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Environmental and molecular regulation of phenazine-1-carboxamide biosynthesis in *Pseudomonas chlororaphis* strain PCL1391

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Environmental and molecular regulation of phenazine-1-carboxamide
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Evert Tjeerd van Rij

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Environmental and molecular regulation of phenazine-1-carboxamide
biosynthesis in *Pseudomonas chlororaphis* strain PCL1391

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“Environmental and molecular regulation of phenazine-1-carboxamide biosynthesis in *Pseudomonas chlororaphis* strain PCL1391” by Tjeerd van Rij

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List of abbreviations

AFM	Anti fungal metabolites
C ₆ -HSL	<i>N</i> -hexanoyl-L-homoserine lactone
DAPG	2,4-diacetylphloroglucinol
FA	Fusaric acid
HCN	Hydrogen cyanide
ISR	Induced systemic resistance
MVB1	Modified Vogel-Bonner medium 1
OD _{620nm}	Optical density at 620 nanometer
ORF	Open reading frame
PCN	phenazine-1-carboxamide
SAR	Systemic acquired resistance

Chapter 1

Introduction

1. Plant microbe interactions

Soil contains a wide diversity of microbes. The most commonly known soil-borne microbes include fungi, bacteria, and viruses. Microbes are concentrated in nutrient rich soil regions including the topsoil layer and the region around the plant root. Roots secrete nutrients that stimulate microbial life and allow fast spreading of microbes through soils (Walker et al., 2003). In 1904 Lorenz Hiltner introduced the term “rhizosphere” for the region of soil that is influenced by plant roots. Soil microbes can either be neutral or determine plant health positively or negatively. Beneficial microbes can promote plant health and growth, cause disease resistance or enhance nutrient availability and uptake.

1.1 Harmful plant-microbe interactions

Plant diseases are caused by microbes able to infect the plant, to cause disease symptoms and to multiply. Microbial plant pathogens include bacteria, viruses, parasitic nematodes, Oomycetes and fungi. Pathogenic bacteria are *Erwinia carotovora* which causes soft rot (Toth and Birch, 2005), *Pseudomonas aeruginosa* which is (opportunistic) pathogenic for animals and plants (Prithiviraj et al., 2005; Rahme et al., 1995) and *Pseudomonas syringae* which causes a wide variety of disease symptoms on plants (Nomura et al., 2005). Well known examples of plant viruses include the tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), and potato virus x (PVX) (Nelson and Citovsky, 2005; Soosaar et al., 2005). Phytoparasitic nematodes feed on plant roots by injecting their stylet (hollow oral spear) into the plant cell and withdrawing cell nutrients (Davis et al., 2004). The most famous and destructive example of a plant pathogenic disease is the Great Irish Famine in 1845-1847. The pathogenic oomycete *Phytophthora infestans* destroyed the potato harvest and caused the hunger death of one million people. The oomycete *Pythium* mainly causes damping-off diseases.

Fungal pathogens cause diseases such as rusts, smuts (black dusty spots), and powdery mildews. Examples of important pathogenic fungi are *Rhizoctonia*, *Gaeumannomyces graminis*, *Alternaria* spp., *Botrytis cinerea*, *Verticillium* spp. and *Fusarium* spp. *Fusarium* causes an extraordinary broad range of plant diseases on economically important host plants. The most important diseases caused by *Fusarium* are crown and root rots, stalk rots, head and grain blights, and vascular wilts (Summerrell et al., 2003). For example, *Fusarium oxysporum* f. sp. *radicis-licopersici* cause's tomato foot and root rot which is a problem in the commercial production of tomatoes.

Fusarium produces many mycotoxins which include; trichothecens, moniliformin, zearalenone, fumonisins and fusaric acid (Conkova et al., 2003). Food contamination with these toxins can be very harmful to human or animal consumers and leads to impaired

immune function, disorders of metabolism, decreased performance, increased disease susceptibility, food refusal, vomiting, and reproduction disorders (Conkova et al., 2003). Production of mycotoxins must be viewed in the ecological perspective and are thought to play a role in habitat protection during saprophytic (feeding on death organic matter) growth (Duffy et al., 2003).

The mycotoxin fusaric acid (Fig. 1) is produced by a wide variety of *Fusarium* species and affects a diversity of organisms including prokaryotes, plants, arthropods and vertebrates. In mammals it affects different organ systems including the nervous, cardiovascular and immune system and is toxic to some mammalian tumour cell lines (Wang and Ng, 1999). In combination with other *Fusarium* toxins, fusaric acid causes synergistic effects (Bacon et al., 1996). High concentrations of fusaric acid inhibit bacterial growth and smaller concentrations repress the production of antifungal metabolites produced by some plant-beneficial soil bacteria (Duffy and Défago, 1997) (Chapter 3).

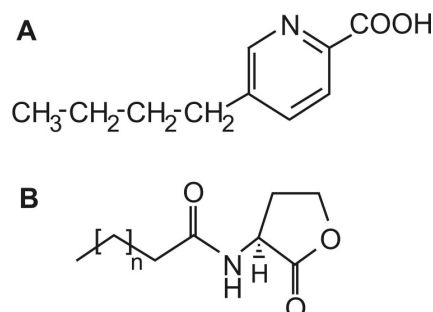


Fig. 1. Chemical structure of the fungal toxin fusaric acid (A) and *N*-acyl-L-homoserine lactones (B). The acyl chain length (n) varies from C4 to C14.

1.2 Plant beneficial interactions

Plants can benefit from soil microbes in many ways. Certain microbes stimulate plant growth, fertilize soils, degrade pollutants, or protect plants against pathogens. All these beneficial characteristics of microbes on plants will be discussed in the following paragraphs.

1.2.1 Phytostimulation

Plant growth can be stimulated by plant hormones that are produced by some soil bacteria. Bacteria of the genus *Azospirillum* are known to stimulate plant growth and increase crop yields. Inoculation of *Azospirillum* alters root morphology by increasing the number of lateral roots and root hairs which is thought to be the result of plant growth promoting hormones (auxins, cytokinins and gibberellins) which can be detected in culture supernatants (Steenhoudt and Vanderleyden, 2000). The most important plant hormone produced by

Azospirillum is auxin. Auxin controls cell elongation and division, tissue differentiation and responses to light and gravity. Many plant growth promoting bacteria including *Pseudomonas* species produce auxin (Costacurta and Vanderleyden, 1995; Lugtenberg et al., 2002; Patten and Glick, 2002).

Plant growth can also be stimulated by bacteria that degrade the plant growth inhibitor ethylene. The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, produced by a number of plant growth promoting rhizobacteria, hydrolyzes the ethylene precursor ACC. Bacteria producing ACC-deaminase were shown to stimulate elongation of plant roots (Glick et al., 1998).

1.2.2 Biofertilization

Plants can form symbiotic interactions with microbes that help them in their nutrient supply. For the supply of nitrogen some plants use microbes that bind atmospheric nitrogen and thereby fertilize soils. Biofertilization accounts for 65% of the nitrogen supply to crops world wide. The nitrogen-fixing *Rhizobiaceae* in combination with legume plants are most often used as green nitrogen fertilizers. They form a host-specific symbiosis with legume plants which results in the formation of nodules that contain bacteroids. The bacteroids containing nodules able to fix atmospheric nitrogen under the physiological conditions present in these nodules (Long, 2001; Spaink, 2000).

A second group of microbes that provides plants with nutrients consists of the *Mycorrhizae* fungi which form an internal symbiosis with roots of most flowering plants and provide the plants with mineral nutrients, predominantly phosphate in exchange for carbohydrates (Harrison, 1999). Arbuscular mycorrhizal fungi and bacteria can interact synergistically to stimulate plant growth by improved nutrient acquisition and inhibition of fungal plant pathogens (Artursson et al., 2006).

1.2.3 Rhizoremediation

Bioremediation makes use of microbes or other biological systems to degrade environmental pollutants such as pesticides, herbicides, and polycyclic aromatic hydrocarbons (Dua et al., 2002). Rhizoremediation describes the degradation of pollutants by microbes in the vicinity of plant roots (rhizosphere) (Kuiper et al., 2004). Rhizoremediation combines the mutual beneficial effects of plants and microbes. Combined selection of good root colonizing and pollutant degrading bacterial strains can result in efficient cleaning of polluted soils (Kuiper et al., 2001a). Rhizobacteria are supported in their growth by the secretion of nutrients by the roots. Efficient root colonization in combination with root growth provides a mechanism to spread the bacterium through the soil and reach inaccessible parts

of contaminated soil. Pollutant degrading bacteria allow plants to grow in soils with phytotoxic pollutant concentrations since the bacteria reduce pollutant concentrations near the plant root (Kuiper et al., 2004).

1.2.4 Biocontrol

Suppression of plant diseases with chemical pesticides is being restricted as a result of increasing public concern caused by fear of danger for human health and the environment. Fungal pathogens can also be suppressed by microbial control agents. These biocontrol strains can be isolated from disease suppressive soils and protect plants against fungal pathogens (Alabouvette, 1986; Haas et al., 2000; Haas and Défago, 2005; Schippers et al., 1987; Schroth and Hancock, 1981). The most common examples of biocontrol strains are found in the genera *Pseudomonas* (Fig. 2), *Bacillus* and *Trichoderma* (Bloemberg and Lugtenberg, 2001; Handelsman and Stabb, 1996; Raaijmakers et al., 2002; Whipps, 2001). The biocontrol mechanisms of these strains include competition for nutrients, induced systemic resistance, parasitism and predation, inhibition of quorum sensing, and the production of anti fungal metabolites. See the following paragraphs for detailed descriptions.



Fig. 2. Biocontrol of the plant pathogen *Fusarium* by a *Pseudomonas* biocontrol strain. Seeds were coated with *Pseudomonas* in pots one and two and the soil was contaminated with *Fusarium* spores in pots two and three. Note the healthy plants in pot two where seeds coated with the biocontrol bacterium were used. (Modified after R. Scheffer, personal communication).

1.2.4.1 Competition for nutrients and niches

Biocontrol can depend on competition for nutrients between the pathogen and the biocontrol strain. Pathogens share with at least some biocontrol strains the same niche on

roots, namely the intracellular junctions between root epidermal cells, a niche which is enriched by nutrients that are thought to leak out between the cells (Fig. 3) (Bloemberg et al., 1997; Bloemberg et al., 2000; Bolwerk et al., 2003; Chin-A-Woeng et al., 1997; Lagopodi et al., 2002). Efficient utilization of these nutrients and occupation of all nutritional sites on the root by the biocontrol strain reduces propagation of the pathogen. Competition for nutrients and niches is illustrated by the biocontrol abilities of the non-pathogenic *Fusarium oxysporum* strain Fo47 against the pathogenic *Fusarium oxysporum* f. sp. *radicis-lycopersici* (F.o.r.l.) (Alabouvette et al., 1993; Alabouvette and Couteaudier, 1992; Bolwerk et al., 2005). Only inoculation with a 10 to 100-fold excess of the non-pathogenic *Fusarium* results in biocontrol and sufficiently reduces the pathogenic *Fusarium* from colonizing the root system (Bolwerk et al., 2005; Lagopodi et al., 2002).

Due to the limitation of soluble iron in the rhizosphere microbes and plants are scavenging for iron with highly sophisticated iron binding and uptake mechanisms which include siderophores (Koster et al., 1995; Schippers, 1993). Siderophores are high affinity iron binding molecules produced by plants, fungi, and bacteria. Mutagenesis suggests that siderophores produced by bacterial biocontrol strains, are involved in biocontrol since siderophore-negative mutants do no longer suppress disease (Audenaert et al., 2002; Handelsman and Stabb, 1996; O'Sullivan and O'Gara, 1992; Raaijmakers et al., 1995; Whipps, 2001).



Fig 3. *Pseudomonas* micro-colony on tomato root epidermis. The micro-colony is aligned along the junction between root epidermal cells. Bar represents 1 μ m (Chin-A-Woeng et al., 1997).

1.2.4.2 Induced systemic resistance (ISR)

Plants protect themselves against pathogens by inducing a resistance response in reaction to attacks by pathogenic microbes and insects. This response is called systemic

acquired resistance (SAR) since the resistance is increased in infected and in non-affected plant parts. Some non-pathogenic rhizobacteria can also induce resistance in plants towards fungi, viruses, and pathogenic bacteria. This mechanism is called induced systemic resistance (ISR) and differs from SAR because different molecular signalling pathways and mechanisms are involved. Root colonization of ISR-inducing rhizobacteria prime plants, which leads to a faster and stronger response to pathogenic attacks. The mechanism of ISR is not yet well understood but detailed molecular studies have started (Verhagen et al., 2004). The bacterial determinants that can induce ISR include lipopolysaccharides, siderophores, and salicylic acid (Haas and Défago, 2005; Harman et al., 2004; van Loon et al., 1998).

1.2.4.3 Predation and Parasitism

Plant pathogenic fungi can be parasitized and degraded by bacteria and other fungi. These biocontrol agents lyse and degrade the fungal cell wall using secreted cellulases, glucanases, chitinases, and proteases (Harman et al., 2004; Whipps, 2001). The fungus *Trichoderma* parasitizes on plant pathogenic fungi such as *Phythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia*, and *Fusarium*. Mycoparasitism by *Trichoderma* involves recognition, attack, penetration and killing of the fungus. Some *Trichoderma* strains coil around the fungus and form an appressorium-like structure to start penetration of the fungal cell wall with the use of cell wall degrading enzymes (Benitez et al., 2004; Harman et al., 2004).

1.2.4.4. Quorum sensing inhibition in plant pathogenic bacteria

Bacteria can sense their population density via a mechanism named quorum sensing. The population density is sensed by the accumulation of small signalling molecules. The quorum sensing signal molecules, *N*-acylhomoserine lactones (AHLs) (Fig. 1) are conserved in many Gram-negative plant pathogenic bacteria (von Bodman et al., 2003). Quorum sensing regulates luminescence, symbiosis, virulence, motility, biofilm formation and secondary metabolite production (Bassler, 1999; Miller and Bassler, 2001; Salmond et al., 1995; Whitehead et al., 2001; Withers et al., 2001). Virulence of pathogenic bacteria is dependant on quorum sensing since this regulates the expression of pathogenicity factors. Recently, AHL degrading enzymes were identified to interfere with the quorum sensing system of pathogenic bacteria (Dong et al., 2004). The plant pathogen *Erwinia carotovora* causes potato soft rot and is dependent on quorum sensing for the spreading on plant tissue. Plant tissue pre-treated with a *Bacillus thuringiensis* strain that produce an AHL degrading enzyme decreases potato soft rot caused by *E. carotovora* (Fig. 4) (Dong et al., 2004). The non effective biocontrol strain *P. fluorescens* strain P3 can repress bacterial pathogens only when it is transformed with a gene encoding an AHL degrading enzyme. The transgenic *P.*

fluorescens P3 reduced potato soft rot caused by *E. carotovora* and crown gall of tomato caused by *Agrobacterium tumefaciens* (Molina et al., 2003). Biocontrol by *P. chlororaphis* strain PCL1391 depends on the production of the antifungal metabolite phenazine-1-carboxamide, which is controlled by AHL dependant quorum sensing. The biocontrol strain *P. chlororaphis* strain PCL1391 failed to repress tomato vascular wilt when co-inoculated with the AHL degrading *P. fluorescens* P3 (Molina et al., 2003). These examples show that AHL degradation can effectively control bacterial pathogens but has undesired negative affects on quorum sensing dependent biocontrol strains that control fungal pathogens.

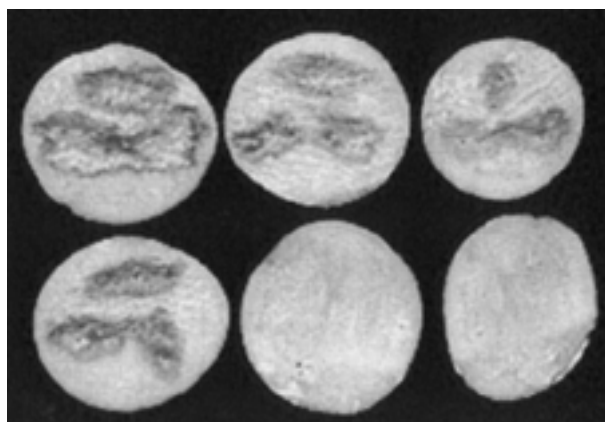


Fig. 4. Soft rot symptoms of *Erwinia carotovora* on potato slices after treatment with the *N*-acyl homoserine lactone degrading *Bacillus thuringiensis*. The cell suspensions of *Erwinia carotovora* at 2×10^8 , 2×10^7 , or 2×10^6 CFU/ml were mixed separately with equal volumes of water (top) or *Bacillus thuringiensis* suspension cultures at 5×10^8 CFU/ml (bottom). The photograph was taken after incubation for 20 h at 28 °C (Dong et al., 2004).

2. Production of anti fungal metabolites by pseudomonads

Production of antifungal metabolites (AFMs) is often found to be essential for the suppression of fungal diseases by biocontrol agents (Haas and Défago, 2005). Antifungal metabolites produced by *Pseudomonas* that are involved in biocontrol are phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides and hydrogen cyanide (Fig. 5) (Haas and Défago, 2005; Price-Whelan et al., 2006; Raaijmakers et al., 2002). The biosynthetic genes of most AFM have been identified. The molecular and environmental regulation of these AFMs is only partially elucidated (see next paragraphs). It must be noted that for efficient delivery of the AFM to the entire root system these biocontrol strains are dependent on efficient root colonization (Lugtenberg and Dekkers, 1999).

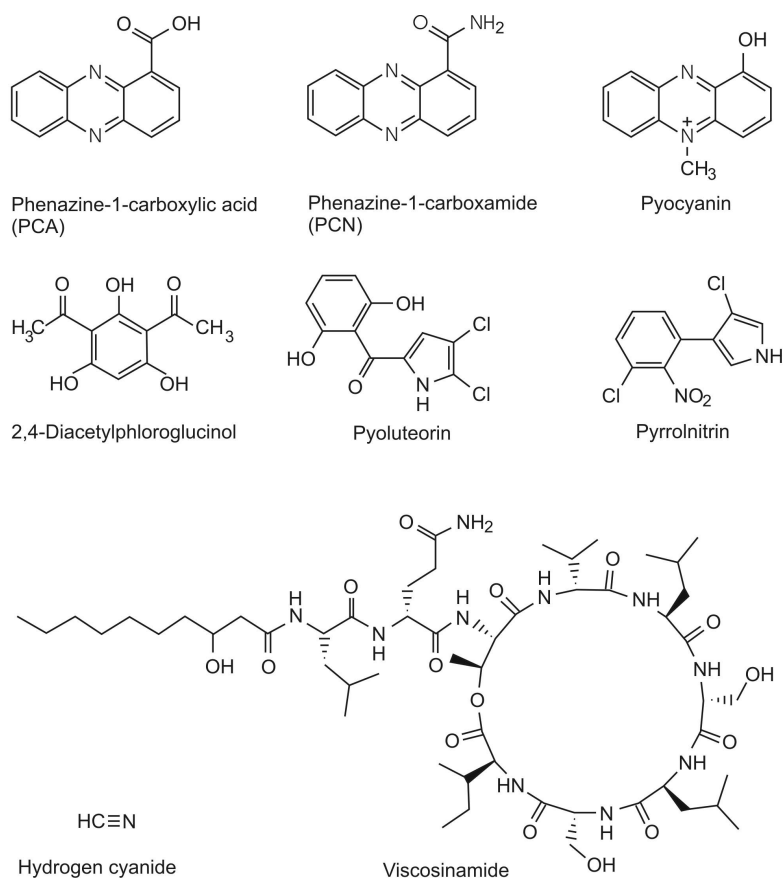


Fig. 5. Antibiotic compounds produced by fluorescent pseudomonads that are relevant for biocontrol modified from (Haas and Défago, 2005).

2.1 Regulation of phenazine production in *Pseudomonas* by environmental factors

Biotic and abiotic environmental conditions in the rhizosphere vary substantially and are dependent on e.g. soil type, season and root exudate composition. Factors that affect the production of anti fungal metabolites (AFM) by biocontrol *Pseudomonads* include minerals, oxygen tension, temperature, pH, osmolality, phosphate, carbon and nitrogen sources, and fungal, bacterial and plant metabolites (Chapter 1) (Campa et al., 1993; Duffy and Défago, 1997; Duffy and Défago, 1999; Haas and Keel, 2003; Messenger and Turner, 1983; Ownley et al., 2003; Raaijmakers et al., 2002; Slininger and Jackson, 1992; Slininger and Shea-Wilbur, 1995; van Rij et al., 2004). Lack of knowledge and variation of these conditions is thought to cause inconsistent biocontrol in the field (Handelsman and Stabb, 1996; Thomashow and Weller, 1988). Chemical analyses of total root exudates and bacterial biosensor strains have identified environmental conditions as they occur in the rhizosphere and indicated that conditions are often nitrogen limited, oxygen limited and rich in organic

acids and sugars (Haas and Keel, 2003; Højberg et al., 1999; Lugtenberg and Bloemberg, 2004; Lugtenberg and Dekkers, 1999; Simons et al., 1997; Vancura, 1964). Identifying conditions that favour the production of AFM could help to improve biocontrol. Production of the AFM phenazine in *P. chlororaphis* strain PCL1391 (Chapter 2), (van Rij et al., 2004), *P. fluorescens* strain 20-79 (Slininger and Shea-Wilbur, 1995), *P. aeruginosa* strain PNA1 (Anjaiah et al., 2006) and *P. aureofaciens* (Korth, 1973) is favoured by growth in rich media and by the presence of aromatic amino acids, glycerol and glucose (Kanner et al., 1978). Ferric iron has a stimulating effect on phenazine production by *P. chlororaphis* strain PCL1391 (Chapter 2; (van Rij et al., 2004) and *P. fluorescens* strain 2-79 (Slininger and Jackson, 1992). Fungal metabolites can suppress the production of AFM (Duffy et al., 2003). Fusaric acid, a fungal metabolite produced by *Fusarium* (Bacon et al., 1996; Schouten et al., 2004), represses the production of 2,4-diacetylphloroglucinol in *P. fluorescens* strain CHA0 (Duffy and Défago, 1997) and of PCN in *P. chlororaphis* strain PCL1391 (Chapter 3) (van Rij et al., 2005). Soils with high zinc concentrations repress the production of fusaric acid by *Fusarium oxysporum* f. sp. *radicis-lycopersici* and improve biocontrol (Duffy and Défago, 1997). Environmental conditions clearly determine the level of phenazine production.

2.2. Genetic regulation of phenazines in pseudomonads

2.2.1 Global regulators *gacS* and *gacA*

The two component regulatory system *gacS/gacA* (global antibiotic and cyanide Sensor/Activator) is highly conserved in pseudomonads and regulates many traits including virulence, survival, motility, biofilm formation, phase variation and the production of extra cellular products (Chancey et al., 2002; Chatterjee et al., 2003; Chin-A-Woeng et al., 2005; de Souza et al., 2003; Heeb and Haas, 2001; Parkins et al., 2001; van den Broek et al., 2005). In *Pseudomonas* biocontrol strains, GacS and GacA are essential for the production of at least some secondary metabolites and extra-cellular enzymes with anti-microbial activity including phenazines, proteases, HCN, diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, chitinase, phospholipase C, all of which can be involved in biocontrol (Chancey et al., 1999; Chin-A-Woeng et al., 2005; Heeb and Haas, 2001; Zuber et al., 2003). GacS is a histidine kinase sensor located in the cytoplasmic membrane. GacA is a transcriptional regulator and can be activated through phosphorylation by GacS (Fig. 6). Between the global regulators *gacS/gacA* and production of secondary metabolites several molecular intermediates are known which include small non coding regulatory RNAs, *psrA*, *rpoS*, and the quorum sensing regulators *phzI/phzR* (for more details see next paragraphs).

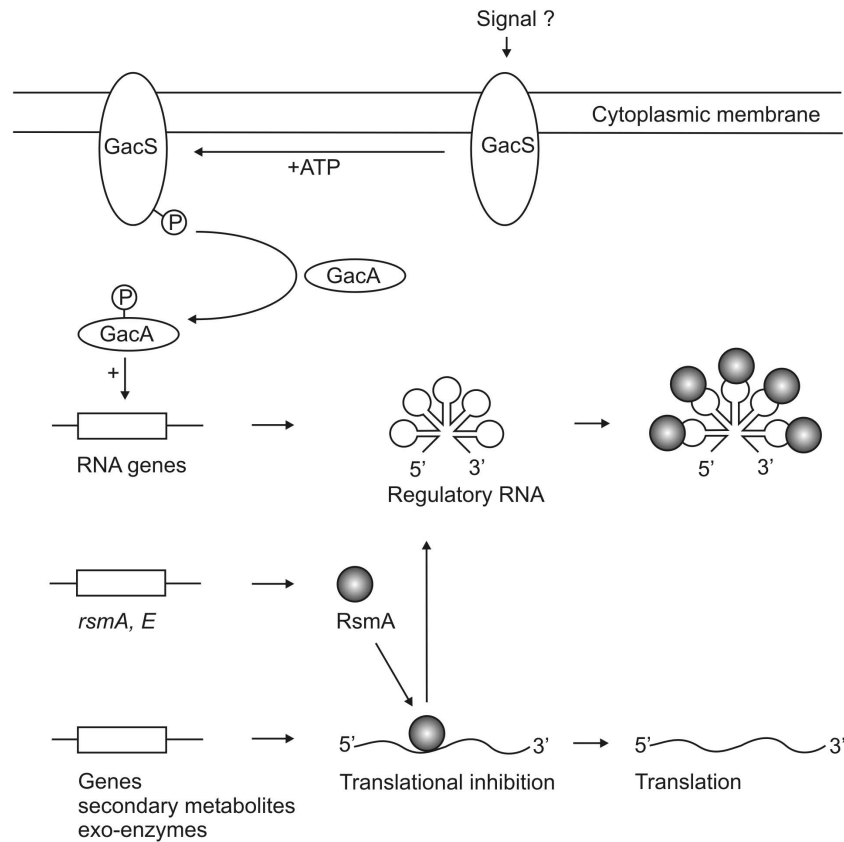


Fig. 6. Signal-transduction pathway model of GacS/GacA, RsmA and small non-coding regulatory RNAs in *Pseudomonas*. The GacS sensor kinase activates the transcription factor GacA by phosphorylation. GacA directly or indirectly activates the transcription of the small regulatory RNA genes. Titration of these RNAs by the RsmA and RsmE proteins relieves the translational repression of mRNAs for secondary metabolites and exo-enzymes (Modified after Haas and Défago, 2005).

2.2.2 Quorum sensing

Quorum sensing is the mechanism by which bacterial cells can sense their population density via the accumulation of small signal molecules that are thought to diffuse freely over the cellular membranes (Bassler, 1999; Fuqua et al., 1994; Miller and Bassler, 2001; Salmond et al., 1995; Whitehead et al., 2001; Withers et al., 2001). Quorum sensing was first described for the bioluminescent marine bacteria *Vibrio fischeri* and *Vibrio harveyi* (Nealson and Hastings, 1979). Emission of light occurs only at high cell density inside organs of eukaryotic hosts like the squid *Euprymna scolopes* (Fig. 7).

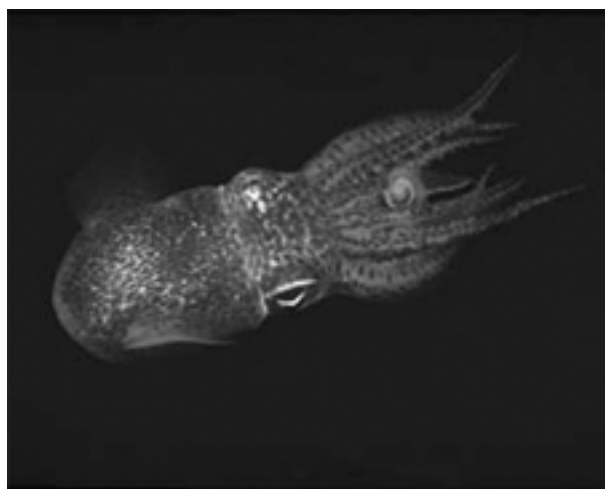


Fig. 7. The luminescent squid *Euprymna scolopes* uses the bioluminescent marine bacterium *Vibrio fischeri* as a source of light (John Hoover).

The quorum sensing system consists of the LuxI/LuxR regulatory system. Similar systems are found in many Gram-negative bacteria where LuxI homologues produce *N*-acylhomoserine lactone signal molecules (Fig. 8) and *luxR* homologues code for transcription factors that are activated when the autoinducers (*N*-acylhomoserine lactones) exceed a certain threshold concentration. The production of *N*-acylhomoserine lactones is often auto-regulated. Once activated, it amplifies its own signal (auto induction) and more *N*-acylhomoserine lactones are produced (Fig. 8) (Salmond et al., 1995). Examples of traits regulated by quorum sensing are luminescence (example above), symbiosis, virulence (paragraph, 1.2.4.4.), plasmid transfer, motility, biofilm formation and secondary metabolite production (Miller and Bassler, 2001; Salmond et al., 1995; Whitehead et al., 2001).

Production of many secondary metabolites by *Pseudomonas* are regulated by quorum sensing, including the production of phenazines by *P. fluorescens* strain 2-79 (Cha et al., 1998), *P. aeruginosa* strain PAO1 (Latifi et al., 1995), *P. aureofaciens* 30-84 (Pierson, III et al., 1994; Wood et al., 1997) and *P. chlororaphis* strain PCL1391 (Chin-A-Woeng et al., 2001b). The *luxI/luxR* homologues in *P. chlororaphis*, *P. fluorescens*, and *P. aureofaciens* are named *phzI/phzR* and regulate the expression of the phenazine (*phz*) operon. *P. aureofaciens* strain 30-84 contains two quorum sensing systems PhzI/PhzR and CsaR/Csrl producing different homoserine lactone molecules (Pierson, III et al., 1994; Wood et al., 1997; Zhang and Pierson, III, 2001). In *P. aeruginosa* there are also two quorum sensing systems LasI/LasR producing *N*-3-oxododecanoyl-homoserine lactone (3-oxo-C₁₂-HSL) and RhII/RhIR producing *N*-butanoyl homoserine lactone (C₄-HSL) (De Kievit and Iglewski, 2000; Juhas et al., 2005; Latifi et al., 1995; Pesci and Iglewski, 1997). Also for *P. chlororaphis* strain

PCL1391 a second quorum sensing system has been indicated to be present. However the genes for the second quorum sensing system have not been identified.

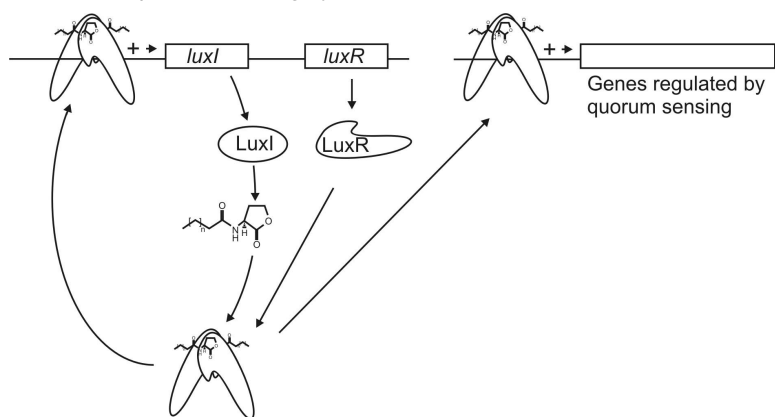


Fig. 8. Quorum sensing model. At low cell density only a low level of *N*-acyl homoserine lactone is produced by LuxI and the amplification loop is not activated. At high cell density the *N*-acyl homoserine lactones accumulate and these signal molecules bind to the transcription factor LuxR resulting in the dimerization and activation of LuxR. The activated LuxR binds to the promoter region of the *luxI* gene and induces the amplification loop. Activated LuxR also induces the transcription of other genes which are regulated by quorum sensing.

2.2.3. The molecular regulators PsrA and RpoS

The regulators *psrA* (*Pseudomonas* sigma regulator) and *rpoS* (stationary phase alternative sigma factor σ^s) affect the antibiotic production of in *P. fluorescens* strain Pf-5 (Sarniguet et al., 1995), *P. aeruginosa* strain PAO1 (Schuster et al., 2004; Suh et al., 1999) and *P. chlororaphis* strain PCL1391 (Chin-A-Woeng et al., 2005; Girard et al., 2006). PsrA acts as a positive regulator of *rpoS* gene expression and RpoS protein levels, which places *rpoS* downstream of *psrA* (Kojic and Venturi, 2001); (Girard et al., 2006). PsrA is a transcriptional activator that contains a typical helix-turn-helix DNA binding motif (Chin-A-Woeng et al., 2005; Girard et al., 2006; Kojic and Venturi, 2001). Sigma factors, including RpoS, determine the specificity of the RNA polymerase. RpoS controls the expression of genes involved in responses to various stress conditions, for example starvation. The expression of *psrA* and *rpoS* is dependant on *gacS/gacA* and places *psrA/rpoS* downstream of *gacS/gacA* in the regulatory cascade of PCN biosynthesis (Chin-A-Woeng et al., 2005; Whistler et al., 1998).

2.2.4. Post transcriptional regulation by RsmA and small non-coding RNAs

RNA binding proteins and small non-coding regulatory RNAs regulate the production of secondary metabolites and exo-enzymes in *Pseudomonas* spp. The translational repressor RNA binding protein RsmA represses the production of pyocyanin (a phenazine derivative; Fig. 5) and *N*-acyl-homoserine lactones in *P. aeruginosa* strain PAO1 (Pessi et al., 2001).

The biocontrol strain *P. fluorescens* strain CHA0 contains two RNA binding proteins, RsmA and its homolog RsmE, both repressing the production of hydrogen cyanide, exoprotease, and 2,4-diacetylphloroglucinol (Blumer et al., 1999; Kay et al., 2005; Reimmann et al., 2005). RsmA like proteins are presumed to interact with the ribosome binding sites of target genes and prevent their transcription. Genome sequence analyses of different sequenced pseudomonads predict that the number of *rsmA* homologues varies between different *Pseudomonas* strains from one to six (Reimmann et al., 2005). A *rsmA* homologue was also identified in *P. chlororaphis* strain PCL1391 (G. Girard, personal communication). Repression by RsmA is relieved by GacS/GacA controlled small non-coding RNAs such as PrrB in *P. fluorescens* strain F113 (Aarons et al., 2000), RsmZ and RsmY in *P. fluorescens* strain CHA0 (Heeb et al., 2002; Valverde et al., 2003), and RsmZ and RsmB in *P. aeruginosa* strain PAO1 (Burrowes et al., 2005; Heurlier et al., 2004). These small non-coding RNAs bind to RsmA, or its homologues, and relieve the repression of the synthesis of secondary metabolites and exo-enzymes (Fig. 6). The observation that *rsmA* is found in *P. chlororaphis* strain PCL1391 suggests that a similar post transcriptional mechanism occurs in this strain.

3. Phenazine biosynthesis in *Pseudomonas*

The molecular backbone of phenazines consists of three aromatic rings with two nitrogen atoms in the middle ring (Fig. 9). The phenazine biosynthetic operon contains 7 conserved genes, *phzABCDEFG*, and is present in phenazine producing *Pseudomonas* strains such as, *P. fluorescens* strain 2-79 (Mavrodi et al., 1998), *P. aereofaciens* strain 30-84 (Pierson, III et al., 1995), *P. aeruginosa* strain PAO1 (Mavrodi et al., 2001; Stover et al., 2000) and *P. chlororaphis* strain PCL1391 (Chin-A-Woeng et al., 2001b).

