRNLCLE SYDRIEQT--LKHCIEAKMLPADLMTRRA
       *       *  * *           *  *     * *          *   * *

Pip   SFCFYRVSNRHTFGEIFQIDLPDEAIKQRHREMICESVLRYLQA                  223
AcrR  AIIMRGYISGLMENWLFAPQSFDLKKEARDYVAILLEMYLLCP -
                         *     *     *    *
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FIG. 2. In silico analysis of the *pip* gene. (A) 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 putative transcriptional regulator was not completely sequenced. The position of the transposon insertion is shown as an arrowhead at the beginning of *pip*. 4-HHPD, 4-hydroxyphenylpyruvate

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 overexpression of *phzR*, PCL1114 was transformed with pMP7447 (10) to obtain PCL2013. For overexpression of *rpoS*, PCL1114 was transformed with pMP7420 (10) to obtain PCL2036. For overexpression of *psrA*, PCL1114 was transformed with pMP7465 (10) to obtain PCL2046. In order to study the effect of overexpression of *pip* on *phzR* expression, PCL1104 (6) was transformed with pMP7455, which resulted in PCL2035.

The *pip* gene was also constitutively expressed in wild-type, *psrA*, and *rpoS* mutant backgrounds. Therefore, PCL1391, PCL1111 (7), and PCL1954 (10) were transformed with the plasmid pMP7455 by triparental mating, and the resulting strains were named PCL2019, PCL2038, and PCL2040, respectively.

Primers oMP1045 (5′-ATATATGAATTCGAGGTCAGCCGGGCCAAGGAG-3′) and oMP817 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 EcoRI 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. Strains PCL1391, PCL1114, PCL1104, and PCL1111 (*pip*, *phzR*, and *psrA* mutants, respectively) were transformed with pMP7487 to obtain strains PCL2089, PCL2085, PCL2086, and PCL2087, respectively.

Extraction and analysis for phenazine and *N*-AHL. 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 (34). For *N*-AHL extraction, supernatants from 50-ml MVB1 cultures were harvested at an optical density at 620 nm (OD_{620}) of 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-C_{18} thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany). The TLC plates were developed in methanol-water (60:40, vol:vol). For detection of *N*-AHLs, the TLC was overlaid with 0.8% agar LC containing a 10-fold dilution of overnight culture of the *Chromobacterium violaceum* indicator strain CV026 (24) 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 Tn*5luxAB* reporter strains. Expression of *pip* was monitored in various derivatives making use of the *luxAB* reporter genes of the Tn*5* derivative in PCL1114. Expression was determined by quantification of bioluminescence during growth. Cells from overnight MVB1 cultures were washed with fresh medium and diluted to an OD_{620} of 0.1 in 10 ml of fresh MVB1 medium. During growth, the 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] in a 2% bovine serum albumin solution) was added, and 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. Ten milliliters of MVB1 medium was inoculated with an overnight culture washed with fresh medium at an OD_{620} of 0.1. Cells were harvested at an OD_{620} of 1.0 or 2.2 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% sodium dodecyl sulfate, 2 mM EDTA, 10% glycerol, 0.01% bromophenol blue, 1% β-mercaptoethanol) and boiled for 3 min. The samples were subsequently loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel, and proteins were separated and blotted following a standard Western blotting procedure (1). A dried aliquot of RpoS antibody was kindly provided by K. Tanaka (Tokyo, Japan). This sample was resuspended in 100 μl of phosphate-buffered saline, diluted 1,000-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 solution of 250 μM sodium luminol (Sigma) in 0.1 M Tris-HCl, (pH 8.6), and 0.01% H_2O_2 mixed with 60 μl of enhancer solution (67 μM *p*-hydroxy coumaric acid [Sigma] in dimethyl sulfoxide). Protein bands were detected using a Super R-X photographic film (Fujifilm, Düsseldorf, Germany).

Shift assays. A PCR product of the upstream region of *pip* was obtained using the primers oMP1116 (5′-CCAAGTTGTAGGAGTTTCGTAAC-3′) and oMP1117 (5′-TGTGGTCATTGTCATTCTGGG-3′) with pMP7444 as template and the Phusion DNA polymerase (Finnzymes). After purification on QIAquick columns (QIAGEN, Westburg, Leusden, The Netherlands), the PCR product was labeled with [γ-^{32}P]ATP using polynucleotide kinase (Fermentas, St. Leon-Rot, Germany) and purified over MicroSpin S-200 HR columns (GE Healthcare, Roosendaal, The Netherlands).

Cellular extracts of several *P. chlororaphis* derivatives were produced using the following method. Fifty milliliters of fresh MVB1 medium was inoculated with washed cells from overnight cultures at an initial OD_{620} of 0.1. Cells were harvested by spinning down cultures at an OD_{620} of 1.0 for 15 min at 3,000 rpm at 4°C. The pellets were resuspended in 2 ml of B-PER bacterial protein extraction reagent (Pierce, Perbio Science, Etten-Leur, The Netherlands) and gently shaken at room temperature for 15 min. The samples were centrifuged at 25,000 rpm for 30 min at 4°C in a Centrikon T-2070 ultracentrifuge (Kontron Instruments, Beun-De Ronde, Abcoude, The Netherlands). Supernatants (S30 fractions) were collected and frozen at −80°C in 10% glycerol for later use in binding reactions.

