

# Influence of Environmental Conditions on the Production of Phenazine-1-Carboxamide by *Pseudomonas chlororaphis* PCL1391

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***Pseudomonas chlororaphis* PCL1391 produces the secondary metabolite phenazine-1-carboxamide (PCN), which is an antifungal metabolite required for biocontrol activity of the strain. Identification of conditions involved in PCN production showed that some carbon sources and all amino acids tested promote PCN levels. Decreasing the pH from 7 to 6 or decreasing the growth temperature from 21 to 16°C decreased PCN production dramatically. In contrast, growth at 1% oxygen as well as low magnesium concentrations increased PCN levels. Salt stress, low concentrations of ferric iron, phosphate, sulfate, and ammonium ions reduced PCN levels. Fusaric acid, a secondary metabolite produced by the soilborne *Fusarium* spp. fungi, also reduced PCN levels. Different nitrogen sources greatly influenced PCN levels. Analysis of autoinducer levels at conditions of high and low PCN production demonstrated that, under all tested conditions, PCN levels correlate with autoinducer levels, indicating that the regulation of PCN production by environmental factors takes place at or before autoinducer production. Moreover, the results show that autoinducer production not only is induced by a high optical density but also can be induced by certain environmental conditions. We discuss our findings in relation to the success of biocontrol in the field.**

Phenazine-1-carboxamide (PCN) and its analogues are being evaluated as DNA intercalating, anticancer agents that inhibit topoisomerase I and II (Gamage et al. 2002; Rewcastle et al. 1987). Both topoisomerases are essential in dividing and proliferating cells because they regulate DNA topology by allowing single- and double-stranded DNA to pass through each other (Wang 2002). PCN used in these studies was synthesized chemically (Gamage et al. 2002; Rewcastle et al. 1987). PCN also occurs as a natural product (e.g., produced in high quantities by the gram-negative bacterium *Pseudomonas chlororaphis* PCL1391) (Chin-A-Woeng et al. 1998); therefore, biotechnological production of PCN seems a promising alternative.

A second application of PCN is its use as an antifungal metabolite (AFM) in microbial control of plant diseases (Chin-A-Woeng et al. 1998). Although biocontrol looks highly promising under laboratory conditions, field experiments do not always give consistent results (Handelsman and Stabb 1996; Thomashow and Weller 1988). The influence of many highly variable environmental factors on various biocontrol

traits (Duffy and Défago 1999; Thomashow and Weller 1988), including PCN production (Chin-A-Woeng 2000; Lugtenberg et al. 2002), are likely to contribute to this phenomenon.

Production of phenazines and many other microbial secondary metabolites are regulated by quorum sensing (Bassler 1999; Chin-A-Woeng et al. 2001b; Pearson et al. 1997; Pierson and Thomashow 1992; Whitehead et al. 2001). Quorum sensing is a mechanism by which cells sense population density via the accumulation of small diffusible molecules. Quorum sensing in gram-negative bacteria often is mediated through levels of N-acyl-homoserine lactone (N-AHL) autoinducers (Fuqua et al. 1994; Miller and Bassler 2001; Salmond et al. 1995; Winzer et al. 2002). Other examples of secondary metabolites of which the biosynthesis is controlled by quorum sensing are carbapenem produced by *Erwinia carotovora* (Bainton et al. 1992) and pyocyanin produced by *P. aeruginosa* (Latifi et al. 1995). The *luxI* and *luxR* homologues of strain PCL1391, *phzI* and *phzR*, regulate the expression of the biosynthetic *phz* operon, and PhzI produces the autoinducer N-hexanoyl-L-homoserine lactone (C<sub>6</sub>-HSL) (Chin-A-Woeng et al. 2001b), which supposedly activates the transcriptional activator PhzR. Activated PhzR is thought to turn on the biosynthetic *phz* genes (Chin-A-Woeng et al. 2001b; Pierson et al. 1994). Quorum sensing is dependent on population density and, in addition, is regulated by other regulatory components (Chin-A-Woeng et al. 2001b; Whitehead et al. 2001).

Identification of conditions which control PCN production will lead to a better understanding of the regulation of the biosynthesis of secondary metabolites. This information may become important for optimizing biocontrol under practical conditions. Therefore, we initiated a study in which the effects of various biotic and abiotic conditions on PCN production by *P. chlororaphis* PCL1391 are investigated. The results are presented in this article.

## RESULTS

### PCN production was growth-phase dependent and influenced by the nature of the carbon source.

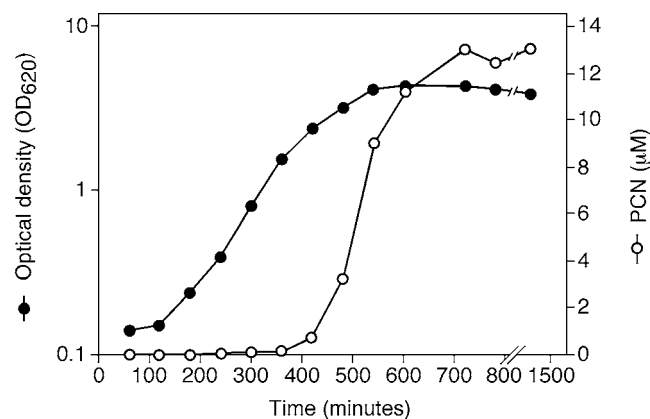
Growth and PCN production of *P. chlororaphis* PCL1391 was monitored in time (Table 1). Modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose (MVB1-glucose-cas) initially was used for the analyses of PCN production. In this medium, PCN production started at the end of the exponential phase and continued to increase until the cells reached stationary phase (Fig. 1). During the first 12 h of stationary phase, PCN levels remained unchanged (Fig. 1). To study the effects of various carbon sources on the level of PCN

production, PCL1391 cells were grown in MVB1-cas supplemented with equal concentrations of carbon atoms for the carbon sources glycerol, and sugars and organic acids present in the root exudate of tomato (Lugtenberg et al. 1999; Lugtenberg and Bloemberg 2004). Generation time and optical density at stationary phase were determined. Short generation times (60 to 74 min) were found for growth on glucose, fumaric acid, lactic acid, malic acid, and succinic acid. Moderate generation times (79 to 101 min) were found for growth on citric acid, glycerol, L-pyroglyutamic acid, and sucrose. Growth on ribose, pyruvic acid, and fructose resulted in the longest generation times (186 to 500 min) (Table 2). Strain PCL1391 did not grow on the carbon sources 2-ketoglutaric acid, maltose, oxalic acid, propionic acid, xylose, and T-aconitic acid. Cultures grown on the carbon sources fructose, glucose, glycerol, and sucrose reached the stationary phase at an optical density at a wavelength of 620 nm ( $OD_{620}$ ) value greater than 4. On the remaining carbon sources, early stationary phase was reached at lower  $OD_{620}$  values, which varied between values of 1.8 and 3.2 (Table 2). MVB1-cas medium supplemented with the carbon sources glucose, glycerol, and L-pyroglyutamic acid resulted in the highest levels of PCN (8.4 to 3.0  $\mu\text{M}/OD_{620}$ ) compared with all other carbon sources tested (Table 2). On MVB1-glucose-cas, a high production of PCN and a short generation time were measured. Therefore, this medium subsequently was used to study the effects of other environmental factors on the production of PCN by PCL1391 cells. Omitting 0.05% casamino acids from the media reduced stationary phase with an  $OD_{620}$  value of 0.4 (data not shown), did not affect generation time (data not shown), but dramatically reduced the levels of PCN production (Table 2).