The pathway for phenazine biosynthesis branches from the shikimate pathway which is essential for the biosynthesis of aromatic amino acids (Turner and Messenger, 1986). PhzC is involved in the synthesis of shikimic acid and bypasses the household DAHP (3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate) synthetase. PhzE converts chorismate into ADIC (2-amino-2-deoxyisochorismic acid), PhzD converts ADIC into DHHA (*trans*-2,3-dihydro-3-hydroxyanthranilic acid) and PhzF dimerizes two DHHA molecules into phenazine-1-carboxylic acid (PCA) (Blankenfeldt et al., 2004). PhzA and PhzB accelerate this dimerization process and PhzG reacts with the phenazine precursor and catalyzes an oxidation/aromatization reaction (Parsons et al., 2004).

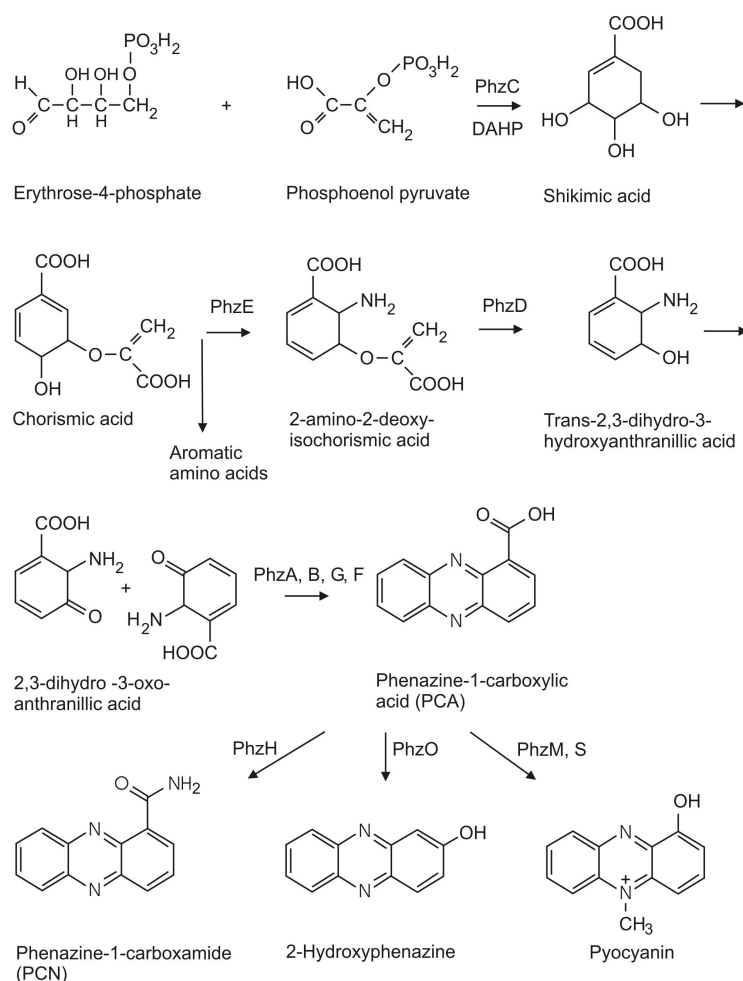


Fig. 9. Model for the biosynthetic pathway of phenazines in *Pseudomonas*. For explanation, see text (Modified after (Chin-A-Woeng et al., 2003)).

Some strains contain additional phenazine modification genes that allow the modification of PCA into other phenazine derivatives. In *P. chlororaphis* strain PCL1391 an additional *phzH* gene converts PCA into PCN (Chin-A-Woeng et al., 2001a). In *P. aereofaciens* strain 30-84 an additional *phzO* leads to the synthesis of 2-hydroxyphenazine (Delaney et al., 2001). In *P. aeruginosa* PhzH converts PCA into PCN and PhzM and PhzS convert PCA into pyocyanin (Fig. 9) (Mavrodi et al., 2001).

4. The biochemical properties of phenazines

The role and mode of action of phenazines synthesised by *Pseudomonas* is diverse and not well understood. Phenazines contribute to the ecological fitness of the producer

strains (Mazzola et al., 1992) and are thought to protect favourable niches. Plant roots colonized with phenazine-producing pseudomonads benefit from this property by preventing pathogens access to the roots. Phenazines have a broad antibiotic spectrum and repress growth of microbes including fungi (Fig. 10) and Gram-positive bacteria and eukaryotes. The mode of action of phenazines is very diverse and includes intercalation with DNA, interaction with topoisomerases, anti-oxidation characteristics, and generation of free radicals (Gamage et al., 2002; Laursen and Nielsen, 2004). One or a combination of these characteristics explains the growth inhibitory action of phenazines. Phenazines also play a role in processes not related to its antibiotic characteristics. Recently, PCN of *P. chlororaphis* strain PCL1391 was shown to be involved in microbial mineral reduction of crystalline iron and manganese oxide and increased the availability by dissolving these minerals. Phenazines can reduce these mineral crystals because of their redox-active characteristics and are thought the function as an electron shuttle, thereby dissolving large amounts of minerals with a small amount of PCN (Hernandez et al., 2004).

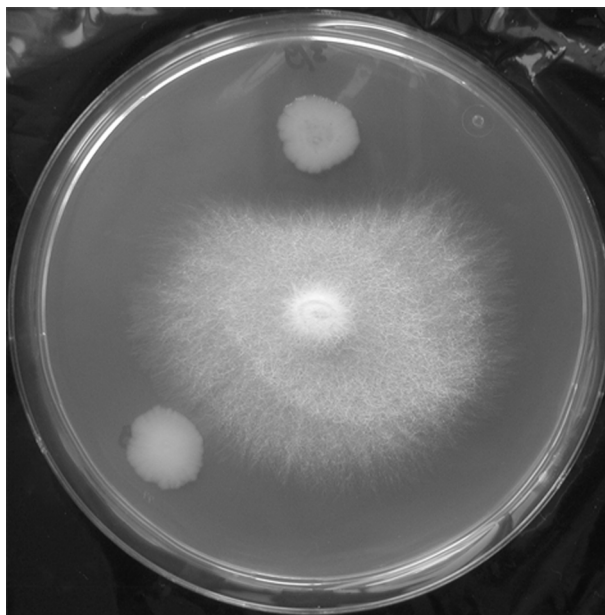


Fig. 10. Growth inhibition assay of the phenazine-1-carboxamide producing *Pseudomonas chlororaphis* strain PCL1391 (top) and *Fusarium oxysporum* (middle).

5. Genomics

Sequencing of genomes and the use of bioinformatics to process this information is a rapidly developing research field. The number of sequenced genomes is constantly increasing and currently the NCBI data base (www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) counts 473 bacterial, 28 archaeal, and 102 eukaryotic genomes (17/02/2006). Bacterial

genomes are relative small compared to eukaryotic genomes. For instance the *Escherichia coli* K-12 genome has $4.6 \cdot 10^6$ base pairs, whereas the human genome is almost one thousand times larger with an estimate of $3.2 \cdot 10^9$ base pairs. This difference in size is one of the explanations for the fact that more bacterial genomes have been sequenced than other genomes. Some members of the *Pseudomonas* genus have been sequenced including the biocontrol strain *P. fluorescens* Pf-5 (Paulsen et al., 2005) (Fig. 11), the human opportunistic pathogen *P. aeruginosa* strain PAO1 (Stover et al., 2000), the toluene degrading *P. putida* strain KT2440 (Nelson et al., 2002), the plant pathogens *P. syringae* pv *syringae* strain B728a and *P. syringae* pv *tomato* strain DC3000 (Buell et al., 2003). *P. cholororaphis* strain PCL1391 is presently being sequenced in the frame work of the EU project "Pseudomics". The sequenced pseudomonads have an average size of around $6 \cdot 10^6$ - $7.1 \cdot 10^6$ base pairs that code for approximately 5,000-6,000 proteins (www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Comparative genome analyses revealed that 85%-68% of the ORFs are homologues among the three sequenced pseudomonads (Buell et al., 2003; Nelson et al., 2002). The predicted percentage of the genome coding for proteins or RNAs is between 85% and 90%.

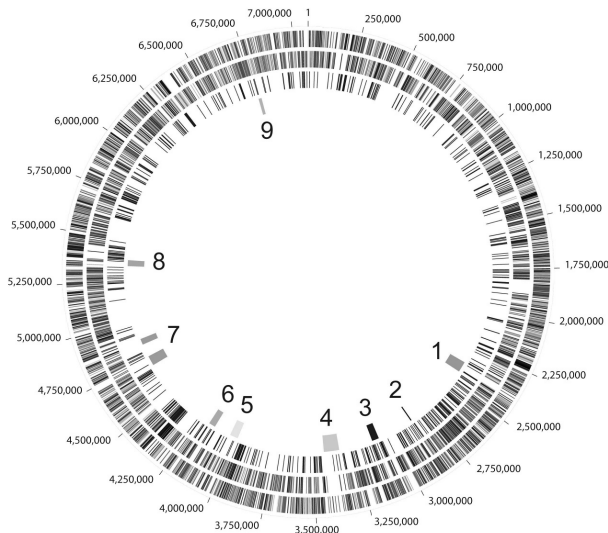


Fig. 11. Circular representation of the *P. fluorescens* Pf-5 genome. The outer circle indicates the chromosomal location in base pairs. The distribution of genes is depicted by grey lines. The first circle shows genes in the plus strand and the second circle shows the genes in the minus strand. The fourth circle shows nine regions encoding secondary metabolism 1, decapeptide biosynthesis; 2, Hydrogen cyanide; 3, pyoluteorin biosynthesis; 4, polyketide biosynthesis; 5, pyochelin biosynthesis; 6, pyrrolnitrin biosynthesis; 7, pyoverdine biosynthesis; 8, nonribosomal peptide synthesis; 9, 2,4-diacetylphloroglucinol synthesis (modified after Paulsen et al., 2005)

A comparative analysis with other microbial genomes demonstrates that pseudomonads have a high proportion of metabolic genes, transporter genes, and regulatory genes. The high percentages of metabolic genes reflect the ability to metabolize a wide variety of organic substrates. The large numbers of transporter systems allows the uptake of many organic substrates, the secretion of secondary metabolites, and contributes to the high intrinsic resistance to antibiotics. The large number of regulatory genes coordinates the regulation of these diverse adaptation abilities of pseudomonads. Genome sequencing also showed that there is plenty left to discover since roughly 40% of the *Pseudomonas* genes have unknown functions and are not related to known proteins (Buell et al., 2003; Paulsen et al., 2005; Stover et al., 2000). Sequencing of biocontrol strains *P. fluorescens* strain SBW25 (http://www.sanger.ac.uk/Projects/P_fluorescens/) is still in progress.

6. Transcriptomics and DNA microarrays

The breakthrough of DNA microarray technology generated the possibility to compare the transcriptional activity of all genes (transcriptome) in one organism (Brown and Botstein, 1999; Lockhart and Winzeler, 2000; Young, 2000). DNA microarrays can contain nucleic acid probes of all the genes from a single organism on a few square centimetres. Relative RNA abundance of each single gene can be analysed by labelling the total RNA pool of one condition with a red fluorescent dye and that of a second condition with a green fluorescent dye. Both coloured RNA pools are mixed and hybridized with the DNA microarray. For each probe (gene) on the microarray the relative colour ratio is determined and translated into a relative difference in messenger RNA level (see cover of this thesis). Microarrays are used to compare transcriptional differences between environmental conditions, mutants and wild types, and tissues.

The first microarrays containing PCR fragments of every gene of the budding yeast *Saccharomyces cerevisiae* was developed by Brown and colleagues. These arrays were used to identify transcriptional changes during sporulation, diauxic shift, and in genetically modified lines (Chu et al., 1998; DeRisi et al., 1997). These experiments identified new sets of genes that were not previously linked with these specific processes. Commercial microarrays of *P. aeruginosa* (GeneChip®) became available from Affymetrix in 2002. These GeneChips® were used to analyse the transcriptional profile of *P. aeruginosa* in response to iron (Ochsner et al., 2002) and identified novel genes involved in iron starvation. Since commercial microarrays are not available for other *Pseudomonas* strains. A *P. chlororaphis* strain PCL1391 microarray was constructed in the Lugtenberg lab. With these microarrays the effect of the fungal toxin fusaric acid on *P. chlororaphis* strain PCL1391 gene expression was studied and revealed an overlap with the transcription profile of iron starvation in *P.*

aeruginosa (Chapter 3), (van Rij et al., 2005). These same arrays were also used to compare the transcription profiles of *rpoS* and *psrA* mutants of *P. chlororaphis* strain PCL1391 and demonstrated an overlapping transcription profile of both regulators and identified novel genes in the *rpoS/psrA* regulon (Girard et al., 2006). Microarrays are very powerful tools and have developed very rapidly over the last years into a molecular biological technique that helps to investigate many biological questions.

7. Aim and outline thesis

This thesis focuses on the biocontrol bacterium *Pseudomonas chlororaphis* strain PCL1391, the biocontrol activity of which depends on the production of the antifungal metabolite phenazine-1-carboxamide (PCN) (Chin-A-Woeng et al., 1998). This thesis aims at elucidating environmental factors that influence PCN biosynthesis and at identifying the molecular regulation of PCN biosynthesis in *P. chlororaphis* strain PCL1391.

The effect of biotic and abiotic conditions, on the production of PCN and autoinducers by *P. chlororaphis* strain PCL1391 was investigated. The conditions tested included carbon sources, nitrogen sources, amino acids, temperature, oxygen levels, pH, salt stress, fusaric acid, ferric iron, phosphate, sulphate, and ammonium ions (Chapter 2), (van Rij et al., 2004). In chapter 3 the repression of PCN biosynthesis by the fungal toxin fusaric acid was investigated in detail, thereby analyzing the molecular mechanisms and environmental conditions which influence PCN production by *P. chlororaphis* strain PCL1391 in the presence of fusaric acid and identifying new genes of which the expression is affected by fusaric acid (van Rij et al., 2005). The transcriptome of three conditions influencing PCN biosynthesis identified in chapter 2 (van Rij et al., 2004), namely iron starvation, salt stress, and additional presence of 1 mM of the amino acid phenylalanine, were analysed using microarrays. Comparison of these environmental transcription profiles resulted in the identification of putative PCN regulated genes (chapter 6). In a second approach to identify more genes involved in the regulation of PCN, a mutant screen was performed that selected for mutants producing reduced levels of PCN. The identified mutants are described in chapters 4 and 5.

Chapter 2

Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* strain PCL1391

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Molecular Plant-Microbe Interactions (2004). 17:557-566

Abstract

Pseudomonas chlororaphis strain PCL1391 produces the secondary metabolite phenazine-1-carboxamide (PCN), which is an anti-fungal 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°C 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 soil-borne fungus *Fusarium*, also reduced PCN levels. Different nitrogen sources greatly influenced PCN levels. Analysis of autoinducer levels during PCN producing 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 is not only induced by a high optical density but can also be induced by certain environmental conditions. We discuss our findings in relation to the success of biocontrol in the field.

Introduction

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 since 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). Since 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), biotechnological production of PCN seems a promising alternative.

A second application of PCN is its use as an anti-fungal 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 III and Thomashow, 1992; Whitehead et al., 2001). Quorum sensing is a mechanism by which cells sense optical density via the accumulation of small diffusible molecules. Quorum sensing in Gram-negative bacteria is often 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 *Pseudomonas 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*₆-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, III 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* strain PCL1391 are investigated. The results are presented in the paper.

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> ; <i>N</i> -acyl-homoserine lactone (AHL) reporter strain	(Milton et al., 1997)
DH5 α	<i>Escherichia coli</i> ; <i>SupE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	(Hanahan, 1983)
JM109	<i>Escherichia coli</i> ; <i>recA1</i> <i>supE44</i> <i>endA1</i> <i>hsdR17</i> <i>gyrA96</i> <i>relA1</i> <i>thi</i> Δ (<i>lac-proAB</i>) F' [<i>traD36</i> <i>proAB</i> ⁺ <i>lacF</i> ⁺ <i>lacZ</i> Δ 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)

Results

PCN production is growth phase dependent and influenced by the nature of the carbon source.

Growth and PCN production of *P. chlororaphis* strain PCL1391 (Table 1) was monitored in time. Modified Vogel Bonner medium amended with 0.05% casamino acids and 30 mM glucose (MVB1-glucose-cas) was initially 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 reach stationary phase (Fig. 1). During the first 12 hours of stationary phase PCN levels remained unchanged (Fig. 1).

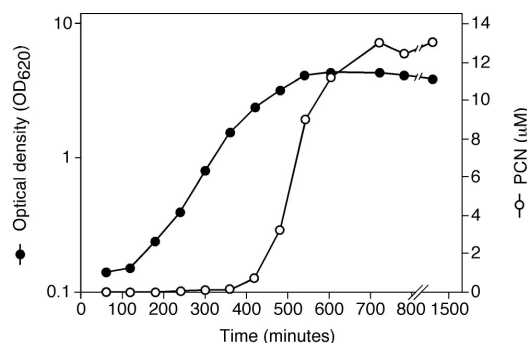


Fig. 1. Growth and PCN production by *P. chlororaphis* strain PCL1391. Cells were grown on MVB1-glucose-cas. Experiments were performed three times with similar results. Data of one of the experiments is shown. Amounts of PCN were determined by high-performance liquid chromatography (HPLC) analyses.

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.

Table 2. Influence of various carbon sources on generation time in exponential phase and optical density and PCN production at stationary phase of *P. chlororaphis* strain PCL1391^a

Carbon source (conc.)	+0.05% cas		-0.05% cas	
	Generation time (minutes)	optical density (OD ₆₂₀)	PCN (μM/OD ₆₂₀)	PCN (μM/OD ₆₂₀)
Citric acid (30 mM)	101 ± 4.4	2.9 ± 0.3	1.0 ± 0.6	0.22 ± 0.07
Fructose (30 mM)	186 ± 22	4.4 ± 0.4	0.35 ± 0.1	0.10 ± 0.1
Fumaric acid (45 mM)	73 ± 25	2.8 ± 0.7	0.11 ± 0.01	0.13 ± 0.07
Glucose (30 mM)	60 ± 9.8	4.3 ± 0.1	7.9 ± 0.2	0.86 ± 0.2
Glycerol (60 mM)	93 ± 17	4.8 ± 0.2	3.0 ± 0.1	0.12 ± 0.1
Lactic acid (60 mM)	70 ± 10	3.2 ± 0.2	1.2 ± 0.08	0.04 ± 0.01
Malic acid (45 mM)	68 ± 2.0	1.8 ± 0.2	0.05 ± 0.01	0.04 ± 0.01
L-Pyroglutamic acid (36mM)	79 ± 0.16	3.0 ± 0.2	8.4 ± 0.7	ND ^b
Pyruvic acid (60 mM)	213 ± 33	2.3 ± 0.1	0.17 ± 0.2	0.01 ± 0.006
Ribose (36 mM)	500 ± 58	2.8 ± 0.3	0.32 ± 0.2	0.02 ± 0.01
Succinic acid (C45 mM)	74 ± 4.8	3.2 ± 0.3	0.13 ± 0.009	0.04 ± 0.006
Sucrose (15 mM)	83 ± 9.8	4.4 ± 0.2	0.79 ± 0.2	0.13 ± 0.06

^a Cells were grown in 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 (HPLC) when cells had reached stationary phase. Average and standard deviations of three experiments are shown.

^bND (not detected)

Short generation times (60 to 74 minutes) were found for growth on glucose, fumaric acid, lactic acid, malic acid, and succinic acid. Moderate generation times (79 to 101 minutes) were found for growth on citric acid, glycerol, L-pyroglutamic acid, and sucrose. Growth on ribose, pyruvic acid, and fructose resulted in the longest generation times (186 to 500 minutes) (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 OD₆₂₀ value higher than 4. On the remaining carbon sources early stationary phase was reached at lower OD₆₂₀ 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-pyroglutamic acid resulted in the highest levels of PCN (8.4 to 3.0 μM/OD₆₂₀) compared to all other carbon sources tested (Table 2). On MVB1-glucose-cas a high production of PCN and a short generation time were measured. This medium was therefore subsequently 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 OD₆₂₀ 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 affect 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 NaNO_3 , urea, or casamino acids. Urea or NaNO_3 resulted in decreased PCN levels (Fig. 2A), whereas growth on 16 mM casamino acids caused a 3-fold increase in the level of PCN and a 50% higher final optical density compared to 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 if NH_4^+ ions stimulated PCN levels, NH_4Cl was tested. Increasing NH_4Cl concentrations appeared to stimulate PCN production indeed (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 was 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 addition of 1 mM of any of the mentioned amino acids increased PCN production at least two fold. 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 analysed 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_{620} value of 1.0 instead of 2.0 (data not shown).

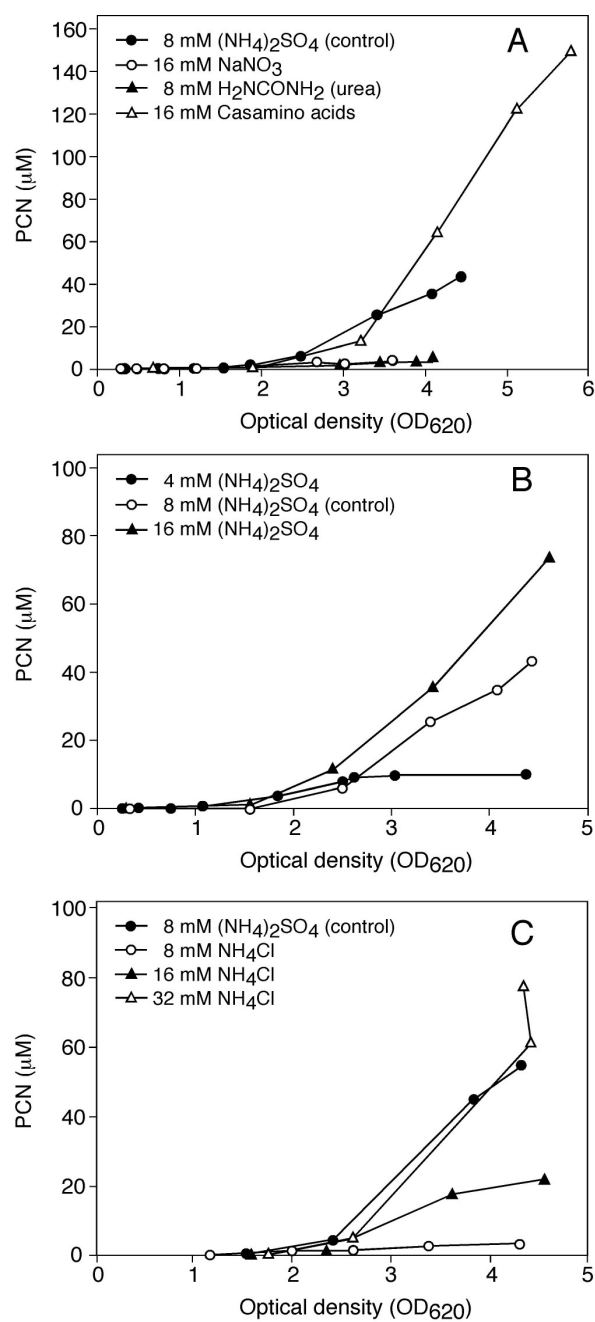


Fig. 2. Influence of various nitrogen sources on the level of PCN. *P. chlororaphis* strain PCL1391 was grown with various nitrogen sources (A) and with various concentrations of (NH₄)₂SO₄ (B) and NH₄Cl (C). Cells were grown in MVB2-glucose-cas. Experiments were performed at least three times with similar results. Data of one of the experiments is shown.

Table 3. Influence of individual amino acids on PCN production in MVB1-glucose by *P. chlororaphis* strain PCL1391

Amino acid added (1 mM)	PCN production ($\mu\text{M}/\text{OD}_{620}$) ^a
None	0.91 $\pm$ 0.21a
Asparagine	3.9 $\pm$ 0.61 b
Aspartic acid	3.6 $\pm$ 2.3 b
Glutamic acid	2.1 $\pm$ 0.78 a
Glutamine	3.7 $\pm$ 0.59 b
Glycine	6.6 $\pm$ 2.3 b
Histidine	3.7 $\pm$ 0.13 b
Isoleucine	6.1 $\pm$ 0.32 b
Leucine	3.9 $\pm$ 1.1 b
Lysine	3.9 $\pm$ 1.1 b
Phenylalanine	21 $\pm$ 2.1 b
Threonine	2.0 $\pm$ 1.2 a
Tryptophan	7.1 $\pm$ 0.22 b
Tyrosine	12 $\pm$ 3.2 b

^a PCN concentration of the supernatant fluid was quantified by HPLC 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 LSD test.

Abiotic factors affect PCN production.

Cells of strain PCL1391 were grown at temperatures between 16°C and 31°C, whereas 28°C is the standard growth temperature. Increasing or decreasing the temperature to 31°C 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 to 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 optical density (Fig. 3B). The growth rate at 1% oxygen was almost 2-fold lower (193 min) compared to 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_2HPO_4 and $\text{Na}(\text{NH}_4)\text{HPO}_4 \cdot 4\text{H}_2\text{O}$ in MVB1-glucose-cas were replaced by K_2HPO_4 , KH_2PO_4 , and the nitrogen source $(\text{NH}_4)_2\text{SO}_4$. This medium was named MVB2-glucose-cas and allowed easy changes in the pH by changing the ratio of K_2HPO_4 and KH_2PO_4 . Growth of strain PCL1391

in MVB2-glucose-cas resulted in a more than 2-fold increase in PCN levels compared to 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 respectively to pH 4.2, 6.5, and 7.1. 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 an extremely low PCN level (Fig. 3C) and a decreased optical density at the stationary phase (not shown). The pH of all other environmental conditions tested in this manuscript 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, 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 affect 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 FeCl₃ leads to equal levels of PCN (data not shown). Concentrations of 0.005 and 0.01 mM FeCl₃ partially restored PCN production in a concentration dependant manner (Fig. 4A). Omitting ferric iron (Fe³⁺) from MVB1 did not change the growth rate during the exponential phase or the final optical density 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 OD₆₂₀ value (Fig. 4B). Decreasing the phosphate concentration to 10 or 1 mM also decreased PCN production and reduced optical density at stationary phase by 22% and 62%, respectively. Decreasing the standard MgSO₄ concentration of 0.8 mM to 0.08 mM resulted in a 90% reduction in PCN production, a 40% reduction in optical density and an increased generation time during exponential phase (203 min.). Increasing the MgSO₄ concentration to 8 mM resulted in a 72% reduction in PCN production but did not affect growth rate or optical density during stationary phase (data not shown).

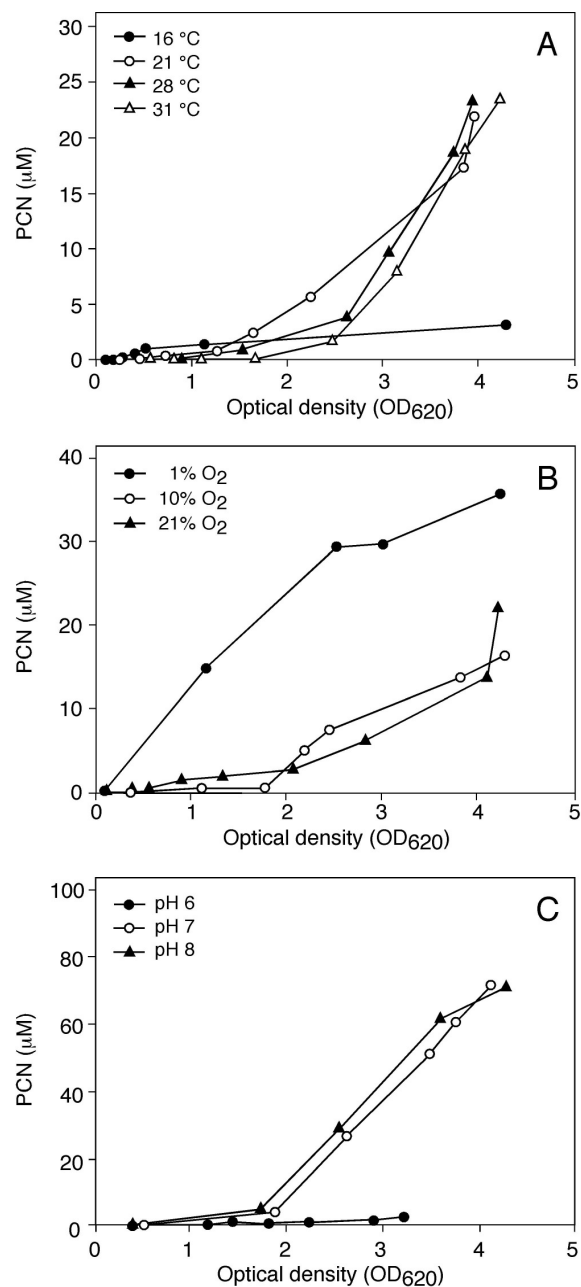


Fig. 3. Influence of abiotic factors on the level of PCN. *P. chlororaphis* strain PCL1391 was grown at various temperatures (A), oxygen concentrations (B), and pH values (C). Cells were grown in MVB1-glucose-cas (A and B) or in MVB2-glucose-cas (C). Experiments were performed at least three times with similar results. Data of one of the experiments is shown.

To test whether these effects were due to Mg^{2+} or to SO_4^{2-} , MgCl_2 and K_2SO_4 were used as separate Mg^{2+} and SO_4^{2-} sources. Decreasing the MgCl_2 concentration ten-fold resulted in almost two-fold decreased growth rate (117 min) during the exponential phase, a start of PCN production at a lower OD_{620} value and in increased levels of PCN. Increasing the MgCl_2 concentration ten-fold abolished PCN production completely (Fig. 4C). Decreasing the K_2SO_4 concentration ten-fold abolished PCN production, whereas a ten-fold increase has little effect on PCN levels (Fig. 4D).

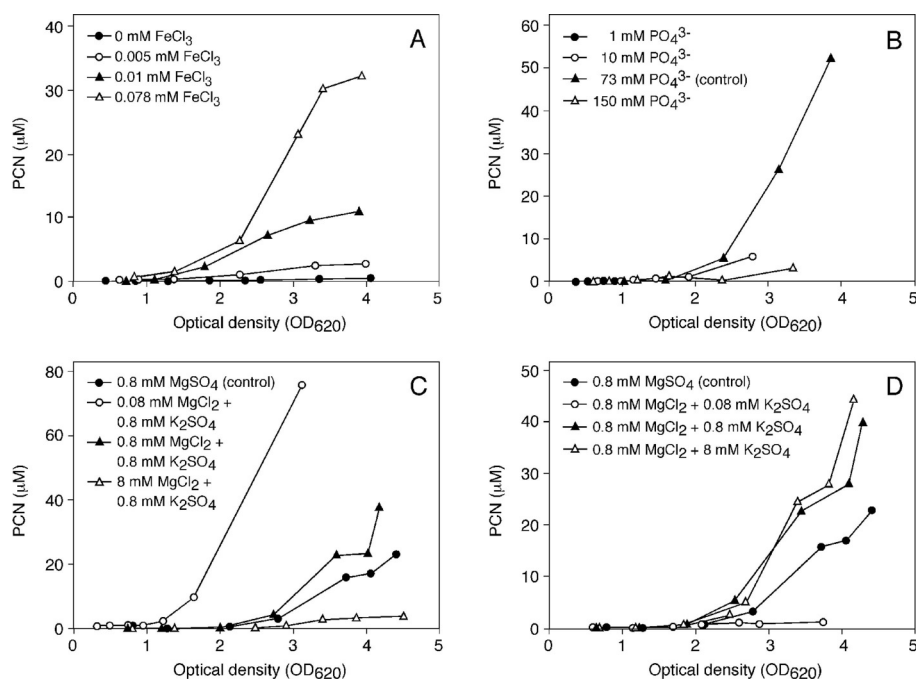


Fig. 4. Influence of ions on the level of PCN. *P. chlororaphis* strain PCL1391 was grown at various FeCl_3 concentrations (A), PO_4^{3-} concentrations (B), MgCl_2 concentrations (C), and K_2SO_4 concentrations (D). Cells were grown in MVB1-glucose-cas (A, C, and D) or in MVB2-glucose-cas (B). Experiments were performed at least three times with similar results. Data of one of the experiments is shown.

Salt stress affects the production of PCN.

The effect of osmolality on PCN production was tested by adding NaCl, KCl, Na_2SO_4 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_2SO_4 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_2SO_4 .

(Table 4). The growth and optical density 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 can 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).

Table 4. Influence of salts, osmolality, and osmoprotectants on PCN production of *P. chlororaphis* strain PCL1391^a

	PCN Production ($\mu\text{M}/\text{OD}_{620}$)	
	MVB1-glucose-cas	MVB1-glucose-cas + NaCl (0.1 M)
Control	7.3 $\pm$ 0.80 a	0.06 $\pm$ 0.05 b
NaCl (0.1 M)	0.06 $\pm$ 0.05 b	-
Xylose (0.2 M)	7.1 $\pm$ 0.07 a	-
KCl (0.1 M)	0.09 $\pm$ 0.03 b	-
Na ₂ SO ₄ (0.5 M)	1.1 $\pm$ 0.07 b	-
Betaine (1 mM)	8.4 $\pm$ 2.5 a	1.1 $\pm$ 0.55 b
Choline (1 mM)	9.9 $\pm$ 2.2 a	1.5 $\pm$ 0.88 b
L-proline (1 mM)	5.8 $\pm$ 1.2 a	0.09 $\pm$ 0.02 b
Trehalose (1 mM)	4.3 $\pm$ 1.5 a	0.08 $\pm$ 0.03 b

^a 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 HSD test.

Fusaric acid reduces PCN production.

P. chlororaphis strain PCL1391 is a biocontrol strain of tomato foot and root rot caused by the fungal pathogen *F. oxysporum* f. sp. *radicis-lycopersici* ZUM2407 (Chin-A-Woeng et al., 1998). *Fusarium oxysporum* f. sp. *radicis-lycopersici* ZUM2407 (de Weert, et al. *in preparation*) and many other *Fusarium* species 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.

Table 5. Influence of fusaric acid on generation time, optical density at stationary phase, and PCN production of *P. chlororaphis* strain PCL1391^a.

Fusaric acid (mM)	Generation time (Minutes)	PCN ($\mu\text{M}/\text{OD}_{620}$)
0	85.3 $\pm$ 5.6 a	7.0 $\pm$ 1.9 a
0.1	91.1 $\pm$ 7.6 a	5.1 $\pm$ 0.82 a
0.3	93.4 $\pm$ 6.3 a	1.8 $\pm$ 0.6 b
0.5	89.0 $\pm$ 6.6 a	1.6 $\pm$ 0.51 b
0.75	97.8 $\pm$ 4.1 a	1.4 $\pm$ 0.64 b
1	99.1 $\pm$ 3.7 a	0.72 $\pm$ 0.21 b
1.5	106.4 $\pm$ 15.1 b	0.28 $\pm$ 0.054 b

^a Cells were grown in MVB1-glucose-cas with different concentrations of fusaric acid. Optical density was measured at stationary phase between OD values 3.09 and 3.83, and PCN concentration of the supernatant fluid was quantified using HPLC 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 HSD test.

