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Phenazine-1-Carboxamide Production in the Biocontrol Strain *Pseudomonas chlororaphis* PCL1391 Is Regulated by Multiple Factors Secreted into the Growth Medium

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***Pseudomonas chlororaphis* PCL1391 controls tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. The production of phenazine-1-carboxamide (PCN) is crucial for this biocontrol activity. In vitro production of PCN is observed only at high-population densities, suggesting that production is under the regulation of quorum sensing. The main autoinducer molecule produced by PCL1391 was identified structurally as *N*-hexanoyl-L-homoserine lactone (C_6-HSL). The two other autoinducers that were produced comigrate with *N*-butanoyl-L-homoserine lactone (C_4-HSL) and *N*-octanoyl-L-homoserine lactone (C_8-HSL). Two PCL1391 mutants lacking production of PCN were defective in the genes *phzI* and *phzR*, respectively, the nucleotide sequences of which were determined completely. Production of PCN by the *phzI* mutant could be complemented by the addition of exogenous synthetic C_6-HSL, but not by C_4-HSL, C_8-HSL, or any other HSL tested. Expression analyses of Tn*5luxAB* reporter strains of *phzI*, *phzR*, and the *phz* biosynthetic operon clearly showed that *phzI* expression and PCN production is regulated by C_6-HSL in a population density-dependent manner. The introduction of multiple copies of the regulatory genes *phzI* and *phzR* on various plasmids resulted in an increase of the production of HSLs, expression of the PCN biosynthetic operon, and consequently, PCN production, up to a sixfold increase in a copy-dependent manner. Surprisingly, our expression studies show that an additional, yet unidentified factor(s), which are neither PCN nor C_4-HSL or C_8-HSL, secreted into the growth medium of the overnight cultures, is involved in the positive regulation of *phzI*, and is able to induce PCN biosynthesis at low cell densities in a growing culture, resulting in an increase of PCN production.**

Additional keywords: biofungicide, *luxI*, phenazine, plant growth-promoting rhizobacteria, signal molecules.

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The tomato rhizosphere isolate *Pseudomonas chlororaphis* PCL1391 exhibits biocontrol activity against *Fusarium oxysporum* (Schlechtend.:Fr.) f. sp. *radicis-lycopersici* (W. R. Jarvis & Shoemaker), the causal agent of tomato foot and root rot. The production of phenazine-1-carboxamide (PCN), including the last step, conversion of phenazine-1-carboxylic acid (PCA) to PCN, was shown to be essential for the biocontrol activity of strain PCL1391 (Chin-A-Woeng et al. 1998). PCN production is maximal at the end of the exponential growth phase, suggesting that the production is mediated through a population density-dependent regulation mechanism known as quorum sensing. In quorum sensing, a key role is played by a small diffusible signal molecule, or autoinducer. In many gram-negative bacteria, the process of quorum sensing is activated when the overall concentration of *N*-acyl-homoserine lactone (HSL) signal molecules exceeds a certain threshold concentration (Fuqua et al. 1994; Salmond et al. 1995). The population density-dependent HSL signal molecule interacts with its cognate transcriptional regulator, a LuxR homolog. In *Pseudomonas aeruginosa*, it was shown that C_4-HSL diffuses freely across the cellular membrane into the local environment, whereas 3-*N*-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) reaches the environment through an active efflux (Pearson et al. 1999). In the *tra* system, binding of the activated complex TraR–3-oxo-C_8-HSL to target promoters subsequently induces gene expression (Zhu and Winans 1999). The autoinducer synthase often is regulated by an autoregulatory loop that, once initiated, results in amplification of the signal (Salmond et al. 1995).

Recently, we described a structural gene cluster *phzA* to *phzH* that is responsible for the biosynthesis of PCN in which the last gene, *phzH*, directs the conversion of PCA to PCN

(Chin-A-Woeng et al. 2001). In this paper, we describe the i) structural identification of C_6-HSL as the main HSL produced by PCL1391; ii) identification and characterization of regulatory genes *phzI* and *phzR* affecting PCN production; iii) construction and expression analyses of *luxAB* reporter strains of *phzI*, *phzR*, and the *phz* biosynthetic operons; iv) identification of C_6-HSL as the autoinducer of PCN biosynthesis; v) overproduction of PCN by the introduction of multiple copies of the *phzI* and *phzR* genes; and vi) indication of the presence of a second, yet unidentified factor in spent growth medium of PCL1391, which can advance and increase PCN biosynthesis.

RESULTS

Purification and identification of autoinducers produced by *P. chlororaphis*.

Production of PCN in *P. chlororaphis* PCL1391 (Table 1) is observed only in cultures with high population density and therefore likely to be subject to quorum sensing. To analyze the production of autoinducer(s) by strain PCL1391, a crude dichloromethane extract of spent culture medium of a 72-h culture was tested for induction of a number of quorum-sensing reporter systems, including those for *Chromobacterium violaceum* pigmentation (*cvi*) (McClean et al. 1997), *Vibrio fischeri* luminescence (*lux*) (Stevens and Greenberg 1997), and *Agrobacterium tumefaciens* conjugation (*tra*) (Hwang et al. 1995). After separation with C_{18} thin-layer chromatography (TLC), three spots were detected in the *Chromobacterium* spp. overlay system (Fig. 1, lane 4). One major spot with R_f value 0.4 migrated to the same position as synthetic C_6-HSL (Fig. 1, lane 2). A second spot with a R_f value of 0.57, which migrated to the same position as synthetic C_4-HSL, was detected (Fig. 1, lane 1). A third spot with a R_f value 0.13 comigrated with synthetic C_8-HSL (Fig. 1, lane 3). Extracts of spent culture supernatant also were able to induce the *lux* system, although they induced only weak signals in the *tra* system (data not shown). None of the reporter systems was induced by dichloromethane extracts of uninoculated King's B (KB) (King et al. 1954) or Luria-Bertani (LB) growth medium.

To overcome the problem of copurification of autoinducer(s) and PCN in the wild type, strain PCL1119 (*phzB*::Tn*5luxAB*) (Chin-A-Woeng et al. 1998), a PCL1391 derivative unable to produce PCN as a result of a mutation in the biosynthetic *phzB* gene, was used for autoinducer purification. PCL1119 is not changed in its autoinducer production compared with the wild-type strain, as analyzed by TLC (data not shown). The compound migrating with R_f value 0.4 was purified with C_{18}-reverse phase high-pressure liquid chromatography (HPLC) and analyzed with positive ion mode nanoelectrospray collision-induced dissociation (CID) tandem mass spectrometry on a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer, which allows exceptional sensitivity in the tandem mode. Instrument conditions were optimized with a solution of 1.3 ng of standard C_6-HSL per μl, having a concentration approximately one-tenth of that necessary to give a response in the bioassay similar to that of the fraction isolated from strain PCL1391. Following optimization, a CID spectrum in which fragmentation is induced on collision with a positive pressure of argon from the standard was recorded (Fig. 2A). The needle containing the standard was then discarded, and a CID spectrum was recorded of the same precur-

