

# Biocontrol by Phenazine-1-carboxamide-Producing *Pseudomonas chlororaphis* PCL1391 of Tomato Root Rot Caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*

Thomas F. C. Chin-A-Woeng,<sup>1</sup> Guido V. Bloemberg,<sup>1</sup> Arjan J. van der Bij,<sup>1</sup> Koen M. G. M. van der Drift,<sup>2</sup> Jan Schripsema,<sup>3</sup> Bernadette Kroon,<sup>4</sup> Rudy J. Scheffer,<sup>4</sup> Christoph Keel,<sup>5</sup> Peter A. H. M. Bakker,<sup>6</sup> Hans-Volker Tichy,<sup>7</sup> Frans J. de Bruijn,<sup>8</sup> Jane E. Thomas-Oates,<sup>2</sup> and Ben J. J. Lugtenberg<sup>1</sup>

<sup>1</sup>Leiden University, Institute of Molecular Plant Sciences, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands; <sup>2</sup>Utrecht University, Department of Mass Spectrometry, F.A.F.C. Wentgebouw, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands; <sup>3</sup>Núcleo de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saude, Bloco H, 21941-590-Ilha do Fundão, Rio de Janeiro-RJ, Brazil; <sup>4</sup>Novartis Seeds BV, Westeinde 62, Postbus 26, 1600 AA Enkhuizen, The Netherlands; <sup>5</sup>Laboratoire de Biologie Microbienne, Université de Lausanne, CH-1015 Lausanne, Switzerland; <sup>6</sup>Utrecht University, Department of Plant Ecology and Evolutionary Biology, Section of Plant Pathology, P.O. Box 800.84, 3508 TB Utrecht, The Netherlands; <sup>7</sup>TÜV Energie und Umwelt GmbH, Niederlassung Freiburg, Robert-Bunsen Strasse 1, 79108 Freiburg i. Br., Germany; and <sup>8</sup>MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing 48824, U.S.A.

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Seventy bacterial isolates from the rhizosphere of tomato were screened for antagonistic activity against the tomato foot and root rot-causing fungal pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici*. One isolate, strain PCL1391, appeared to be an efficient colonizer of tomato roots and an excellent biocontrol strain in an *F. oxysporum*/tomato test system. Strain PCL1391 was identified as *Pseudomonas chlororaphis* and further characterization showed that it produces a broad spectrum of antifungal factors (AFFs), including a hydrophobic compound, hydrogen cyanide, chitinase(s), and protease(s). Through mass spectrometry and nuclear magnetic resonance, the hydrophobic compound was identified as phenazine-1-carboxamide (PCN). We have studied the production and action of this AFF both in vitro and in vivo. Using a PCL1391 transposon mutant, with a *lux* reporter gene inserted in the phenazine biosynthetic operon (*phz*), we showed that this phenazine biosynthetic mutant was substantially decreased in both in vitro antifungal activity and biocontrol activity. Moreover, with the same mutant it was shown that the *phz* biosynthetic operon is expressed in the tomato rhizosphere. Comparison of the biocontrol activity of the PCN-producing strain PCL1391 with those of phenazine-1-carboxylic acid (PCA)-producing strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 showed that the PCN-producing strain is able to suppress disease in the tomato/*F. oxysporum* system,

whereas the PCA-producing strains are not. Comparison of in vitro antifungal activity of PCN and PCA showed that the antifungal activity of PCN was at least 10 times higher at neutral pH, suggesting that this may contribute to the superior biocontrol performance of strain PCL1391 in the tomato/*F. oxysporum* system.

*Additional keywords:* microbiological control.

In recent years, various plant root-colonizing *Pseudomonas* spp. have been shown to be potent microbiological control agents in various plant-pathogen systems (Thomashow and Weller 1995). The use of such plant growth-promoting rhizobacteria (PGPR) is considered an addition to and an alternative for chemical pesticides, several of which are a threat to our health and environment (Lugtenberg et al. 1994). Inconsistent performance in the field has, however, delayed commercial development and general acceptance of the use of biocontrol bacteria. Therefore, more insights into the mechanisms that govern the interactions between bacteria, plant, and pathogen are needed. Biocontrol bacteria can mediate their role in disease suppression through various mechanisms including competition for nutrients and niches (Paulitz 1990), production of antimicrobial metabolites (Lugtenberg et al. 1991; Dunne et al. 1996; Keel and Defago 1997; O'Sullivan and O'Gara 1992; Thomashow and Weller 1995), and induced systemic resistance (ISR) in the host plant (van Peer et al. 1991; Leeman et al. 1995a; Pieterse et al. 1996).

*Fusarium oxysporum* Schlechtend.:Fr. f. sp. *radicis-lycopersici* W. R. Jarvis & Shoemaker, the causal agent of to-

mato foot and root rot, is responsible for substantial crop losses in commercial greenhouses in The Netherlands. Since chemical seed treatments act for only a limited period of time, microbiological control is considered a preferred strategy to control the disease. Moreover, *Fusarium* spp. are pathogens for many crops and biocontrol of tomato root rot can be used as a general model for diseases caused by *Fusarium* spp.

In this paper, we report the screening of tomato rhizosphere bacteria for in vitro antagonistic activity toward the fungal phytopathogen *F. oxysporum* f. sp. *radicis-lycopersici*. The most active isolate, strain PCL1391, was found able to suppress the disease substantially. Its biocontrol activity was characterized at the molecular level and found to be mediated through the production of phenazine-1-carboxamide (PCN).