Combinations of environmental factors affect 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 pair-wise and PCN production was analysed. Phenylalanine strongly increased PCN production at 16°C, with 0.1 mM NaCl, and during iron starvation. Striking is that 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 PCN compared to 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).

Table 6. Cumulative effect of environmental conditions on PCN production of *P. chlororaphis* strain PCL1391^a

	PCN production ($\mu\text{M}/\text{OD}_{620}$)			
	MVB1-glucose-cas	Phenylalanine (1 mM)	NaCl (0.1 M)	Fe(III) (0 M)
28°C	7.3 $\pm$ 0.8 a	28.7 $\pm$ 1.6 a	0.06 $\pm$ 0.05 a	0.27 $\pm$ 0.1 a
16°C	2.6 $\pm$ 1.1 b	9.9 $\pm$ 1.8 bc	0.07 $\pm$ 0.02 a	0.20 $\pm$ 0.23 a
1% O ₂	9.2 $\pm$ 1.8 a	7.7 $\pm$ 2.0 bcd	6.1 $\pm$ 2.4 b	3.0 $\pm$ 2.2 ac

Phenylalanine (1 mM)	28.7 ± 1.6 d	-	3.1 ± 0.8 c	6.3 ± 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	-

^a Cells grown at 28°C in MVB1-glucose-cas served as a control. PCN concentration of the supernatant was quantified by HPLC 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 HSD test.

Autoinducer levels are affected by environmental conditions.

The levels of the major autoinducer C₆-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 OD₆₂₀ value of 3.0 and analyzed with a *Chromobacterium violaceum* 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, C₆-HSL appeared to be the only detectable autoinducer (Fig. 5A). Quantitative analyses of the isolated autoinducer showed that PCL1391 produced less C₆-HSL when grown on MVB2 at pH 6, MVB1-glucose-cas with 0.1 M NaCl, and MVB1-glucose-cas without iron (III) compared to 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 to growth on MVB1-glucose-cas (Fig. 5). The stimulating effect of phenylalanine was further analysed and autoinducers were also isolated at an OD₆₂₀ value of 1.0 and 2.0 from cultures with and without 1 mM phenylalanine. At an OD₆₂₀ value of 1.0 C₆-HSL was already detected with 1 mM phenylalanine and not in the control, MVB1-glucose-cas. At an OD₆₂₀ value of 2.0 higher amounts of C₆-HSL were detected in the presence of 1mM phenylalanine (Data not shown). These results demonstrate that the production of C₆-HSL was advanced and increased by the addition of 1 mM phenylalanine. Autoinducer production was also 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 OD₆₂₀ 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).

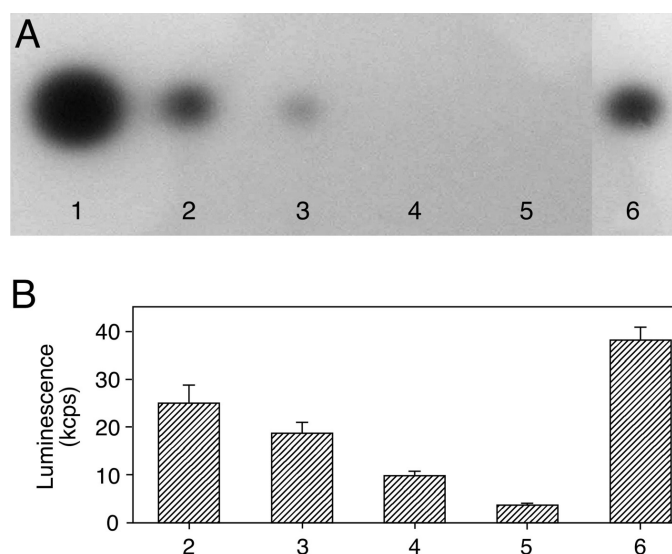


Fig. 5. Analyses of C_6 -HSL production by *P. chlororaphis* PCL1391. Fig. A, lane 1 synthetic C_6 -HSL standard ($2.5 \cdot 10^{-7}$ mol); lane 2: standard MVB1-glucose-cas (pH 7); lane 3: MVB1-glucose-cas lacking Fe^{3+} ; lane 4: MVB1-glucose-cas supplemented with 0.1 M NaCl; lane 5: MVB2-glucose-cas, pH 6; lane 6: MVB1-glucose-cas supplemented with 1 mM phenylalanine. Samples were separated on a C18-reverse phase TLC and visualized by *C. violaceum* strain CV026. (B) C_6 -HSL concentrations compared by bioluminescence induction assay based on *E. coli* strain JM109 containing plasmid pSB401. Lane 1: figure was of scale and is not shown, values were 230 ± 16 (kcps). Both experiments A and B were performed with the same extracts, isolated at OD_{620} 3.0. The experiment was performed twice with similar results.

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 Bloemberg, 2004). From these carbon sources glucose, L-pyroglutamic acid, and glycerol yielded the highest PCN levels in MVB1-cas. The six most common carbon sources found in tomato root exudate, namely citric acid, malic acid, lactic acid, succinic acid, oxalic acid, and pyruvic acid, resulted in limited PCN levels compared to glucose and pyroglutamic acid (Table 2). The later two compounds are less abundant in the tomato rhizosphere. It therefore 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) is probably not due to their precursor role since available evidence suggests that glutamine is the source of the nitrogen in PCN (Pierson III 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 Na₂SO₄ reduced the production of PCN by PCL1391 (Table 4). In contrast, high osmolality caused by the sugar xylose (0.2 M) does not inhibit PCN production. Xylose was chosen above sucrose to increase the osmolality of the medium, since xylose cannot be metabolised 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 compensate 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), O₂ concentrations (Fig. 3B), Fe³⁺ (Fig. 4A), PO₄³⁻ (Fig. 4B), Mg²⁺ (Fig. 4C), and SO₄²⁻ (Fig.4D).

Transcription of the *phz* operon is regulated via quorum sensing and PCN synthesis is dependant on C₆-HSL (Chin-A-Woeng et al., 2001b). From all conditions analyzed for C₆-HSL production, PCN levels correlated with those of C₆-HSL levels (Fig. 5 and Results section). Growth at low oxygen and the addition of 1 mM phenylalanine resulted in elevated levels of C₆-HSL and PCN at lower optical densities.

In the hope to find 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* species. 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) was found to be stimulated by glucose and glycerol. This suggests a similar response of phenazine producing pseudomonads to these carbon sources. Like in PCL1391 (Fig. 2A), phenazine production in *P. aeruginosa* is also regulated by nitrogen sources in that NH₄⁺ supports a higher production of PCN than urea, asparagine or peptone (Kanner et al., 1978). PCA production by *P. fluorescens* strain 2-79 was not found to be affected by different nitrogen sources (Slininger and Shea-Wilbur, 1995). Like in strain PCL1391 (Table 3) aromatic amino acids stimulate 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 non-aromatic amino acids on PCN levels (Table 3) has not been reported previously. The pH optimum for PCA production in PCL1391 (Fig.3) differs 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), Fe³⁺ has a positive effect on the production of PCA in *P. fluorescens* 2-79, whereas magnesium ions positively affect PCA production in *P. fluorescens* 2-79 (Slininger and Jackson, 1992). The production of pyocyanin, a phenazine derivative produced by *P.*

aeruginosa is 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 anti-fungal metabolites (AFM) producing *Pseudomonas* strains we can conclude that some environmental factors have similar effects whereas others have opposite effects on different strains. It would therefore 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* strain CHA0 fusaric acid repressed the expression of another AFM, namely 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 are also likely to affect biocontrol and can at least partially explain 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). The 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; Ownley et al., 2003). This demonstrates that the pH of the rhizosphere is an important factor for successful biocontrol. The concentration of Fe^{3+} is low in neutral and alkaline soil which is due to its insolubility. Therefore pseudomonads use siderophores for the acquisition of Fe^{3+} and the competition for these ions is an established mechanism in biocontrol of soil born plant pathogens (Duijff et al., 1993; Koster et al., 1994; Loper and Buyer, 1991; Moënne-Loccoz et al., 1996). Free amino acids in the rhizosphere can be derived from root exudates (Simons et al., 1997; Vancura, 1964) but can also be the result of protein degradation by proteolytic enzymes produced by microbes in the rhizosphere such as *P. chlororaphis* strain 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. As the environmental regulation seems to differ between strains, 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 stabilised 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 unravelling 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

Micoorganisms and culture conditions.

The microbial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium (Sambrook and Russel, 2001) was used as the standard medium for culturing *Escherichia coli* and *Chromobacterium 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 μM $NaFeEDTA(III)$, 6.8 μM $MnSO_4 \cdot H_2O$, 0.35 μM $CuSO_4 \cdot 5H_2O$, 4.1 μM $Na_2MoO_4 \cdot 2H_2O$, 0.85 μM $ZnSO_4 \cdot 7H_2O$, 51 μM 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-pyroglutamic 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, sulphate, 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 culture 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, MI, USA). 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. Optical density of cultures were measured at a wavelength of 620 nm. Growth analyses were made in a 100 ml flask with 10 ml 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 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 l/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 et al. (1998) with minor modifications. Culture samples (250 μl) were taken at various time intervals, centrifuged, and the culture supernatants were acidified to pH 2 using 6 M HCl. They were subsequently extracted with an equal volume of toluene by shaking on an Eppendorff 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 μl acetonitrile and the obtained solution was mixed with 400 μl water. PCN concentrations were determined by high-performance liquid chromatography (HPLC) (DIONEX, Sunnyvale, CA, chromeleon software version 6.20) and the peak areas were calculated with a calibration curve. HPLC was performed using an econosphere C18 5u, 259 mm x 4.6 mm column (Alltech Associates, Inc, Deerfield, IL) at 30°C with a linear gradient of 20-80% 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, shaken for one hour and the solvent was collected. Each supernatant was extracted twice, extracts were pooled and the solvent was removed by rotary evaporation (McClean 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) (v/v). After development, the TLC was overlaid with LB 0.8% agar containing a 10-fold diluted overnight culture of the *Chromobacterium violaceum* indicator strain CV026 (Milton et al., 1997) and kanamycin (50 µg/ml). After incubation for 48 h at 28°C, chromatograms were analysed 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 water and loaded in triplicate on an Optiplate-96 (Packard, Meriden, CT). An overnight culture of *E. coli* strain JM109 containing pSB401 was diluted 10-fold in fresh LB and 100 µl was added to each sample. After incubation for 4 hrs at 28°C bioluminescence was determined using the luminescence counter MicroBeta 1450 TriLux, (Wallac, Turku, Finland).

Acknowledgements

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Chapter 3

Influence of fusaric acid on phenazine-1-carboxamide synthesis and gene expression of *Pseudomonas chlororaphis* strain PCL1391

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Summary

Production of the antifungal metabolite phenazine-1-carboxamide (PCN) by *P. chlororaphis* strain PCL1391 is essential for the suppression of tomato foot and root rot caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. We have shown that fusaric acid, a phytotoxin produced by *Fusarium oxysporum*, represses the production of PCN and of the quorum sensing signal *N*-hexanoyl-L-homoserine lactone (C₆-HSL). Here we report that PCN repression by fusaric acid is maintained even during PCN stimulating environmental conditions such as additional phenylalanine, additional ferric iron and a low Mg²⁺ concentration, which suggest that PCN repression by fusaric acid takes place under various environmental conditions. Constitutive expression of *phzI* or *phzR* increases the production of C₆-HSL and abolishes the repression of PCN production by fusaric acid. Transcriptome analysis using *P. chlororaphis* PCL1391 microarrays showed that fusaric acid represses expression of the phenazine biosynthetic operon (*phzABCDEFGH*) and of the quorum sensing regulatory genes *phzI* and *phzR*. Fusaric acid does not alter expression of the PCN regulators *gacS*, *rpoS*, and *psrA*. In conclusion, reduction of PCN levels by FA is due to direct or indirect repression of *phzR* and *phzI*. Microarray analyses identified genes of which the expression is strongly influenced by fusaric acid. Genes highly up-regulated by fusaric acid are also up-regulated by iron starvation in *P. aeruginosa*. This remarkable overlap in the expression profile suggests an overlapping stress response to fusaric acid and iron starvation.

Introduction

P. chlororaphis strain PCL1391 gives excellent biocontrol of tomato foot and root rot (TFRR), a disease caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* ZUM 2407 (Chin-A-Woeng et al., 1998). In the tomato rhizosphere PCL1391 colonizes the same niche as occupied by *Fusarium oxysporum* hyphae, and closely interacts with *Fusarium oxysporum* by attaching to, and forming micro-colonies on, hyphae (Bolwerk et al., 2003). Secretion of toxic and growth inhibiting compounds by both the fungus and the bacterial biocontrol strain is part of the complex interaction between fungi and bacteria.

PCL1391 produces the antifungal metabolite phenazine-1-carboxamide (PCN), which inhibits hyphal growth (Chin-A-Woeng et al., 1998). Using a PCN biosynthetic mutant it was shown that PCN is essential for the biocontrol ability of PCL1391 (Chin-A-Woeng et al., 1998). PCN production is regulated by the quorum sensing regulatory genes *phzI* and *phzR* which are homologous to *luxI* and *luxR* (Chin-A-Woeng et al., 2001b). In addition, biosynthesis of PCN is dependent on the two-component regulatory system formed by GacS

and GacA and is regulated by PsrA and the stationary phase sigma factor encoded by *rpoS* (Chin-A-Woeng et al., 2005); G. Girard, *in preparation*).

Plant pathogenic and non-pathogenic *Fusarium* spp. produce fusaric acid (5-butylpicolinic acid) (Bacon et al., 1996; Notz et al., 2002; Schouten et al., 2004). Fusaric acid (FA) is toxic for eukaryotes and prokaryotes (Bochner et al., 1980; Wang and Ng, 1999). In addition, FA was shown to be involved in fungal defense against *Pseudomonas* spp. biocontrol strains by repressing the production of antifungal metabolites. FA represses the production of 2,4-diacetylphloroglucinol in *P. fluorescens* CHA0 (Duffy and D  fago, 1997) and the synthesis of PCN in *P. chlororaphis*, PCL1391 (van Rij et al., 2004). The latter repression is correlated with a reduction of the level of the auto inducer, C₆-HSL (van Rij et al., 2004).

In this study we investigate the repression of PCN biosynthesis by FA in more detail and aim at (i) analyzing the molecular mechanisms and environmental conditions which influence PCN production in the presence of FA and (ii) identifying new genes of which the expression is affected by the presence of FA.

Table 1. Microorganisms and plasmids used in this study

Strains or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
PCL1391	Wild-type biocontrol strain <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide	(Chin-A-Woeng et al., 1998)
PCL1111	PCL1391 <i>psrA</i> ::Tn5 <i>luxAB</i>	(Chin-A-Woeng et al., 2005)
PCL1119	PCL1391 <i>phzB</i> ::Tn5 <i>luxAB</i>	(Chin-A-Woeng et al., 1998)
PCL1103	PCL1391 <i>phzI</i> ::Tn5 <i>luxAB</i>	(Chin-A-Woeng et al., 2001b)
PCL1104	PCL1391 <i>phzR</i> ::Tn5 <i>luxAB</i>	(Chin-A-Woeng et al., 2001b)
PCL1960	Derivative of PCL1391 containing pBBR1-MCS5, Gm ^r	(G. Girard, <i>in preparation</i>)
PCL1969	PCL1391 <i>gacS</i> ::Tn5 <i>luxAB</i>	This study
PCL1993	Derivative of PCL1391, pMP7447 containing P _{tac} - <i>phzR</i> in pBBR1-MCS5, Gm ^r	(G. Girard, <i>in preparation</i>)

PCL1999	Derivative of PCL1391, pMP7450 containing P_{lac} - <i>phzI</i> In pBBR1-MCS5, Gm^r	This study
CV026	<i>Chromobacterium violaceum</i> . <i>N</i> -acyl-homoserine lactone (HSL) reporter strain, Km^r	(McClellan et al., 1997)
DH5 α	<i>SupE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1 endA1 gyrA96 thi1 relA1</i>	(Boyer and Roulland-Dussoix, 1969)
Plasmids		
pBluescript	General purpose cloning vector, Cb^r	Stratagene, La Jolla, CA
pBBR1MCS-5	General purpose cloning vector, Gm^r	(Kovach et al., 1995)
pRK2013	Helper plasmide for tri-parental mating	(Ditta et al., 1980)
pMP7447	pBBR1MCS-5 containing the <i>phzR</i> gene of PCL1391 downstream of the P_{lac} promoter, inserted between the <i>XhoI</i> and <i>EcoRI</i> sites	(G. Girard, <i>in preparation</i>)
pMP7450	pBBR1MCS-5 containing the <i>phzI</i> gene of PCL1391 downstream of the P_{lac} promoter, inserted between the <i>EcoRI</i> sites	This study

Results

FA represses PCN production under different environmental conditions

The effect of 1 mM FA on PCN production by *P. chlororaphis* PCL1391 (Table 1) was evaluated under environmental conditions that stimulate PCN production (van Rij et al., 2004). The latter conditions included 1 mM phenylalanine, a 10-fold reduction of Mg^{2+} to a final concentration of 0.08 mM Mg^{2+} , and a 23-fold increase of ferric ions (Fe^{3+}) with a final concentration of 2 mM $FeCl_3$ (Table 2).

A combination of FA and phenylalanine increased PCN production compared to FA alone but decreased PCN production compared to phenylalanine alone. FA suppressed the positive effect of a low Mg^{2+} or high ferric ion concentration on PCN production. The fact that FA has divalent cation chelating characteristics which include iron (Bochner et al., 1980) and iron limitation is known to repress PCN production (van Rij et al., 2004), suggests that iron chelation by FA is a possible mechanism by which FA represses PCN production. To test that hypothesis, the effect of 0.1 mM and 1.0 mM of the strong iron chelator EDDHA was added to the growth medium. A concentration of 1.0 mM EDDHA did not alter the production of PCN compared to that in the control. A ten time's lower concentration of EDDHA almost doubled PCN production compared to the control.

Table 2. Cumulative effect of fusaric acid and other conditions on phenazine-1-carboxamide production by *P. chlororaphis* strain PCL1391*

PCN production (μM)						
	-†	Phenylalanine (1mM)	MgCl ₂ (0.08 mM)	FeCl ₃ (2 mM)	EDDHA (0.1 mM)	EDDHA (1.0 mM)
-†	34 ± 11 ^a	103 ± 8.3 ^c	116 ± 4.3 ^c	57 ± 17 ^d	60 ± 20 ^d	46 ± 19 ^d
Fusaric acid (1 mM)	6.5 ± 1.4 ^b	32 ± 11 ^a	2.5 ± 1.5 ^b	5.9 ± 3.9 ^b	n.d.	n.d.

* Cells grown at 28 °C in MVB1 served as a control. The PCN concentration in the supernatant fluid was measured by HPLC when cells reached stationary phase. Average and standard deviations of at least three experiments are shown. Values followed by different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test. Not determined (n.d.)

† MVB1

Another divalent cation which forms a possible candidate for FA chelation is zinc. Reduced zinc levels caused by FA chelation could cause PCN repression since zinc is structurally important in the ferric up take regulator (Fur) protein (Pohl et al., 2003). The effect of different zinc concentrations in combination with FA was investigated (Table 3). Zinc concentrations of 10 μM, 100 μM, and 2 mM ZnSO₄ did not increase PCN production with or without FA. To rule out interference of SO₄²⁻, the effect of ZnCl₂ investigated in an identical experiment which had similar results (Data not shown).

Table 3. Cumulative effect of fusaric acid and zinc on phenazine-1-carboxamide production by *P. chlororaphis* strain PCL1391*

PCN production (μM)				
	-†	ZnSO ₄ (10 μM)	ZnSO ₄ (100 μM)	ZnSO ₄ (2 mM)
-†	34 ± 11 ^{ab}	30 ± 12 ^{ab}	39 ± 14 ^{abc}	48 ± 15 ^{ac}
Fusaric acid (1 mM)	6.5 ± 1.4 ^b	6.1 ± 2.2 ^b	2.8 ± 2.0 ^b	2.7 ± 1.2 ^b

* Cells grown at 28 °C in MVB1 served as a control. The PCN concentration in the supernatant fluid was measured by HPLC when cells reached stationary phase. Average and standard deviations of at least three experiments are shown. Values followed by different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test.

† MVB1

Expression *phzI* and *phzR* is repressed by FA under different environmental conditions

The quorum sensing regulators *phzI* and *phzR* are regulating PCN production (Chin-A-Woeng *et al.*, 2001b). Transcription of these regulators was monitored during growth in standard MVB1 medium. Transcription of *phzI* in the *phzI*::Tn5*luxAB* mutant (PCL1103) remained at basal level with and without FA. Because PCL1103 does not contain a functional *phzI* there is no production of C₆-HSL and the auto-induction of *phzI* can not take place (Fig. 1). Additional synthetic C₆-HSL (5 μ M) induced *phzI* transcription. Adding both C₆-HSL and FA resulted in the repression of *phzI* transcription. Transcription of *phzR* in PCL1104 (*phzR*::Tn5*luxAB*) was also repressed by FA (Fig. 1). Transcription of *phzI* and *phzR* was reduced by FA during growth under PCN stimulating conditions which included additional phenylalanine, a low Mg²⁺ concentration, and additional ferric iron (Fig. 2). MVB1 with phenylalanine results in *phzI* and *phzR* transcription reaching a maximum between OD₆₂₀ 2 and OD₆₂₀ 3 and decreases when cells reach OD₆₂₀ 4. Additional phenylalanine and FA advances the peak of *phzI* and *phzR* transcription towards OD₆₂₀ 1 (Fig. 2a). Additional ferric iron or a low Mg²⁺ concentration shows the largest repression of *phzI* and *phzR* transcription by FA (Fig. 2b and c).

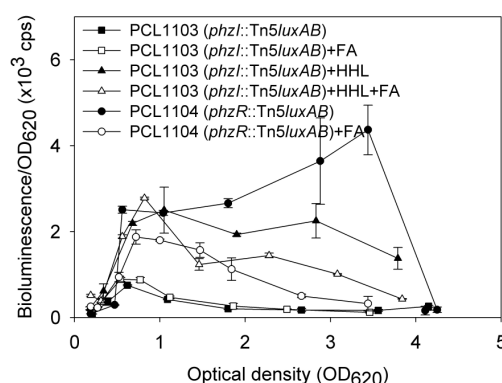


Fig. 1. Expression of *phzI* (PCL1103) with and without 5 μ M synthetic C₆-HSL (HHL) and *phzR* (PCL1104) in *P. chlororaphis* PCL1391. Cells were grown in MVB1 in the absence (black symbols) and in the presence of fusaric acid (FA) (white symbols). Luminescence values were measured in counts per second (cps) per optical density unit during growth. Averages of two samples are shown.

Constitutive expression of *phzI* or *phzR* restores PCN production in the presence of FA

It can be predicted that PhzI is responsible for the synthesis of the autoinducer *N*-hexanoyl-L-homoserine lactone (C₆-HSL) (Chin-A-Woeng *et al.*, 2001b), the production of which is reduced by the presence of FA (van Rij *et al.*, 2004). The *phzI* gene is constitutively

expressed in PCL1999, which is a PCL1391 derivative containing plasmid pMP7450 with *phzI* under the control of the constitutive *lac* promoter. This strain overproduces C₆-HSL more than ten-fold in comparison with PCL1391 (Fig. 3). It appeared that PCN production by PCL1999 was not affected by the presence of FA (Fig. 4).

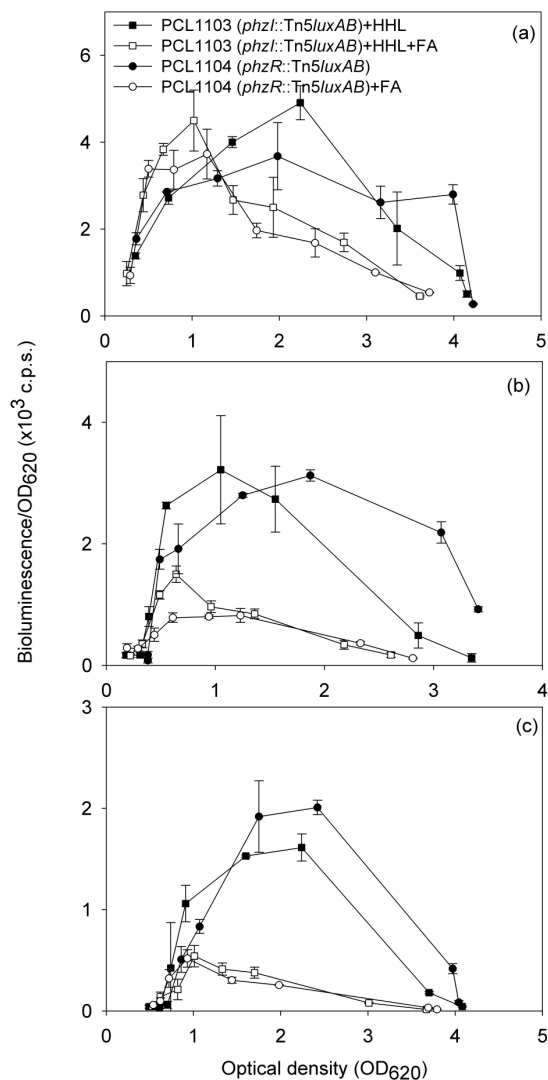


Fig. 2. Expression of *phzI* (PCL1103) with and without 5 μ M synthetic C₆-HSL (HHL) and *phzR* (PCL1104) grown in MVB1 with (a) additional 1 mM phenylalanine, (b) reduced Mg²⁺ concentration, or (c) additional 2 mM FeCl₃. Cells were grown in the absence (black symbols) and in the presence of 1 mM fusaric acid (FA) (white symbols). Luminescence values were measured in counts per second (cps) per optical density unit during growth. Averages of two samples are shown.

Constitutive expression of the second quorum sensing regulator, *phzR* was achieved by introducing pMP7447 into PCL1391. The plasmid pMP7447 contains *phzR* under constitutive expression of P_{tac} . Introduction of pMP7447 into the wild type strain PCL1391 resulted in PCL1993. FA did not reduce PCN production in PCL1993 whereas it did in strain PCL1960 which contains the empty cloning vector (Fig. 4). PCL1993 produce increased amounts of C_6 -HSL compared to PCL1391 and FA did not detectably reduce these high levels of C_6 -HSL (Fig. 3). PCL1960 increased PCN production compared to the wild type strain PCL1391 for unknown reasons but served as the correct control for the *phzI* and *phzR* over-expressing strains as it contains the empty vector.

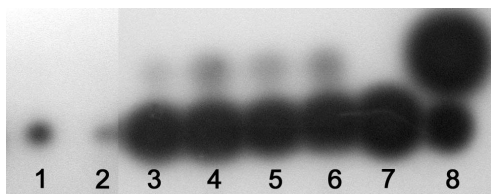


Fig. 3. Influence of fusaric acid on the production of homoserine lactone (HSL) by *P. chlororaphis* strain PCL1391. Lane 1 and 2: PCL1391; lane, 3 and 4: PCL1999 (PCL1391 containing a constitutive *phzI* gene *in trans*); lane, 5 and 6: PCL1993 (PCL1391 containing a constitutive *phzR* gene *in trans*); lane 7: synthetic C_6 -HSL standard ($2.5 \cdot 10^{-7}$ mole); lane 8: synthetic C_4 -HSL ($3 \cdot 10^{-6}$ mole) and C_6 -HSL ($2.5 \cdot 10^{-8}$ mole). Samples in lanes 2, 4 and 6 were grown in the presence of 1 mM FA. Samples were separated on C18-reverse phase TLC and visualized using *C. violaceum* strain CV026. Extracts were isolated at OD_{620} 3.0. The experiment was performed three times with similar results.

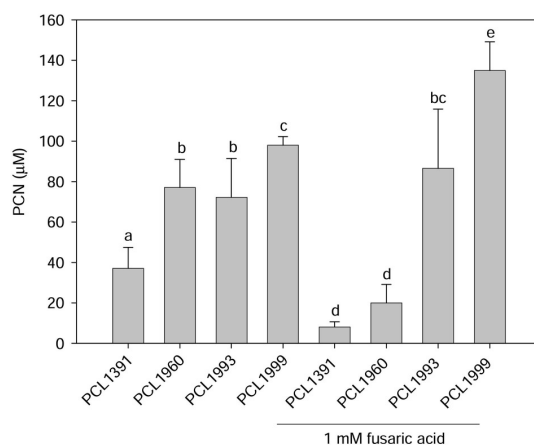


Fig. 4. Influence of constitutive expression of *phzR* and *phzI* on the inhibition of phenazine-1-carboxamide (PCN) by fusaric acid (FA). Cells of *P. chlororaphis* PCL1391 were grown on MVB1 and the PCN concentrations of the supernatant fluids were quantified by HPLC when cells reached stationary phase. Wild type strain: PCL1391, PCL1960 (PCL1391 with empty vector), PCL1993 (PCL1391 containing a constitutive *phzR* gene *in trans*), and PCL1999 (PCL1391 containing a constitutive *phzI* gene *in trans*) were grown in the absence and presence of 1 mM FA. Average and standard deviations of at least three experiments are shown. Bars followed by different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test.

Effect of FA on the expression of the PCN biosynthetic and regulatory genes

Microarray analysis allowed the identification of changes in gene transcription of the *phz* operon and the regulatory PCN-genes *phzI*, *phzR*, *gacS*, *rpoS* and *psrA* after the addition of FA. RNA was isolated from a PCL1391 culture at OD₆₂₀ 2.0, at which density the start of PCN production is detected in the medium, and was used for cDNA synthesis and fluorescent labeling. The labeled cDNA was hybridized with PCL1391 microarrays. Expression profiling showed that the expression of PCN biosynthetic genes and the quorum sensing regulatory genes *phzI* and *phzR* was severely reduced in the presence of 1 mM FA (Table 4). The highest reduction (six-fold) was observed for the PCN biosynthetic genes *phzB*, *phzC* and *phzH*. The expression of *phzI* was reduced four-fold and the expression of the transcription factor *phzR* was reduced two-fold (Table 4). Expression of the PCN regulators *rpoS*, *gacS* and *psrA* was not affected by FA (Table 4). Transcription of the PCN regulators *psrA*, *gacS*, and the biosynthetic gene *phzB* were followed during growth with the use of Tn5 *luxAB* reporter mutants (Fig. 5). Transcription of *psrA* in PCL1111 (*psrA*::Tn5*luxAB*) and transcription of *gacS* in PCL1969 (*gacS*::Tn5*luxAB*) were not influenced by FA. Transcription of the biosynthetic operon (*phzB*::Tn5*luxAB*) in PCL1119 increased following optical density whereas it did not increase in the presence of FA (Fig. 5).

Table 4. Identified genes of *P. chlororaphis* PCL1391 with altered expression caused by the presence of fusaric acid

Clone number *	Change of expression	Closest homologous gene, homologous gene in <i>P. aeruginosa</i> †	Bit score §	E-Value #	Gene accession number, organism ‡
Phenazine regulator genes of <i>P. chlororaphis</i> PCL1391					
- 6.4 ±2.2		<i>phzB</i> , biosynthetic gene for PCN			AAF17496 <i>P. chlororaphis</i>
- 6.2 ±2.7		<i>phzC</i> , biosynthetic gene for PCN			AAF17497 <i>P. chlororaphis</i>
-6.4 ±3.2		<i>phzH</i> , biosynthetic gene for PCN			AAF17502 <i>P. chlororaphis</i>
- 3.6 ±0.7		<i>phzI</i> , autoinducer synthase			AAF17493 <i>P. chlororaphis</i>
- 2.1 ±0.5		<i>phzR</i> , transcriptional activator			AAF17494 <i>P. chlororaphis</i>
+ 1.1 ±0.4		<i>gacS</i> , sensor kinase			AAM52311 <i>P. chlororaphis</i>

	- 1.1	<i>rpoS</i> , stationary phase alternative			AAT46075
	±0.2	sigma factor			<i>P. chlororaphis</i>
	1.0	<i>psrA</i> , transcriptional regulator			AAM52309
	±0.2				<i>P. chlororaphis</i>
Outer membrane ferripyoverdin receptor					
36_B6	+ 17	Outer membrane ferripyoverdin	258	9e-68	NP_746334.1
	±8.6	receptor, <i>fpvA</i> (PA2398)			<i>P. putida</i>
40_C11	+ 19	Membrane ferripyoverdin	224,	4e-	NP_746334.1
	±7.3	receptors, <i>pvdE</i> (PA2397) and	200	57,	<i>P. putida</i> ,
		<i>fpvA</i> (PA2398)		7e-50	NP_251087.1
					<i>P. aeruginosa</i>
62_H8	+ 19	Outer membrane ferripyoverdin	265	8e-70	NP_746334.1
	±3.6	receptor, <i>fpvA</i> (PA2398)			<i>P. putida</i>
109_H1	+ 20	Outer membrane ferripyoverdin	228	2e-58	NP_746334.1
0	±16	receptor, <i>fpvA</i> (PA2398)			<i>P. putida</i>
110_D7	+ 18	Outer membrane ferripyoverdin	446	e-124	NP_746334.1
	±12	receptor, <i>fpvA</i> (PA2398)			<i>P. putida</i>
60_D12	+ 26	Outer membrane ferripyoverdin	227	2e-82	NP_252857.1
	±10	receptor B, <i>fpvB</i> (PA4168)			<i>P. aeruginosa</i>
Non ribosomal peptide and pyoverdin synthetase					
3_C9	+ 9.2	Non-ribosomal peptide synthetase,	584	e-165	ZP_00140124.1
	±3.0	<i>pvdI</i> (PA2402)			<i>P. aeruginosa</i>
21_C4	+ 11	Non-ribosomal peptide synthetase,	438	e-121	ZP_00092691.1
	±2.8	<i>pvdD</i> (PA2399)			<i>Azotobacter</i>
					<i>vinelandii</i>
127_A3	+ 12	Non-ribosomal peptide synthetase,	162	3e-39	ZP_00092691.1
	±6.7	<i>pvdD</i> (PA2399)			<i>Azotobacter</i>
					<i>vinelandii</i>
68_C11	+ 9.3	Non-ribosomal peptide synthetase,	459	e-128	ZP_00265634.1
	±7.5	<i>pvdI</i> (PA2402)			<i>P. fluorescens</i>
89_H9	+ 9.0	Non-ribosomal peptide synthetase,	437	e-121	ZP_00265634.1
	±8.2	<i>pvdI</i> (PA2402)			<i>P. fluorescens</i>
116_G1	+ 8.9	Non-ribosomal peptide synthetase,	385	e-105	ZP_00265634.1
0	±8.4	<i>pvdI</i> (PA2402)			<i>P. fluorescens</i>
118_C1	+15.3	Non-ribosomal peptide synthetase,	282	e-113	ZP_00265634.1
0	±17	<i>pvdI</i> (PA2402)			<i>P. fluorescens</i>
53_G8	+11	Siderophore non-ribosomal	257	4e-87	CAC32046.1
	±4.3	peptide synthetase, <i>pvdJ</i>			<i>P. putida</i>
		(PA2400)			

68_H4	+13 ± 11	Non-ribosomal peptide synthetase, <i>pvdJ</i> (PA2400)	307	2e-82	ZP_00092691.1 <i>Azotobacter vinelandii</i>
65_C5	+42 ±21	L-ornithine <i>N</i> ⁵ -hydroxylase, <i>pvdA</i> (PA2386)	284	9e-76	AAG27518.1 <i>Pseudomonas</i> sp.
Cytochrome C					
12_B5	-6.0 ±2.4	Cbb3-type cytochrome oxidase, subunit 1, (PA1557)	765	0.0	ZP_00265650.1 <i>P. fluorescens</i>
39_H8	-2.9 ±0.5	Cytochrome C (PA1552) and Cbb3-type cytochrome c oxidase, subunit1, (PA1554)	290, 214	6e- 77, 4e-54	NP_250243.1 <i>P. aeruginosa</i> , NP_746371.1 <i>P. putida</i>
77_B7	-3.2 ±0.8	Cytochrome C, (PA1552)	276	3e-89	ZP_00265653.1 <i>P. fluorescens</i>
104_H4	-6.3 ±3.0	Cbb3-type cytochrome oxidase, subunit 1, (PA1554)	177	5e-43	ZP_00263212.1 <i>P. fluorescens</i>
Fumarase					
50_A5	+27 ±13	Fumarase, <i>fumC1</i> (PA4470)	452	e-126	ZP_00266219.1 <i>P. fluorescens</i>
83_B11	+10 ±6	Fumarase C, <i>fumC1</i> (PA4470)	253	9e-66	AAD28162.1 <i>P. putida</i>
tRNA synthetase					
59_H6	-5.6 ±2.9	Valyl-tRNA synthetase, -	110	3e-23	ZP_00124212.1 <i>P. syringae</i>
82_H9	-6.9 ±4.0	Alanyl-tRNA synthetase, <i>alaS</i> (PA0903)	401	e-111	NP_746585.1 <i>P. putida</i>
Upregulated by FA					
75_B12	+5.2 ±1.9	Hypothetical protein and hypothetical protein,-	357, 82	2e- 97, 1e-14	ZP_00087993.1 <i>P. fluorescens</i> , ZP_00127496.1 <i>P. syringae</i>
77_C5	+10 ±9.1	Polyferredoxin, (PA4131)	273	1e-71	ZP_00265347.1 <i>P. fluorescens</i>
Downregulated by FA					
14_C10	-5.9 ±2.7	Conserved hypothetical protein and cation ABC transporter, (PA2406) and periplasmic cation-binding protein, (PA2407)	191, 418	5e- 47, e-115	NP-791964.1 <i>P. syringae</i> , NP_791963.1 <i>P. syringae</i>

15_H6	-6.2 ±3.1	Oxygen-independent coproporphyrinogen III dehydrogenase, <i>hemN</i> (PA1546) and putative membrane protein, (PA1547)	267, 249	2e- 70, 7e-65	AF321091_1 <i>P. putida</i> , NP_746379.1 <i>P. putida</i>
82_H10	-3.7 ±0.9	Methyl-accepting chemotaxis protein (PA2867), and hypothetical protein, (PA2868)	421, 171	e- 116, 6e-41	ZP_00264179.1 <i>P. fluorescens</i> , ZP_00264180.1 <i>P. fluorescens</i>
5_D11	-6.1 ±1.5	Hypothetical protein, -	258	1e-67	ZP_00263210.1 <i>P. fluorescens</i>
20_E8	-5.0 ±2.9	Hypothetical protein, (PA4292)	133	4e-30	ZP_00085666.1 <i>P. fluorescens</i>
116_D5	-5.3 ±1.9	Hypothetical protein,-	115	1e-24	NP_233235.1 <i>Vibrio cholerae</i>

* The clone number refers to the number in the library (plate number, row and column). All clones selected in this table corresponded to genes of which the expression was decreased (-) or increased (+) by 1 mM fusaric acid.

