



Universiteit
Leiden
The Netherlands

Genetic regulation of phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* strain PCL1391

Girard, G.

Citation

Girard, G. (2006, June 6). *Genetic regulation of phenazine-1-carboxamide synthesis by Pseudomonas chlororaphis strain PCL1391*. Retrieved from <https://hdl.handle.net/1887/4406>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/4406>

Note: To cite this publication please use the final published version (if applicable).

Genetic regulation of phenazine-1-carboxamide
synthesis by *Pseudomonas chlororaphis*
strain PCL1391

Geneviève Girard

Cover: The picture on the front cover is used with the gracious authorization of Yann Arthus-Bertrand. Legend translated from his website (www.yannarthusbertrand.com): *"Tree of life", Tsavo National Park, Kenya (2°11' S, 38°25' E). This acacia is a symbol of life in vast expanses of thorny savanna, where tracks of wild animals taking advantage of its leaves or its shade converge. Tsavo National Park in southeastern Kenya (...) is the country's largest protected area (8,200 square miles of 21,000 km²) and was declared a national park in 1948. (...) The prohibition of international trading in ivory and rhino horns has enabled certain wild animal populations to increase in number. However, poaching and disappearing natural habitats remain disturbing threats. World trade in wild flora and fauna provides between \$12 billion and \$19 billion in revenues each year.*

This picture was chosen for two reasons. Firstly, it displays a complex network of animal tracks that can symbolize the complex networks present through the thesis (genetic networks regulating secondary metabolism or complex relationships between microorganisms in the rhizosphere). Secondly, the tree in the center of the picture strikingly resembles an aging colony of *Pseudomonas chlororaphis* PCL1391, which itself looks dark yellow and displays a green central area due to precipitation of phenazines. In analogy to the picture on the front cover, on the back cover a picture is shown of such two aging colonies of *P. chlororaphis* at different enlargements.

Cover designed by Peter Hock.

Similarly to the front cover illustration, the picture present on the first page of each chapter represents a complex plant network and can symbolize complex networks studied in the thesis. Drawing from Olivier Girard.

Printed by: Ridderprint, Ridderkerk, The Netherlands

ISBN-10: 90-9020621-3

ISBN-13: 978-90-9020621-9

Pour mes parents

Genetic regulation of
phenazine-1-carboxamide synthesis by
Pseudomonas chlororaphis
strain PCL1391

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D. D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 6 juni 2006
klokke 15.15 uur

door

Geneviève Anne Odile Girard
geboren te Les Lilas, Frankrijk, in 1979

Promotiecommissie

Promotor:	Prof. Dr. E. J. J. Lugtenberg
Co-promotor:	Dr. G. V. Bloemberg
Referent:	Dr. B. Kraal
Overige leden:	Prof. Dr. P. J. J. Hooykaas Prof. Dr. H. P. Spaink Prof. Dr. C. A. M. J. J. van den Hondel Prof. Dr. J. A. van Veen

J.E. Jurriaanse Foundation financially contributed to the print costs of this thesis.

TABLE OF CONTENTS

	List of abbreviations	9
Introduction	Aims of this thesis	13
Chapter 1	Regulatory mechanisms governing secondary metabolism of <i>Pseudomonas</i> species	17
Chapter 2	Regulatory roles of <i>psrA</i> and <i>rpoS</i> in phenazine-1-carboxamide synthesis by <i>Pseudomonas chlororaphis</i> PCL1391	45
Chapter 3	Development of a microarray of <i>Pseudomonas chlororaphis</i> PCL1391 and transcriptome analyses of its <i>psrA</i> and <i>rpoS</i> mutants	65
Chapter 4	The novel regulator Pip acts between PsrA/RpoS and quorum-sensing in the regulatory cascade of phenazine synthesis in <i>Pseudomonas chlororaphis</i> PCL1391	93
Chapter 5	The transcriptional regulator Pip is involved in the repression of phenazine synthesis under stress conditions in <i>Pseudomonas chlororaphis</i> PCL1391	117
Chapter 6	Summary and discussion	145
	References	157
	Résumé et samenvatting	175
	Curriculum vitae	189

LIST OF ABBREVIATIONS

Acyl-ACP: acyl-acyl carrier protein

Acyl-CoA: acyl-coenzyme A

AcrAB: multidrug export pump in *E. coli*

AcrR: TetR homologue, regulator of *acrAB* expression

ANR: anaerobic regulator (see Table 1 in Chapter 1)

AprA: secreted alkaline protease

Bp: base pairs

C₄-HSL: *N*-butanoyl-homoserine lactone, cognate *N*-AHL of RhII/RhIR

C₆-HSL: *N*-hexanoyl-L-homoserine lactone, cognate *N*-AHL of PhzI/PhzR

3O-C₁₂-HSL: *N*-(3-oxododecanoyl)-homoserine lactone cognate *N*-AHL of LasI/LasR

CF: cystic fibrosis

CFU: colony forming units

CsrA: small regulatory protein (carbon storage regulator) (see Table 1 in Chapter 1)

CsrB: small regulatory RNA (see Table 1 in Chapter 1)

FixJ: regulator of nitrogen fixation (see Table 1 in Chapter 1)

Fur: ferric uptake regulator (see Table 1 in Chapter 1)

GacS/GacA: two-component system including a sensor and a transcriptional activator (global antibiotic control) (see Table 1 in Chapter 1)

galETK: galactose operon regulated by Spot42

glgC: ADP-glucose pyrophosphorylase gene regulated by CsrA.

Gm: gentamicin

4-HPPD: 4-hydroxyphenylpyruvate dioxygenase

H₂O₂: hydrogen peroxide

HCN: hydrogen cyanide

Hfq: small regulatory protein (see Table 1 in Chapter 1)

IppA: regulator of phenazine synthesis in *P. chlororaphis* PCL1391 (inducer of phenazine production)

IsmB: regulator of phenazine synthesis and other traits of the secondary metabolism in *P. chlororaphis* PCL1391 (inducer of secondary metabolism)

ISR: induced systemic resistance

Kb: kilo base-pairs

Km: kanamycin

LasI/LasR: quorum-sensing system (see Table 1 in Chapter 1)

LuxI/LuxR: quorum-sensing system (see Table 1 in Chapter 1)

Mb: mega base-pairs

MIC: minimum inhibitory concentration

N-AHL: *N*-acyl homoserine lactone

nlpD: lipoprotein gene

NMR: nucleic magnetic resonance

ORF: open reading frame

PCA: phenazine-1-carboxylic acid

PCN: phenazine-1-carboxamide

PGPR: plant-growth promoting rhizobacteria

PHA: polyhydroxyalkanoic acid

Phl: 2,4-diacetylphloroglucinol

phlACBD: biosynthetic operon for Phl

PhlF: TetR homologue, regulator of *phlABCD* (see Table 1 in Chapter 1)

PhlH: TetR homologue, regulator of *phlABCD* (see Table 1 in Chapter 1)

phzABCDEFGH: phenazine (*phz*) operon responsible for the synthesis of PCN

PhzI/PhzR: quorum-sensing system regulator of *phzABCDEFGH* (see Table 1 in Chapter 1)

Pip: TetR homologue indirectly regulating phenazine synthesis (phenazine inducing protein)

PsrA: TetR homologue regulating *rpoS* expression (*Pseudomonas* sigma regulator) (see Table 1 in Chapter 1)

ppGpp: guanosine tetraphosphate (see Table 1 in Chapter 1)

PQS: 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal) (see Table 1 in Chapter 1)

PvdS: alternative sigma factor involved in the response to changes in iron concentrations in the environment

QscR: LuxR homologue (see Table 1 in Chapter 1)

RhlR/RhlI: quorum-sensing system (see Table 1 in Chapter 1)

RNAP: RNA polymerase

RpoS: alternative sigma factor (also called σ^S) that is recruited under stress conditions or during stationary phase of growth (see Table 1 in Chapter 1).

RsmA: small regulatory protein (regulator of secondary metabolites) (see Table 1 in Chapter 1)

RsmE: small regulatory protein (see Table 1 in Chapter 1)

RsmX: small regulatory RNA (see Table 1 in Chapter 1)

RsmY: small regulatory RNA (see Table 1 in Chapter 1)

RsmZ: small regulatory RNA (see Table 1 in Chapter 1)

RyhB: small regulatory RNA (see Table 1 in Chapter 1)

SAR: systemic acquired resistance

sdhCDAB: operon involved in iron metabolism, regulated by RyhB

Spot42: small regulatory RNA (see Table 1 in Chapter 1)

sRNA: small RNA

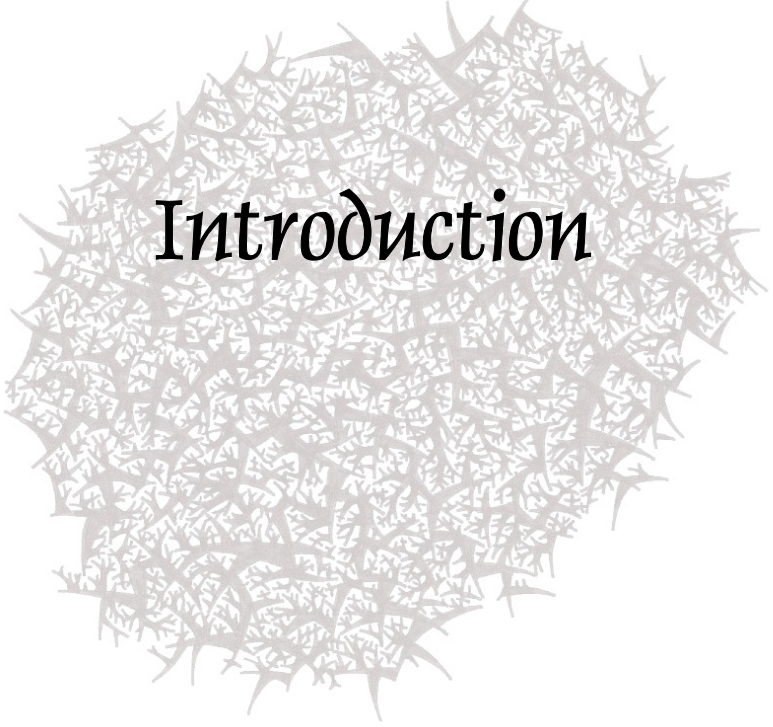
TetAB: pump exporting tetracycline

tetO: operator in the promoter of *tetAB* and *tetR*

TetR: transcriptional regulator of *tetAB* expression by binding to *tetO* operator in its promoter (tetracycline regulator)

VfR: cyclic AMP receptor protein (see Table 1 in Chapter 1)

VqsR: LuxR homologue (see Table 1 in Chapter 1)



Introduction

Aims of this thesis

Aims of this thesis

Pseudomonas chlororaphis strain PCL1391 is a plant-beneficial rhizobacterium that protects tomato plants from tomato foot and root rot caused by the fungus *Fusarium oxysporum* f. sp. *radicis lycopersici*. Previously, it was shown that both microorganisms compete for colonization of the junctions between epidermic cells of tomato roots and that root colonization is required for the delivery of the antifungal metabolite phenazine-1-carboxamide (PCN) produced by strain PCL1391 (Chin-A-Woeng *et al.*, 2000; Bolwerk *et al.*, 2003) (Fig. 1). PCN is a secondary metabolite that inhibits the growth of *Fusarium oxysporum* and its synthesis is necessary for the biocontrol ability of PCL1391 (Chin-A-Woeng *et al.*, 1998).

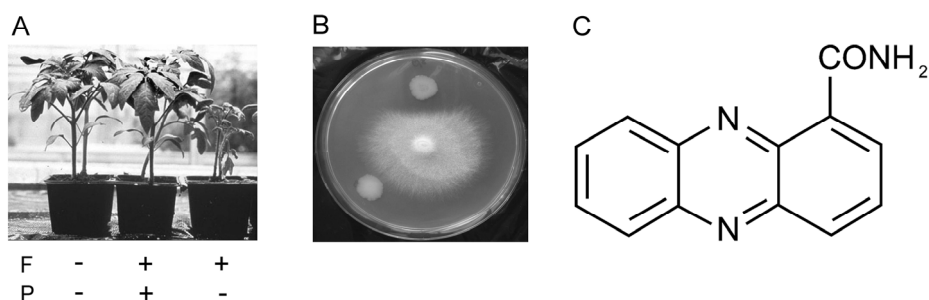


Figure 1. Biocontrol activity of *Pseudomonas chlororaphis* strain PCL1391.

Panel A. Presence of the fungus *Fusarium oxysporum* f. sp. *radicis lycopersici* (F) causes foot and root rot on the tomato plant. Coating of the tomato seeds with *P. chlororaphis* PCL1391 (P) protects the tomato plant against the fungus.

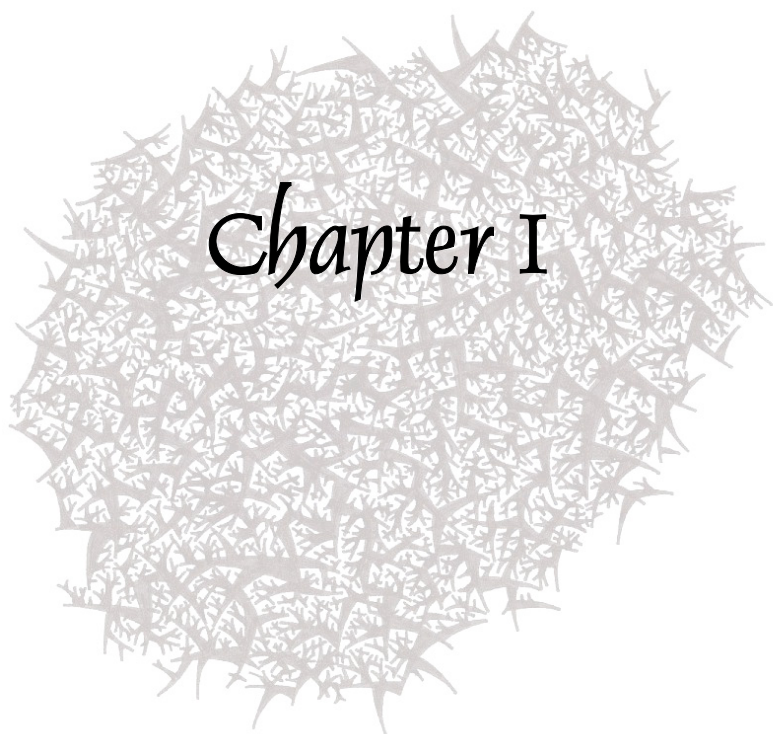
Panel B. On agar medium, strain PCL1391 (top) inhibits radial growth of *F. oxysporum* (center). However, the mutant strain PCL1119 (bottom left) derivative of strain PCL1391 is unable to inhibit the growth of the fungus. PCL1119 is mutated in *phzB*, the second gene of the *phzABCDEFGH* operon responsible for the biosynthesis of PCN by strain PCL1391 (Picture from T. van Rij).

Panel C. Structure of phenazine-1-carboxamide.

The major goal of the studies presented in this thesis is to elucidate the complex mechanisms regulating PCN production in *P. chlororaphis* strain PCL1391. So far three main genes or gene pairs have been isolated that regulate the PCN biosynthetic operon *phzABCDEFGH*. These include the two-component system *gacS/gacA*, the *tetR* homologue *psrA* and the quorum sensing system *phzI/phzR* (Chin-A-Woeng *et al.*, 2001b; Chin-A-Woeng *et al.*, 2005). Another regulator of phenazine synthesis, *ippA*, was recently characterized by van Rij *et al.* (van Rij *et al.*, *in preparation*). Regulation of secondary metabolites synthesis in other bacterial

species indicated that additional genes could be involved in the regulation of PCN synthesis and that their interactions might be very complex.

A general overview of regulation of secondary metabolism in *Pseudomonas* species is given in Chapter 1. Several approaches were combined to identify novel genes involved in the regulation of PCN synthesis and to study their interactions with other regulators. Site-directed mutagenesis was used to test the hypothesis that *rpoS* is a regulatory gene of PCN synthesis (Chapter 2). To discover additional genes in the regulatory cascade, which already contains *psrA* and *rpoS*, a random DNA-fragment microarray of the PCL1391 genome was constructed and used for transcriptomics of the *psrA* and *rpoS* mutants (Chapter 3). A random mutagenesis approach resulted in the identification of *pip*, a novel gene that stimulates PCN production in PCL1391 (Chapter 4). Analyses on the role of Pip as a switch of PCN production depending on environmental conditions are described in Chapter 5. The results described in this thesis are summarized in Chapter 6, where in addition the regulatory network of PCN synthesis in *P. chlororaphis* PCL1391 is compared to regulatory networks of secondary metabolism in other *Pseudomonas* species.



**Regulatory mechanisms governing
secondary metabolism of *Pseudomonas*
species**

Geneviève Girard and Guido V. Bloemberg

ABSTRACT

Among Gram-negative bacteria, pseudomonads produce a wide variety of secondary metabolites, including simple organic molecules such as hydrogen cyanide, enzymes such as chitinases, elastase and exoproteases, and antibiotics such as 2,4-diacetyl-phloroglucinol (Phl) and phenazines. Many of these metabolites are involved in interactions with other organisms, including pathogenesis of plants, animals and humans, direct antibiosis against other microorganisms, and plant growth promotion. Many secondary metabolites are of biotechnological, pharmaceutical and agricultural interest. Genomes of various *Pseudomonas* species have recently been sequenced. Two striking features of *Pseudomonas* genomes are their relatively large size and the high proportion of regulatory genes among the identified ORFs. It is proposed that this reflects an evolutionary adaptation to colonize a high diversity of ecological niches and to adequately react on changes in the environment. Regulation of secondary metabolism in *Pseudomonas* species involves environmental factors, transcriptional regulators among which several sigma factors and quorum-sensing genes, and regulatory mechanisms acting at the post-transcriptional level, such as several small RNAs that interfere with gene expression at the mRNA level. The importance of small RNAs was until recently under-estimated for *Pseudomonas* species although studies in *E. coli* show that they play a major role in regulatory events of the cell.

TABLE OF CONTENTS

INTRODUCTION	21
REGULATION OF SECONDARY METABOLISM BY ENVIRONMENTAL FACTORS	23
pH, temperature and water flow	23
Oxygen	24
Ions	24
<i>Salts</i>	24
<i>Iron</i>	24
<i>Other ions</i>	25
Carbon and nitrogen sources	26
Signal molecules and inhibitors	26
TRANSCRIPTIONAL CONTROL MECHANISMS INVOLVED IN SECONDARY METABOLITE SYNTHESIS	27
Sigma factors	27
<i>Regulatory roles of sigma factors</i>	27
<i>Different sigma factors compete for binding to RNA polymerase</i>	28
Quorum sensing	29
<i>General features of quorum-sensing</i>	29
<i>N-acyl homoserine lactone synthesis</i>	30
<i>LuxR-type proteins</i>	30
<i>Regulation of quorum-sensing</i>	33
TetR homologues	33
POST-TRANSCRIPTIONAL CONTROL MECHANISMS	34
GacS/GacA two-component global regulatory system	34
<i>Regulatory roles of GacS/ GacA</i>	34
<i>Molecular features of the GacS/ GacA system</i>	35
Rsm family of small RNAs and proteins	36
<i>RsmA homologues act at mRNA level</i>	37
<i>sRNAs modulate the activity of RsmA homologues</i>	37
<i>Structural features</i>	38
Small RNAs and Hfq	38
<i>Small RNAs and Hfq in Pseudomonas species</i>	38
<i>Small RNAs and Hfq in E. coli</i>	40
CONCLUDING REMARKS	41

INTRODUCTION

Pseudomonas species are Gram-negative bacteria, which colonize diverse environments such as surface water, soil, fungi, plants, animals and humans. They interact with organisms, resulting in direct antibiosis against other microorganisms, diseases of plants, animals or humans, and plant growth promotion. The opportunistic pathogen *Pseudomonas aeruginosa* is the best studied *Pseudomonas* species. *P. aeruginosa* is the most common Gram-negative bacterium found in nosocomial (hospital-acquired) infections (Van Delden & Iglewski, 1998) and causes premature death of most cystic fibrosis (CF) patients by colonizing their lungs (Costerton, 2001). Some other *Pseudomonas* species are beneficial to organisms. For example, several non-pathogenic rhizobacterial pseudomonads can induce a systemic resistance (ISR) response in plants that differs from systemic acquired resistance (SAR) (Van Loon *et al.*, 1998), resulting in the protection against pathogens. Some also protect plants from diseases by directly inhibiting the growth of pathogens (Weller *et al.*, 2002). Other biocontrol mechanisms include competition for nutrients and niches and direct antibiosis (Haas & Defago, 2005). The discovery of these protective abilities forms the basis for the commercial application of microbes for biocontrol purposes (Compant *et al.*, 2005).

Secondary metabolites play a central role in interactions between *Pseudomonas* species and other organisms. In contrast to primary metabolites, secondary metabolites are not essential for growth and reproduction. They are not directly derived from nutrients, but from primary metabolites and are usually produced at the entry of the stationary growth phase. Many secondary metabolites are involved in pathogenicity and direct antibiosis, such as hydrogen cyanide, enzymes like chitinases and exoproteases, and antibiotics like 2,4-diacetyl-phloroglucinol (Phl) and various phenazines (Raaijmakers *et al.*, 2002; Haas & Defago, 2005). Chitinases, for example, can be used by bacteria to degrade fungal cell walls. Pyocyanin, a phenazine molecule produced by *P. aeruginosa*, was indicated to play a role in lung infections of CF patients (Lau *et al.*, 2004). HCN was also shown to be involved in virulence of *P. aeruginosa* (Gallagher & Manoil, 2001). Some secondary metabolites are part of the molecular bacterial determinants that induce systemic resistance in plants, such as siderophores and salicylic acid (van Loon *et al.*, 1998). Others are involved in nutrition and competition for the uptake of nutrients, like siderophores that chelate iron (O'Sullivan & O'Gara, 1992).

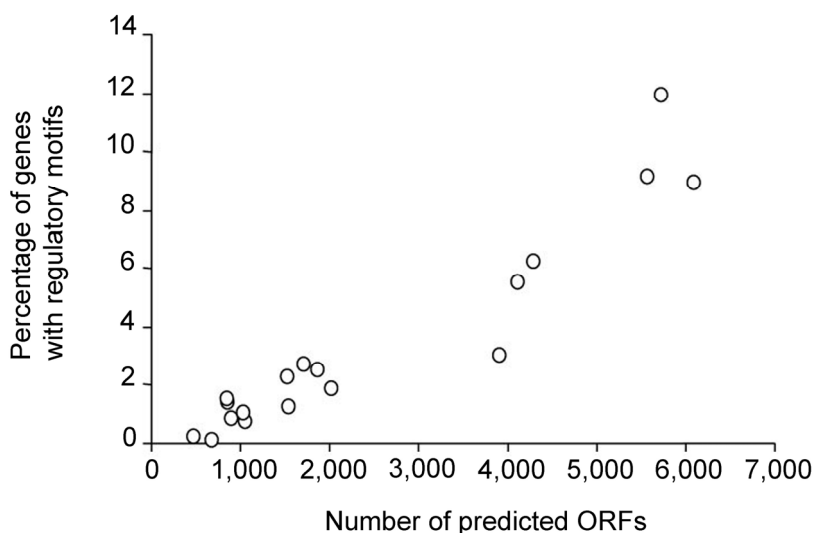


Figure 1. The percentage of genes with regulatory motifs increases with the size of the genome. The genomes analyzed were those of *Mycoplasma genitalium* (480 predicted ORFs), *Mycoplasma pneumoniae* (677), *Rickettsia prowazekii* (834), *Borrelia burgdorferi* (850), *Chlamidia trachomatis* (894), *Treponima pallidum* (1031), *Chlamydomophila pneumoniae* (1052), *Aquifex aeolicus* (1522), *Helicobacter pylori* 26695 (1553), *Haemophilus influenzae* (1709), *Methanocaldococcus jannaschii* (1715), *Pyrococcus abyssi* (1765), *Thermotoga maritima* (1846), *Methanothermobacter thermoautotrophicum* (1869), *Neisseria meningitidis* MC58 (2025), *Pyrococcus horikoshii* (2064), *Archaeoglobus fulgidus* (2407), *Synechocystis* PCC6803 (3169), *Mycobacterium tuberculosis* (3918), *Bacillus subtilis* (4100), *Escherichia coli* (4289), *Pseudomonas aeruginosa* (5570), *Pseudomonas syringae* pv *tomato* DC3000 (5763) and *Pseudomonas fluorescens* Pf-5 (6144). Adapted from Stover *et al.* (2000).

Genomes of various *Pseudomonas* species have recently been sequenced. Two striking features of these genomes are their large size (6.3 Mb, 6.5 Mb and 7.1 Mb for *P. aeruginosa* PAO1, *P. syringae* pv. *tomato* DC3000 and *P. fluorescens* Pf-5, respectively, compared to 4.6 Mb for *Escherichia coli*) and the high proportion of (predicted) regulatory genes among the identified ORFs. In general, the proportion of the genome devoted to regulatory proteins increases with the size of bacterial genome, whereas highly specialized organisms like *Mycobacterium tuberculosis* and *Helicobacter pylori* have a smaller proportion of regulatory proteins in their genome (Stover *et al.*, 2000). Analysis of the sequenced genomes predicts that for *P. aeruginosa* PAO1 8.4 % of the genes are involved in regulation (Stover *et al.*, 2000), for *P. syringae* pv. *tomato* DC3000 12 % (Buell *et al.*, 2003) and for *P. fluorescens* Pf-5 8.7 % (Paulsen *et al.*, 2005) compared to 5.8 % of the genes for *E. coli* (Blattner *et al.*, 1997; Stover *et al.*, 2000) (Fig. 1). It is proposed that the large size of *Pseudomonas* species genomes and the high proportion of regulatory genes reflect an evolutionary adaptation to establish themselves in a high diversity of ecological niches and to react efficiently to changes in environments (Stover *et al.*, 2000).

This review considers first the influence of ecological factors on secondary metabolism of *Pseudomonas* species and subsequently reviews the molecular mechanisms involved in the regulation of secondary metabolism. Since secondary metabolism is of utmost relevance for pathogenic as well as beneficial effects of pseudomonads, we have chosen to review the regulatory processes with a focus on the human opportunistic pathogen *P. aeruginosa* and plant associated pseudomonads, mostly beneficials. A table of all the regulators described below is inserted at the end of this chapter.

REGULATION OF SECONDARY METABOLISM BY ENVIRONMENTAL FACTORS

A niche represents an environment that is defined by various abiotic and biotic parameters, both of which influence the production of secondary metabolites by pseudomonads. Physical and chemical parameters include pH, temperature, water flow, concentrations of various molecules, particularly those of oxygen and iron including salts, iron and carbon sources. In their living environment pseudomonads also encounter other organisms, which affect the regulatory mechanisms of secondary metabolite production.

pH, temperature and water flow

Soil pH varies substantially between different soil types and was shown to influence the biocontrol abilities of *P. fluorescens* 2-79 (Ownley *et al.*, 2003). In *P. chlororaphis* PCL1391, the acidification of the growth medium from pH 7 to pH 6 decreases the production of phenazine-1-carboxamide (PCN) (van Rij *et al.*, 2004), which is an essential component of its biocontrol ability (Chin-A-Woeng *et al.*, 1998). pH can also affect quorum-sensing (for details see below) of Gram-negative bacteria in general, since high pH destabilizes the lactone ring of most *N*-acyl homoserine lactones (*N*-AHLs) (Yates *et al.*, 2002), thereby abolishing its inducing activity. pH was also shown to influence iron availability in the soil (Loper & Henkels, 1997), which is an important determinant in regulating secondary metabolism (see below).

Pseudomonads are able to survive and grow in a wide temperature range, with a variation in optimal growth temperature between different species. Several reports show that temperature influences the production of secondary metabolites. For example lowering the growth temperature from 21 to 16°C decreases the production of PCN in *P. chlororaphis* PCL1391 (van Rij *et al.*, 2004). In contrast, the lipopeptide surfactant production by *P. putida* PCL1445 is increased by temperatures lower than 21°C (Dubern *et al.*, 2005).

Finally, water flow in the soil, by affecting the accumulation of molecules, influences the concentrations of different signals and therefore the processes regulated by these signals (Fuqua *et al.*, 2001).

Oxygen

Oxygen concentrations can strongly vary in the living environment. In *P. aeruginosa*, a link was shown between the genes involved in oxygen and iron regulation and the quorum-sensing regulatory protein LasR (Kim *et al.*, 2005). Genetic regulation by oxygen is particularly important for *P. aeruginosa*, since for example in the lungs of CF patients, this bacterium forms biofilms, which are mostly anaerobic (Yoon *et al.*, 2002).

In soil, oxygen concentrations depend on soil compactness and water content. Several reports show that decreased oxygen levels increases secondary metabolism. Oxygen depletion stimulates production of PCN by *P. chlororaphis* PCL1391 (van Rij *et al.*, 2004). In *P. fluorescens* CHAO, the production of HCN is optimal in micro-aerophilic conditions. This is due to the activated transcriptional regulator ANR (anaerobic regulator), which upregulates the transcription of the *hcnABC* operon (Blumer & Haas, 2000). This global regulator is a FeS protein that switches between active and inactive states depending on oxygen concentration (Haas & Keel, 2003).

Ions

Salts

In soil pseudomonads encounter salt stress depending on the soil type and drought conditions. Salt stress reduces the production of PCN by *P. chlororaphis* PCL1391 (van Rij *et al.*, 2004). In the salt tolerant bacterium *P. mendocina*, an accumulation of compatible solutes such as glucosylglycerol, *N*-acetylglutaminylglutamine amide and L- α -glutamate was observed in response to osmotic stress (Pocard *et al.*, 1994). The resistance to salt stress is mediated by the alternative sigma factor RpoS in *P. fluorescens* Pf5 (Sarniguet *et al.*, 1995), but also by the sigma factor RpoN in *P. fluorescens* CHAO (Péchy-Tarr *et al.*, 2005).