Table 1. Microorganisms and plasmids

Strains–plasmids	Relevant characteristics	Reference or source
Bacterial strains		
PCL1391	Wild-type *Pseudomonas chlororaphis*, producing phenazine-1-carboxamide and biocontrol strain of tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*	Chin-A-Woeng et al. 1998
PCL1392	PCL1391 derivative tagged with Tn*5lacZ*, Km^R, with wild-type colonizing ability	Chin-A-Woeng et al. 2000
PCL1103	PCL1391 derivative in which a Tn*5*-promoterless *luxAB* has been inserted in *phzI*	This study
PCL1104	PCL1391 derivative in which a Tn*5*-promoterless *luxAB* has been inserted in *phzR*	This study
PCL1119	PCL1391 derivative in which a Tn*5*-promoterless *luxAB* has been inserted in *phzB*	Chin-A-Woeng et al. 1998
DH5α	*supE44* Δ*lac*U169 (Φ80 *lacZ*ΔM15) *hsdR*17 *recA1 endA1 gyrA*96 *thi*1 *relA*1; general-purpose *Escherichia coli* host strain used for transformation and propagation of plasmids	Boyer and Roulland-Dussoix 1969
CV026	*Chromobacterium violaceum N*-acyl-homoserine lactone (HSL) reporter strain	Milton et al. 1997
NT1(pJM749)	*Agrobacterium tumefaciens* NT1 HSL reporter strain harboring pJM749 containing a *lacZ* reporter fused to a *tra* gene of which expression is dependent on TraR	Piper et al. 1993
Plasmids		
pRL1063a	Plasmid harboring Tn*5* transposon containing promoterless *luxAB*	Wolk et al. 1991
pBBR1MCS-5	Gentamycin-resistant derivative of the broad-host-range cloning vector pBBR1MCS	Kovach et al. 1995
pME6010	*E. coli*/*Pseudomonas* spp. shuttle vector, stably maintained in *Pseudomonas* spp., Tc^R	Heeb et al. 2000
pMP6003	pRL1063a-based plasmid recovered from chromosomal DNA of PCL1103 after digestion with *Eco*RI	This study
pMP6004	pRL1063a-based plasmid recovered from chromosomal DNA of PCL1104 after digestion with *Eco*RI	This study
pMP6007	pBluescript containing a 4.5-kb chromosomal fragment of strain PCL1391 with the *phzI* and *phzR* genes and the first part of the *phzA* gene	This study
pMP4062	Rhizosphere-stable plasmid pME6010 containing a 4.5-kb *Eco*RI fragment with the *phzI* and *phzR* genes of strain PCL1391	This study
pMP4065	pBBR1MCS-5 containing a 2.9 kb *Eco*RI/*Bam*HI fragment with the *phzI* and *phzR* genes of strain PCL1391 subcloned from the 4.5-kb chromosomal fragment in pMP6007	This study
pMP4067	The stable plasmid pME6010 containing a 2.9-kb *Hin*dIII with the *phzI* and *phzR* genes of strain PCL1391	This study
pSB401	Autoinducer reporter construct based upon the *Vibrio* bioluminescence (*lux*) system	Winson et al. 1998
pBluescript	General-purpose cloning vector, Cb^R	Stratagene, La Jolla, CA, U.S.A.

sor ion mass, yet only introduced solvent in order to be certain that no contamination of the instrument had occurred with the standard and to identify any background signals arising from the solvent. No ions corresponding to C_6-HSL were observed in this blank spectrum, although an ion at *m/z* 107, also observed in the C_6-HSL spectrum, was present. The bioactive fraction was then introduced into the mass spectrometer in a fresh needle, and a mass spectrum was recorded. Very weak pseudomolecular ions were observed at *m/z* 200 ($M+H^+$) and 222 ($M+Na^+$) for a species with a molecular mass corresponding to C_6-HSL. These ions were barely discernible above the background. A CID spectrum of the $M+H^+$ ion at *m/z* 200, however, was recorded and generated a good-quality spectrum (Fig. 2B) that was very similar to the one obtained from the standard C_6-HSL (Fig. 2A). Fragment ions characteristic of C_6-HSL were observed at *m/z* 99, 102, 172, and 182 (Fig. 2A inset). The ions at *m/z* 99 and 102 were generated on fragmentation of the amide bond between the hexanoyl moiety and the homoserine lactone, with the ion at *m/z* 99 corresponding to the hexanoyl substituent and that at *m/z* 102 being characteristic of the homoserine lactone. The ions at *m/z* 172 and 182 arise by the loss of small neutrals (*m/z* $172 = M+H^+ - C_2H_4$; *m/z* $182 = M+H^+ - H_2O$). These results clearly demonstrate that the component in the major bioactive fraction is *N*-hexanoyl-L-homoserine lactone (C_6-HSL).

In dichloromethane extracts of spent culture supernatant, the other two activities were present in small amounts that were not sufficient for mass spectrometric analyses and, thus, tentatively assigned as *N*-butanoyl-L-homoserine lactone (C_4-HSL) and *N*-octanoyl-L-homoserine lactone (C_8-HSL) on the basis of their TLC migration behavior (Fig. 1) and their biological activities in the *cvi*, *lux*, and *tra* reporter systems.

Isolation and characterization of mutants affected in PCN biosynthesis.

Screening of 18,000 Tn*5luxAB* PCL1391 transconjugants on agar plates and in liquid cultures resulted in the isolation of eight mutants altered in PCN production, as judged by their altered pigmentation. Four mutants were identified in which the transposon was not inserted in a PCN biosynthetic gene, two of which are described in this paper. The regions flanking the Tn*5* insertion in these two mutants, PCL1103 and PCL1104, were recovered in plasmids pMP6003 and pMP6004, respectively. Nucleotide sequence analyses of the flanking regions showed that the transposons in these mutants were inserted in homologs of *luxI* and *luxR*, respectively. Because the homologous genes are involved in PCN biosynthesis, we named them *phzI* and *phzR*, respectively. The transposons were inserted at nucleotide position 348 and 620 of the *phzI* and *phzR* genes, respectively. These sequence data have been submitted to the DDBJ, EMBL, and GenBank databases under accession no. AF195615.

Characterization of *phzI* and *phzR* mutants.

The production of PCN by PCL1103 (*phzI*::Tn*5luxAB*) and PCL1104 (*phzR*::Tn*5luxAB*) was determined with TLC and HPLC. PCN was not detected in the toluene extracts of strain PCL1103 (*phzI*::Tn*5luxAB*) and PCL1104 (*phzR*::Tn*5luxAB*) (data not shown), nor were any autoinducers detected in dichloromethane extracts of these strains (data not shown). PCN production in the *phzI* mutant was restored to wild-type levels by the addition of either spent growth medium from PCL1119 (*phzB*::Tn*5luxAB*) or synthetic C_6-HSL (data not shown). The *phzI* and *phzR* mutants were not altered in the production of hydrogen cyanide (HCN), protease, and chitinase (Table 2). Neither mutant was impaired in motility or in their ability to colonize the tomato root tip in competition with the parental strain, as judged after coinoculation of seeds with a *lacZ*-tagged derivative of wild-type strain PCL1391 in a 1:1 ratio (data not shown). Strains PCL1103 (*phzI*::Tn*5luxAB*) and PCL1104 (*phzR*::Tn*5luxAB*) were unable to inhibit growth of *F. oxysporum* f. sp. *radicis-lycopersici* in an in vitro antifungal assay (data not shown), which is consistent with the observed lack of PCN production.

Isolation and characterization of *luxI* and *luxR* homologs of strain PCL1391.