† Closest homologous gene to the sequenced microarray clone and the closest homologous gene in *P. aeruginosa* PAO1. If a clone is not homologous to a gene in *P. aeruginosa* this is indicated by (-).

§ Higher Bit score indicates larger region of similarity with the homologues gene shown in column three.

Lower E-values indicate higher similarity of the homologues gene shown in column three.

‡ Gene accession number and organism of the homologues gene shown in column three.

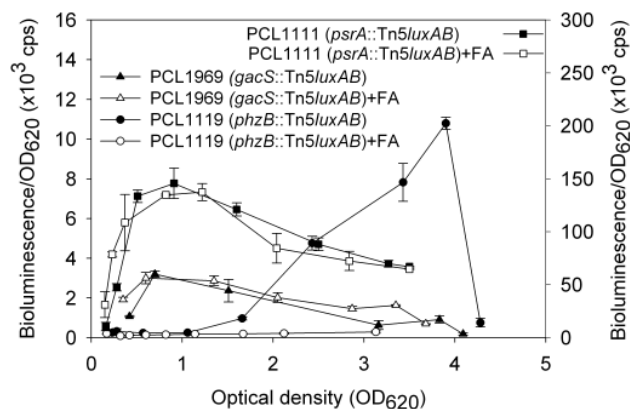


Fig. 5. Expression of *psrA* (PCL1111, right Y-axis), *gacS* (PCL1969, left Y-axis) and *phzB* (PCL1119, left Y-axis) in *P. chlororaphis* PCL1391. Cells were grown in MVB1 in the absence (black symbols) and in the presence of FA (white symbols). Luminescence values were measured in counts per second (cps) per optical density unit during growth. Averages of two samples are shown.

Effects of FA on the transcriptome of *P. chlororaphis* PCL1391

Microarrays containing genomic fragments of PCL1391 were used to reveal the effect of FA on its transcriptome. Expression of 17 % (2115 clones) of the 12,000 clones spotted was detected. Clones with two-fold difference in expression after the addition of FA were selected resulting in 98 clones (4.6 %) that were up-regulated and 112 clones (5.3 %) that were down-regulated. The raw microarray data are shown in the electronic supplement (<http://www.ibl.leidenuniv.nl/index.php3?c=212>). A total of 32 clones with altered expression ratios was sequenced, ORF's were identified and analyzed using BLAST (Altschul et al., 1990). Genes with the highest similarity to the sequenced clones, gene accession numbers and corresponding organisms are shown in Table 4. Column three includes the most similar gene and annotation in *P. aeruginosa* behind the name of the gene with highest similarity in order to compare our results with microarray studies of *P. aeruginosa*. Clones that were up- or down-regulated by FA can be divided in seven main classes. Class one has the highest similarity with a putative outer membrane ferripyoverdin receptor from *P. putida* KT2440 and is also similar to the main ferripyoverdin receptor *fpvA* of *P. aeruginosa*. Clone 60_D12 was also included in this sub-group as it is highly homologous to the second outer membrane ferripyoverdin receptor *fpvB* in *P. aeruginosa* (Ghysels et al., 2004). The second class contains genes that show homology to non-ribosomal peptide synthetase (NRPS) and pyoverdin synthetase genes. Clones 21_C4 and 127_A3 belong to the same ORF which has the highest similarity with a NRPS of *Azotobacter vinelandii* and to a lesser extent with the NRPS for pyoverdin biosynthesis *pvdD* from *P. aeruginosa*. Clones 3_C9, 68_C11, 89_H9, 116_G10, and 118_C10 have the highest similarity with a NRPS of *P. fluorescens* and reduced similarity with the NRPS for pyoverdin *pvdI* from *P. aeruginosa*. Clone 65_C5 is similar to a L-ornithine *N*⁵-hydroxylase gene which is involved in the modification of the peptide chain of pyoverdin. The outer membrane ferripyoverdin receptor and the pyoverdin synthetases are up-regulated by FA and involved in production or uptake of pyoverdin in *P. aeruginosa* (Ravel and Cornelis, 2003). Class three contains ORF's with similarity to cytochrome C genes, which are all down-regulated by FA. Class four consists of two clones which have similarity with fumarase genes. The two clones with similarity to valyl and alanyl-tRNA synthetase were grouped in class five. Up-regulated clones that occurred once were gathered in class six, which contains ORF's with similarity to two hypothetical proteins on clone 75_B12 and a polyferredoxin on clone 77_C5. Class seven contains the remaining down-regulated clones. Genes on these clones are similar to a cation ABC transporter, a periplasmic cation-binding protein, an oxygen-independent coproporphyrinogen III dehydrogenase, a putative membrane protein, a methyl-accepting chemotaxis protein, and three hypothetical proteins, respectively (Table 4).

Discussion

In the rhizosphere, biocontrol strains are constantly interacting with their environment which includes other microbes. In this study we focused on the effect of the fungal toxin fusaric acid (FA) on *P. chlororaphis* PCL1391. One of our questions was whether FA could repress PCN biosynthesis under environmental conditions that favor PCN production. Under all three PCN stimulating conditions, 1 mM phenylalanine, 2 mM FeCl₃ and a low Mg²⁺, FA reduced PCN levels. The PCN production in the presence of FA was not elevated by an additional 2 mM FeCl₃ or a low Mg²⁺ concentration. Combining both phenylalanine and FA resulted in intermediate PCN levels compared to only FA or phenylalanine (Table 2). This shows that FA represses PCN production under different environmental conditions and can be influenced by some environmental changes. Transcriptional analyses of *phzI* and *phzR* under these different environmental conditions demonstrated that FA repressed their transcription in all conditions (Fig. 2) and confirmed that *phzI* and *phzR* expression is necessary for optimal PCN production. Repression of *phzI* and *phzR* by FA was highest in MVB1 with a low Mg²⁺ concentration or additional iron. These two conditions also resulted in the largest repression of PCN production by FA (Table 2).

When the C₆-HSL concentration was increased by constitutive expressing *phzI* (strain PCL1999), PCN levels were no longer reduced by the addition of FA (Fig. 3 and 4). Constitutive expression of *phzR* also led to an increased production of HSL's (Fig. 3). Increasing concentrations of the transcriptional regulator PhzR stimulate expression of *phzI* and therefore increase C₆-HSL production. Transcription studies with Tn5*luxAB* reporter mutants and microarray analyses showed that the expression of the biosynthetic phenazine-operon and of the quorum sensing regulators *phzI* and *phzR* are reduced by FA (Table 4, Fig. 1 and 5). This reduction in gene expression explains the reduced levels of PCN in the presence of FA and suggests that repression of PCN production occurs upstream of the biosynthetic *phz* operon and of *phzI*. FA could directly or indirectly repress *phzR* expression and thereby reduce the expression of *phzI* and biosynthesis of C₆-HSL. Expression of the PCN regulators *gacS*, *rpoS* and *psrA* was not altered by FA (Table 4 and Fig. 5), which suggests that FA acts parallel or downstream of these genes.

The microarray allowed identification of genes differentially expressed by FA. Because the genome of PCL1391 has not been sequenced, a microarray containing only random fragments of genomic DNA and control genes was available (see Methodes). Sequencing revealed that the same genes were present in different selected clones spotted several times on the microarray, thereby internally validating the analyses (Table 4).

Comparison of our data with those of the literature suggests a correlation between treatments with FA and iron limitation. Genes up-regulated by FA in *P. chlororaphis* were induced by iron limitation in *P. aeruginosa*. Clones of class one are homologous to outer membrane ferripyoverdin receptors *fpvA* (PA2398) and *fpvB* (PA4168) (Ghysels et al., 2004) of *P. aeruginosa* and are up-regulated by iron limitation (Ochsner et al., 2002; Palma et al., 2004). These outer membrane ferripyoverdin receptors have similarity with the TonB system that was shown to be involved in tolerance to solvents and drugs and thought to play a role in drug exclusion (Godoy et al., 2001). This could explain the up-regulation of the clones in this class in response to FA. The pyoverdin-biosynthetases genes found to be up-regulated by FA are homologous to the non-ribosomal peptide synthetases *pvdI* (PA2402) *pvdJ* (PA2400), and *pvdD* (PA2399) and the pyoverdin modification gene *pvdA* (PA2386) (Ochsner et al., 2002; Palma et al., 2003). All of these genes are induced by iron limitation in *P. aeruginosa*. Up-regulation of these genes involved in the biosynthesis and uptake of the pyoverdin can be explained in relation to iron starvation but is less obvious in relation to FA stress. Iron limitation increased *fumC1* (PA4470) and decreased *hemN* (PA1546) expression in *P. aeruginosa* (Ochsner et al., 2002; Palma et al., 2003) and FA treatment also increase fumarase (*fumC1*) and decreased oxygen-independent coproporphyrinogen III dehydrogenase (*hemN*) expression in *P. chlororaphis*. Remarkably, clone 14_C10 is repressed by FA but has similarity with PA2406 and PA2407 which are also part of the pyoverdin cluster of *P. aeruginosa* and that are up-regulated by iron limitation (Ochsner et al., 2002). This is the only sequenced clone that is regulated oppositely when comparing the expression profile of FA stress and iron limitation. Interestingly, FA does not seem to stimulate the transcription of all the genes induced by iron limitation, but seems to stimulate the pyoverdin system more specifically.

Although FA induces many genes involved in the uptake of iron, an increased production of siderophores was only detected during iron limitation and not during FA stress of *P. chlororaphis* PCL1391 (data not shown). This suggests that FA stress does not result in a complete iron starvation response. FA has ferric ion chelating characteristics (Bochner et al., 1980) which could explain the repression of PCN by FA since iron limitation represses PCN production. However, an excess of ferric ions (2 mM FeCl_3) did not restore PCN production. A second experiment also contradicts this hypothesis, since high affinity iron chelator EDDHA (0.1 and 1.0 mM) did not mimic FA stress and did not repress PCN biosynthesis (Table 2). FA also chelates other divalent cations including zinc and this could cause PCN repression by FA. However, an excess of 2mM ZnSO_4 or ZnCl_2 does not restore PCN production in the presence of FA (Table 3) and refutes this possibility.

FA and iron limitation reduce PCN production in PCL1391 (van Rij et al., 2004) and have partially overlapping expression profiles but FA is not causing or mimicking iron limitation. This suggests that the two stress conditions FA and iron limitation trigger partially over-lapping regulatory cascades that result in the repression of PCN biosynthesis.

Methods

Microorganisms and culture conditions.

The microbial strains and plasmids used are listed in Table 1. Luria-Bertani (LB) medium (Sambrook and Russel, 2001) was used as standard medium for culturing *Escherichia coli* and *Chromobacterium violaceum*. A modified Vogel Bonner medium (van Rij et al., 2004; Vogel and Bonner, 1956) was used to culture *Pseudomonas* cells. Modified Vogel-Bonner salts # 1 (MVB1) contained final concentrations of 30 mM glucose, 0.05 % (w/v) casamino acids, 57 mM K₂HPO₄, 16 mM Na(NH₄)HPO₄, 81 mM MgSO₄, 78 µM NaFeEDTA(III), 6.8 µM MnSO₄, 0.35 µM CuSO₄, 4.1 µM Na₂MoO₄, 0.85 µM ZnSO₄, 51 µM H₃BO₃ and the pH was adjusted to 6.6 using HCl (van Rij et al., 2004). If indicated phenylalanine or fusaric acid (Acros, Geel, Belgium) was added to MVB1 at a final concentration of 1.0 mM. The magnesium concentration was modified by replacing MgSO₄ by 0.8 mM K₂SO₄ and 0.08 mM MgCl₂. Solidified growth media contained 1.8 % (w/v) agar (Difco Laboratories, Detroit, MI). If appropriate, media were supplemented with the antibiotics kanamycin or gentamycin at final concentrations of 50 and 30 µg ml⁻¹, respectively. Cultures were shaken at 195 rpm on a Janke und Kunkel shaker KS501D (Staufen, Germany) at 28 °C and growth was monitored by measuring OD₆₂₀. Growth analyses and RNA isolation were conducted in 100 ml flasks with 10 ml media after inoculation from an appropriate 5 ml overnight culture to an OD₆₂₀ of 0.1.

Analyses of bioluminescent Tn5/*luxAB* reporter strains

Expression of Tn5/*luxAB*-tagged genes was determined by quantification of *luxAB* activity during culturing. Samples of 100 µl were taken in duplicate and a volume of 100 µl *n*-decyl-aldehyde substrate solution that contains 0.2 % (v/v) *n*-decyl-aldehyde (Sigma, St. Louis) and 2.0 % (w/v) bovine serum albumin. Bioluminescence was determined using a luminescence counter (MicroBeta 1450 TriLux, Wallac, Turku, Finland).

DNA manipulation

Polymerase chain reactions were carried out with Super Taq enzyme (Enzyme Technologies Ltd, UK). The primers were synthesized by Isogen Life Science (Maarsse, The Netherlands). Restriction enzymes were purchased from New England BioLabs Inc. (Westburg, Leusden, The Netherlands) and ligase from Promega (Leiden, The Netherlands). To PCR the *phzI* gene under the *P_{lac}* promoter two primers (oMP812: 5'-ATATATGAATTCAATTGTGAGCGGATAACAATTT CACACAGGAAACAGGATCCTAAAATGATGCACATGGAAGAGCACACACTGAACGG-3'

and oMP813: 5'-ATATATGAATTCCGGCGTGATCATGGGGGTGTGCACCG-3') were used. This PCR fragment was digested with *EcoRI* and ligated into *EcoRI*-digested pBBR1-MCS5 to obtain pMP7450. A PCR fragment with the *phzR* gene under control of the P_{lac} promoter was constructed (Girard, *in preparation*) by using two primers (oMP777: 5'-ATATATCTCGAGTTGACAATTAATCATCGGCTC GTATAATGTGTGGAATTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGAG TTAGGGCAGCAGTTGGGATGGG-3' and oMP778: 5'-ATATATGAATTCCC CCTCAGATATAGCCCATCGCAACTGCG-3'). This PCR fragment was digested with *XhoI* and *EcoRI* and ligated into *XhoI* and *EcoRI* digested pBBR-MCS5 to obtain pMP7447.

Analysis of phenazine-1-carboxamide (PCN) production

For monitoring PCN production in time, PCN was extracted according to Chin-A-Woeng *et al.* (1998) with minor modifications. Culture samples (250 μ l) were centrifuged and the culture supernatants were acidified to pH 2 using 6 M HCl. They were subsequently extracted with an equal volume of toluene by shaking on an Eppendorff mixer 5432 for 5 min. After centrifugation, the toluene phase was collected and dried in a rotary evaporator. The dry residue was dissolved in 100 μ l acetonitrile and the obtained solution was mixed with 400 μ l water.

PCN concentrations were determined by high-performance liquid chromatography (HPLC) (DIONEX, Sunnyvale, CA, chromeleon software version 6.20) using a calibration curve. HPLC was performed using an econosphere C18 5u, 259 mm x 4.6 mm column (Alltech Associates, Inc, Deerfield, IL) at 30 °C with a linear gradient of 20-80 % (v/v) acetonitrile acidified with 0.1 % (v/v) trifluoroacetic acid in water and a flow rate of 1 ml min⁻¹.

Analysis of *N*-acyl-homoserine lactone (AHL) production

Culture supernatants were adjusted to pH 9.0 by NaOH. Supernatants were extracted with 0.5 volume of ethyl acetate, shaken for half an hour and the solvent phase was collected. Subsequently the solvent phase was dried by rotary evaporation. The dried residue was dissolved in 25 μ l acetonitrile and analyzed using TLC. Samples spotted on C18 TLC plates (Merck, Darmstadt, Germany) were developed in methanol-water (60:40) (v/v). After development, the TLC was overlaid with LB 0.8 % (w/v) agar containing a 10-fold diluted overnight culture of the *Chromobacterium violaceum* indicator strain CV026 (McClellan *et al.*, 1997) and kanamycin (50 μ g ml⁻¹). After incubation for 48 h at 28 °C, chromatograms were analyzed for appearance of violet spots.

Construction of a chromosomal microarray of *P. chlororaphis* PCL1391

Microarray construction, RNA isolation, and data analyses were performed according to Girard *et al.* (*In preparation*). Microarray analyses were performed according to the MIAME standards (Brazma *et al.*, 2001). The chromosomal microarray consists of approximately 12,000 random chromosomal *Saullia*, DNA fragments from PCL1391 varying in size between 0.4 kb and 2 kb. After an initial cloning of the chromosomal fragments into pBluescript, each insert was amplified with amino labeled primers annealing to the multicloning site flanking the insert. PCR products were spotted by on poly-L-lysine coated glass slides with a Genemachines Omnigrid 100 spotter (Genomic Solutions, Isogen Life Science, Maarssen, The Netherlands). The microarrays were designed to contain the following controls: empty spots (neither DNA, nor buffer), spots with only 50 % (v/v) DMSO, a negative control with λ phage DNA (Westburg, Leusden, The Netherlands), and PCR products of several known genes of PCL1391: *phzB*, *phzH*, *phzR*, *phzI*, *sss*, *gacS*, and *psrA* (Chin-A-Woeng *et al.*, 2000; Chin-A-Woeng *et al.*, 2001a; Chin-A-Woeng *et al.*, 2001b; Chin-A-Woeng *et al.*, 2005).

RNA preparation and microarray processing

RNA was extracted from cultures at an OD₆₂₀ of 2.0 with an adapted protocol developed by Jon Bernstein (URL http://bugarrays.stanford.edu/protocols/rna/Total_RNA_from_Ecoli.pdf). After phenol/chlorophorm extraction, the supernatant was applied on columns from the RNeasy Midi kit (Qiagen, Westburg, Leusden, The Netherlands), and the RNA was extracted following the protocol supplied by the manufacturer, including the DNase step. RNA purity was verified on 1.2 % (w/v) agarose gel following the protocol of the RNeasy Midi kit. RNA was immediately used for cDNA probe generation using the CyScribe post-labelling kit (Amersham Biosciences, Roosendaal, The Netherlands). Each reaction was performed with 30 μ g of total RNA and random nanomers. After purification, the efficiency of Cy label incorporation into the cDNA and the quality and amounts of labeled cDNA were verified with a Ultrospec 2100 pro spectrophotometer (Amersham Biosciences). The amounts of Cy-labeled cDNA were calculated using http://www.pangloss.com/seidel/Protocols/percent_inc.html. Equal amounts of cDNA for both Cy3 and Cy5 labels, with a minimum of 40 pM of each dye, were hybridized. Before hybridization, the microarrays were rehydrated by H₂O steam at 50 °C and snap-cooled on a hot plate. Then they were UV-crosslinked at 250 mJoules cm⁻² (Amersham LifeSciences UV cross linker). They were subsequently prehybridized with 100 μ l prehybridization buffer (0.4 μ g μ l⁻¹ herring sperm DNA (Gibco, Invitrogen, Breda, The Netherlands), 0.4 μ g μ l⁻¹ yeast tRNA (Gibco, Invitrogen, Breda, The Netherlands), 5X Denhardt's (Denhardt, 1966), 3.2X

SSC (sodium chloride/sodium citrate buffer, 3 M NaCl - 0.3 M (Na)₃-citrate, 0.4 % (w/v) SDS) for 2 minutes at 80 °C and 30 minutes at 65 °C. Finally, the slides were washed at room temperature in 2X SSC, twice in 70 % (v/v) ethanol, once in 90 % (v/v) and 100 % (v/v) ethanol (5 minutes per wash step) and air dried. The Cy-labeled cDNA was hybridized overnight at 65 °C in a GeneTAC Hybstation (Genomic Solutions). After hybridization, the slides were washed in 2X SSC, 0.1 % (w/v) SDS for 5 minutes at 30 °C, in 0.5X SSC for 5 min at 25°C and in 0.2X SSC for 5 min at 25 °C. The slides were dehydrated by washing in 70 %, 90 % and 100 % (v/v) ethanol (1 minute per wash step), dried by compressed air and scanned in a G2565AA Microarray Scanner (Agilent, Amstelveen, The Netherlands). The experiment was repeated at least 4 times, including dye swaps.

Microarray data analysis

After scanning, the microarrays were analyzed in GenePix Pro version 5.0 (Molecular Devices Corporation, Union City, USA). The following criteria were implemented to select spots corresponding to genes assumed to be affected significantly in their expression by FA: spots were considered as interesting if the mean of the ratio of red and green laser intensities was higher than 2 (Ratio of Medians (650/550) > 2) or lower than 0.5, but positive, (Ratio of Medians (650/550) < 0.5 and Ratio of Medians (650/550) > 0). In both cases, the spots were selected only if they had at least 80% of their feature pixels more than two standard deviations above background in both the green or red channel ([% > B550+2SD] > 80 and [% > B650+2SD] > 80). Results of four identical microarray experiments were compared and spots were categorized in three groups (i) unaltered expression by FA (ii) more than two fold up-regulated by FA (iii) more than two fold down regulated by FA. The values were normalized assuming that most genes of the array are not differentially expressed. Results of four microarray experiments were used in our analyses. Raw microarray data, microarray image, and sequences of clones are available at <http://www.ibl.leidenuniv.nl/index.php3?c=212>.

ippA, a novel gene involved in the regulation of phenazine-1-carboxamide production by *Pseudomonas chlororaphis* strain PCL1391

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Summary

The antifungal metabolite phenazine-1-carboxamide (PCN) produced by the biocontrol bacterium *Pseudomonas chlororaphis* PCL1391 is essential for the suppression of tomato foot and root rot caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Here we describe the identification and characterization of a novel gene, designated as *ippA*, which is involved in the induction of PCN production. Homologues of *ippA* and an identical organization of genes flanking *ippA* were also found in other sequenced *Pseudomonas* spp. genomes, suggesting an evolutionary conserved locus. In strain PCL1971, an *ippA* mutant of *P. chlororaphis* PCL1391, production of PCN and the quorum sensing signal *N*-hexanoyl-L-homoserine lactone (C₆-HSL) is strongly decreased. Constitutive expression of one of the quorum sensing genes *phzI* or *phzR* increases the production of C₆-HSL and complements for the loss of PCN production. Previously, the *Pseudomonas* sigma regulator *psrA* and the stationary phase sigma factor *rpoS* were shown to stimulate PCN production in PCL1391, but constitutive expression of these genes in the *ippA* mutant does not restore PCN production. In contrast, constitutive expression of the recently discovered phenazine inducing gene *pip* restores PCN levels in the *ippA* mutant. This suggests that *ippA* acts down stream of *psrA* and *rpoS* and upstream of *pip* in the PCN regulatory cascade.

Introduction

The soil bacterium *Pseudomonas chlororaphis* PCL1391 is an excellent biocontrol strain of tomato foot and root rot caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Chin-A-Woeng et al., 1998). Production of the antifungal metabolite phenazine-1-carboxamide (PCN) and root colonization are essential traits for the biocontrol ability of *P. chlororaphis* PCL1391 (Chin-A-Woeng et al., 1998). Production of phenazines in *Pseudomonas* biocontrol strains is quorum-sensing dependent (Chin-A-Woeng et al., 2001b; Khan et al., 2005; Wood et al., 1997). The quorum-sensing regulatory genes *phzI* and *phzR*, which are homologous to *luxI* and *luxR* respectively, are essential for the production of PCN. PhzI is supposed to be the enzyme that produces the quorum sensing signal molecule *N*-hexanoyl-L-homoserine lactone (C₆-HSL). Binding of C₆-HSL to the transcriptional regulator PhzR is thought to induce the expression of the biosynthetic *phz* operon and to increase *phzI* expression by binding to conserved palindromic sequences in the promoter regions called *phz* boxes (Chin-A-Woeng et al., 2001b). The stimulation of *phzI* expression results in the amplification of C₆-HSL production. Previously, constitutive expression of *phzI* and *phzR* was shown to induce the production of PCN under conditions that repress PCN biosynthesis (van Rij et al., 2005).

Quorum sensing in PCL1391 is controlled by a regulatory pathway, which starts with the global regulatory GacS/GacA (Chin-A-Woeng et al., 2005; Girard et al., 2006). The Gac system also controls phenazine production in strains *P. fluorescens* Pf-5 (Sarniguet et al., 1995) and *P. aeruginosa* (Suh et al., 1999). The GacS/A system stimulates expression of *psrA* (*Pseudomonas* sigma regulator) (Kojic and Venturi, 2001) and subsequently *PsrA* stimulates expression of the stationary phase sigma factor *rpoS* in PCL1391 (Girard et al., 2006). Recently *pip*, the gene product of which shows homology to members of the TetR family of regulatory proteins, was identified to stimulate phenazine production in *P. chlororaphis* PCL1391 (G. Girard, unpublished). Expression of *pip* was shown to be controlled by RpoS, and Pip stimulates expression of *phzI* and *phzR* (G. Girard, unpublished).

In this paper we describe the identification and characterization of *ippA*, a novel gene that stimulates PCN production in *P. chlororaphis* strain PCL1391. The role of *ippA* in the regulatory pathway of PCN production was investigated by using constructs that constitutively express previously identified PCN regulatory genes.

Table 1. Micro-organisms and plasmids used in this study.

Strains or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
PCL1114	PCL1391 <i>psrA</i> ::Tn5 <i>luxAB</i> , Km ^r	Chin-A-Woeng et al. (2005)
PCL1391	Wild-type biocontrol strain <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide	(Chin-A-Woeng et al., 1998)
PCL1957	Derivative of PCL1391 <i>rpoS</i> ::Tn5 <i>luxAB</i> containing pBBR1MCS-5, Km ^r Gm ^r	(Girard et al., 2006)
PCL1962	Derivative of PCL1391 <i>psrA</i> ::Tn5 <i>luxAB</i> containing pBBR1MCS-5, Km ^r Gm ^r	(Girard et al., 2006)
PCL1960	Derivative of PCL1391 containing the cloning vector pBBR1MCS-5, Gm ^r	(Girard et al., 2006)
PCL1971	PCL1391 <i>ippA</i> ::Tn5 <i>luxAB</i> , Km ^r	This study
PCL1990	PCL1391 derivative mutated in <i>ippA</i> by single homologous recombination using pMP7585, Km ^r	This study
PCL1993	Derivative of PCL1391 harboring pMP7447 (<i>P</i> _{tac} <i>phzR</i> in pBBR1MCS-5), Gm ^r	(Girard et al., 2006)
PCL1999	Derivative of PCL1391 harboring pMP7450 (<i>P</i> _{tac} <i>phzI</i> in pBBR1MCS-5), Gm ^r	(van Rij et al., 2005)

PCL2011	Derivative of PCL1391 <i>pip::Tn5luxAB</i> containing pBBR1MCS-5, Km ^r Gm ^r	G. Girard, (unpublished)
PCL2027	Derivative of PCL1971 containing pMP7447 (<i>P_{tac} phzR</i> in pBBR1MCS-5), Km ^r Gm ^r	This study
PCL2028	Derivative of PCL1971 containing pMP7450 (<i>P_{tac} phzI</i> in pBBR1MCS-5), Km ^r Gm ^r	This study
PCL2029	Derivative of PCL1971 containing pBBR1MCS-5, Km ^r Gm ^r	This study
PCL2053	Derivative of PCL1971 containing pMP7420 (<i>P_{tac} rpoS</i> in pBBR1MCS-5), Km ^r Gm ^r	This study
PCL2055	Derivative of PCL1971 containing pMP7455 (<i>P_{tac} pip</i> in pBBR1MCS-5), Km ^r Gm ^r	This study
PCL2057	Derivative of PCL1971 containing pMP7465 (<i>P_{tac} psrA</i> in pBBR1MCS-5), Km ^r Gm ^r	This study
PCL2061	Derivative of PCL1971 containing pMP7464 (<i>P_{tac} ippA</i> in pBBR1MCS-5), Km ^r Gm ^r	This study
PCL2060	Derivative of PCL1391 containing pMP7464 (<i>P_{tac} ippA</i> in pBBR1MCS-5), Gm ^r	This study
PCL2070	Derivative of PCL1391 <i>psrA::Tn5luxAB</i> containing pMP7464 (<i>P_{tac} ippA</i> in pBBR1MCS-5), Km ^r Gm ^r	This study
PCL2071	Derivative of PCL1114 containing pMP7464 (<i>P_{tac} ippA</i> in pBBR1MCS-5), Km ^r Gm ^r	This study
PCL2072	Derivative of PCL1391 <i>pip::Tn5luxAB</i> containing pMP7464 (<i>P_{tac} ippA</i> in pBBR1MCS-5), Km ^r Gm ^r	This study
PCL2075	PCL1391 derivative mutated in <i>cop</i> by single homologous recombination using pMP7480, Km ^r	This study
CV026	<i>Chromobacterium violaceum</i> . <i>N</i> -acyl-homoserine lactone (HSL) reporter strain, Km ^r	(McClellan et al., 1997)
DH5 α	<i>Escherichia coli</i> SupE44 Δ lacU169(Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi1 relA1</i>	(Boyer and Roulland-Dussoix, 1969)
Plasmids		
pRL1063a	Plasmid harboring a Tn5 transposon containing a promoterless <i>luxAB</i>	(Wolk et al., 1991)
pBBR1MCS-5	General purpose cloning vector, Gm ^r	(Kovach et al., 1995)
pRK2013	Helper plasmid for tri-parental mating	(Ditta et al., 1980)
pMP5285	pME3049 derivative, lacking the Hg ^r gene used for single homologous recombination; Km ^r	(Kuiper et al., 2001b)
pMP7420	pBBR1MCS-5 containing the <i>rpoS</i> gene of PCL1391 under control of the <i>tac</i> promoter.	(Girard et al., 2006)

pMP7447	pBBR1MCS-5 containing the <i>phzR</i> gene of PCL1391 under control of the <i>tac</i> promoter, inserted between the <i>XhoI</i> and <i>EcoRI</i> sites	(Girard et al., 2006)
pMP7450	pBBR1MCS-5 containing the <i>phzI</i> gene of PCL1391 downstream of the <i>lac</i> promoter, inserted between the <i>EcoRI</i> sites	(van Rij et al., 2005)
pMP7455	pBBR1MCS-5 containing the <i>pip</i> gene of PCL1391 under control of the <i>tac</i> promoter.	G. Girard, (unpublished)
pMP7464	pBBR1MCS-5 containing the <i>ippA</i> gene of PCL1391 under control of the <i>tac</i> promoter	This study
pMP7465	pBBR1MCS-5 containing the <i>psrA</i> gene of PCL1391 under control of the <i>tac</i> promoter	(Girard et al., 2006)
pMP7480	pMP5285 based suicide plasmid containing a PCR fragment from <i>cop</i> , Km ^r	This study
pMP7585	pMP5285 based suicide plasmid containing a PCR fragment from <i>ippA</i> , Km ^r	This study

Results

Isolation of PCL1971, a mutant strain of *P. chlororaphis* impaired in the production of PCN and C₆-HSL

Production of PCN is visible by pigmentation of colonies grown on agar medium plates. *P. chlororaphis* PCL1391 (Table 1) colonies that produce PCN are yellow, whereas a reduced PCN production results in a less yellow or white colony. In order to identify genes involved in PCN production, a Tn5/*luxAB* mutant library of *P. chlororaphis* PCL1391 consisting of 5,000 mutants was screened for impaired PCN production by plating the mutant library on MVB1 agar and selecting for colonies with reduced or lacking yellow pigmentation. This procedure resulted in the isolation of the white colored mutant strain PCL1971, which was subsequently characterized in detail. Quantification of PCN in liquid MVB1 cultures of PCL1971 showed that PCN production was almost abolished (Fig. 1). PCN production in *P. chlororaphis* PCL1391 is controlled by the quorum sensing signal *N*-hexanoyl homoserine lactone (C₆-HSL) (Chin-A-Woeng et al., 1998). Quantification of C₆-HSL in spent growth medium of PCL1971 showed that the production C₆-HSL in PCL1971 was severely reduced as compared with wild-type PCL1391 (Fig. 2).