Reactions were performed in a 10-μl final volume, containing 50 mM Tris-HCl (pH 7.6), 60 mM NH_4Cl, 7 mM $MgCl_2$, 0.9 ng of ^{32}P-labeled PCR product (1 nM), and purified Pip-His_6 protein and/or S30 extracts as indicated. A 1,000-fold excess of genomic DNA was present in the samples to avoid nonspecific DNA-protein interactions. After a 20-min incubation at room temperature, samples were supplemented with 10% glycerol and loaded on an 8% polyacrylamide gel electrophoresis gel in 20 mM Tris-borate (pH 7.6) and run in the same buffer. Radioactivity was visualized by phosphor imaging (Bio-Rad, Veenendaal, The Netherlands).

Computational prediction of Pip target genes. Search for conserved motifs in the upstream region of *pip* orthologues *of P. aeruginosa* PAO1, *P. chlororaphis* PCL1391, *P. fluorescens* Pf-5, *P. fluorescens* PfO1, *Pseudomonas putida* KT2440, *Pseudomonas syringae* B728a, and *P. syringae* pv. *tomato* str. DC3000, was performed using the MEME program (2) (available at http://meme.sdsc.edu/meme/meme.html). A 47-nucleotide (nt) sequence (with the consensus CGCCATCG CGGCTTCCTTCGCTGGGCGGCGCGCCCCATAATCGCCCG) was proposed as the best-conserved motif among orthologous *pip* upstream regions. In order to identify similar conserved patterns and therefore potential Pip target genes in genomes of pseudomonads, we generated a position weight matrix from the set of the seven conserved sequences (see Table S1 in the supplemental material) deduced from the MEME program using the Target Explorer web tool (33) (available at http://trantor.bioc.columbia.edu/Target_Explorer/). The maximum score that a 47-nt sequence could obtain with the "Pip" scoring matrix is 39.44 bits, and the minimum score is −95.04 bits. We used the generated matrix to scan the partial genome of *P. chlororaphis* PCL1391, and scoring of potential binding sites is based on the program PATSER (14). The cutoff score was fixed to 10 bits, 10.23 bits below the minimum score for the training set of sequences (20.23 bits for Pip of *P. putida* KT2440) in order to allow identification of sequences with several mismatches versus the consensus described above. No sequences with significant scores (similar to those obtained by the 47-nt motifs of upstream *pip* genes) were recovered. Identical results were obtained from *P. aeruginosa* PAO1 and *P. fluorescens* Pf-5 genome scans. As an alternative, the same in silico approach was applied using the Predict Regulon Server (37) (available at http://210.212.212.6/cgi-bin/regsites/predictregulonv1.pl). This program, although similar to Target Explorer, is less restrictive, but predictions contain many more false-positive hits than the former one. This program predicted in *P. aeruginosa* PAO1 eight sequences that contain patterns similar to the one observed upstream of Pip (see Table S2 in the supplemental material).

dioxygenase. (B) 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), *A. vinelandii* (ZP_00091468), *Burkholderia pseudomallei* K96243 (YP_111478), and *S. meliloti* 1021 (NP_436576). The amino acids that are conserved in all the Pip homologues are indicated in bold. The amino acids that are conserved in all the Pip homologues and in the TetR N terminus are indicated by asterisks. The numbers at right indicate the amino acid numbering of Pip in strain PCL1391. (C) Alignment of Pip and AcrR from *E. coli* (AAC73566). Conserved amino acids are indicated by asterisks. The numbers at right indicate the amino acid numbering of Pip.