### Nitrogen sources and amino acids affected the production of PCN.

PCN levels were studied after replacement of the nitrogen source  $(\text{NH}_4)_2\text{SO}_4$  in MVB2-glucose-cas by  $\text{NaNO}_3$ , urea, or

casamino acids. Urea or  $\text{NaNO}_3$  resulted in decreased PCN levels (Fig. 2A), whereas growth on 16 mM casamino acids caused a threefold increase in the level of PCN and a 50% higher final OD compared with 8 mM  $(\text{NH}_4)_2\text{SO}_4$  (Fig. 2A). Increasing the standard  $(\text{NH}_4)_2\text{SO}_4$  concentration increased PCN levels, whereas decreasing the  $(\text{NH}_4)_2\text{SO}_4$  concentration reduced PCN levels (Fig 2B). To test whether or not  $\text{NH}_4^+$  ions stimulated PCN levels,  $\text{NH}_4\text{Cl}$  was tested. Increasing  $\text{NH}_4\text{Cl}$  concentrations appeared to stimulate PCN production (Fig. 2C). To test whether the stimulatory effect of casamino acids on PCN levels described earlier can be ascribed to individual amino acids, casamino acids in MVB1-glucose-cas were



**Fig. 1.** Growth and phenazine-1-carboxamide (PCN) production by *Pseudomonas chlororaphis* strain PCL1391. Cells were grown on modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose (MVB1-glucose-cas). Experiments were performed three times with similar results. Data of one of the experiments is shown. Amounts of PCN were determined by high-performance liquid chromatography analyses.

**Table 1.** Microorganisms and plasmids used in this study

Strains or plasmid	Relevant characteristics	Reference
Bacterial strains		
PCL1391	Wild-type biocontrol strain <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide	Chin-A-Woeng et al. 1998
CV026	<i>Chromobacterium violaceum</i> ; N-acyl-homoserine lactone (AHL) reporter strain	Milton et al. 1997
DH5 $\alpha$	<i>Escherichia coli</i> ; supE44 $\Delta$ lacU169( $\Phi$ 80 lacZ $\Delta$ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Hanahan 1983
JM109	<i>E. coli</i> ; recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB) F' [traD36 proAB <sup>+</sup> lac <sup>R</sup> lacZ M15]	Yanisch-Perron et al. 1985
Plasmids		
pSB401	Autoinducer reporter plasmid based upon the <i>Photobacterium</i> bioluminescence ( <i>lux</i> ) system	Winson et al. 1998

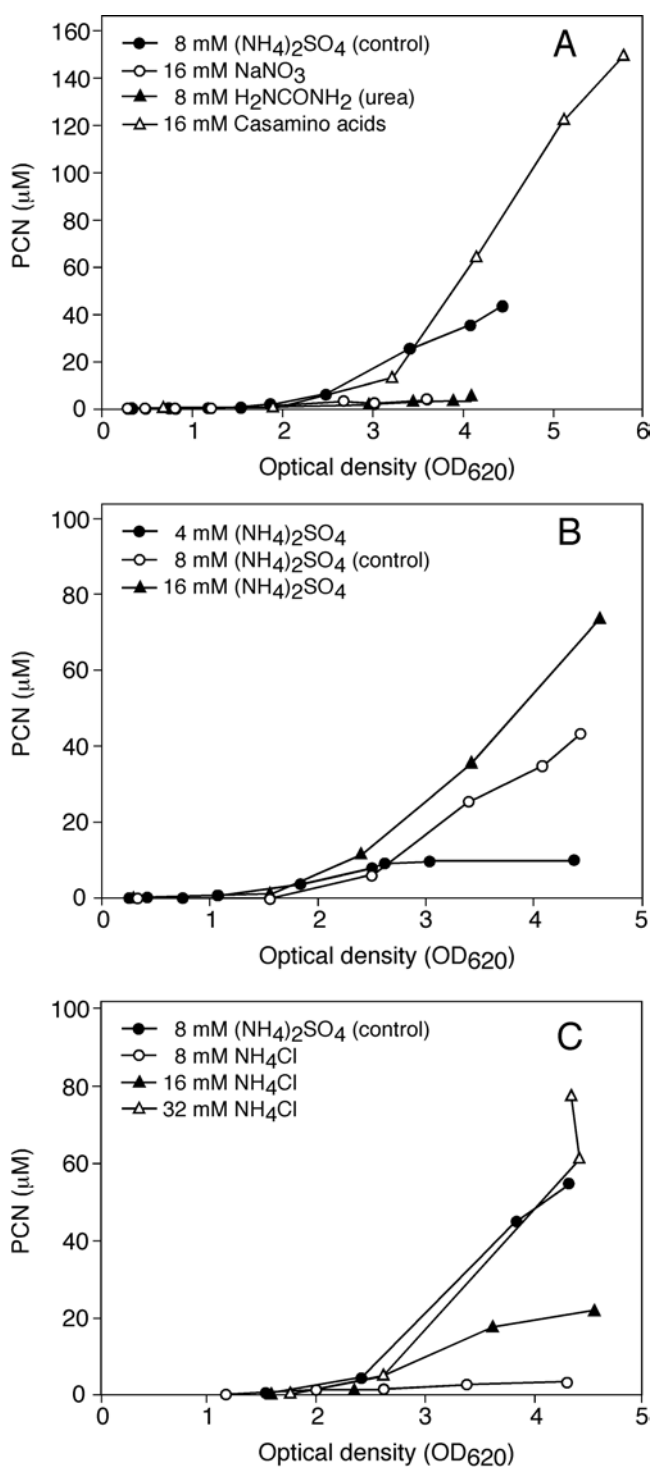
**Table 2.** Influence of various carbon sources on generation time in exponential phase and optical density and phenazine-1-carboxamide (PCN) production at stationary phase of *Pseudomonas chlororaphis* PCL1391<sup>y</sup>

Carbon source (concentration)	+0.05% cas <sup>z</sup>			-0.05% cas
	Generation time (min)	Optical density ( $OD_{620}$ )	PCN ( $\mu\text{M}/OD_{620}$ )	PCN ( $\mu\text{M}/OD_{620}$ )
Citric acid (30 mM)	101 $\pm$ 4.4	2.9 $\pm$ 0.3	1.00 $\pm$ 0.6	0.22 $\pm$ 0.07
Fructose (30 mM)	186 $\pm$ 22	4.4 $\pm$ 0.4	0.35 $\pm$ 0.1	0.10 $\pm$ 0.1
Fumaric acid (45 mM)	73 $\pm$ 25	2.8 $\pm$ 0.7	0.11 $\pm$ 0.01	0.13 $\pm$ 0.07
Glucose (30 mM)	60 $\pm$ 9.8	4.3 $\pm$ 0.1	7.90 $\pm$ 0.2	0.86 $\pm$ 0.2
Glycerol (60 mM)	93 $\pm$ 17	4.8 $\pm$ 0.2	3.00 $\pm$ 0.1	0.12 $\pm$ 0.1
Lactic acid (60 mM)	70 $\pm$ 10	3.2 $\pm$ 0.2	1.20 $\pm$ 0.08	0.04 $\pm$ 0.01
Malic acid (45 mM)	68 $\pm$ 2.0	1.8 $\pm$ 0.2	0.05 $\pm$ 0.01	0.04 $\pm$ 0.01
L-Pyroglyutamic acid (36 mM)	79 $\pm$ 0.16	3.0 $\pm$ 0.2	8.40 $\pm$ 0.7	ND
Pyruvic acid (60 mM)	213 $\pm$ 33	2.3 $\pm$ 0.1	0.17 $\pm$ 0.2	0.01 $\pm$ 0.006
Ribose (36 mM)	500 $\pm$ 58	2.8 $\pm$ 0.3	0.32 $\pm$ 0.2	0.02 $\pm$ 0.01
Succinic acid (C45 mM)	74 $\pm$ 4.8	3.2 $\pm$ 0.3	0.13 $\pm$ 0.009	0.04 $\pm$ 0.006
Sucrose (15 mM)	83 $\pm$ 9.8	4.4 $\pm$ 0.2	0.79 $\pm$ 0.2	0.13 $\pm$ 0.06

<sup>y</sup> Cells were grown in modified Vogel Bonner medium (MVB1) in the presence or absence of 0.05% casamino acids with various carbon sources, such that equal concentrations of carbon atoms were present. Optical density was measured at stationary phase and PCN concentrations of the supernatant fluids were quantified using high-performance liquid chromatography when cells had reached stationary phase. Average and standard deviations of three experiments are shown. ND = not detected.