## RESULTS

### Isolation and characterization of *P. chlororaphis* strain PCL1391.

Screening of 70 *Pseudomonas* strains isolated from Spanish tomato rhizosphere resulted in the identification of three isolates, PCL1391, PCL1393, and PCL1394, that displayed substantial in vitro growth inhibitory activity against *F. oxysporum* f. sp. *radicis-lycopersici*. The isolate PCL1391, which formed the largest growth inhibition zone, appeared to possess the best biocontrol activity in a tomato/*F. oxysporum* test system. In these tests, in which the 2,4-diacetylphloroglucinol-producing *P. fluorescens* strain F113 (Shanahan et al. 1992) and *P. fluorescens* strain WCS374, which can cause ISR in radish (Leeman et al. 1995a), were compared, strain PCL1391 caused a significant reduction in the number of diseased plants (Fig. 1). Based on amplified ribosomal DNA-restriction analysis and comparison with reference strains, two strains, PCL1393 and PCL1394, were identified as *P. fluorescens* (results not shown). With eight different restriction enzymes, it was shown that ribosomal DNA patterns of PCL1391 were identical to patterns of *P. chlororaphis* and *P. aureofaciens* (results not shown). Since strain PCL1391 produces a blue pigment after prolonged growth on KB agar plates, and since the orange colony coloration characteristic for *P. aureofaciens* (Krieg 1984) is absent, we conclude that strain PCL1391 belongs to the species *P. chlororaphis*. Strain PCL1391 is a fluorescent pseudomonad, colonies of which show a bright yellow coloration on KB agar and, after prolonged incubation, a green-blue pigment in the colony center. Addition of iron to the medium enhances the production of blue pigment on solid and in liquid medium. Strain PCL1391 was also able to inhibit mycelial growth of other important plant-pathogenic fungi, including *Rhizoctonia solani*, *Botrytis cinerea*, *Pythium ultimum*, *Verticillium albo-atrum*, and *Alternaria dauci* in a plate assay (data not shown), indicating a broad spectrum of antifungal activity. Strain PCL1391 secreted a hydrophobic antifungal compound (see below), hydrogen cyanide, chitinase(s), protease(s), lipase(s), and siderophore(s). Pyochelin, salicylic acid, phloroglucinols, pyoluteorin, glucanase, and cellulase, as determined by high-performance liquid chromatography (HPLC) and enzyme assays, were not produced by this strain.

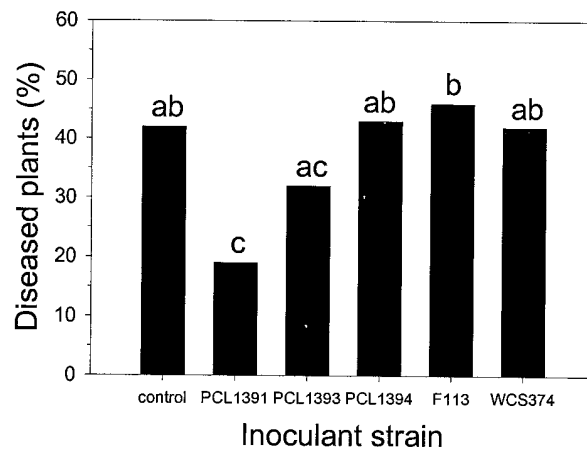
Furthermore, strain PCL1391 was shown to be an efficient colonizer of the tomato root system since it is able to compete with the excellent rhizosphere-colonizing strain *P. fluorescens*

WCS365 (Simons et al. 1996). Seven days after inoculation of pre-germinated tomato seeds with equal numbers of strain PCL1391 and a Tn5-*lacZ*-marked derivative of strain WCS365 (van der Bij et al. 1996), analysis of the tips of 12 roots revealed the presence of equal numbers of both strains ( $4.3 \pm 0.5 \log_{10}$  [CFU+1]/cm of root tip and  $4.4 \pm 0.4 \log_{10}$  [CFU+1]/cm of root tip, respectively).