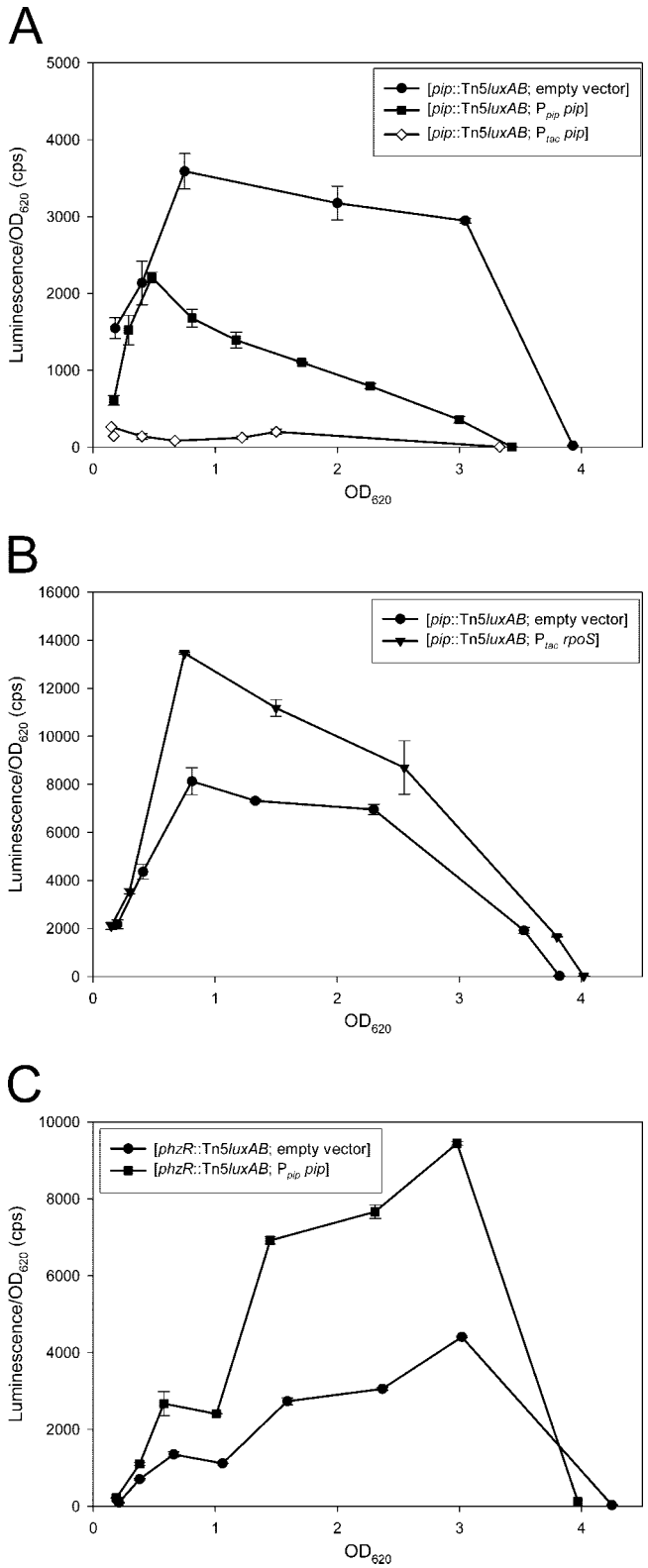

FIG. 3. Expression analyses of *P. chlororaphis* PCL1391 *pip*, *psrA*, and *phzR* derivative strains. Each panel corresponds to a particular chromosomal background, and the genes expressed in the different backgrounds are indicated in the legend. Cell cultures were grown in 10 ml of MVB1 medium, and samples were taken at regular time intervals. Activity of the *luxAB* reporter was determined by quantifying bioluminescence. Measurements were performed in duplicate, and averages are plotted. The bars represent the standard deviation. The strains used in these experiments are as indicated on the graphs.

Nucleotide sequence accession number. The *pip* sequence determined in this study was given accession number DQ311664.

RESULTS

For clarity in the presentation of results, strains will be described as, for example, the *phzR* P_{tac} *pip* strain, where the first gene indicates a genomic modification and the second is the gene cloned in the vector pBBR1MCS-5 and added in *trans*. In general, genomic mutations are due to transposon insertion of the *luxAB* gene, via the plasmid pRL1063a (Table 1). Some mutations were made by single homologous recombination (SHR) and are indicated as, for example, $rpoS_{SHR}$ (Table 1).

General characteristics of *pip*. (i) Isolation of mutant unable to produce PCN (strain PCL1114). A transposon library containing 18,000 mutants of *P. chlororaphis* PCL1391, established using pRL1063a (6), was screened for mutants exhibiting reduced PCN production. After growth on LC agar, mutant colonies producing PCN appear yellow. Among 20 white transposon colonies, one mutant (Fig. 1A) was tested for PCN production after growth in liquid, complex LC medium. Quantitative high-pressure liquid chromatography (HPLC) analysis shows that this mutant, named PCL1114, is severely affected in PCN production (<1% of wild-type [wt] strain PCL1391 production). When mutant PCL1114 was grown in King's B medium, another complex medium, PCN production was reduced to 2.5% compared to wt. PCN production by PCL1114 was not detected (<1% of wt) during growth in the poorer synthetic MVB1 medium (Fig. 1B), which was used as the standard growth medium in subsequent experiments. For convenience, the results of the experiment in Fig. 1 are summarized in Table 2. In addition, analysis of *N*-AHL production showed that C_6-HSL could not be detected in the supernatant of PCL1114 (Fig. 1C, lane 7). The PCL1114 mutant is therefore unable to synthesize both PCN and its associated *N*-AHL signaling molecule, suggesting a mutation within a gene involved in the signaling cascade.