To isolate a chromosomal fragment of strain PCL1391 containing *luxI* and *luxR* homologs, an *Eco*RI chromosomal library was transformed to DH5α harboring pSB401, a HSL-dependent reporter construct based upon the *Vibrio lux* system (McClean et al. 1997). Plasmid pMP6007 appeared to induce the reporter and contained a 4.5-kb chromosomal fragment of strain PCL1391. Nucleotide sequencing of this clone revealed the complete *phzI* and *phzR* genes and the promoter and first

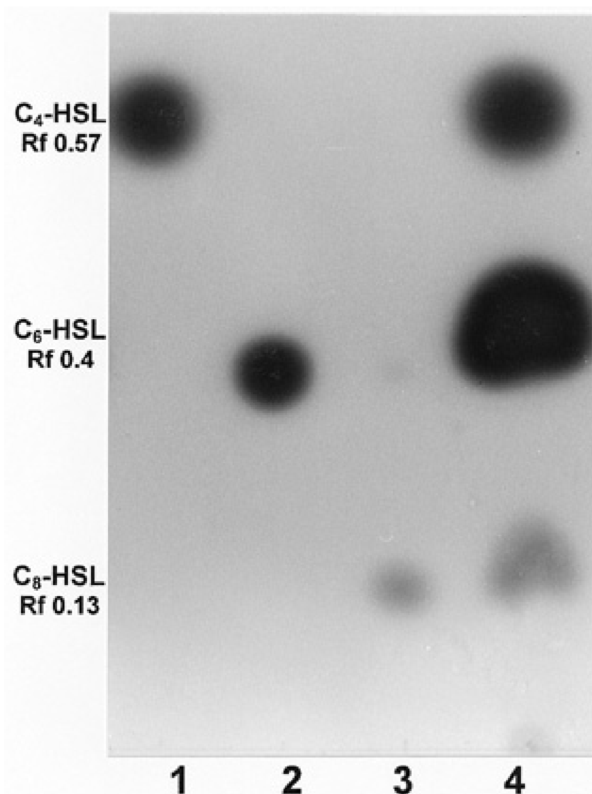

Fig. 1. C_{18}-reverse phase thin-layer chromatography analysis of *N*-acyl-L-homoserine lactones produced by *Pseudomonas chlororaphis* strain PCL1391 visualized with the *Chromobacterium violaceum* reporter assay. Lane 1: synthetic C_4-HSL standard; lane 2: synthetic C_6-HSL standard; lane 3: synthetic C_8-HSL standard; lane 4: autoinducer profile of strain *P. chlororaphis* PCL1391. Note that the major spot visible in lane 4 moved to exactly the same position as C_6-HSL when a smaller amount of sample was applied.

part of *phzA*, the first gene of the PCN biosynthetic operon (Fig. 3) (Chin-A-Woeng et al. 1998).

Escherichia coli strain DH5α harboring pMP6007 induced the DH5α[pSB401] and *Chromobacterium* spp. CV026 reporter strains when streaked in the vicinity of the reporter, indicating the production of a diffusible HSL. To test whether *phzI* and *phzR* are responsible for the synthesis of C_6-HSL, culture supernatants of *E. coli* DH5α, with and without pMP6007, were extracted with dichloromethane and subjected to TLC analysis. Autoinducer activity with R_f value identical to C_6-HSL produced by wild-type PCL1391 was detected only in strains harboring pMP6007 (data not shown).

To transfer the *phzI* and *phzR* genes to PCL1391 mutant derivatives, plasmid pMP4062 was constructed by transfer of the 4.5-kb *Eco*RI chromosomal fragment containing the *phzI* and *phzR* genes of strain PCL1391 from pMP6007 to pME6010, a shuttle vector maintained stably in *Pseudomonas* spp., without antibiotic pressure (Heeb et al. 2000). After pMP4062 was introduced into strains PCL1103 (*phzI*::Tn*5luxAB*) and PCL1104 (*phzR*::Tn*5luxAB*), PCN production was restored to wild-type levels and resulted in increased production in both mutants, as analyzed on TLC analysis of culture supernatants (data not shown). This result, together with the mutant studies of strain PCL1103 (*phzI*::Tn*5luxAB*) and PCL1104 (*phzR*::Tn*5luxAB*), shows that PhzI and PhzR are required for autoinducer synthesis.

Sequence analysis of the open reading frames shows that the *phzI* and *phzR* genes are convergently transcribed, with *phzI* being transcribed in the same direction as the *phz* biosynthetic operon (Fig. 3A). The deduced *phzI* and *phzR* gene products of PCL1391 were most homologous with the *phzI* (Fig. 3B) and *phzR* (Fig. 3C) gene products in the PCA-producing strains *Pseudomonas aureofaciens* 30-84 (95 and 96% identity, respectively) and *Pseudomonas fluorescens* 2-79 (85 and 87% identity, respectively) (Mavrodi et al. 1997; Wood et al. 1997). Similarity to additional LuxI and LuxR homologs in other *Pseudomonas* and bacterial species was much less (20 to 40%).

In the promoter regions of the *phzA* homologous genes of *P. chlororaphis*, *P. aureofaciens*, and *P. fluorescens*, a strictly conserved 18-bp palindromic sequence was identified (Fig. 3D). A similar conserved sequence also was observed in the promoter regions of the *phzI* genes of the three species (Fig. 3D). In *V. fischeri*, a 20-bp palindromic sequence was thought to constitute the binding site for the LuxR regulatory protein. Because the palindromic sequences lack similarity with the *lux* box in the promoter regions of the *lux* and *las* genes, we named the *lux* box-like sequences *phz* boxes.

Influence of exogenous autoinducers on the expression of the *phzI* and *phzR* genes and the biosynthetic operon.

Previously, a number of biosynthetic mutants have been identified from which mutant PCL1119 contains a promoterless *luxAB* reporter in the *phzB* gene. Strain PCL1119 was used for expression studies in the tomato rhizosphere, showing that the biosynthetic operon is expressed in vivo (Chin-A-