### Purification and structural identification of the hydrophobic antifungal compound produced by strain PCL1391.

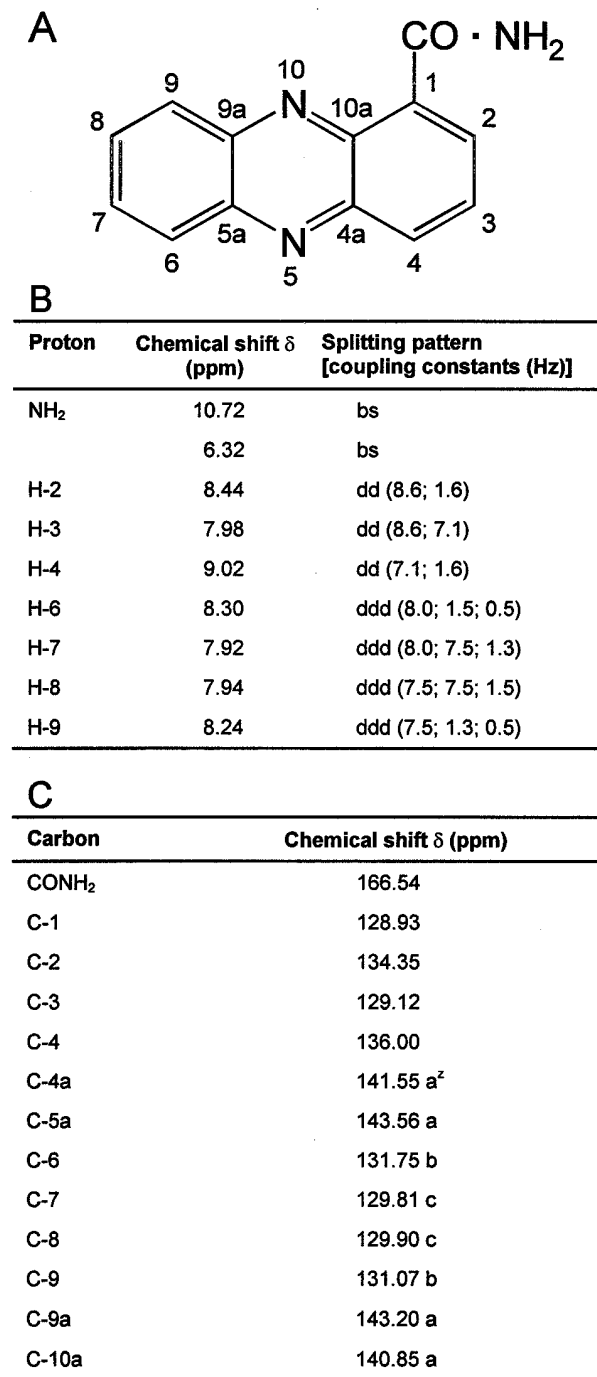
To identify compounds with antifungal properties, iron-supplemented KB medium of a 72-h culture supernatant of strain PCL1391 was extracted with toluene. After evaporation of the solvent, the residue was analyzed by silica thin layer chromatography (TLC). Antifungal activity was identified with an *F. oxysporum* TLC bioassay, and one growth-inhibiting spot migrating with an  $R_f$ -value of 0.47 was detected. This spot appeared to inhibit both *F. oxysporum* spore germination and mycelial growth. To elucidate its structure, the activity was purified by HPLC analysis. The activity eluted from the HPLC column after 20.0 min as a single peak, and its UV spectrum (data not shown) indicated that the compound was a phenazine derivative. The active fraction was further analyzed by mass spectrometry and nuclear magnetic resonance (NMR).

The electrospray spectrum of the fraction recorded with a cone voltage of 18 V contained an ion at  $m/z$  224 and a much less abundant ion at  $m/z$  246, corresponding to the protonated  $[M+H]^+$  and the sodiated  $[M+Na]^+$  molecule for a compound with a molecular mass of 223, consistent with phenazine carboxamide. In the spectrum recorded with the higher cone voltage of 53 V, in addition to the pseudo-



**Fig. 1.** Biocontrol by various *Pseudomonas* isolates of tomato foot rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Strains isolated from the tomato rhizosphere, PCL1391, PCL1393, PCL1394, and the *P. fluorescens* strains F113 and WCS374 were tested in an *F. oxysporum*/tomato test system. Bacteria were coated on pre-germinated tomato seeds, and plants were grown in potting soil infected with *F. oxysporum* spores ( $3.0 \times 10^6$  spores per kg) under controlled conditions. Percentage of diseased plants was determined 3 weeks after inoculation. Per strain, 100 plants in 10 trays of 10 plants were tested. Data were analyzed for significance after arcsine square root transformations with analysis of variance followed by Fisher's least significant difference test ( $\alpha = 0.05$ ,  $n = 10$ ). Values with different letter indications denote a statistically significant difference.

molecular ions, a very intense fragment ion was observed at  $m/z$  207, corresponding to the loss of  $\text{NH}_3$ , a fragmentation typical of amide-containing compounds. A less abundant fragment ion was observed at  $m/z$  179, corresponding to the protonated phenazine ring from which the carboxamide functional group has been eliminated.



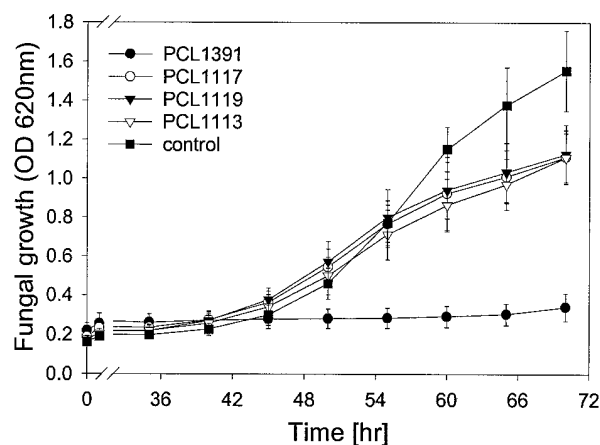
<sup>z</sup> Chemical shift values followed by the same letter might be interchanged.

**Fig. 2.** Nuclear magnetic resonance (NMR) signals from phenazine-1-carboxamide (PCN). A, Structure of PCN as deduced from (B)  $^1\text{H}$ -NMR and (C)  $^{13}\text{C}$ -NMR signals.

With NMR, the compound was definitively identified as PCN (Fig. 2A). The  $^1\text{H}$ -NMR spectrum (Fig. 2B) showed the characteristic two broad singlets for the amide protons, at  $\delta$  10.72 for the proton involved in hydrogen bonding with the aromatic nitrogen and at  $\delta$  6.32 for the other proton. The position of the substituent at C-1 appears clearly from the coupling patterns of the protons at positions 2, 3, and 4. The  $^{13}\text{C}$ -NMR spectrum is also in full accordance with the proposed structure (Fig. 2C).