(ii) Pip is essential to PCN synthesis. Plasmid rescue from chromosomal DNA of PCL1114 showed that the Tn*5luxAB* transposon is inserted in a small open reading frame (ORF) of 669 bp in between positions 71 and 72 (Fig. 2A). The gene corresponding to this ORF was named *pip*. The protein that shows highest overall similarity with Pip is a putative transcriptional regulator of the TetR family in *P. fluorescens* (ZP_00262623). Orthologues of Pip were found in other *Pseudomonas* species (94% homology in *P. fluorescens* Pf-5, 83% homology in *P. putida* KT2440, and 79% homology in *P. aeruginosa* PAO1), as well as homologues in a large variety of other gram-negative species, such as *Azotobacter vinelandii*, *Burkholderia* spp., *Sinorhizobium meliloti*, *Agrobacterium tumefasciens*, and *Ralstonia* spp. To our knowledge, no function has been published for any of these homologues. A domain homology search on the Pip sequence showed that Pip is homol-

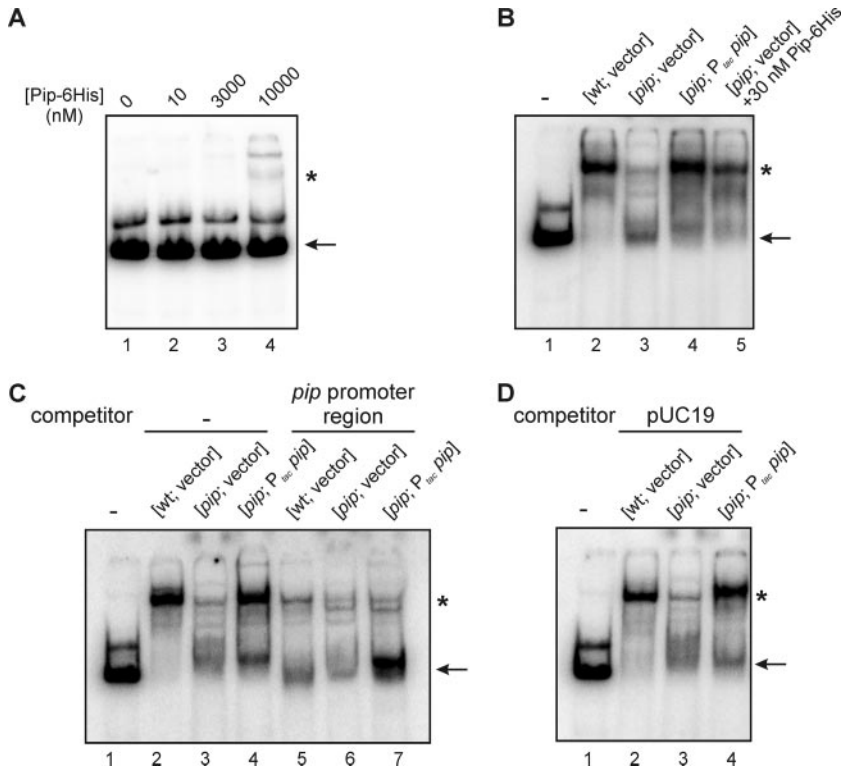

FIG. 4. Binding of Pip to its own promoter region. One nanogram of a ^{32}P-labeled DNA fragment of 120 bp, corresponding to the *pip* promoter region, was used as a probe for band shift assays with either purified Pip protein (A) with cell extracts from different strains (B, C, and D) or a combination of both (B). Competition assays were performed with 50 ng of the *pip* promoter region (C) and with 50 ng of pUC19 plasmid (D). Samples were separated by 8% native polyacrylamide gel electrophoresis at 120 V for 20 min, and bands were visualized by phosphor imaging. The arrows indicate the positions of the free probes and the asterisks indicate the positions of the complex.

ogous to members of the TetR/AcrR family, which are transcriptional regulators (11). In *E. coli*, TetR regulates a pump involved in tetracycline resistance (3), and AcrR regulates a pump involved in multidrug resistance (26). A multiple alignment between Pip orthologues and the TetR N-terminal domain (Pfam 00440) shows that many amino acids are conserved within the region that contains a helix-turn-helix motif (Fig. 2B). Although no homology with the TetR C-terminal effector-binding domain was found, full-length alignments are possible with AcrR-like proteins, and Pip has an overall similarity of 42% with AcrR (accession number AAC73566) (Fig. 2C). A putative promoter sequence was found upstream of the *pip* ORF (putative −10 box [GCCCATAAT] and −35 box [TTT CCT]). No *rho*-independent terminator could be detected downstream of *pip*, although a putative gene is located there in the opposite direction of transcription (see below).

The chromosomal organization around *pip* was determined by sequencing and analyzed by BLAST search. An ORF of 1,908 nucleotides is present upstream of *pip* (Fig. 2A), which has the same transcription orientation as *pip* and encodes a protein showing 92% homology to a putative 4-hydroxyphenylpyruvate dioxygenase 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 (GTAACGGCGGCGGCAAAGGGC CGCCGTCCTGC), followed by the putative promoter sequence upstream of the *pip* ORF. Downstream of *pip*, an ORF is present (Fig. 2A), of which the predicted protein product shows 58% homology with a putative transcriptional regulator of *C. violaceum* ATCC 12472 (accession number AAQ59192).