Iron

Iron is an important element for growth of all organisms since it is a cofactor for many enzymes of the primary metabolism. Iron exists in two ionic forms, either ferrous (Fe²⁺) or ferric (Fe³⁺), the first being the most soluble. However,

in most environments Fe^{3+} is the predominant form of iron, due to the rapid oxidation of ferrous to ferric irons. Its low solubility is not sufficient for efficient uptake by microorganisms and for optimal growth. Many pseudomonads secrete siderophores that chelate ferrous iron (O'Sullivan & O'Gara, 1992), providing a competitive advantage in the environment. It was shown that production of the siderophore ornibactin by *Burkholderia cepacia*, an opportunistic human pathogen that infects lungs already colonized by *P. aeruginosa*, can be detected by *P. aeruginosa*, resulting in the induction of the iron-responsive gene PA4467 (Weaver & Kolter, 2004). The rhizobacterium *P. putida* is able to utilize siderophores produced by other bacterial species to increase its own iron availability (Loper & Henkels, 1999). In general, *Pseudomonas* species are able to use a large number of heterologous siderophores via different TonB-dependent receptors (Cornelis & Matthijs, 2002). Some results indicate that bacterial siderophores can even inhibit iron uptake by higher plants (O'Sullivan & O'Gara, 1992). On the other hand, some plants have been described to be able to divert bacterial siderophores for their own iron uptake (Yehuda *et al.*, 1996). Therefore iron is also involved in cross regulation between species.

Iron availability inhibits siderophore production via the Fur protein (ferric uptake regulation). In *P. aeruginosa*, Fur not only regulates the expression of siderophore genes, but also other regulatory genes and genes that contribute to virulence among others (Vasil & Ochsner, 1999). Recently it was found that small RNAs (sRNAs) act as intermediates of the regulation by Fur in *P. aeruginosa* (Wilderman *et al.*, 2004). In *P. fluorescens* CHAO, iron regulates the production of hydrogen cyanide (Keel *et al.*, 1989) via ANR (Blumer & Haas, 2000). In *P. chlororaphis* PCL1391, a decrease of iron concentrations results in a reduction of the production of *N*-AHL and PCN (van Rij *et al.*, 2004).

Other ions

Important ions such as phosphate, sulfate, ammonium, magnesium and zinc also play a role in the regulation of secondary metabolism. Low concentrations of phosphate, sulfate and ammonium result in reduced PCN levels in *P. chlororaphis*, whereas low magnesium stimulates PCN production (van Rij *et al.*, 2004). The presence of zinc stimulates production of Phl by *P. fluorescens* CHAO (Duffy & Defago, 1999). Magnesium is an important co-factor for several enzymes and zinc is known for example to be structurally important in the Fur protein (Pohl

et al., 2003) Sulfate and ammonium are important nutrient sources for the synthesis of proteins.

Carbon and nitrogen sources

Plant roots exude many compounds which feed pseudomonads in the rhizosphere. The composition of root exudates varies depending on the plant species, but it generally consist mainly of carbon sources such as organic acids and sugars (Lugtenberg *et al.*, 1999; Kuiper *et al.*, 2002; Lugtenberg & Bloemberg, 2003). Several studies showed that carbon sources affect the production of antibiotics in root colonizing pseudomonads. For example the production of PCN by *P. chlororaphis* PCL1391 is stimulated by glucose and glycerol (van Rij *et al.*, 2004). Similarly, the production of Phl and pyoluteorin by *P. fluorescens* is increased by glucose and glycerol respectively, but glucose inhibits the production of pyoluteorin. Pyrrolnitrin production is increased by fructose, and pyochelin by fructose and glucose (Duffy & Defago, 1999).

Amino acids strongly influence the production of various metabolites, because they are an important source of nitrogen and sometimes because they are direct precursors of these metabolites. However, in *P. chlororaphis* PCL1391, the strong stimulation of PCN production by phenylalanine does not have an evident explanation (van Rij *et al.*, 2004).

Signal molecules and inhibitors

Fusaric acid is a phytotoxin excreted by *Fusarium* fungal species (Bacon *et al.*, 1996). Fusaric acid was shown to repress the production of the antifungal compound Phl in *P. fluorescens* CHAO (Duffy & Defago, 1997). Similarly, fusaric acid affects *P. chlororaphis* PCL1391 by inhibiting the synthesis of *N*-AHL and the antifungal PCN (van Rij *et al.*, 2005).

N-AHLs are signal molecules that are excreted by many Gram-negative bacteria. They allow bacteria to communicate with each other, not only with the same species, but also with other ones (Riedel *et al.*, 2001). *N*-AHLs are key molecules of the quorum-sensing regulatory mechanism, as described in more detail below. Different species can influence each other by the production of *N*-AHLs. For example, *N*-(3-oxododecanoyl)-homoserine lactone (3O-C₁₂-HSL) produced by *P. aeruginosa* inhibits filamentation of the fungus *Candida albicans*, an opportunistic pathogen which co-infects the host with *P. aeruginosa* (Hogan *et al.*, 2004). Some bacteria, particularly *Bacillus* species, have the ability to degrade *N*-AHLs

synthesized by other species, and therefore influence the secondary metabolism of their putative competitors (Leadbetter & Greenberg, 2000; Dong *et al.*, 2002). Finally, quorum-sensing inhibitors can also be synthesized by higher organisms, such as furanones from algae that influence the secondary metabolism of *P. aeruginosa* (Hentzer *et al.*, 2003).

TRANSCRIPTIONAL CONTROL MECHANISMS INVOLVED IN SECONDARY METABOLITE SYNTHESIS

Sigma factors

The RNA polymerase (RNAP) is composed of a catalytic core of around 400 kDa including five subunits ($\alpha_2\beta\beta'\omega$) and a sigma (σ) subunit that is required for promoter-specific transcription initiation in bacteria. The number of σ factor genes present in the genome varies among different bacterial species, ranging from seven in *E. coli* (Gruber & Gross, 2003) to twenty (predicted) in *P. aeruginosa* PAO1 (Stover *et al.*, 2000) and thirty-two in *P. fluorescens* Pf-5 (Paulsen *et al.*, 2005), each of them recognizing different specific promoter sequences. The majority of genes (house-keeping genes) are transcribed during exponential growth by the RNAP containing the σ^{70} subunit encoded by *rpoD*. The alternative σ factors direct transcription of specific regulons during unusual physiological conditions, such as stress. In *E. coli*, these factors are similar to σ^{70} , except σ^{54} encoded by *rpoN* (Lonetto *et al.*, 1992).

Regulatory roles of sigma factors

The sigma factor σ^S encoded by *rpoS* was originally identified in *E. coli* as an alternative sigma factor that activates gene expression under stress conditions, such as starvation, heat shock, increased osmolarity or increased H_2O_2 concentration (Hengge-Aronis, 1993). In *Pseudomonas* species an *rpoS* mutation also decreases resistance to stress, although to a lower level than in *E. coli* (Ramos-González & Molin, 1998; Jorgensen *et al.*, 1999; Suh *et al.*, 1999). However, RpoS is an important regulator of secondary metabolism in various *Pseudomonas* strains. For example RpoS positively regulates production of exotoxin A and alginate and inhibits production of the phenazine pyocyanin and the siderophore pyoverdine by *P. aeruginosa* (Suh *et al.*, 1999). RpoS also inhibits the production of the antibiotics pyoluteorin and Phl by *P. fluorescens* Pf-5 (Sarniguet *et al.*, 1995), and it stimulates production of PCN in *P. chlororaphis* (Girard *et al.*, 2006). In several species RpoS was shown to exert its effect by influencing the production of *N*-AHLs. RpoS has a moderate effect on quorum sensing in *P. aeruginosa* (Whiteley *et al.*, 2000; Schuster

et al., 2004a) and also regulates the *N*-AHL synthase *ppuI* in *P. putida* (Bertani & Venturi, 2004). The effect of RpoS on quorum-sensing seems to be very sensitive to growth conditions, since apparently conflicting results were obtained in both *P. aeruginosa* (Latifi *et al.*, 1996; Whiteley *et al.*, 2000) and in *P. putida* (Kojic *et al.*, 1999; Bertani & Venturi, 2004). In many strains, *rpoS* is under the control of the GacS/GacA system (Whistler *et al.*, 1998; Schmidt-Eisenlohr *et al.*, 2003; Chatterjee *et al.*, 2003; Girard *et al.*, 2006), a regulatory system that is discussed below.

The sigma factor σ^{54} or RpoN has been less intensively studied than σ^S , but has a significant effect on the production of secondary metabolites. RpoN negatively regulates the two quorum sensing systems in *P. aeruginosa* PAO1 (Pesci *et al.*, 1997), as well as the production of elastase, rhamnolipids and HCN. These effects could be mediated by GacA (Heurlier *et al.*, 2003). There could be a mutual regulation of *gacA* and *rpoN*, since a *gacA* mutant of *P. syringae* pv. *tomato* DC3000 shows reduced levels of *rpoN* transcript (Chatterjee *et al.*, 2003). In *P. fluorescens* CHAO, σ^{54} was shown to inhibit the production of Phl, whereas it activates motility and pyoluteorin production (Péchy-Tarr *et al.*, 2005).

Different sigma factors compete for binding to RNA polymerase

In *E. coli*, most sigma factors belong to the family of the σ^{70} subunit. They are modular proteins consisting of up to four domains. DNA binding determinants are located in regions 2 and 4 that recognize the -10 and -35 DNA sequences of the promoter regions, respectively (Lonetto *et al.*, 1992). Structural conservation indicates that the general mechanism of DNA binding to promoters is similar among all σ^{70} type sigma factors (Gruber & Gross, 2003). All sigma factors bind to the same RNAP and compete for a limited pool of RNAP (Nystrom, 2004). Specific mechanisms ensure that the appropriate σ factor is associated to RNAP under a particular condition.

Competition between σ^{70} and σ^S for binding to promoter sequences was studied in detail in *E. coli*. The functioning of σ^{70} and σ^S in *E. coli* is probably similar to their functioning in *Pseudomonas* species, since *rpoS* in *P. aeruginosa* for example is highly homologous to *rpoS* of *E. coli* and can functionally replace it (Tanaka & Takahashi, 1994). It appeared that the consensus DNA sequences for optimal binding of σ^{70} and σ^S are identical, but σ^S selectivity could be less stringent than the one of σ^{70} (Hengge-Aronis, 2002b). In addition, binding of σ^S could be influenced by other features around the -35 and -10 boxes (Gruber & Gross, 2003). It was also shown in *P. putida* that elements of the sequence downstream of the -10

box influence σ^S -dependent transcription (Ojangu *et al.*, 2000). Additional *trans*-acting regulators play a role in promoter selectivity for σ^S . One of them is the alarmone guanosine tetraphosphate (ppGpp) that is produced during stationary phase and under starvation conditions. Data suggest that ppGpp regulates both the production and activity of σ^S in *E. coli*. ppGpp could act by weakening the interactions between σ^{70} and RNAP. This would result in increased availability of free RNAP for promoters of stress genes, which are relatively poor in recruiting RNAP (Nystrom, 2004). A link also exists between ppGpp and RpoS in *P. aeruginosa*, although ppGpp may not be strictly required for expression of RpoS-regulated genes (Erickson *et al.*, 2004). Another *trans*-acting factor in *E. coli* is the small RNA 6S that specifically interferes with RNAP- σ^{70} (Trotochaud & Wassarman, 2005) and therefore could favor transcription by RNAP- σ^S .

Quorum sensing

General features of quorum-sensing

Quorum sensing was originally discovered in the bioluminescent marine bacterium *Vibrio fischeri*, which colonizes the light organ of fishes and squids, and from which the first quorum-sensing system (LuxR/LuxI) was isolated (Ruby, 1996). Quorum sensing is a common mechanism used by *Pseudomonas* species to regulate the production of secondary metabolites, such as virulence traits and antibiotics. The biochemical mechanisms involved in quorum sensing have been studied in detail for *P. aeruginosa*. Two quorum sensing systems were identified in *P. aeruginosa*, which influence each other (Latifi *et al.*, 1996) and regulate different genes and operons. LasR/LasI regulate the production of elastase, alkaline protease and toxin A, whereas RhlR/RhlI regulate the production of rhamnolipid, pyocyanin, cyanide, lipase, protease and elastase (Gambello *et al.*, 1993; Ochsner & Reiser, 1995; Brint & Ohman, 1995; Latifi *et al.*, 1995; Pearson *et al.*, 1997). Recent microarray studies showed that in *P. aeruginosa*, over 300 genes are controlled by quorum-sensing (Schuster *et al.*, 2003; Wagner *et al.*, 2003).

N-AHLs are constitutively synthesized at a low level by an *N*-AHL synthase, usually a LuxI homologue. They can traffic back and forward across the cell membranes and are released in the environment, where they can be sensed by other bacteria. At a high density of bacterial cells, *N*-AHLs accumulate, and after reaching an intracellular threshold concentration (quorum), *N*-AHLs induce the expression of quorum-sensing regulated genes by binding specifically to a transcription factor, often a LuxR homologue. In many species, the LuxR-*N*-AHL complex stimulates the

transcription of *luxI*, thereby creating a positive autoinducing feedback in the quorum-sensing system LuxI/LuxR (Fuqua *et al.*, 2001). Therefore, bacteria can use *N*-AHL as an indicator of their cell density, which provides for example many pathogens with a mechanism to decrease detection by the host by delaying production of virulence factors until bacteria reach a sufficient concentration to cause a substantial effect. However, *N*-AHLs could have other roles. For example it was recently discovered that products deriving from 3O-C₁₂-HSL have bactericidal activity against Gram-positive bacteria and might solubilize iron (Kaufmann *et al.*, 2005).

N-acyl homoserine lactone synthesis

An *N*-AHL molecule consists of a homoserine lactone ring and an acyl chain. Diversity of the *N*-AHL family is linked to the structural variety of acyl chains, which can differ in length, saturation or degree of substitution. Up to now the longest acyl chain observed in an *N*-AHL molecule contains eighteen C-atoms (Fuqua *et al.*, 2001). The homoserine lactone ring is hydrophilic, whereas the acyl chain is hydrophobic. This amphipatic characteristic of the molecule probably allows it to diffuse through the lipid bilayer of membranes as well as in the aqueous cytoplasm and the extracellular environment. Long acyl chains might trap the *N*-AHLs in the membrane bilayer and therefore decrease diffusion over the membrane (Fuqua *et al.*, 2001). In *P. aeruginosa*, an efflux system was shown to export long chain *N*-AHL, but not *N*-AHL with short acyl chains (Evans *et al.*, 1998).

Synthesis of *N*-AHL by LuxI homologues requires S-adenosylmethionine (SAM) and acyl-acyl carrier protein (acyl-ACP) as precursors for the homoserine lactone ring and the acyl chain, respectively. The catalytic site in RhII (a Lux-I homologue in *P. aeruginosa*) seems to be located in the N-terminus (Fuqua *et al.*, 2001). Recently, LasI, the second LuxI homologue in *P. aeruginosa*, was crystallized (Gould *et al.*, 2004), giving new insights concerning *N*-AHL-synthase acyl-chain-length selectivity.

LuxR type proteins

LuxR homologues from *V. fischeri* and *Agrobacterium tumefaciens* are the best studied of this family of regulators, although data concerning LuxR homologues in *P. aeruginosa* have recently been accumulating. There are two known LuxR homologues in *P. aeruginosa*, LasR and RhIR, that function with their cognate *N*-AHL synthase LasI and RhII, respectively. QscR is a third LuxR homologue of *P.*

aeruginosa, of which no cognate *N*-AHL synthase has been identified yet (Chugani *et al.*, 2001). Recently, a fourth LuxR homologue of *P. aeruginosa* strain TB, VqsR, was characterized that controls a wide range of quorum-sensing-regulated and virulence genes (Juhas *et al.*, 2004).

LasR forms an oligomer, possibly a dimer, only in the presence of its cognate *N*-AHL, 3O-C₁₂-HSL (Kiratisin *et al.*, 2002) (Fig. 2, panel A). This oligomerization determines the ability of LasR to activate transcription of its target genes, as explained in Figure 2A. Data indicate that some LuxR homologues might bind to the membrane in the absence of *N*-AHL (Fuqua *et al.*, 2001) (Fig. 2, panel A). There are no clear data yet to indicate whether LasR functions in such a manner (Kiratisin *et al.*, 2002).

Data show a different situation with RhlR (Fig. 2, panel B). RhlR seems to exist as a dimer with or without its cognate *N*-AHL *N*-butanoyl-homoserine lactone (C₄-HSL). Binding to C₄-HSL would induce a conformational change of the RhlR dimer (Ventre *et al.*, 2003). When RhlR is bound to C₄-HSL it indeed seems to bind DNA with a different conformation than when it is not in complex with C₄-HSL (Medina *et al.*, 2003). In the absence of C₄-HSL RhlR inhibits the transcription of its target *rhlAB*, whereas in the presence of C₄-HSL it activates the transcription of *rhlAB* (Ventre *et al.*, 2003).

For several LuxR homologues, a conserved DNA sequence is present in the promoter of the target genes, which is referred to as the *lux* box. The *lux* box is required for LuxR binding resulting into transcriptional activation (Fuqua *et al.*, 2001). Many studies have been conducted to understand what features define target promoters of quorum-sensing in *P. aeruginosa* (Whiteley & Greenberg, 2001; Schuster *et al.*, 2003). These studies tried to establish a consensus for the *las* and *rhl* boxes, *lux* boxes for LasR and RhlR, respectively. It was hypothesized that these boxes are relatively well conserved and that they have a dyad symmetry. Finally, it was also suggested that differential binding (due to different affinities) of LasR to quorum-controlled promoters would govern the timing of quorum-controlled gene expression during growth. However, a recent study using purified LasR shows that binding affinities of various promoter sequences do not correlate with timing or magnitude of the response, and that the recognition sequences for LasR exhibit little conservation and no dyad symmetry (Schuster *et al.*, 2004b). It is possible that the target sequences of RhlR show a limited degree of dyad symmetry. They also cluster in a different group than target sequences of LasR (Schuster *et al.*, 2004b).

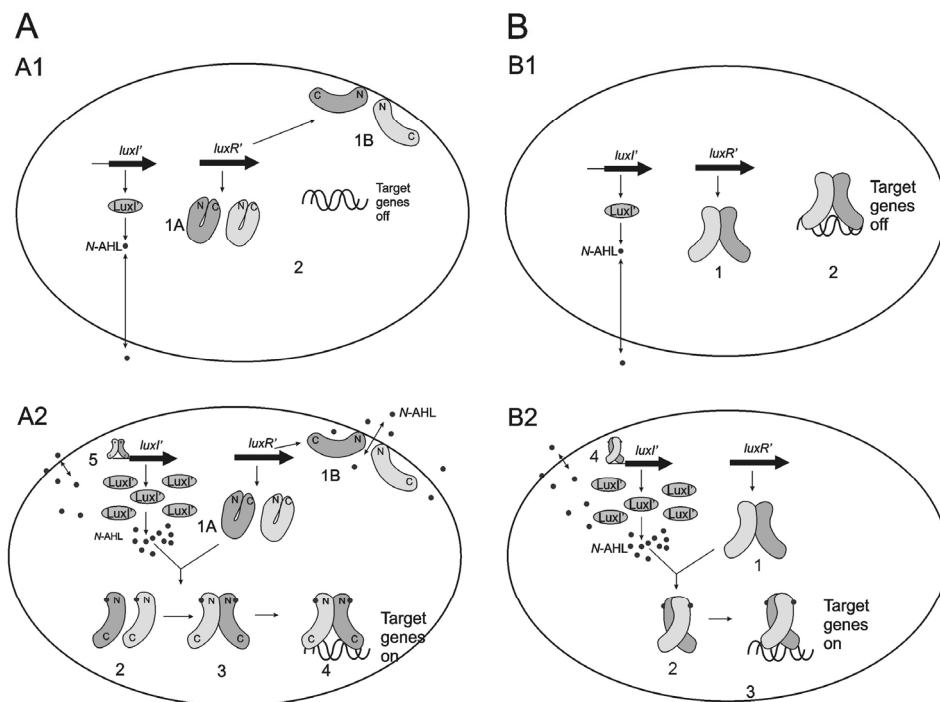


Figure 2. Models for the molecular mechanisms of quorum-sensing.

Two general models dependent on the functioning of LuxR homologs.

Panel A. Binding of the N-AHL to the LuxR homologue (LuxR') is required for its multimerization and activity. This mechanism seems to be the most common one and applies to the LasR/LasI system in *P. aeruginosa* PAO1 (Fuqua *et al.*, 2001; Kiratisin *et al.*, 2002).

A1. At low cell density, *luxI'* is constitutively expressed at a low level resulting in a basal level of N-AHL production (small spheres). Expression of *luxR'* results in the production of LuxR' monomers. The C-terminus of LuxR' could be masked by the N-terminus (1A) or alternatively the LuxR' N-terminus could be locked in the membrane (1B). In both situations, the access to target DNA by the C-terminus DNA-binding domain of LuxR' is impossible. Therefore the target genes are off (2).

A2. At high cell density, the local concentration of N-AHLs increases. The N-AHL molecules cross the membrane and bind to the N-terminus of LuxR', thereby inducing a conformational change that exposes the C-terminus and N-terminus (1A) or releases LuxR' from the membrane (1B). In both situations, the N-terminus is exposed (2) and dimerization occurs (3). The dimer of LuxR' can bind DNA at the level of target genes via its C-terminus, thereby activating their expression (4). The expression of *luxI'* (one of the target genes of LuxR') increases and more N-AHLs are synthesized, creating a positive feedback (autoinducing) loop (5).

Panel B. The LuxR homologue exists as a dimer independently of the N-AHL. This mechanism has been shown for RhlR in *P. aeruginosa* PAO1.

B1. At low cell density, *luxI'* expression is constitutively low and results in a basal level of N-AHL production (small spheres). Expression of *luxR'* results in the production of LuxR' that is able to dimerize (1). Dimers of LuxR' bind to the target genes, thereby blocking their expression (2).

B2. At high cell density, the local concentration of N-AHLs increases. The molecules of N-AHL cross the membrane and bind to LuxR', thereby inducing a conformational change (2). Activated LuxR' binds the target genes in a new manner that stimulates their expression (3). The expression of *luxI'* (one among the targets of LuxR') increases and more N-AHLs are synthesized, creating a positive feedback loop (4).

Regulation of quorum-sensing

Quorum-sensing is often part of complex regulatory cascades. For many *Pseudomonas* species it was shown that quorum sensing is under strict regulation of the GacA/GacS two-component signaling system (Reimmann *et al.*, 1997; Ledgham *et al.*, 2003; Chin-A-Woeng *et al.*, 2005). RpoS is also involved in control of quorum-sensing, but the effect depends on growth conditions and species (Schuster *et al.*, 2004a). In *P. aeruginosa* PAO1, greater complexity arises from the existence of two quorum-sensing systems and a third LuxR homologue. There is a hierarchy between the two quorum-sensing systems, since LasR regulates the expression of *rhlR* and *rhlI* (Latifi *et al.*, 1996). It was also shown that 3O-C₁₂-HSL, the cognate N-AHL of LasR, can dissociate the RhlR homodimer into non-effective monomers (Ventre *et al.*, 2003). In addition, QscR can form heterodimers with LasR and RhlR *in vivo* and interact with both 3O-C₁₂-HSL and C₄-HSL, adding further complexity to quorum-sensing regulation (Ledgham *et al.*, 2003). In *P. aeruginosa*, 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS), produced at the end of the exponential phase, regulates both *lasI* and *rhlI* (Diggle *et al.*, 2003). Interestingly, PQS was shown to actively mediate its own packaging into vesicles that are secreted and serve to traffic this molecule within a population (Mashburn & Whiteley, 2005). Vfr, a cyclic AMP receptor protein, is another regulator of *lasR* (Albus *et al.*, 1997). Post-transcriptional control of quorum-sensing was also shown in *P. aeruginosa*, where the small protein RsmA inhibits expression of *lasI* and *rhlI* (Pessi *et al.*, 2001). Finally, as mentioned before, quorum-sensing was shown to be influenced by other bacterial species.

TetR homologues

The TetR protein regulates the resistance mechanism against the antibiotic tetracycline in *E. coli* (Hillen & Berens, 1994). Located in the membrane, TetA exports the tetracycline-magnesium [MgTc]⁺ complex. TetR recognizes two palindromic operator sites (*tetO*_{1,2}) in the promoter region of the divergently oriented genes *tetR* and *tetA*, thereby inhibiting their expression. Binding of [MgTc]⁺ to TetR relieves this inhibition by reducing the affinity of TetR for *tetO* (Hillen & Berens, 1994). Several regulators of *Pseudomonas* species showing homology to TetR were shown to be regulators of secondary metabolism.

In *P. fluorescens*, the *phlACBD* operon responsible for the synthesis of the antifungal compound Phl is controlled in a similar way as *tetA*. The TetR homologue PhlF, encoded by *phlF* upstream of *phlACBD* and transcribed divergently, binds to

the *phlA* promoter region. This binding is prevented by the addition of Phl (Schnider-Keel *et al.*, 2000). PhlF was shown to recognize the 26-nucleotide palindromic sequence *phO* upstream of *phlA*. There are indications that PhlF may bind *phO* as an oligomer, probably a dimer. Salicylate, an enhancer of repression of *phlA* expression by PhlF, was shown to stabilize the PhlF-*phO* complex (Abbas *et al.*, 2002). In *P. fluorescens* CHAO a second TetR homologue was identified that regulates Phl synthesis. It is encoded by *phlH* situated just downstream of *phlF*. PhlH is indicated to be an activator or anti-repressor of *phlACBD* (Schnider-Keel *et al.*, 2000).

Another TetR homologue, PsrA, was identified in *P. putida* and *P. aeruginosa*, which regulates *rpoS* expression (Kojic & Venturi, 2001). It was shown that PsrA directly binds to the promoter of *rpoS* and its own promoter by recognizing a palindromic sequence (Kojic *et al.*, 2002). PsrA inhibits *N*-AHL synthesis in *P. putida* WCS358 (Bertani & Venturi, 2004). In *P. chlororaphis* PCL1391, it represses or stimulates *N*-AHL synthesis as well as PCN production depending on the growth conditions (Chin-A-Woeng *et al.*, 2005; Girard *et al.*, 2006).

POST-TRANSCRIPTIONAL CONTROL MECHANISMS

GacS/GacA two-component global regulatory system

Regulatory roles of GacS/GacA

Two-component regulatory systems are very common in bacteria and abundantly present in *Pseudomonas* species as is shown from the analyses of their complete genome sequences (Stover *et al.*, 2000; Buell *et al.*, 2003; Paulsen *et al.*, 2005). These systems are usually composed of a sensor kinase that can detect environmental signals, and a cognate response regulator that is phosphorylated by the activated sensor kinase (Rodrigue *et al.*, 2000). Phosphorylation of the response regulator enables it to activate gene expression (Zuber *et al.*, 2003). In the GacS/GacA system, the sensor kinase GacS is localized in the membrane and activates the response regulator GacA (Heeb & Haas, 2001). In *Pseudomonas* species, GacS and GacA are global regulators of the secondary metabolism, since they are situated at the top position of many regulatory cascades and function as master regulators. GacS and GacA are involved in the regulation of a broad set of genes and multiple traits, for example production of HCN and Phl (Laville *et al.*, 1992), production of exoprotease and phospholipase C (Sacherer *et al.*, 1994), production of lipopeptides (Dubern *et al.*, 2005) and production of various phenazines (Reimmann *et al.*, 1997; Chin-A-Woeng *et al.*, 2005). GacS and GacA

exert their effect on secondary metabolism at both transcriptional and post-transcriptional levels (Blumer *et al.*, 1999; Pessi & Haas, 2001) by modulating the expression of various regulators (Heeb & Haas, 2001; Chatterjee *et al.*, 2003; Haas & Defago, 2005) including quorum-sensing (Reimmann *et al.*, 1997; Bertani & Venturi, 2004; Girard *et al.*, 2006), σ^s (Whistler *et al.*, 1998; Girard *et al.*, 2006), RsmA and homologues (Pessi *et al.*, 2001; Reimmann *et al.*, 2005) and small RsmA-binding RNAs (Heeb *et al.*, 2002; Valverde *et al.*, 2003). It seems that RsmA homologues and their sRNA partners are central regulatory intermediates of the effects of GacS/GacA in pseudomonads (see below). GacS/GacA stimulate the expression of the two sRNAs RsmZ and RsmY in *P. fluorescens* CHAO and of RsmZ in *P. aeruginosa* PAO1 (Heeb *et al.*, 2002; Valverde *et al.*, 2003; Heurlier *et al.*, 2004). In *P. fluorescens* PfO1, the sRNAs in turn inhibit the activity of the small proteins RsmA and RsmE (Reimmann *et al.*, 2005). The GacS/GacA system was also shown to be involved in phase variation of rhizosphere *Pseudomonas* species, a mechanism inducing phenotypic variation, which plays an important role in competition in the rhizosphere and affects biocontrol (van den Broek *et al.*, 2005).

Molecular features of the GacS/GacA system

GacS has two N-terminal transmembrane domains separated by a periplasmic loop and preceding a linker region and three putative phosphotransfer domains: a transmitter, a receiver and a C-terminal secondary transmitter (Fig. 3). Experimental data support the following model for the mode of action. After activation by a so far unknown environmental signal, GacS is autophosphorylated at a histidine residue (His294) in the transmitter domain. The phosphate is then transferred to an aspartate residue (Asp717) in the receiver domain and to a histidine residue (His863) in the C-terminal secondary transmitter domain. Finally, the phosphate is transferred to aspartate 54 of the receiver domain of GacA (Zuber *et al.*, 2003). The linker domain seems to be crucial for proper GacS functioning, which could be explained by its role in a putative conformational change of GacS following binding of the environmental signal. On the contrary, the periplasmic loop was shown to be non-essential for activation of the GacS/GacA pathway (Zuber *et al.*, 2003).

The N-terminal domain of GacA contains the receiver domain that is phosphorylated by GacS, whereas the C-terminus contains a helix-turn-helix motif that is involved in DNA binding. GacA is a FixJ homologue and might therefore be

active as a dimer (Heeb & Haas, 2001). So far, no gene was shown to be a direct target of GacA and the DNA consensus sequence recognized by GacA is unknown.