#### Antifungal activity of PCN-nonproducing mutants of strain PCL1391.

A PCL1391 Tn5-*luxAB* transposon library, consisting of 10,000 exconjugants, was screened for PCN-deficient mutants by selection for the absence of pigment production after growth on LB agar medium and in 250- $\mu\text{l}$  liquid KB cultures. In this library, three mutants, PCL1113, PCL1117, and PCL1119, were identified that are impaired in phenazine production. This was confirmed by HPLC of toluene extracts of spent growth medium. The site of the transposon insertion was determined and DNA sequence analysis of the flanking regions revealed that the transposons are inserted in homologs of the *phzF*, *phzC*, and *phzB* genes, respectively, which are part of the phenazine biosynthetic operon of *P. fluorescens* strain 2-79 (Mavrodi et al. 1998). Regions of 600 bp adjacent to the transposon insertions show approximately 80% homology at the nucleotide level to the genes of *P. fluorescens*. None of the phenazine mutants was found to be altered in HCN, chitinase, lipase, or protease production. Neither did we detect a decrease in tomato root-colonizing ability in comparison with the wild-type strain (data not shown). In vitro microtiter plate fungal inhibition experiments show that the ability of spent growth medium of phenazine-negative mutants to inhibit growth of *F. oxysporum* was strongly reduced (Fig. 3). For mutant PCL1119, in which the *luxAB* genes of the Tn5 transposon are inserted in the same transcription direction as the phenazine biosynthetic operon, it was shown that this operon was expressed in the rhizosphere of tomato (Fig. 4).



**Fig. 3.** In vitro antifungal activity of culture supernatants of strain PCL1391 and its phenazine biosynthetic mutants PCL1113, PCL1117, and PCL1119. *Fusarium oxysporum* f. sp. *radicis-lycopersici* was grown in the presence of spent growth medium of the bacterial strains. Fungal growth was followed by measuring increase of absorbance ( $\text{OD}_{620\text{nm}}$ ) due to fungal mycelial growth. Eight replicates of each strain were tested.

### Biological control of PCN production mutants of strain PCL1391.

With the tomato/*F. oxysporum* foot and root rot system it was shown that the wild-type *P. chlororaphis* strain PCL1391 is able to reduce the number of diseased plants from 78% in the untreated control to 33% (Fig. 5). Strain PCL1119, a PCL1391 derivative impaired in PCN biosynthesis, did not suppress disease formation. Likewise, of the two other PCN biosynthetic mutants, strain PCL1113 and PCL1117 carrying transposon insertions in the *phzF* and *phzC* genes of the bio-



**Fig. 4.** Expression of the phenazine biosynthetic operon in the rhizosphere of tomato. Autoradiograph of two tomato roots grown for 7 days in a gnotobiotic sand system after inoculation with strain PCL1119 harboring promoterless *luxAB* genes inserted in the *phzB* gene of the phenazine biosynthetic operon. Bacteria were inoculated on pregerminated tomato seeds and seedlings were grown in a gnotobiotic sand system. After 7 days, roots were removed from the sand, and light was detected after addition of *n*-decyl aldehyde as a substrate. Position of the complete plant root is indicated by a black line.

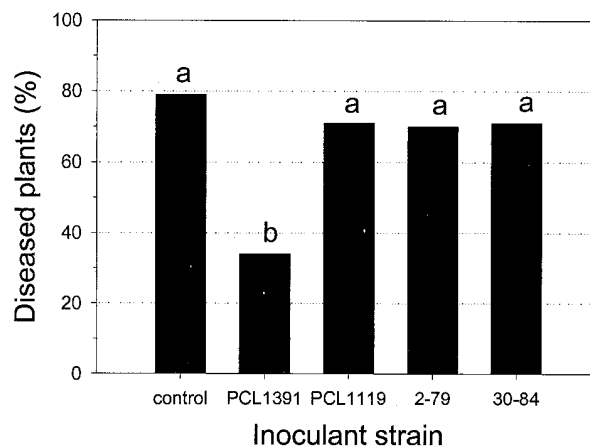
synthetic operon, respectively, neither was able to suppress disease formation (data not shown). In the same experiment, the biocontrol activities of the phenazine-1-carboxylic acid (PCA)-producing strain *P. fluorescens* 2-79 (Thomashow and Weller 1988), and *P. aureofaciens* 30-84 (Pierson and Thomashow 1992) were tested. No significant biocontrol activity was observed for either of these strains in the tomato/*F. oxysporum* test system (Fig. 5).

### Influence of pH on in vitro antifungal activity of PCN and PCA.

The antifungal activities of PCN and PCA were compared in an in vitro 96-well microtiter plate bioassay with *F. oxysporum* spores and HPLC-purified PCN from spent culture medium of strain PCL1391 and PCA from *P. fluorescens* 2-79 (Thomashow and Weller 1988). Equimolar concentrations of HPLC-purified phenazine antibiotics were serially diluted (0.5 to 0.02  $\mu$ M) in malt extract medium and adjusted to pH values ranging from 3.1 to 7.0 with phosphate-citrate buffer. Within this range, growth of the fungus was not influenced by pH (Fig. 6B). At pH 5.7 and a concentration of 0.25  $\mu$ M, the activity of PCN was 10 times higher than that of PCA (Fig. 6A). PCA activity was completely abolished under less acidic conditions whereas PCN exhibited antifungal activity at all of the pH values tested.