<sup>z</sup> Optical density at a wavelength of 620 nm =  $OD_{620}$ .

replaced by individual amino acids detected in tomato root exudate, namely phenylalanine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, leucine, asparagine, glutamine, and threonine (Simons et al. 1997), as well as by the aromatic amino acids tryptophan and tyrosine. As mentioned earlier, strain PCL1391 produced hardly any PCN when grown in MVB1-glucose without added amino acids. The addi-



**Fig. 2.** Influence of various nitrogen sources on the level of phenazine-1-carboxamide (PCN). *Pseudomonas chlororaphis* strain PCL1391 was grown with **A**, various nitrogen sources and various concentrations of **B**, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and **C**, NH<sub>4</sub>Cl. Cells were grown in modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose (MVB2-glucose-cas). Experiments were performed at least three times with similar results. Data of one of the experiments is shown.

tion of any of the mentioned amino acids at 1 mM increased PCN production at least twofold. The largest increases in PCN levels, between 8- and 23-fold, occurred after the addition of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Table 3). The increased PCN production caused by phenylalanine was analyzed in time and PCN production was advanced in relation to the optical cell density. The addition of 1 mM phenylalanine caused the PCN production to start at an OD<sub>620</sub> value of 1.0 instead of 2.0 (data not shown).

### Abiotic factors affected PCN production.

Cells of strain PCL1391 were grown at temperatures between 16 and 31°C, whereas 28°C is the standard growth temperature. Increasing or decreasing the temperature to 31 or 21°C, respectively, had no major effect on PCN levels. Incubation at 21°C decreased the growth rate by 30% (data not shown) compared with that at 28°C. Lowering the growth temperature to 16°C practically abolished PCN production (Fig. 3A) and decreased the growth rate by 80% (data not shown).

The influence of oxygen limitation on the levels of PCN was investigated by aerating MVB1-glucose-cas with gas mixtures containing 21, 10, and 1% oxygen. Decreasing the oxygen concentration from 21 to 10% had little effect on PCN levels and growth rate. However, aeration with 1% oxygen resulted in a strong increase in PCN levels and in initiation of PCN production at a much lower OD<sub>620</sub> (Fig. 3B). The growth rate at 1% oxygen was almost twofold lower (193 min) compared with that with 21% oxygen (105 min) in the same experimental setup.

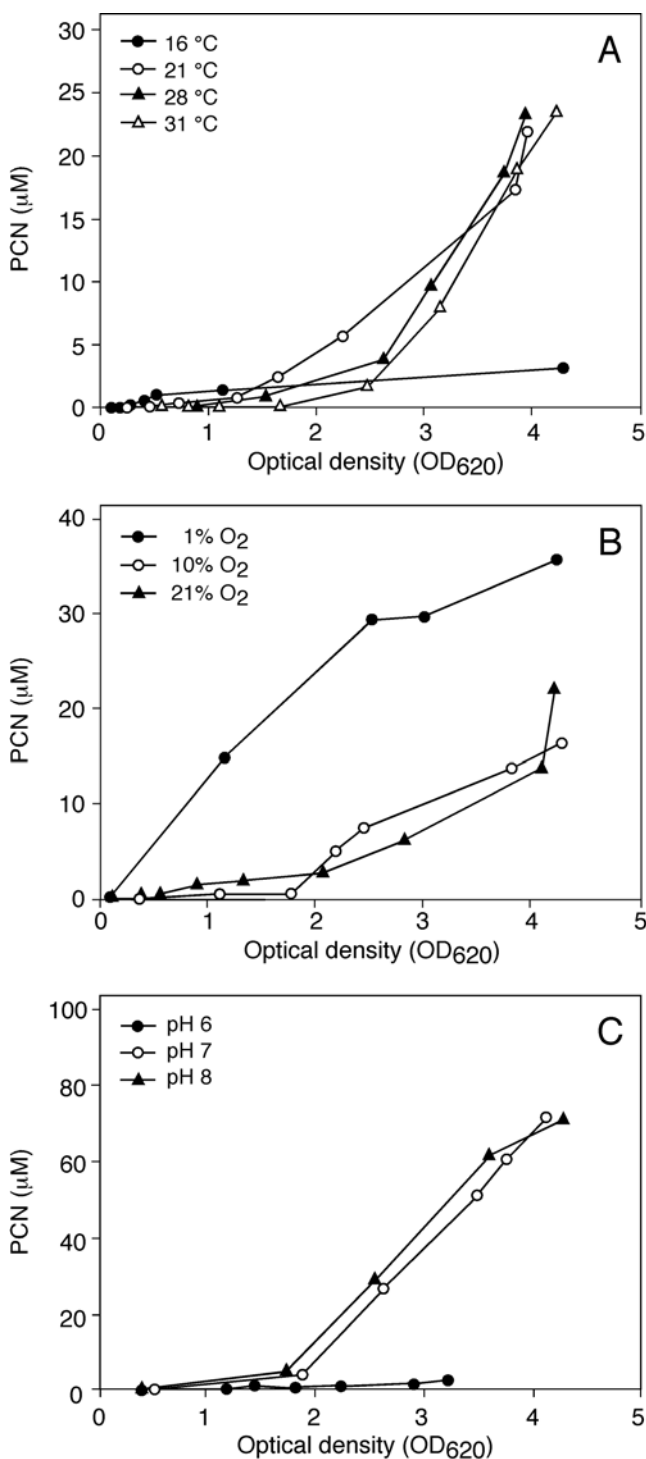
To test the influence of the pH of the medium on PCN levels, K<sub>2</sub>HPO<sub>4</sub> and Na(NH<sub>4</sub>)HPO<sub>4</sub> · 4H<sub>2</sub>O in MVB1-glucose-cas were replaced by K<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, and the nitrogen source (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. This medium was named MVB2-glucose-cas and allowed easy changes in the pH by changing the ratio of K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub>. Growth of strain PCL1391 in MVB2-glucose-cas resulted in a greater than twofold increase in PCN levels compared with growth in MVB1-glucose-cas. MVB2-glucose-cas media with pH values of 6.0, 7.0, and 8.0 were used to monitor PCN production during growth. The pH of these cultures was measured during growth and the pH decreased to pH 4.2, 6.5, and 7.1, respectively. Growth in MVB2 with pH 7.0 or 8.0 resulted in equal levels of PCN. However, growth in MVB2 with a pH value of 6.0 resulted in

**Table 3.** Influence of individual amino acids on phenazine-1-carboxamide (PCN) production in modified Vogel Bonner medium (MVB1)-glucose by *Pseudomonas chlororaphis* PCL1391