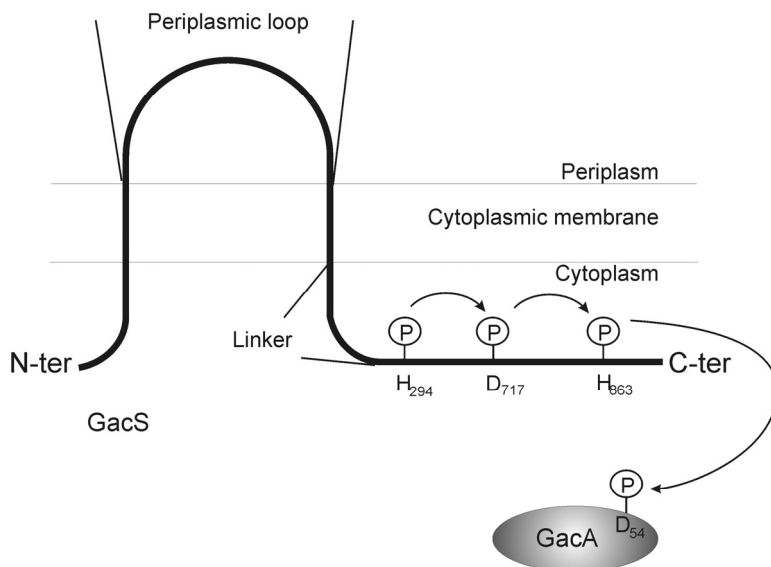


Figure 3. Model of the GacS/GacA signal transduction pathway in *Pseudomonas fluorescens* CHAO. The postulated phosphate relay indicated by P and arrows is based on the comparison with the other two-component systems involving a tripartite sensor kinase and supported by in vitro data in *E. coli* and mutational analysis performed in *P. fluorescens* (Zuber *et al.*, 2003). See text for details. Adapted from Zuber *et al.* (2003).

Rsm family of small RNAs and proteins

In several *Pseudomonas* species it was shown that the phosphorylation of GacA activates the transcription of small RNA genes that belong to the Rsm mixed family of small regulatory proteins and small regulator RNAs (Haas & Defago, 2005). These small RNAs interact with RsmA to relieve the translational repression exerted by this small protein. Most molecular studies on these interactions were conducted with the RsmA homologue CsrA of *E. coli*. These data provide an informative background for the study of RsmA. Therefore data from *E. coli* are presented here, in parallel with more recent data available from *Pseudomonas* species. CsrA (carbon storage regulator) of *E. coli* was first characterized as a regulator of carbon storage, particularly of *glgC*, the ADP-glucose pyrophosphorylase gene (Romeo, 1998). CsrA homologues are present in various Gram-negative bacteria and a second CsrA

homologue, called RsmE, was even found in *P. fluorescens* CHAO (Reimmann *et al.*, 2005).

RsmA homologues act at mRNA level

CsrA is a 62-amino-acid protein that contains a putative RNA-binding sequence (GVKG) (Romeo, 1998). In case of GlgC mRNA, the *cis*-acting region for negative regulation via CsrA is close to the ribosome binding site (RBS) (Romeo, 1998). In a similar manner, RsmA of *P. fluorescens* CHAO inhibits hydrogen cyanide and exoprotease production *via* a binding site overlapping with the RBS (Blumer *et al.*, 1999). RsmE and RsmA interfere with the expression of several biosynthetic genes of the secondary metabolism, including *hcnA* (involved in the synthesis of HCN), *aprA* (exoprotein) and *phlA* (Phl) (Blumer *et al.*, 1999; Reimmann *et al.*, 2005).

CsrA destabilizes GlgC mRNA (Romeo, 1998), but does not act itself as a nuclease. The mechanism inducing mRNA decay remains unclear but data indicate that CsrA primarily interferes with translation. CsrA and RsmA were shown to bind to a conserved GGA-containing motif around the RBS of their targets (Romeo, 1998; Blumer *et al.*, 1999), thereby inhibiting expression of these genes by sterically interfering with ribosome binding. mRNA decay could be a consequence of this translation inhibition since lack of translation can result into mRNA degradation (Vytvytska *et al.*, 2000). Increased mRNA decay can be explained by exposure of RNase E cleavage sites that are usually shielded by translating ribosomes.

sRNAs modulate the activity of RsmA homologues

The CsrB small RNA is able to sequester CsrA, thereby preventing it from binding to its target mRNAs. CsrA was even co-purified from *E. coli* with CsrB RNA. CsrA proteins do not form a large multimer but spread out to restricted domains on the RNA (Romeo, 1998). Small RNAs were also found to be partners for the CsrA homologue in several pseudomonads, including RsmZ, RsmY and RsmX in *P. fluorescens* CHAO (Heeb *et al.*, 2002; Valverde *et al.*, 2003; Haas & Defago, 2005; Kay *et al.*, 2005), and RsmZ in *P. aeruginosa* PAO1 (Heurlier *et al.*, 2004). This redundancy is assumed to facilitate fine-tuning of GacS/GacA controlled traits (Kay *et al.*, 2005). In *P. fluorescens* CHAO, the three small RNAs bind specifically RsmA and RsmE, which in turn restores the expression of genes inhibited by the small proteins interfering with ribosome binding, for example genes involved in the production of Phl, HCN or pyoluteorin (Blumer *et al.*, 1999; Heeb *et al.*, 2002; Reimmann *et al.*, 2005).

Structural features

A striking feature found in various bacterial species is that, although the RNA partners of CsrA and RsmA homologues are poorly conserved in their overall sequence, they all contain short repeated sequences with a GGA motif (Romeo, 1998; Haas & Defago, 2005). RsmY RNA (Fig. 4, panel A) with deleted GGA repeats showed reduced ability to derepress the expression of target genes *in vivo* and failed to bind the RsmA protein efficiently *in vitro* (Valverde *et al.*, 2004). Eighteen of these repeat sequences are present in CsrB RNA (Fig. 4, panel B), which exactly correlates with the number of CsrA units bound to one RNA molecule (Romeo, 1998). CsrB and RsmY are assumed to fold in a succession of small hairpin structures, most of which harbor the GGA sequence in their loop. Other single-stranded regions in these sRNAs are intercalated between the hairpins, and some of them have the conserved GGA motif. CsrA and RsmA would bind to these small single-stranded regions along the length of the sRNA. Interestingly, the GGA motif is also present in known binding sites in the mRNA targets of CsrA and is frequently present in Shine-Dalgarno sequences. It is likely that CsrA traffics between the GGA motifs on mRNA targets and those of its sRNA antagonists, the latter acting as a reservoir for CsrA (Romeo, 1998) (Fig. 4, panel C).

Very recently a nuclear magnetic resonance (NMR)-based structure of CsrA was described, which is very similar to the crystal structure obtained for RsmA in *P. aeruginosa* PAO1 (Gutierrez *et al.*, 2005). In this model, an upstream GGA-motif-containing hairpin serves as the second signal for CsrA binding (besides the SD sequence), which discriminates target mRNAs from other mRNAs.

Small RNAs and Hfq

Small RNAs and Hfq in Pseudomonas species

Many sRNAs in *E. coli* have been shown to play regulatory roles at post-transcriptional level. Many of these sRNAs require the presence of the small protein Hfq to function (Gottesman, 2004). In *Pseudomonas* species the role of Hfq is far less studied than in *E. coli*. However it is likely that it functions in a similar way as it does in *E. coli*, since Hfq of *P. aeruginosa* is able to functionally replace Hfq in *E. coli* (Sonnleitner *et al.*, 2002). A *hfq* mutant of *P. aeruginosa* shows reduced virulence and is affected in its growth and primary metabolism (Sonnleitner *et al.*, 2003). It was recently shown that Hfq positively regulates the quorum-sensing gene *rhlI* via stabilization of the sRNA RsmY in *P. aeruginosa* PAO1 (Sonnleitner *et al.*, 2006), thereby showing an effect on secondary metabolism. However a *hfq* mutant of

that sRNAs play a central role in the regulatory events in the *Pseudomonas* cells as they do in *E. coli* (Gottesman, 2004). Therefore, a concise description of how sRNAs exert their regulatory roles with Hfq in *E. coli* is given below.

Small RNAs and Hfq in E. coli

Binding of sRNAs to mRNAs has various consequences (Fig. 5, panel A). The annealing of RyhB sRNA downstream of *sdhC* on the full-length SdhCDAB mRNA (involved in iron metabolism) (Masse & Gottesman, 2002) results in degradation of the SdhDAB mRNA. Meanwhile, RhyB is also degraded. The simultaneous degradation of sRNA and mRNA could be a common phenomenon (Masse *et al.*, 2003). However, inhibition of translation initiation does not always result in mRNA decay. For example, the GalETK (galactose operon) mRNA seems to remain stable after binding of Spot 42 sRNA downstream of GalET (Moller *et al.*, 2002), although translation is blocked between GalET and GalK. This elucidates one way by which genes in an operon can be expressed differently from each other. Binding of sRNAs can also have a positive effect on gene expression. For example, the RBS of *rpoS* mRNA in *E. coli* is predicted to be buried in a stable hairpin structure, which inhibits translation initiation. Binding of DsrA sRNAs disrupts the hairpin structure and stimulates translation (Repoila *et al.*, 2003).

Hfq is necessary for the functioning of many sRNAs *in vivo* (Gottesman, 2004), including the ones described above. The main role of Hfq is probably promoting interactions of sRNAs with target mRNA (Mikulecky *et al.*, 2004). The structure of Hfq was recently elucidated. Biochemical and structural data indicate that monomers of the protein assemble into hexamers forming a donut structure through which RNA might thread (Schumacher *et al.*, 2002). It is not well understood how the Hfq protein facilitates sRNA-mRNA interactions. Previously, several hypotheses were reviewed (Storz *et al.*, 2004) (Fig. 5, panel B). One model hypothesizes that Hfq acts as an RNA chaperone that unfolds one RNA to render it accessible to the second one. Another model proposes that Hfq binds both RNAs to increase the probability of their interaction. A third model invokes two hexamers of Hfq, one binding the sRNA, the other one the mRNA. The two RNAs would be subsequently brought together by protein-protein interactions. All three models might be represented among the many different mRNA-Hfq-sRNA combinations. Recent data from mutational analyses showed that Hfq has two independent RNA-binding surfaces (Mikulecky *et al.*, 2004).

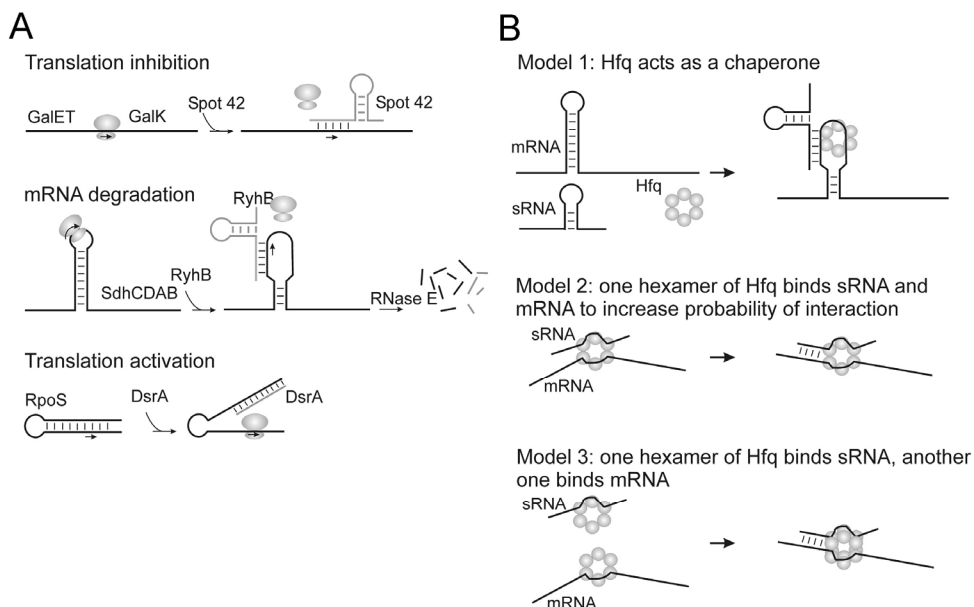


Figure 5. Mechanisms underlying regulation by Hfq-sRNA in *E. coli*.

Panel A. Possible consequences of sRNA binding to mRNA. Hfq is necessary for these interactions but was omitted from the drawing for clarity. See text for details. Adapted from Geissmann & Touati (2004) and Storz *et al.* (2004).

Panel B. Three models that could explain the role of Hfq in promoting mRNA/sRNA interaction. See text for details. Adapted from Storz *et al.* (2004).

CONCLUDING REMARKS

The regulation of secondary metabolism in *Pseudomonas* species is extremely complex and many differences exist among them. In most species, quorum-sensing play a central role, particularly when several quorum-sensing systems interact like in *P. aeruginosa* (Fig. 6, panel A). However, in some species no quorum-sensing system could be characterized, like in *P. fluorescens* CHAO, where small RNAs and proteins are then the central regulators (Fig. 6, panel B). A common point in most of the species discussed in this review is the important function of the GacS/GacA two-component system, as a master regulator of many secondary traits.

In the near future, these schemes are likely to be complicated by new regulators probably including many sRNAs. Although many sRNAs were isolated in *E. coli*, characterization of sRNAs in *Pseudomonas* species is still challenging because they display very low sequence conservation (Gottesman, 2004). Screening for sRNAs will be allowed by the rapidly growing number of completely sequenced *Pseudomonas* genomes, which gives the possibility to perform genome-wide sequence searches based on parameters common to sRNAs, comparative genomics

and microarrays, followed by confirmation with Northern blot analysis or RT-PCR, binding studies using RNA-binding proteins and mutant analyses as described previously (Wassarman *et al.*, 2001; Zhang *et al.*, 2003). Additionally, proteomics and metabolomics will extend the genetic tools for linking ecological variations to molecular responses in the cell.

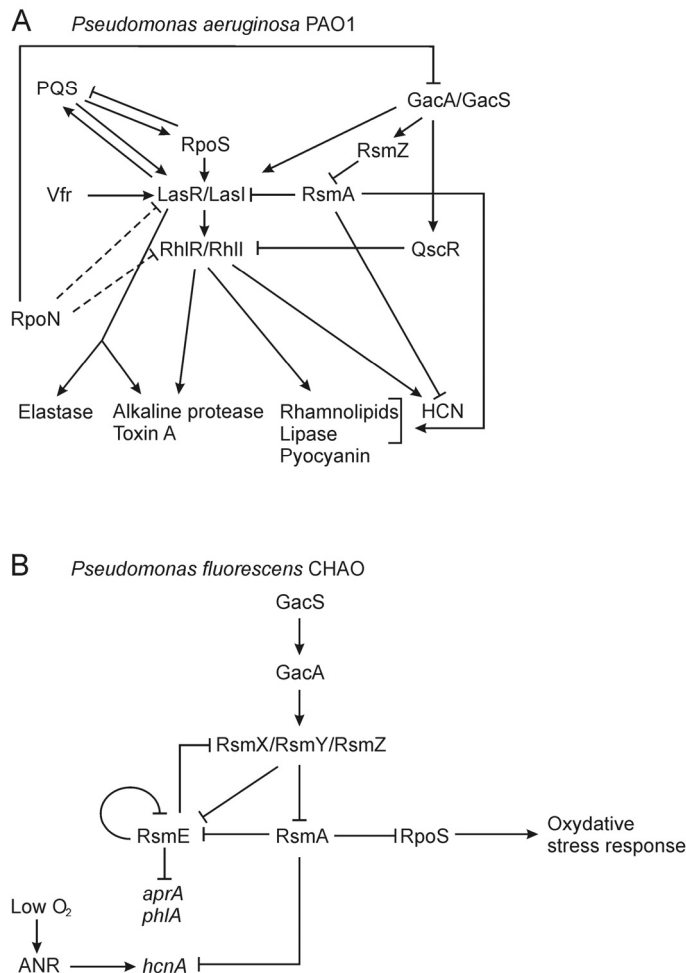
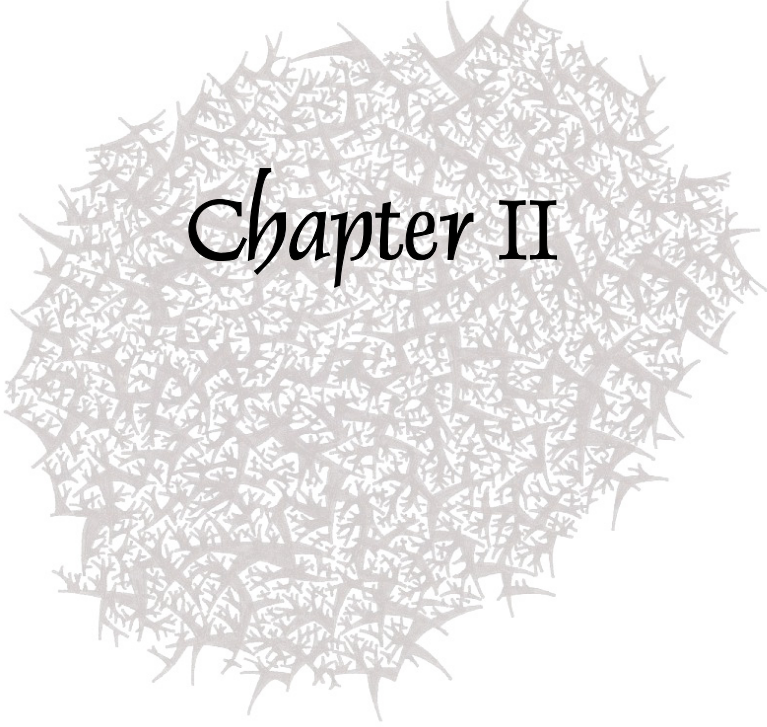


Figure 6. Regulation of secondary metabolism in *Pseudomonas* species. The non-exhaustive schemes result from the compilation of published data. All the interactions were shown to happen but may not always co-exist, depending on physiological conditions. The triangular arrow heads indicate a positive regulation, whereas the flat arrow heads indicate a negative regulation. Panel A. Regulation of secondary metabolism in *P. aeruginosa* PAO1. It is not always clear whether the effects of various regulators on *rhlR* expression are direct or mediated via LasR. Panel B. Regulation of secondary metabolism in *P. fluorescens* CHAO. The sigma factor RpoS is not involved as an intermediate between the GacS/GacA system and secondary metabolites production. However, it regulates oxidative stress resistance downstream of RsmA (Heeb *et al.*, 2005).

Table 1. Regulators of secondary metabolism in *Pseudomonas* species.

Regulators	Functions	References
ANR	Anaerobic regulator, FeS protein, active at low oxygen concentrations. Upregulates HCN production in <i>P. fluorescens</i> .	(Blumer & Haas, 2000; Haas & Keel, 2003)
CsrA	Carbon storage regulator, small RNA-protein. Can inhibit translation by binding to the RBS of target mRNAs.	(Romeo, 1998)
CsrB	Small RNA. Represses the activity of CsrA by sequestering it.	(Romeo, 1998)
FixJ	Regulator of nitrogen fixation in rhizobia. Transcriptional activator activated by phosphorylation.	(Fischer, 1994)
Fur	Ferric uptake regulator, responds to variations in iron concentrations, inhibits siderophore production when iron is available.	(Vasil & Ochsner, 1999)
GacS/GacA	Two component system including the sensor kinase GacS (global antibiotic control) and the transcriptional activator GacA. GacS binds to a so far unknown environmental signal, is subsequently phosphorylated and activates GacA, a FixJ homologue.	(Heeb <i>et al.</i> , 2002; Zuber <i>et al.</i> , 2003)
Hfq	Small regulatory protein that mediates interactions between small regulatory RNAs and their mRNA target.	(Gottesman, 2004; Valentin-Hansen <i>et al.</i> , 2004)
LasI/LasR	LuxI/LuxR homologues, respectively, in <i>P. aeruginosa</i> .	(Gambello <i>et al.</i> , 1993; Kiratisin <i>et al.</i> , 2002; Gould <i>et al.</i> , 2004)
LuxI/LuxR	Quorum-sensing system isolated in <i>Vibrio fischeri</i> . LuxI is an <i>N</i> -AHL synthase. <i>N</i> -AHL binds to the LuxR transcriptional regulator, thereby activating it.	(Fuqua <i>et al.</i> , 1996)
PhlF	TetR homologue, downregulates the expression of the <i>phlACBD</i> operon, responsible for the synthesis of Phl.	(Abbas <i>et al.</i> , 2002)
PhlH	TetR homologue, upregulates the expression of <i>phlACBD</i> .	(Schnider-Keel <i>et al.</i> , 2000)
PhzI/PhzR	LuxI/LuxR homologues, respectively, in <i>P. chlororaphis</i> .	(Chin-A-Woeng <i>et al.</i> , 2001)
ppGpp	Guanosine tetraphosphate. Alarmone that regulates the activity of RpoS.	(Nystrom, 2004; Erickson <i>et al.</i> , 2004)
PQS	2-heptyl-3-hydroxy-4-quinolone (<i>Pseudomonas</i> quinolone signal). Regulates <i>lasI</i> and <i>rhlI</i> expression.	(Diggle <i>et al.</i> , 2003)
PsrA	TetR homologue. Upregulates <i>rpoS</i> expression.	(Kojic & Venturi, 2001; Kojic <i>et al.</i> , 2002)
QscR	LuxR homologue, no cognate LuxI homologue identified yet. Represses <i>lasI</i> expression. Forms heterodimers with	(Chugani <i>et al.</i> , 2001; Ledgham <i>et al.</i> , 2003)

	LasR and RhlR, binds both C ₄ -HSL and 3O-C ₁₂ -HSL.	
RhlI/RhlR	LuxI/LuxR homologues, respectively, in <i>P. aeruginosa</i> . Upregulated by LasI/LasR.	(Brint & Ohman, 1995; Latifi <i>et al.</i> , 1996; Medina <i>et al.</i> , 2003; Lamb <i>et al.</i> , 2003; Ventre <i>et al.</i> , 2003)
RpoD	Sigma subunit of the RNA polymerase (also called σ^{70}). Directs the transcription of the house-keeping genes during exponential phase of growth.	(Lonetto <i>et al.</i> , 1992)
RpoN	Alternative sigma subunit of the RNA polymerase (also called σ^{54}). Regulates several traits of the secondary metabolism.	(Pesci <i>et al.</i> , 1997; Chatterjee <i>et al.</i> , 2003; Péchy-Tarr <i>et al.</i> , 2005)
RpoS	Alternative sigma subunit of the RNA polymerase (also called σ^S). Involved in stress response and regulation of secondary metabolism.	(Sarniguet <i>et al.</i> , 1995; Ramos-González & Molin, 1998; Jorgensen <i>et al.</i> , 1999; Suh <i>et al.</i> , 1999; Hengge-Aronis, 2002a; Schuster <i>et al.</i> , 2004a; Girard <i>et al.</i> , 2006)
RsmA	Small RNA-binding protein, CsrA homologue (regulator of secondary metabolism). Important regulatory intermediate of GacS/GacA.	(Blumer <i>et al.</i> , 1999; Heeb <i>et al.</i> , 2002)
RsmE	Small RNA-binding protein, CsrA homologue (regulator of secondary metabolism). Important regulatory intermediate of GacS/GacA.	(Reimmann <i>et al.</i> , 2005)
RsmX	Small regulatory RNA, CsrB homologue.	(Kay <i>et al.</i> , 2005)
RsmY	Small regulatory RNA, CsrB homologue.	(Valverde <i>et al.</i> , 2003)
RsmZ	Small regulatory RNA, CsrB homologue.	(Heurlier <i>et al.</i> , 2004)
RyhB	Small regulatory RNA, regulator of genes involved in iron metabolism in <i>E. coli</i> . RyhB homologues also were found in <i>P. aeruginosa</i> .	(Masse & Gottesman, 2002; Masse <i>et al.</i> , 2003; Wilderman <i>et al.</i> , 2004)
Spot42	Small regulatory RNA, regulator of the <i>galETK</i> operon.	(Moller <i>et al.</i> , 2002)
TetA/TetB	Proteins forming a pump that exports tetracyclin in <i>E. coli</i> .	(Miller & Sulavik, 1996; Grkovic <i>et al.</i> , 2002)
TetR	Regulators of <i>tetAB</i> expression.	(Hillen & Berens, 1994; Grkovic <i>et al.</i> , 2002)
Vfr	Cyclic AMP receptor protein. Regulates <i>lasR</i> expression.	(Albus <i>et al.</i> , 1997)
VqsR	LuxR homologue, no cognate LuxI homologue identified yet.	(Juhas <i>et al.</i> , 2004)



Chapter II

Regulatory roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL1391

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

Adapted from Girard *et al.*, *Microbiology* (2006) **152**: 43-58

ABSTRACT

Production of the secondary metabolite phenazine-1-carboxamide (PCN) by *Pseudomonas chlororaphis* PCL1391 is crucial for biocontrol activity against the phytopathogen *Fusarium oxysporum* f. sp. *radicis lycopersici* on tomato roots. Regulation of PCN production involves the two-component signaling system GacS/GacA, the quorum-sensing system PhzI/PhzR, and the regulator PsrA. Here we report that a functional *rpoS* is required for optimal PCN and *N*-hexanoyl-L-homoserine lactone (C₆-HSL) production. Constitutive expression of *rpoS* is able to complement partially the defect of a *psrA* mutant for PCN and *N*-acyl-homoserine lactone (*N*-AHL) production. Western blotting shows that *rpoS* is regulated by *gacS*. All together, these results suggest the existence of a cascade consisting of *gacS/gacA* upstream of *psrA* and *rpoS*, which influence expression of *phzI/phzR*. Overproduction of *phzR* complements the effects on PCN and C₆-HSL production of all mutations tested in the regulatory cascade, which shows that a functional quorum-sensing system is essential and sufficient for PCN synthesis. In addition, the relative amounts of PCN, phenazine-1-carboxylic acid (PCA) and C₆-HSL produced by *rpoS* and *psrA* mutants harboring a constitutively expressed *phzR* indicate an even more complex network of interactions, probably involving other genes.

INTRODUCTION

Secondary metabolites secreted by Gram-negative bacteria are key elements in various interactions with other organisms in the rhizosphere (Bakker *et al.*, 2002; Bassler, 1999; Lugtenberg *et al.*, 2002). In *Pseudomonas chlororaphis* PCL1391, the production of the antifungal metabolite PCN (Chin-A-Woeng *et al.*, 2003) is synthesized through expression of the biosynthetic *phzABCDEFGH* operon (Chin-A-Woeng *et al.*, 1998). Previous work led to a model of regulation of PCN production involving three different groups of genes: the *phzI/phzR* quorum sensing system (Chin-A-Woeng *et al.*, 2001b), *gacS/gacA* (global antibiotic and cyanide control), and the regulatory *psrA* gene (*Pseudomonas* sigma regulator) (Chin-A-Woeng *et al.*, 2005).

The *phzI* gene is responsible for the synthesis of autoinducers, of which *N*-hexanoyl-L-homoserine lactone (C₆-HSL) is the main product (Chin-A-Woeng *et al.*, 2001b). C₆-HSL is supposed to bind to PhzR, thereby activating it. Subsequently, the PhzR-C₆-HSL complex probably binds to the *lux* (or *phz*) box upstream of the *phz* biosynthetic operon, which results in initiating the transcription of the *phz* operon. The PhzR-C₆-HSL complex also upregulates *phzI* via a second *lux* box. A similar regulation of phenazine synthesis by quorum-sensing was shown in *P. aureofaciens* 30-84 (Pierson III *et al.*, 1994).

The GacS/GacA system is composed of a sensor kinase, responding to an unknown (possibly environmental) factor (Heeb *et al.*, 2002; Zuber *et al.*, 2003), and a response regulator belonging to the Fix J family. In *Pseudomonas* species, GacS and GacA are global regulators of the secondary metabolism, since they are situated upstream of many regulatory cascades. GacS and GacA are involved in the regulation of a substantial set of genes and of multiple traits, such as production of metabolites like HCN and Phl in *P. fluorescens* CHAO (Laville *et al.*, 1992), of enzymes like exoprotease and phospholipase C in *P. fluorescens* CHAO (Sacherer *et al.*, 1994) and of various phenazines in *P. aureofaciens* 30-84 and *P. aeruginosa* PAO1 (Chancey *et al.*, 1999; Reimmann *et al.*, 1997). GacS and GacA exert their effect on secondary metabolism by modulating the expression of various regulators (Chatterjee *et al.*, 2003; Haas & Defago, 2005) including quorum-sensing (Bertani & Venturi, 2004; Chancey *et al.*, 1999; Reimmann *et al.*, 1997) and σ^s (Schmidt-Eisenlohr *et al.*, 2003; Whistler *et al.*, 1998). In *P. chlororaphis* strain PCL1391, a mutation in *gacS* results in a severe decrease of PCN production to undetectable levels, while the *N*-AHL production is also much lower than in the wild-type (Chin-A-Woeng *et al.*, 2005).

GacS/GacA is also required for *psrA* expression in PCL1391 (Chin-A-Woeng *et al.*, 2005). The *psrA* gene of *P. putida* was shown to regulate the transcription of the *rpoS* gene (Kojic & Venturi, 2001) by directly binding to the *rpoS* promoter (Kojic *et al.*, 2002). *rpoS* encodes the stationary phase alternative sigma factor σ^s , which is responsible for the switch in gene expression occurring upon exposure of cells to starvation and/or various stresses (Lange & Hengge-Aronis, 1991). In *Pseudomonas* species, *rpoS* mutants are often affected in their secondary metabolism, and particularly in their antibiotic production (Sarniguet *et al.*, 1995; Suh *et al.*, 1999). However, the results are different depending on the species and the antibiotic considered. For example, an *rpoS* mutation results in a decrease of pyrrolnitrin production by *P. fluorescens*, but in an increase of pyoluteorin and 2,4-diacetylphloroglucinol production by the same strain (Sarniguet *et al.*, 1995) and of pyocyanin in *P. aeruginosa* (Suh *et al.*, 1999).

Here we describe *rpoS* in *P. chlororaphis* PCL1391 and its role in the synthesis of PCN. A significant number of PCL1391 derivatives were constructed that are affected in the expression of the following genes: *psrA*, *rpoS* and *phzR*. Quantification of PCN and C₆-HSL showed that the *phz* operon is regulated by a cascade involving GacS, PsrA, RpoS and PhzI/PhzR.

RESULTS

Identification of *rpoS* in *P. chlororaphis* PCL1391

Using degenerate primers based on known *Pseudomonas rpoS* sequences, a PCR fragment was obtained with chromosomal DNA of PCL1391 as a template. After sequencing of this fragment, flanking chromosomal regions were isolated by PCR using a pBlueScript chromosomal library from PCL1391 as the template (for details see Experimental Procedures). Sequence analyses of the *P. chlororaphis* PCL1391 fragments confirmed the presence of an ORF (accession number AY586457) encoding a protein of 335 amino acids, which showed an identity of 99% with *rpoS* of *P. chlororaphis* strain 06 (Kang *et al.*, 2004), 97% to *rpoS* of *P. fluorescens* PfO1 (accession number ZP_00266495.1), 93% to *rpoS* of *P. putida* (Kojic *et al.*, 1999), and 93% to *rpoS* of *P. syringae* pv. *tomato* str. DC3000 (accession number NP_791390).