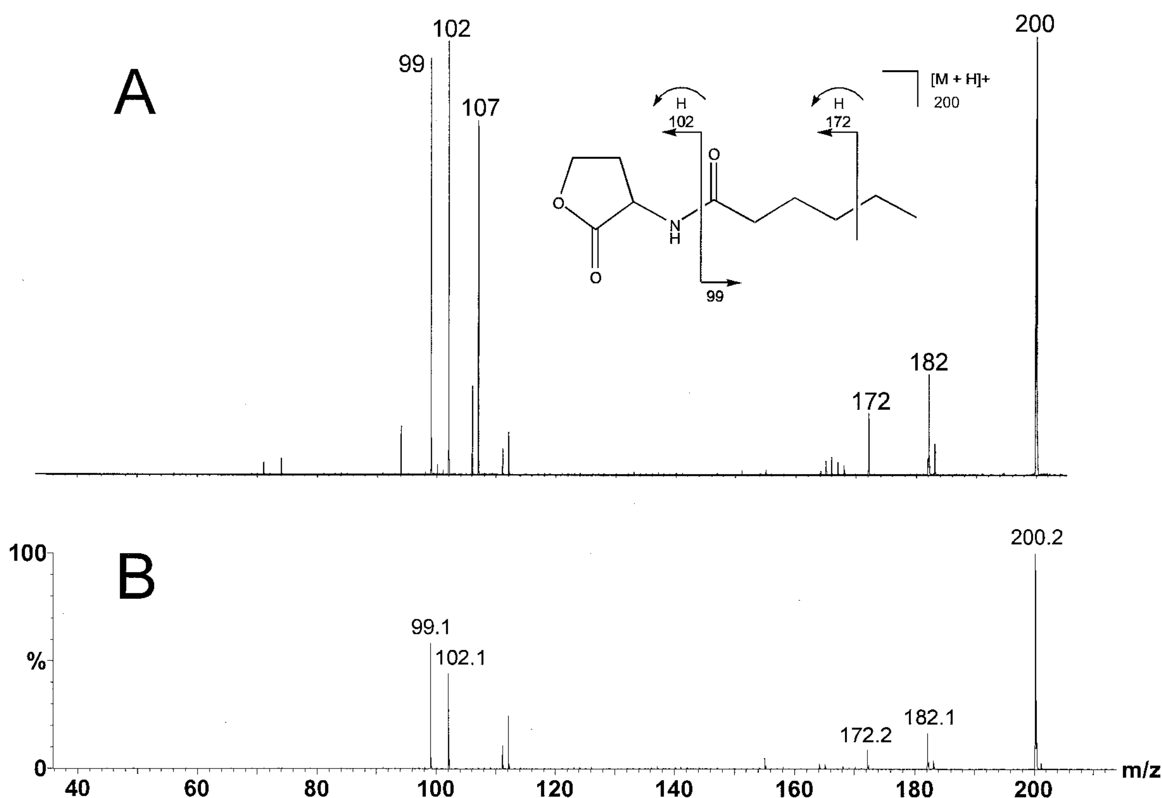

Fig 2. Structural identification of the major compound exhibiting autoinducer activity produced by *Pseudomonas chlororaphis* PCL1391 by mass spectrometry. **A,** Collision-induced dissociation (CID) spectrum obtained from a 1.3 ng of standard solution of synthetic C_6-HSL per ml. **Insert,** Fragmentation scheme for C_6-HSL. **B,** CID spectrum obtained from a high-pressure liquid chromatography fraction corresponding with the autoinducer activity with R_f 0.4 on C18 reverse-phase thin-layer chromatography (Fig. 1).

Woeng et al. 1998). This strain was used to quantify the expression of the PCN biosynthetic operon during growth in liquid cultures under various conditions. Similarly, expression of the *phzI* and *phzR* genes that mutated in PCL1103 (*phzI*::Tn*5luxAB*) and PCL1104 (*phzR*::Tn*5luxAB*) could be quantified because the promoterless *luxAB* genes were inserted in the same orientation as the direction of transcription. Expression of the *phzI* gene in mutant PCL1103 remained at a basal level during growth, indicating that inducing activity is absent without a functional *phzI*. The addition of synthetic C_6-HSL or spent growth medium of an overnight culture of PCL1391 to a cell culture of optical density (OD) at 620 nm of 0.1 of PCL1103 resulted in an induction of *phzI* expression, once the cell culture grew to OD_{620} of 0.6 or more (Fig. 4A). Expression of *phzR* in mutant PCL1104 was constitutive in the presence of exogenous C_6-HSL as well as in the absence of endogenous autoinducers, although the presence of exogenous C_6-HSL increased expression (Fig. 4B). Expression of the PCN biosynthetic gene cluster (*phzB*::Tn*5luxAB*) in PCL1119 was increased greatly in the late exponential–early stationary phase, and the moment of induction could be advanced by the addition of synthetic C_6-HSL (Fig. 4C), but not by the addition of other synthetic HSLs (C_4-HSL, C_8-HSL, C_{10}-HSL, C_{12}-HSL, 3-oxo-C_6-HSL, 3-oxo-C_8-HSL, or 3-oxo-C_{10}-HSL) (data not shown).

The addition of cell-free spent culture medium of the HSL-producing strain PCL1119 (*phzB*::Tn*5luxAB*) to a fresh culture of strain PCL1103 (*phzI*::Tn*5luxAB*) resulted in a twofold and earlier induction of the *phzI* gene than what occurred after the addition of synthetic C_6-HSL (Fig. 4C). Although the addition of spent culture medium of an overnight culture of strain PCL1103 did not induce the *phzI* gene, a synergistic effect was observed when it was added with C_6-HSL (Fig. 4D). Expression induction in the presence of the culture supernatant of strain PCL1103 was already in midexponential-phase growth (OD_{620} of 0.35), much earlier than in the presence of C_6-HSL alone (Fig. 4D). Combinations of autoinducers produced by strain PCL1391 were tested for their possible synergistic effect on expression. None of the combinations, C_4-HSL–C_6-HSL, C_6-HSL–C_8-HSL, or C_4-HSL–C_6-HSL–C_8-HSL resulted in a different level or point of onset of induction than what was observed after the addition of C_6-HSL only (data not shown).

Influence of increased *phzI* and *phzR* copy numbers on the expression of the *phz* operon.

To analyze the possibility of increasing PCN production by introducing additional copies of the *phzI* and *phzR* regulatory genes, a 2.9-kb PCL1391 chromosomal fragment containing these genes was subcloned from pMP6007 into plasmids pME6010 (resulting in pMP4067) and pBBR1MCS-5 (resulting in pMP4065). In *Pseudomonas* spp., pME6010 is maintained at approximately four to eight copies per cell (Heeb et al. 2000) and pBBR1MCS derivatives are assumed to be present in 30 to 40 copies per cell in *E. coli* and *Bordetella bronchiseptica* (Antoine and Locht 1992). An increased production of C_6-HSL produced by PCL1391 harboring pME4067 or pME4065 was observed from which the latter produced the largest amount (an estimated tenfold) compared with the wild type, as indicated by the analyses of spent growth supernatant extracts with *Chromobacterium* spp. indicator assay (data not shown). Subsequently, plasmids were transferred to the bioluminescent reporter PCL1103 (*phzI*::Tn*5luxAB*) for *phzI* expression studies and to reporter PCL1119 (*phzB*::Tn*5luxAB*) to analyze expression of the *phz* operon. Strain PCL1103 harboring pMP4065 (a "high" copy number) already was induced at a population density of OD_{620} of 0.2 and expression was four times higher than strain PCL1103 harboring pMP4067 (an "intermediate" copy number) (Fig. 4E). The use of the PCL1103 (*phzI*::Tn*5luxAB*) reporter harboring pMP4067 also shows that *phzI* is much higher expressed than is the control with synthetic C_6-HSL added (Fig. 4E). The introduction of multiple copies of the *phzI* and *phzR* genes into strain PCL1119 (*phzB*::Tn*5luxAB*) resulted in an elevated and earlier expression of the *phz* operon (Fig. 4F). Expression of the *phz* operon in PCL1119 harboring pMP6065 (a high copy number) was slightly higher than PCL1119 harboring pMP4067 (an intermediate copy number). In both strains, the *phz* genes were at least ten times higher expressed than they were in the empty PCL1119. Because the highest production of C_6-HSL and the highest expression of the *phzI* and *phz* genes was observed in PCL1391 harboring pMP4065, spent culture supernatants of this strain were analyzed with HPLC to quantify the amount of PCN produced. A 6.3-fold production increase was observed compared with the wild type (data not shown).