In order to test if the inhibition of PCN production was indeed due to the defect in *pip*, we tested whether PCL1114 could be complemented by expression of *pip*. The *pip* P_{tac} *pip* strain is PCL1114 harboring *pip* under control of the *tac* promoter in *trans*. This strain produced 1.4-fold more PCN (Fig. 1B) than wt (empty vector) strain as analyzed after 12 h of growth. The *pip* P_{tac} *pip* strain also produces high amounts of C_6-HSL (Fig. 1C, lane 2). The *pip* gene was also expressed under its own promoter in *trans* in the *pip* P_{pip} *pip* mutant strain and showed restored production of PCN and *N*-AHL (Fig. 1B and C). These results clearly show that the impaired production of PCN and *N*-AHL in PCL1114 is only caused by disruption of *pip*.

Role of Pip in the regulation of PCN synthesis. (i) Autoregulation of *pip* expression. Since both TetR and AcrR repress their own expression (15, 21), we tested whether Pip shows a similar autoregulatory mechanism. Analysis of the orientation of the Tn*5luxAB* in PCL1114 showed that the *luxAB* genes and *pip* have the same direction of transcription, which allows measurements of *pip* transcription by quantifying the *luxAB* activity. The expression of *pip* was measured in three *pip* derivatives containing P_{tac} *pip*, P_{pip} *pip*, or the empty cloning vector

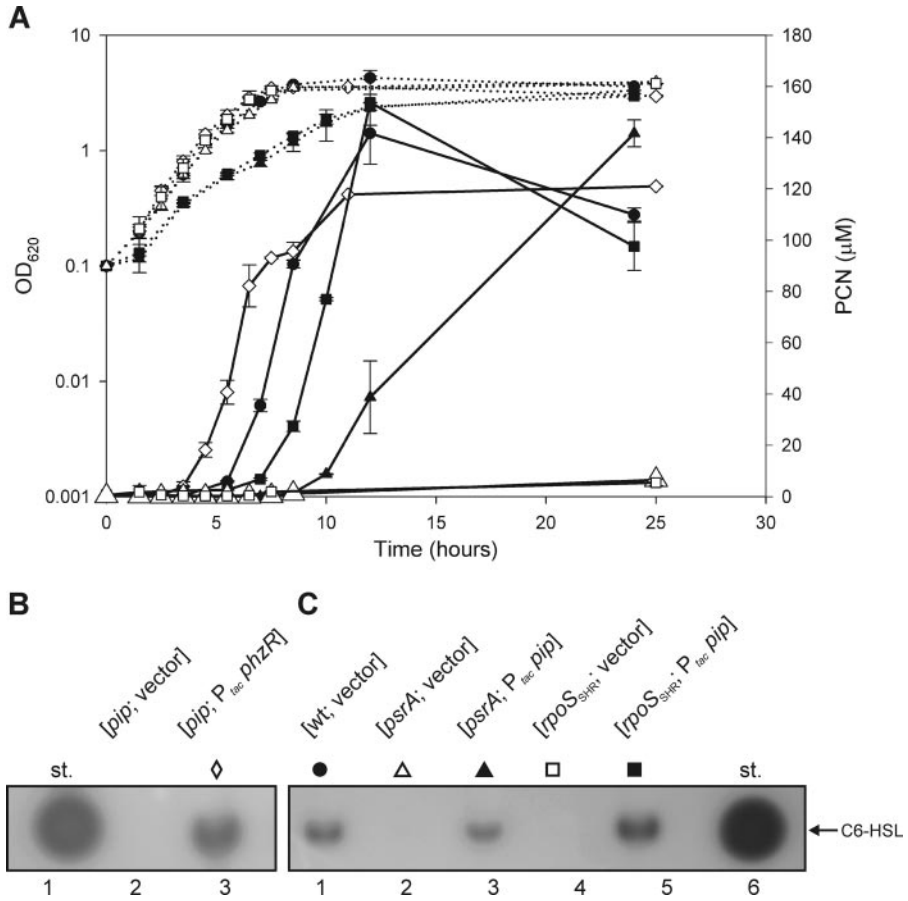

FIG. 5. Analysis of PCN and *N*-AHL production in *P. chlororaphis* PCL1391 derivative strains. (A) Extractions were carried out from at least three independent cultures in 10 ml of MVB1 medium in a time course, and the PCN concentration was determined by HPLC analysis. On each graph, the absorbance is plotted along the left axis (dotted lines), and the PCN concentration is plotted along the right axis (solid lines). The error bars indicate the standard deviations. Symbols correspond to those of panels B and C. The symbol for the *psrA* strain (empty vector) (△) was magnified for better visualization. (B and C) C_{18} reverse-phase TLC analysis of *N*-AHL produced by the *pip* (empty vector) strain (lane 2) and other derivatives. st, 2.5 nmol of synthetic C_6-HSL. Extractions were performed on supernatants of cultures that reached an OD_{620} value of 3.0. For the detection of *N*-AHL, see Materials and Methods.

(Fig. 3A). The *pip* P_{pip} *pip* strain showed an intermediate *lux* activity (2,210 ± 67 cps) compared to the *pip* (empty vector) strain (3,590 ± 231 cps) and to the *pip* P_{tac} *pip* strain (262 ± 1 cps). These results suggest that Pip, like TetR and AcrR, represses its own transcription.