A putative Shine-Dalgarno sequence was detected starting 12 nucleotides upstream of the start codon, and a putative Rho-independent terminator sequence is present 22 nucleotides downstream of the stop codon. In addition a sequence (GAAACTGCACTTTG) was identified close to the ATG codon in the promoter of the

PCL1391 *rpoS* homologous gene, identical to the PsrA binding box consensus of *P. putida* (Kojic *et al.*, 2002).

The ORF upstream of PCL1391 *rpoS* is homologous (98% identity) to the lipoprotein gene *nlpD* of *P. chlororaphis* 06 (Kang *et al.*, 2004). The ORF sequence identified downstream of *rpoS*, shows homology (50% identity) to a transposase gene of *Ralstonia solanacearum* (accession number NP_520694.1).

In contrast, *rpoS* of other *Pseudomonas* strains is followed by the small RNA regulator *rsmZ* (regulator of secondary metabolites) and *fdxA* (ferredoxine A) (Heurlier *et al.*, 2004). Neither a repetitive GGA motif (Heurlier *et al.*, 2004), nor a conserved upstream element (Heeb *et al.*, 2002) indicating the presence of an *rsmZ* homologue downstream of *rpoS* were found in PCL1391. Alignment analysis using Vector NTI with *rsmZ* sequences of several *Pseudomonas* species with a 500-nucleotides sequence downstream of *rpoS* in PCL1391 did not show any homology (not shown).

Effect of *rpoS* on PCN and *N*-AHL production

A 500-bp internal fragment of *rpoS* was generated by PCR and used for single homologous recombination in strain PCL1391 resulting into PCL1954 (for details see Experimental Procedures). Western blot analysis showed that the RpoS protein was absent in PCL1954 (Fig. 1, lane 6). The production of PCN by the *rpoS* mutant PCL1954 was decreased by 99% compared to that by PCL1391 (Fig. 2, panels A and B). Constitutive expression of *rpoS* was established by cloning *rpoS* under control of the *tac* promoter in the vector pBBRMCS-5 resulting in pMP7420 (see Experimental Procedures). In the derivative PCL1955 (*rpoS* mutant with P_{tac} *rpoS*), the production of RpoS was shown using Western blot analysis (Fig. 1, lane 7) and PCN production was restored between 35% (Fig. 2, panel B) and 70% (Fig. 3, panel A) of that of the control strain PCL1960 (wt + pBBR1MCS-5).

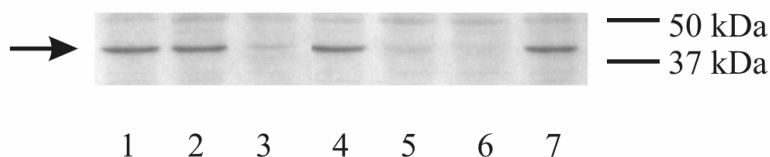


Figure 1. Western blot analysis of RpoS production in *P. chlororaphis* PCL1391 and derivative strains. Lane 1: PCL1391. Lane 2: PCL1103 (*phzI::Tn5luxAB*). Lane 3: PCL1111 (*psrA::Tn5luxAB*). Lane 4: PCL1119 (*phzB::Tn5luxAB*). Lane 5: PCL1123 (*gacS::Tn5luxAB*). Lane 6: PCL1954 (*rpoS::pMP7418*). Lane 7: PCL1955 (*rpoS::pMP7418* + P_{tac} *rpoS*). On the right side of the blot two markers are shown. The arrow on the left indicates the position of RpoS.

The production of C₆-HSL by the *rpoS* mutant was very decreased, but detectable (Fig. 3, panel A). C₆-HSL levels in an *rpoS* mutant background were restored by the constitutive production of RpoS in PCL1955 (Fig. 3, panel A).

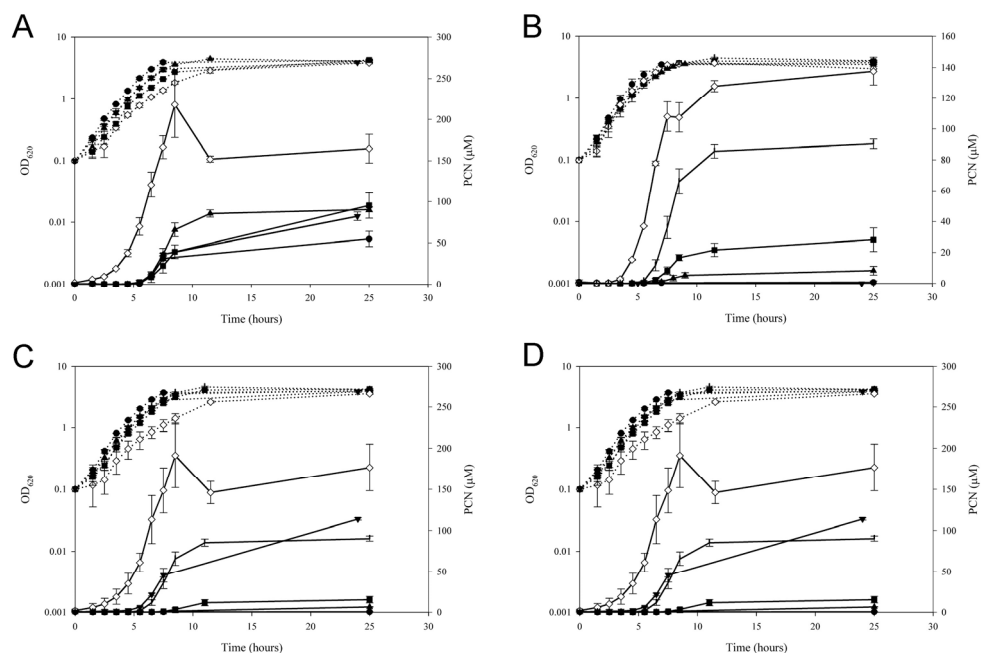


Figure 2. PCN production by *P. chlororaphis* PCL1391 and derivative strains.

Extractions were made from at least three independent cultures in 10 ml MVB1 during time and the PCN concentration was determined by HPLC. On each graph, the absorbance is plotted following the left axis (dotted lines) and the PCN concentration is plotted following the right axis (plain lines). For easier reading, the same symbols were used in the different panels for the following constructs: original strain (●), derivative containing pBBR1MCS-5 (▲), derivative containing *P_{tac} rpoS* (■), derivative containing *P_{tac} phzR* (◇), derivative containing *P_{tac} psrA* (▼). For panels B, C, and D, the values for PCL1960 (PCL1391 + pBBR1MCS-5) are plotted as a control (|).

Panel A. Wild-type derivatives. Panel B. *rpoS* derivatives. Panel C. *psrA* derivatives. Panel D. *gacS* derivatives.

Since a defect in *rpoS* decreased PCN production, the effect of over-expression of *rpoS* in the wild-type strain PCL1391 was also analyzed by transforming pMP7420 to PCL1391. No major difference was observed between the amounts of PCN produced by wt PCL1391 with constitutive expression of *rpoS* and wt PCL1391 containing the empty cloning vector (Fig. 2, panel A).

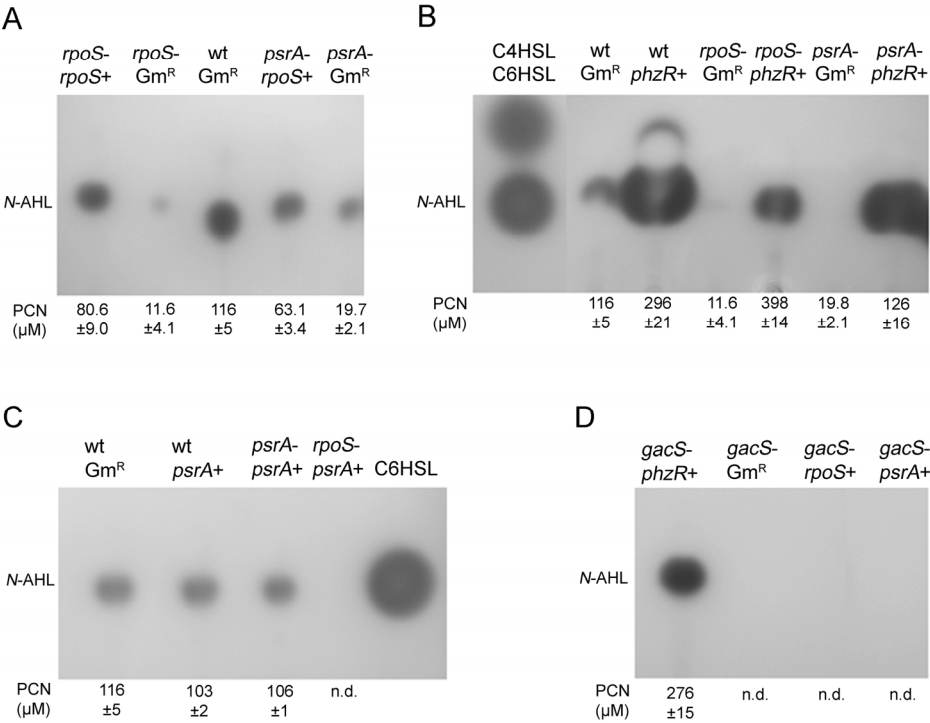


Figure 3. C18-reverse phase TLC analysis of *N*-AHLs produced by *P. chlororaphis* strain PCL1391 derivatives in 50 ml MVB1. On each panel a group of PCL1391 derivatives is analyzed. The strains are described above the TLC image: wt indicates a wild-type derivative, *psrA*- a PCL1111 derivative and *rpoS*- a PCL1954 derivative; on the second line, Gm^R indicates the presence of the empty vector pBBR1MCS-5 in the derivative, *phzR*⁺ the presence of pMP7444 overexpressing *phzR*, *rpoS*⁺ the presence of pMP7420 overexpressing *rpoS* and *psrA*⁺ the presence of pMP7465 overexpressing *psrA*. The lines under the TLC image indicate the PCN production after overnight growth in 50 ml MVB1 for each PCL1391 derivative. These numbers are averages and standard deviations calculated from extractions made in at least three independent cultures. The PCN concentration was determined by HPLC.

Interactions between quorum sensing and *rpoS* and their influence on PCN production

The observations that the amounts of PCN and C₆-HSL are decreased in an *rpoS* mutant, and restored by the constitutive expression of *rpoS*, indicate that RpoS regulates the *phz* operon via *phzR* and/or *phzI*. Therefore the effect of constitutive expression of *phzR* in the *rpoS* mutant was tested. For this purpose *phzR* was cloned under the control of the *tac* promoter resulting in plasmid pMP7447 and transformed to PCL1954 (*rpoS*) The resulting strain PCL1986 showed complementation for PCN production of the *rpoS* mutation, as it produced 1.5-fold

higher PCN than PCL1391 harboring empty pBBR1-MCS5 (Fig. 2, panel B) and showed an increased C₆-HSL production (Fig. 3, panel B).

Regulation of RpoS synthesis by genes involved in PCN synthesis

The effect of mutations in *gacS*, *psrA*, *phzI* or *phzB* of PCL1391 on the production of RpoS protein was tested by Western blot analysis. The experiments were performed in MVB1 medium and samples for RpoS analysis were harvested during logarithmic phase (OD₆₂₀ 1.0) and at the beginning of the stationary phase (OD₆₂₀ 2.2). The amounts of RpoS appeared to be similar at the two time points. A blot of the results at OD₆₂₀ 1.0 is shown in Figure 1. RpoS amounts were severely reduced as a result of mutations in *psrA* (PCL1111, lane 3) and *gacS* (PCL1123, lane 5). Mutations in *phzI* (PCL1103, lane 2) and in *phzB* (PCL1119, lane 4) did not affect the production of RpoS.

Relationships between *psrA*, *rpoS* and *gacS*

A *psrA* mutant of PCL1391 showed low production of PCN and *N*-AHL when grown in MVB1 medium as compared to PCL1391 (decrease of 99%) (Fig. 2, panel C and Fig. 3, panel A). It was shown that *psrA* regulates *rpoS* in other *Pseudomonas* species (Kojic & Venturi, 2001), probably *via* binding to the promoter of the *rpoS* gene at a PsrA-binding box. Therefore, an attempt was made to complement the *psrA* mutant PCL1111 with constitutively expressed *rpoS*. For this purpose, the vector pMP7420 (*P_{tac}* *rpoS*) was transformed into PCL1111, which resulted into PCL1961. PCL1961 showed increased PCN and *N*-AHL levels compared to the *psrA* mutant and produced up to 55% of the amount of PCN produced by the wild-type (Fig. 2, panel C and Fig. 3, panel A). PCL 2048, the *rpoS* mutant over-expressing *psrA*, was unable to produce PCN (Fig. 2, panel B) or *N*-AHL (Fig. 3, panel C). As a control, we transformed pMP7465 (pBBR1MCS-5 harboring *P_{tac}* *psrA*) into PCL1111 (*psrA*). The resulting strain, PCL2045, showed restored levels of PCN and C₆-HSL (Fig. 2, panel C, and Fig. 3, panel C). Constitutive expression of *phzR* also restored production of PCN and *N*-AHL in a *psrA* background (strain PCL1996, Fig. 2, panel C and Fig. 3, panel B).

Since it was shown that GacA/GacS regulate PCN and *N*-AHL production in KB medium, as well as *psrA* expression (Chin-A-Woeng *et al.*, 2005) and that RpoS is severely decreased in the *gacS* mutant PCL1123, the relationship between GacS, PsrA/RpoS, quorum-sensing and PCN was studied in more detail. The *gacS* mutant did not produce any detectable PCN or *N*-AHL in MBV1 (Fig. 2, panel D and Fig. 3,

panel D). Neither constitutive *rpoS* expression, nor constitutive expression of *psrA* (Fig. 2, panel D and Fig. 3, panel D), were sufficient to compensate for the *gacS* mutation. Only the constitutive *phzR* gene restored PCN and AHL production in a *gacS* mutated background (Fig. 2, panel D and Fig. 3, panel D). Surprisingly, after overnight growth (see Fig. 1 in Supplementary Materials), high amounts of PCA are present in strains PCL1986 and PCL1998 (*rpoS* and *gacS* mutant, respectively, both over-expressing *phzR*), but not in PCL1993 or PCL1996 (wt and *psrA* mutant, respectively, both over-expressing *phzR*).

DISCUSSION

***psrA* and *rpoS* control PCN production in strain PCL1391**

The organization of the *rpoS* gene in strain PCL1391 is comparable to that observed in other *Pseudomonas* species (Fujita *et al.*, 1994; Heeb & Haas, 2001; Kojic *et al.*, 1999; Kojic *et al.*, 2002; Ramos-González & Molin, 1998). A substantial difference is the presence of a putative transposase downstream of *rpoS* in PCL1391, whereas in many other *Pseudomonas* species *rpoS* is followed by *rsmZ* and the ferredoxin gene *fdxA* (Heurlier *et al.*, 2004). No indication could be found of the presence of an *rsmZ* gene downstream of *rpoS*. Measurements of the production of PCN and *N*-AHL in various derivatives (Fig. 2 and 3) show that *rpoS* activates the synthesis of these two metabolites.

This study was started with the assumption that *psrA* and *rpoS* would constitute two components of a cascade regulating the *phz* operon, according to results in other strains (Kojic & Venturi, 2001). Previous work in rich growth medium indicated that PsrA inhibits *N*-AHL and PCN production in PCL1391 (Chin-A-Woeng *et al.*, 2005). In our study using a poor MVB1 medium, *psrA* was shown to activate PCN and *N*-AHL production in PCL1391.

Our data confirm the key role of *gacS* for PCN synthesis (Fig. 2, panel D). Constitutive *rpoS* expression did not restore PCN synthesis in a *gacS* mutant (PCL2010), which indicates that next to *rpoS* in the regulatory cascade, other factors affected by *gacS* are necessary for PCN production (Fig. 4). However, constitutive expression of the *phzR* gene restores PCN and *N*-AHL synthesis in a *gacS* mutant (PCL1998). This could be surprising considering that in *P. aureofaciens* 30-84, which is closely related to *P. chlororaphis* PCL1391, GacS/GacA affect mostly the transcription of *phzI* and not that of *phzR* (Chancey *et al.*, 1999). Additionally, phenazine synthesis is regulated in a comparable way in both strains by PhzI/PhzR/C₆-HSL and GacS/GacA (Chancey *et al.*, 1999; Pierson III *et al.*, 1994;

Wood *et al.*, 1997; Wood & Pierson III, 1996). The role of PsrA and RpoS in phenazine synthesis was so far not studied in strain 30-84. The following hypothesis would conciliate results in both strains: GacS/GacA could regulate *phzI* at the transcriptional level and *phzR* at the post-transcriptional level, since it was shown that GacA acts at both levels (Blumer *et al.*, 1999; Pessi & Haas, 2001). In our *gacS* mutant, the presence of constitutively expressed *phzR* strongly increased PCN and *N*-AHL production in a *psrA* mutant (strain PCL1961, Fig. 2, panel C and Fig. 3, panel A). The fact that the complementation was only partial (also in the *rpoS* mutant) could be explained by two hypotheses. (i) PsrA regulates other genes not downstream of *rpoS* that are necessary for full activation of the *phz* genes. (ii) A fine-tuning of *rpoS* expression might be necessary for wild-type amounts of PCN, which is not possible when the gene is under the control of a constitutive promoter. It was previously shown for *P. putida* that PsrA regulates the expression of *rpoS* (Kojic & Venturi, 2001) by binding to its promoter (Kojic *et al.*, 2002). Our results indicate a similar regulation in PCL1391. Our results show to our knowledge for the first time that this interaction is relevant for a particular phenotypic trait, the production of the secondary metabolite PCN.

Interestingly, our results show that the effect of a *psrA* mutation is dependent on the growth conditions. Additionally, it is remarkable that constitutive expression of *rpoS* does not restore PCN production in *rpoS* and *psrA* mutants to the same level between different volumes of cultures conditions (Fig. 2 and 3). Although this looks peculiar, it is not unique, since conditional results were also reported for another phenazine regulator, RpeA (repressor of phenazine expression) (Whistler & Pierson III, 2003). RpeA was shown to regulate PCN production mostly in minimum medium, not in complex medium. Similarly, RpoS could have a role in controlling secondary metabolism mostly under nutrient-limiting conditions. It could act as a controller of energy distribution in the cell when the nutritional conditions are more stringent, as indicated by the high sensitivity to external conditions of the strains constitutively expressing *rpoS* (see also below). It is also likely that other unidentified factors sensing environmental changes are involved in PCN regulation that would explain the switch in the role of PsrA between KB medium and MVB1 medium. Conditions in the soil are known to be nutrient-limiting. Therefore the choice of a relatively poor medium as MVB1 seemed more relevant for this study.

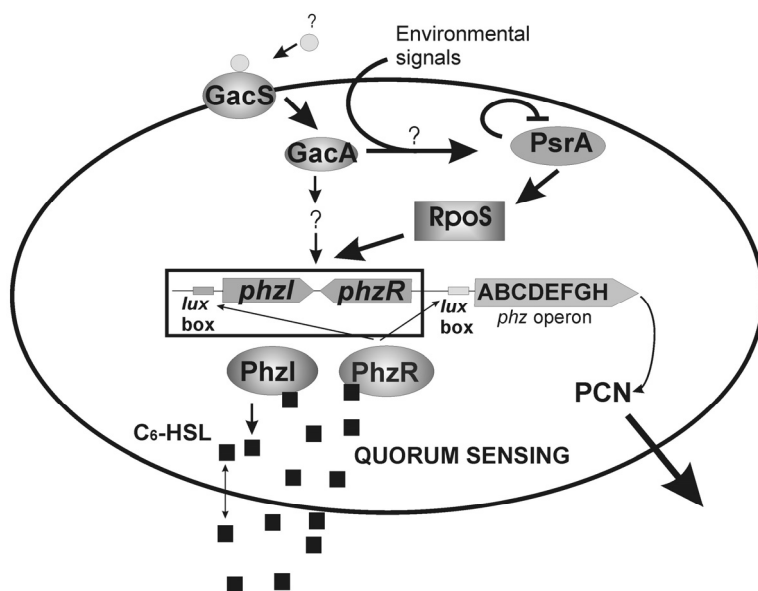


Figure 4. Model for the regulatory cascade governing PCN production in *P. chlororaphis* PCL1391 in MVB1 medium. Upstream in the cascade, the sensor GacS is activated by a putative environmental factor. Subsequently, GacS stimulates its cognate kinase GacA. GacA activates a cascade of genes including PsrA and RpoS. Next to GacA, unknown environmental factors probably affect PsrA. This part of the regulation is so far not understood. In a second cascade, unknown factors are regulated by GacA. These unknown factors, together with RpoS, activate the quorum-sensing system *phzI/phzR*, which in turn switches on expression of the *phz* operon. The *phz* operon is responsible for the synthesis of phenazine-1-carboxamide (PCN).

A regulation cascade between *gacS* and the *phz* operon involves *psrA*, *rpoS* and the quorum sensing system *phzI/phzR*

Under various growth conditions the amounts of C₆-HSL present in PCL1391 spent culture medium were shown to be correlated with the amounts of PCN produced (Chin-A-Woeng *et al.*, 2001b; Chin-A-Woeng *et al.*, 2003; van Rij *et al.*, 2004). In our study, the correlation of PCN and C₆-HSL levels among the various PCL1391 derivatives (Fig. 3) and the restoration of PCN production by constitutive *phzR* expression in the *rpoS* and *psrA* mutants (Fig. 2, panels B and C) show that *rpoS* stimulates PCN production via *phzI/phzR*. Conversely, *phzI* does not regulate *rpoS* expression (Fig. 1). A role of *rpoS* in antibiotic production has been reported (Sarniguet *et al.*, 1995; Suh *et al.*, 1999), but not for regulating PCN production. The inhibitory effect of RpoS on quorum sensing and pyocyanin in *P. aeruginosa* (Whiteley *et al.*, 2000) or of PsrA and RpoS on quorum-sensing in *P. putida* WCS358 (Bertani & Venturi, 2004) is the opposite of what we observed for strain PCL1391.

Surprisingly, RpoS was previously shown not to be involved in homoserine lactone production by *P. putida* WCS358 (Kojic *et al.*, 1999). This is interesting for our study, because in the latter case *P. putida* was grown in minimal medium (Kojic *et al.*, 1999), whereas in the most recent study *P. putida* was grown in the complex LB medium (Bertani & Venturi, 2004). In *P. aeruginosa*, the effect of quorum sensing on *rpoS* transcription in *P. aeruginosa* is mild (Schuster *et al.*, 2004). Thus, very diverse relationships exist between PsrA/RpoS and quorum-sensing/antibiotic production depending on the bacterial species and environmental conditions within one species.

Western blot analysis showed that a mutation in *phzB* does not affect the RpoS level, which suggests that there is no feedback from PCN production on *rpoS* expression. A defect in the regulatory genes *psrA* or *gacS* results in a severe decrease of the amounts of RpoS. Similar observations would result in an excess of PhzR mRNA that would overcome negative post-transcriptional regulation. The PhzR protein produced in turn would bind the low amounts of C₆-HSL resulting from leakage of the *phzI* promoter and restart the positive regulatory loop of PhzI/PhzR by binding to the *lux* box upstream of *phzI*. It would be of great interest to test if a constitutive expression of *phzR* could restore phenazine production in a *gacS* mutant of strain 30-84.

Restoration of PCN and C₆-HSL production by constitutive expression of *phzR* in all the tested mutants is very striking and indicates that for expression of the *phz* operon, a functional expressed quorum sensing system is sufficient. However, it is surprising that after overnight growth, over-expression of *phzR* induces high amounts of PCA only in *rpoS* and *gacS* mutants (see Fig. 1 in Supplementary Materials). Conversely, only PCL1993 and PCL1996 (wt and *psrA* mutant, respectively, both over-expressing *phzR*) show a peak of PCN production (Fig. 2, panels A and C). These observations could be explained by precipitation of PCN, indicated by the presence of numerous crystals in overnight cultures observed only in case of PCL1993 and PCL1996. Since there is still a low amount of RpoS present in the *psrA* mutant (Fig. 1), an explanation for the high production of PCA could be that *rpoS* regulates (probably indirectly) the *phzH* gene, responsible for the conversion of PCA to PCN. The *phzH* gene is the last gene of *phz* operon in PCL1391. It is remarkable that the distances between the *phz* genes (*phzA*, *B*, *C*, *D*, *E*, *F*, and *phzG*) do not exceed 15 nucleotides, whereas there are 111 nucleotides between *phzG* and *phzH* (Chin-A-Woeng *et al.*, 2001a), which could provide a binding site for

regulatory proteins. Various computer analyses did not point to any particular sequence within these 111 nucleotides.

Our results show that a cascade involving GacS/GacA, PsrA, RpoS and quorum-sensing regulates the *phz* operon and that several regulators downstream of GacS/GacA must exist in addition to PsrA/RpoS to activate expression of the *phz* operon. The results presented in this study set a frame for the future work which shall consist in filling the gaps in the regulatory network.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Pseudomonas* strains were cultured at 28°C in liquid MVB1 (van Rij *et al.*, 2004) and shaken at 195 rpm on a Janke und Kunkel shaker KS501D (IKA Labortechnik, Staufen, Germany). *E. coli* strains were grown at 37°C in Luria-Bertani medium (Sambrook & Russel, 2001) under vigorous aeration. Media were solidified with 1.8% Bacto agar (Difco, Detroit, MI, USA). When appropriate, growth media were supplemented with kanamycin (50 µg/ml), carbenicillin (200 µg/ml), gentamicin (10 µg/ml for *E. coli* and 30 µg/ml for *P. chlororaphis*), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 µl/ml). To follow growth, the absorbance of liquid cultures was measured at 620 nm.

The strains and plasmids used in this study are listed in Table 1.

Construction of vectors and PCL1391 mutant strains

Polymerase chain reactions (PCRs) were carried out in general with Super Taq enzyme (Enzyme Technologies Ltd, Cambridge, UK). Only for the production of genes under *P_{tac}* promoter for complementations, PCRs involved Proof Start from Qiagen (Westburg, Leusden, The Netherlands). Primers were synthesized by Isogen Life Science (Maarssen, The Netherlands). Restriction enzymes were purchased from New England BioLabs Inc. (Westburg) and ligase from Promega (Leiden, The Netherlands). The plasmids and primers used in this study are listed in Tables 1 and 2, respectively.

Degenerate primers were designed from the *rpoS* genes of *P. aeruginosa*, *P. fluorescens* and *P. putida*. These primers (oMP768 and oMP769) were used for PCR of chromosomal DNA of PCL1391 and resulted in the amplification of a DNA fragment of 0.7 kb. Sequencing showed that this fragment shared high homologies (see Results) with *rpoS* genes of other *Pseudomonas* strains. Subsequently, primers

Table 1. Bacterial strains and plasmids used

Bacterial strains and plasmids	Description	Reference or source
<i>Pseudomonas chlororaphis</i>		
PCL1391	Wild-type <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide and biocontrol strain of tomato foot and root rot caused by <i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	(Chin-A-Woeng <i>et al.</i> , 1998)
PCL1392	Derivative of PCL1391 tagged with <i>lacZ</i> with wild-type colonizing ability; Km ^r	(Chin-A-Woeng <i>et al.</i> , 2000)
PCL1103	Derivative of PCL1391 in which a promoterless Tn5 <i>luxAB</i> is inserted in <i>phzI</i> ; Km ^r	(Chin-A-Woeng <i>et al.</i> , 2001b)
PCL1104	Derivative of PCL1391 in which a promoterless Tn5 <i>luxAB</i> is inserted in <i>phzR</i> ; Km ^r	(Chin-A-Woeng. <i>et al.</i> , 2001b)
PCL1111	Derivative of PCL1391 in which a promoterless Tn5 <i>luxAB</i> is inserted in <i>psrA</i> ; Km ^r	(Chin-A-Woeng <i>et al.</i> , 2005)
PCL1123	Derivative of PCL1391 in which a promoterless Tn5 <i>luxAB</i> is inserted in <i>gacS</i> ; Km ^r	(Chin-A-Woeng <i>et al.</i> , 2005)
PCL1954	Derivative of PCL1391, <i>rpoS</i> ::pMP7418; Km ^r	This study
PCL1955	Derivative of PCL1954, <i>rpoS</i> ::pMP7418, containing pMP7420; Km ^r , Gm ^r	This study
PCL1957	Derivative of PCL1954, <i>rpoS</i> ::pMP7418, containing pBBR1-MCS5; Km ^r , Gm ^r	This study
PCL1958	Derivative of PCL1391 derivative, containing pMP7420; Gm ^r	This study
PCL1960	Derivative of PCL1391 containing pBBR1-MCS5; Gm ^r	This study
PCL1961	Derivative of PCL1111, containing pMP7420; Km ^r , Gm ^r	This study
PCL1962	Derivative of PCL1111, containing pBBR1-MCS5; Km ^r , Gm ^r	This study
PCL1986	Derivative of PCL1954, containing pMP7447; Km ^r , Gm ^r	This study
PCL1993	Derivative of PCL1391, containing pMP7447; Gm ^r	This study
PCL1996	Derivative of PCL1111, containing pMP7447; Km ^r , Gm ^r	This study
PCL1998	Derivative of PCL1123, containing pMP7447; Km ^r , Gm ^r	This study
PCL2000	Derivative of PCL1104, containing pMP7447; Km ^r , Gm ^r	This study
PCL2001	Derivative of PCL1104, containing pBBR1-MCS5; Gm ^r	This study
PCL2004	Derivative of PCL1123, containing pBBR1-MCS5; Gm ^r	This study
PCL2010	Derivative of PCL1123, containing pMP7420; Km ^r , Gm ^r	This study
PCL2044	Derivative of PCL1391 containing pMP7465; Gm ^r	This study

PCL2045	Derivative of PCL1111 containing pMP7465; Km ^r , Gm ^r	This study
PCL2047	Derivative of PCL1123 containing pMP7465; Km ^r , Gm ^r	This study
PCL2048	Derivative of PCL1954 containing pMP7465; Km ^r , Gm ^r	This study
<i>Chromobacterium violaceum</i>		
CV026	Double mini-Tn5 mutant from <i>C. violaceum</i> ATCC 31532, AHL biosensor; Km ^r	Milton <i>et al.</i> , 1997
<i>Escherichia coli</i>		
DH5α	<i>Escherichia coli</i> ; <i>supE44</i> Δ <i>lacU169</i> (Φ80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi1</i> <i>relA1</i>	(Hanahan, 1983)
Plasmids		
pRK2013	Helper plasmid for tri-parental mating; Km ^r	(Ditta <i>et al.</i> , 1980)
pGEM-T easy	Plasmid designed for direct ligation of PCR fragments; Cb ^r	Promega
pBBR1MCS-5	Cloning vector; Gm ^r	(Kovach <i>et al.</i> , 1995)
pMP4030 (previously pMP6007)	pBluescript containing a 4.5 chromosomal fragment of strain PCL1391 with the <i>phzI</i> and <i>phzR</i> genes and the first part of the <i>phzA</i> gene; Cb ^r	(Chin-A-Woeng <i>et al.</i> , 2001b)
pMP5285	Suicide plasmid for <i>Pseudomonas</i> spp. Used for homologous recombination; Km ^r , Cb ^r	(Kuiper <i>et al.</i> , 2001)
pMP7418	pMP5285 containing a 0.5-kb fragment of <i>rpoS</i> from pMP7425 by <i>EcoRI</i> digestion; Km ^r	This study
pMP7420	pBBR1MCS-5 containing the <i>rpoS</i> gene of PCL1391 downstream of the <i>P_{tac}</i> promoter, obtained by <i>EcoRI</i> digestion of pMP7424; Km ^r	This study
pMP7424	pGEM-T containing <i>rpoS</i> of PCL1391 downstream of the <i>P_{tac}</i> promoter, obtained by PCR; Cb ^r	This study
pMP7425	pGEM-T containing a 0.5-kb PCR product of the central part of <i>rpoS</i> of PCL1391; Cb ^r	This study
pMP7447	pBBR1MCS-5 containing <i>phzR</i> of PCL1391 downstream of the <i>P_{tac}</i> promoter, inserted between the <i>XhoI</i> and <i>EcoRI</i> sites; Gm ^r	This study
pMP7465	pBBR1MCS-5 containing <i>psrA</i> of PCL1391 downstream of the <i>P_{tac}</i> promoter, inserted in the <i>EcoRI</i> site; Gm ^r	This study

oMP770 and oMP771 were designed based on the partial *rpoS* sequence and used in PCR on a pBlueScript chromosomal library of PCL1391 (Chin-A-Woeng *et al.*, 2001b) in combination with oMP49 and oMP50, which anneal close to the multi-cloning site of pBlueScript. oMP770 in combination with oMP49 produced a fragment of 1.2 kb containing the flanking region upstream of *rpoS*. oMP771 in combination with oMP50 produced a PCR fragment of 1.8 kb that contained the flanking regions downstream of *rpoS*. A third primer was designed for further

sequencing of the 3' downstream region with the same method (oMP772) which in combination with oMP50 produced a 1.2 kb PCR fragment.