DISCUSSION

PCN production is essential for the biocontrol activity of *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998). The first indication that PCN biosynthesis in strain PCL1391 is population density regulated came from the observation that initiation of its production was at the start of the stationary phase. This was confirmed by the observed induction of expression upon reaching the stationary phase with reporter strain PCL1119 (*phzB*::Tn*5luxAB*) (Fig. 4C). Quorum sensing usually is regulated by two proteins that belong to the LuxI and LuxR family of two-component regulatory systems. LuxI homologs are assumed to be HSL synthases, which utilize *S*-adenosylmethionine and specific acylated carrier proteins to synthesize HSL signal molecules (Hanzelka and Greenberg 1996). Indeed, homologs of *luxI* and *luxR,* designated *phzI* and

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

	Bacterial strains		
Traits	**PCL1391**	**PCL1103**	**PCL1104**
Mutated gene	None	*phzI*	*phzR*
PCN production	+[a]	–[a]	–
Antifungal activity[b]	+	–	–
Autoinducer production	+	–	–
HCN production	+	+	+
Protease production	+	+	+
Chitinase production	+	+	+
Motility	+	+	+
Tomato root tip colonization[c]	+	+	+

[a] +: wild-type level; –: absent

[b] Activity was tested in a petri dish assay for antifungal activity (Geels and Schippers 1983) against *Fusarium oxysporum* f. sp. *radicis-lycopersici*

[c] Colonizing ability was tested after seedling inoculation in competition with PCL1392, a Tn*5lacZ* tagged derivative of PCL1391 not impaired in root colonization, in a gnotobiotic sand system (Simons et al. 1996).

phzR, respectively, were identified in strain PCL1391 (Fig. 3) and involved in population density-dependent regulation of PCN biosynthesis (Table 2).

Autoinducer production was shown with the use of HSL reporter assays (Fig. 1). The major autoinducer was identified structurally as *N*-hexanoyl-L-homoserine lactone by nanoelectrospray Q-TOF mass spectrometry (Fig. 2). In addition, strain PCL1391 produces two other autoinducers that comigrate with synthetic C_4-HSL and C_8-HSL. The latter two compounds evidently do not play a role in PCN production and may be synthesized as the result of a somewhat loose specificity for the acyl–acyl carrier protein (ACP) substrate during C_6-HSL biosynthesis. In vitro work with TraI and LuxI has shown that these synthases can synthesize acyl–HSL signals with different chain lengths and even C-3 substitutions (Parsek et al. 1999; Schaefer et al. 1997). In strain PCL1391, the *phzI* and *phzR* genes are transcribed convergently and their organization, localization (Fig. 3A), and nucleotide and deduced

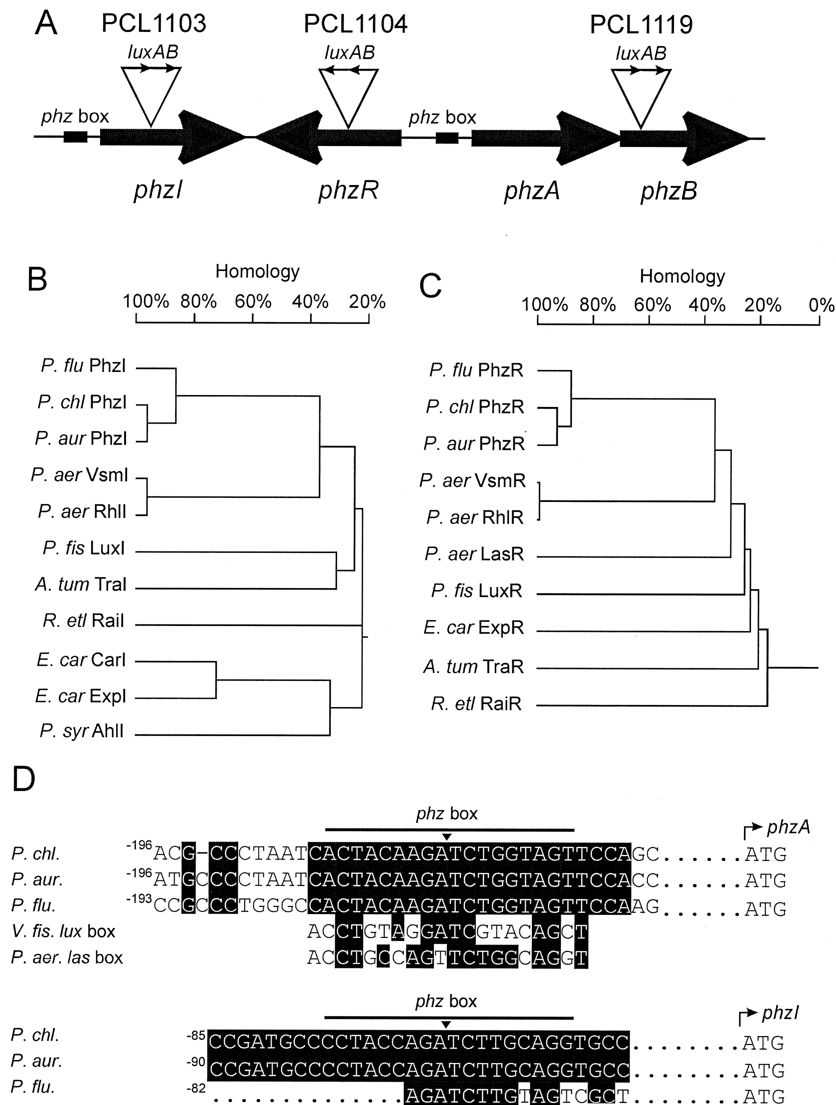

Fig. 3. Chromosomal localization, regulatory elements, and homology of gene products regulating phenazine-1-carboxamide (PCN) production in *Pseudomonas chlororaphis* PCL1391. **A,** Genetic analysis of mutants of *P. chlororaphis* strain PCL1391 lacking PCN production. Positions and orientations of the Tn*5luxAB* transposon of strains PCL1103, PCL1104, and PCL119 are indicated with arrows. The *phzI* and *phzR* and the first two genes of the PCN biosynthetic operon, *phzA* and *phzB*, are shown. **B,** Tree of homology of the LuxI-homologous proteins of strain *P. chlororaphis* PCL1391 (*P. chl.* PhzI), *Pseudomonas aureofaciens* 30-84 (*P. aur.* PhzI), *Pseudomonas fluorescens* 2-79 (*P. flu.* PhzI), *Pseudomonas aeruginosa* (*P. aer.* VsmI and RhlI), *Pseudomonas syringae* pv. *syringae* (*P. syr.* AhlI), *Vibrio fischeri* (*V. fis.* LuxI), *Erwinia carotovora* (*E. car.* ExpI, CarI), *Agrobacterium tumefaciens* (*A. tum.* TraI), and *Rhizobium etli* (*R. etl.* RaiI). **C,** Homology tree of of the LuxR-homologous proteins of *P. chlororaphis* PCL1391 (*P. chl.* PhzI), *P. aureofaciens* 30-84 (*P. aur.* PhzR), *P. fluorescens* 2-79 (*P. flu.* PhzR), *P. aeruginosa* PAO1 (*P. aer.* VsmR, RhlR, LasR), *V. fischeri* (*V. fis.* LuxR), *E. carotovora* (*E. car.* ExpR), *A. tumefaciens* (*A. tum.* TraR), and *R. etli* (*R. etl.* RaiR). **D,** Identification of palindromic sequences in the *phzI* and *phzA* promoter regions of *P. chlororaphis*, *P. aureofaciens* 30-84, and *P. fluorescens* 2-79. The sequences and relative positions of the *phz* boxes and the ATG start codons of *phzI* and *phzA* are indicated. The *lux* box of *V. fischeri* and *lux*-like box of *P. aeruginosa* are aligned for comparison. Nucleotide sequences of the *phzA* promoter regions of *P. aureofaciens* 30-84 and *P. fluorescens* 2-79 were obtained from GenBank as accession nos. L48616 and AF007801, respectively. Nucleotide sequences of the *phzI* promoter regions are from GenBank sequences, accession nos. L33724 and L48616, respectively. Note that the *P. fluorescens phzI* promoter sequence was not completely available.