Since Pip shows homologies with DNA-binding transcriptional regulators and autoregulates its own expression, we searched for a Pip-specific *cis*-acting element within its upstream region. An in silico search for conserved motifs in several upstream regions of *pip* orthologues retrieved a 47-nt sequence (see Materials and Methods) located 36 bp upstream of the *pip* start codon as the best-conserved motif among orthologous *pip* upstream regions. The putative −35 and −10 boxes suggested above are included in this sequence. A 120-bp PCR product of the Pip upstream region containing the conserved sequence was obtained and used for band-shift analysis. Purified Pip-His_6 did not seem to be able to bind and shift the labeled DNA (Fig. 4A). However, S30 fractions (see Materials and Methods) of wt and of the *pip* P_{tac} *pip* strain were able to bind the ^{32}P-labeled PCR product as shown by band retardation, whereas the S30 fraction of the *pip* mutant was not (Fig. 4B, lanes 2, 4, and 3, respectively). Interestingly, the addition of purified Pip-His_6 to the S30 fraction of the *pip* mutant resulted in the shifting of the DNA (Fig. 4B, lane 5). Competition with the unlabeled PCR product inhibited the shift, whereas competition with unlabeled pUC19 plasmid did not (Fig. 4C and D, respectively). These results show that Pip is able to specifically recognize and bind a DNA sequence within the 120 bp upstream of the start codon. However, an additional factor present in S30 fractions obtained from *P. chlororaphis* cultures is apparently necessary for DNA-binding activity of Pip.

(ii) Position of Pip in the regulatory network of PCN synthesis. Several genes, including *psrA*, *rpoS*, and *phzI*/*phzR*, are known to play a role in the regulation of PCN synthesis (6, 10), and therefore experiments were conducted in order to understand how *pip* fits into the PCN biosynthesis signaling cascade.

In MVB1 medium the *psrA* and *rpoS* genes positively regulate PCN and *N*-AHL production (10). To test whether Pip could regulate PCN and *N*-AHL production downstream of *psrA* and/or *rpoS*, *pip* was overexpressed in strains PCL1111 (*psrA* mutant) and PCL1954 (*rpoS* mutant). Both strains showed restored production of PCN (Fig. 5A) and C_6-HSL