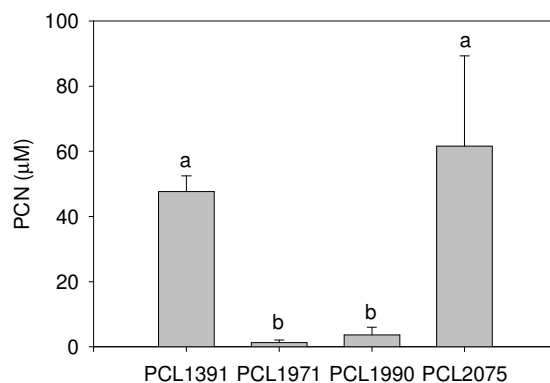


Fig. 1. Phenazine-1-carboxamide (PCN) production by *P. chlororaphis* PCL1391, PCL1971 (*ippA::Tn5luxAB*), PCL1990 (*ippA*) and PCL2075 (*cop*). Cells were grown in MVB1 medium and the PCN concentration in the supernatant fluid was quantified by HPLC analysis when cells had reached the stationary phase around an OD_{620nm} value of 4. Average and standard deviations of at least three experiments are shown. Bars followed by different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test.

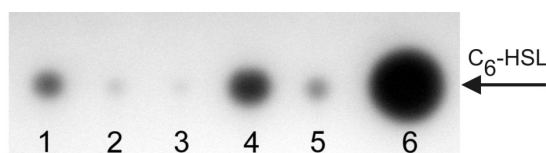


Fig. 2. C18-Reverse Phase TLC analysis of *N*-hexanoyl-L-homoserine lactone (C_6 -HSL) production by *P. chlororaphis* strain PCL1391 and mutant derivatives. Cell culture supernatants were harvested at OD_{620nm} 3.0 and extracted with ethylacetate. Samples were separated on C18-reverse phase TLC and the presence of C_6 -HSL was visualized using *C. violaceum* strain CV026. Lane 1, PCL1391; lane, 2 PCL1971 (*ippA::Tn5luxAB*); lane 3, PCL1990 (*ippA*); lane 4, PCL2061 (PCL1971 containing pMP7464 harboring *ippA* under control of the *tac* promoter); lane 5, PCL2029 (PCL1971 carrying the cloning vector pBBR1MCS-5); lane 6, $2.5 \cdot 10^{-5}$ mol synthetic C_6 -HSL. The experiment was performed three times with similar results.

Genetic characterization of strain PCL1971

In order to identify the chromosomal location of the transposon insertion in PCL1971, the chromosomal regions flanking the *Tn5luxAB* were obtained by re-isolation of the pRL1063a *Tn5* transposon plasmid including flanking regions of the adjacent chromosomal DNA from the PCL1971 genome. Sequencing of these genomic regions revealed that the *Tn5* transposon was inserted in an open reading frame of 828 bp, which encodes a 276 amino acid protein with a predicted localization in the cytoplasm. The mutated open reading frame was tentatively designated as *ippA* (inducer of phenazine production). BLAST (Altschul et al., 1990) analyses showed that *IppA* has the highest similarity (92 % identity) to a putative GTP cyclohydrolase I from *P. fluorescens* Pf-5 (Paulsen et al., 2005). However, only one third of the putative GTP cyclohydrolases from *P. fluorescens* Pf-5 has

similarity with a domain found in GTP cyclohydrolases. The first gene in PCL1391 upstream of *ippA* has 77 % identity with an uncharacterized copper binding protein from *P. fluorescens* PfO-1, which was named *cop* (DQ339483). The PCL1391 gene adjacent to *cop* was partially analyzed and shows 88 % identity to a response regulator from *P. fluorescens* PfO-1 (DQ339483). Downstream of *ippA* a conserved hypothetical protein of 87 amino acids is present which has the highest similarity (80 % identity) with a predicted protein from *P. syringae* B728a (Feil et al., 2005) (Fig. 3a). The chromosomal organization of *ippA* and the two flanking genes is conserved in *P. aeruginosa* POA1 (PA2805-PA2807) (Stover et al., 2000), *P. putida* KT2440 (Nelson et al., 2002), *P. fluorescens* Pf-5 (Paulsen et al., 2005). The *cop* gene, upstream of *ippA* is not conserved in *P. syringae* strains DC3000 (Buell et al., 2003) and B728a (Feil et al., 2005) (Fig. 3b).

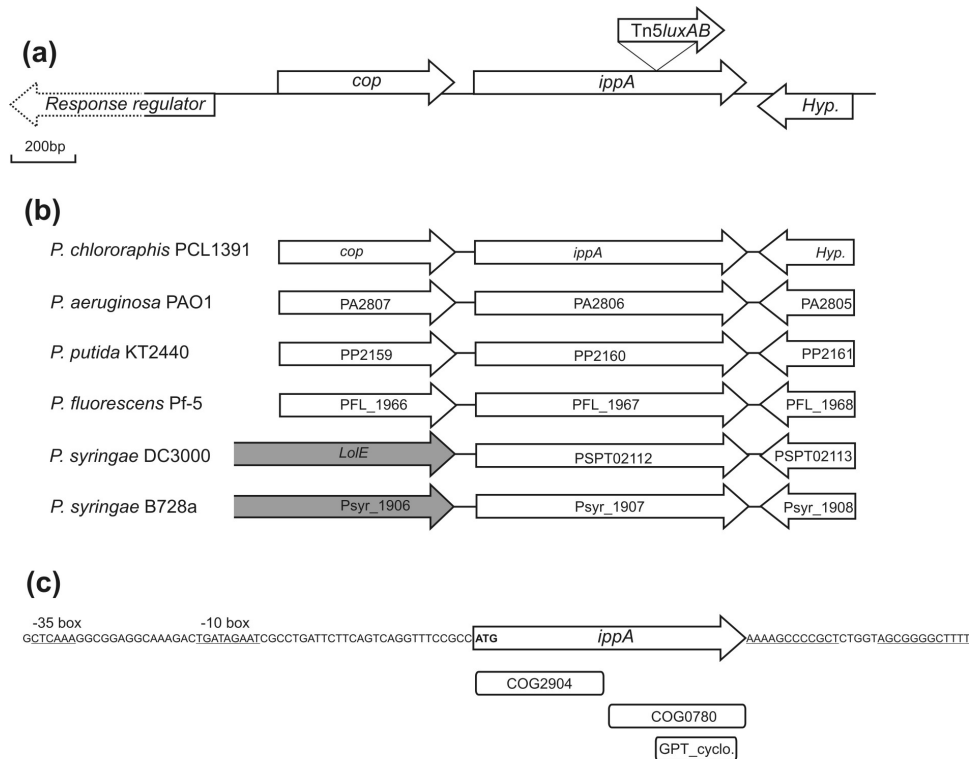


Fig. 3. Chromosomal analysis of the *ippA* region in *P. chlororaphis* strain PCL1391.

(a) Schematic representation of the genomic locus of the Tn5 insertion in *P. chlororaphis* PCL1391 mutant derivative PCL1971 (*ippA*::Tn5luxAB). (b) Genomic context of *ippA* and its homologs in *P. aeruginosa* POA1, *P. putida* KT2440, *P. fluorescens* Pf-5, *P. syringae* strains DC3000, and B728a. The grey genes in *P. syringae* strains DC3000, and B728 are not have similarity with *cop* (c) Nucleotide sequence of the promoter (indicating the putative -35 and -10 boxes) and terminator region (complementary sequences forming a hairpin are underlined) of *ippA*. Localization of homologous protein domains are shown under the complete *ippA* open reading frame. The sequence is available in GenBank under accession number DQ339483.

A conserved domain search (Marchler-Bauer and Bryant, 2004) identified three similarity domains in *IppA* (Fig. 3c). Domain 1 formed by the N-terminus shows similarity to an uncharacterized protein conserved domain (COG2904). Domain 2 formed by the C-terminal part of *IppA* shows similarity to a domain from an enzyme related to GTP cyclohydrolase I (COG0780). Domain 3, which is positioned within domain 2, shows similarity to a GTP cyclohydrolase I domain (pfam01227). The bacterial promoter prediction program PBROM identified a putative -10 box: 5'-TGATAGAAT-3' and a -35 box: 5'-CTCAAA-3' located at 58 bp and upstream of the *ippA* start codon. Two nucleotides after the stop codon of *ippA* a conserved Rho independent termination sequence AAAAGCCCCGCTCTGGT AGCGGGGCTTTTT with a dG of -16.8 Kcal/mol was detected (Fig. 3c).

Complementation and characterization of *ippA*

To test whether the mutant phenotype of PCL1971 (*ippA::Tn5luxAB*) is indeed due to the *Tn5luxAB* insertion, the *ippA* gene was disrupted using the constructed *ippA* suicide plasmid pMP7585 (see Methods section for construction). The newly generated *ippA* mutant PCL1990 produced severely reduced amounts of PCN and C₆-HSL (Figs. 1 and 2), which were similar to PCL1971 (*ippA::luxABTn5*). Complementation of PCL1971 (*ippA::luxABTn5*) was achieved by introducing a *P_{tac} ippA* construct (see Methods section for construction) into PCL1971. The generated strain was named PCL2061 and its PCN and C₆-HSL production in spent growth medium was analyzed. PCL2061 produced comparable amounts of PCN as the wild-type strain carrying the empty cloning vector control. The *ippA* mutant harboring the empty cloning vector control, referred to as strain PCL2029, produces ten times less PCN than the complemented *ippA* mutant (PCL2061) (Fig. 4). C₆-HSL levels in the complemented mutant PCL2061 are also restored as compared to the *ippA* mutants PCL1971 (*ippA::luxABTn5*) and PCL2029 (*ippA*) (Fig. 2). C₆-HSL levels increase in PCL2029 (PCL1971 with pBBR1MCS-5) compared to PCL1971 (*ippA::luxABTn5*) for unknown reasons but served as the correct control for the complemented *ippA* mutant (PCL2061). To test whether *ippA* and the upstream *cop* gene are part of one operon, *cop* was mutated (see Methods section for details). The *cop* mutant, strain PCL2075, was not impaired in its ability to produce PCN (Fig.1).

PCL1971 (*ippA::Tn5luxAB*) and the wild-type strain PCL1391 were analyzed for growth in MVB1 medium. Generation times were measured during exponential growth. PCL1391 had a generation time of 72±1.4 minutes and PCL1971 (*ippA::Tn5luxAB*) had a longer generation time of 83±2.1 minutes. Both strains reached similar cell densities after 24 hours.

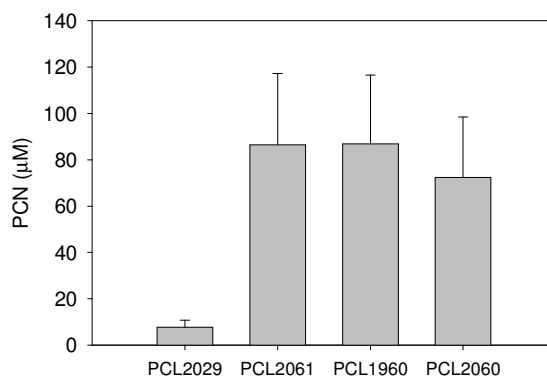


Fig. 4. Complementation of strain PCL1971 (*ippA::Tn5/luxAB*) by constitutive *ippA* expression. Phenazine-1-carboxamide (PCN) production by PCL2029 (PCL1971 with the empty vector pBBR1MCS-5), PCL2061 (PCL1971 harboring pMP7464 containing P_{lac} *ippA*), PCL1960 (PCL1391 with the vector pBBR1MCS-5) and PCL2060 (PCL1391 containing pMP7464) are shown. Cells were grown in MVB1 medium and the PCN concentrations of the supernatant fluids were quantified by HPLC when cells reached stationary phase around an OD_{620nm} value of 4. Average and standard deviations of at least three experiments are shown.

Constitutive expression of *phzI* and *phzR* restores PCN production in the *ippA* mutant PCL1971

The auto-inducer signal C₆-HSL produced by PhzI regulates PCN production in *P. chlororaphis* PCL1391 (Chin-A-Woeng et al., 2001b). In order to artificially increase the production of C₆-HSL in PCL1971 (*ippA::Tn5/luxAB*) plasmid pMP7450 harboring *phzI* under control of the constitutive *lac* promoter was introduced, resulting in strain PCL2028. This strain overproduced C₆-HSL in comparison with the control strain PCL2091 (PCL1971 containing the cloning vector) (Fig. 6). Constitutive expression of *phzI* in the *ippA* mutant background (PCL2028) restored PCN levels to that of the PCL1391 wild-type strain containing pMP7450 (PCL1999). The *ippA* mutant strain with the vector control (PCL2029) produces a minor amount of PCN compared to the wild-type with the cloning vector (PCL1960) (Fig. 5).

Constitutive expression of the quorum sensing regulator, *phzR* was achieved by introducing pMP7447 into PCL1971. The plasmid pMP7447 contains *phzR* under control of the *tac* promoter (Girard et al., 2006). Introduction of pMP7447 into PCL1971 resulted in PCL2027. PCN production was restored in PCL2027 and comparable to the wild type control strains PCL1993 (wild-type with constitutive *phzR*) and PCL1960 (wild-type with cloning vector) (Fig. 5). In addition, C₆-HSL production was restored in PCL2027 (Fig. 6).

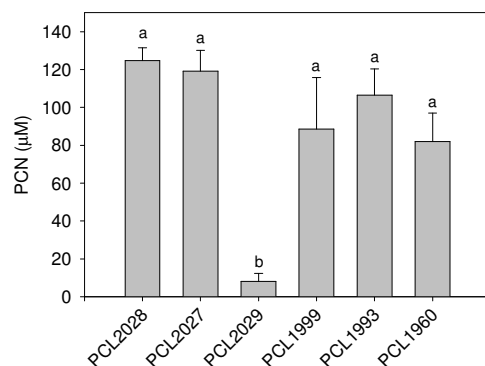


Fig. 5. The influence of constitutive *phzR* and *phzI* expression on phenazine-1-carboxamide (PCN) production by *P. chlororaphis* PCL1391 and mutant derivatives. Cells were grown in MVB1 medium and the PCN concentrations of the culture supernatant were quantified by HPLC analysis when cells reached the stationary phase around an OD_{620nm} value of 4. The following strains were analyzed: PCL2028 (PCL1971 containing pMP7450 harboring $P_{lac} phzI$), PCL2027 (PCL1971 with pMP7447, containing $P_{tac} phzR$), PCL2029 (PCL1971 with pBBR1MCS-5), PCL1999 (PCL1391 containing pMP7450 harboring $P_{lac} phzI$), PCL1993 (PCL1391 with pMP7447, containing $P_{tac} phzR$) and PCL1960 (PCL1391 with pBBR1MCS-5). Average and standard deviations of at least three experiments are shown. Bars followed by different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test.

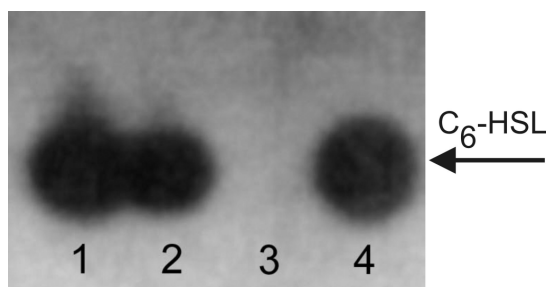


Fig. 6. Effect of constitutive expression of *phzI* and *phzR* on production of *N*-hexanoyl-L-homoserine lactone (C_6 -HSL) by strain PCL1971 (*ippA::Tn5luxAB*). Cell culture supernatants were isolated at OD_{620nm} 3.0 and extracted with ethylacetate. Samples were separated on C18-reverse phase TLC and the presence of C_6 -HSL was visualized using *C. violaceum* strain CV026. Lane 1, PCL2028 (PCL1971 harboring pMP7450, which contains $P_{lac} phzI$); lane 2, PCL2027 (PCL1971 harboring pMP7447, which contains $P_{tac} phzR$); lane 3, PCL2029 (PCL1971 with the empty vector pBBR1MCS-5); lane 4, $2.5 \cdot 10^{-9}$ mol synthetic C_6 -HSL. The experiment was performed in duplicate with similar results.

Constitutive expression of *pip* restores PCN production in an *ippA* mutant.

Since constitutive expression of *phzI* and *phzR* complements the *ippA* mutant phenotype and restores PCN production, a similar experiment was performed with three other identified regulators of PCN production, namely *rpoS* (Girard et al., 2006), *psrA* (Chin-A-Woeng et al., 2005) and *pip* (G. Girard, unpublished). Plasmids that constitutively express *rpoS*, *psrA*, and *pip* under control of the *tac* promoter were introduced into PCL1971 (*ippA*::Tn5*luxAB*), resulting in strains PCL2057, PCL2053 and PCL2055, respectively. Constitutive expression of *psrA* or *rpoS* did not increase the PCN production in PCL1971 (*ippA*::Tn5*luxAB*). In contrast, constitutive expression of *pip* increased PCN production in PCL1971 (*ippA*::Tn5*luxAB*) to a concentration comparable to the wild-type strain carrying the empty vector control (PCL1960) (Fig. 7).

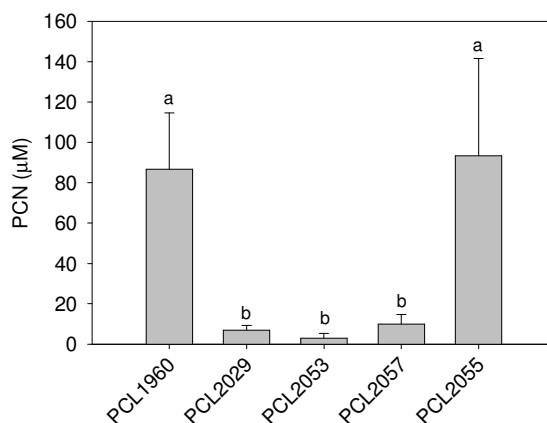


Fig. 7. The influence of constitutive *rpoS*, *psrA* and *pip* expression on phenazine-1-carboxamide (PCN) production by strain PCL1971 (*ippA*::Tn5*luxAB*). Cells were grown in MVB1 medium and the PCN concentrations of the culture supernatant were quantified by HPLC analysis when cells reached the stationary phase around an OD_{620nm} value of 4. The following strains were analyzed: PCL1960 (PCL1391 with pBBR1MCS-5), PCL2029 (PCL1971 with pBBR1MCS-5), PCL2053 (PCL1971 harboring pMP7420, which contains P_{tac}*rpoS*), PCL2057 (PCL1971 containing pMP7465, which contains P_{tac}*psrA*), and PCL2055 (PCL1971 containing pMP7455, containing *pip* under control of the *tac* promoter). Average and standard deviations of at least three experiments are shown. Bars followed by different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test.

In order to determine if constitutive expression of *ippA* could complement PCN production in the *psrA*, *rpoS* and *pip* mutants, the construct pMP7464 (P_{tac} *ippA*) was introduced into these mutants, resulting in strains PCL2070, PCL2072 and PCL2071, respectively. PCN analyses of the culture supernatants showed that PCN production was not restored in these strains as compared with the strains carrying the empty vector control (strains PCL1962, PCL1957 and PCL2011) (Fig. 8), which are all impaired in the production of PCN.

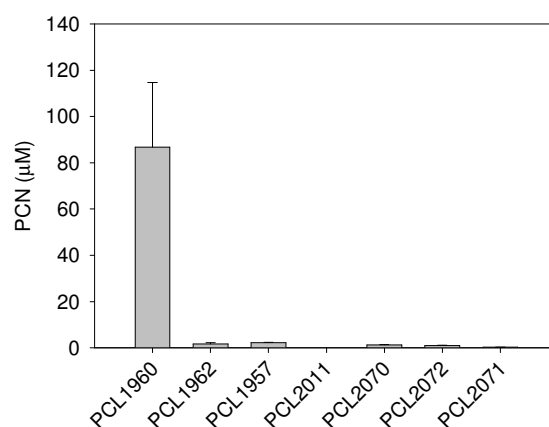


Fig. 8. Effect of constitutive *ippA* expression on phenazine-1-carboxamide production by *psrA*, *rpoS* and *pip* mutant derivatives of *P. chlororaphis* PCL1391.

Strains were grown in MVB1 medium until they reached the stationary phase around an OD_{620nm} value of 4. Phenazine-1-carboxamide (PCN) concentration of the harvested culture supernatants was quantified by HPLC. The following strains were analyzed: PCL1960 (PCL1391 with pBBR1MCS-5), PCL1962 (*psrA* mutant with pBBR1MCS-5), PCL1957 (*rpoS* mutant with pBBR1MCS-5), PCL2011 (*pip* mutant pBBR1MCS-5), PCL2070 (*psrA* mutant containing pMP7464 harboring P_{tac} *ippA*), PCL2072 (*rpoS* mutant containing pMP7464 harboring P_{tac} *ippA*), PCL2071 (*pip* mutant containing pMP7464 harboring P_{tac} *ippA*). Average and standard deviations of three independent experiments are shown.

Discussion

In this paper we describe the identification and characterization of a novel regulatory gene, *ippA*, which is involved in the regulation of PCN production in *P. chlororaphis* PCL1391. A mutation in *ippA* results in severely reduced production of PCN as well as of C₆-HSL (Figs. 1 and 2). Directed mutagenesis of *ippA* and complementation of the *ippA* mutant confirmed that the observed phenotype of PCL1971 was due to the Tn5 integration in this gene and not the result of a secondary mutation (Figs. 1 and 4).

BLAST analyses of the predicted amino acid sequence of IppA showed that it is highly conserved in many *Pseudomonas* species (Nelson et al., 2002; Paulsen et al., 2005; Stover et al., 2000) (Fig. 3b). Although IppA has 92 % identity with a putative GTP cyclohydrolase, only one third of both proteins align with a GTP cyclohydrolase related domain (Fig. 3c). Genomes of five sequenced *Pseudomonas* spp. contain one *ippA* homologue and one or two highly conserved GTP cyclohydrolase homologues. This suggests that *ippA* fulfills another unknown function. The genomic organization of the upstream and downstream genes is also conserved in all sequenced *Pseudomonas* genomes. Since *ippA* and the gene upstream (*cop*) are in the same orientation they could be part of one operon. However, directed mutagenesis of *cop* did not result in reduced PCN levels, which

demonstrates that *cop* and *ippA* are not transcriptionally coupled. Furthermore, detailed DNA sequence analyses indicate that *ippA* has its own promoter and terminator of transcription and is not part of an operon.

Since IppA interferes with quorum sensing and stimulates C₆-HSL production, the effect of constitutive *phzI* and *phzR* expression was investigated in the *ippA* mutant. Constitutive expression of the HSL synthase gene *phzI* increased the production of C₆-HSL (Fig. 6). Constitutive expression of *phzR* is thought to stimulate *phzI* expression and thereby to increase the C₆-HSL levels. Activating the quorum sensing system with constitutive expression of either gene restores PCN biosynthesis and suggests that IppA directly or indirectly stimulates *phzI* or *phzR* expression.

PsrA and RpoS are subsequent components of the regulatory cascade that controls PCN synthesis in *P. chlororaphis* PCL1391 (Fig. 9) (Chin-A-Woeng et al., 2005; Girard et al., 2006). Constructs with *psrA* and *rpoS* under the control of the *tac* promoter were introduced into the *ippA* mutant. Constitutive expression of either gene did not increase PCN biosynthesis and these results suggest that both genes are not influenced by *ippA* (Fig. 7). The phenazine inducing protein (Pip) stimulates PCN production in *P. chlororaphis* PCL1391 (G. Girard, unpublished). Introduction of *pip* behind the *tac* promoter in the *ippA* mutant background induced PCN production (Fig. 7). The ability of P_{*tac*} *pip* to complement the *ippA* mutant suggests that *ippA* controls *pip* or that P_{*tac*} *pip* bypasses the negative effect of the *ippA* mutation on PCN production. Constitutive expression of *ippA* in the *psrA*, *rpoS* and *pip* mutants did not restore the production of PCN in these mutants (Fig. 8). This result suggests that *ippA* is not located downstream of any of these three regulators in the regulatory cascade that controls PCN production. The role of IppA in relation with PCN production and other known PCN regulators is depicted in a model (Fig. 9). This model hypothesizes that *ippA* regulates PCN and C₆-HSL synthesis by influencing expression of *pip*.

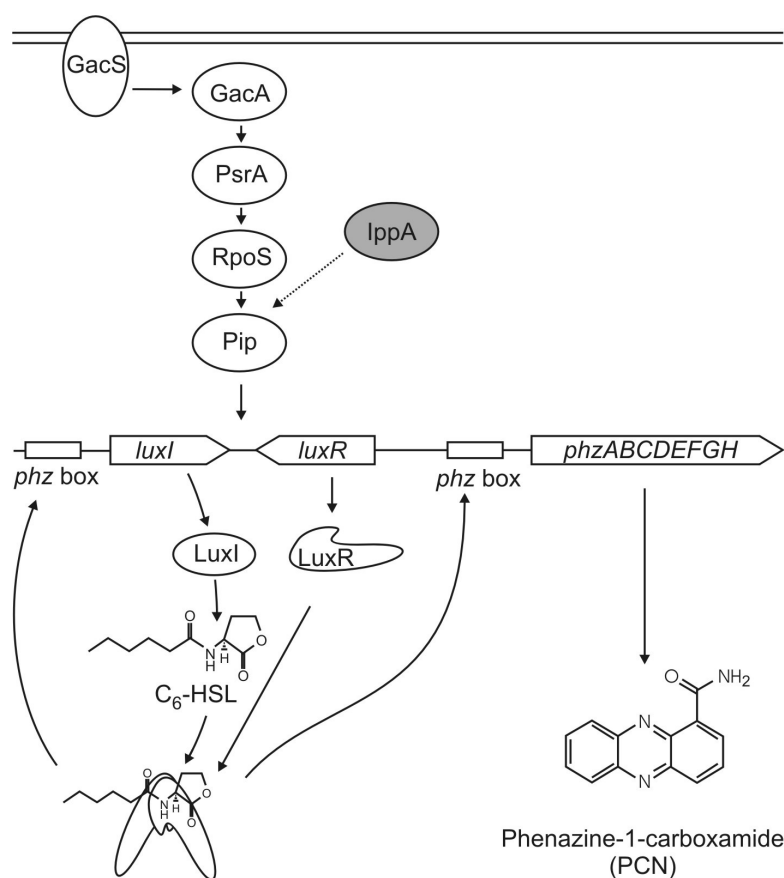


Fig. 9. Model for the regulation of PCN biosynthesis in *P. chlororaphis* PCL1391.

The GacS/A two component system is positioned on top of the PCN regulatory cascade. GacS is located in the membrane and autophosphorylated and transfers its phosphate to GacA. Subsequently, phosphorylated GacA stimulates expression of *psrA*. *PsrA* stimulates *rpoS* expression and *RpoS* stimulates *pip* expression. *Pip* stimulates expression of the quorum sensing system *phzI/phzR*, which controls the production of PCN. Our data show that *IppA* has a positive effect on quorum sensing and indicate that *IppA* is located upstream of *Pip* and not down stream of *PsrA*, *RpoS* and *Pip* in the regulatory cascade of PCN production.

Methods

Microorganisms and culture conditions

The microbial strains and plasmids used are listed in Table 1. Luria-Bertani (LB) medium (Sambrook and Russel, 2001) was used as standard medium for culturing *Escherichia coli* and *Chromobacterium violaceum*. A modified Vogel Bonner medium (van Rij et al., 2004; Vogel and Bonner, 1956) was used to culture *Pseudomonas* cells. Modified Vogel-Bonner # 1 (MVB1) consisted of 30 mM glucose, 0.05 % (w/v) casamino acids, 57 mM K_2HPO_4 , 16 mM $Na(NH_4)HPO_4$, 81 mM $MgSO_4$, 78 μM $NaFeEDTA(III)$, 6.8 μM $MnSO_4$, 0.35 μM $CuSO_4$, 4.1 μM Na_2MoO_4 , 0.85 μM $ZnSO_4$, 51 μM H_3BO_3 and the pH was adjusted to 6.6 using HCl (van Rij et al., 2004). Solidified growth media contained 1.8 % (w/v) agar (Difco Laboratories, Detroit, MI). If appropriate, media for *P. chlororaphis* were supplemented with the antibiotics kanamycin or gentamycin at final concentrations of 50 and 30 $\mu g\ ml^{-1}$, respectively. Media for *E. coli* were supplemented with the antibiotics kanamycin or gentamycin at final concentrations of 50 and 10 $\mu g\ ml^{-1}$, respectively. Cultures were shaken at 195 rpm on a Janke und Kunkel shaker KS501D (Staufen, Germany) at 28 °C and growth was monitored by measuring OD_{620nm} . Growth analyses were conducted in 100 ml flasks with 10 ml media after inoculation from a 5 ml overnight culture to an OD_{620nm} of 0.1. Optical densities (OD_{620nm}) were diluted ten times and measured with a Novospec II 4040 using cuvettes with a path length of 1 cm.

Isolation of mutants impaired in PCN biosynthesis

A mutant library of PCL1391 was generated with pRL1063a (Wolk et al., 1991) which resulted in random insertions of Tn5 transposons carrying promoterless *luxAB* reporter genes and a kanamycin selection marker gene. Transposon mutant colonies were screened for absence of the yellow PCN pigmentation on MVB1 agar plates. Flanking regions of Tn5 insertions were recovered by digesting chromosomal DNA with *EcoRI* and subsequently ligating the chromosomal fragments. Selection on kanamycin resistance resulted in the isolation of the pRL1063a plasmid with the flanking chromosomal regions. Sequencing of the flanking regions was performed with oMP458 (5'-TACTAGATTCAATGCTATCAATGAG-3') and oMP459 (5'-AGGAGGTCACATGGAATATCAGAT-3'). Sequencing of the flanking regions of PCL1971 was continued with oMP952 (5'-GGCTGACGGTCAGCTTGCCG-3'). The obtained sequences were analyzed using BLASTX in GenBank (Altschul et al., 1990) and deposited in Genbank with accession number DQ339483.

DNA manipulation

Polymerase chain reactions were carried out with Super Taq enzyme (Enzyme Technologies, UK) or ProofStart DNA polymerase (Qiagen, Germany). The primers were synthesized by Isogen Life Science (Maarssen, The Netherlands). Restriction enzymes were purchased from New England BioLabs Inc. (Westburg, Leusden, The Netherlands) and T4 DNA ligase from Promega (Leiden, The Netherlands). For mutagenesis of *ippA* an internal PCR fragment of 493 bp, generated by PCR using oMP947 (5'-CGGAATTCCGAACATTCGCCGCTGGGC-3') and oMP948 (5'-CGGAATTCCGCGACCTGTCGCACTGCAGC-3'), was cloned into the *EcoRI* site of the suicide plasmid pMP5285, which generated pMP7585. Mutants were verified by PCR on the genomic DNA using oMP947 and oMP949 (5'-CAGCGCCGG GCCACGGTACTC-3'). For the mutagenesis of *copA* a 325 bp PCR fragment was generated with oMP1004 (5'-CCGGAATTCTACGATTTCCGGTCAGGCGGCC-3') and oMP1005 (5'-CCGGAATTCTGTTCGGATCGTCGTGCTTC-3'). This fragment was cloned into pMP5285 with *EcoRI* and resulted in pMP7480. Obtained mutants were checked by PCR using oMP986, oMP985 and oMP952.

For the complementation of the *ippA* mutant a vector containing a P_{tac} *ippA* construct was generated. The *ippA* gene was amplified by PCR using oMP1014 (5'-ATGCATCCCGCAGCCGAACATTCGCCGCTGGG-3') and oMP967 (5'-CGGAATTCTGTCAAACCCTCGGCAACATGGGG-3') and the PCR product was used as a template for positioning the *tac* promoter in front of *ippA* using the P_{tac} containing primer oMP1013 (5'-CGGAATTCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGCATCCCGCAGCCGAACATTCGCCGCTGGG-3') and oMP967. The P_{tac} *ippA* fragment was cloned into pBBR1MCS-5 with *EcoRI* and this new vector was named pMP7464.

Analysis of phenazine-1-carboxamide (PCN) production

For quantification of PCN, spent culture supernatants were extracted according to Chin-A-Woeng *et al.* (1998) with minor modifications. Culture samples (250 μ l) were centrifuged and the culture supernatants were acidified to pH 2 using 6 M HCl. They were subsequently extracted with an equal volume of toluene by shaking on an Eppendorff mixer 5432 for 5 min. After centrifugation, the toluene phase was collected and dried in a rotary evaporator. The dry residue was dissolved in 100 μ l acetonitrile and the obtained solution was mixed with 400 μ l water.

PCN concentrations were determined by high-performance liquid chromatography (HPLC) (DIONEX, Sunnyvale, CA, Chromeleon software version 6.20) using a calibration curve. HPLC was performed using an econosphere C18 5 μ m, 259 mm x 4.6 mm column

(Alltech Associates, Inc, Deerfield, IL) at 30 °C with a linear gradient of 20-80 % (v/v) acetonitrile acidified with 0.1 % (v/v) trifluoroacetic acid in water and a flow rate of 1 ml min⁻¹.

Analysis of *N*-acyl-homoserine lactone (HSL) production

Supernatants were extracted with 0.5 volume of ethyl acetate, shaken for half an hour and the ethyl acetate phase was collected and subsequently dried by rotary evaporation. The dried residue was dissolved in 25 µl acetonitrile and analyzed using TLC. Samples spotted on C18 TLC plates (Merck, Darmstadt, Germany) were developed in methanol-water (60:40) (v/v). After development, the TLC was overlaid with LB 0.8 % (w/v) agar containing a 10-fold diluted overnight culture of the *Chromobacterium violaceum* indicator strain CV026 (McClellan *et al.*, 1997) and kanamycin (50 µg ml⁻¹). After incubation for 48 h at 28 °C, chromatograms were analyzed for appearance of violet spots representing the presence of *N*-acyl-homoserine lactone molecules

Acknowledgements

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Characterization of a novel locus involved in the regulation of
phenazine-1-carboxamide biosynthesis by *Pseudomonas*
chlororaphis strain PCL1391

E. Tjeerd van Rij, Geneviève Girard, Ben J.J. Lugtenberg, and Guido V. Bloemberg

Summary

Pseudomonas chlororaphis strain PCL1391 controls tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. The ability to produce the antifungal metabolite phenazine-1-carboxamide (PCN) is crucial for biocontrol by strain PCL1391. In this study the identification and characterization of two PCL1391 mutants that produce reduced amounts of PCN is described. The corresponding adjacent and equally orientated genes were named *iopA* and *iopB* (inducer of phenazine). Mutation and complementation studies suggest that *iopA* and *iopB* are not part of the same operon. The *iopA* and *iopB* mutants are also impaired in the production of the quorum sensing signal *N*-hexanoyl-*L*-homoserine lactone (C₆-HSL) which control PCN synthesis. Remarkably, both *iopA* and *iopB* have the highest similarity with genes from *Ralstonia solanacearum* and *Streptomyces glaucescens* and not with any genes from *Pseudomonas* sp. The position of *iopB* in the regulatory cascade of PCN production was characterized in more detail. The PCN production in the *iopB* mutant could be restored by constitutive expression of each one of the two quorum sensing genes *phzI* and *phzR* individually. Constitutive expression of two other genes regulating PCN production, *rpoS* and *pip*, also restored PCN production in the *iopB* mutant background. Constitutive expression of *psrA* in the *iopB* mutant did not restore PCN production. These results indicate that *iopB* is located higher in the regulatory cascade controlling PCN production than the four regulatory genes that could complement the *iopB* mutant. Biocontrol tests with the *iopB* mutant showed that a functional *iopB* is essential for biocontrol by strain PCL1391 which can be explained by the impaired PCN production by the *iopB* mutant.