### DISCUSSION

Strain PCL1391, the most active of the three *F. oxysporum* antagonistic isolates identified out of 70 tomato rhizosphere bacterial strains analyzed, was found to have a highly significant biocontrol activity against foot and root rot of tomato (Figs. 1 and 5). Further characterization showed that strain PCL1391 has broad-spectrum antifungal activity and produces a variety of potential antifungal metabolites, including a phenazine derivative, chemically characterized as PCN (Fig.



**Fig. 5.** Biocontrol activities of phenazine-1-carboxamide (PCN) and phenazine-1-carboxylic acid (PCA)-producing strains in biocontrol of tomato foot rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. PCN-producing strain PCL1391, its phenazine biosynthetic mutant PCL1119, and PCA producers *Pseudomonas fluorescens* 2-79 and *P. aureofaciens* 30-84 were tested in an *F. oxysporum*/tomato test system. For experimental setup, see Figure 1 caption. Bars with the same letter are not significantly different at  $\alpha = 0.05$ ,  $n = 10$ , according to Fisher's least significant difference test.

2). The use of a PCN-negative mutant, strain PCL1119, showed that PCN is involved in antifungal action, both in vitro (Fig. 3) and in disease suppression in vivo (Fig. 5). This is the first report of a *Pseudomonas* strain that produces PCN in which the production of this compound is shown to be crucial for biocontrol activity. Consistent with the role of PCN in biocontrol are the observations that the *phz* biosynthetic operon is expressed in the rhizosphere (Fig. 4) and that strain PCL1391 displays an efficient tomato root-colonizing ability.

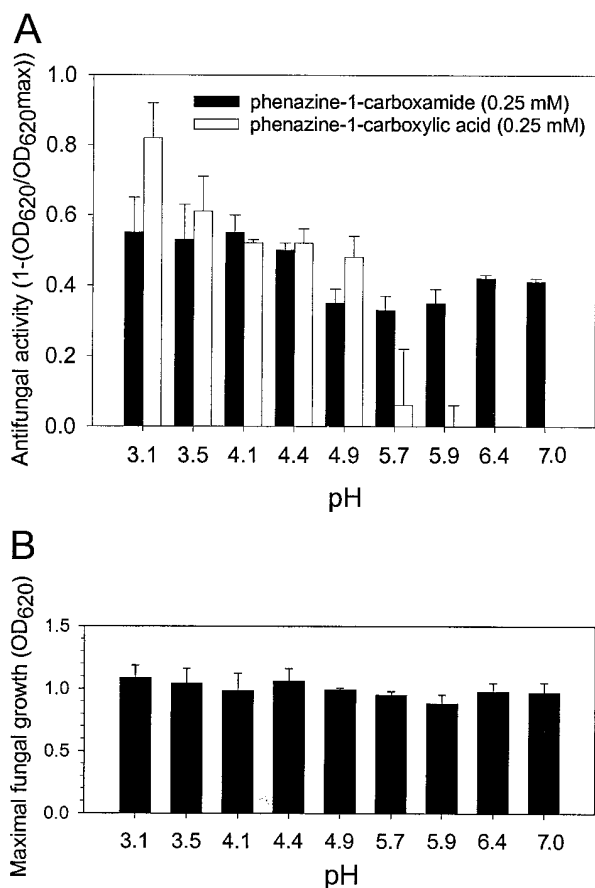
A number of strains that have been shown to have antifungal activity in vitro do not show suppression in the tomato/*F. oxysporum* test system used in this study. These strains include the two strains isolated from the tomato rhizosphere, PCL1393 and PCL1394, the 2,4-diacetylphloroglucinol-producing strain F113, the systemic resistance-inducing strain WCS374 (Leeman et al. 1995a) (Fig. 1), and the two PCA-producing *Pseudomonas* strains, 2-79 (Thomashow and Weller 1988) and 30-84 (Pierson and Thomashow 1992) (Fig. 5). Strains 2-79 and 30-84 produce PCA as the main phenazine compound and this metabolite has been shown to

be involved in the suppression of take-all disease of wheat, caused by *Gaeumannomyces graminis* var. *tritici* and other fungal pathogens. Both strains inhibit growth of *F. oxysporum* in in vitro agar plate assays. These observations indicate that in vitro antifungal activity is not always correlated with in vivo biocontrol activity.

A comparison of antifungal activity of PCA and PCN by an in vitro antifungal assay (Fig. 6) showed that PCN remains active at pH 5.7 and higher, at which the antagonistic activity of PCA drastically decreases. Under more acidic conditions, both derivatives have similar antifungal activity. Studies in which the PCA-producing *P. fluorescens* strain 2-79 was tested on pH-controlled agar medium against the wheat pathogen *G. graminis* var. *tritici* gave similar results (Brisbane et al. 1987). These authors suggested that the antimicrobial activity of PCA against the wheat pathogen was related to the concentration of protonated PCA, suggesting that as the soil pH or rhizosphere pH increase, and the anionic form of PCA predominates, PCA-producing pseudomonads are less effective in biocontrol. The wider range of action of PCN can be explained by the nearly neutral carboxamide group attached to the basic ring structure, which, in contrast to PCA, will remain protonated under more basic conditions. It is therefore likely that the difference in nature of the phenazine derivatives produced contributes to the difference in biocontrol activity between the PCA-producing strains 2-79 and 30-84 and the PCN-producing strain PCL1391.