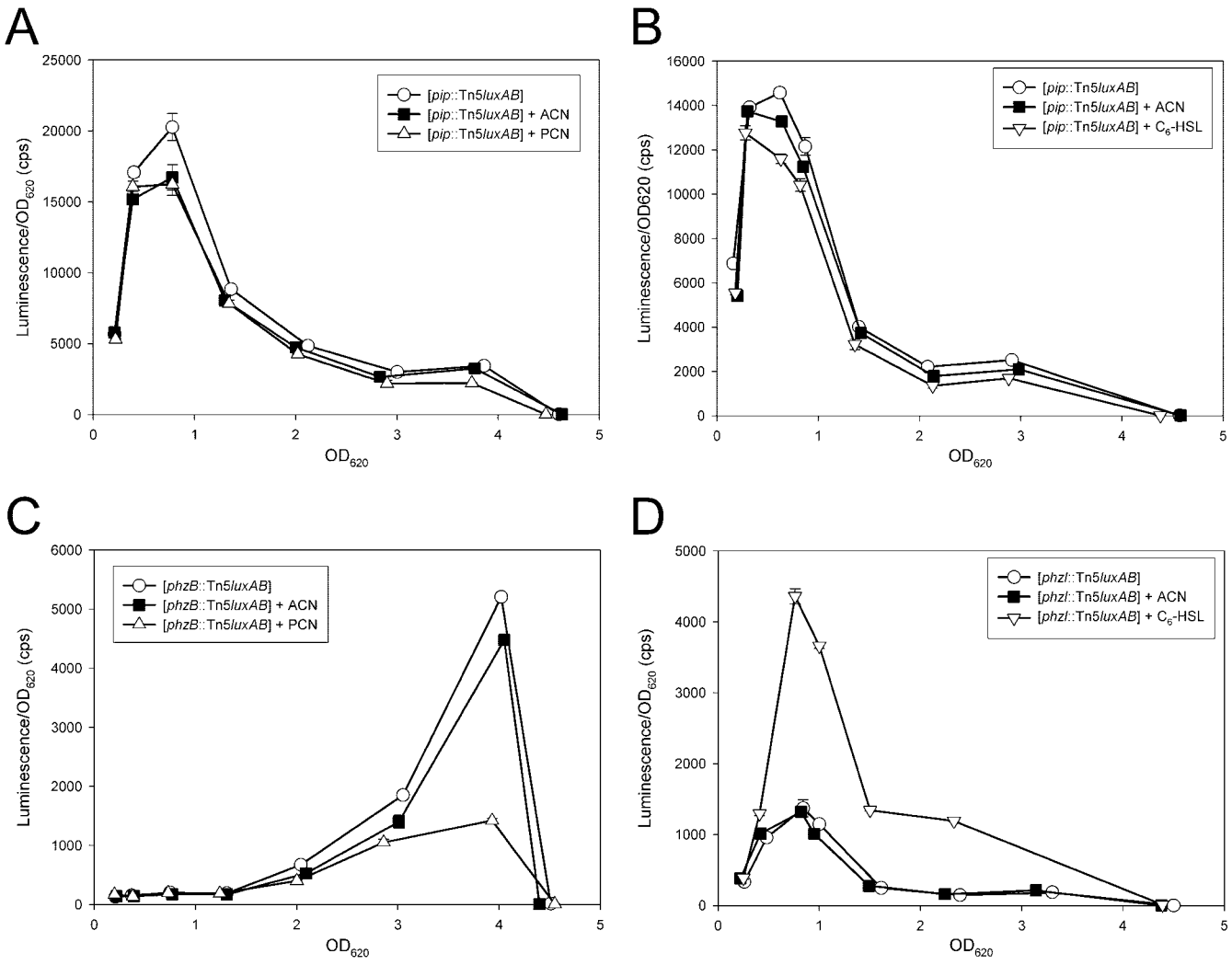

FIG. 6. Influence of C_6-HSL and PCN on P_{pip} activity. Cell cultures were grown in 10 ml of MVB1 medium supplemented with acetonitrile (ACN), 2 μM PCN, or 5 μM C_6-HSL. Samples were taken at regular time intervals. Activity of the *luxAB* reporter was determined by quantifying bioluminescence. Measurements were performed in duplicate, and averages are plotted. The bars represent the standard deviations, and some are too small to be seen. The following strains were used in these experiments: the *pip*::Tn*5luxAB* strain (A and B), the *phzB*::Tn*5luxAB* strain (C), and the *phzI*::Tn*5luxAB* strain (D).

(Fig. 5C; compare lanes 3 and 2 and 5 and 4). In addition, constitutive expression of *rpoS* in the mutant *pip* P_{tac} *rpoS* strain resulted in a 25% increase of *lux* activity compared to the *pip* (empty vector) strain (Fig. 3B), showing that RpoS can influence *pip* expression. In contrast, constitutive production of PsrA and RpoS in the *pip* mutant PCL1114 (*pip* P_{tac} *psrA* strain and *pip* P_{tac} *rpoS* strain, respectively) was not able to restore PCN production (results not shown). Western blot analysis confirmed that similar amounts of RpoS were isolated in the extracts from the wt and *pip* mutant (see Fig. S1, lanes 1 and 2, in the supplemental material). These cross-complementation assays position Pip downstream of PsrA and RpoS in the regulatory pathway leading to PCN production. For convenience, results of the experiment shown in Fig. 5 are summarized in Table 2.

To test the relationship between Pip and quorum sensing, a *pip* mutant derivative was constructed that constitutively expresses *phzR*. The resulting *pip* P_{tac} *phzR* strain showed restored production of both PCN and C_6-HSL (Fig. 5A and B). Transformation with a plasmid containing *pip* under its own promoter showed a positive effect on *phzR*::Tn*5luxAB* expression (Fig. 3C). These results confirm that Pip regulates PCN synthesis *via* the PhzI/PhzR quorum-sensing system, downstream of *psrA* and *rpoS*. Pip is therefore the fifth transcription factor shown to be involved in the regulation of PCN biosynthesis in strain PCL1391, along with GacA, PsrA, RpoS, and PhzR.

(iii) Pip, an efflux-pump regulator? Based on the homology between Pip and AcrR/TetR, we considered the hypothesis that Pip might directly regulate the expression of a gene encoding an efflux pump, analogously to AcrR and TetR. Results described above could suggest that this pump would secrete PCN or even more likely *N*-AHL. To test this hypothesis, the effect of PCN and *N*-AHL on *pip* transcription was measured, since it was shown that the expression of *acrR* and *tetR* is under the regulation of the molecules secreted by their target pumps.

Results show that neither PCN nor *N*-AHL regulates the activity of P_{pip} (Fig. 6A and B). The same results were obtained with an intact *pip* gene in *trans* by using the *pip*::Tn*5luxAB* P_{pip} *pip* strain (data not shown). Importantly, the concentrations of PCN and *N*-AHL we used were sufficient to inhibit transcription of *phzB* (Fig. 6C) and stimulate that of *phzI* (Fig. 6D). These results indicate that the transcription of *pip* is not influenced by *N*-AHL or PCN.

DISCUSSION

In this study we identified a new gene (*pip*) required for the production of the antifungal metabolite PCN in *P. chlororaphis* PCL1391. A *pip* mutant does not produce any detectable amounts of PCN, while increasing the copy number of *pip* results in an increased transcription of the PCN activator *phzR* (Fig. 3C), which is now confirmed by preliminary microarray data analyses (data not shown). The identity of Pip as a transcriptional regulator was suggested in silico (AcrR/TetR family) and demonstrated in vitro (Fig. 4) and in vivo (*luxAB* expression), which enables us to insert a new control point into the signaling pathway leading to PCN production.