Table 2. Oligonucleotides used

Name	Nucleotide sequence
oMP49	5'-CAGGAAACAGCTATGACCATGATTAC-3'
oMP50	5'-CCCAGTCACGACGTTCTAAAACG-3'
oMP500	5'-CCCAAGCTTCGGTGGACTTCACTGGC-3'
oMP501	5'-CCCAAGCTTGGCACACGTACCTCAAGGCT-3'
oMP582	5'-GGAATTCGGTGAAATAGCCTCCCAACA-3'
oMP583	5'-GGAATTCGGTCATCTTCGATGGTCAGG-3'
oMP604	5'-CCCAAGCTTTCGGCGTAGATCATGGGGGTGTGC-3'
oMP605	5'-CCCAAGCTTGCGCCGGGGCGCCGCCCAAGCATCC-3'
oMP652	5'-GGAATTCGCCCGCCTGCAYCARCARGGSCARTCC-3'
oMP653	5'-GGAATTCAGCAGATGGCTGGCGAAGGAGTGYCG-3'
oMP686	5'-TTAAGTTTATTCTTATCAATATAGG-3'
oMP689	5'-CGCGGATCCGGCTGCTGGAACGCTACACA-3'
oMP690	5'-CGCGGATCCACGGTCGAGCAATATATGCG-3'
oMP768	5'-AAMGAAGBGCCGGAGTTTGAC-3'
oMP769	5'-GTRTCSAGCAGGGTCTTGTC CGA-3'
oMP770	5'-CAGTGGCGATGTCCGTCTCC-3'
oMP771	5'-CTCGACCATGAACCCCTCCCC-3'
oMP772	5'-TCCTGCCGTTGAAAACCCCG-3'
oMP773	5'-AAGCAACCTGCGTCTGGTGG -3'
oMP774	5'-CGATGCTTTCGACAGGTCG -3'
oMP775	5'-ATATATGAATTCCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAAT TGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGCTCTCAGT AAAGAAGTGCCGGAGTTTGACATCGACG-3'
oMP776	5'-ATATATGGATCCGGGATTCCGTGAAGAACCAATAAAAAGCCCC-3'
oMP777	5'-ATATATCTCGAGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAA TTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGAGTTAG GGCAGCAGTTGGGATGGG-3'
oMP778	5'-ATATATGAATTCCTCCCTCAGATATAGCCCATCGCAACTGCG-3'
oMP783	5'-GGGGTACCGGCGGTGAATTATCATTTTCG-3'
oMP784	5'-GGGAATTCGGAATACCTTGCCGTACATG-3'
oMP689	5'-CGCGGATCCGGCTGCTGGAACGCTACACA-3'
oMP690	5'-CGCGGATCCACGGTCGAGCAATATATGCG-3'
oMP836	5'-ATATATGAATTCCTCAGAAGAACTCGTCAAGAAGGCG-3'
oMP837	5'-ATATATCTCGAGATGATTGAACAAGATGGATTGCACG-3'
oMP859	5'-ATGGCCCAGTCGGAACCGTTGAACGC-3'
oMP860	5'-ATATATGAATTCCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAAT TGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGCCCAGTCG GAAACCGTTGAACGC-3'
oMP861	5'-ATATATGAATTCCTCGCGCCCAACCGGACGGTCAGGCC-3'

In order to construct an *rpoS* mutant derivative of PCL1391, two primers (oMP773 and oMP774) were used in PCR on the chromosomal DNA of PCL1391 and an internal fragment of 0.5 kb of the *rpoS* gene was obtained. This fragment was ligated in pGEM-T easy (Promega) to obtain pMP7425. An *EcoRI* digestion of pMP7425 produced an *EcoRI* internal fragment of *rpoS* which was cloned into the

suicide plasmid pMP5285, resulting in pMP7418. This vector was introduced into PCL1391 for single homologous recombination by triparental mating using pRK2043 as helper. The obtained *rpoS* mutant of PCL1391 was checked by Southern blot and by PCR with oMP686 primer annealing on pMP5285 close to the multi-cloning site (MCS) and oMP776 (annealing on the *rpoS* 3' end which is not present in pMP7418) and named PCL1954.

Two primers were designed according to the sequence of the newly characterized *rpoS* gene of PCL1391 (oMP775 and oMP776), in order to produce a PCR fragment containing the whole *rpoS* under control of the P_{tac} promoter and a part of the 3' downstream region of *rpoS* including the putative terminator. The obtained PCR fragment had the expected size of 1.2 kb and was checked by restriction analysis and sequencing. Subsequently it was cloned into pGEM-T easy, which yielded pMP7424. The P_{tac} *rpoS* fragment was isolated from pMP7424 by *EcoRI* digestion and ligated into pBBR1-MCS5 to produce pM7420, which was introduced into PCL1391 and PCL1954 by triparental mating to produce PCL1958 and PCL1955, respectively. The control strains PCL1960 and PCL1957 were obtained by transforming the cloning vector pBBR1-MCS5 into PCL1391 and PCL1954 respectively. pMP7420 and pBBR1-MCS5 were also introduced into PCL1111 (*psrA*) to obtain PCL1961 and PCL1962, respectively.

Two primers (oMP777 and oMP778) were used with pMP4030 as template to produce a PCR fragment containing the *phzR* gene under P_{tac} control. This product was digested by *XhoI* and *EcoRI* and ligated into the *XhoI*-*EcoRI*-digested pBBR1-MCS5 to obtain pMP7447, which was validated by sequencing and its ability to restore a wild-type PCN production in the *phzR* mutant PCL1104. The resulting strain PCL2000 was able to produce PCN (not shown), in contrast to the PCL1104 derivative PCL2001 which contained the cloning vector pBBR1-MCS5. pMP7447 was also introduced into PCL1391, PCL1954, PCL1111 and PCL1123 to obtain PCL1993, PCL1986, PCL1996 and PCL1998, respectively.

Primers oMP859 and oMP861 were used with chromosomal DNA as template to produce a PCR fragment containing the *psrA* gene. This fragment was used as template for PCR with oMP860 and oMP861 to obtain the *psrA* gene under P_{tac} promoter control. This fragment was digested with *EcoRI* and ligated into the *EcoRI* site of pBBR1MCS-5, to obtain pMP7465. pMP7465 was validated by sequencing. pMP7465 was subsequently introduced into PCL1391, PCL1111, PCL1123 and PCL1954 to obtain PCL2044, PCL2045, PCL2047 and PCL2048, respectively.

Extraction and analysis of phenazine and *N*-acyl homoserine lactones

Phenazine extraction was carried out from 10 ml MVB1 liquid cultures in 100 ml Erlenmeyer flasks at regular time points during growth and/or after overnight growth of bacterial strains as described previously (van Rij *et al.*, 2004).

For extraction of *N*-AHLs, supernatants from 50 ml MVB1 liquid MVB1 cultures in 500 ml Erlenmeyer flasks were mixed with 0.7 volume of dichloromethane, and shaken for one hour, after which the organic phase was collected. Each supernatant was extracted twice and the pooled extracts were dried using a rotary evaporator. The dried residue was dissolved in 25 μ l of acetonitrile and spotted on C18 TLC plates (Merck, Darmstadt, Germany). As a control, 0.5 μ l of synthetic hexanoyl-homoserine lactone (C₆-HSL) (5 μ M) (Fluka, Sigma-Aldrich, Zwijndrecht, the Netherlands) was spotted on the TLC. The plates were developed in methanol-water (60:40, v:v). For detection of *N*-AHLs, the TLC was overlaid with 0.8% LC agar containing a 10-fold diluted overnight culture of the *Chromobacterium violaceum* indicator strain CV026 and supplemented with kanamycin (50 μ g/ml). After incubation for 48 h at 28°C, chromatograms were judged for appearance of violet spots.

Western blot analysis

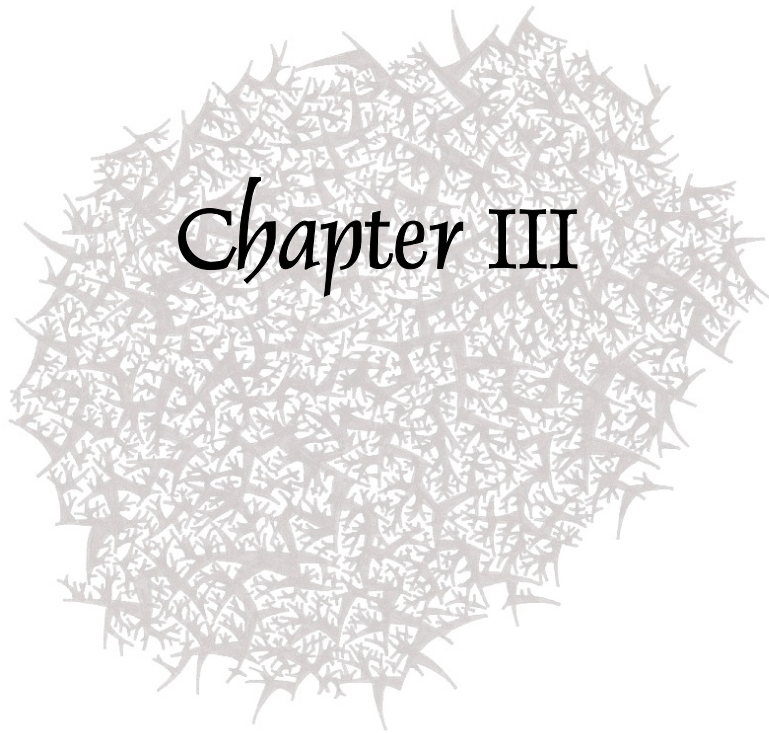
Cells were grown after inoculation of 10 ml MVB1 from an overnight culture diluted to OD₆₂₀ of 0.1. Cells were harvested at OD₆₂₀ 1.0 or 2.2 (cultures are diluted 10-fold before OD₆₂₀ measurement) in volumes of culture corrected for their differences in OD₆₂₀ to obtain similar amounts of cells. Cell pellets were suspended in 200 μ l of cracking buffer (50 mM Tris HCl pH 6.8, 1% SDS, 2 mM EDTA, 10% glycerol, 0.01% bromophenol blue, 1% β -mercaptoethanol) and boiled for 3 minutes. The samples were subsequently loaded on a 10% SDS-PAGE gel and proteins were separated and transferred on a blot following a standard Western blot procedure (Ausubel *et al.*, 1997). A dry aliquot of RpoS antibodies was kindly provided by Prof. K. Tanaka (Tokyo, Japan). The pellet was suspended in 100 μ l PBS and diluted 1000 times for reaction with immobilized protein, as recommended. Peroxidase-labeled goat anti-rabbit antiserum (Amersham Biosciences) was subsequently incubated with the blots. Finally, blots were incubated in luminal solution (250 μ M sodium luminol (Sigma), 0.1 M Tris-HCl, pH 8.6, 0.01% H₂O₂) mixed with 60 μ l enhancer solution (67 μ M p-hydroxy coumaric acid (Sigma) in DMSO). Hybridizing

protein bands were visualized on Super R-X photographic film (Fujifilm) after chemiluminescence detection.

ACKNOWLEDGEMENTS

The authors thank K. Tanaka (Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) for providing the anti-RpoS rabbit serum.

This project was financially supported by the FW6 EU R&D project QRLT-2002-00914 (acronym “Pseudomics”) and by the BioScience Initiative from Leiden University.



Chapter III

Development of a microarray of *Pseudomonas chlororaphis* PCL1391 and transcriptome analyses of its *psrA* and *rpoS* mutants

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

Adapted from Girard *et al.*, *Microbiology* (2006) **152**: 43-58

ABSTRACT

Production of the secondary metabolite phenazine-1-carboxamide (PCN) by *Pseudomonas chlororaphis* PCL1391 is crucial for biocontrol of tomato foot and root rot caused by the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis lycopersici*. Regulation of PCN production involves the two-component signaling system GacS/GacA, the TetR-homologue PsrA, the sigma factor RpoS and the quorum-sensing system PhzI/PhzR. Microarray technology for strain PCL1391 was developed to allow identification of regulons of genes governing PCN synthesis. After spotting of the microarray and testing several protocols for hybridization of cDNA, the microarray was validated by showing that transcriptomes of *psrA* and *rpoS* mutants confirm our model of regulation of PCN synthesis by strain PCL1391 grown in MVB1, our standard medium for studying PCN synthesis. In addition, the microarray survey allowed us to identify new genes of the *psrA*/*rpoS* regulon that might be directly or indirectly involved in secondary metabolism.

INTRODUCTION

Secondary metabolites secreted by bacteria are key elements in various interactions with other organisms in the rhizosphere (Bassler, 1999; Blumer & Haas, 2000; Lugtenberg *et al.*, 2002). In *Pseudomonas chlororaphis* PCL1391, the production of the antifungal metabolite phenazine-1-carboxamide (PCN) (Chin-A-Woeng *et al.*, 2003) is synthesized through expression of the biosynthetic *phzABCDEFHG* operon (Chin-A-Woeng *et al.*, 1998). Previous work led to a model of regulation of PCN production involving four different genes or pairs of genes.

Completely upstream in the regulatory cascade, the GacS/GacA master regulator system is composed of a GacS sensor kinase, responding to an unknown (possibly environmental) factor (Heeb *et al.*, 2002; Zuber *et al.*, 2003), and a GacA response regulator belonging to the Fix J family. A mutation in *gacS* results in a severe decrease of PCN production to undetectable levels and in *N*-acyl-homoserine lactone (*N*-AHL) levels much lower than those in the wild-type (Chapter 2).

GacS/GacA is also required for *psrA* expression in PCL1391 (Chin-A-Woeng *et al.*, 2005). The *psrA* gene is a TetR homologue that was shown to regulate the transcription of the *rpoS* gene in *P. putida* (Kojic & Venturi, 2001) by directly binding to the *rpoS* promoter (Kojic *et al.*, 2002). *rpoS* encodes the stationary phase alternative sigma factor σ^s , which is responsible for the switch in gene expression occurring upon exposure of cells to starvation and/or various stresses (Lange & Hengge-Aronis, 1991). In *P. chlororaphis* PCL1391, *PsrA* was shown to regulate PCN production positively via *RpoS* in our standard synthetic poor medium MVB1 (Chapter 2).

GacS/GacA, *PsrA* and *RpoS* were all shown to act via the *PhzI/PhzR* quorum-sensing system (Chapter 2, p. 58, Fig. 4). *phzI* is responsible for the synthesis of several autoinducers, of which *N*-hexanoyl-L-homoserine lactone (C_6 -HSL) is the main one (Chin-A-Woeng *et al.*, 2001). C_6 -HSL is supposed to bind to *PhzR*, thereby activating it. Subsequently, the *PhzR*- C_6 -HSL complex presumably binds to the *lux* (or *phz*) box upstream of the *phz* biosynthetic operon, which results in initiating the transcription of the *phz* operon. The *PhzR*- C_6 -HSL complex also upregulates *phzI* via a second *lux* box. Interestingly, previous work showed that a constitutively expressed quorum-sensing system is sufficient for synthesis of PCN in all regulatory mutants tested, which indicates that the role of PCN-regulatory genes would ultimately be to modulate the quorum-sensing system.

The genes introduced above are part of a complex network of factors regulating the synthesis of PCN. However, previous work established that more

unknown genes must be involved in these regulatory cascades (Chapter 2). In an attempt to fill in the gaps of our model, microarray technology for transcriptome analysis was developed. This allows quantification of the expression of a genome-wide set of genes in a culture at a precise time point. It also provides a tool to measure the impact of a genetic mutation or an environmental change on the expression of this set of genes.

After the construction of a PCL1391 microarray containing random chromosomal fragments, a protocol was developed for the isolation of RNA and labeling of cDNA before hybridization on the microarray and scanning. The microarray was tested with our *psrA* and *rpoS* mutants by comparing transcriptomics results with the previously established model of regulation of PCN production in PCL1391 (Chapter 2, p. 58, Fig. 4). After validation of the microarray, data were analyzed to identify genes of the PsaA/RpoS regulon.

RESULTS

Set up of the protocol for microarray analysis

RNA extraction

RNA extraction was successful with the protocol described in detail in the Experimental Procedures section. The RNA extraction kit from Qiagen would in theory be sufficient for RNA extraction. However, the amounts of RNA required for the following step of the procedure, cDNA indirect labeling, were very high. The absolute maximum amount of bacteria per column recommended by the manufacturer is 10^{10} , whereas 7 ml of culture at $OD_{620}=2$ corresponding to approximately 5×10^{10} bacteria is required for the labeling step. In order to load a relatively clean fraction on the column, a phenol-chloroform purification step was performed to remove cell membranes and proteins. There are two reasons why phenol-chloroform extracted RNA without column-step purification would not be sufficient. Firstly, the phenol-chloroform extracted RNA still contains a high amount of small sized RNA, probably 5S rRNA (Fig. 1). The column removes this short RNA (Fig. 1), which is important for microarray analysis since labeled cDNA generated from 5S rRNA increases the background on the scanned slide by non-specific binding. Secondly, the RNA purification with the Qiagen kit involves a DNase step which is necessary before cDNA synthesis to prevent false positives.

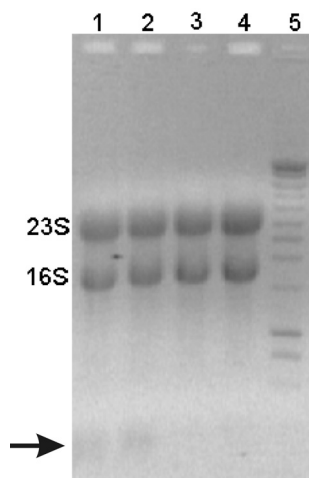


Figure 1. Total RNA extracted from *P. chlororaphis* PCL1391.

The RNA was extracted from PCL1391 cells and separated on 1.2% agarose gel following recommendations of QIAGEN (for details see Experimental Procedures section). For preparations loaded in lanes 1 and 2, the RNA extraction was stopped after the phenol/chloroform extraction. For preparations loaded in lanes 3 and 4, the RNA was purified on QIAGEN columns after the phenol/chloroform extraction. Lanes 2 and 4 correspond to a duplicate (from independent cultures) of lanes 1 and 3, respectively. Lane 5 contains the DNA marker Smart Ladder (Promega). The arrow indicates the migration distance of small sized RNA.

Generation of a labeled probe and microarray analysis

Three procedures were tested for the labeling and production of a suitable probe for microarray analysis (see Experimental Procedures). Only the indirect labeling of cDNA gave positive results. For both the RNA labeling and the cDNA direct labeling, the signal detected on the microarray was very low and only a limited amount of spots were hybridized, showing that these procedures were not suitable for the experiments.

During our various attempts, measuring of the efficiency of labeling proved to be an important step. These measurements required a spectrophotometer with a wavelength range from 230 to 700 nm. The visualization of the absorption spectrum of the Cy-cDNA was important to judge the quality of the labeling process (Fig. 2). In addition, the values obtained at 260, 550 or 650 nm were used to calculate the amount of synthesized cDNA and the efficiency of the labeling step. These measurements were also necessary for hybridizing equal amounts of each dye on the microarray. Experience proved that hybridizing less than 40 pmol of each dye was not sufficient to obtain a proper signal from the microarray.

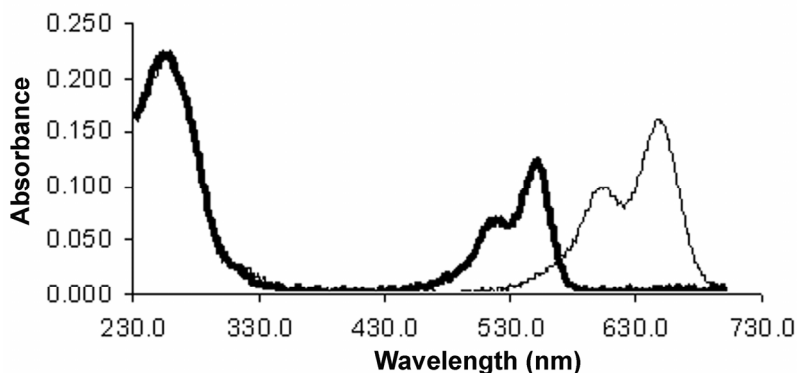


Figure 2. Photospectrometric analysis of dye label incorporation in Cy-cDNA.

After labeling of cDNA with Cy3 or Cy5, samples were analyzed in a photospectrometer and the spectrum determined. The thick line represents the absorption spectrum of Cy3 labeled cDNA. The thin line represents the absorption spectrum of Cy5-labeled cDNA.

The analysis of data after scanning of the microarray was performed using the GenePixPro software. As described in detail in the Experimental Procedures section, a first step was to normalize the data so that the average of the spots shows a ratio (ratio of medians of intensities in the red and the green channels) of 1. Subsequently, spots showing a ratio higher than 2 or lower than 0.5 were carefully selected. These spots correspond to clones containing genes or fragments of genes of which the regulation is affected by the condition tested. To increase the probability that the selection of spots does not include artifacts, the experiments were repeated and the labels were swapped. Selected spots showed for example a ratio higher than 2 with Cy3-cDNA from the mutant hybridized with Cy5-cDNA from the wild-type and a ratio lower than 0.5 with Cy3-cDNA from the wild-type hybridized with Cy5-cDNA from the mutant (Fig. 3).

Transcriptomics in *psrA* and *rpoS* mutants: a preliminary survey

In order to evaluate the pathways regulated by *psrA* and *rpoS*, the gene expression profiles of *psrA* mutant PCL1111 and *rpoS* mutant PCL1954 were compared to the gene expression of wild type strain PCL1391 on microarrays. As an example for the reader and in order to comply to the MIAME standards (Brazma *et al.*, 2001), one representative experiment among four, for both sets of microarrays, was selected for each mutant and the corresponding data, i.e. images, raw output of image analysis, normalized and flagged data are available on a website for supplementary material ([http://rulbim.leidenuniv.nl/girard/suppl material.htm](http://rulbim.leidenuniv.nl/girard/suppl%20material.htm)).

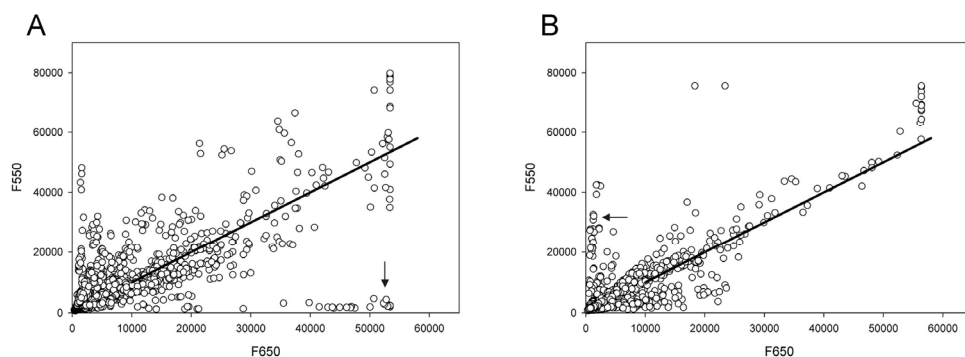


Figure 3. Plot of the intensities of spots of two microarrays.

Each spot of the microarrays is plotted for its intensity in the green channel (F550, absorption measured at 550 nm) and in the red channel (F650, absorption measured at 650 nm). On both graphs the normalization line ($F650=F550$) is indicated.

Panel A: cDNA from wild-type strain PCL1391 was labeled with Cy3 (green) and cDNA from *psrA* mutant strain PCL1111 was labeled with Cy5 (red). The spots (duplicate) corresponding to the clone 116_B10 are shown as an example by an arrow ($F650=53385$ or 53384 and $F550=2222$ or 2324 , respectively).

Panel B: cDNA from wild-type strain PCL1391 was labeled with Cy5 (red) and cDNA from *psrA* mutant strain PCL1111 was labeled in with Cy3 (green). The spots (duplo) corresponding to the clone 116_B10 are shown as an example by an arrow ($F650=1250$ or 1255 and $F550=32618$ or 30853 , respectively).

The data filter (see Experimental Procedures section for the details about the filtering of the data) selected a total of 190 spots for the experiments with the *psrA* mutant, of which 157 had a stronger intensity in the wild-type than in the *psrA* mutant, and 33 a lower intensity. Two hundred thirty-four spots were selected from the experiments involving the *rpoS* mutant, of which 211 had a stronger intensity in the wild-type, and 23 a lower intensity. A total of 108 spots were common to the group of 190 spots from *psrA* arrays and the group of 234 spots from *rpoS* arrays. They were all more intense in the wild-type than in the *psrA* and *rpoS* mutants. Among these 108 spots, the 57 spots that were most strongly affected by both mutations were selected and the corresponding DNA was sequenced. The sequences of the clones are also available on the website of supplementary material. The analysis of sequences and the variations of expression due to *rpoS* and *psrA* mutations are presented in Table 1. The clones were grouped according to the predicted function of the ORF in the insert.

The microarray data reveal that the expression of *phz* biosynthetic genes is decreased at least 7-fold in *rpoS* mutant PCL1954 and around 15-fold in *psrA* mutant PCL1111 (clones 4_D1, 119_D12 and 126_G12 in Table 1, and controls shown in the supplementary material). In addition, some clones sequenced after microarray analysis containing parts of the *phzI/phzR* genes (24_C5 and 93_G11) have a decreased expression of approximately 5-fold in the *psrA* mutant and of

approximately 7-fold in the *rpoS* mutant. Some spots showing very high ratios on the *psrA* microarray were also sequenced (see Supplementary materials).