The exact functions of the genes in the *phz* biosynthetic operon have not yet been fully characterized, although genetic studies on the phenazine biosynthetic pathway, in which the biosynthetic operon for the main phenazine compounds was identified, have shed more light on the individual reactions in this pathway (Pierson and Thomashow 1992; Mavrodi et al. 1998, 1997). From the observations that the PCA-producing strains do not produce PCN, and that PCA can be converted to PCN by cultures of *P. chlororaphis* (Ingram et al. 1970), we expect that strain PCL1391 possesses an additional gene different from the known phenazine biosynthetic genes.

Secretion of phenazine in the culture medium by strain PCL1391 starts in the early stationary phase and reaches a maximum during the late stationary phase (data not shown). For strain 30-84, it has been shown that phenazine biosynthesis is regulated by quorum sensing (Wood and Pierson 1996). Our previous studies with scanning electron microscopy have shown that pseudomonads colonize the tomato root system as micro-colonies between the junctions of root epidermal cells (Chin-A-Woeng et al. 1997). We hypothesized that these might be the sites where quorum sensing-regulated mechanisms, such as the regulation of phenazine biosynthesis, play a role. The observation that expression of the *phz* operon in the tomato rhizosphere is concentrated in discrete spots on the root (Fig. 4) supports our notion that micro-colonies are the sites of PCN production.



**Fig. 6.** Effect of pH on the antifungal activities of phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN). **A**, Effect of pH on the ability of PCN and PCA to inhibit growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *F. oxysporum* was grown in the presence of purified phenazine antibiotic (0.25  $\mu$ M) in a pH-range from 3.1 to 7.0. Mycelial growth was determined by measuring optical density at 620 nm after 72 h of incubation. **B**, As a control, mycelial growth of *F. oxysporum* was determined under different pH conditions without addition of phenazine compounds. Each condition was tested with two replicates.

## MATERIALS AND METHODS

### Microorganisms, growth conditions, and plasmids.

The microorganisms and plasmids used are listed in Table 1. *Pseudomonas* strains were routinely cultured in King's medium B (KB) (King et al. 1954) at 28°C. *Escherichia coli* was grown in Luria-Bertani (LB) medium (Sambrook et al. 1989)

at 37°C. *F. oxysporum* f. sp. *radicis-lycopersici* was stock cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) and grown in Czapek-Dox liquid medium (Thom and Raper 1945) at 28°C. Media were solidified with 1.8% agar (Difco), when necessary. Kanamycin was added to the medium to a concentration of 50 µg/ml when appropriate.

### Methods for screening and characterization of the biocontrol strains.

Tomato plants from a commercial field near Granada, Andalusia, Spain, were kindly provided by J. Olivares. Bulk soil was removed from the root systems in order to isolate the roots and directly adhering rhizosphere soil particles. Twenty-five grams of root material was shaken at 160 rpm in 50 ml sterile water for 45 min at 28°C (Orbital open air shaker 3591-3, Lab-Line Instruments, Melrose Park, IL) and samples of the suspension were plated on KB medium containing carbenicillin (10 µg/ml), chloramphenicol (12.5 µg/ml), and cycloheximide (200 µg/ml). Screening for in vitro antifungal activity on KB agar plates against *F. oxysporum* f. sp. *radicis-lycopersici* was performed by stabbing a 0.5 × 0.5 cm agar plug containing the fungus in the center of an LB agar plate and inoculating the bacterial strain at a distance of 3.0 cm from the fungus

(Geels and Schippers 1983). Bacterial strains inhibiting mycelial growth, as evidenced by a growth inhibition zone, were selected for further characterization. In vitro inhibition of a number of other plant pathogens, including *Rhizoctonia solani*, *Botrytis cinerea*, *Pythium ultimum*, *Verticillium albo-atrum*, and *Alternaria dauci* was performed in the same way. Strains of interest were typed by amplified ribosomal DNA-restriction analysis (ARDRA), which is based on sequences encoding 16S ribosomal RNA. The polymerase chain reaction (PCR) is used to amplify these DNA sequences specifically, and subsequently restriction patterns are compared with those in a data base (Williams et al. 1990; Vaneechoutte et al. 1998). Eight different restriction enzymes were used to obtain ribosomal DNA patterns and these were compared with an ARDRA data base containing 35 *Pseudomonas* and *Comamonas* spp. Identification of secreted potential antifungal metabolites was performed by previously described assays. Briefly, hydrogen cyanide (HCN) was detected by cyanide indicator paper (Castric 1975), protease with 3% milk agar plates, β-glucanase with plates containing lichenan (Sigma, St. Louis, MO) (Walsh et al. 1995), lipase with Tween 80 agar plates (Howe and Ward 1976), chitinase with plates containing colloidal chitin (Shimahara and Takiguchi 1988), and cel-