Introduction

Pseudomonas chlororaphis strain PCL1391 controls tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Chin-A-Woeng et al., 1998). Essential for biocontrol by *P. chlororaphis* PCL1391 are the production of the anti-fungal metabolite phenazine-1-carboxamide (PCN) and the ability to efficiently colonize the rhizosphere for the delivery of PCN (Chin-A-Woeng et al., 2000). Root colonization by *Pseudomonas* sp. depends on various traits, including chemotaxis and prototrophy for amino acids (de Weert et al., 2002; Lugtenberg and Dekkers, 1999). The phenazine biosynthetic operon has been identified in the phenazine producing strains *P. fluorescens* 2-79 (Mavrodi et al., 1998), *P. aereofaciens* 30-84 (Pierson III et al., 1995), *P. aeruginosa* PAO1 (Stover et al., 2000) and *P. chlororaphis* PCL1391 (Chin-A-Woeng et al., 2001b) and consists of the seven conserved genes *phzABCDEFG*. In *P. chlororaphis* strain PCL1391 the additional *phzH* gene is responsible for the conversion of phenazine-1-carboxylic acid (PCA) to PCN (Chin-A-Woeng

et al., 2001a). The production of PCN is regulated by environmental factors (van Rij et al., 2004). High cell density stimulates phenazine biosynthesis via the phenazine quorum sensing genes *phzI* and *phzR* (Chin-A-Woeng et al., 2001b; Khan et al., 2005; Pierson III et al., 1994). The main quorum sensing signal present in spent growth medium of *P. chlororaphis* strain PCL1391 is *N*-hexanoyl-L-homoserine lactone (C₆-HSL) which is produced by PhzI. The transcriptional regulator PhzR is activated upon binding of C₆-HSL, similarly to activation of TraR in *Agrobacterium tumefaciens* (Zhu and Winans, 1999). Activated PhzR stimulates the expression of *phzI* and the phenazine biosynthetic (*phz*) operon via conserved *phz* boxes found in the two promoter regions (Chin-A-Woeng et al., 2001a). Quorum sensing and PCN biosynthesis are dependant on the GacS/GacA two component signaling system, which regulates the production of homoserine lactones, PCN, cyanide, proteases and chitinases (Chin-A-Woeng et al., 2005). GacS is a membrane localized sensor kinase that activates the transcriptional regulator GacA through a phosphorelay mechanism (Haas and Défago, 2005; Heeb and Haas, 2001). Other regulators that are known to play a role in the regulation of phenazines include the *Pseudomonas* sigma factor regulator (PsrA) and the stationary phase alternative sigma factor RpoS (σ^S) (Chin-A-Woeng et al., 2005; Girard et al., 2006). PsrA was shown to be a positive regulator of RpoS in *P. putida* strain WCS358, *P. aeruginosa* strain PAO1 (Kojic and Venturi, 2001) and *P. chlororaphis* strain PCL1391 (Girard et al., 2006) by directly binding to the *rpoS* promoter (Kojic et al., 2002). The *rpoS* promoter of *P. chlororaphis* strain PCL1391 contains a similar PsrA binding site (Girard et al., 2006).

This study describes the characterization of two *P. chlororaphis* PCL1391 genes, *iopA* and *iopB*, which are involved in the regulation of PCN and C₆-HSL. Complementation of PCN production in the *iopB* mutant was tested after constitutive expression of *phzI*, *phzR*, *psrA*, *rpoS*, and *pip*. Finally, the *iopB* mutant was tested for biocontrol.

Table 1. Microorganisms and plasmids used in this study

Strains or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
PCL1391	Wild-type biocontrol strain <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide	(Chin-A-Woeng et al., 1998)
PCL1975	PCL1391 <i>iopB</i> ::Tn5/ <i>luxAB</i>	This study
PCL1960	Derivative of PCL1391 containing pBBR1MCS-5, Gm ^r	(Girard et al., 2006)
PCL1991	PCL1391 derivative, mutated in <i>iopB</i> mutated by homologous recombination, Km ^r	This study
PCL1993	Derivative of PCL1391 containing pMP7447 (<i>P_{tac}</i> - <i>phzR</i> in pBBR1MCS-5), Gm ^r	(Girard et al., 2006)

PCL1999	Derivative of PCL1391 containing pMP7450 (P_{lac} - <i>phzI</i> in pBBR1MCS-5), Km ^r	(van Rij et al., 2005)
PCL2030	Derivative of PCL1975 containing pMP7447 (P_{lac} - <i>phzR</i> in pBBR1MCS-5), Km ^r , Gm ^r	This study
PCL2031	Derivative of PCL1975 containing pMP7450 (P_{lac} - <i>phzI</i> in pBBR1MCS-5), Km ^r , Gm ^r	This study
PCL2032	Derivative of PCL1975 containing pBBR1-MCS5, Km ^r	This study
PCL2043	PCL1391 derivative mutated in <i>iopA</i> by homologous recombination, Km ^r	This study
PCL2054	Derivative of PCL1975 containing pMP7420 (P_{lac} - <i>rpoS</i> in pBBR1MCS-5), Km ^r , Gm ^r	This study
PCL2056	Derivative of PCL1975 containing pMP7455 (P_{lac} - <i>pip</i> in pBBR1MCS-5), Km ^r , Gm ^r	This study
PCL2058	Derivative of PCL1975 containing pMP7465 (P_{lac} - <i>psrA</i> in pBBR1-MCS5), Km ^r , Gm ^r	This study
PCL2059	PCL1391 derivative mutated by homologous recombination in the putative amidinotransferase gene adjacent of <i>iopB</i> , Km ^r	This study
PCL2066	Derivative of PCL2043 containing pMP7478 (P_{lac} - <i>iopA</i> in pBBR1-MCS5), Km ^r Gm ^r	This study
PCL2067	Derivative of PCL2043 containing pBBR1-MCS5, Gm ^r	This study
PCL2068	PCL1391 derivative mutated by homologous recombination in the putative lysine exporter gene adjacent of the putative amidinotransferase gene, Km ^r	This study
PCL2092	Derivative of PCL1975 containing pMP7589 (<i>iopB</i> including the 887 bp putative upstream promoter region in pBBR1MCS-5), Km ^r , Gm ^r	This study
CV026	<i>Chromobacterium violaceum</i> . N-acyl-homoserine lactone (HSL) reporter strain, Km ^r	(McClellan et al., 1997)
DH5 α	<i>E. coli</i> , <i>SupE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi1</i> <i>relA1</i>	(Boyer and Roulland-Dussoix, 1969)
Fungus		
ZUM2407	<i>Fusarium oxysporum</i> f.sp. <i>radicis-lycopersici</i> (F.o.r.l.). Cause of foot and root rot of tomato. CSB 101.587	IPO-DLO, Wageningen, The Netherlands
Plasmids		
pRL1063a	Plasmid harboring a Tn5 transposon containing a promoterless <i>luxAB</i> reporter genes, Km ^r	(Wolk et al., 1991)

pBluescript	General purpose cloning vector, Cb ^r	Stratagene, La Jolla, CA
pBBR1MCS-5	General purpose cloning vector, Gm ^r	(Kovach et al., 1995)
pRK2013	Helper plasmid for tri-parental mating, Km ^r	(Ditta et al., 1980)
pMP5285	pME3049 derivative, lacking the Hg ^r gene used for single homologous recombination, Km ^r	(Kuiper et al., 2001b)
pMP7420	pBBR1MCS-5 containing <i>rpoS</i> of PCL1391 under control of the <i>tac</i> promoter.	(Girard et al., 2006)
pMP7447	pBBR1MCS-5 containing <i>phzR</i> of PCL1391 under control of the <i>tac</i> promoter.	(Girard et al., 2006)
pMP7450	pBBR1MCS-5 containing <i>phzI</i> of PCL1391 under control of the <i>lac</i> promoter.	This study
pMP7455	pBBR1MCS-5 containing <i>pip</i> of PCL1391 under control of the <i>tac</i> promoter.	(G. Girard, <i>unpublished results</i>)
pMP7465	pBBR1MCS-5 containing <i>psrA</i> of PCL1391 under control of the <i>tac</i> promoter.	(Girard et al., 2006)
pMP7476	Suicide plasmid pMP5285 containing a 413 bp fragment of a predicted amidinotransferase gene.	This study
pMP7478	pBBR1MCS-5 containing <i>iopA</i> of PCL1391 under control of the <i>tac</i> promoter.	This study
pMP7479	Suicide plasmid pMP5285 containing a 388b bp fragment of a predicted lysine exporter gene.	This study
pMP7580	Suicide plasmid pMP5285 containing a 615 bp fragment of <i>iopA</i> .	This study
pMP7586	Suicide plasmid pMP5285 containing a 510 bp fragment of <i>iopB</i> .	This study
pMP7589	pBBR1MCS-5 containing <i>iopB</i> including the 887 bp putative upstream promoter region.	This study

Results

Isolation and genetic characterization of a *P. chlororaphis* strain PCL1391 mutant defective in PCN production

A Tn5 mutant library of *P. chlororaphis* strain PCL1391 was screened for mutants producing reduced amounts of phenazine-1-carboxamide (PCN). The library was plated on MVB1 agar and colonies lacking a yellow color, caused by PCN in wild-type colonies, were selected. Genomic regions flanking the Tn5 insertions were sequenced and a number of previously characterized genes involved in PCN production, including *gacS*, *rpoS*, and *phzH*, were identified. In addition, one mutant, named PCL1975, appeared to have its Tn5 transposon inserted in an uncharacterized gene that was named inducer of phenazine (*iopB*) (Table 1). The mutated open reading frame consists of 2220 bp and the predicted protein shows highest similarity (33% identity) with a hypothetical protein (RS03054) from *Ralstonia solanacearum*. Additional sequencing revealed that *iopB* is the second gene in a series of at least five equally oriented open reading frames (Fig. 1). The gene upstream of *iopB* consists of 1002 bp and its predicted gene product shows highest similarity (41% identity) with a hypothetical protein from *Streptomyces* (KCTC 0041BP). This gene was named *iopA* because it preceded *iopB*. Homologues of *iopA* or *iopB* could not be detected in other sequenced genomes of *P. fluorescens* Pf-5 (Paulsen et al., 2005), *P. aeruginosa* strain PAO1 (Stover et al., 2000), *P. putida* strain KT2440 (Nelson et al., 2002), *P. syringae* pv *syringae* strain B728a and strain DC3000 (Buell et al., 2003).

The gene downstream of *iopB* consists of 1044 bp and the encoded protein has similarity (52% identity) with an amidinotransferase from *Streptomyces glaucescens* (CAA55571.1). The fourth gene consists of 654 bp and its predicted protein shows similarity (51% identity) with a lysine exporter protein (LyseE/YggA) from *P. fluorescens* strain Pf-5 (YP_261245.1). The fifth gene encodes a protein which has similarity (55% identity) with a putative transferase protein from *Ralstonia solanacea* (NP_523050.1). The partial sequence upstream of *iopA* suggests that this gene codes for a protein similar (77% identity) to a putative transcriptional regulator of *P. aeruginosa* strain PAO1 (NP_250722.1) that is divergently transcribed from *iopA*. Promoter analyses with PBROM (<http://www.softberry.com>) identified two inverted -10 and -35 promoter regions between *iopA* and the putative transcriptional regulatory gene (Fig. 1). No obvious promoter sequences have been identified in the intergenic regions between the five equally orientated genes.

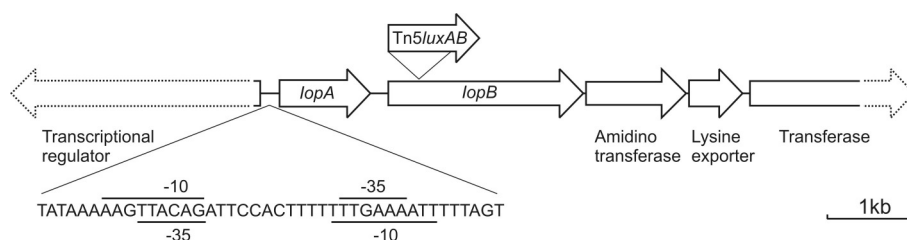


Fig. 1. Schematic representation of the genomic locus with the Tn5 insertion in *P. chlororaphis* mutant strain PCL1975 (*iopB*::Tn5/*luxAB*). The nucleotide sequence illustrates the position of the two -10 and -35 promoter regions for the transcriptional start in two directions. Sequence is available in GenBank under accession number DQ367408.

In order to quantify the observed PCN deficient phenotype of strain PCL1975 (*iopB*::Tn5/*luxAB*), the PCN production of this strain was analyzed in MVB1 liquid medium. Comparison with its wild-type showed that strain PCL1975 (*iopB*::Tn5/*luxAB*) produced 76% less PCN (Fig. 2). To confirm that the reduction in PCN production is due to the Tn5 insertion, a second *iopB* mutant was created by homologous recombination. This second *iopB* mutant PCL1991 also produced less PCN (78% reduction) than the wild-type strain (Fig. 2).

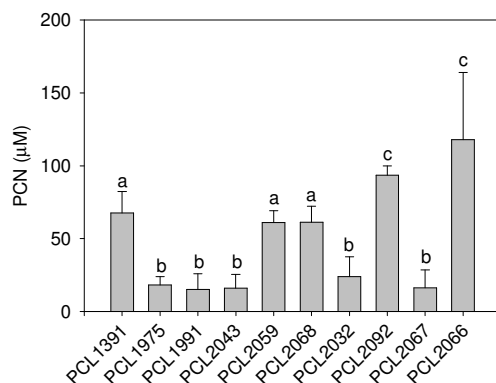


Fig. 2. Phenazine-1-carboxamide (PCN) production by *P. chlororaphis* strains PCL1391, PCL1975 (*iopB*::Tn5/*luxAB*), PCL1991 (*iopB*), PCL2043 (*iopA*), PCL2059 (mutated in a putative amidino transferase), PCL2068 (mutated in a putative lysine exporter), PCL2032 (PCL1975, containing pBBR1MCS-5), PCL2092 (*iopB*::Tn5/*luxAB* complemented with pMP7589 with *iopB* and 887 bp upstream), PCL2067 (*iopA*, containing pBBR1-MCS5) and PCL2066 (*iopA* mutant harboring pMP7478 with *P_{tac}-iopA*). Cells were grown in MVB1 medium and the PCN concentrations of the supernatant fluids were quantified by HPLC when cells reached stationary phase ($OD_{620nm}=4$). The average and the standard deviation of at least three experiments are shown. Bars followed by different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test.

Complementation of strain PCL1975 (*iopB*::*luxAB*Tn5) was achieved by introducing *iopB* including the 887 bp upstream region (see Methods section for construction) into strain PCL1975. The generated strain was named PCL2092 and its PCN production in spent growth medium was analyzed. The complemented *iopB* mutant (strain PCL2092) produced

almost four times more PCN than strain PCL2032 (empty vector control) and considerably more than the wild type strain PCL1391 (Fig. 2).

PCN production in *P. chlororaphis* strain PCL1391 depends on the presence and concentration of the C₆-HSL signal molecule (Chin-A-Woeng et al., 2001b). Analysis of HSLs isolated from spent growth medium of strain PCL1975 (*iopB*::Tn5*luxAB*) and PCL1391 demonstrated that the C₆-HSL concentration is strongly reduced in strain PCL1975 (*iopB*::Tn5*luxAB*) (Fig. 3, lanes 1 and 2). The complemented *iopB* mutant strain PCL2092 was also restored in the production of C₆-HSL compared to the empty vector control strain PCL2032 (Fig. 3, lanes 6 and 7)

Possible involvement in PCN production of genes adjacent of *iopB* was further analyzed by mutating *iopA* and the two genes downstream of *iopB*. PCN concentrations in the spent growth medium of the three generated mutants PCL2043 (*iopA*⁻), PCL2059 (putative amidinotransferase⁻) and PCL2068 (putative lysine exporter⁻) were quantified. PCN production by the *iopA* mutant was comparable to that of the *iopB* mutant (Fig. 2). Mutation of the putative amidinotransferase or the putative lysine exporter did not affect PCN production (Fig. 2). In addition, it was shown that production of C₆-HSL was also impaired in the *iopA* mutant (Fig. 3, lane 3). In the second *iopB* mutant (Fig. 3, lane 4) C₆-HSL production was also impaired although minor amount were still detected due to overloading of the TLC.

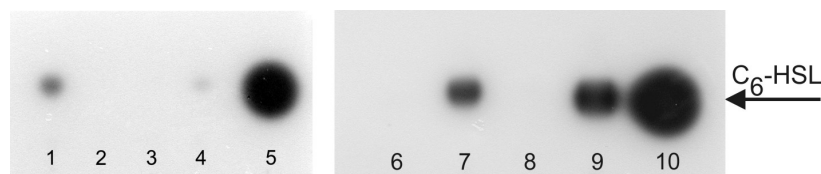


Fig. 3. Analysis of hexanoyl homoserine lactone (C₆-HSL) production by *P. chlororaphis* strain PCL1391 and mutant derivative strains. Lane 1, PCL1391; lane, 2 PCL1975 (*iopB*::Tn5*luxAB*); lane, 3 PCL2043 (*iopA*⁻); lane, 4 PCL1991 (*iopB*⁻); lane 5, synthetic C₆-HSL standard (2.5·10⁻⁶ mol); lane, 6 PCL2032 (PCL1975, containing pBBR1MCS-5); lane, 7 PCL2092 (*iopB*::Tn5*luxAB* complemented with pMP7589 with *iopB* and 887 bp upstream); lane, 8 PCL2067 (*iopA*⁻, containing pBBR1-MCS5); lane, 9 PCL2066 (*iopA* mutant harboring pMP7478 with P_{tac}-*iopA*); lane 10, synthetic C₆-HSL standard (2.5·10⁻⁶ mol). Samples were separated on C18-reverse phase TLC and C₆-HSL visualized using the *C. violaceum* reporter strain CV026. Extracts were isolated at OD_{620nm} 3.0.

Since mutating *iopA* or *iopB* resulted in reduced levels of both PCN and C₆-HSL, it was necessary to determine if both genes are essential for normal PCN production and whether *iopA* and *iopB* are part of one operon. This was tested by the introduction of a plasmid containing *iopA* under control of the *tac* promoter into the *iopA* mutant (PCL2066). The complemented *iopA* strain was restored in PCN and C₆-HSL production compared to the *iopA* mutant empty vector control (PCL2067) (Figs. 2 and 3). This demonstrated that *iopA* regulates PCN and C₆-HSL production. The fact that the *iopB* mutant (PCL1975) could be

complemented with a plasmid containing *iopB* with 887 bp upstream which lacks the promoter region of *iopA*, suggest that both genes are not part of one operon.

Growth of the *iopA* and *iopB* mutants was analyzed because growth defects also influence PCN and C₆-HSL production. Comparison of the *iopA* and *iopB* mutants with the parental strain PCL1391 showed that these mutants are not affected in their growth (Fig. 4).

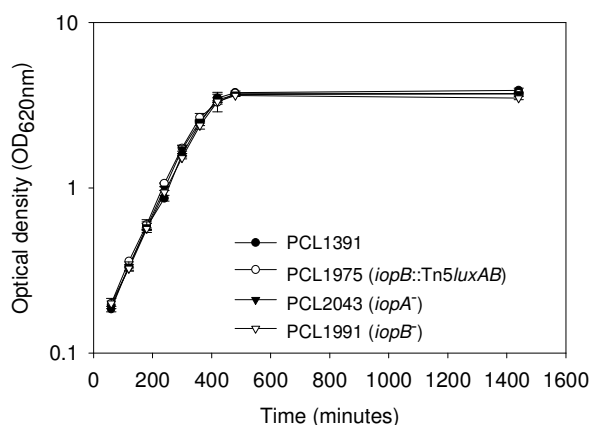


Fig. 4. Growth of PCL1975 (*iopB*::Tn5*luxAB*) and derivatives in MVB1-medium during time. The following strains were analyzed: *P. chlororaphis* strain PCL1391, PCL1975 (*iopB*::Tn5*luxAB*), PCL2043 (*iopA*⁻) and PCL1991 (*iopB*⁻). Means and standard deviations are shown of two replicates.

Constitutive expression of *phzI* and *phzR* restores PCN production in *iopB* mutant PCL1975

Quorum sensing determines the initiation of PCN production by regulating the expression of the *phz* operon. Constitutive expression of either *phzI* or *phzR* restored PCN production when tested during various PCN limiting conditions (van Rij et al., 2005) and in various PCN regulatory mutant strains (Girard et al., 2006). Plasmid pMP7450 contains a *lac* promoter followed by *phzI* (van Rij et al., 2005). Introduction of pMP7450 into *iopB* mutant PCL1975 resulted in strain PCL2031. It increased the production of HSLs as compared with the control strain PCL2032 that contains the empty vector (Fig. 5, lanes 1 and 3). Measuring the PCN concentration in the spent growth medium of strain PCL2031 showed that PCN production was higher than wild-type levels. Constitutive expression of *phzR* was achieved by introducing *P_{tac}-phzR* (pMP7447) (Girard et al., 2006) into PCL1975. The obtained strain PCL2030 produces increased amounts of HSLs and PCN as compared to the *iopB* mutant empty vector control strain PCL2032 (Fig. 5, lanes 2 and 3 and Fig. 6). PCN production by the wild-type strain containing the empty vector control (PCL1960), the *P_{lac}-phzI* plasmid

(PCL1999), and the P_{tac} -*phzR* plasmid (PCL1993) were included as controls and produced similar amounts of PCN (Fig. 6).

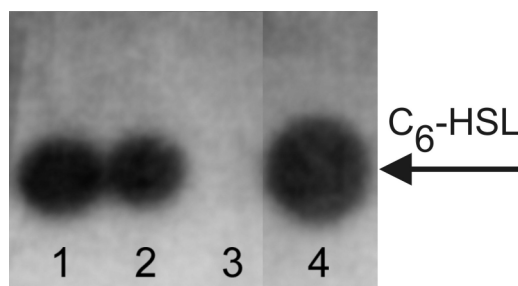


Fig. 5. Analyses of hexanoyl homoserine lactone (C_6 -HSL) production by strain PCL1975 (*iopB*::Tn5/*luxAB*) containing P_{lac} -*phzI* or P_{tac} -*phzR*. Lane 1, PCL2031 (PCL1975 containing pMP7450 with *phzI* under control of the *lac* promoter); lane 2, PCL2030 (PCL1975 containing pMP7447 harboring *phzR* under control of the *tac* promoter); lane 3, PCL2032 (PCL1975 with pBBR1-MCS5); lane 4, synthetic C_6 -HSL standard ($2.5 \cdot 10^{-6}$ mole). Samples were separated on C18-reverse phase TLC and visualized using *C. violaceum* strain CV026. Extracts were isolated at OD_{620} 3.0. The experiment was repeated with similar results.

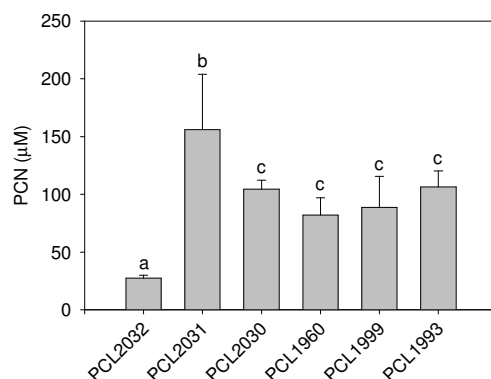


Fig. 6. The influence of constitutive *phzR* and *phzI* expression on phenazine-1-carboxamide (PCN) production by *P. chlororaphis* strain PCL1391 and mutant derivatives. Bacterial strains were grown in MVB1-medium and the PCN concentrations of the culture supernatant were quantified by HPLC when cells reached stationary phase ($OD_{620nm}=4$). The following strains were analyzed: PCL2032 (PCL1975 containing pBBR1-MCS5), PCL2031 (PCL1975 containing pMP7450 with *phzI* under control of the *lac* promoter), PCL2030 (PCL1975 containing pMP7447 harboring *phzR* under control of the *tac* promoter), PCL1960 (PCL1391 with pBBR1-MCS5), PCL1999 (PCL1391 containing pMP7450), PCL1993 (PCL1391 containing pMP7447). The average and standard deviations of at least three experiments are shown. Bars followed by different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test.

Constitutive expression of *rpoS* and *pip* restores PCN production in *iopB* mutant PCL1975

PCN production by *P. chlororaphis* strain PCL1391 is regulated by *psrA*, *rpoS* and *pip* (Girard et al., 2006; G. Girard, unpublished results). The relationship between *iopB* and these three regulators was investigated by using plasmid constructs harboring these genes under control of the constitutive *tac* promoter. Introduction of pMP7465 (P_{tac} -*psrA*) into the

iopB mutant strain PCL1975 resulted in strain PCL2058. Comparison of PCN levels of PCL2058 and the *iopB* mutant empty vector control strain PCL2032 showed that both strains produce similar amounts of PCN and that these PCN levels are significantly lower than those of the wild-type empty vector control strain PCL1960 (Fig. 7). Constitutive expression of *rpoS* was achieved by introducing pMP7420 containing P_{tac} -*rpoS* in PCL1975 resulting in PCL2054. PCN production by PCL2054 was comparable with that of the wild type (PCL1960) and was significantly higher than in the *iopB* mutant empty vector control (PCL2032) (Fig. 7). For the constitutive expression of *pip*, vector pMP7455 (P_{tac} -*pip*) (Girard *et al.*, unpublished results) was transformed into PCL1975, resulting in strain PCL2056. PCN production by PCL2056 was not significantly higher than the wild-type PCL1960. However, the PCN levels produced by PCL2056 were significantly higher than those of the *iopB* mutant strain PCL2032 containing the empty cloning vector (Fig. 7).

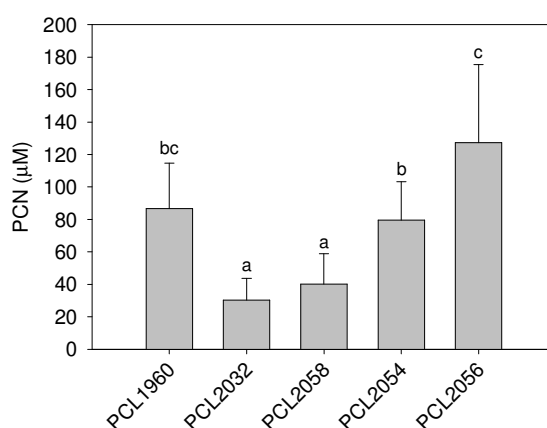


Fig. 7. The influence of constitutive *rpoS*, *psrA* and *pip* expression on phenazine-1-carboxamide (PCN) production by PCL1975 (*iopB*::Tn5*luxAB*). Cells were grown in MVB1 medium and the PCN concentrations of the supernatant fluids were quantified by HPLC when cells reached stationary phase ($OD_{620nm}=4$). The following strains were analyzed: PCL1960 (PCL1391 with pBBR1-MCS5), PCL2032 (PCL1975 with pBBR1-MCS5), PCL2058 (PCL1975 containing pMP7465 with *psrA* controlled by the *tac* promoter), PCL2054 (PCL1975 containing pMP7420 harboring P_{tac} -*rpoS*), and PCL2056 (PCL1971 containing pMP7455, P_{tac} -*pip*). Average and standard deviations of at least three experiments are shown. Bars followed by different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test.

***iopB* mutant PCL1975 is impaired in biocontrol**

The significance of PCN production in biological control by *P. chlororaphis* strain PCL1391 was demonstrated by Chin-A-Woeng, et al., (1998), who showed that a biosynthetic PCN mutant of strain PCL1391 did not suppress tomato foot and root rot in contrast to the wild-type strain. In order to determine if *iopB* is important for biocontrol, the

iopB mutant PCL1975 was tested in a tomato foot and root rot biocontrol assay. Tomato seeds were planted and grown in *Fusarium oxysporum* f. sp. *radicis-lycopersici* infested soil and after three weeks plants were scored for the presence of disease symptoms. Coating the seeds with *P. chlororaphis* PCL1391 reduced the percentage of diseased plants from 56% (non-coated control) to 25%. Coating the seeds with strain PCL1975 (*iopB*::Tn5luxAB) did not significantly reduce plant disease (55% diseased plants) as compared to the negative control and demonstrated that strain PCL1975 has lost the ability to control tomato foot and root rot (Fig. 8).

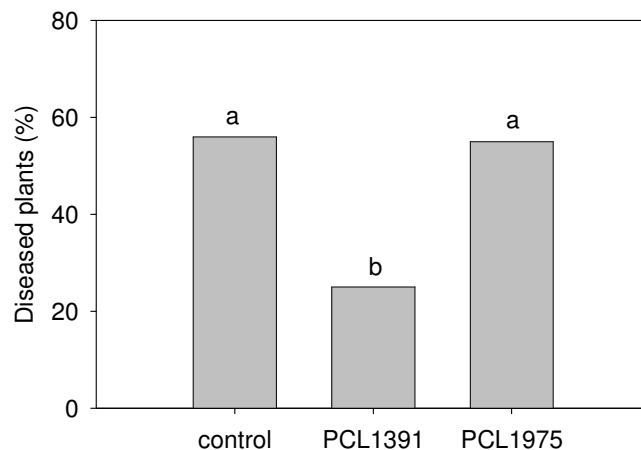


Fig. 8. Biocontrol of tomato foot and root rot by *P. chlororaphis* strain PCL1391 and strain PCL1975 (*iopB*::Tn5luxAB).

Tomato seeds coated with the bacterial strain were sown in non-sterile potting soil infested with spores of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. After 16 days the number of diseased plants was determined and the disease percentage is shown on the Y-axis. Bars with different letters are significantly different at $P=0.05$ according to Fisher's least significant difference test.

Discussion

The biocontrol strain *P. chlororaphis* strain PCL1391 suppresses tomato foot and root rot (Chin-A-Woeng et al., 1998), wheat take all (van den Broek et al., 2003) and white root rot in Avocado (Cazorla et al., 2006) which are all caused by fungal pathogens. Production of the anti-fungal metabolite phenazine-1-carboximide (PCN) is crucial for biocontrol (Chin-A-Woeng et al., 1998). This study describes two adjacent genes (*iopA* and *iopB*) that positively influence PCN and C₆-HSL production. For this reason the genes were named inducer of phenazine A and B (*iopA* and *iopB*). Strain PCL1975 (*iopB*::Tn5luxAB) and the fact that a mutation in the gene down stream of *iopB*, amidinotransferase gene, is not involved in the PCN biosynthesis, demonstrates that *iopB* is essential for the biosynthesis of PCN.

Sequencing of the *iopB* locus identified the genes flanking *iopB* and demonstrated that *iopB* is the second gene of at least five genes, which are transcribed in the same orientation. The facts that the *iopB* mutant PCL1975 is impaired in the production of PCN and that mutating the third and fourth gene of this locus does not affect PCN production (Fig. 2), demonstrates that *iopB* is essential for PCN production and that the *iopB* mutant phenotype is not caused by a downstream transcription effect on other genes. The gene advancing *iopB* in this locus was named *iopA*. The *iopA* mutant is also impaired in the production of PCN and C₆-HSL. The genes *iopA* and *iopB* could be part of one operon and a mutation in *iopA* could result in a decreased PCN and C₆-HSL production due to a downstream transcription effect on *iopB*. However, the *iopA* mutation could be complemented with a plasmid containing *iopA* under control of the *tac* promoter and the *iopB* mutation could be complemented by introducing *iopB* under control of its own promoter in the *iopB* mutant (Fig. 2). This demonstrates that *iopA* is involved in the regulation of PCN and *iopA* and *iopB* are both affecting PCN production and are not part of one operon. The increased production of PCN and C₆-HSL by the complemented *iopA* and *iopB* mutant strains PCL2066 and PCL2092 compared to the wild-type strain PCL1391 could be caused by the increased copy number of *iopA* and *iopB* in the complemented strains as a result of pBBR1MCS-5.

Remarkably, *iopA*, *iopB* and the putative amidinotransferase gene have closest homology with genes from *Ralstonia solanacearum* and *Streptomyces* strains and not with *Pseudomonas* sequences. The *iopA* and *iopB* homologues in *Streptomyces* are also two adjacent genes and in *Ralstonia solanacearum* they are separated by two genes. This could suggest that this genomic region was acquired by *P. chlororaphis* PCL1391 and not by any of the sequenced *Pseudomonas* strains. As far as we know, functions of these genes have not been described.

The genomic region between *iopA* and the upstream predicted transcriptional regulator contains two inversed -10 and -35 promoter sequences that could initiate transcription in two directions (Fig. 1). This region could possibly form a molecular switch between the transcriptional regulator and *iopA* transcribing in one direction and thereby blocking transcription in the other direction.