amino acid sequences are highly homologous with the locus in the PCA-producing biocontrol strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 (Fig. 3B and C). *P. fluorescens* 2-79 produces PCA (Mavrodi et al. 1997), whereas *P. aureofaciens* 30-84 produces hydroxy-phenazines in addition to PCA (Pierson and Thomashow 1992). Despite the high similarities observed in the nucleotide sequences of the *phzI* genes in the three phenazine-producing species, the identities of the quorum-sensing signals appear to differ. *P. aureofaciens* 30-84 was reported only to produce C_4-HSL and C_6-HSL (Pierson

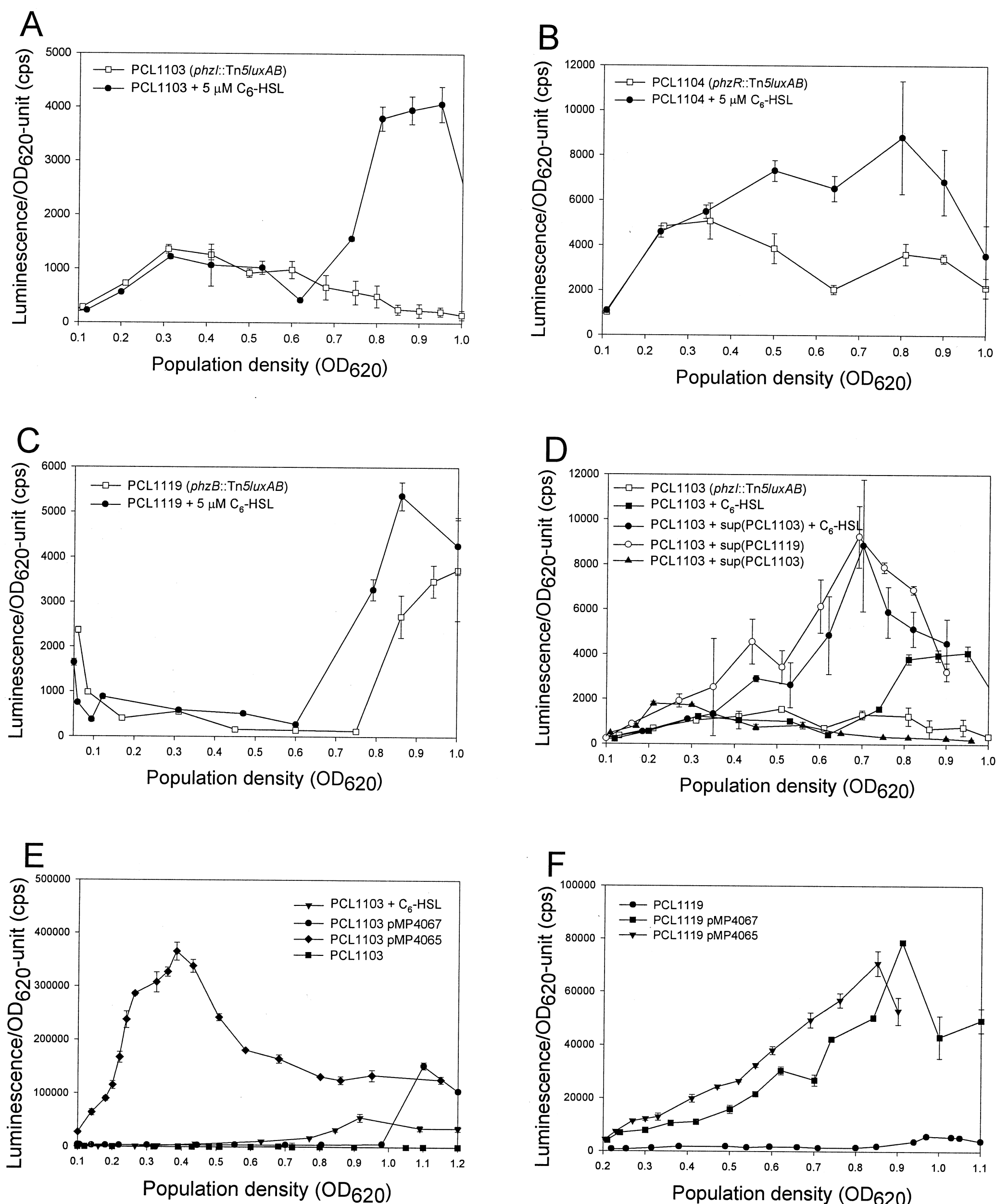

Fig 4. Expression analyses of the *phzI* gene, *phzR* gene, and the phenazine-1-carboxamide (PCN) biosynthetic operon of *Pseudomonas chlororaphis* strain PCL1391. Strains were grown in Luria-Bertani medium. *N*-hexanoyl-L-homoserine lactone (C_6-HSL) was added at the initial optical density (OD) at 620 nm of 0.1. Values depicted in the panels are values for the luminescence measured in counts per seconds (cps) per OD unit during growth in time. **A,** Expression of *phzI* in strain PCL1103 (*phzI*::Tn*5luxAB*) in the absence and presence of 5 μM synthetic C_6-HSL. **B,** *phzR* expression in the absence and presence of 5 μM synthetic C_6-HSL with strain PCL1104 (*phzR*::Tn*5luxAB*). **C,** Expression of the PCN biosynthetic operon of strain PCL1119 (*phzB*::Tn*5luxAB*) in the absence and presence of 5 μM synthetic C_6-HSL. **D,** Induction of *phzI* by synthetic C_6-HSL, cell-free spent culture supernatant of strain PCL1103 (*phzI*::Tn*5luxAB*), spent growth supernatant of strain PCL1119 (*phzB*::Tn*5luxAB*), and a combination of spent growth supernatant of PCL1103 and C_6-HSL. **E,** Expression of the PCN biosynthetic operon of strain PCL1119 (*phzB*::Tn*5luxAB*) harboring additional copies of *phzI* and *phzR*. Multicopy plasmids pMP4065 (estimated copy number 30-40) and pMP4067 (estimated copy number 4-8) contain a 2.9-kb chromosomal fragment with the *phzI* and *phzR* genes. **F,** Expression of the *phzI* gene of strain PCL1103 (*phzI*::Tn*5luxAB*) harboring additional copies of *phzI* and *phzR* (pMP4065 and pMP4067) and expression of *phzI* in the absence and presence of 5 μM synthetic C_6-HSL.

and Pierson 1996), whereas *P. fluorescens* 2-79 produced 3-OH–C_6-HSL, 3-OH–C_8-HSL, 3-OH–C_{10}-HSL, and C_8-HSL, yet hardly any C_6-HSL (Cha et al. 1998).