Table 1. Genes of which the mRNA levels were affected by *rpoS* and *psrA* as shown by microarray analyses

Clone number *	Change of expression in PCL1111 (<i>psrA</i>) §	Change of expression in PCL1954 (<i>rpoS</i>) §	Gene homology and/or accession number #	Bacterium corresponding to the gene homology ‡	Predicted function #†
<i>phz</i> genes					
2_A5	20±6	13±3	<i>phzR</i> AAF17494	<i>Pseudomonas chlororaphis</i>	Transcriptional activator
93_G11	6±2	8±2	<i>phzI</i> AAF17493	<i>Pseudomonas chlororaphis</i>	Autoinducer synthase
24_C5	3±0.7	6±0.8	<i>phzI</i> AAF17493 and <i>phzR</i> AAF17494	<i>Pseudomonas chlororaphis</i>	Autoinducer synthase and transcriptional activator
4_D1	21±8	12±0.8	<i>phzB/C</i> AAF17496 and AAF17497	<i>Pseudomonas chlororaphis</i>	Biosynthetic genes for PCN
97_D1	29±5	18±3	<i>phzD</i> AAF17498	<i>Pseudomonas chlororaphis</i>	Biosynthetic gene for PCN
119_D12	10±3	7±1	<i>phzE</i> AAF17499	<i>Pseudomonas chlororaphis</i>	Biosynthetic gene for PCN
126_G12	14±4	9±2	<i>phzH</i> AAF17502	<i>Pseudomonas chlororaphis</i>	Biosynthetic gene for PCN
Membrane protein genes					
2_C4	3.6±0.4	10±3	ZP_002628 06	<i>Pseudomonas fluorescens</i>	Autotransporter adhesin
13_B1	3.8±0.5	9±2	ZP_002628 06	<i>Pseudomonas fluorescens</i>	Autotransporter adhesin
36_A12	3±0.4	7±2	ZP_002628 06	<i>Pseudomonas fluorescens</i>	Autotransporter adhesin
38_G7	3.3±0.3	8±2	ZP_002628 06	<i>Pseudomonas fluorescens</i>	Autotransporter adhesin
105_A12	3.5±0.2	8±2	ZP_002628 06	<i>Pseudomonas fluorescens</i>	Autotransporter adhesin
115_A2	3±0.4	5±1	ZP_002631 90 and NP_745085	<i>Pseudomonas fluorescens</i> and <i>Pseudomonas putida</i>	Integral membrane protein (1-59/223) and conserved hypothetical protein (169-253/261)
4_H7	4.7±0.7	2±0.3	<i>nlpD</i> AAP97085	<i>Pseudomonas chlororaphis</i>	Lipoprotein (86-265/294)
Primary metabolism genes					
4_C1	4±1	4±2	NP_762166	<i>Vibrio</i>	Deoxycytidyla

				<i>vulnificus</i>	te deaminase (139-514/622)
4_G11	4±2	7±4	NP_901074	<i>Chromobacterium violaceum</i>	Aminotransferase (4-268/367)
36_F1	0.034±0.01	1.1±0.1	ZP_002623 98.1	<i>Pseudomonas fluorescens</i>	Acyl-CoA dehydrogenase
38_B12	0.016±0.01	1±0.07	ZP_002623 98.1	<i>Pseudomonas fluorescens</i>	Acyl-CoA dehydrogenase
116_B10	0.025±0.01	1±0.08	ZP_002623 98.1	<i>Pseudomonas fluorescens</i>	Acyl-CoA dehydrogenase
Intermediate metabolism genes					
2_B3	3±0.7	7±1	<i>phaC2</i> BAB78721	<i>Pseudomonas chlororaphis</i>	PHA-synthase 2 (225-560/560)
41_G2	3±0.7	7±2	<i>phaC2</i> BAB78721	<i>Pseudomonas chlororaphis</i>	PHA-synthase 2 (1-376/560)
53_F2	3±1	5±0.8	<i>phaG</i> BAB32432	<i>Pseudomonas</i> sp 61-3	3-hydroxyacyl-acyl carrier protein CoA transferase (131-294/294)
60_E1	3±0.9	5±0.9	Clone identical to 53_F2	<i>Pseudomonas</i> sp 61-3	Clone identical to 53_F2
71_A4	2±0.3	4±0.6	Clone identical to 53_F2	<i>Pseudomonas</i> sp 61-3	Clone identical to 53_F2
74_B4	3±0.6	5±1	Clone identical to 53_F2	<i>Pseudomonas</i> sp 61-3	Clone identical to 53_F2
74_E7	3±0.5	5±2	Clone identical to 53_F2	<i>Pseudomonas</i> sp 61-3	Clone identical to 53_F2
93_D8	2.5±0.5	6±2	<i>phaG</i> BAB32432	<i>Pseudomonas</i> sp 61-3	3-hydroxyacyl-acyl carrier protein CoA transferase (124-294/294)
Secondary metabolism genes					
11_G8	4±0.5	6±1	<i>chic</i> NP_250990	<i>Pseudomonas aeruginosa</i>	Chitinase (293-373/483)

121_H3	3±0.3	6±0.8	<i>chiC</i> NP_250990	<i>Pseudomonas aeruginosa</i>	Chitinase (165-479/483)
100_B7	3±0.4	7±0.6	NP_746359	<i>Pseudomonas putida</i>	Pyoverdine synthase? (665-987/4317)
86_H8	6±4	10±0.8	NP_901071 and NP_901070	<i>Chromobacterium violaceum</i>	Probable dihydrorhizobitoxine desaturase (248-353/369) and probable 5'-methylthioadenosine phosphorylase (31-186/302)
Regulatory genes					
65_B7	5±0.7	3±0.3	<i>rpoS</i> AAP97086 and <i>nlpD</i> AAP97085	<i>Pseudomonas chlororaphis</i>	RNA polymerase sigma factor (1-155/334) and lipoprotein (96-294/294)
98_B2	6±0.5	3±0.4	<i>rpoS</i> (AAP97086)	<i>Pseudomonas chlororaphis</i>	RNA polymerase sigma factor (40-334/334)
42_G8	4±1	7±2	ZP_002640 29	<i>Pseudomonas fluorescens</i>	GGDEF/EAL domains containing regulator (260-617/624)
76_G2	3±0.9	4±1	ZP_002638 82 and ZP_002638 83	<i>Pseudomonas fluorescens</i>	Hypothetical protein (378-454/454) and ATP-dependent transcriptional regulator (1-236/911)
Hypothetical protein genes					
72_D12	7±2	40±12	ZP_002628 03, ZP_002628 02 and ZP_002627 96	<i>Pseudomonas fluorescens</i>	Microcystin-dependent protein (1-191/191), hypothetical protein (1-

119_A8	7±1	32±5	ZP_00262803 and ZP_00262802	<i>Pseudomonas fluorescens</i>	103/103) and histone acetyltransferase (71-161/163) Microcystin-dependent protein (1-191/191) and hypothetical protein (1-92/103)
1_B9	3±0.6	5±1	ZP_00266535	<i>Pseudomonas fluorescens</i>	Hypothetical protein (58-170/170)
3_F11	3±0.9	4±0.4	ZP_00128106	<i>Pseudomonas syringae</i> pv. <i>syringae</i>	Hypothetical protein (31-170/170)
11_E2	3±0.5	5±0.5	ZP_00263881.1 and ZP_00263880	<i>Pseudomonas fluorescens</i>	Hypothetical protein (1-184/629) and Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II (528-551/560)
21_E2	3±0.6	4±0.3	Clone identical to 11_E2	<i>Pseudomonas fluorescens</i>	Clone identical to 11_E2
12_G9	4±0.8	6±2	PP2941 NP_745085	<i>Pseudomonas putida</i>	Conserved hypothetical protein (187-253/261)
41_A12	6±2	12±2	ZP_00262372 and ZP_00262371	<i>Pseudomonas fluorescens</i>	Uncharacterized conserved protein (1-153/423) and putative Ser protein kinase (525-640/640)
47_F5	4±0.7	8±3	ZP_00263881 and ZP_00263882	<i>Pseudomonas fluorescens</i>	Hypothetical protein (524-629/629) and hypothetical protein (1-335/454)
72_H6	5±1	7±2	Clone	<i>Pseudomonas</i>	Clone

93_H9	4±0.4	5±0.6	identical to 47_F5 Clone identical to 47_F5	<i>fluorescens</i> <i>Pseudomonas fluorescens</i>	identical to 47_F5 Clone identical to 47_F5
112_C1	4±0.4	6±0.5	Clone identical to 47_F5	<i>Pseudomonas fluorescens</i>	Clone identical to 47_F5
59_C10	4±0.5	4±0.6	NP_929618	<i>Phototrhobdus luminescens</i> subsp. <i>laumondii</i>	Hypothetical protein (2-93/93)
65_H9	4±1	8±2	ZP_002673 18 and ZP_002669 17	<i>Pseudomonas fluorescens</i>	RTX toxin and related Ca ²⁺ binding protein (428-468/468) and hypothetical protein (34-248/300)
74_H8	7±1	25±4	NP_929844	<i>Phototrhobdus luminescens</i> subsp. <i>laumondii</i>	Hypothetical protein (12-281/325)
81_G2	4±1	7±2	ZP_001281 06	<i>Pseudomonas syringae</i> pv. <i>syringae</i>	Hypothetical protein (58-170/170)
101_H9	4±0.4	4±1	NP_929618	<i>Phototrhobdus luminescens</i> subsp. <i>laumondii</i>	Hypothetical protein (2-70/93)
114_H5	3±0.4	5±0.7	ZP_002638 21	<i>Pseudomonas fluorescens</i>	Hypothetical protein (19-163/390)
119_C6	3±0.4	4±1			No homology

* The clone number refers to the number in the library (plate number, row and column).

§ All the spots selected in this table correspond to genes of which the expression was lower in the mutant than in the wild-type, except for the spots corresponding to the acyl-CoA dehydrogenase gene. Thus the ratios represent the intensity of the spots in the wild-type over the intensity in the mutant.

‡ The precise strains are: *P. chlororaphis* strain 06 except for the *phz* genes which are from strain PCL1391 and *phaC2* which is homologous to *phaC2* of strain IFO 3521, *P. fluorescens* Pf01, *P. putida* KT2440, *P. aeruginosa* PAO1, *P. syringae* pv. *syringae* B728a, *V. vulnificus* CMCP6, *C. violaceum* ATCC 12472 and *Phototrhobdus luminescens* subsp. *laumondii* TTO1.

Because the microarray was spotted from a random genomic library, some clones appeared to be spotted several times. In this case, it is indicated in the last columns ("clone identical to").

† In brackets, the region of the protein encoded on the insert of the clone is indicated (first aminoacid-last aminoacid/total aminoacid length).

Sequencing of 19 selected clones and homology studies identified the presence of hypothetical proteins genes encoded in the clones. On several clones, two or three ORFs could be identified since the microarrays were constructed from a library of random PCL1391 chromosomal fragments of approximately 1 to 2 kb in

size. Additional RT-PCR experiments should be performed to show which gene or operon is responsible for the ratio measured. For most genes (in clones 76_G2, 47_F5, 86_H8, 11_E2 for example), it was observed that they correspond to homologues that are also adjacent to each other in other sequenced *Pseudomonas* genomes. Several genes were sequenced that give homology to genes which cannot be linked obviously to *rpoS* and *psrA* functions, like an aminotransferase (clone 4_G11), a deoxycytidylate deaminase (clone 4_C1) and a putative adhesin (Pflu3629) which is recurrent in the clones. However, many clones (12) show homology to genes that could be related to intermediate and secondary metabolism (see Table 1). Other interesting clones (4) show homology to regulators.

In order to test several of the genes that were selected by microarray analyses, three mutants were constructed. (i) PCL2009 is mutated in a putative transcriptional regulator gene identified in microarray clone 76_G2. (ii) PCL2050 is mutated in a putative GGDEF/EAL regulator identified in microarray clone 42_G8. (iii) PCL2052 is mutated in a hypothetical protein identified in microarray clones 76_G2 and 47_F5. Various phenotypic traits of these mutants were analyzed (see Experimental Procedures). The mutants showed wild-type production of HCN, chitinase and exoprotease. They were all able to swim and swarm although PCL2052 showed a decreased swimming ability and PCL2050 seemed to be affected in its swarming (not shown). The PCN production of PCL2009 ($465 \pm 28 \mu\text{M}$) and PCL2052 ($435 \pm 14 \mu\text{M}$) appeared to be 2-fold increased compared to PCL1391 ($237 \pm 9 \mu\text{M}$).

We also selected three clones that showed strikingly high ratios on the *psrA* microarray but not on the *rpoS* microarray (36_F1, 38_B12 and 116_B10). In contrast to the other sequenced clones, these show a higher intensity in the *psrA* mutant than in the wild-type. Remarkably, all these clones contained a chromosomal insert showing homology with an acyl-CoA dehydrogenase. Two derivatives of PCL1391 mutated in this acyl-CoA dehydrogenase were constructed and named PCL1981 and PCL1982. In 50 ml MVB1 overnight culture, PCL1981 produced $81.1 \pm 4.1 \mu\text{M}$ PCN whereas PCL1391 produced $51.8 \pm 2.5 \mu\text{M}$. Synthesis of C₆-HSL of both strains was visualized by TLC analysis (Fig. 4).

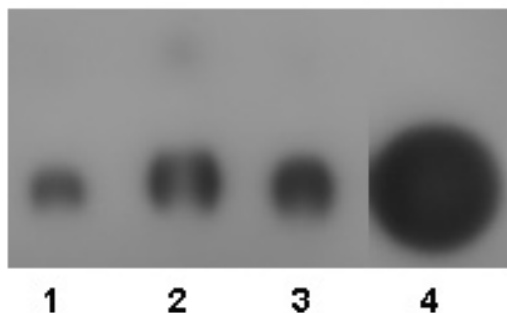


Figure 4. C18-reverse phase TLC analysis of *N*-AHLs produced by *P. chlororaphis* PCL1391 derivatives. Lane 1: PCL1391. Lanes : PCL1981. Lane 3: PCL1982. Lane 4: 2.5 nmol synthetic C₆-HSL

DISCUSSION

Microarray analysis

In case of eukaryotic total RNA it is possible to generate a labeled probe for microarray analysis from only mRNA among total RNA by selecting the polyA tailed RNA. In general, probe generation for prokaryotic RNA is more difficult than for eukaryotic RNA because of the absence of this polyA in the prokaryotic mRNA. An important part of label is therefore lost in the generation of probe for the ribosomal RNA, which represents up to 90% of the total RNA. Of the three methods tested for probe generation, only the cDNA indirect labeling gave positive results. The failure of the RNA labeling could be explained by the instability of the RNA, particularly in this case since prokaryotic mRNA is less stable. In addition, the protocol takes a relatively long time, due to the enrichment step. This increases the probability for the RNA to degrade. Finally, it is possible that the amounts of RNA used for labeling were not sufficient. The reason why the cDNA direct labeling did not succeed is not very clear. It is likely that cDNA was not synthesized in sufficient amounts, since only 1 µg of total RNA was used as a template instead of the 30 µg used for the indirect labeling. It was the experience of other laboratories that the indirect labeling is more efficient than the direct labeling (N. Kramer, *personal communication*). According to the manufacturer itself, indirect labeling generates higher yields. Another advantage of this method is that the labeling is more even between the two dyes as compared with other methods. Concerning the RNA labeling and the cDNA direct labeling, low signal on the microarray was probably also due to numerous and long washes after hybridization, particularly in ethanol. It was shown that incubation of the hybridized slide in ethanol degrades the signal

and it is recommended to dry the slides by centrifugation after washes in SSC buffer (recommendation of Genomic Solutions).

With the “home-made” microarray of strain PCL1391, the main disadvantage is that it is derived from a random chromosomal bank of the organism, consisting of fragments between 0.4 and 2 kb. This is due to the fact that the genome of strain PCL1391 is not sequenced. Therefore a classical microarray spotted with probes derived from the genome is not possible yet. This causes several problems. Firstly, it is likely that the whole genome is not covered. Secondly, the scanning of the slides does not give any direct results, since sequencing of the clones corresponding to the selected spots is required to identify the differentially expressed genes. Thirdly, a spot can represent several genes, of which only one is differentially expressed. Finally, a lot of the selected spots are present in duplicate or even in higher numbers. The latter is also an advantage since it provides an internal control in every microarray. In our selection of spots (Table 1), several clones were found to be spotted several times and several clones, although not identical, contained portions of the same genes. This validates our procedure.

In our previous study using a synthetic MVB1 medium, *psrA* and *rpoS* were shown to activate PCN and *N*-AHL production in PCL1391 (Chapter 2). The microarray data from cells grown in MVB1 medium confirmed that the expression of the *phz* genes is strongly reduced by the *psrA* and *rpoS* mutations (Table 1). This is important for two reasons: it supports our model (Chapter 2), which was different from the one previously published in which experiments were performed in rich medium (Chin-A-Woeng *et al.*, 2005); and it also validates our microarray procedure. It was to be expected that microarray analyses of the *psrA* and *rpoS* mutants, which are altered in PCN production, would result in the selection of clones containing genes of the *phz* operon.

Transcriptome analyses of *psrA* and *rpoS* mutants of *P. chlororaphis* PCL1391

Functional genomics provides a high throughput analysis possibility to identify the genes of the cascade downstream of *rpoS*. However, the expected large amount of genes due to very downstream effects of *rpoS* would hamper the selection of genes of interest. *rpoS* and *psrA* are predicted to be close to each other in the regulatory cascade for PCN synthesis. Therefore the data of microarray analyses of *psrA* and *rpoS* were crossed. This approach increases the probability to select genes that are part of the *psrA/rpoS* regulatory cascade.

Our selection method revealed 13 clones containing parts of genes from the *phz* operon and *phz* quorum sensing system (not all of them are shown in the Table 1 for conciseness), which strongly validates our method. Besides, many of the genes sequenced from the positive clones were also present on other selected clones spotted elsewhere on the microarray (like *phaG* in clones 53_F2, 60_E1, 71_A4, 74_B4, 74_E7 or *chiC* in clones 11_G8 and 121_H3). These observations contribute to the validation of our microarray analyses.

Many clones carry genes that show homology to genes related to intermediate and secondary metabolism, such as *phaC2*, *phaG*, *chiC*, pyoverdine synthase and a probable dihydrorhizobitoxine desaturase (Table 1). *phaC2* was reported to be involved in polyhydroxyalkanoic acid (PHA) synthesis (Nishikawa *et al.*, 2002; Qi *et al.*, 1997). *phaG* is also involved in PHA synthesis (Rehm *et al.*, 1998). PHAs are polymers used for carbon and energy storage in bacteria in response to environmental stress, which would explain their regulation by *rpoS*. *chiC*, encoding a chitinase, was shown to be regulated by quorum-sensing in *P. aeruginosa* PAO1 (Foldes *et al.*, 2001).

One clone (76_G2) contains a putative regulatory gene with a HTH-LuxR domain (SMART accession SM00421) and therefore might respond to *N*-AHLs. A mutation in this regulator, as well as in the hypothetical protein upstream of it, resulted in a two-fold increase in PCN production. The function of these genes is to our knowledge not yet characterized in other strains. Our data show that these genes are affecting PCN production in strain PCL1391. The third gene of interest located on clone 42_G8 contains GGDEF and EAL domains, which are found in two-component signaling systems (Galperin *et al.*, 2001). A recent study shows the involvement of such a protein (RocS) in regulation of the rugose phenotype and biofilm formation in *Vibrio cholerae* (Rashid *et al.*, 2003). A mutation in this putative regulatory gene did not change PCN production.

Remarkably, we also isolated three clones that all contain an acyl-CoA dehydrogenase gene homologous to the ORF PA0506 of *P. aeruginosa* PAO1. According to the results (Table 1), the expression of this gene is highly repressed by *psrA*. However, it is not at all under the regulation of *rpoS*. Interestingly, a strain mutated in this gene showed a relative increase in PCN synthesis and in *N*-AHL production (Fig. 4). This gene was also shown to be under negative regulation of *psrA* in *P. aeruginosa* (Kojic *et al.*, 2005). This work by Kojic *et al.* (2005) used proteomics analysis, which is nicely complementary to our work. In their work it was shown that *rpoS* does not regulate PA0506. Acyl-CoA dehydrogenases are

involved in the β -oxidation of fatty acids. It is also possible that, together with a protein like HdtS, this acyl-CoA dehydrogenase is involved in adaptation to diverse environments (Cullinane *et al.*, 2005).

Our previous results showed that a cascade involving GacS/GacA, PsrA, RpoS and quorum-sensing regulates the *phz* operon and that several regulators downstream of GacS/GacA must exist in addition to PsrA/RpoS to activate expression of the *phz* operon. Preliminary microarray analyses, by allowing measurement of the effect of *psrA* and *rpoS* mutations on the *phz* genes, support our model of the regulation of PCN production. In addition, these data led to the identification of novel genes involved in regulatory fine-tuning of PCN production. The microarray analyses form a solid basis for future studies on identifying the role of other novel genes and their relation to *psrA*, *rpoS* and secondary metabolism, particularly PCN production.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Pseudomonas* strains were grown at 28°C in liquid MVB1 (van Rij *et al.*, 2004) and shaken at 195 rpm on a Janke und Kunkel shaker KS501D (IKA Labortechnik, Staufen, Germany). *E. coli* strains were grown at 37°C in Luria-Bertani medium (Sambrook & Russel, 2001) under vigorous aeration. Media were solidified with 1.8% Bacto agar (Difco, Detroit, MI, USA). When appropriate, growth media were supplemented with kanamycin (50 $\mu\text{g/ml}$), carbenicillin (200 $\mu\text{g/ml}$), gentamicin (10 $\mu\text{g/ml}$ for *E. coli* and 30 $\mu\text{g/ml}$ for *P. chlororaphis*), or 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (40 $\mu\text{l/ml}$). To follow growth, the absorbance of liquid cultures was measured at 620 nm.

Construction of vectors and PCL1391 mutant strains

Polymerase chain reactions (PCRs) were carried out in general with Super Taq enzyme (Enzyme Technologies Ltd, Cambridge, UK). 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 plasmids and primers used in this study are listed in Tables 2 and 3, respectively.

Table 2. Bacterial strains and plasmids used

Bacterial strains and plasmids	Description	Reference or source
<i>Pseudomonas chlororaphis</i>		
PCL1391	Wild-type <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide and biocontrol strain of tomato foot and root rot caused by <i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	(Chin-A-Woeng <i>et al.</i> , 1998)
PCL1111	Derivative of PCL1391 in which a promoterless Tn5luxAB is inserted in <i>psrA</i> ; Km ^r	(Chin-A-Woeng <i>et al.</i> , 2005)
PCL1123	Derivative of PCL1391 in which a promoterless Tn5luxAB is inserted in <i>gacS</i> ; Km ^r	(Chin-A-Woeng <i>et al.</i> , 2005)
PCL1954	Derivative of PCL1391, <i>rpoS</i> ::pMP7418; Km ^r	Chapter 2
PCL1981	Derivative of PCL1391 mutated in a putative acyl-CoA dehydrogenase; Km ^r	This study
PCL2009	Derivative of PCL1391 mutated in a putative transcriptional regulator by recombination of pMP7452; Km ^r	This study
PCL2050	Derivative of PCL1391 mutated in a putative GGDEF/EAL regulator by recombination of pMP7467; Km ^r	This study
PCL2052	Derivative of PCL1391 mutated in a hypothetical protein by recombination of pMP7470; Km ^r	This study
<i>Escherichia coli</i>		
DH5α	<i>Escherichia coli</i> ; <i>supE44</i> Δ <i>lacU</i> 169(Φ80 <i>lacZ</i> Δ <i>M</i> 15) <i>hsdR17 recA1 endA1 gyrA96 thi1 relA1</i>	(Hanahan, 1983)
Plasmids		
pRK2013	Helper plasmid for tri-parental mating; Km ^r	(Ditta <i>et al.</i> , 1980)
pGEM-T easy	Plasmid designed for direct ligation of PCR fragments	Promega
pMP5285	Suicide plasmid for <i>Pseudomonas</i> spp. Used for homologous recombination; Km ^r	(Kuiper <i>et al.</i> , 2001)
pMP7426	pGEM-T easy containing a 0.5-kb PCR product of an internal part of a putative acyl-CoA dehydrogenase sequenced in clones 38_B12 and 36_F1; Cb ^r	This study
pMP7428	pMP5285 containing the insert from pMP7426; Km ^r	This study
pMP7452	pMP5285 containing a 0.4-kb PCR product of an internal part of a putative transcriptional regulator gene sequenced in microarray clone 76_G2; Km ^r	This study
pMP7467	pMP5285 containing a 0.4-kb PCR product of an internal part of a putative GGDEF/EAL regulator gene sequenced in microarray clone 42_G8; Km ^r	This study
pMP7470	pMP5285 containing a 0.5-kb PCR product of an internal part of a hypothetical protein gene sequenced in microarray clones 76_G2 and 47_F5; Km ^r	This study

Four mutants were constructed in genes selected by microarray analyses. The selected genes include a putative transcriptional regulator gene partially found in microarray clone 76_G2, a putative GGDEF/EAL regulator gene partially found in microarray clone 42_G8, a hypothetical protein gene partially found in microarray clones 76_G2 and 47_F5 and an acyl-CoA dehydrogenase gene found in clones 38_B12 and 36_F1. Primers oMP810 and oMP811, oMP972 and oMP973, oMP977 and oMP978, oMP1041 and oMP1042 (or oMP1043) were used with clone 76_G2, clone 42_G8 and chromosomal DNA as a template, respectively, to produce an internal fragment of 0.4 kb for the putative transcriptional regulator gene, 0.4kb for the putative GGDEF/EAL regulator gene, 0.5 kb for the hypothetical protein gene and 0.45 kb (and 0.6 kb) for the acyl-CoA dehydrogenase gene, respectively. The obtained PCR products were cloned in the *EcoRI* site of pMP5285, resulting into pMP7452, pMP7467, pMP7470 and pMP7428 (and pMP7429), respectively. These vectors were introduced into PCL1391 to obtain PCL2009, PCL2050, PCL2052 and PCL1981 (and PCL1982), respectively. The mutations were verified by PCR and/or sequencing. Two independent mutants were made with different suicide vectors for the acyl-coA dehydrogenase gene (PCL1981 and PCL1982).

Table 3. Oligonucleotides used

Name	Nucleotide sequence
oMP810	5'-CAAGAGTTCGCTGGCGGTGG3'
oMP811	5'-GATTCGTCGTAGGTCAGGCG3'
oMP972	5'-ATATATGAATTCCTCGGTATTCGCTACGGTTCGG3'
oMP973	5'-ATATATGAATTCCCCAGCCATGGCCGGGCCGG3'
oMP977	5'-ATATATGAATTCGGGAAACTACAAGATGCCGG3'
oMP978	5'-ATATATGAATTCTCGAGGGTTTCGTGCACCAG3'
oMP1041	5'-CGCCTGCCGGATGCGCCGG3'
oMP1042	5'-CCTGCTTGGCGGTGAAGTAGACC3'
oMP1043	5'-GCCCCACTCGGCGATGAAGCC3'

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

Chromosomal DNA from PCL1391 was digested with *Sau3AI* (New England Bio Labs, Westburg). Fragments smaller than 2 kb and larger than 0.4 kb were purified out of gel using a PCR purification kit (Qiagen, Westburg) and cloned into the *XhoI* site of pIC20H after partial filling with Klenow DNA polymerase (New England BioLabs Inc.). The resulting plasmids were transformed into *E. coli* DH5a.

The bacteria were subsequently plated on X-gal LC agar and single white colonies were separated in 96-well plates. PCR was conducted on the PCL1391 bank in 96-well plates with the primers oMP779 and oMP780 in a total volume of 100 μ l per well. The resulting PCR fragments were precipitated by adding 10 μ l of 3 M NaAc and 110 μ l isopropanol, cooled at -80°C for one hour and centrifuged at 3,250 rpm for 30 min at 4°C in a Multifuge 3 S-R (Heraeus, Dijkstra Vereenigde B.V., Lelystad, The Netherlands). After random verification on gel of the presence of fragments for 4 PCR samples per 96-well plate, the PCR fragments were washed with 70% ethanol, air-dried, redissolved in 50% DMSO and transferred into 384-well plates. In total 128 96-well plates with PCR fragments were pooled into 32 384-well plates. The resulting 12,000 clones correspond to a theoretical 3-fold coverage of the genome of PCL1391, which is estimated between 6 and 6.5 Mb. Finally, poly-L-lysine coated glass slides were spotted with a Genemachines Omnigrid 100 spotter (Genomic Solutions, Isogen Life Science, Maarssen, The Netherlands). The library was divided in two parts and each part was spotted in duplicate on one set of slides. So for each experiment, two slides were used.

The microarrays contained the following controls: some empty spots (neither DNA nor buffer), spots with only 50% DMSO, a negative control with λ phage DNA (Westburg), and several PCRs products of known genes of PCL1391: *psrA* (0.6 kb fragment with the primers oMP783 and oMP784), *phzR* (0.45 kb fragment), *phzI* (0.5 kb fragment with the primers oMP604 and oMP605), *sss* (0.45 kb fragment with the primers oMP652 and oMP653), *gacS* (1.6 kb fragment with the primers oMP582 and oMP583), *phzH* (0.7 kb fragment with the primers oMP500 and oMP501) and *phzB* (0.5 kb fragment with the primers oMP689 and oMP690). These latter DNA fragments were made by PCR on the chromosomal DNA of PCL1391 with primers specific for the known genes.

Isolation of RNA

A volume of 12ml of MVB1 medium was inoculated in 50 ml flasks to an OD₆₂₀ 0.1 from overnight cultures of *P. chlororaphis* PCL1391 or derivatives. The fresh cultures were shaken at 28°C at a speed of 195 rpm on a Janke und Kunkel shaker KS501D (IKA Labortechnik) until the optical density reached a value of 2.0. This absorbance corresponds to the beginning PCN synthesis (Chapter 2), and was chosen on the presumption that the genes regulating the *phz* operon are then probably highly expressed. Volumes of 7 ml of the cultures were mixed with 3 ml

PBS and 1.25 ml 5% phenol-ethanol. After a short centrifugation step, the cells were resuspended in 800 μ l of lysozyme (1 mg/ml) in TE buffer, subsequently 40 μ l of 20% SDS was added and the cells were allowed to lyse for 2 minutes at 65°C. A volume of 30 μ l of 3M NaAc, pH 5.4, was subsequently added and the lysate was mixed in 1 ml of acidic phenol at 65°C (adapted from the protocol developed by Jon Bernstein, URL http://bugarrays.stanford.edu/protocols/rna/Total_RNA_from_Ecoli.pdf). After phenol/chloroform extraction, the water phase was applied on columns from the RNeasy Midi kit (Qiagen), and the RNA was extracted following the protocol supplied by the manufacturer, including the DNase step. RNA purity was verified on 1.2% agarose gel following the protocol of the RNeasy Midi kit (Qiagen).

Probe labeling and microarray processing

Three methods were tested for probe labeling with Cy3 and Cy5 fluorescent dyes. They are illustrated in Figure 5.

RNA labeling

mRNA enrichment was performed following the procedure of Affymetrix GeneChip Expression Analysis Manual (Affymetrix, High Wycombe, UK). Briefly, this procedure started with the synthesis of cDNA of 16S and 23S rRNAs. The rRNA was subsequently digested by RNaseH (Roche, Mannheim, Germany) and the cDNA by DNaseI. After enrichment, the enriched RNA was purified on QIAGEN RNeasy mini columns following instructions of the manufacturer. Finally the RNA was labeled using the Ulysis Cy3 and Cy5 nucleic acid labeling kit (Kreatech Diagnostics, Amsterdam, The Netherlands). Briefly, this labeling kit uses a platinum compound with two free binding sites, one of which is bound to the marker group and the other one is used to link the Pt-Cy3 or Pt-Cy5 complex to the purines of RNA. The labeled RNA was subsequently purified using the RNeasy mini kit (Qiagen). The labeled RNA was dissolved in 50 μ l H₂O.