PCN production in *P. chlororaphis* strain PCL1391 depends on quorum sensing and the production of C₆-HSL has been observed to be strongly correlated with PCN production (Chin-A-Woeng et al., 2001b; van Rij et al., 2004). Also the *iopA* and *iopB* mutants produced less C₆-HSL than PCL1391 (Fig. 3). This is probably caused by a reduced expression of the quorum sensing genes *phzI* and/or *phzR* (van Rij et al., 2005) since constitutive expression of *phzI* increased HSLs and PCN production in the *iopB* mutant (Figs. 5 and 6). Constitutive expression of *phzI* resulted in higher production of PCN in the *iopB* mutant background

(PCL2031) compared to the wild-type strain containing the same construct (PCL1999) for unknown reasons (Fig. 6). Constitutive expression of *phzR* increases the production of HSLs and restores PCN production (Figs. 5 and 6) since PhzR stimulates the expression of *phzI* and therefore increases C₆-HSL production. This suggests that *iopB* directly or indirectly regulates transcription of *phzI* and or *phzR*

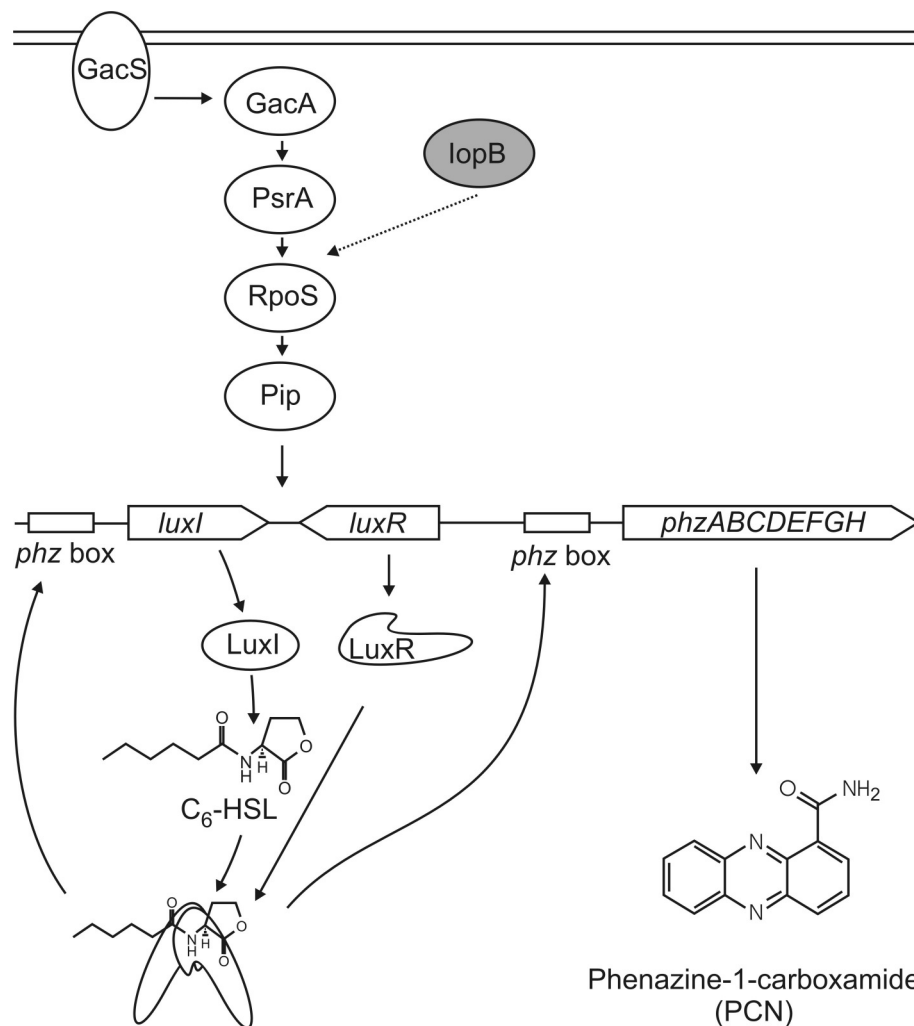


Fig. 9. Model for the regulation of PCN biosynthesis by *P. chlororaphis* strain PCL1391. On top of the PCN regulatory cascade the global regulatory two component signaling system formed by GacS and GacA is positioned, which is regulating secondary metabolism including PCN. *psrA* expression is activated by GacA, PsrA activates *rpoS* expression and RpoS stimulates *pip* transcription. Pip activates expression of the quorum sensing system *phzI/phzR*, which controls the production of PCN by controlling the expression of the *phz* operon. Results presented in this paper show that *iopB* has a positive effect on quorum sensing and PCN production. Our data indicated that *iopB* influences *rpoS* and *pip* expression.

The two regulatory genes, *rpoS* and *pip*, complement the *iopB* mutation when expressed constitutively (Fig. 7). Remarkably, constitutive expression of *psrA* did not increase PCN levels in the *iopB* mutant whereas that of *rpoS* did (Fig. 7). Since *rpoS* was shown to be controlled by *psrA* (Girard et al., 2006; Kojic et al., 2002; Kojic and Venturi, 2001) similar results from constitutive expression of both genes could have been expected. However, negative regulatory feed back loops by RpoS on its own transcription could prohibit the stimulatory effect of constitutive expression of *psrA* (Girard et al., 2006). The results suggest that *iopB* regulates *rpoS* and *pip* since constitutive expression of these genes complement the *iopB* mutant phenotype. The results from the studies presented in this paper position *iopA* and *iopB* upstream of *phzI*, *phzR*, *pip*, and *rpoS* in the regulatory cascade of PCN production in *P. chlororaphis* strain PCL1391 (Fig. 9.) However, further genetic characterization is required to show the regulatory function of *lopB* and its direct target genes.

The *iopB* mutation results in loss of biocontrol of tomato foot and root rot (Fig. 8). This is probably caused by the impaired PCN production of the *lopB* mutant, and suggests that *lopB* stimulated the production of PCN and C₆-HSL in rhizosphere relevant conditions since PCN production is essential for biocontrol by *P. chlororaphis* strain PCL1391.

Materials and Methods

Microorganisms and culture conditions

The microbial strains and plasmids used are listed in Table 1. Luria-Bertani (LB) medium (Sambrook and Russel, 2001) was used as standard medium for culturing *Escherichia coli* strains and *Chromobacterium violaceum* CV026. A modified Vogel Bonner medium (van Rij et al., 2004; Vogel and Bonner, 1956) was used to culture *Pseudomonas* cells. Modified Vogel-Bonner # 1 (MVB1) contained final concentrations of 30 mM glucose, 0.05 % (w/v) casamino acids, 57 mM K₂HPO₄, 16 mM Na(NH₄)HPO₄, 81 mM MgSO₄, 78 µM NaFeEDTA(III), 6.8 µM MnSO₄, 0.35 µM CuSO₄, 4.1 µM Na₂MoO₄, 0.85 µM ZnSO₄, 51 µM H₃BO₃ and the pH was adjusted to 6.6 using HCl (van Rij et al., 2004). Solidified growth media contained 1.8 % (w/v) agar (Difco Laboratories, Detroit, MI). If appropriate, media were supplemented with the antibiotics kanamycin or gentamycin at final concentrations of 50 and 30 µg ml⁻¹, respectively. Cultures were shaken at 195 rpm on a Janke und Kunkel shaker KS501D (Staufen, Germany) at 28 °C and growth was monitored by measuring OD_{620nm}. Growth analyses were conducted in 100 ml flasks with 10 ml culture media after inoculation from a 5 ml overnight culture to an OD_{620nm} of 0.1.

Isolation of mutants impaired in PCN biosynthesis

A mutant library of PCL1391 was generated with pRL1063a (Wolk et al., 1991) which resulted in random insertions of Tn5 transposons carrying promoterless *luxAB* reporter genes and a kanamycin selection marker. Mutants were screened for absence of the yellow PCN color on MVB1 agar plats. Flanking regions of Tn5 insertions were recovered by digesting chromosomal DNA with *EcoRI* (of which the target sequence is not present in pRL1063A), subsequent ligation of the fragments and selection of the pRL1063a insert with the flanking chromosomal regions using kanamycin. Sequencing of the flanking chromosomal regions was performed with oMP458 (5'-TACTAGATTCAATGCTATCAATGAG-3') and oMP459 (5'-AGGAGGTCACATGGAATATCAGAT-3'). Sequencing of the flanking regions of PCL1975 was continued with primers oMP942 (5'-GCGCGCCAGGCACTCGCG-3'), oMP943 (5'-CTGATGGGCAAGATGATCC-3'), oMP956 (5'-ACCGCCACGGCCAGGAGTTCCG-3'), oMP958 (5'-GTTGATCCAGGGTTTGCTGTCC-3'), oMP959 (5'-GCACACTGGCAGCGG GTCC-3'), oMP966 (5'-CCGGAATTCGGCCTGTTCCGCCCTGGATTAC-3'). Obtained sequences were analyzed using BLASTX in GeneBank (Altschul et al., 1990) and are accessible under number DQ367408.

DNA manipulation

Polymerase chain reactions were carried out with Super *Taq* enzyme (Enzyme Technologies Ltd, UK). The primers were synthesized by Isogen Life Science (Maarsse, The Netherlands). Restriction enzymes were purchased from New England BioLabs Inc. (Westburg, Leusden, The Netherlands) and ligase from Promega (Leiden, The Netherlands). Directed mutagenesis of *iopB* was performed by generating the suicide plasmid pMP7586 by inserting a 510 bp *iopB* PCR fragment generated by oMP944 (5'-CGGAATTCGTACAGGCGGGGCTGACCGC-3') and oMP945 (5'-CGGAATTCTGCGCC AGGCTGCGGCCTTC-3') into pMP5285. Mutants in *iopB* were verified by PCR on the genomic DNA using oMP944 and oMP946 (5'-GCACGATCAGGCGGCTGCGTG-3'). The *iopA* gene was mutated by the suicide plasmid pMP7580 which is a derivative of pMP5285 containing a 615 bp *iopA* PCR fragment generated by oMP921 (5'-CCCATCTTCTGTGCGCCCTCG-3') and oMP920 (5'-GTTCCGGATGCCTGGACACTGCC-3'). The open reading frame coding for a putative amidino transferase was mutated by the suicide plasmid pMP7476 which was a derivative of pMP5285 containing a 413 bp fragment generated by oMP964 (5'-CCGGAATTCGGCCTGTTCCGCCCTGGATTAC-3') and oMP965 (5'-CGGAATTCGATCCGGTAGGTGCTGTCCAG-3'). Mutants were confirmed by PCR using oMP957 (5'-CGCCATCGACGCCTACATGCG-3') in combination with either oMP985 (5'-CCTGCAGGTGCGACTCTAGAGGATCC-3') or oMP986 (5'-TATTAAGTTTATTCTTATC

AATATAGGAGC-3'). The open reading frame coding for a putative lysine exporter was mutated by the suicide plasmid pMP7479, which is a derivative of pMP5285 containing a 388 bp fragment generated by oMP998 (5'-CCGGAATTCGACTACTACGCCGTCATCCTG-3') and oMP999 (5'-CCGGAATTCGTGGTGTAGGCCAGGACCGC-3'). Mutants were confirmed by PCR using oMP966 combined with either oMP985 or oMP986.

A *P_{tac}-iopA* fragment was generated by a PCR with oMP1002 (5'-GATGGGCCCTCATGGCAGACTCCTTTAAAG-3') and oMP983 (5'-GTGAGCGGATAACAATTTTCACACAGGAAACAGCTAACCCGCATCGCGCTGTACTGGTTG-3') and a second PCR using the former PCR as a template with oMP1002 and oMP1000 (5'-GATGGGCCCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTTC-3'). The fragment was cloned with *Apal* into pBBR1MCS-5 resulting in Pmp7478.

An *iopB* fragment including the 887bp upstream putative promoter region was generated by a PCR with oMP1066 (5'-CGGGATCCGGACAGCAAACCCTGGATCAAC-3') and oMP1115 (5'-CGGGATCCAGGCGCTGTAACCAGGACG-3'). The fragment was cloned with *BamHI* into pBBR1MCS-5 resulting in Pmp7589.

Analysis of phenazine-1-carboxamide (PCN) production

For monitoring PCN production in time, PCN was extracted according to Chin-A-Woeng *et al.* (1998) with minor modifications. Culture samples (250 µl) were centrifuged and the culture supernatants were acidified to pH 2 using 6 M HCl. They were subsequently extracted with an equal volume of toluene by shaking on an Eppendorff mixer 5432 for 5 min. After centrifugation, the toluene phase was collected and dried in a rotary evaporator. The dry residue was dissolved in 100 µl acetonitrile and the obtained solution was mixed with 400 µl water.

PCN concentrations were determined by high-performance liquid chromatography (HPLC) (DIONEX, Sunnyvale, CA, chromeleon software version 6.20) using a calibration curve. HPLC was performed using an econosphere C18 5u, 259 mm x 4.6 mm column (Alltech Associates, Inc, Deerfield, IL) at 30 °C with a linear gradient of 20-80 % (v/v) acetonitrile acidified with 0.1 % (v/v) trifluoroacetic acid in water and a flow rate of 1 ml min⁻¹.

Analysis of *N*-acyl-homoserine lactone (AHL) production

Culture supernatants were extracted with 0.5 volume of ethyl acetate, shaken for half an hour and the solvent phase was collected. Subsequently the solvent phase was dried by rotary evaporation. The dried residue was dissolved in 25 µl acetonitrile and analyzed using TLC. Samples spotted on C18 TLC plates (Merck, Darmstadt, Germany) were developed in methanol-water (60:40) (v/v). After development, the TLC was overlaid with LB

0.8 % (w/v) agar containing a 10-fold diluted overnight culture of the *Chromobacterium violaceum* indicator strain CV026 (McClellan et al., 1997) and kanamycin ($50 \mu\text{g ml}^{-1}$). After incubation for 48 h at 28 °C, chromatograms were analyzed for appearance of violet spots.

Biocontrol assays

Tomato foot and root rot biocontrol tests using bacterial strains were performed according to Chin-A-Woeng et al. (1998). Tomato seeds (*Lycopersicon esculentum* Mill cv. Carmello) were coated with 1% (w/v) methylcellulose containing $1 \cdot 10^9$ CFU/ml, dried and put into non sterile potting soil infested with *Fusarium oxysporum* f.sp. *radicis-lycopersici* ($3 \cdot 10^6$ spores/kg). Roots were scored for disease symptoms (brown lesions) between 14 and 21 days after sowing

Acknowledgements

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Comparative studies of transcription profiles obtained from *Pseudomonas chlororaphis* strain PCL1391 in relation to phenazine-1-carboxamide production during growth under iron limitation, salt stress and in presence of phenylalanine

E. Tjeerd van Rij, Ben J.J. Lugtenberg, and Guido V. Bloemberg

Abstract

Production of the antifungal metabolite phenazine-1-carboxamide (PCN) by *Pseudomonas chlororaphis* strain PCL1391 is controlled by environmental conditions and a cascade of regulatory genes. This study was initiated to understand how environmental conditions influence PCN production and to identify novel putative regulatory genes. *P. chlororaphis* strain PCL1391 microarrays were used to obtain transcription profiles under conditions of salt stress, ferric iron limitation and after the addition of phenylalanine to the growth medium. The results show that the *phz* biosynthetic operon and the quorum sensing genes *phzI* and *phzR* are repressed during salt stress and under iron limitation and are induced by the presence of phenylalanine. Among the many genes which were differentially expressed, six were mutated. These included genes encoding a putative antioxidant protein, a heat shock protein, and four hypothetical proteins of which one is predicted to be a membrane protein. The obtained mutants were not affected in their PCN production and the corresponding genes do therefore not play a major role in the regulation of PCN synthesis.

Introduction

Many plant diseases caused by soil-borne pathogenic fungi can be controlled by plant root colonizing *Pseudomonas* strains (Haas and Défago, 2005; Lugtenberg et al., 2002; Lugtenberg et al., 2005; O'Sullivan and O'Gara, 1992; Thomashow and Weller, 1995). The molecular mechanisms by which these strains protect plants include induced systemic resistance (ISR) (Haas and Défago, 2005; Kamilova et al., 2005), the coordinated production of antifungal compounds (Chin-A-Woeng et al., 2003; Raaijmakers et al., 2002), and an efficient competition with the pathogenic fungi for nutrients and niches (Kamilova et al., 2005). The biocontrol strain *P. chlororaphis* strain PCL1391 controls tomato foot and root rot caused by *Fusarium* f. sp. *radicis-lycopersici*. Biocontrol by this strain depends on the production of the antifungal metabolite phenazine-1-carboxamide (PCN) (Chin-A-Woeng et al., 1998). Phenazine producing *Pseudomonas* spp. have at least one biosynthetic operon of minimally seven conserved genes *phzABCDEFG* (Chin-A-Woeng et al., 1998; Mavrodi et al., 1998; Pierson, III et al., 1995). In *P. chlororaphis* the *phzG* gene is followed by the additional gene *phzH*, which is required to convert phenazine-1-carboxylic acid into PCN (Chin-A-Woeng et al., 2001a).

PCN production is strongly regulated by cell density and the quorum sensing regulatory genes *phzI* and *phzR*, both of which are essential for PCN production (Chin-A-Woeng et al., 2001b). In addition, expression of the quorum sensing system is regulated by a cascade which starts with the two component global regulatory system GacS and GacA. Production of secondary metabolites and exo-enzymes in many *Pseudomonas* spp. and

other Gram-negative bacteria are regulated by GacS and GacA (Heeb and Haas, 2001). In *P. chlororaphis* strain PCL1391, the GacS/A system stimulates subsequently expression of *psrA*, the gene product of which stimulates expression of the alternative sigma factor *rpoS* (Chin-A-Woeng et al., 2005; Girard et al., 2006). RpoS stimulates expression of *pip*, which directly or indirectly stimulates expression of the quorum sensing system and consequently expression of the *phz* operon.

PCN production by *P. chlororaphis* strain PCL1391 is affected by various growth conditions including the carbon nutrient source, the nitrogen concentration and the growth temperature. PCN production is stimulated by low oxygen concentrations and by phosphate, sulfate and magnesium ions. Amino acids also stimulate PCN production. The strongest effect was observed with phenylalanine. Iron limitation and salt stress repress the production of PCN (van Rij et al., 2004). In addition, it was shown that the fungal toxin fusaric acid, which is commonly produced by *Fusarium* species, reduces the production of the antifungal metabolite 2,4-diacetylphloro-glucinol by biocontrol strain *P. fluorescens* CHA0 (Duffy and D  fago, 1997) and PCN production by *P. chlororaphis* strain PCL1391 (van Rij et al., 2004; van Rij et al., 2005). Microarray analyses of PCL1391 demonstrated that fusaric acid and iron limitation result in overlapping expression profiles (van Rij et al., 2005). In this study we aimed at understanding the effect of environmental conditions on the regulation of PCN production and the identification of novel genes regulating the production of PCN. For this purpose microarray analyses were performed to determine the expression profiles of three environmental conditions affecting the production of PCN. These three conditions are salt stress, iron limitation and the addition of phenylalanine. A number of genes with differential expression during PCN repressing or stimulating conditions were mutated and their PCN production was analyzed.

Table 1. Microorganisms and plasmids used in this study

Strains or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
PCL1391	Wild-type biocontrol strain <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide	(Chin-A-Woeng et al., 1998)
PCL2076	PCL1391 derivative mutated in a hypothetical membrane homologue using pMP7481, Km ^r	This study
PCL2077	PCL1391 derivative mutated in an antioxidant homologue using pMP7482, Km ^r	This study

PCL2078	PCL1391 derivative mutated in a hypothetical protein homologue using pMP7483, Km ^r	This study
PCL2079	PCL1391 derivative mutated in a heat shock protein homologue using pMP7484, Km ^r	This study
PCL2080	PCL1391 derivative mutated in a hypothetical protein homologue using pMP7485, Km ^r	This study
PCL2081	PCL1391 derivative mutated in a iron dependant peroxidase homologue using pMP7586, Km ^r	This study
CV026	<i>Chromobacterium violaceum</i> . N-acyl-homoserine lactone (HSL) reporter strain, Km ^r	(McClellan et al., 1997)
DH5 α	<i>SupE44 ΔlacU169(Φ80 <i>lacZ</i>ΔM15) <i>hsdR17 recA1 endA1 gyrA96 thi1 relA1</i></i>	(Boyer and Roulland-Dussoix, 1969)
Plasmids		
pBluescript	General purpose cloning vector, Cb ^r	Stratagene, La Jolla, CA
pRK2013	Helper plasmid for tri-parental mating	(Ditta et al., 1980)
pMP7481	Suicide plasmid pMP5285 containing a 360 bp fragment of a hypothetical membrane protein gene (clone 15_H6).	This study
pMP7482	Suicide plasmid pMP5285 containing a 404 bp fragment of a antioxidant gene (clone 40_B1)	This study
pMP7483	Suicide plasmid pMP5285 containing a 388 bp fragment of hypothetical protein gene (clone 48_F2)	This study
pMP7484	Suicide plasmid pMP5285 containing a 402 bp fragment of heat shock protein gene (clone 49_C12)	This study
pMP7485	Suicide plasmid pMP5285 containing a 303 bp fragment of hypothetical protein gene (clone 49_H2)	This study
pMP7486	Suicide plasmid pMP5285 containing a 406 bp fragment of a hypothetical protein gene (clone 75_B12)	This study

Results and Discussion

PCN production in relation to salt stress, iron limitation, high phenylalanine levels, and cell density

The production of PCN by *P. chlororaphis* strain PCL1391 (Table 1) is influenced by its growth conditions. A microarray similar to the one described in Chapter 3, except that the *P. chlororaphis* PCL1391 genomic PRC fragment library was spotted on three instead of two microscopic slides, was used to generate the transcription profiles of *P. chlororaphis*

PCL1391. Transcription profiles of cells grown under three different environmental conditions, salt stress, iron limitation and the presence of additional phenylalanine, were obtained. Previously, it was shown that phenylalanine stimulates the production of PCN, whereas salt stress and iron limitation repress the production of PCN (van Rij et al., 2004). The production of PCN under these growth conditions and the control (standard MVB1) is shown in Fig. 1. Growth in MVB1 results in the start of PCN production at an optical cell density of OD_{620nm} 2.0 and PCN concentrations continue to increase until the culture reaches stationary phase, which is at approximately OD_{620nm} 4.0 (Chapter 2, Fig. 1) (van Rij et al., 2004). Expression of the biosynthetic *phz* operon during growth in MVB1 was analyzed by using the *P. chlororaphis* strain PCL1391 reporter derivative strain PCL1119 (*phzB::Tn5luxAB*) (Chin-A-Woeng et al., 1998). The expression of the *phz* operon precedes PCN production and starts at an OD_{620nm} value of 1.6, increases with increasing cell density and stops once the stationary phase is reached (Chapter 3, Fig. 5) (van Rij et al., 2005). The start of PCN production (OD_{620nm} 2.0) was assumed to be the best moment to isolate RNA because at this moment the expression of the *phz* operon is highly stimulated by putative PCN regulators.

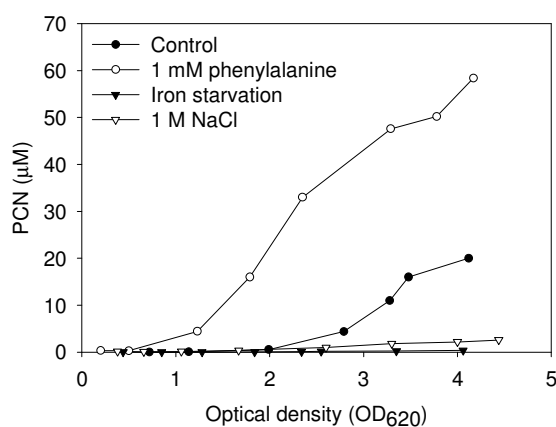


Fig. 1. Influence of additional phenylalanine, salt stress and iron limitation on the production of PCN by *P. chlororaphis* strain PCL1391 during growth. Cells were grown on MVB1 medium with or without ferric iron ions, 1.0 M NaCl or 1 mM phenylalanine. Amounts of PCN were determined by HPLC analysis. Experiments were performed at least three times with similar results. Data of one of the experiments is shown.

The transcription profile of salt stress was obtained by comparing the transcript abundance of RNA isolated at OD_{620nm} 2.0 during growth in MVB1 with additional salt and RNA isolated at OD_{620nm} 2.0 during growth on MVB1. The transcription profile of iron limitation was obtained in an identical manner. Since phenylalanine causes PCN production to start at an earlier OD_{620nm} of 1.2 (Fig. 1), the transcription profile of cultures with additional

phenylalanine was obtained by comparing the transcript abundance of RNA isolated at OD_{620nm} 1.2 during growth on MVB1 supplemented with additional phenylalanine and RNA isolated at OD_{620nm} 1.2 during growth on MVB1. At this early OD_{620nm} PCN production or *phz* operon expression is not yet initiated when grown on MVB1. Putative PCN regulators whose expression is advanced due to the addition of phenylalanine might be identified. The isolated RNA was transcribed to cDNA and labeled with different fluorescent dyes. The two fluorescent cDNA batches were hybridized with the microarray.

Comparison of transcriptomes of *P. chlororaphis* PCL1391 obtained during salt stress, iron limitation, and in the presence of additional phenylalanine

A selection of the most differentially expressed PCL1391 genes based on the results obtained from the transcription profiles during salt stress, iron limitation and additional phenylalanine are shown in Table 2. From a total of 12,924 genomic fragments spotted on three different glass slides, the percentage of detected spots was 59% for the culture with iron limitation, 43% for salt stress and 37% for additional phenylalanine. Note that for phenylalanine the data of only two slides are included. This difference in overall expression between iron limitation and salt stress on the one hand and phenylalanine addition on the other hand can be explained by the isolation of RNA at different cell densities. The number of genes being expressed at OD_{620nm} 2.0 (transition log/stationary phase) is could be higher than the number of genes expressed at OD_{620nm} 1.2 (log phase) because both the stationary phase and exponential phase genes will be transcribed at OD_{620nm} 2.0. Furthermore, specific sets of genes will be transcribed only during certain conditions and not during others. Iron limitation for instance induces various genes that are specifically involved in the adaptation to an iron limiting environment (Table 2).

Table 2. Differential expression of clones located on *P. chlororaphis* strain PCL1391 microarrays after hybridization with RNA isolated from cells grown under salt stress, iron limitation or addition of phenylalanine to the medium.^a

	Number of clones		
	NaCl	Fe ³⁺	Phenylalanine ^b
Total up regulated	26	301	25
Total down regulated	86	232	22
Percentage of expressed clones	(43%) 5616/12924	(59%) 7668/12924	(37%) 3222/8616

^aThe DNA array consisted of three slides which included 12924 different genomic clones induplicate.

^bFrom the phenylalanine treatment only the data from slide 1 and 2 was included and not those of slide 3.

Spots with a more than two-fold ratio difference were considered to represent genes that are differentially transcribed in comparison to the control condition (standard MVB1

medium). Salt stress induced the transcription of 26 clones and repressed the expression of 86 clones. Iron limitation resulted in the largest number of differentially transcribed clones: 301 were up-regulated and 232 were down-regulated. Addition of phenylalanine induced the transcription of 25 clones and repressed the expression of 22 (Table 2). This demonstrates that the *Pseudomonas* transcriptome undergoes large changes, especially as a result of iron limitation and underlines the importance of iron metabolism (Palma et al., 2003). Salt stress and additional phenylalanine cause less transcriptional changes than iron limitation. This suggests that these two conditions require less adaptation from *P. chlororaphis* strain PCL1391.

In order to identify which genes are differentially transcribed, a selection of clones with a more than two-fold ratio difference was sequenced and the identified genes are shown in Table 3. From the 53 sequenced clones, 12 contain a fragment with genes of the biosynthetic *phz* operon or of the quorum sensing genes *phzI* or *phzR* (Table 3). Both salt stress and iron limitation repress the production of PCN and reduce the transcription of the *phz* operon. Phenylalanine induces the production of PCN and increased the transcription of the *phz* operon as compared to the control. The changes in gene expression correlated with the observed changes in PCN production (van Rij et al., 2005). Identification of PCN biosynthetic genes and quorum sensing genes in differently expressed clones validated the experimental set up of our microarray study.

Table 3. Genes of *P. chlororaphis* PCL1391 with altered expression caused by salt stress, iron limitation and additional phenylalanine^a.

Clone number ^b	Change of expression			Most homologues gene	Organism and Gene accession number ^c
	Growth condition				
	NaCl	Fe ⁺	Phe		
Phenazine biosynthetic and regulatory genes					
97_D1	0.055	0.036	x ^d	<i>phzD</i>	<i>P. chlororaphis</i> AAF17498.1
119_D12	0.15	0.1	2.2	<i>phzE</i>	<i>P. chlororaphis</i> AAF17499.1
126_G12	0.15	0.062	3.5	<i>phzH</i>	<i>P. chlororaphis</i> AAF17502.1
23_B10	0.051	0.15	9.5	<i>phzH</i>	<i>P. chlororaphis</i> AAF17502.1

30_G2	0.053	0.13	13.6	<i>phzB/C</i>	<i>P. chlororaphis</i> AAF17496/7.1
34_G1	0.045	0.11	23.7	<i>phzC</i>	<i>P. chlororaphis</i> AAF17497.1
4_D1	0.064	0.15	8	<i>phzB/C</i>	<i>P. chlororaphis</i> AAF17496/7.1
6_F9	0.045	0.09	15.2	<i>phzC/D</i>	<i>P. chlororaphis</i> AAF17497/8.1
57_E12	0.14	0.074	x ^d	<i>phzH</i>	<i>P. chlororaphis</i> AAF17502.1
69_E8	0.12	0.038	x ^d	<i>phzC</i>	<i>P. chlororaphis</i> AAF17497.1
93_G11	0.3	0.12	x ^d	<i>phzI</i>	<i>P. chlororaphis</i> AAF17493.1
2_A5	0.11	0.3	9.5	<i>phzR</i>	<i>P. chlororaphis</i> AAF17494.1
NaCl affected clones					
53_F2	0.26	1.2	1.2	3-hydroxyacyl-acyl carrier CoA transferase	<i>Pseudomonas</i> sp. BAB32432
77_B7	0.43	0.6	x ^d	Cytochrome <i>c</i>	<i>P. fluorescens</i> ZP_00265653.1
Iron limitation affected clones					
62_H8	- ^e	54	x ^d	Outer membrane ferripyoverdin receptor	<i>P. putida</i> NP_746334.1
36_B6	- ^e	56	- ^e	Outer membrane ferripyoverdin receptor, <i>fpvA</i>	<i>P. putida</i> NP_746334.1
60_D12	- ^e	60	x ^d	Outer membrane ferripyoverdin receptor <i>fpvB</i>	<i>P. aeruginosa</i> NP_252857.1
3_C9	- ^e	3.7	0.7	Siderophore non-ribosomal peptide synthetase, <i>pvdI</i>	<i>P. aeruginosa</i> ZP_00140124.1
21_C4	- ^e	17	- ^e	Siderophore non ribosomal peptide synthetase, <i>pvdD</i>	<i>Azotobacter vinelandii</i> ZP_00092691.1
127_A3	- ^e	16	- ^e	Siderophore non ribosomal peptide synthetase	<i>Azotobacter vinelandii</i> ZP_00092691.1
68_C11	- ^e	14	x ^d	Siderophore non-ribosomal peptide synthetase	<i>P. fluorescens</i> ZP_00265634.1

89_H9	0.7	0.2	x ^d	Siderophore non-ribosomal peptide synthetase	<i>P. fluorescens</i> ZP_00265634.1
118_C10	- ^e	12	- ^e	Siderophore non-ribosomal peptide synthetase	<i>P. fluorescens</i> ZP_00265634.1
53_G8	0.7	19	- ^e	Siderophore non-ribosomal peptide synthetase	<i>P. putida</i> CAC32046.1
68_H4	- ^e	10	x ^d	Siderophore non-ribosomal peptide synthetase	<i>Azotobacter vinelandii</i> ZP_00092691.1
65_C5	- ^e	27	x ^d	L-ornithine N5-hydroxylase	<i>Pseudomonas</i> sp. AAG27518.1
35_D7	0.8	23	-	Pyoverdine synthetase A	<i>P. fluorescens</i> AF237701_1
12_B5	- ^e	0.05	1.1	Cbb3-type cytochrome oxidase, subunit 1	<i>P. fluorescens</i> ZP_00265650.1
104_H4	0.8	0.05	1	Cbb3-type cytochrome oxidase, subunit 1	<i>P. fluorescens</i> ZP_00263212.1
50_A5	- ^e	85	-	Fumarase, fumC1	<i>P. fluorescens</i> ZP_00266219.1
83_B11	- ^e	70	x ^d	Fumarase C, fumC1	<i>P. putida</i> AAD28162.1
59_H6	1	0.17	x ^d	Valyl-tRNA synthetase	<i>P. syringae</i> ZP_00124212.1
14_C10	- ^e	2	- ^e	Conserved hypothetical protein and cation ABC transporter, and periplasmic cation-binding protein,	<i>P. syringae</i> NP-791964.1 <i>P. syringae</i> NP_791963.1
102_B2	- ^e	0.14	- ^e	Acyl-CoA dehydrogenase family protein	<i>P. fluorescens</i> YP_262743.1
39_A9	0.8	0.37	0.7	Dipeptide ABC transporter, periplasmic dipeptide-binding protein	<i>P. fluorescens</i> YP_258010.1
82_H10	1	0.2	x ^d	Methyl-accepting chemotaxis protein	<i>P. fluorescens</i> ZP_00264179.1
106_A10	1.2	0.16	1.6	Hypothetical protein, =128_G10	<i>P. aeruginosa</i> AAG07294.1
128_G10	1.2	0.18	1.6	Hypothetical protein, 106_A10	<i>P. aeruginosa</i> AAG07294.1

Phenylalanine affected clones					
49_C12 ^f	1.4	0.6	7.1	Heat shock protein, <i>htpG</i>	<i>P. syringae</i> NP_792029.1
50_F5	1.8	0.7	7.1	Heat shock protein, <i>ibpA</i>	<i>P. putida</i> NP_744133.1
Salt stress and iron limitation affected clones					
109_H10	0.49	23	0.6	Membrane ferripyoverdin receptor, <i>fpvA</i>	<i>P. putida</i> NP_746334.1
40_C11	0.5	23	x ^d	ABC transporter <i>pvdE</i> and membrane ferripyoverdin receptors <i>fpvA</i>	<i>P. putida</i> , NP_746334.1 <i>P. aeruginosa</i> NP_251087.1
118_B9	0.39	0.28	0.6	Outer membrane porin, <i>oprD</i> family	<i>P. fluorescens</i> YP_258011.1
39_H8	0.44	0.26	1.1	Cytochrome <i>c</i> and Cbb3-type cytochrome <i>c</i> oxidase, subunit1	<i>P. aeruginosa</i> NP_250243.1 <i>P. putida</i> NP_746371.1
82_H9	0.03	0.04	x ^d	Alanyl-tRNA synthetase, <i>alaS</i>	<i>P. putida</i> NP_746585.1
49_H2 ^f	0.47	8.3	0.4	Hypothetical protein	<i>P. fluorescens</i> ZP_000833396.1
75_B12 ^f	2.1	10.5	x ^d	Hypothetical protein and hypothetical protein	<i>P. fluorescens</i> , ZP_00087993.1 <i>P. syringae</i> ZP_00127496.1
Iron limitation and phenylalanine affected clones					
40_B1 ^f	1.1	0.18	4.3	Anti oxidant protein, <i>IsfA</i>	<i>P. putida</i> NP_742403.1
20_E8	0.8	0.28	2.3	Hyp. protein	<i>P. fluorescens</i> ZP_00085666.1
15_H6 ^f	0.8	0.19	2.4	Hypothetical membrane protein and oxygen-independent coproporphyrinogen synthase	<i>P. putida</i> ZP_00127239.1 <i>P. putida</i> AF321091_1

Salt stress, iron limitation and phenylalanine affected clones

48_F2 ^f	0.072	0.4	8.6	Hypothetical protein	<i>Ralstonia</i> <i>metallidurans</i> ZP_00022895.1
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^a All clones selected in this table correspond to genes of which the expression was decreased (-) or increased (+) by at least one of the three test conditions: salt stress, iron limitation or phenylalanine addition.

^b The clone number refers to the number in the chromosomal fragment library (plate number, row and column).

^c Organisms belonging to the genes shown in column five.

^d Data missing due to the incomplete set of phenylalanine slides (x).

^e Signal for this clone was not detected (-)

^f The genes found on these clones were mutated and the PCN production of the mutants was analyzed in Fig. 2.