Amino acid added (1 mM)	PCN production (μM/OD <sub>620</sub> ) <sup>z</sup>
None	0.91 ± 0.21a
Asparagine	3.90 ± 0.61 b
Aspartic acid	3.60 ± 2.3 b
Glutamic acid	2.10 ± 0.78 a
Glutamine	3.70 ± 0.59 b
Glycine	6.60 ± 2.3 b
Histidine	3.70 ± 0.13 b
Isoleucine	6.10 ± 0.32 b
Leucine	3.90 ± 1.1 b
Lysine	3.90 ± 1.1 b
Phenylalanine	21.00 ± 2.1 b
Threonine	2.00 ± 1.2 a
Tryptophan	7.10 ± 0.22 b
Tyrosine	12.00 ± 3.2 b

<sup>z</sup> Optical density at a wavelength of 620 nm = OD<sub>620</sub>. PCN concentration of the supernatant fluid was quantified by high-performance liquid chromatography at stationary phase. Average and standard deviations of three experiments are shown. Values followed by different letters are significantly different from no added amino acids at *P* = 0.05 according to Fisher's least significant difference test.

an extremely low PCN level (Fig. 3C) and a decreased OD<sub>620</sub> at the stationary phase (data not shown). The pH of all other environmental conditions tested in this study was measured at the stationary phase and was between pH 6.4 and 6.7. Growth on the organic acids carbon sources resulted in higher pH values of 9.5 for citric acid, 8.3 for fumaric acid, 8.9 for malic acid,



**Fig. 3.** Influence of abiotic factors on the level of phenazine-1-carboxamide (PCN). *Pseudomonas chlororaphis* strain PCL1391 was grown at various **A**, temperatures, **B**, oxygen concentrations, and **C**, pH values. Cells were grown in **A** and **B**, modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose (MVB1-glucose-cas) or **C**, MVB2-glucose-cas. Experiments were performed at least three times with similar results. Data of one of the experiments is shown.

7.0, pyruvic acid, and 9.3 for succinic acid. Lactic acid was the exception, with a pH value of 6.5.

#### Ions in the medium affected PCN production.

Omitting NaFeEDTA(III) from MVB1-glucose-cas resulted in a loss of PCN production (Fig. 4A). Replacing 0.078 mM NaFeEDTA(III) by 0.078 mM FeCl<sub>3</sub> led to equal levels of PCN (data not shown). Concentrations of 0.005 and 0.01 mM FeCl<sub>3</sub> partially restored PCN production in a concentration-dependant manner (Fig. 4A). Omitting ferric iron (Fe<sup>3+</sup>) from MVB1 did not change the growth rate during the exponential phase or the final OD<sub>620</sub> during the stationary phase (data not shown). Increasing the phosphate concentration to 150 mM resulted in a reduction of PCN levels and a small reduction in stationary phase OD<sub>620</sub> value (Fig. 4B). Decreasing the phosphate concentration to 10 or 1 mM also decreased PCN production and reduced OD<sub>620</sub> at stationary phase by 22 and 62%, respectively. Decreasing the standard MgSO<sub>4</sub> concentration of 0.8 mM to 0.08 mM resulted in a 90% reduction in PCN production, a 40% reduction in OD<sub>620</sub>, and an increased generation time during exponential phase (203 min). Increasing the MgSO<sub>4</sub> concentration to 8 mM resulted in a 72% reduction in PCN production but did not affect growth rate or OD<sub>620</sub> during stationary phase (data not shown). To test whether these effects were due to Mg<sup>2+</sup> or to SO<sub>4</sub><sup>2-</sup>, MgCl<sub>2</sub> and K<sub>2</sub>SO<sub>4</sub> were used as separate Mg<sup>2+</sup> and SO<sub>4</sub><sup>2-</sup> sources. Decreasing the MgCl<sub>2</sub> concentration 10-fold resulted in almost twofold decreased growth rate (117 min) during the exponential phase, a start of PCN production at a lower OD<sub>620</sub> value, and increased levels of PCN. Increasing the MgCl<sub>2</sub> concentration 10-fold abolished PCN production completely (Fig. 4C). Decreasing the K<sub>2</sub>SO<sub>4</sub> concentration 10-fold abolished PCN production, whereas a 10-fold increase had little effect on PCN levels (Fig. 4D).

#### Salt stress affected the production of PCN.

The effect of osmolality on PCN production was tested by adding NaCl, KCl, Na<sub>2</sub>SO<sub>4</sub>, or xylose to MVB1-glucose-cas. Increasing NaCl concentrations resulted in a decrease of PCN levels and 0.1 M NaCl abolished PCN levels almost completely (Table 4). A concentration of 0.2 M xylose, which corresponds with the osmolality of 0.1 M NaCl, did not reduce PCN levels. Concentrations of 0.1 M KCl or 0.5 M Na<sub>2</sub>SO<sub>4</sub> were added to MVB1-glucose-cas to test the effects of the sodium and chloride ions separately. Both salts caused a severe reduction in PCN production, with a more drastic reduction for KCl than for Na<sub>2</sub>SO<sub>4</sub> (Table 4). The growth and OD<sub>620</sub> at stationary phase of PCL1391 were not affected by the addition of these salts or by xylose (data not shown). The osmoprotectants betaine, choline, L-proline, and trehalose were added to MVB1-glucose-cas in order to test whether they could compensate for the reduction in PCN level caused by 0.1 M NaCl. None of the osmoprotectants significantly increased PCN production in the presence of 0.1 M NaCl (Table 4).