The microarrays were prehybridized with 60 μ l of prehybridization buffer (60% formamide, 5X Denhardt's and 50 μ g/ml herring sperm DNA). The prehybridization started with a step of 2 minutes at 80°C and a step of 30 minutes at 37°C. The slides were subsequently washed in 2X SSC [sodium chloride/sodium citrate buffer, 3M NaCl - 0.3M Na₃-citrate], then in 70% ethanol and 90% ethanol, each time for 2 minutes. Finally, the slides were washed for 5 minutes in 96% ethanol and vertically air-dried.

A volume of 8.25 μl of the labeled RNA was mixed with 41.25 μl of hybridization buffer (60% formamide, 35 mM Na_2HPO_4 , 35 mM NaH_2PO_4 , 3 mM EDTA, 2X SSC and 50 $\mu\text{g}/\text{ml}$ herring sperm DNA; all final concentrations). The slides were incubated for 16 hours at 37°C. After hybridization, the slides were washed 3 times for 10 min in 2X SSC at 37°C, once 10 min in 2X SSC at 50°C, once 2 min in 70% ethanol, once 2 min in 90% ethanol and once 5 min in 96% ethanol. Finally, the slides were vertically air-dried and scanned on a GMS418 scanner (Genetic Microsystems, Woburn, Massachusetts, USA).

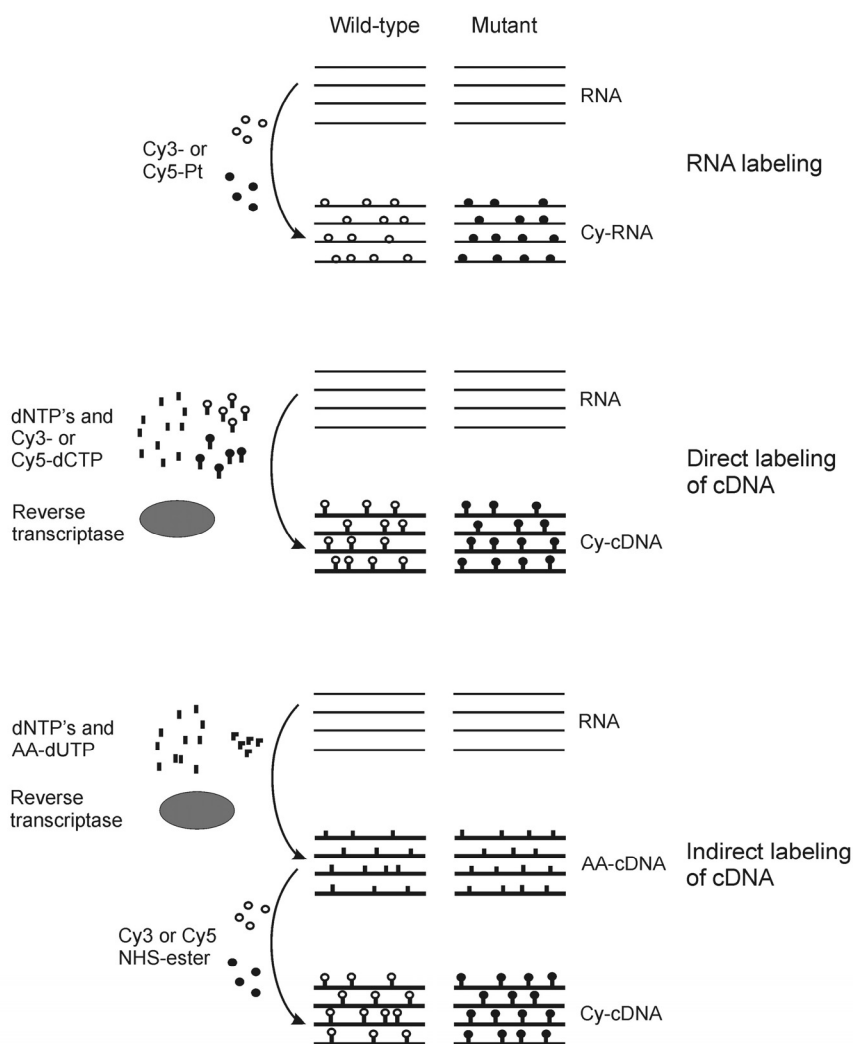


Figure 5. Three methods tested for probe labeling with Cy3 or Cy5 dyes before hybridization on the microarray. For details see Experimental Procedures.

cDNA direct labeling

RNA was used for cDNA probe generation using the CyScribe First Strand cDNA labeling kit (Amersham Biosciences, Roosendaal, The Netherlands). Each reaction was performed with 1 µg of total RNA. The labeling kit was also tested with enriched RNA (see above) as a template. After labeling, the labeled cDNA was purified using the QIAquick PCR purification kit (QIAGEN) and eluted from the column with 30 µg H₂O. The amount of cDNA and label was evaluated using a LKB Ultraspec Spectrophotometer (Amersham, previously Pharmacia) at 260 nm, 550 nm and 650 nm. Prehybridization and hybridization of the microarray were performed as explained above.

cDNA indirect labeling

RNA was immediately used for cDNA probe generation using the CyScribe post-labeling kit (Amersham Biosciences). Each reaction was performed with 30 µg of total RNA and 1 µl of random nucleotide nanomers. After purification, the efficiency of Cy label incorporation into the cDNA and the quality and amounts of labeled cDNA were verified with an Ultraspec 2100 pro spectrophotometer (Amersham Biosciences). The absorption of the samples was measured by a wavescan from 200 nm to 700 nm. The amounts of Cy-labeled cDNA were calculated on the following website: http://www.pangloss.com/seidel/Protocols/percent_inc.html. Volumes of cDNA for both Cy3 and Cy5 labels were pipetted so that equal amounts of each dye would be hybridized on the microarray. A minimum of 45 pmol of each dye was hybridized. The volume of Cy-labeled cDNA was reduced by evaporation and rediluted in a solution of TE, yeast tRNA (0.192 µg/µl) (Gibco BRL, Breda, the Netherlands), 3.3X SSC and 0.3% SDS (all final concentrations) to reach a final volume of 135 µl when both probes were mixed.

Before hybridization, the microarrays were rehydrated by a H₂O steam at 50°C and snap-dried on a hot plate. Then DNA was UV-crosslinked at 250 mJoules/cm² (Amersham LifeSciences UV cross linker). The microarrays were subsequently prehybridized with 100 µl prehybridization buffer (containing 0.4 µg/µl herring sperm DNA (Gibco BRL), 0.4 µg/µl yeast tRNA (Gibco BRL), 5X Denhardt's reagent (Denhardt, 1966), 3.2X SSC, 0.4% SDS; all final concentrations) 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% ethanol, once in 90% and once 100% ethanol (5 minutes per wash step) and air dried. The prehybridized slides were stored in the dark in airtight boxes. The Cy-labeled cDNA was hybridized on

the microarrays overnight at 65°C in a GeneTAC Hybstation (Genomic Solutions). After hybridization, the slides were washed in 2X SSC/0.1% 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 dried by centrifugation at 1000 rpm for 3 min and scanned in a G2565AA Microarray Scanner (Agilent, Amstelveen, The Netherlands).

Each experiment was repeated at least 4 times, including at least two independent ones and a dye swap. Each experiment included “as test” the Cy-labeled cDNA derived from the RNA of a mutant, and “as reference” the Cy-labeled cDNA derived from the RNA of the wild-type.

Microarray data analysis

After scanning, the microarrays were analyzed in GenePix Pro version 4.0. The values were normalized assuming that most genes of the array are not differentially expressed. Several criteria were implemented to select spots corresponding to genes assumed to be significantly affected in their expression by the gene mutation: spots were selected 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 and red channels ([% > B550+2SD] > 80 and [% > B650+2SD] > 80). This condition prevents the selection of spots from which the feature intensity is too close to the background. As an additional safety condition concerning spots of low intensity, all the spots that had intensities lower than the intensity of the λ control in both the red and green channel were eliminated. In order to avoid false positives because of a problem of uniformity of the background and/or the spot, all the selected spots were controlled directly on the image of the scan by verifying their aspect before any further analysis.

Phenotypic analyses of mutants deriving from microarray analyses

In order to test protease production, bacteria were tested on LC-milk agar plates as previously described (Chin-A-Woeng *et al.*, 1998), except that the concentration of milk was increased to 10% in MVB1 agar plates.

To test swimming and swarming ability, the method described previously (Deziel *et al.*, 2001) was used, in which 1/20 KB-0.3% agar plates were used for

analysis of swimming, and 1/20 KB-0.5% agar plates were used for analysis of swarming.

For measuring the production of chitinase, plates were poured with 2% agar dissolved in 0.05 M sodium acetate and Cm-Chitin-RBV solution (Loewe Biochemica GmbH, Sauerlach, Germany) following recommendations of the manufacturer. Two hundred microliters of supernatant of 3-day old LC cultures were applied in wells made in the plates. After overnight incubation at 28°C, the formation of a halo was judged by eye.

The production of hydrogen cyanide (HCN) was measured as described previously (Castric, 1975). Whatman 3MM paper was soaked into a chloroform solution containing copper(II) ethyl acetoacetate (5 mg/ml) and 4,4'-methylene-bis-(N,N-dimethylanilin) (5 mg/ml), and subsequently dried and stored in the dark. A piece of paper was placed in the lid of a Petri dish in which bacteria had been plated on MVB1-agar (1%). The Petri dishes were incubated overnight at 28°C. Bacteria which produced HCN turned the paper blue.

Extraction and analysis of phenazine and *N*-acyl homoserine lactones

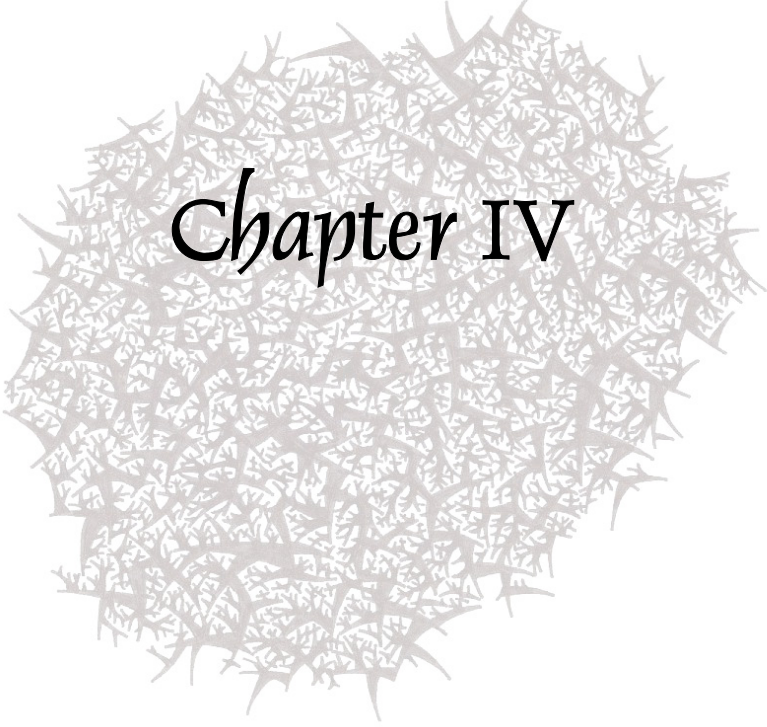
Phenazine extraction was carried out from 10 ml MVB1 liquid cultures in 100 ml Erlenmeyer flasks at regular time points during growth and/or after overnight growth of bacterial strains as described previously (van Rij *et al.*, 2004).

For extraction of *N*-AHL, supernatants from 50 ml MVB1 liquid MVB1 cultures in 500 ml Erlenmeyer flasks were mixed with 0.7 volume of dichloromethane, and shaken for one hour, after which the organic phase was collected. Each supernatant was extracted twice and the pooled extracts were dried using a rotary evaporator. The dried residue was dissolved in 25 µl of acetonitrile and spotted on C18 TLC plates (Merck, Darmstadt, Germany). As a control, 0.5 µl of synthetic hexanoyl-homoserine lactone (C₆-HSL) (5µM) (Fluka, Sigma-Aldrich, Zwijndrecht, the Netherlands) was spotted on the TLC. The plates were developed in methanol-water (60:40, v:v). For detection of *N*-AHLs, the TLC was overlaid with 0.8% LC agar containing a 10-fold diluted overnight culture of the *Chromobacterium violaceum* indicator strain CV026 and supplemented with kanamycin (50 µg/ml). After incubation for 48 h at 28°C, chromatograms were judged for appearance of violet spots.

ACKNOWLEDGEMENTS

The authors thank K. Tanaka (Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) for providing the anti-RpoS rabbit serum and Naomi Kramer (RUG, Groningen, the Netherlands), Joanna Cardoso, Ellen Sterrenburg, Rolf Turk and André Wijfes (LGTC, Leiden, the Netherlands) for technical advice concerning microarray. We thank also André Wijfes for the construction of the random chromosomal fragment library. Thomas Chin-A-Woeng set up the data filter that was used for microarray analyses.

This project was financially supported by the FW6 EU R&D project QRLT-2002-00914 (acronym “Pseudomics”) and by the BioScience Initiative from the Faculty of Mathematics and Natural Sciences of Leiden University.



Chapter IV

**The novel regulator Pip acts between
PsrA/RpoS and quorum-sensing in the
regulatory cascade of phenazine synthesis
in *Pseudomonas chlororaphis* PCL1391**

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

Submitted

ABSTRACT

Secondary metabolites are important factors for interactions of bacteria with other microorganisms in their natural environment. *Pseudomonas chlororaphis* PCL1391 produces the antifungal metabolite phenazine-1-carboxamide (PCN) and is used as a model organism to study regulation of secondary metabolism in *Pseudomonas* species. Our previous work showed that a cascade of regulators controls PCN synthesis, starting with the GacA/GacS two-component signaling system, *via* the regulator PsrA, the stationary phase sigma factor RpoS and the quorum-sensing system PhzI/PhzR down to the phenazine biosynthetic (*phz*) operon. Here we describe the identification of a novel regulator, named Pip (phenazine inducing protein), which shows similarity to members of the TetR/AcrR family of regulators. Highly conserved homologues of *pip* were found in a large diversity of bacterial species, of which the functions are unknown. Expression studies and quantification of *N*-AHL and PCN in various mutant strains identified Pip as acting downstream of PsrA and RpoS and upstream of PhzI/PhzR.

INTRODUCTION

Understanding interactions between organisms is critical for the study of bacterial processes such as pathogenicity and competition, which take place in their natural habitat, such as the rhizosphere. Interactions of Gram-negative bacteria with other microorganisms often involve the production of secondary metabolites with various functions. For example, some Gram-negative bacteria produce *N*-acyl-homoserine lactones (*N*-AHLs), which are able to traffic across membranes. The *N*-AHL extracellular concentration reflects the number of bacteria present in a (semi)closed environment. *N*-AHLs enable bacteria to sense the density of their population and to induce specific (sets of) genes beyond a threshold concentration (quorum). This phenomenon, called quorum-sensing (Bassler, 1999; Fuqua *et al.*, 2001), controls the production of other secondary metabolites, for example in pseudomonads the production of toxins, exoenzymes, (Winson *et al.*, 1995) rhamnolipids (Ochsner & Reiser, 1995; Pearson *et al.*, 1997), hydrogen cyanide (Pessi & Haas, 2000) and phenazines (Chin-A-Woeng *et al.*, 2001). Many of these metabolites contribute to the competition with other microorganisms and to the protection of plants against phytopathogens.

Phenazine-1-carboxamide (PCN) is produced by *Pseudomonas chlororaphis* PCL1391, which suppresses tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis lycopersici* (Chin-A-Woeng *et al.*, 1998). PCN production, combined with efficient root colonization to deliver PCN in the rhizosphere, are crucial traits for the biocontrol ability of strain PCL1391 (Chin-A-Woeng *et al.*, 2000). Understanding the components regulating the synthesis of PCN is likely to contribute to insights in regulation of bacterial secondary metabolism in general.

Production of PCN was shown to be regulated by different factors, including environmental biotic and abiotic factors (van Rij *et al.*, 2004; van Rij *et al.*, 2005), and intrinsic regulatory systems. The GacS/GacA system is a classical two-component system (Rodrigue *et al.*, 2000) including a sensor (GacS) that recognizes an unknown environmental signal and a transcriptional regulator (GacA) that activates a cascade of regulators upstream of the *phz* operon (Chin-A-Woeng *et al.*, 2001; Girard *et al.*, 2006). PsrA was shown to be part of this regulatory cascade, and controls the expression of the stationary phase sigma factor encoded by *rpoS* (Girard *et al.*, 2006). Downstream of RpoS, PhzI synthesizes C₆-HSL that is supposed to bind to the transcriptional regulator PhzR. Activated PhzR binds in turn to the *lux* box upstream of the *phz* operon, which is responsible for the synthesis of PCN at the onset of the stationary phase. Our previous results showed

that a constitutively activated quorum-sensing system PhzI/PhzR is sufficient for synthesis of PCN when other regulators are mutated (Girard *et al.*, 2006).

Here we describe the identification of *pip* (phenazine inducing protein), a novel gene that is involved in controlling PCN synthesis. Our results show that Pip, a putative transcriptional regulator, acts downstream of PsrA and RpoS and stimulates the expression of the *phz* operon via the quorum-sensing system.

RESULTS

Isolation of mutant strain PCL1114

A transposon library of *P. chlororaphis* PCL1391 consisting of 18,000 transposants and established using pRL1063a (Chin-A-Woeng *et al.*, 2001) was screened for mutants exhibiting reduced PCN production on LC agar. On LC agar, PCN production is visually detectable by the yellow color of the colonies. Among 21 transposant colonies isolated for altered coloration, one was selected and tested for PCN production after growth in liquid complex LC medium. Quantitative HPLC analysis shows that this mutant, named PCL1114, is severely affected in PCN production (0.03% compared to the parental strain PCL1391). When strain PCL1114 was grown in King's B medium, another complex medium, similar results were obtained: 97.5% of decrease in PCN production. PCN production by PCL1114 was not detected during growth in poorer MVB1 medium (Fig. 1, panel A), which was used as a standard in the next experiments.

Genetic analysis of PCL1114

Plasmid rescue from chromosomal DNA of PCL1114 showed that the Tn5*luxAB* transposon is inserted in a small ORF of 669 bp in between positions 71 and 72. The gene corresponding to this ORF was named *pip* (phenazine inducing protein).

The chromosomal organization around *pip* was analyzed by further sequencing, which showed the presence of an ORF of 1908 nucleotides upstream of *pip* (Fig. 2). This OFR (accession number DQ311664) has the same transcription orientation as *pip* and the protein shows 92% homology to a putative 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) of *P. fluorescens* PfO1 (accession number ZP_00262624). Computer analysis shows the presence of a putative *rho*-independent transcription terminator for the 4- hydroxyphenylpyruvate dioxygenase gene, seven nucleotides downstream of its stop codon (GTAACGGCGGCGGCAAAGGGCCGCGTCTGCTGC), followed by a putative promoter

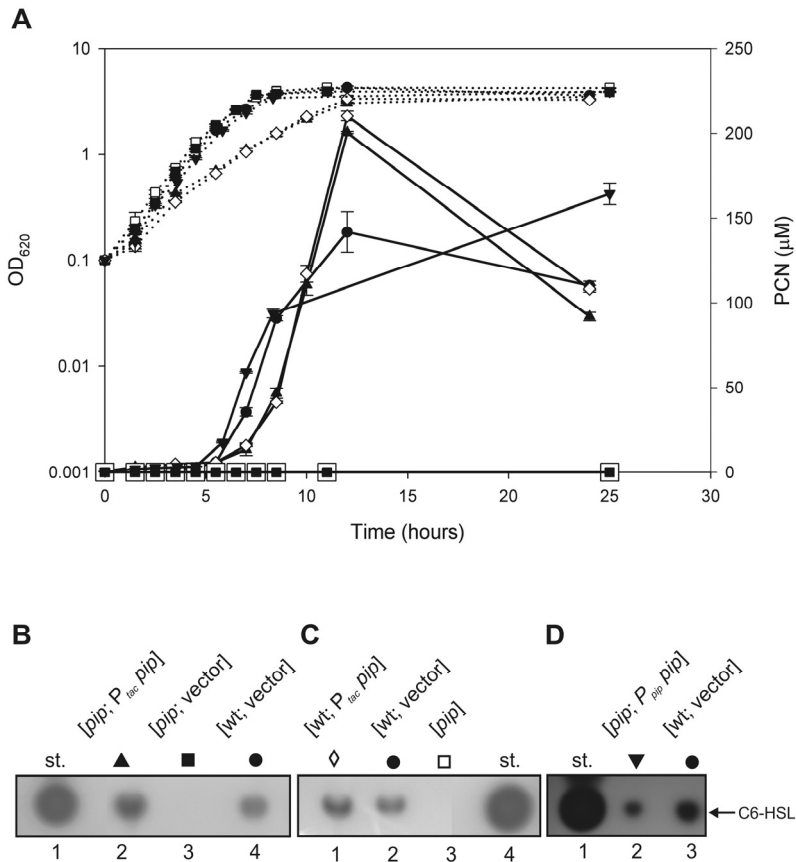


Figure 1. Analysis of PCN and *N*-AHL production by *P. chlororaphis* PCL1391 and PCL1114 derivatives. Panel A. Extractions were made from at least three independent cultures in 10 ml MVB1 in a time course and the PCN production level was determined by HPLC. The error bars indicate the standard deviations. On each graph, the OD at 620 nm (see left axis; dotted lines) and the PCN concentration (see right axis; solid lines) are plotted. Meaning of the symbols is indicated in panels B-D. The symbol for [pip] (□) was magnified for better visualization. Panels B-D. C18-reverse phase TLC analysis of *N*-AHL production by various PCL1391 derivatives at OD₆₂₀ = 3.0. Symbols are the same as in panel A and st indicates a standard of 2.5 nmol of synthetic C₆-HSL.

sequence upstream of the *pip* ORF. The start of transcription is predicted to be 35 nucleotides upstream of the *pip* start codon, downstream of a putative -10 box (GCCATAAT) and a putative -35 box (TTCCTT). No *rho*-independent terminator could be detected downstream of *pip*, however a putative gene is located there in the opposite direction of transcription. Its predicted protein product shows 58% homology with a putative transcriptional regulator of *Chromobacterium violaceum* ATCC 12472 (accession number AAQ59192).

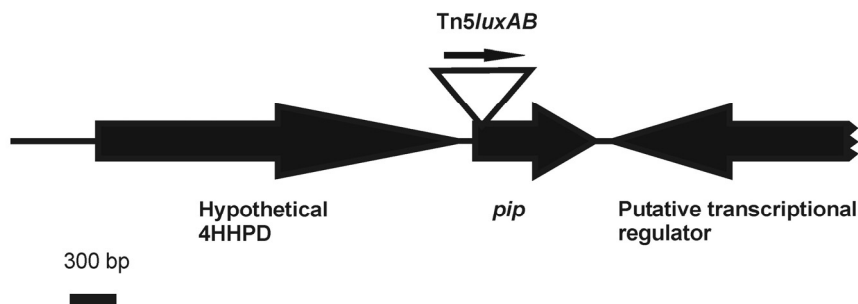


Figure 2. Genomic organization of the chromosomal region of *P. chlororaphis* PCL1391 surrounding *pip*. Each ORF is represented by an arrow which indicates the direction of transcription. The interrupted part of the arrow for the probable transcriptional regulator indicates that this ORF was not completely sequenced. The position of the transposon insertion is shown as an arrow head at the beginning of *pip* and the transcriptional directions are indicated. 4-HHPD stands for 4-hydroxyphenylpyruvate dioxygenase

Homologues of Pip were found in many pseudomonads (94% homology in *P. fluorescens* Pf-5; 83% homology in *P. putida* KT2440 and 79% homology in *P. aeruginosa* PAO1), as well as in a large variety of other Gram-negative species, such as *Azotobacter vinelandii*, *Burkholderia* sp., *Sinorhizobium meliloti*, *Agrobacterium tumefaciens*, *Ralstonia* sp. To our knowledge, no function has been published for any of these homologues. Domain homology search on the Pip sequence showed that Pip is homologous to members of the TetR/AcrR family, which are transcriptional regulators. In *E. coli*, TetR regulates a pump involved in tetracycline resistance (Beck *et al.*, 1982) and AcrR regulates a pump involved in multidrug resistance (Olliver *et al.*, 2004). This search retrieved a TetR N-terminal domain (Pfam00440) for the stretch of amino-acids from position 31 to 77 (53 bits, E value of $3e^{-8}$). Alignments between Pip homologues of various species and the TetR N-terminal domain (Fig. 3) show that many amino acids are conserved in the N-terminal region, which is similar to the TetR N-terminus. In addition, towards the C-terminal two small regions corresponding to amino acids 107-130 and 172-191 from Pip are conserved. No homology with the TetR C-terminal domain was found. The Pip protein has an overall similarity of 42% with the AcrR transcriptional regulator of *E. coli* (accession number AAC73566).

```

PCL1391      -----MTTELSVVPEQSVNEPRKSRKNNPEKTRENILQEAIVEFVQQGLSGARVDA 51
DC3000      -----MKKIDS AVAVEPAATVPKGRKNNPEKTREDILKAAVAEFVAHGLTGARVDA
KT2440      -----MSDSVVLGQPETEGGRKTRKNNPEKTREDILQAAINEFVQQGLAGARVDA
Pfo1        -----MTSELPAASALPAVEPRKSRKNNPEKTRENILQEAIVEFVQQGLSGARVDA
PAO1        -----MSKPDPAVVEESAPRG-KGRKNNPEKTRQDILRAAIDEFVAQGLSGARVDA
Azotobacter -----MPDTPKVA--DPAARPRQPRKNNPEKTRQDILLAAIREFAEQGLSGARVDA
K96243      MPPSDHAKMKQGSKAAAPDAEARRDEARRKYDPEQTKRNILQVATQEFSAMGLSGARVDA
1021        -----MAERQANGRKNDPQRTQDDILEVATEEFSTHGLAGARVDQ
TetR_N      -----ILDAALELFAERYDATVRE
                ** * * * *

PCL1391      IAERIHTSKRMIYYFSGSKEQLYVEVLEKLYGDIRSTESRLHLAELAPRDAIRRLVEFTF 111
DC3000      IAERTKTSKRMIYYFSGSKEQLYVEVLEKLYGAIRNTESELNLGELEPVEAHRVVEFTF
KT2440      IAERTATSKRMIYYFSGSKEQLYCECLVKLYGDIRKTEQSLDLESLPAEQAIRRLVEFTF
Pfo1        IAERIHTSKRMIYYFSGSKEQLYVEVLEKLYGDIRNTENRHLAELPPVEAIRRLVEFTF
PAO1        IAERTHTSKRMIYYFSGSKEQLYQAVLEKLYSDIRGIEGTLRLGALPPAKAMEKLVFEFSF
Azotobacter IAERTSTSKRMIYYFSGSKEQLYLAVLEKLYGDIRATEATLRLDELEPEAAIRRLVEFTF
K96243      IAERTNTTKRMLYYFDSKEGLYEAVLEKVGDIRTLERELNVGELEPREGLSRLVEFTF
1021        IAERTRTSKRMIYYFSGSKEALYLAVLERSYRKIRTLEADLELANLPPEEALRTLVAFTF
TetR_N      IAKEAGVSKGALYRHFPSKEELLAL-----
                ** * * * *

PCL1391      DHHDRNVDFVRIICTENIHYGEYVKQSPAIREMSSSLVLEALGNTLRRGVQEGVFRAGIEV 171
DC3000      DHHDRNVDFVRIVSENIHNAEYVKRSEAIKAMNNTILHALGEILQRGVDEGVFRAGLDP
KT2440      DHHDRNVDFVRIVSENIHNAEYVKRSDAIKAMNNTILDSLGEILRRGAEEGVFRAGLDA
Pfo1        DYHDRHVDFVRIISENIHKGEHIASSELVQSVNSSIVQSIAEILRRGEAEGVFRAGLEA
PAO1        DHDSNVDFVRIVCENIHNGENVKQSDTIQAKSQNIIRALDGLIRRGAEAGLFRDGVHP
Azotobacter DHHANVDFIRLVSIENIHGGEHIARSPSIRLSKSVSQIAAILARGADAGVFRAGIDP
K96243      DYHDRHRDFVRLVTIENIHGAKYIEQLKSFKNRVNSIIKTLEELLARGVESGVFRDDVDP
1021        DHDEANPDFVRLVSENIHRAAHMLRSDAIRDLNVSVIQMIEAIIERGLDGTFRRKADP
TetR_N      -----

PCL1391      LDVHLLISSFCFYRVSNRHTFGEIFQIDLPDEAIKQRHREMICESVLRYLQA----- 223
DC3000      VDLHLMISSFCFYRISNRHTFSEIFQIELWSEEVKQRHKAMICDAVLRYLKR-----
KT2440      IDLHMLMSFCFYRVSNRHTFGEIFQIDLSDEQIKLRHKAMICDAVLRYIQLEPRT----
Pfo1        LDVHLLISSFCFYRVSNRHTFGEIFQIDLPDESIKQRHREMICESVLRYLQA-----
PAO1        LDVHMLISSFCFYRVSNRYTVSRIFRTDLHDAEVRQRHRRMIGEAVLRYIRA-----
Azotobacter LDHLLISSFCFFRVSNRYTFTSTHQIDLGGAEARHREMICEAVIRVHRA-----
K96243      FDLHLLISSFCFHRVANRYTFGTAFGRDPSSPRLRARHRAMITDTVLRVY-----AR-
1021        IDVHMLISAFCFRVSNRYTFGTIFRRDLSEPKTIARHRTMIADAVSVYLSKDEPRAARR
TetR_N      -----

```

Figure 3. Alignment of Pip homologues from various bacterial species with the TetR N-terminal domain of *E. coli*.

Homologues of Pip from *P. chlororaphis* PCL1391 were found in *P. syringae* pv. *tomato* str. DC3000 (NP_792164), *P. putida* KT2440 (NP_745664), *P. fluorescens* PfO-1 (ZP_00262623), *P. aeruginosa* PAO1 (AAG03632), *Azotobacter vinelandii* (ZP_00091468), *Burkholderia pseudomallei* K96243 (YP_111478) and *Sinorhizobium meliloti* 1021 (NP_436576). The aminoacids that are conserved in all the Pip homologues are indicated in bold. The aminoacids that are conserved in all the Pip homologues and in the TetR N-terminal domain are indicated by an asterisk. The numbers on the right margin indicate the aminoacid counting of Pip in strain PCL1391.