Identification and characterization of differentially expressed genes during salt stress, iron limitation, and in the presence of additional phenylalanine

Clones containing sequences other than *phz* genes were analyzed using BLAST software (Altschul et al., 1990) for similarity prediction (Table 3, column five). The sequences of the clones of table 3 are available at (<http://biology.leidenuniv.nl/ibl/S4/data>). Clones that were only induced by salt stress include two genes. The first one encodes a protein that has similarity with a 3-hydroxyacyl carrier CoA transferase and the second one has similarity with a cytochrome *c*. The relationship of these two genes with salt stress is unclear and cannot be explained by previous studies reported in the literature. Many sequenced clones were up-regulated by iron limitation and contain genes with similarity to ferripyoverdin receptors, siderophore peptide synthetases, *cbb3*-type cytochrome oxidase subunit 1, fumarase, valyl-tRNA synthetase, two ABC transporters, acyl-coA dehydrogenase family protein, methyl accepting chemotaxis protein and two hypothetical proteins. Many of these genes are homologous to genes that were identified in iron limitation studies performed in *P. aeruginosa* (Ochsner et al., 2002; Palma et al., 2003). A large number of these iron induced genes from *P. chlororaphis* strain PCL1391 were previously identified as being differentially transcribed due to the addition of fusaric acid to the medium (van Rij et al., 2005). Phenylalanine induces two clones predicted to encode two different heat shock proteins with similarity to HtpG and IbpA. This suggests a specific response to phenylalanine involving heat shock proteins, however there seems to be no data in the literature which confirm this notion. Clones affected by both iron limitation and salt stress contain sequences coding for proteins with similarity to a ferripyoverdine receptor, a porin protein, a cytochrome *c*, *cbb3*-type cytochrome oxidase, a alanyl-tRNA synthetase and two clones, 49_H2 and 75_B12, show homology to hypothetical proteins. The transcription of the ferripyoverdine receptor is known to be induced by iron limitation. All of these clones, with the exception of porin and clone 49_H2, were also

differentially transcribed during growth with additional fusaric acid (van Rij et al., 2005). It is unclear why these clones and the corresponding genes are differentially expressed by the presence of extra salt in the medium. Three sequenced clones were induced by phenylalanine and repressed by iron limitation. These sequences of these clones coded for genes with similarity to an anti oxidant protein, an oxygen independent coproporphyrinogen synthase and two hypothetical proteins. Nothing seems to be known about environmental regulation of these genes. Clones affected by salt stress and phenylalanine and not by iron limitation were not sequenced and therefore not included in Table 3. Clone 48_F2 is repressed by salt stress and iron limitation and induced by phenylalanine. The transcription profile of clone 48_F2 correlates with the production of PCN under the three different environmental conditions. This correlation makes the gene found on clone 48_F2 a good candidate to be involved in the regulation PCN in *P. chlororaphis* strain PCL1391.

Generation and characterization of *P. chlororaphis* PCL1391 derivatives mutated in putative *phz* regulating genes

In order to identify novel genes regulating the production of PCN in *P. chlororaphis* strain PCL1391, six genes present on independent differentially transcribed clones were mutated. The genes selected for mutagenesis were chosen from clones in four different categories listed in Table 3. One gene coding for a heat shock protein was selected from clone 49_C12 and was induced by phenylalanine. Two genes encoding hypothetical proteins were selected from two different clones, 49_H2 and 75_B12. Both clones are induced by iron limitation. Clone 49_H2 is repressed by salt stress and clone 75_B12 is induced by salt stress. Two genes identified on clones 40_B1 and 15_H6 code for an anti oxidant protein and a hypothetical membrane protein, respectively, and are repressed by iron and induced by phenylalanine. The latter gene, encoding a hypothetical protein, is identified on clone 48_F2 and was repressed by salt stress and iron limitation and induced by phenylalanine. The six generated mutants PCL2076, PCL2077, PCL2078, PCL2079, PCL2080, and PCL2081 were analyzed for PCN production, which varied for all mutants between 65 and 84 μM . These PCN levels are similar to the amounts of PCN produced by the wild-type strain PCL1391 (Fig. 2). It was therefore concluded that all six genes do not play a major role in the regulation of PCN production.

One possible reason that no PCN regulator was identified could be that the RNA was isolated at the wrong moment to identify transcriptional changes of PCN regulators, since expression of the *phz* operon initiates at the $\text{OD}_{620\text{nm}}$ value of 1.6 which is earlier than the production of PCN and the isolation of RNA ($\text{OD}_{620\text{nm}}$ value 2.0). A second possibility is that the microarray was insufficiently sensitive to detect expression differences of weakly

expressed regulatory genes. However, these microarray studies are informative with respect to *phzI*, *phzR* and *phz* operon expression during the three environmental conditions that were studied because it showed that environmental conditions determine the transcription of these genes and affect the production of PCN. Another informative aspect of the microarray is the demonstrated environmental regulation of numerous genes in *P. chlororaphis* strain PCL1391. This contributes to the understanding of gene expression during iron limitation, salt stress and phenylalanine addition in *P. chlororaphis* strain PCL1391 which probably can be extended to gene regulation in pseudomonads in general.

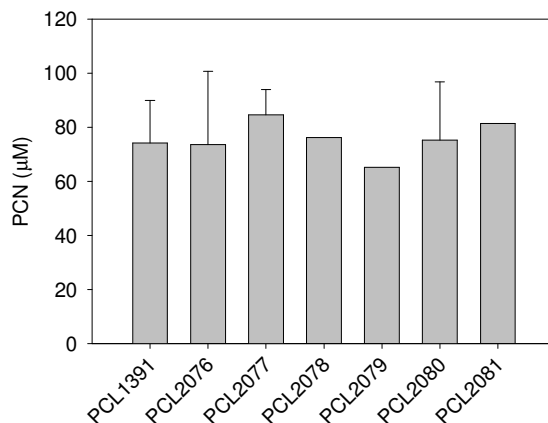


Fig. 2. Phenazine-1-carboxamide (PCN) production by *P. chlororaphis* PCL1391 and mutant derivatives. PCL1391, PCL2076 (hypothetical membrane protein mutant, clone15_H6), PCL2077 (antioxidant mutant, clone 40_B1), PCL2078 (hypothetical protein mutant, clone 48_F2), PCL2079 (heat shock protein mutant, 49_C12), PCL2080 (hypothetical protein mutant, clone 49_H2), and PCL2081 (iron independent peroxidase mutant, clone75_B12). Cells were grown on MVB1 and the PCN concentrations of the supernatant fluids were quantified by HPLC when cells reached stationary phase. When possible, average and standard deviations are shown.

Materials and Methods

Microorganisms, culture conditions and microarray

The microbial strains and plasmids used are listed in Table 1. Luria-Bertani (LB) medium (Sambrook and Russel, 2001) was used as standard medium for culturing *Escherichia coli* and *Chromobacterium violaceum*. A modified Vogel Bonner medium (van Rij et al., 2004; Vogel and Bonner, 1956) was used to culture *Pseudomonas* cells. For establishing iron limitation, NaFeEDTA(III) was omitted from MVB1. When appropriate MVB1 was supplemented with phenylalanine or NaCl at final concentrations of 1.0 mM and 1.0 M, respectively. Solidified growth media contained 1.8 % (w/v) agar (Difco Laboratories, Detroit,

MI). If appropriate, media were supplemented with the antibiotic kanamycin at final concentrations of 50 µg ml⁻¹. Cultures were shaken at 195 rpm on a Janke und Kunkel shaker KS501D (Staufen, Germany) at 28 °C and growth was monitored by measuring OD_{620nm}. Growth analyses and RNA isolation were conducted in 100 ml flasks with 10 ml medium after inoculation from a 5 ml overnight culture to a start OD_{620nm} of 0.1. For monitoring PCN production during growth, PCN was extracted as described by van Rij *et al.* (2004). Microarray construction, RNA isolation, and data analyses were developed by G. Girard as previously described by van Rij *et al.* (2005).

DNA manipulation

Polymerase chain reactions were carried out with Super Taq enzyme (Enzyme Technologies Ltd, UK). The primers were synthesized by Isogen Life Science (Maarsse, The Netherlands). Restriction enzymes were purchased from New England BioLabs Inc. (Westburg, Leusden, The Netherlands) and ligase from Promega (Leiden, The Netherlands). The hypothetical membrane protein identified on clone 15_H6 was mutated by using a pMP5258 based plasmid containing a 360 bp PCR fragment obtained with oMP1031 (5'-GCTGGAATTCAGCCAGACCGGT CGCCAGCAGCACCG-3') and oMP1032 (5'-GCCGGAATTCGGCCTGGCCGGCTG GGCGGTGGCCAG-3'). The gene coding for a putative antioxidant protein on clone 40_B1 was mutated by a pMP5285 derivative containing a 404 bp PCR fragment generated with oMP1033 (5'-GCCGGAATTCGACATCGCCCCGACTTCGAACA GG-3') and oMP1034 (5'-GCTGGAATTCGGCCGATAAGTGATGGTCAGGC-3'). The gene coding for a putative hypothetical protein identified on clone 48_F2 was mutated by a pMP5285 based vector containing a 388 bp PCR fragment obtained with oMP1018 (5'-CGGAATTCGTATGCTCGATTCCCCCTATGTTCG-3') and oMP1019 (5'-CGGAATTCGGCCGCCGCAATTGCCGCGAACC-3'). The gene coding for a putative heat shock protein identified on clone 49_C12 was mutated by insertion of a 402 bp PCR fragment generated by Omp1020 (5'-CGGAATTCGAGTGTGGAACTCAAAAGGAAACCC-3') and oMP1021 (5'-CGGAATTCGATGAAGGCGGAATAGAAGCCACG-3') into pMP5285. The hypothetical protein identified on clone 49_H2 was mutated by using a pMP5258 based plasmid containing a 303 bp PCR fragment obtained with oMP1027 (5'-CGGAATTCGATAAAGAGTGGCACCAGTCACCCG-3') and oMP1028 (5'-CGGAATTCGAGTTCAAGCTGCCAGGCCATCAGG-3'). The hypothetical protein identified on clone 75_B12 was mutated by using a pMP5258 based plasmid containing a 406 bp PCR fragment obtained with oMP1029 (5'-CGGAATTCGCCTCTCGCTGTGTTGCTGACCC-3') and oMP1030 (5'-CGGAATTCCTTGTTTCAGGCGGTGGCCAGGGC-3').

Chapter 7

Summary and general discussion

***Pseudomonas chlororaphis* PCL1391, the rhizosphere guard-dog**

Soil in the close vicinity of plant root and influenced by plant root is called the rhizosphere (Hiltner, 1904). For example, roots are changing the soil by taking up water and ions, and by exuding various compounds, including organic acids, sugars and amino acids. Microbes benefit from these exuded organic compounds and this explains the high microbial concentration in the rhizosphere as compared to bulk soil. Competition for the “nutrient rich” rhizosphere is fierce and various microbes produce toxic compounds and antibiotics to protect their niche.

Some soil microbes and fungi are plant pathogenic, which leads to substantial crop losses in agriculture and horticulture. Chemical pesticides are used world-wide to control these pathogens. However, the number of registered chemical pesticides continues to decrease due to tighter legislation caused by the polluting effect of some pesticides and the increasing concern for our environment and human health. A promising alternative is the use of bacterial biocontrol strains that are able to protect plants against fungal pathogens. *Pseudomonads* are abundantly present in soil and are able to (i) efficiently utilize root exudates as nutrient source, (ii) colonize the rhizosphere and (iii) control plant diseases (Bloemberg and Lugtenberg, 2001; Lugtenberg et al., 1999; Raaijmakers et al., 2002).

The biocontrol strain studied in this thesis is *P. chlororaphis* strain PCL1391, which controls tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-licopersici* (Chin-A-Woeng et al., 1998). Production of the anti-fungal metabolite phenazine-1-carboxamide (PCN) and an efficient root colonizing ability (Chin-A-Woeng et al., 2000) are crucial for efficient biocontrol by *P. chlororaphis* strain PCL1391. The PCN biosynthetic operon was identified and contains seven genes *phzABCDEFGH* (Chin-A-Woeng et al., 1998; Chin-A-Woeng et al., 2001a). PCN production is regulated by quorum sensing and the quorum sensing genes *phzI* and *phzR* are located upstream of the biosynthetic operon (Chin-A-Woeng et al., 2001b). In addition, PCN production is dependant on the GacS/GacA global regulatory system (Chin-A-Woeng et al., 2005). The *pseudomonas* σ regulator (PsrA) and the stationary phase sigma factor RpoS are also involved in the regulation of PCN production (Chin-A-Woeng et al., 2005; Girard et al., 2006). Although some genes involved in the regulation of PCN production have been identified and described, our knowledge of molecular and environmental regulation of PCN production is far from complete and, as indicated by previous studies, very complex.

This thesis aims at the identification and analysis of environmental conditions affecting the production of PCN and the identification of novel genes involved in the regulation of PCN production.

PCN production is regulated by environmental conditions (Chapter 2)

Chapter 2 (van Rij et al., 2004) aims at analyzing the effect of various environmental growth conditions on PCN production. Environmental conditions in the rhizosphere are dependant on the soil-type, climate, plant species and other organisms that are present in the soil. Some nutrients can be limiting and others can be present in excessive amounts. The results (van Rij et al., 2004) show that the carbon sources glucose, L-proline, glutamic acid and glycerol, have a stimulating effect on the production of PCN. The majority of the sugars and organic acids found in tomato root exudates components (Lugtenberg et al., 1999; Lugtenberg and Bloemberg, 2004) that were tested, do not stimulate PCN production. Plant roots also secrete amino acids, although in tomato root exudates only low amounts were detected (Simons et al., 1997). Amino acids stimulate PCN production when added in relatively small amounts to the growth medium (van Rij et al., 2004). The aromatic amino acid phenylalanine causes the largest stimulation (23-fold) of PCN production. This implies that the majority of plant root exudates (organic acids) stimulate growth and not the production of PCN. Whether PCN production can be induced upon interaction with other microbes including pathogenic fungi is unknown but a very interesting possibility. A specific induction of PCN in the presence of fungi would be very efficient to inhibit pathogenic fungi. Many abiotic factors such as temperature, oxygen tension and pH are influencing PCN production. Optimal PCN production occurs between 21 and 31 °C. The the production of PCN is reduced when the temperature drops to 16 °C. Low oxygen tension, which is thought to occur in the rhizosphere (Højberg et al., 1999), stimulates the production of the quorum sensing signal C₆-HSL as well as PCN production. Remarkably, a low oxygen concentration seems to be the only factor that reduces growth rate and stimulates the production and accumulation of C₆-HSL. Ferric iron and SO₄²⁻ stimulate the production of PCN and PO₄³⁻ concentrations around 70 mM are optimal for PCN production. Salt stress represses the production of PCN, and this effect is not due to a higher osmolality. To investigate the effect of environmental conditions on quorum sensing, the levels of the major auto-inducer of PCL1391, C₆-HSL, were analyzed. These results demonstrate that in all tested conditions the PCN levels correlate with the C₆-HSL levels. This shows that C₆-HSL production is not only dependant on the cell density but also on the environmental conditions.

Studies with other phenazine producing *Pseudomonas* strains demonstrate that phenazine production is regulated differently in every strain (Kanner et al., 1978; Labeyrie and Neužil, 1981; Slininger and Jackson, 1992; Slininger and Shea-Wilbur, 1995; Turner and Messenger, 1986). This indicates that the success of phenazine producing biocontrol strains, to control pathogenic fungi, depends on the environmental conditions of the agricultural field or greenhouse. It is therefore desirable to identify the critical parameters of phenazine

producing biocontrol strains to be able to select strains with the characteristics suitable for the agricultural conditions in which they will be applied.

Fungi can repress the production of PCN (Chapter 3)

Chapter 3 investigates the effect of fusaric acid, a fungal toxin, on PCN production and gene expression in *P. chlororaphis* strain PCL1391 (van Rij et al., 2005). In the rhizosphere, bacteria and fungi are interacting with each other and colonizing plant roots at similar sites (Bolwerk et al., 2003) probably in order to benefit from the nutritious root exudate. Both bacteria and fungi produce toxic and growth inhibitory compounds to increase their competitive strength. *P. chlororaphis* strain PCL1391 produces proteases, chitinases, lipases, HCN and the anti-fungal metabolite PCN (Chin-A-Woeng et al., 1998; Chin-A-Woeng et al., 2000). Many *Fusarium* species produce fusaric acid (FA), which is toxic for eukaryotes and prokaryotes (Bochner et al., 1980; Wang and Ng, 1999).

FA represses the production of the anti-fungal metabolites 2,4-diacylphloroglucinol in biocontrol strain *P. fluorescens* strain CHA0 (Duffy and Défago, 1997) and PCN in *P. chlororaphis* strain PCL1391 (Chapter 3) (van Rij et al., 2005). The repression of PCN by FA was studied in detail with transcription reporter strains and microarray analyses. Results show that the transcription of the PCN biosynthetic *phz* operon and the transcription of the quorum sensing regulatory genes *phzI* and *phzR* is repressed by FA. Constitutive expression of *phzI* or *phzR* increases the production of C₆-HSL and PCN. This suggests that FA directly or indirectly repress the expression of *phzI* or *phzR*. The ability of FA producing fungi to repress the production of anti-fungal metabolites PCN and 2,4-diacylphloroglucinol seems to be a great advantage to the fungi. However, *P. chlororaphis* strain PCL1391 exhibits biocontrol of tomato foot and root rot caused by the FA producing *F. oxysporum* f. sp. *radicis-lycopersici* (de Weert et al., 2004). These results can be explained by a concentration effect since 1 mM FA inhibits PCN production, whereas a ten-fold lower concentration did not reduce PCN production (van Rij et al., 2005).

Microarray data demonstrated that the transcription profile of *P. chlororaphis* strain PCL1391, when grown with FA in the medium, has a remarkable overlap with transcription profiles of *P. aeruginosa* grown under iron starvation (Ochsner et al., 2002; Palma et al., 2003). FA and iron limitation both induce the expression of the pyoverdine receptor genes *fpvA* and *fpvB* (Ghysels et al., 2004) and the pyoverdine biosynthetic and modification genes, *pvdI*, *pvdJ*, *pvdD* and *pvdA*. The fact that the transcription profiles are not identical, suggests that FA is not causing an iron limitation response caused by the iron chelating abilities of FA (Bochner et al., 1980). This notion was confirmed by experiments in which we added an excess of iron or using the strong iron chelator EDDHA. It therefore can be concluded that FA

and iron limitation trigger partially overlapping but different regulatory cascades which result in the repression of PCN production.

Identification of novel PCN regulators (Chapters 4 and 5)

The production of PCN in *P. chlororaphis* strain PCL1391 is regulated by a complex molecular network of which some genes have been identified (Chin-A-Woeng et al., 2001b; Chin-A-Woeng et al., 2003; Chin-A-Woeng et al., 2005; Girard et al., 2006). In order to identify more genes involved in the regulation of PCN two approaches were performed. In the first approach *P. chlororaphis* strain PCL1391 microarrays were used. Comparisons of transcription profiles of conditions favorable and non-favorable for PCN production were used to identify putative regulators of PCN production (Chapter 6). In the second approach a Tn5 mutant library was screened for mutants impaired in PCN production (Chapter 4 and 5). The previously identified genes that are involved in the regulation of PCN production namely, *phzI*, *phzR*, *gacS*, *psrA* and *rpoS* (Chin-A-Woeng et al., 2001b), were identified by screens using “rich” KB growth medium. As demonstrated in chapter 2, the production of PCN is regulated by a wide variety of environmental factors. This suggested that additional genes regulating PCN could be identified by screening mutants under different growth conditions. Therefore a *P. chlororaphis* strain PCL1391 mutant screen was performed in which the transposants were grown on the “poor” medium MVB1. This resulted in the identification of two mutants impaired in the production of PCN.

The mutated gene, disrupted by a Tn5*luxAB* transposon, was named inducer of PCN production (*ippA*). The *ippA* mutant (Chapter 4) is impaired in the production of PCN and the quorum sensing signal C₆-HSL. The protein encoded by *ippA* has the highest similarity at the amino acid level (92% identity) to a putative GTP cyclohydrolase I from *P. fluorescens* strain Pf-5 (Paulsen et al., 2005) that it is highly conserved in many *Pseudomonas* species (Nelson et al., 2002; Paulsen et al., 2005; Stover et al., 2000). This similarity is based on one domain that spans one third of the entire protein. Constitutive expression of the quorum sensing genes *phzI* and *phzR* in the *ippA* mutant induces the production of C₆-HSL and restores the production of PCN. This demonstrates that IppA positively regulates quorum sensing in *P. chlororaphis* strain PCL1391, which is essential for the production of PCN. Constitutive expression the PCN regulator *pip* also restores PCN levels, whereas constitutive expression of *psrA* or *rpoS* does not induce the production of PCN in the *ippA* mutant. Constitutive expression of *ippA* in the PCN impaired *psrA*, *rpoS* and *pip* mutants does not restore PCN production. These results suggest that *ippA* is acting upstream of *pip*, thereby controlling PCN production. The *ippA* mutant is impaired in biocontrol which could be explained by the lack of PCN production. However, this mutant is

also reduced in its growth which is resulting in impaired competitive root colonization. This implies *lppA* regulates additional cellular processes in addition to PCN and C₆-HSL production.

The *iopB* gene (Chapter 5) is part of a genomic locus of at least five equally orientated genes. Mutagenesis of the first (*iopA*) or second (*iopB*) gene of this locus resulted in strains that were impaired in the production PCN. Growth analyses demonstrated that the *iopA* and *iopB* mutants are not impaired in growth as compared with its wt. Both *iopA* and *iopB* encode hypothetical proteins that show similarity with hypothetical proteins from *Ralstonia* and *Streptomyces*, respectively. The *iopA* and *iopB* mutants produce reduced levels of the quorum sensing signal C₆-HSL. The similar phenotype of the *iopA* and *iopB* mutants suggests that both genes are positioned at the same location in the PCN regulation network. Constitutive expression of the quorum sensing genes *phzI* and *phzR* restored C₆-HSL and PCN production in the *iopB* mutant, which shows that *iopB* is a positive regulator of quorum sensing. Constitutive expression of *rpoS* and *pip* complemented the *iopB* mutant and suggest that *iopB* is acting upstream of these two regulators. Constitutive expression of *psrA* did not complement the *iopB* mutant, which could be due to its negative autoregulation, thereby not affecting *rpoS* and *pip* expression. The *iopB* mutant was impaired in biocontrol, which could be the result of the reduced production of PCN.

Identification of PCN regulators using microarrays (Chapter 6)

The second method used to identify genes involved in the regulation of PCN was based on the use of *P. chlororaphis* strain PCL1391 microarrays. This microarray contains several known control genes (based on previously identified genes) but the majority of the microarray consists of random genomic DNA fragments of PCL1391. Three different environmental conditions additional phenylalanine, salt stress and iron limitation (which were identified and studied in Chapter 2) were used in Chapter 5 to identify differently expressed genes. The transcription profiles of these three conditions; were obtained and analyzed. The analyses confirmed that transcription of the quorum sensing genes *phzI* and *phzR* and the biosynthetic PCN operon were repressed by iron starvation and by salt stress and induced by phenylalanine. Additionally, the majority of the random genomic fragments with a similar transcription profile turned out to contain fragments of *phzI*, *phzR* or the *phz* biosynthetic operon. The fact that their expression profiles correlates with the production of PCN during these conditions validates the microarray. Genes differentially regulated by iron limitation in *P. chlororaphis* are homologous to iron limitation studies performed in *P. aeruginosa* (Ochsner et al., 2002; Palma et al., 2003) and confirm our results. Further analyses of the microarray data resulted in the selection of six genes, which were mutated and analyzed for

differential production of PCN. None of the generated mutants was affected in the production of PCN. These results suggest that comparing transcription profiles obtained under different environmental conditions to identify genes involved in the regulation of PCN is not very efficient to identify novel PCN regulators. Screening for mutants using different environmental conditions, as used to identify the mutant described in Chapter 4, seems to be a more effective approach to identify genes involved in the complex molecular network regulating the production of PCN.

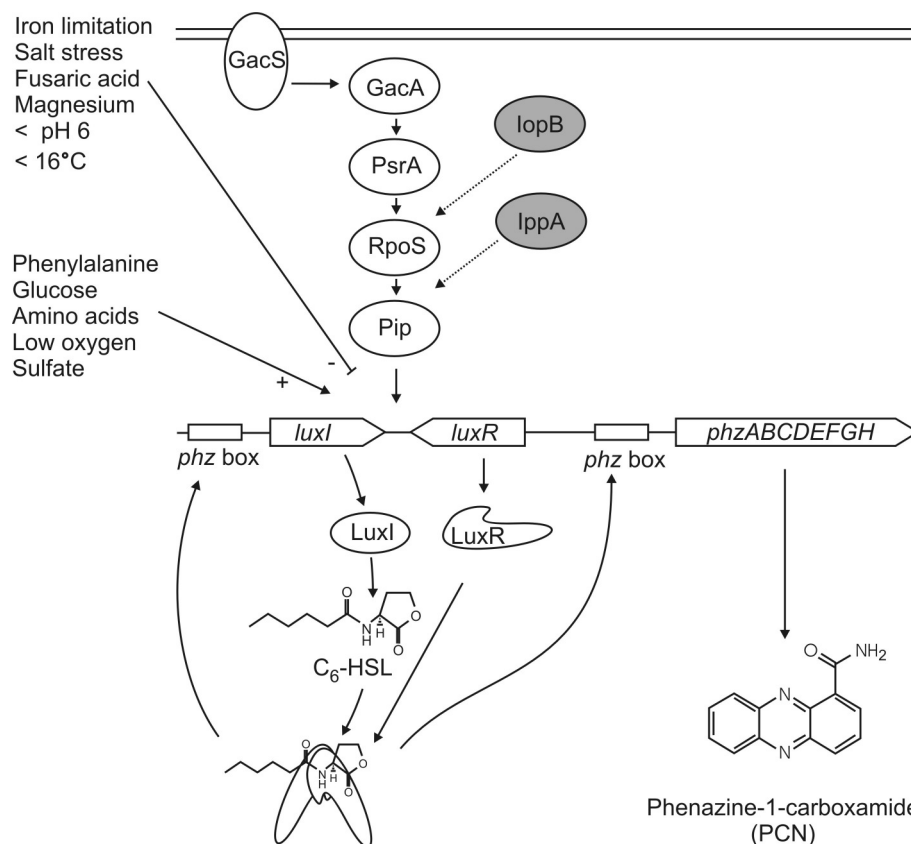


Fig. 1. Model for environmental and molecular PCN regulation in *P. chlororaphis* strain PCL1391. Lines ending with arrows indicate positive regulation. Lines ending with a small square line indicate negative regulation. The novel regulators involved in PCN production, lppA, lppB and lppC, are shown in gray shading.

Concluding remarks

This thesis contributes to the understanding of the environmental and molecular regulation of PCN production in *P. chlororaphis* strain PCL1391 as described in chapters 2 through 5. The obtained results are summarized in a model which includes the effect of

environmental conditions and lppA in the regulation of PCN production (Fig. 1). However the mechanism by which environmental stimuli are perceived and integrated in the molecular regulation network remains largely unknown and is a challenging field of research for the future. Understanding the regulation of PCN production could contribute to the development of more efficient biocontrol strains. Moreover, it contributes to the fundamental understanding of the regulation of secondary metabolism in Gram-negative bacteria in general.

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Samenvatting

De bacterie *Pseudomonas chlororaphis* stam PCL1391 helpt bij het bestrijden van plantenziektes

Ziekteverwekkende schimmels in de land- en tuinbouw zijn een groot probleem doordat zij de kwaliteit en kwantiteit van de oogst verminderen. Deze pathogene schimmels kunnen bestreden worden met chemische pesticiden, waarvan sommigen een groot gevaar vormen voor de volksgezondheid en het milieu. Pathogene schimmels kunnen ook op een biologische manier bestreden worden (biocontrole), namelijk door gebruik te maken van bepaalde bodembacteriën, die op en rond de wortels van planten leven (Fig. 3, Introduction). Pseudomonaden zijn bodembacteriën, die bekend staan om hun waardevolle biocontrole eigenschappen.

De biocontrole bacterie *Pseudomonas chlororaphis* stam PCL1391 produceert de anti-schimmelstof phenazine-1-carboxamide (PCN) (Fig. 5, introduction). De productie van PCN draagt bij aan competieren voor voedselrijke niches met andere micro-organismen. De productie van PCN is essentieel voor de bestrijding van plantpathogene schimmels, zoals *Fusarium oxysporum f.sp. radicis lycopersici*, die wortelrot bij tomaat veroorzaakt. PCN wordt niet constant geproduceerd en de productie wordt nauwkeurig gereguleerd. Bij lage celdichtheden wordt er geen PCN geproduceerd terwijl er bij hoge celdichtheden veel PCN geproduceerd wordt. Sommige bacteriën waaronder pseudomonaden kunnen hun celdichtheid waarnemen door het uitscheiden van kleine signaalmoleculen (acylhomoserine lactonen). Concentraties van deze signaalmoleculen worden waargenomen en wanneer deze een bepaalde drempelwaarde (concentratie) bereiken wordt de productie van PCN geïnitieerd. De rationale hier achter is dat de productie van PCN pas een effect heeft bij een concentratie die alleen gezamenlijk bereikt kan worden. Door op deze manier samen te werken kunnen bacteriën de strijd aan gaan met competierende organismen waaronder pathogene schimmels. Het PhzI eiwit produceert acylhomoserine lactonen en de transcriptionele regulator PhzR activeert de transcriptie van het PCN biosynthetisch operon. Behalve celdichtheid wordt PCN-productie nog op tal van andere manieren gereguleerd. In dit proefschrift wordt onderzocht hoe omgevingsfactoren de productie van PCN beïnvloeden en welke genen de PCN-productie reguleren.

In **hoofdstuk 2** wordt beschreven welke omgevingsfactoren en groeicondities de productie van PCN en acylhomoserine lactonen beïnvloeden. Er wordt aangetoond dat stikstof- en koolstofbronnen een grote invloed hebben op de groei en de productie van PCN. Ijzer-, fosfaat-, sulfaat-, en ammoniumionen, aminozuren, een lage zuurstofconcentratie en een lage magnesiumconcentratie stimuleren de productie van PCN. Daarentegen reduceren

zoutstress, lage temperatuur en de schimmelmetaboliet fusaarzuur de productie van PCN. Ook blijkt productie van acylhomoserine lactonen afhankelijk te zijn van deze omgevingsfactoren en een vermindering van PCN correleert met een verminderde productie van deze signaalmoleculen. De conclusie van de resultaten is dat de productie van PCN door *P. chlororaphis* stam PCL1391 sterk wordt beïnvloed door vele verschillende omgevingsfactoren, die de efficiëntie van het biocontroleproces sterk kunnen beïnvloeden.

Hoofdstuk 3 behandelt de invloed van het schimmelmetaboliet fusaarzuur op de PCN-productie en genexpressie van *P. chlororaphis* stam PCL1391. Fusaarzuur wordt geproduceerd door veel *Fusarium* stammen en is toxisch voor micro-organismen, planten en dieren. Productie van de toxische verbindingen PCN en fusaarzuur, door twee organismen, die elkaar daardoor beïnvloeden in groei en genexpressie illustreert de onderlinge competitie van micro-organismen in de bodem met behulp van chemische stoffen. Repressie van fusaarzuur op PCN- en acylhomoserine lactonsynthese wordt onder verschillende groeiomstandigheden waargenomen. Door een kunstmatig verhoogde en constante expressie van *phzI* en *phzR* kan verhinderd worden dat fusaarzuur de productie van acylhomoserine lactonen en PCN onderdrukt. Een uitgebreide genactiviteitsanalyse met behulp van DNA-microarrays laat zien dat genen die geactiveerd worden door fusaarzuur ook geactiveerd worden tijdens ijzerlimiterende groeiomstandigheden. Een verklaring hiervoor moet nader onderzocht worden.

Een mutant van *P. chlororaphis* stam PCL1391 die gestoord is in de productie van PCN is beschreven in **hoofdstuk 4**. Deze mutant is geïdentificeerd middels een selectie waarbij gekeken werd naar het ontbreken van de karakteristieke gele kleur van bacteriële kolonies, die veroorzaakt wordt door de productie van PCN. Het gemuteerde gen is genoemd *ippA* (*inducer of phenazine production A*) en is gestoord in de productie van PCN en acylhomoserine lactonen. Een kunstmatige hoge en constante expressie van *phzI* and *phzR* in de *ippA* mutant herstelt de productie van acylhomoserine lactonen en herstelt daardoor de productie van PCN. Deze mutant is niet langer in staat om tomatenplanten te beschermen tegen *Fusarium* infecties. Dit is mogelijk een direct gevolg van de verminderde productie van PCN.

In **hoofdstuk 5** worden twee genen beschreven (*iopA* en *iopB*) die de productie van PCN bij *P. chlororaphis* PCL1391 stimuleren. Stammen die gemuteerd zijn in *inducer of phenazine A of B* (*iopA* of *iopB*) produceren minder PCN en minder acylhomoserine lactonen. Ook bij *iopB* herstelt een kunstmatige hoge en constante expressie van *phzI* and *phzR* de productie van acylhomoserine lactonen en PCN. De *iopB* mutant is gestoord in biocontrole van tomatenplanten tegen *Fusarium* infecties. Dit is mogelijk een direct gevolg van de verminderde productie van PCN bij deze mutant.

In **hoofdstuk 6** zijn de transcriptieprofielen van *P. chlororaphis* stam PCL1391 na groei onder drie verschillende condities met elkaar vergeleken. De drie gebruikte transcriptieprofielen waren afkomstig van condities die de productie van PCN beïnvloeden (Hoofdstuk, 2). Deze groei condities zijn zoutstress, ijzertekort en toevoeging van het aminozuur phenylalanine, waarbij de eerste twee condities de PCN-productie remmen en de derde conditie de PCN-productie stimuleert. De data verkregen middels de microarrayanalyses laten zien dat de transcriptie van *phzI*, *phzR* en de PCN-biosynthesegenen vermindert tijdens zoutstress en ijzerlimitatie, terwijl deze transcriptie juist verhoogd is tijdens de groei in aanwezigheid van phenylalanine. Verder is gedemonstreerd in welk opzicht verschillende genen gereguleerd worden onder de drie gebruikte groeicondities. De functie van de betreffende genen en hun rol in *P. chlororaphis* moet in de toekomst verder opgehelderd worden.

Dit proefschrift beschrijft de invloed op de regulatie van PCN-productie door milieu- en moleculaire factoren. Op basis van deze data is een model gemaakt van alle genen en omgevingsfactoren, waarvan nu aangetoond is dat die de productie van PCN beïnvloeden (**hoofdstuk 7**). Het bestuderen van PCN-productie helpt bij het begrijpen en mogelijk verbeteren van biocontrole door *P. chlororaphis* stam PCL1391 en draagt bij aan de kennis over de complexe regulatie van de productie van secundaire metabolieten in bacteriën in het algemeen.

Curriculum vitae

Evert Tjeerd van Rij was born on the 14th of May 1976, in Leiden, The Netherlands. In 1994 he obtained his VWO certificate at the Rembrandt SG in Leiden. After one year as a sailing instructor and working holidays in Australia he started to study biology at the University of Amsterdam in 1995. As a student he participated in the biology student association Congo and was chairman during 1997. In 2000 he participated in the organization of the international biology student symposium SymBioSE. During his first internship the yeast cell wall was studied at the Plant physiology department, University of Amsterdam. During his second internship he studied the genetics of nuclear organelles called Cajal bodies in *Arabidopsis thaliana* at the John Innes Centre, Norwich, UK. After graduation in 2000 he worked for one year in the Plant physiology department at the University of Amsterdam on phospholipid signalling in tomato.

In 2001 he started as a PhD student at Leiden University in the molecular microbiology group of Prof. Dr. Lugtenberg and under the supervision of Dr. Bloemberg. His results on the environmental and molecular regulation of the antifungal metabolite, phenazine-1-carboxamide, produced by *Pseudomonas chlororaphis* strain PCL1391 are described in the thesis. In April 2006 he started as a Scientist Genetics at DSM Food Specialties in Delft.

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