#### Fusaric acid reduced PCN production.

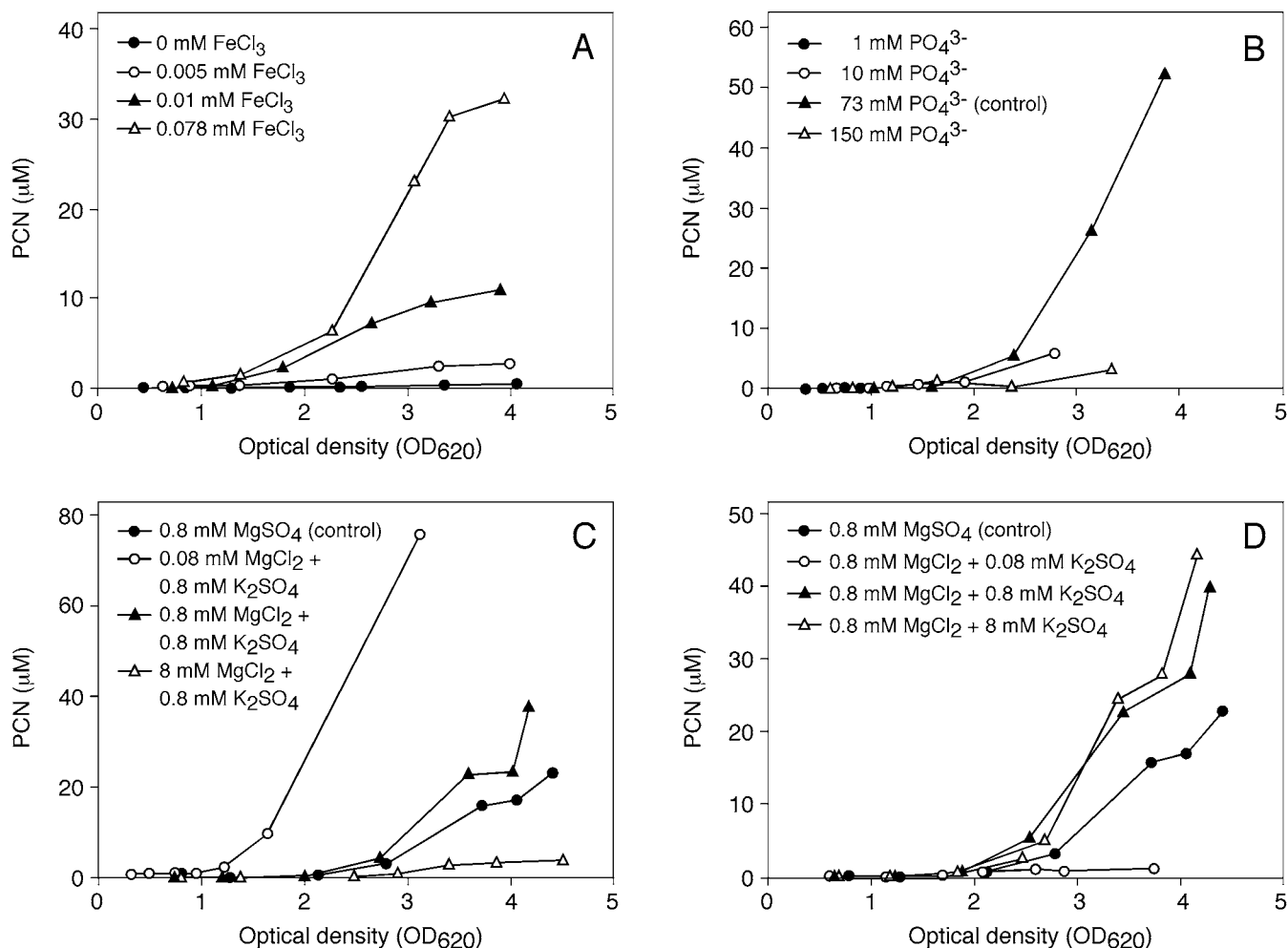
*P. chlororaphis* PCL1391 is a biocontrol strain of tomato foot and root rot caused by the fungal pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* ZUM2407 (Chin-A-Woeng et al. 1998). *F. oxysporum* f. sp. *radicis-lycopersici* ZUM2407 (S. de Weert, et al. *unpublished data*) and many other *Fusarium* spp. produce the fungal toxin fusaric acid (Bacon et al. 1996; Notz et al. 2002). Between 0.1 and 1.5 mM fusaric acid was added to MVB1-glucose-cas and, subsequently, generation time and PCN levels were analyzed (Table 5). Generation time appeared to be increased by the addition of a fusaric acid concentration of 1.5 mM or higher. The production of PCN started to decrease at a concentration of 0.3

mM fusaric acid. Comparing the influence of fusaric acid in terms of percentage on generation time and PCN production showed that the effect on PCN levels is by far the largest. Addition of 1.5 mM fusaric acid resulted in a 25% increase in generation time and a 97% decrease in PCN production.

### Combinations of environmental factors affected the production of PCN.

Addition of 1 mM phenylalanine (Table 3) and low oxygen (Fig. 3B) stimulated PCN production, whereas 0.1 M NaCl

(Table 4), iron starvation (Fig. 4A), and growth at 16°C (Fig. 3A) reduced PCN production. To investigate possible synergistic effects of these five factors, they were combined pairwise and PCN production was analyzed. Phenylalanine strongly increased PCN production at 16°C, with 0.1 mM NaCl, and during iron starvation. Strikingly, low oxygen decreased the effects of phenylalanine, salt stress, and iron starvation. Combining low oxygen with 1 mM phenylalanine did not increase PCN production. Combining 0.1 M NaCl or iron starvation with 1% oxygen resulted in a relatively small reduction of



**Fig. 4.** Influence of ions on the level of phenazine-1-carboxamide (PCN). *Pseudomonas chlororaphis* strain PCL1391 was grown at various **A**, FeCl<sub>3</sub> concentrations, **B**, PO<sub>4</sub><sup>3-</sup> concentrations, **C**, MgCl<sub>2</sub> concentrations, and **D**, K<sub>2</sub>SO<sub>4</sub> concentrations. Cells were grown in **A**, **C**, and **D**, modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose (MVB1-glucose-cas) or **B**, MVB2-glucose-cas. Experiments were performed at least three times with similar results. Data of one of the experiments is shown.

**Table 4.** Influence of salts, osmolality, and osmoprotectants on phenazine-1-carboxamide (PCN) production of *Pseudomonas chlororaphis* PCL1391<sup>2</sup>

Variables	PCN production (µM/OD <sub>620</sub> )	
	MVB1-glucose-cas	MVB1-glucose-cas + NaCl (0.1 M)
Control	7.30 ± 0.80 a	0.06 ± 0.05 b
NaCl (0.1 M)	0.06 ± 0.05 b	...
Xylose (0.2 M)	7.10 ± 0.07 a	...
KCl (0.1 M)	0.09 ± 0.03 b	...
Na <sub>2</sub> SO <sub>4</sub> (0.5 M)	1.10 ± 0.07 b	...
Betaine (1 mM)	8.40 ± 2.5 a	1.10 ± 0.55 b
Choline (1 mM)	9.90 ± 2.2 a	1.50 ± 0.88 b
L-proline (1 mM)	5.80 ± 1.2 a	0.09 ± 0.02 b
Trehalose (1 mM)	4.30 ± 1.5 a	0.08 ± 0.03 b

<sup>2</sup> Optical density at a wavelength of 620 nm = OD<sub>620</sub>; modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose = MVB1-glucose-cas. PCN concentration of the supernatant was quantified by HPLC when cells reached early stationary phase. Average and standard deviations of three experiments are shown. Values followed by different letters are significantly different at P=0.05 according to Tukey's honestly significant difference test.

PCN compared with normal oxygen levels. Combining two of the three PCN production-suppressing conditions, 0.1 M NaCl, iron starvation, and growth at 16°C, reduced PCN production stronger (Table 6).

#### Autoinducer levels were affected by environmental conditions.

The levels of the major autoinducer C<sub>6</sub>-HSL of PCL1391 (Chin-A-Woeng et al. 2001b) were determined under selected conditions that promote or repress the production of PCN. Autoinducers were isolated at an OD<sub>620</sub> value of 3.0 and analyzed with a *Chromobacterium violaceum* thin-layer chromatography (TLC) overlay assay (McClellan et al. 1997) and a bioluminescence induction assay based on the autoinducer reporter plasmid pSB401 (Winson et al. 1998), allowing both qualitative and quantitative analyses. Using the *C. violaceum* TLC overlay assay, C<sub>6</sub>-HSL appeared to be the only detectable autoinducer (Fig. 5A). Quantitative analyses of the isolated autoinducer showed that PCL1391 produced less C<sub>6</sub>-HSL when grown on MVB2 at pH 6, MVB1-glucose-cas with 0.1 M NaCl, and MVB1-glucose-cas without iron (III) compared with growth on the control MVB1-glucose-cas (Fig. 5). Furthermore, PCL1391 produced higher amounts of autoinducers when grown on MVB1-glucose with 1 mM phenylalanine, compared with growth on MVB1-glucose-cas (Fig. 5). The stimulating effect of phenylalanine was further analyzed and autoinducers also were isolated at an OD<sub>620</sub> value of 1.0 and 2.0 from cultures with and without 1 mM phenylalanine. At an OD<sub>620</sub> value of 1.0, C<sub>6</sub>-HSL already was detected with 1 mM phenylalanine and not in the control, MVB1-glucose-cas. At an OD<sub>620</sub> value of 2.0, higher amounts of C<sub>6</sub>-HSL were detected in the presence of 1 mM phenylalanine (data not shown). These