**Table 1.** Microorganisms and plasmids used

Strains and plasmids	Relevant characteristics	Reference or source
<b>Bacterial strains</b>		
PCL1391	Wild-type <i>Pseudomonas chlororaphis</i> , isolated from Spanish tomato rhizosphere	This study
PCL1393	Wild-type <i>Pseudomonas fluorescens</i> , isolated from Spanish tomato rhizosphere	This study
PCL1394	Wild-type <i>P. fluorescens</i> , isolated from Spanish tomato rhizosphere	This study
PCL1113	PCL1391 derivative in which a promoterless Tn 5- <i>luxAB</i> has been inserted in a <i>phzF</i> homolog	This study
PCL1117	PCL1391 derivative in which a promoterless Tn 5- <i>luxAB</i> has been inserted in a <i>phzC</i> homolog	This study
PCL1119	PCL1391 derivative in which a promoterless Tn 5- <i>luxAB</i> has been inserted in a <i>phzB</i> homolog	This study
PCL1500	Tn5- <i>lacZ</i> marked derivative of the efficient root colonizing <i>P. fluorescens</i> biocontrol strain WCS365	van der Bij et al. 1996
2-79	<i>P. fluorescens</i> strain whose biocontrol activity in a <i>Gaeumannomyces graminis</i> var. <i>tritici</i> /wheat system is partly due to phenazine-1-carboxylic acid (PCA) production	Thomashow and Weller 1988
30-84	<i>P. aureofaciens</i> strain whose biocontrol activity in a <i>G. graminis</i> var. <i>tritici</i> /wheat system is partly due to PCA production	Pierson and Thomashow 1992
F113	<i>P. fluorescens</i> strain whose biocontrol activity in a <i>Pythium ultimum</i> /sugar beet system is partly due to 2,4-diacetylphloroglucinol production	Shanahan et al. 1992
WCS374	<i>P. fluorescens</i> biocontrol strain causing induced systemic resistance (ISR) in radish	Leeman et al. 1995a
<b>Fungi</b>		
<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i> ZUM2407	Cause of crown and root rot of tomato	IPO-DLO, Wageningen, The Netherlands
<i>Rhizoctonia solani</i> 3R4FNA	Cause of damping-off and fruit rot	IPO-DLO, Wageningen, The Netherlands
<i>Botrytis cinerea</i> ZUM2076	Cause of gray mold of tomato	Novartis Seeds, Enkhuizen, The Netherlands
<i>Pythium ultimum</i> LBOP17	Cause of damping-off and fruit rot of tomato	IPO-DLO, Wageningen, The Netherlands
<i>Verticillium albo-atrum</i>	Cause of wilt of tomato	IPO-DLO, Wageningen, The Netherlands
<i>Alternaria dauci</i> ZUM2372	Isolated from carrot seeds; pathogen of carrot	Novartis Seeds, Enkhuizen, The Netherlands
<b>Plasmids</b>		
pRL1063a	Plasmid harboring promoterless Tn 5- <i>luxAB</i> transposon	Wolk et al. 1991
pMP6001	pRL1063a-based plasmid recovered from chromosomal DNA of PCL1113 after digestion with <i>EcoRI</i>	This study
pMP6002	pRL1063a-based plasmid recovered from chromosomal DNA of PCL1119 after digestion with <i>EcoRI</i>	This study
pMP6003	pRL1063a-based plasmid recovered from chromosomal DNA of PCL1117 after digestion with <i>Clal</i>	This study

lulase with plates containing 1-carboxymethylcellulose (Hankin and Anagnostakis 1977). The presence of pyochelin, salicylic acid, phloroglucinols, and pyoluteorin was detected by HPLC analyses (Keel et al. 1992) of ethylacetate extracts of 16-, 48-, or 72-h cultures of the bacterial strain grown on KB, PDA, or malt (Oxoid, London) media.

Root tip colonization by strain PCL1391 and mutants of PCL1391 in competition with PCL1500, a Tn5-*lacZ*-marked derivative of *P. fluorescens* WCS365, was determined by inoculation of pre-germinated seeds with a mixture of the bacteria in a 1:1 ratio. Plants were grown in a gnotobiotic sand system as described previously (Simons et al. 1996) and, after 7 days, colonization of the root tip was analyzed by dilution plating.

#### **Purification and structural identification of the hydrophobic antifungal compound of strain PCL1391.**

After growth in KB broth medium supplemented with a final concentration of 100  $\mu$ M FeCl<sub>3</sub> for 72 h at 28°C, the cell-free supernatant fluid was extracted with an equal volume of toluene and the extracts were concentrated by evaporation in vacuo. Samples were dissolved in acetonitrile and fractionated by TLC or HPLC. Silica plates (Merck, Darmstadt, Germany) were developed in a solvent mixture of butanol/acetone (90/10, vol/vol). After development, antifungal activity was detected by an *F. oxysporum* silica TLC bioassay (Keen et al. 1971). The TLC plate was dried, sprayed with an *F. oxysporum* spore suspension ( $5 \times 10^5$ /ml) in malt extract broth (Sigma), and incubated in a closed container above a layer of water for 72 h at 28°C. Antifungal activity on the TLC plate was evidenced by the absence of growth of fungal mycelium.

HPLC was performed with an Alltech Hypersil ODS 5  $\mu$ m 250  $\times$  4.6 mm column (Alltech Associates, Deerfield, IL) and a linear 18 to 80% (vol/vol) gradient of acetonitrile in water, with 0.1% (vol/vol) trifluoroacetic acid, and a flow rate of 1 ml/min (Watson et al. 1986; Fernandez and Pizarro 1997). UV detection was performed with a Pharmacia RSD 2140 diode array detector (Pharmacia, Uppsala, Sweden) with wavelength scanning from 190 to 400 nm, and 1.0-ml fractions were collected for testing antifungal activity with the *F. oxysporum* TLC bioassay. Fractions with antifungal activity were collected for mass spectrometry and NMR analyses.

Positive ion mode electrospray mass spectra were obtained with a VG Platform II single quadrupole mass spectrometer. Aliquots (10  $\mu$ l) of each fraction dissolved in 100  $\mu$ l of toluene were infused into the electrospray source in ACN/H<sub>2</sub>O (50:50 vol/vol) acidified with formic acid (1% vol/vol) at a flow rate of 5  $\mu$ l/min. In the positive mode, spectra were scanned at a speed of 10 s for *m/z* 50 to 1000, with a cone voltage of 18 or 53 V. Spectra were recorded and processed with the MassLynx 2.0 software.