Because members of the *luxI* and *luxR* family usually show weak homologies, the high similarities between the *phzI–phzR* homologs indicate that this set of genes, which regulates antifungal metabolite production within these three *Pseudomonas* species, is evolutionary well conserved. Within or directly adjacent to the domain proposed to contain the amino acids involved in acyl–ACP selection in *V. fischeri* (amino acids 133 to 164 of LuxI) (Hanzelka et al. 1997), the amino acid sequence of *P. chlororaphis* is identical to that of *P. aureofaciens*, whereas the amino acids at positions 150, 156, 158, and 159 differ in *P. fluorescens*. This is consistent with the observation that *P. chlororaphis* and *P. aureofaciens* mainly produce the C_6-HSL signal (Wood and Pierson 1996), whereas *P. fluorescens* produces other autoinducer signals (Cha et al. 1998). In *P. chlororaphis*, PhzI and PhzR are more similar to the *phzI* and *phzR* gene products of *P. aureofaciens* than they are to those of *P. fluorescens*, which is consistent with the apparent closer relationship between the former two species (Krieg 1984).

C_6-HSL clearly is involved in the regulation of PCN biosynthesis. First, PCN production was restored in the *phzI* mutant strain by the addition of either synthetic C_6-HSL or of spent growth medium of strain PCL1391 to the cells or by providing *phzI* in *trans*. Second, from the series of synthetic HSLs tested (see below), only C_6-HSL was able to advance expression of the reporter in PCL1119 (*phzB*::Tn*5luxAB*), showing that C_6-HSL is involved in the expression of *phzI* (Fig. 4C). Nevertheless, this does not exclude the fact that other HSLs do play a role in regulating PCN biosynthesis and that some may even act as antagonists. Experiments in the *lux*, *las*, and *tra* systems have shown that noncognate HSLs or analogs have an agonistic or antagonistic effect when supplied in high concentrations (Puskas et al. 1997). We used multicopy plasmids to show that PCN production can be increased sixfold by introducing additional copies of the *phzI* and *phzR* genes. This resulted in an increased expression of the *phzI* gene (Fig. 4F), which was accompanied by an increased production of autoinducers secreted into the growth medium. We therefore explain the increased expression of the *phz* operon and increased amounts of PCN secreted into the growth medium are caused by the increased production of autoinducers. We believe that this is the first time that an increase of antibiotic production through the overproduction of HSLs is shown, which offers a new approach for increased antibiotic production in bacteria.

A palindromic sequence that is highly conserved in the promoter regions of the *phzI* and *phzR* genes of phenazine-producing *Pseudomonas* species was identified. We speculate that these *phz* boxes are binding sites for PhzR such as the palindromic *lux* box in *V. fischeri*, which was identified as a binding region for the transcriptional activator LuxR (Fig. 3D) (Devine et al. 1989; Shadel et al. 1990).

In addition to the dependence of PCN production on C_6-HSL, the expression of *phzI* apparently requires so-far-unidentified additional factor(s) secreted into the culture supernatant, as judged from the synergistic effect of spent growth medium from strain PCL1103 (*phzI*::Tn*5luxAB*) and synthetic C_6-HSL (Fig. 4D). Upon the addition of C_6-HSL at a density of 10^8 CFU/ml, the same delay in induction as seen with *A. tumefaciens* was observed, where induction occurred only after reaching late-exponential growth phase. This contrasts with the *V. fischeri lux* system. where induction is observed immediately (Eberhard et al. 1986; Nealson 1977). Wild-type expression levels of the PCN biosynthetic genes were reached only in the presence of the assumed second factor in the growth supernatant (Fig. 4D). Because strain PCL1391 also produces small amounts of C_4-HSL and C_8-HSL, mixtures of C_4-HSL, C_6-HSL, and C_8-HSL were tested for induction of the *phzB*::Tn*5luxAB* reporter. Neither C_4-HSL nor C_8-HSL influenced the induction in combination with C_6-HSL at the concentrations added, indicating that the additional factor is different from these HSLs. No signals in addition to the ones that were detected in the *Chromobacterium* spp. *cvi* system were observed with the *Agrobacterium* spp. *tra* sensor, which is more sensitive overall and responds to a broader spectrum of HSL molecules. This makes it less likely that oxo- or hydroxy-HSLs are responsible for the additional inducing activity. We speculate that the signal may be relayed into the cell by a GacS homolog, which is required for optimal production of autoinducers (Chin-A-Woeng et al., *submitted*).

GacS is a sensor kinase that operates closely together with the global regulator GacA (Rich et al. 1994). We also exclude the possibility that PCN itself is the additional factor needed to induce the expression of *phzI* at low cell densities because spent growth supernatant of strain PCL1119 (*phzB*::Tn*5luxAB*), which does not produce PCN, was able to induce *phzI* expression. Apparently, the regulation mechanism differs from that of 2,4-diacetylphloroglucinol production, an antifungal factor produced by *P. fluorescens* CHA0 and other biocontrol strains, where the expression was induced by the final product (Schnider-Keel et al. 2000).

MATERIALS AND METHODS

Microorganisms and media.

The bacterial strains and plasmids used are listed in Table 1. KB medium was used for routine culturing of *Pseudomonas* spp. strains. *E. coli* and *C. violaceum* were grown in LB medium (Sambrook et al. 1989). *A. tumefaciens* was grown on yeast-extract mannitol broth (YMB) medium (Smit et al. 1987). Solid growth media contained 1.8% (wt/vol) agar (Difco Laboratories, Detroit, MI, U.S.A.). The antibiotic selection added, per ml, was 50 µg of kanamycin, 80 µg of tetracycline, 20 µg of chloramphenicol, and 50 µg of carbenicillin, where applicable.

DNA modifications.

Digestions with restriction endonucleases, ligation, and transformation of *E. coli* cells with plasmid DNA were performed with the use of standard molecular biological protocols (Sambrook et al. 1989). Nucleotide sequencing was performed by Eurogentec (Herstal, Belgium) with AB1377-based fluorescent sequencing technology. Computer analysis of protein and nucleotide sequences was achieved with the Wisconsin software package, version 10.0 (Genetics Computer Group, Madison, WI, U.S.A).

PCN quantification, bioassays for hydrolytic activities, and root colonization.

PCN production was determined by extracting spent culture supernatants with an equal volume of toluene. The water

phase was again extracted with toluene, the organic phases were pooled, and toluene was removed. Extracts were analyzed with HPLC, as described previously (Chin-A-Woeng et al. 1998). HCN was detected by cyanide-indicator paper (Castric 1975), protease with 5% milk agar plates, lipase with Tween 80 agar plates (Howe and Ward 1976), and chitinase with agar plates containing colloidal chitin (Shimahara and Takiguchi 1988). The tomato root colonizing ability of the strains was performed as previously described (Simons et al. 1996). The strains were tested in competition with PCL1392, a *lacZ*-tagged derivative of strain PCL1391, with the same colonizing ability as the wild type (Chin-A-Woeng et al. 2000).

Bioassays for autoinducer activity.