**Table 5.** Influence of fusaric acid on generation time and phenazine-1-carboxamide (PCN) production of *Pseudomonas chlororaphis* strain PCL1391<sup>z</sup>

Fusaric acid (mM)	Generation time (min)	PCN (μM/OD <sub>620</sub> )
0	85.3 ± 5.6 a	7.0 ± 1.9 a
0.1	91.1 ± 7.6 a	5.1 ± 0.82 a
0.3	93.4 ± 6.3 a	1.8 ± 0.6 b
0.5	89.0 ± 6.6 a	1.6 ± 0.51 b
0.75	97.8 ± 4.1 a	1.4 ± 0.64 b
1	99.1 ± 3.7 a	0.72 ± 0.21 b
1.5	106.4 ± 15.1 b	0.28 ± 0.054 b

<sup>z</sup> Optical density at a wavelength of 620 nm = OD<sub>620</sub>. Cells were grown in modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose (MVB1-glucose-cas) with different concentrations of fusaric acid. OD was measured at stationary phase between OD values 3.09 and 3.83, and PCN concentration of the supernatant fluid was quantified using high-performance liquid chromatography when cells had reached stationary phase. Average and standard errors of four experiments are shown. Values followed by different letters are significantly different from 0 mM fusaric acid at *P* = 0.05 according to Tukey's honestly significant difference test.

**Table 6.** Cumulative effect of environmental conditions on phenazine-1-carboxamide (PCN) production of *Pseudomonas chlororaphis* strain PCL1391<sup>z</sup>

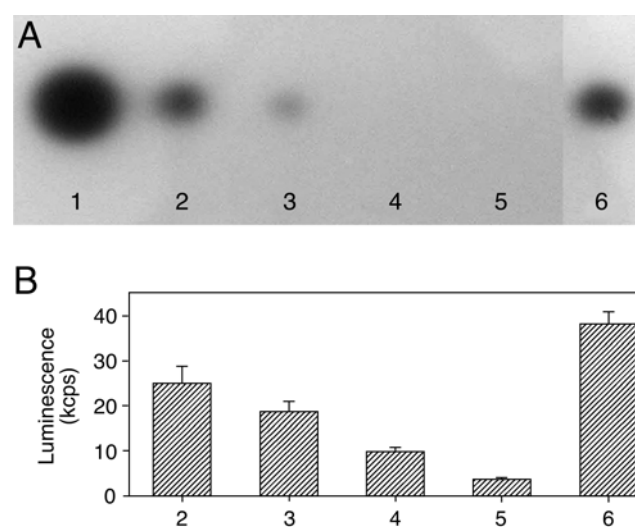
Conditions	PCN production (μM/OD <sub>620</sub> )			
	MVB1-glucose-cas	Phenylalanine (1 mM)	NaCl (0.1 M)	Fe(III) (0 M)
Temperature, 28°C	7.30 ± 0.8 a	28.7 ± 1.6 a	0.06 ± 0.05 a	0.27 ± 0.1 a
Temperature, 16°C	2.60 ± 1.1 b	9.9 ± 1.8 bc	0.07 ± 0.02 a	0.20 ± 0.23 a
1% O <sub>2</sub>	9.20 ± 1.8 a	7.7 ± 2.0 bcd	6.10 ± 2.4 b	3.00 ± 2.2 ac
Phenylalanine (1 mM)	28.70 ± 1.6 d	...	3.10 ± 0.8 c	6.30 ± 0.1 bc
NaCl (0.1 mM)	0.06 ± 0.05 b	3.1 ± 0.8 cd	...	0.25 ± 0.05 a
Fe (III) (0 μM)	0.27 ± 0.1 b	6.3 ± 0.1 bcd	0.25 ± 0.05 a	...

<sup>z</sup> Optical density at a wavelength of 620 nm = OD<sub>620</sub>. Cells grown at 28°C in modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose (MVB1-glucose-cas) served as a control. PCN concentration of the supernatant was quantified by high-performance liquid chromatography when cells reached stationary phase. Average and standard deviations of three experiments are shown. Within a column values followed by different letters are significantly different at *P* = 0.05 according to Tukey's honestly significant difference test.

results demonstrate that the production of C<sub>6</sub>-HSL was advanced and increased by the addition of 1 mM phenylalanine. Autoinducer production also was analyzed in cultures grown at 16°C and in cultures aerated with 1% oxygen (data not shown). Growth at 16°C reduced the production of autoinducers, cultures aerated with 1% oxygen produced higher amounts of autoinducers, and the production started at an OD<sub>620</sub> value of 1.5 instead of 3. Cultures supplemented with 1 mM fusaric acid did not show any autoinducer production when analyzed by the *C. violaceum* TLC overlay assay or by the bioluminescence induction assay (data not shown).

## DISCUSSION

The work presented here is the most extensive study on the effects of environmental factors on PCN production. The choice of the 16 tested carbon sources, with the exception of glycerol, was based on their presence in tomato root exudate (Lugtenberg et al. 1999; Lugtenberg and Bloembergen 2004).



**Fig. 5.** Analyses of N-hexanoyl-L-homoserine lactone (C<sub>6</sub>-HSL) production by *Pseudomonas chlororaphis* strain PCL1391. **A**, Lane 1, synthetic C<sub>6</sub>-HSL standard (2.5 × 10<sup>-7</sup> mol); lane 2, standard modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose (MVB1-glucose-cas) (pH 7); lane 3, MVB1-glucose-cas lacking Fe<sup>3+</sup>; lane 4, MVB1-glucose-cas supplemented with 0.1 M NaCl; lane 5, MVB2-glucose-cas, pH 6; lane 6, MVB1-glucose-cas supplemented with 1 mM phenylalanine. Samples were separated on a C18-reverse phase thin-layer chromatography plate and visualized by *Chromobacterium violaceum* strain CV026. **B**, C<sub>6</sub>-HSL concentrations compared by bioluminescence induction assay based on *Escherichia coli* strain JM109 containing plasmid pSB401. Lane 1: figure was of scale and is not shown, values were 230 ± 16 (kcps). Experiments A and B were performed with the same extracts, isolated at an optical density at a wavelength of 620 nm value of 3.0. The experiment was performed twice with similar results.

From these carbon sources glucose, L-pyroglyutamic acid, and glycerol yielded the highest PCN levels in MVB1-cas. The six most common carbon sources found in tomato root exudate (citric acid, malic acid, lactic acid, succinic acid, oxalic acid, and pyruvic acid) resulted in limited PCN levels compared with glucose and pyroglyutamic acid (Table 2). The later two compounds are less abundant in the tomato rhizosphere. Therefore, it must be concluded that the tomato root exudate composition is far from optimal for PCN production. Monitoring PCN production as a function of growth (Fig. 1) showed that it accumulated during the transition of the exponential to the stationary phase. Comparison of PCN production and generation time for the various carbon sources did not show a relationship between these two parameters (Table 2). This demonstrates that the growth rate does not regulate PCN production, at least not in a direct way. Ammonium ions and amino acids (Fig. 2) have a strong effect on PCN production. The fact that PCN contains nitrogen is a logical explanation for the stimulatory effect of N-containing compounds on PCN production. The strong stimulatory effect of aromatic amino acids (Table 3) probably is not due to their precursor role because available evidence suggests that glutamine is the source of the nitrogen in PCN (Pierson and Thomashow 1992). Surprisingly, glutamine had only a moderate stimulatory effect on PCN production (Table 3).