NMR spectra were obtained on a Bruker AM 500 spectrometer operating at 500 MHz for protons and 125 MHz for carbon-13. Samples were dissolved in CDCl<sub>3</sub> and the solvent signal was used as internal standard (7.26 ppm for <sup>1</sup>H-NMR and 77.00 ppm for <sup>13</sup>C-NMR).

#### **Isolation of mutants impaired in PCN biosynthesis.**

A Tn5 transposon library was generated with pRL1063a harboring a promoterless Tn5-*luxAB* transposon (Wolk et al. 1991), as described by Kragelund et al. (1995) Phenazine bio-

synthetic mutants were identified by loss of pigment production when grown on LB agar or in 250  $\mu$ l of liquid KB cultures in 96-well microtiter plates. Regions flanking the site of the transposon insertion were recovered by digestion of chromosomal DNA with either *EcoRI* or *ClaI* and subsequent recircularization of the excised fragments by ligation. Nucleotide sequencing of the flanking regions was performed with unique primers homologous to the left (5' TAC-TAGATTCAATGCTATCAATGAG 3') and right (5' AG-GAGGTCACATGGAATATCAGAT 3') ends of the Tn5 transposon. Nucleotide sequencing was performed with the Thermo Sequenase kit according to methods described by the supplier (Amersham Life Science, Cleveland, OH), and sequences were analyzed with the Wisconsin Package from the Genetics Computer Group (GCG; Madison, WI).

#### **Detection of *lux* expression in the tomato rhizosphere.**

Expression of *lux* in the tomato rhizosphere was assayed in a gnotobiotic sand system (Simons et al. 1996) in which seedlings were grown after inoculation of pre-germinated tomato seeds with Tn5-*luxAB*-marked strains. After 7 days, the seedlings were removed from the sand and most adhering rhizosphere sand, which is virtually devoid of bacteria (Chin-A-Woeng et al. 1997), was removed with sterile forceps. Subsequently, the roots were placed onto a filter paper that had been drenched in a phosphate buffer containing 0.2% (vol/vol) *n*-decyl aldehyde as a substrate for the luciferase enzyme. Light production was detected by placing a photographic film over the root for 24 h (de Weger et al. 1991).

#### **In vitro microtiter-plate antifungal assay.**

In vitro inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* was assayed by mixing filtered spent growth medium (KB) of 16-h cultures with malt extract broth containing 0.75% low melting point agarose (FMC BioProducts, Rockland, ME) or HPLC-purified phenazine antibiotics and  $1 \times 10^3$  spores per well in a microtiter plate (Cuppers et al. 1997). When necessary, the pH in the wells was altered by preparing malt extract broth in a citrate-phosphate buffer (Dawson et al. 1969) instead of in water. The plates were sealed with oxygen-permeable plate sealers (Merlin Diagnostic Systems, Rotterdam, The Netherlands) and incubated at 28°C. The growth of the fungal mycelium was followed by measuring the optical density at 620 nm of the wells every hour with a microtiter plate reader (Bio-Rad, Hercules, CA) for 72 h. Maximal fungal growth and background were determined by growing without inhibitors and without fungal spores, respectively.

#### **Biocontrol tests.**

One third of a 10-day-old PDA petri dish culture of *F. oxysporum* f. sp. *radicis-lycopersici* was homogenized and inoculated in 200 ml of Czapek-Dox medium in a 1-liter Erlenmeyer flask. After growth for 3 days at 28°C under shaking (110 rpm; Orbital open air shaker 3591-3, Lab-Line Instruments), the fungal material was placed on top of sterile glass wool and the filtrate was adjusted to a concentration of  $5 \times 10^5$  spores/ml. For inoculation, spores were mixed thoroughly with potting soil ( $3.0 \times 10^6$  spores per kg).

Tomato (*Lycopersicon esculentum* Mill.) seeds (cv. Carmello) were coated with bacteria by dipping the seeds in a mixture of 1% (wt/vol) methylcellulose (Sigma) and  $1 \times 10^9$

CFU/ml bacteria in phosphate-buffered saline (PBS) buffer (Leeman et al. 1995b). Coated seeds were dried in a sterile air stream. The number of bacteria recovered from the coated seeds after shaking the bacteria into PBS, followed by dilution plating onto KB medium, was approximately  $1 \times 10^4$  CFU/seed. In a pot containing 25 g of soil (Jongkind grond, Aalsmeer, The Netherlands), one seed was sown per pot at a depth of approximately 1.5 cm. One hundred plants were tested per treatment in series of 10 plants in order to be able to apply statistical analysis to the results. Seedlings were grown in a greenhouse at 21°C, 70% relative humidity, and 16 h of daylight. Plants were watered from the bottom. The number of diseased plants was determined when a substantial number of the plants in the untreated control were diseased, usually between 14 and 21 days after sowing. Plants were removed from the soil and washed, and the plant roots were examined for foot and root rot indicated by browning and lesions. Roots without any disease symptoms were classified as healthy. Data were analyzed for significance after arcsine square root transformations with analysis of variance followed by Fisher's least significant difference test ( $\alpha = 0.05$ ), with SAS software (SAS Institute, Cary, NC). All experiments were performed at least twice.

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