Several experiments were conducted to assess where Pip was positioned in the regulatory cascade of PCN synthesis. The relative position of Pip was deduced from the ability of Pip to restore in *trans* PCN production in mutants of other genes known to be involved in the control of PCN production, i.e., *psrA* (activator of *rpoS* transcription) and *rpoS* (stationary-phase sigma factor). Opposite in *trans* complementation assays were also performed for testing the ability of PsrA, RpoS, and PhzR to restore PCN production in the *pip* mutant. Production of PCN and *N*-AHL was fully restored in *psrA* and *rpoS* mutants constitutively expressing *pip*, while the *pip* mutation could only be suppressed by constitutive production of Pip or PhzR (Table 2). These results justify the position of Pip downstream of PsrA and RpoS and upstream of PhzR in our model (Fig. 7).

This new model raises the questions of why there are so many checkpoints for PCN biosynthesis and, in the focus of this particular study, what the effector molecule of Pip is and how the information further is delivered to initiate PCN production. The identification of a direct Pip target gene should provide crucial information to answer these questions. A first hypothesis is that Pip could regulate an efflux pump, as the TetR and AcrR regulators do. To test if Pip, like TetR, regulates an antibiotic efflux-pump, the resistance of *pip* to several antibiotics was compared to that of the wt, but no difference could be shown (data not shown). In analogy to AcrR/TetR, the molecules secreted by the Pip-regulated pump would modulate, in turn, the activity of Pip. It was shown that a factor present in the cell is necessary for Pip to bind DNA (Fig. 4A and B). However, it cannot be either *N*-AHL or PCN, since both are absent in a *pip* mutant background, the cellular extract of which seems to contain the predicted Pip-interacting factor (Fig. 4B, lane 5). In addition, we showed that the expression of *pip* is not under the influence of PCN or *N*-AHL metabolites (Fig. 6). Taken together, these results do not support the notion that *pip* directly regulates a pump for *N*-AHL or PCN; rather, they suggest either that an additional metabolite participates in this regulation or that Pip does not directly control genes encoding an efflux pump.

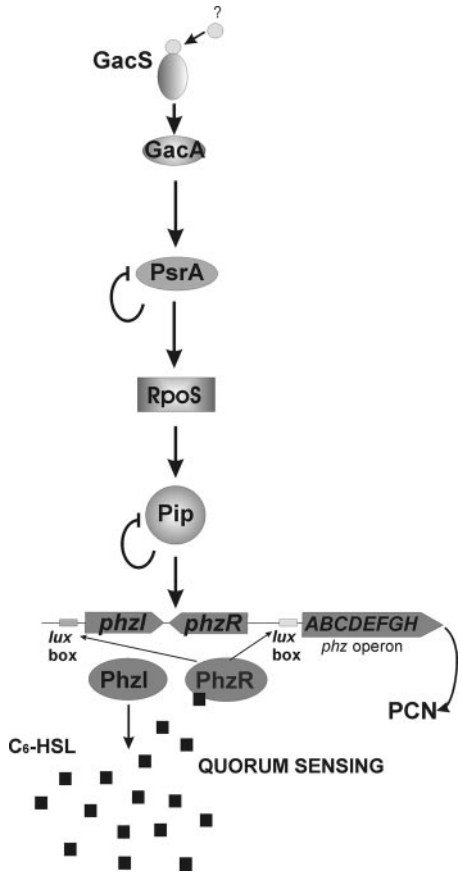

FIG. 7. Schematic model showing the role of Pip in the genetic cascade regulating PCN synthesis in *P. chlororaphis* PCL1391 and in the presence of several stress factors. The regulatory cascade of PCN starts with the sensing of an as yet unidentified environmental signal by GacS and subsequent activation of GacA. The TetR homologue PsrA regulates *rpoS*, probably by binding to its promoter. The alternative sigma factor RpoS positively regulates *pip*, the product of which stimulates expression of 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 PCN.

As a first attempt to identify putative Pip target genes, we used the conserved sequence upstream of *pip*, shown to interact with Pip, to generate a position weight matrix and scan *Pseudomonas* genomes for similar DNA patterns. Computational prediction programs predicted in *P. aeruginosa* PAO1 eight sequences that contain patterns similar to the one observed upstream of *pip* (see Table S3 in the supplemental material). The best scoring hit is located 83 nt upstream of the *epd* gene coding for the D-erythrose 4-phosphate dehydrogenase that connects the pentose-phosphate pathway to the vitamin B6 metabolism. The other seven potential target genes all encode hypothetical proteins with unknown functions. It is also possible that the motif recognized by Pip is not well conserved, as is the case for other known transcriptional regulators, for example LasR in *P. aeruginosa* (32). A large number of additional experiments will be required to precisely identify

the primary target of Pip. Particularly, identification of possible partner(s) of Pip, as indicated by the shift experiments (Fig. 4), is crucial to a better understanding of how Pip interacts with target DNA. Future work could also include a broader analysis of genes regulated by *pip*, using the microarray developed for strain PCL1391 (10). We are currently investigating preliminary data that suggest that Pip connects the PCN biosynthetic pathway to the stress response in *P. chlororaphis* PCL1391.

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