An acidic pH reduces PCN production by PCL1391 (Fig. 3C). A slightly alkaline pH, starting at pH 8 and ending at pH 7.1, does not effect PCN production; higher initial pH values were not tested due to the limiting range of the phosphate buffer. However, growth on the organic acids caused higher pH values and could explain the poor PCN production for these carbon sources. Salt stress in the form of 0.1 M NaCl, 0.1 M KCl, or 0.5 M Na<sub>2</sub>SO<sub>4</sub> reduced the production of PCN by PCL1391 (Table 4). In contrast, high osmolality caused by the sugar xylose (0.2 M) did not inhibit PCN production. Xylose was chosen above sucrose to increase the osmolality of the medium, because xylose cannot be metabolized by PCL1391, in contrast to sucrose. Osmoprotectants, exogenously provided organic solutes that enhance bacterial growth in media of high osmolality (Kempf and Bremer 1998), such as choline, trehalose, L-proline, and betaine, were added in the presence or absence of 0.1 M NaCl. None of these compounds compensated for the inhibitory effect of NaCl to a major extent, demonstrating that PCN production is negatively affected by salt stress and not by osmotic stress. This probably implies that a mechanism for salt concentration sensing is integrated into the regulation of PCN production. Other environmental factors which strongly influence PCN production in PCL1391 appeared to be temperature (Fig. 3A), O<sub>2</sub> concentrations (Fig. 3B), Fe<sup>3+</sup> (Fig. 4A), PO<sub>4</sub><sup>3-</sup> (Fig. 4B), Mg<sup>2+</sup> (Fig. 4C), and SO<sub>4</sub><sup>2-</sup> (Fig. 4D).

Transcription of the *phz* operon is regulated via quorum sensing and PCN synthesis is dependant on C<sub>6</sub>-HSL (Chin-A-Woeng et al. 2001b). From all conditions analyzed for C<sub>6</sub>-HSL production, PCN levels correlated with those of C<sub>6</sub>-HSL levels (Fig. 5; discussed above). Growth at low oxygen and the addition of 1 mM phenylalanine resulted in elevated levels of C<sub>6</sub>-HSL and PCN at lower ODs.

In the hope of finding synergistic effects of environmental factors which strongly influence PCN production, we combined pairs of these factors. Unfortunately, none of the combinations yielded more PCN than the standard medium supplemented with phenylalanine (Table 6). However, combining conditions which affect PCN levels demonstrated that low oxygen reduced the stimulatory effect of phenylalanine as well as the inhibitory effects of salt stress and iron starvation on PCN production. This shows that low oxygen can reduce the regulatory effects of other environmental factors (Table 6).

Phenazine production may not be regulated in the same way in various *Pseudomonas* spp. Although the results obtained for other strains are difficult to compare with ours because of different culture conditions which, as is clear from our results, can have an enormous impact on phenazine production, there are some striking similarities and differences between our results and some literature data. Similar to our strain, the production of phenazine-1-carboxylic acid (PCA) by *P. fluorescens* 2-79 (Slininger and Shea-Wilbur 1995) and PCN production by *P. aeruginosa* (Kanner et al. 1978) were found to be stimulated by glucose and glycerol. This suggests a similar response of phenazine-producing pseudomonads to these carbon sources. As in PCL1391 (Fig. 2A), phenazine production in *P. aeruginosa* also is regulated by nitrogen sources in that NH<sub>4</sub><sup>+</sup> supported a higher production of PCN than urea, asparagine, or peptone (Kanner et al. 1978). PCA production by *P. fluorescens* 2-79 was not found to be affected by different nitrogen sources (Slininger and Shea-Wilbur 1995). As in strain PCL1391 (Table 3), aromatic amino acids stimulated phenazine production in *P. aureofaciens* 517 (Labeyrie and Neuzil 1981); in *P. aeruginosa* A237, only tyrosine and phenylalanine were reported to stimulate PCA production (Korth 1973). The stimulatory effect of nonaromatic amino acids on PCN levels (Table 3) has not been reported previously. The pH optimum for PCN production in PCL1391 (Fig. 3) differed from that in *P. fluorescens* 2-79, in which the optimum is pH 7, with only a small reduction at pH 6, and a severe reduction at pH 8 (Slininger and Shea-Wilbur 1995). Similar to PCL1391 (Figs. 4A and 4D), Fe<sup>3+</sup> had a positive effect on the production of PCA in *P. fluorescens* 2-79, whereas magnesium ions positively affected PCA production in *P. fluorescens* 2-79 (Slininger and Jackson 1992). The production of pyocyanin, a phenazine derivative produced by *P. aeruginosa*, was stimulated by low phosphate concentrations (Turner and Messenger 1986), which is different from PCN production in PCL1391 (Fig. 4B). From these comparisons between PCN production in PCL1391 and other AFM-producing *Pseudomonas* strains, we can conclude that some environmental factors have similar effects whereas others have opposite effects on different strains. Therefore, it would be advisable to study such effects in detail before applying a biocontrol strain. The fungal toxin fusaric acid affects growth and PCN production of *P. chlororaphis* PCL1391 (Table 5). In *P. fluorescens* CHA0, fusaric acid repressed the expression of another AFM, 2,4-diacetylphloroglucinol (Notz et al. 2002). These data indicate that fusaric acid reduces the production of the two most important groups of AFMs produced by *Pseudomonas* biocontrol strains.

All these factors affecting PCN production also are likely to affect biocontrol and can explain, at least partially, the inconsistency of biocontrol in field experiments. Decreased oxygen concentrations were reported in the rhizosphere and are dependant on water content and compaction of the soil (Højberg et al. 1999). Soil pH varies greatly between different soil types (Ownley et al. 2003) and biocontrol by *P. fluorescens* 2-79 against take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* increases with an increasing pH (Ownley et al. 1992, 2003). This demonstrates that the pH of the rhizosphere is an important factor for successful biocontrol. The concentration of Fe<sup>3+</sup> is low in neutral and alkaline soil due to its insolubility. Therefore, pseudomonads use siderophores for the acquisition of Fe<sup>3+</sup> and the competition for these ions is an established mechanism in biocontrol of soilborne plant pathogens (Duijff et al. 1993; Koster et al. 1994; Loper and Buyer 1991; Moënné-Loccoz et al. 1996). Free amino acids in the rhizosphere can be derived from root exudates (Simons et al. 1997; Vancura 1964) but also can be the result

of protein degradation by proteolytic enzymes produced by microbes in the rhizosphere such as *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 2001b). Temperature and salts stress are important factors under practical plant production conditions and influence PCN production severely (Fig. 3A and Table 4). For successful biocontrol by pseudomonads, one needs to understand which and how environmental factors affect the production of AFMs in potential biocontrol products. Environmental regulation seems to differ between strains; therefore, different strains may have to be selected for different field conditions. Selection of strains that match with certain field conditions could be a strong tool in achieving successful biocontrol. Alternatively, when legislation allows it, PCN production may be stabilized by genetic engineering. Although many environmental factors have an effect on PCN production by PCL1391, only a fraction of the molecular cascade that perceives these stimuli and determines PCN production is known (Chin-A-Woeng et al. 2001a). Further unraveling of the molecular network that integrates PCN production is needed for successful biocontrol and will be a challenging field for future research.