To detect autoinducer activity with the *C. violaceum* HSL reporter assay (Milton et al. 1997), overnight cultures of *C. violaceum* CV026 were grown in LB medium supplemented with 50 µg of kanamycin per ml. LB agar plates were overlaid with a 0.8% LB top agar layer mixed with a 16-h culture of 200 µl of the *C. violaceum* CV026 indicator strain per ml (McClean et al. 1997). A 20-µl volume of 100 µl of extract or HPLC fraction was tested for autoinducer activity in wells punched into the agar. The activity of fractions was judged after 16 h of growth at 28°C by the appearance of a violet halo around the well caused by violascein production, which resulted from the activation of reporter genes in the *C. violaceum* strain. Autoinducer activity on C18 reverse-phase TLC (Merck, Darmstadt, Germany), developed in methanol–water (60:40, vol/vol), was detected by overlaying the TLC plate with a 0.8% LB top agar layer containing CV026 cells, as described above, followed by incubation at 28°C for 16 h and analyzed for the appearance of violet spots.

The autoinducer indicator assay, developed on the basis of the *Vibrio* (*lux*) system with plasmid pSB401 (Winson et al. 1998), was conducted similarly to the assay described above for *C. violaceum*. A 50-µl volume of a 16-h culture of DH5α[pSB401] was mixed with 3.0 ml of 0.8% LB top agar and poured onto LB agar plates containing 20 µg of chloramphenicol per ml. A 20-µl volume of sample was placed in the wells, and the plates were incubated for 16 h at 28°C. Luminescence was detected by placing photographic film (Fuji, Tokyo, Japan) against the bottom of the assay plates.

The induction of the *Agrobacterium* spp. *tra* system was detected with *A. tumefaciens* strain NT1 harboring plasmid pJM749, which contained a *lacZ* reporter fused to a *tra* gene, the expression of which is dependent on TraR (located on plasmid pSVB33) and the presence of a sufficient concentration of an autoinducer (Piper et al. 1993). *Agrobacterium* spp. was scraped from YMB plates 3 days after streaking and resuspended in sterile water to OD_{620} of 0.1. A 100-µl volume of the bacterial suspension was mixed with 3.0 ml of top agar and poured onto YMB plates containing 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) to detect expression as blue pigmentation. Samples were tested as described above for the *C. violaceum* bioassay. Blue zones were visible after 24 to 48 h of incubation at 28°C.

Purification of autoinducers.

To isolate autoinducer activity, 3 volumes of dichloromethane were added to 7 volumes of supernatant of a 72-h KB culture of PCL1119 and shaken for 1 h at 120 rpm. Following extraction, the organic phase was removed and dried by evaporation in vacuo (McClean et al. 1997). Crude supernatant extracts were redissolved in 100 µl of 100% acetonitrile and fractionated with either C_{18} TLC or HPLC. C_{18}-TLC plates (Merck) were developed in a solvent mixture of methanol–water (60:40, vol/vol). After development, the plates were dried and overlaid with a top agar layer containing one of the indicator strains and incubated for 16 h at 28°C. HPLC was performed with a Hypersil octadecyl silane, 5 µm, 250 × 4.6 mm column (Alltech Associates Deerfield, IL, U.S.A.) and a linear 20 to 90% (vol/vol) gradient of acetonitrile in water with a flow rate of 1 ml per min. After active fractions were selected, samples were pooled and reapplied to the column and eluted with an isocratic gradient of 35% acetonitrile. UV detection was performed with a RSD 2140 diode array detector (Pharmacia, Uppsala, Sweden) with wavelength scanning from 190 to 400 nm, and 1.0 ml fractions were collected and analyzed for the presence of autoinducer activity with the CV026 biosensor. Finally, active fractions were pooled for mass spectrometry analyses.

Nanoelectrospray Q-TOF mass spectrometric analysis.

Electrospray CID tandem mass spectra were obtained on a hybrid Q-TOF tandem mass spectrometer (Micromass, Wythenshawe, U.K.) equipped with a Z-Spray sample introduction system in a nanoflow electrospray ion source. The mass spectrometer was operated in the positive ion mode. The cone voltage was set at approximately 25 V, and a capillary voltage of 1.5 kV was used. Argon was used as the collision gas, and the spectra were obtained with a collision energy of 10 eV. Spectra were acquired via the TOF analyzer and were integrated every 2.4 s from m/z 35 to 250. Data were recorded and processed with MassLynx software, version 3.1 (Micromass). Mass calibration was performed by multiple ion monitoring of singly charged sodium and cesium iodide signals. The samples were dissolved in 10 µl of methanol, 3 µl of which were loaded into the nanospray gold-coated glass capillary for sample delivery.

Isolation of mutants impaired in PCN biosynthesis.

A mutant library of PCL1391 was established with pRL1063a (Wolk et al. 1991), harboring a Tn*5* transposon carrying promoterless *luxAB* reporter genes. Mutants were screened for the absence of or a change in pigment production on either LB agar plates or in 200-µl liquid KB cultures grown in 96-well microtiter plates for 3 days.

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

Isolation and identification of *luxI* and *luxR* homologs.

A plasmid library of chromosomal fragments of strain PCL1391 was constructed by cloning chromosomal DNA

digested with *Eco*RI into the multicloning site of pBluescript (Stratagene, La Jolla, CA, USA) (Kragelund et al. 1995). After electroporation of this fragment library to an *E. coli* strain harboring the *lux* reporter pSB401 and overnight growth on LB agar plates, clones that induced the luciferase reporter were identified with photographic film. To cure the *E. coli* strain from the pSB401 reporter construct, chloramphenicol selection was omitted, whereas carbenicillin selection was maintained. The nucleotide sequence of the chromosomal insert in the remaining plasmid was determined with standard primers such as the universal and –40 reverse primer flanking the multiple cloning site of pBluescript. The plasmids pMP4067 and pMP4065 with the *phzI* and *phzR* genes were obtained by transferring 2.9-kb *Eco*RI–*Bam*HI and *Hin*dIII subcloned fragments of pMP6007 to plasmids pME6010 and pBBR1MCS-5, respectively.

Expression of bioluminescent Tn*5luxAB* reporter strains. Expression of Tn*5luxAB*-tagged genes was determined by quantification of bioluminescence during culturing. Cells from overnight cultures were washed with fresh medium and diluted to OD_{620} of 0.1. Cultures were grown in LB medium in a volume of 10 ml under vigorous shaking. Growth was followed by measurement of OD_{620} at regular intervals, and 100-µl samples were taken in triplicate to quantify luminescence. A volume of 100 µl of an *n*-decyl-aldehyde substrate solution (0.2% *n*-decyl-aldehyde) (Sigma, St. Louis, MO, U.S.A.) in a 2.0% bovine serum albumin solution) was added. After thorough mixing, bioluminescence was determined with a MicroBeta 1450 TriLux luminescence counter (Wallac, Turku, Finland) and normalized to the luminescence per OD_{620} unit.

The synthetic HSL molecules *N*-butanoyl-L-homoserine lactone (C_4-HSL), *N*-hexanoyl-L-homoserine lactone (C_6-HSL), *N*-octanoyl-L-homoserine lactone (C_8-HSL), *N*-decanoyl-L-homoserine lactone (C_{10}-HSL), *N*-dodecanoyl-L-homoserine lactone (C_{12}-HSL), *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C_6-HSL), *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C_8-HSL), and *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C_{10}-HSL) were tested for the ability to induce Tn*5luxAB* reporter strains. Cells were grown overnight in LB medium, washed, and resuspended to OD_{620} of 0.1 in fresh medium supplemented with either 5 µM synthetic HSL(s) or 10% (vol/vol) spent growth supernatant and grown to stationary phase. Luminescence was determined after every 0.1 U increase of OD_{620} and compared with the control without added HSL.

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