## MATERIALS AND METHODS

### Microorganisms and culture conditions.

The microbial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium (Sambrook et al. 1989) was used as the standard medium for culturing *Escherichia coli* and *C. violaceum*. Modified Vogel Bonner medium (Vogel and Bonner 1956) was used to culture *Pseudomonas* cells. Modified Vogel-Bonner salts #1 (MVB1) contained final concentrations of 57 mM  $K_2HPO_4$ , 16 mM  $Na(NH_4)HPO_4 \cdot 4H_2O$ , 81 mM  $MgSO_4 \cdot 7H_2O$ , 78  $\mu M$   $NaFeEDTA(III)$ , 6.8  $\mu M$   $MnSO_4 \cdot H_2O$ , 0.35  $\mu M$   $CuSO_4 \cdot 5H_2O$ , 4.1  $\mu M$   $Na_2MoO_4 \cdot 2H_2O$ , 0.85  $\mu M$   $ZnSO_4 \cdot 7H_2O$ , and 51  $\mu M$   $H_3BO_3$ , and the pH was adjusted to 6.6 using HCl. MVB1 supplemented with 30 mM glucose, (MVB1-glucose), 0.05% casamino acids (MVB1-cas), or both (MVB1-glucose-cas) was used for monitoring growth and PCN production. To test the influence of different carbon sources on the production of PCN, each carbon source was added to MVB1 in concentrations corresponding with 180 mM carbon atoms and used in the following concentrations: T-aconitic acid (30 mM), citric acid (30 mM), fumaric acid (45 mM), lactic acid (60 mM), 2-ketoglutaric acid (36 mM), malic acid (45 mM), oxalic acid (90 mM), propionic acid (60 mM), L-pyroglytamic acid (36 mM), pyruvic acid (60 mM), ribose (36 mM), succinic (45 mM), fructose (30 mM), glucose (30 mM), glycerol (60 mM), maltose (15 mM), sucrose (15 mM), and xylose (36 mM). The effect of magnesium, sulfate, and iron ions on the production of PCN was analyzed by using concentrations of 0.078, 0.01, or 0.005 mM  $NaFeEDTA(III)$  or  $FeCl_3$  and 0.08, 0.8, or 8 mM  $MgSO_4$ ,  $MgCl_2$ , or  $K_2SO_4$ . Osmolality of MVB1-glucose-cas was altered by adding 0.05 or 0.1 M NaCl, 0.2 M xylose, 0.1 M KCl, or 0.5 M  $Na_2SO_4$ . The osmoprotectants betaine, choline, L-proline, and trehalose were added at a final concentration of 1 mM to MVB1-glucose-cas in the absence or presence of 0.1 M NaCl. The amino acids phenylalanine, tryptophan, tyrosine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, leucine, asparagine, glutamine, and threonine were added to MVB1-glucose at a concentration of 1 mM. Fusaric acid (Acros, Geel, Belgium) was added to MVB1-glucose-cas in a final concentration of 0.1, 0.3, 0.5, 0.75, 1.0, and 1.5 mM.

MVB2-glucose-cas was used for testing the influence of pH, nitrogen source, and  $PO_4^{3-}$  concentration on PCN production. The nitrogen and phosphate sources used in MVB1 were changed in MVB2 from 57 mM  $K_2HPO_4$  and 16 mM

$Na(NH_4)HPO_4 \cdot 4H_2O$  into 45 mM  $K_2HPO_4$ , 28 mM  $KH_2PO_4$ , and 8 mM  $(NH_4)_2SO_4$ . Standard MVB2-glucose-cas was set at pH 7, whereas pH values of 6 and 8 were set by altering the ratio of  $K_2HPO_4$  to  $KH_2PO_4$ . The pH of all cultures was measured at the stationary phase. The pH of the cultures that were set to pH 6, 7, and 8 were measured throughout growth. The nitrogen sources  $(NH_4)_2SO_4$ ,  $NaNO_3$ , urea,  $NH_4Cl$ , and casamino acids were normalized to 16 mM of nitrogen atoms. Normalization of nitrogen atoms in casamino acids was done according to Slininger and Shea-Wilbur (1995): 0.3% corresponds with 16 mM. Phosphate concentrations were altered by changing the total concentration of phosphate buffer. Solidified growth media contained 1.8% agar (Difco Laboratories, Detroit). If appropriate, media were supplemented with the antibiotics kanamycin, tetracycline, or carbenicillin in final concentrations of 50, 80, and 50  $\mu g/ml$ , respectively. All cultures were shaken at 195 rpm on a Janke und Kunkel shaker KS501D (Staufen, Germany) at 28°C. ODs of cultures were measured at a wavelength of 620 nm. Growth analyses were made in a 100-ml flask with 10 ml of media after inoculation with an appropriate 5-ml overnight culture to an  $OD_{620}$  of 0.1.

Growth under various oxygen concentrations was performed as described previously (Camacho Carvajal et al. 2002). Briefly, cells were cultured in 40 ml of MVB1-glucose-cas amended with 0.005% silicon antifoam agent (BDH Limited, Poole, UK). Gas mixtures of oxygen and nitrogen were pumped through a sterilized glass filter immersed in the culture with a gas flow rate of 0.25 liters/min, controlled by a gas mixer (model 5878; Brooks Instruments B. V, Veenendaal, The Netherlands). Culture samples were taken after punching a sterile injection needle through a silicon tube.

### PCN extraction and analyses.

For monitoring PCN production during growth, PCN was extracted according to Chin-A-Woeng and associates (1998) with minor modifications. Culture samples (250  $\mu l$ ) were taken at various time intervals and centrifuged, and the culture supernatants were acidified to pH 2 using 6 M HCl. They were extracted subsequently with an equal volume of toluene by shaking on an Eppendorf mixer 5432 for 5 min. After centrifugation, the toluene phase was taken and dried in a rotary evaporator. The dry residue was dissolved in 100  $\mu l$  of acetonitrile and the obtained solution was mixed with 400  $\mu l$  of water. PCN concentrations were determined by high-performance liquid chromatography (HPLC) (chromeleon software version 6.20; DIONEX, Sunnyvale, CA) and the peak areas were calculated with a calibration curve. HPLC was performed using an econosphere C18 5u 259-by-4.6-mm column (Alltech Associates, Inc, Deerfield, IL) at 30°C with a linear gradient of 20 to 80% acetonitrile acidified with 0.1% trifluoroacetic acid in water and a flow rate of 1 ml/min.

### AHL extraction and analyses.

Culture supernatants were mixed with 0.7 volume of dichloromethane and shaken for 1 h, after which the solvent was collected. Each supernatant was extracted twice, extracts were pooled, and the solvent was removed by rotary evaporation (McClellan et al. 1997). The dried residue was dissolved in acetonitrile and analyzed using TLC or a bioluminescence induction assay. Samples spotted on C18 TLC plates (Merck, Darmstadt, Germany) were developed in methanol-water (60:40, vol/vol). After development, the TLC was overlaid with LB 0.8% agar containing a 10-fold diluted overnight culture of the *C. violaceum* indicator strain CV026 (Milton et al. 1997) and kanamycin (50  $\mu g/ml$ ). After incubation for 48 h at 28°C, chromatograms were analyzed for appearance of violet



spots. For quantification of the AHL concentration, the bioluminescence induction assay (Winson et al. 1998) was used with some modifications. AHL samples dissolved in acetonitrile were taken up in 100 µl of water and loaded in triplicate on an Optiplat-96 (Packard, Meriden, CT). An overnight culture of *E. coli* JM109 containing pSB401 was diluted 10-fold in fresh LB and 100 µl was added to each sample. After incubation for 4 h at 28°C, bioluminescence was determined using the luminescence counter MicroBeta 1450 TriLux, (Wallac, Turku, Finland).

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