Phenotypic characterization of *pip* mutant PCL1114

pip mutant PCL1114 was characterized for motility and production of HCN, siderophore, exoprotease and chitinase. The wild-type strain PCL1391 and the *gacS* mutant PCL1123 (Chin-A-Woeng *et al.*, 2005) were used as control strains. Measurements showed no difference between the wild type and the *pip* mutant in motility. After overnight incubation, a blue coloration was observed for both

PCL1391 and PCL1114 on filter paper used for HCN detection. Similarly, a halo of comparable size for PCL1391 and PCL1114 was measured in the chitinase, exoprotease and siderophore tests (Table 1).

Table 1. Phenotypic tests

Property	Strains		
	PCL1391 wt	PCL1114 <i>pip</i> mutant	PCL1123 <i>gacS</i> mutant
PCN production	+	-	-
C ₆ -HSL production	+	-	-
Swarming diameter (mm)	17.3 (±3.0)	18.0 (±1.0)	1.3 (±0.2)
Swimming diameter (mm)	20.3 (±5.5)	21.7 (±1.5)	1.3 (±0.2)
Chitinase production (mm)	9.3 (±0.6)	9.3 (±0.3)	0.7 (±0.3)
Exoprotease activity	+	+	-
Siderophore production	+	+	++
HCN production	+	+	+
MIC* for gentamicin (µg/ml)	15	15	n. d. **
MIC for tetracyclin (µg/ml)	40	40	n. d.
MIC for rifampicin (µg/ml)	16	16	n. d.
MIC for carbenicillin (µg/ml)	>1,600	>1,600	n. d.

*MIC, minimum inhibitory concentration

**n. d., not determined

Since Pip shows homology with TetR, which is involved in tetracycline resistance (Beck *et al.*, 1982) and with AcrR, which regulates multidrug resistance (Olliver *et al.*, 2004), we tested if the *pip* mutant had a modified resistance to various antibiotics as compared to its wild-type strain. No difference was found between PCL1114 and PCL1391 in their sensitivity towards gentamicin, tetracycline, rifampicin and carbenicillin (Table 1).

Effect of a *pip* mutation on production of PCN and *N*-AHL

Mutant strain PCL1114 was selected for its impaired PCN production (see above). In addition, analysis of *N*-AHL production showed that C₆-HSL could not be detected in the supernatant of PCL1114 (Fig. 1, panel C, lane 3).

In order to test if the inhibition of PCN production was indeed due to the defect in *pip*, several new mutant strains were constructed. PCL2008 contains a suicide vector, which recombined into *pip* by homologous recombination (see Experimental Procedures), and is impaired in PCN and C₆-HSL production, like PCL1114 (data not shown). A second strain, PCL2012, is derived from PCL1114 and contains the vector pMP7455, which harbors *pip* under control of the *tac* promoter, resulting in constitutive expression of *pip*. This strain produced 1.4-fold more PCN (Fig. 1, panel A) than strain PCL1960 (wt PCL1391 containing the empty cloning

vector as a control) as analyzed after 12 hours of growth. Strain PCL2012 (*pip*; P_{tac} *pip*) also produces high amounts of C₆-HSL (Fig. 1, panel B, lane 2). The *pip* gene was also expressed under its own promoter *in trans* in the *pip* mutant (PCL2085). This new strain showed restored production of PCN and *N*-AHL (Fig. 1, panels A and D). Over-expression of *pip* in a wild-type background (transformation of pMP7455 into PCL1391 resulting in PCL2019) gave the same phenotype as in the *pip* mutated background (Fig. 1, panel A and panel C, lane 1). These results clearly show that the impaired production of PCN and *N*-AHL in PCL1114 is indeed caused by the disruption of *pip*.

Analysis of autoregulation of *pip* expression

Both TetR and AcrR negatively auto-regulate their own expression (Hillen & Berens, 1994; Ma *et al.*, 1996). We tested whether Pip shows a similar autoregulatory activity. Analysis of the orientation of the Tn5*luxAB* in PCL1114 showed that the *luxAB* genes and *pip* have the same direction of transcription, which allows measurements of *pip* expression by quantifying the *luxAB* activity (Fig. 2). The expression of *pip* was measured in three derivatives, containing P_{tac} *pip*, P_{pip} *pip*, and the empty cloning vector, respectively (Fig. 4, panel A). PCL2085 (*pip*::Tn5*luxAB*; P_{pip} *pip*) showed a maximum of expression (2210±67 cps), which is intermediate between that of PCL2011 (*pip*::Tn5*luxAB*; empty vector) (3590±231 cps) and that of PCL2012 (*pip*::Tn5*luxAB*; P_{tac} *pip*) (262±1 cps). These results demonstrate that Pip regulates negatively its own transcription.

Regulatory relationships between Pip and other regulators of PCN production

Several genes, including *psrA*, *rpoS* and *phzI/phzR*, are known to play a role in the regulation of PCN synthesis (Chin-A-Woeng *et al.*, 2001; Girard *et al.*, 2006). Experiments were conducted to understand if and, if so, how *pip* interacts with these genes. For easier reading below, the insertion of Tn5*luxAB* is not always mentioned, but in the strains used in the studies, all *psrA* and *pip* mutants are actually *psrA*::Tn5*luxAB* and *pip*::Tn5*luxAB*, respectively.

In MVB1 medium the *psrA* and *rpoS* genes positively regulate PCN and *N*-AHL production (Girard *et al.*, 2006). To test whether *pip* could be regulating these two genes, the effect of a *pip* mutation on the production of RpoS in strain PCL1391 protein was tested by Western blot analysis. Samples were taken during the logarithmic growth phase (OD₆₂₀ 1.0) and at the beginning of the stationary phase (OD₆₂₀ 2.2) of cells grown in MVB1 medium. The amounts of RpoS appeared to be

similar at the two time points for all the tested strains. A blot of the results at OD₆₂₀ 1.0 is shown (Fig. 5). PCL1954 (*rpoS* mutant), used as a negative control, showed no RpoS production (Fig. 5, lane 3), whereas PCL1955 (*rpoS* mutant with constitutive *rpoS* expression *in trans*) showed restored RpoS production (Fig. 5, lane 4). The amounts of RpoS were similar for PCL1391 and PCL1114 (Fig. 5, lanes 1 and 2). Subsequently, the ability of constitutively produced PsrA and RpoS to restore PCN production in *pip* mutant PCL1114 was tested. Measurements showed that PCL2036 (*pip*; *P_{tac} rpoS*) and PCL2046 (*pip*; *P_{tac} psrA*) do not produce detectable amounts PCN (results not shown).

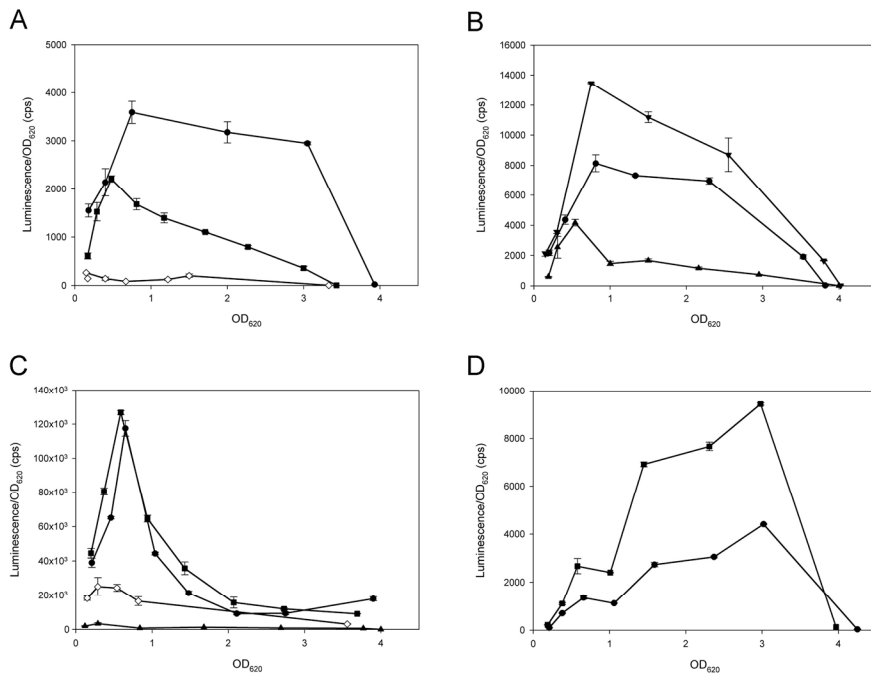


Figure 4. Expression analyses of *P. chlororaphis* PCL1391 *pip*, *psrA* and *phzR* derivative strains. Each panel corresponds to a particular chromosomal background, and only the genes in the vector pBBR1MCS-5, added to the background, are indicated in the detailed legend. Cell cultures were grown in MVB1 medium and samples were taken at regular time intervals. Activity of the *luxAB* reporter was determined by quantifying bioluminescence. Measurements were performed in duplicate. Panel A. Strains with a *pip*::Tn5*luxAB* background: PCL2011 (pBBR1-MCS5, ●), PCL2085 (*P_{pip} pip* ■), PCL2012 (*P_{tac} pip* ◇). Panel B. Strains with a *pip pip*::Tn5*luxAB* background: PCL2011 (pBBR1-MCS5, ●), PCL2036 (*P_{tac} rpoS* ▼) and PCL2046 (*P_{tac} psrA* ▲). Panel C. Strains with a *psrA*::Tn5*luxAB* background: PCL1962 (pBBR1-MCS5 ●), PCL2087 (*P_{pip} pip* ■), PCL2038 (*P_{tac} pip* ◇) and PCL2045 (*P_{tac} psrA* ▲). Panel D. Strains with a *phzR*::Tn5*luxAB* background: PCL2001 (pBBR1-MCS5 ●) and PCL2086 (*P_{pip} pip* ■).

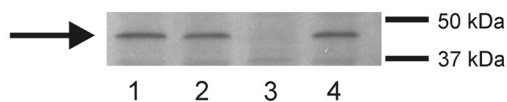


Figure 5. Western-blot analysis of RpoS production in *P. chlororaphis* PCL1391 and derivative strains. Cells were grown in MVB1 until OD_{620} 1.0. Lane 1: PCL1391. Lane 2: PCL1114 (*pip*::Tn5*luxAB*). Lane 3: PCL1954 (*rpoS*::pMP7418). Lane 4: PCL1955 (*rpoS*::pMP7418 + P_{tac} *rpoS*). On the right side of the blot two markers are shown. An arrow on the left indicates the position of RpoS.

Constitutive expression of *rpoS* in *pip* mutant (PCL2036) resulted in a 25% increase of P_{pip} activity compared to PCL2011. In contrast, constitutive expression of *psrA* in PCL2046 had a negative influence (45% reduction) on P_{pip} activity (Fig. 4, panel B). The reverse experiments were performed to evaluate if *pip* had an effect on *psrA* expression. Like for constitutive expression of *pip* in *pip*::Tn5*luxAB*, constitutive expression of *psrA* in *psrA*::Tn5*luxAB* resulted in complete inhibition of luminescence (Fig. 4, panel C, compare PCL1962 [*psrA* with the empty vector] and PCL2045 [*psrA*; P_{tac} *psrA*]). Constitutive expression of *pip* in the same *psrA*::Tn5*luxAB* background (PCL2038) resulted also in a severe decrease (60%) of *lux* activity, although the level remained higher than in PCL2045. The vector containing the *pip* gene under its own promoter was also used for transformation of the *psrA* mutant. Activity of P_{psrA} was comparable between the derivative strain containing P_{pip} *pip* and the derivative strain with the empty cloning vector (Fig. 4, panel C).

Previously it was shown that *psrA* and *rpoS* mutants produce only low amounts of PCN and *N*-AHL (Girard *et al.*, 2006). To test whether *pip* could regulate PCN and *N*-AHL production downstream of *psrA* and/or *rpoS*, *pip* was overexpressed in PCL1111 (*psrA* mutant) and PCL1954 (*rpoS* mutant) by transformation with pMP7455, resulting into PCL2038 and PCL2040, respectively. These two strains showed a restored production of PCN (Fig. 6, panel A) and C₆-HSL, as compared to PCL1962 (*psrA* mutant with empty vector) and PCL1957 (*rpoS* mutant with empty vector) (Fig. 6, panel C; compare lanes 3 and 2, 5 and 4).

To test the relationship between Pip and quorum-sensing further, a *pip* mutant derivative was constructed that constitutively expresses *phzR*. The resulting strain, PCL2013, showed a restored production of both PCN and C₆-HSL (Fig. 6, panels A and B). Transformation with a plasmid containing *pip* under its own promoter showed a positive effect on P_{phzR} activity (Fig. 5, panel D). A summary of all these results is shown in Fig. 7.

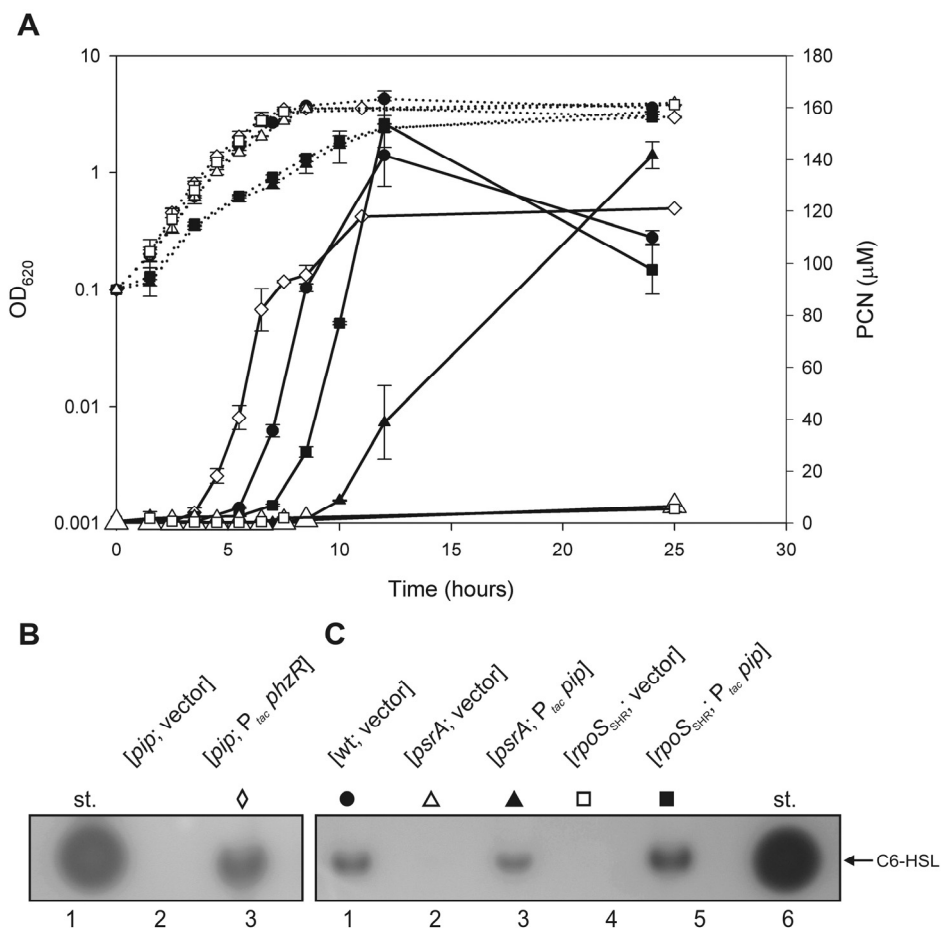


Figure 6. Analysis of PCN and *N*-AHL production in *P. chlororaphis* PCL1391 derivative strains. Panel A. Extractions were carried out from at least three independent cultures in 10 ml MVB1 in a time course and the PCN concentration was determined by HPLC analysis. On each graph, the absorbance is plotted along the left axis (dotted lines) and the PCN concentration is plotted along the right axis (closed lines). Meaning of the symbols is indicated in panels B-C. Since measurements were made at the same time points for the cultures, the points indicating PCN amounts in PCL1962 (Δ) are hidden by the points indicating the amounts of PCN for PCL1957 ($\square$). Panels B and C. C18-reverse phase TLC analysis of *N*-AHL produced by PCL2011, *pip*::Tn5*luxAB* + pBBR1MCS-5 (lane 2) and other derivatives indicated by the symbols of panel A. st: 2.5 nmol of synthetic C₆-HSL.

DISCUSSION

In order to identify new genes involved in the regulation of PCN production, a mutant library was screened for decreased PCN production, which yielded mutant strain PCL1114 (Fig. 1). The analysis of the flanking regions of the Tn5 transposon showed an interrupted ORF, which we named *pip* (Fig. 2). Homologues of *pip* are found in a wide variety of bacterial species (Fig. 3). To our knowledge this is the first time that a phenotype is described as a result of mutating *pip*.

Location of *pip* in the genome

Sequence analysis showed upstream of *pip* the presence of a gene encoding a putative 4-HPPD, in the same direction of transcription as *pip* (Fig. 2). In principle this enzyme is not linked to the metabolism of *N*-AHL or PCN, since it catalyzes the second step in the pathway for the catabolism of the aminoacid tyrosin (Moran, 2005). Searches for reciprocal best BLAST hits in the sequenced genome database version 2 of *P. aeruginosa* PAO1 showed that the *pip* homologue is downstream of a putative 4-HPPD gene in at least *P. fluorescens* Pf-5, *P. aeruginosa* PAO1, *P. putida* KT2440, *P. syringae* B728a and *P. syringae* pv. *tomato* DC3000. This is also the case for *Azotobacter vinelandii*. This conserved genomic organization could indicate that *pip* is functionally coupled to the 4-HPPD. However, a terminator of transcription was found for the putative 4-HPPD and a putative promoter just upstream of *pip* supports the hypothesis that the two genes are independently transcribed. Downstream of *pip* an ORF was detected with an opposite direction of transcription (Fig. 2), indicating that no downstream effect of the Tn5 insertion in *pip* is expected that would influence PCN production. Complementation of the *pip* mutant confirmed this (Fig. 1). Interestingly, the ORF downstream of *pip* encodes a putative transcriptional regulator, whereas in all the other *Pseudomonas* species displayed in the sequenced genome database of *P. aeruginosa* PAO1, the *pip* homolog is located upstream of a putative shikimate 5-dehydrogenase. Similar observations were made concerning the flanking regions of *rpoS* in strain PCL1391 (Girard *et al.*, 2006), which seems to indicate that the chromosome of strain PCL1391 is organized in a different way as compared to closely related pseudomonads.

Pip belongs to the AcrR/TetR family of regulators

Alignments showed that Pip contains an N-terminal domain similar to that of the TetR transcriptional regulator (Fig. 3). The TetR N-terminal is a domain that

contains a helix-loop-helix (HLH) motif (Isackson & Bertrand, 1985), which indicates that Pip would be a transcriptional regulator. Pip also shows overall homology with the transcriptional regulator AcrR. AcrR belongs to the same family of regulatory proteins as TetR (Grkovic *et al.*, 2002). TetR and AcrR have similar functions. The TetR protein regulates the resistance mechanism against the antibiotic tetracycline in *E. coli* (Hillen & Berens, 1994). Located in the membrane, TetA exports the tetracycline-magnesium $[MgTc]^+$ complex. TetR recognizes two palindromic operator (*tetO*_{1,2}) sites in the promoter region of the divergently oriented genes *tetR* and *tetA*, thereby inhibiting their expression. Binding of $[MgTc]^+$ to TetR relieves this inhibition by reducing the affinity of TetR for *tetO*. (Hillen & Berens, 1994). AcrR regulates the production of the AcrAB pump (Ma *et al.*, 1996) that is responsible for multidrug resistance (Ma *et al.*, 1995; Okusu *et al.*, 1996). Similarly to TetR, AcrR represses the *acrAB* operon and its own transcription (Ma *et al.*, 1996).

The negative feedback of Pip on its own expression was also clearly shown: increased expression of *pip* *in trans* in PCL1114 derivatives results in decreased expression of chromosomal *pip*, since P_{pip} *pip* inhibits *pip* expression less than P_{tac} *pip* and more than the presence of the empty cloning vector (Fig. 4, panel A). The homology with AcrR and TetR and the negative feedback of Pip on its own expression (Fig. 4) suggest that Pip might also regulate the production of an efflux pump. However, *tetR* is located next to *tetA* in the chromosome of *E. coli*, as is *acrR* located next to *acrA* (Hillen & Berens, 1994; Ma *et al.*, 1996). In the case of strain PCL1391, no gene encoding a putative pump could be found next to *pip* in the genome (Fig. 2). It also does not share its promoter region with the promoter region of another gene, as *tetR* and *acrR* do. Also unlike TetR and AcrR (Beck *et al.*, 1982; Okusu *et al.*, 1996), Pip does not seem to be involved in resistance to antibiotics (Table 1). We considered the idea that Pip might regulate an *N*-AHL or PCN pump (see also Chapter 5). It seems unlikely in the case of PCN, because then a *pip* mutation should not affect the amounts of *N*-AHL, but it does (Fig. 1). In the case of *N*-AHL, it would explain the effect of *pip* mutation on both *N*-AHL and PCN (Fig. 1). However, it is assumed that C₆-HSL, with such a short acyl chain, is able to diffuse through membranes (Fuqua *et al.*, 2001) and does not traffic *via* a pump: an efflux pump was found in *P. aeruginosa* that exports long chain *N*-AHLs, but not *N*-AHLs with short acyl chains (Evans *et al.*, 1998).

Pip is a new regulator of PCN synthesis

Since *pip* stimulates *N*-AHL and PCN synthesis (Fig. 1), we tried to clarify how Pip interacts with other proteins known to regulate PCN synthesis. Firstly, several experiments were conducted to test if Pip is positioned upstream of RpoS in the regulatory cascade of PCN production. Western blot analysis showed that Pip does not influence *rpoS* expression (Fig. 5). Neither constitutive expression of *psrA* nor constitutive expression of *rpoS* could restore PCN and *N*-AHL production in the *pip* mutant. All together, these results suggest that Pip does not regulate *psrA* and *rpoS*.

In contrast, the stimulation of P_{pip} activity by RpoS (Fig. 4, panel B) indicates that Pip is located downstream of RpoS in the PCN regulatory cascade. However, constitutively produced PsrA has a negative effect on P_{pip} activity (Fig. 4, panel B). Since PsrA positively regulates *rpoS* (Girard *et al.*, 2006), it seems contradictory that RpoS and PsrA have opposite effects on the expression of *pip*. An explanation could be given by the fact that PsrA and Pip show an inhibitory effect on their own expression (Fig. 4, panels A and C). Since PsrA and Pip are both TetR homologues, they are most likely to exert their negative autoregulation in similar ways. In a situation of constitutive expression, Pip or PsrA is present in excess and therefore might bind to other targets, although less specifically. This would result for example in PsrA inducing negative feedback on *pip*, although to a lower level than the negative feedback of Pip itself. This hypothesis was verified by the reverse experiment, that showed that Pip induces negative feedback on *psrA*, although to a lower level than the negative feedback of PsrA itself (Fig. 4, panel C). These results support the hypothesis that the negative effect of PsrA on *pip* expression would be the result of a non-physiological high concentration of PsrA in the cell.

If so, the previous results do not contradict the notion that PsrA and RpoS regulate *pip* expression. This hypothesis was confirmed by the fully restored production levels of PCN and *N*-AHL in *psrA* and *rpoS* mutants constitutively expressing *pip* (Fig. 6). In addition, PCN and *N*-AHL levels were correlated in all the *pip* derivatives and a *pip* mutation was suppressed by constitutive production of PhzR in strain PCL2013. Finally, increasing the copy number of *pip* results in an increased transcription of *phzR* (Fig. 4, panel D). Taken together, these results show that Pip acts in the cascade of PCN synthesis, downstream of PsrA/RpoS and upstream of PhzI/PhzR and the *phz* operon. Subsequent studies showed that Pip does not regulate exoprotease, chitinase, HCN and siderophore production or motility (Table 1), indicating that Pip is regulating PCN rather specifically. Negative

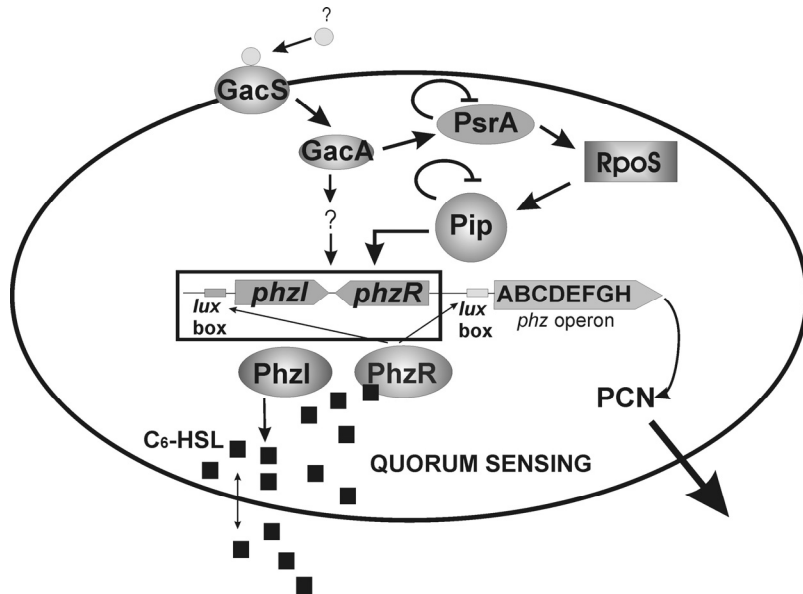


Figure 7. Schematic model showing the role of Pip in the genetic cascade regulating PCN synthesis in *P. chlororaphis* PCL1391. The regulatory cascade of PCN starts with the sensing of a so far unidentified environmental signal by GacS and subsequent activation of GacA. The TetR-homologue PsrA regulates *rpoS* probably by binding to its promoter. The alternative sigma factor RpoS is positively regulates *pip*, the product of which stimulates expression the quorum-sensing system *phzI/phzR*. Both *pip* and *psrA* exhibit negative autoregulation. PhzI is responsible for the production of C₆-HSL, which is supposed in turn to bind to PhzR. The PhzR-C₆-HSL complex binds to *lux* boxes in the promoter sequences of *phzI* and the *phz* operon. Subsequently *phzI* is upregulated and expression of the *phz* operon is switched on, which finally results in the synthesis of phenazine-1-carboxamide (PCN).

The discovery of *pip* is an important step in unraveling the regulatory mechanism of PCN regulation. A complex regulatory model for regulation of the *phz* operon arises from previous results and the results of this study, as depicted in Fig. 7. The autoregulation of Pip adds complexity to this model. This study also brings new insights into the role of homologues of the TetR/AcrR family, which are widely distributed in bacterial species, as far as in *Thermus thermophilus* and in *Streptomyces coelicolor*, two organisms with considerable biotechnological potential (Challis & Hopwood, 2003; Henne *et al.*, 2004).

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Pseudomonas* strains were cultured at 28°C in liquid MVB1 (van Rij *et al.*, 2004) or LC (Girard *et al.*, 2006) or King's Medium B (King *et al.*, 1954) and shaken at 195 rpm on a Janke und Kunkel shaker KS501D (IKA Labortechnik, Staufen, Germany). *Escherichia coli* strains were grown at 37°C in LC medium under vigorous aeration. Media were solidified with 1.8% Bacto agar (Difco, Detroit, MI). When appropriate, growth media were supplemented with kanamycin (50 µg/ml), carbenicillin (200 µg/ml), gentamicin (30 µg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 µg/ml) or hexanoyl-homoserine lactone (C₆-HSL) (5 µM) (Fluka, Sigma-Aldrich, Zwijndrecht, The Netherlands). To follow growth, the absorbance of liquid cultures was measured at 620 nm.

Isolation and sequence analysis of chromosomal regions flanking the transposon in the *pip* mutant PCL1114

A transposon library was obtained by transformation of strain PCL1391 with the plasmid pRL1063a (Chin-A-Woeng *et al.*, 2001). The Tn5 transposon of pRL1063A contains an origin of replication that functions in *E. coli* (Wolk *et al.*, 1991). Chromosomal DNA was isolated from PCL1114, digested with *Eco*RI, religated and transferred into *E. coli* by transformation. One clone was picked among the colonies obtained after kanamycin resistance selection. The plasmid containing the regions flanking the transposon was named pMP7444, and sequenced using primers oMP458 (5'-TACTAGATTCAATGCTATCAATGAG-3') and oMP459 (5'-AGGAGGTCACATGGAATATCAGAT-3'). Similarity and domain searches were done using BLAST (<http://www.ncbi.nih.gov/BLAST>). Search for bacterial promoters and terminators were done using Softberry (<http://www.softberry.com>). Alignments of amino-acid sequences were obtained using the ClustalW software (<http://www.ch.embnet.org/software/ClustalW.html>).

Phenotypic analyses

To test swimming and swarming ability, the method described previously (Deziel *et al.*, 2001) was used, in which 1/20 KB-0.3% agar plates were used for the analysis of swimming, and 1/20 KB-0.5% agar plates were used for analysis of swarming.

Table 2. Bacterial strains and plasmids used

Bacterial strains and plasmids	Description	Reference or source
Bacteria		
<i>Pseudomonas chlororaphis</i>		
PCL1391	Wild-type <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide; biocontrol strain of tomato foot and root rot caused by <i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	(Chin-A-Woeng <i>et al.</i> , 1998)
PCL1104	Derivative of PCL1391 in which a promoterless Tn5luxAB has been inserted in <i>phzR</i> ; Km ^r	(Chin-A-Woeng <i>et al.</i> , 2001)
PCL1111	Derivative of PCL1391 in which a promoterless Tn5luxAB has been inserted in <i>psrA</i> ; Km ^r	(Chin-A-Woeng <i>et al.</i> , 2005)
PCL1114	Derivative of PCL1391 in which a promoterless Tn5luxAB has been inserted in <i>pip</i> ; Km ^r	This study
PCL1123	Derivative of PCL1391 in which a promoterless Tn5luxAB has been inserted in <i>gacS</i> ; Km ^r	(Chin-A-Woeng <i>et al.</i> , 2005)
PCL1954	Derivative of PCL1391; <i>rpoS</i> ::pMP7418; Km ^r	(Girard <i>et al.</i> , 2006)
PCL1962	Derivative of PCL1111 containing pBBR1-MCS5; Km ^r , Gm ^r	(Girard <i>et al.</i> , 2006)
PCL2001	Derivative of PCL1104 containing pBBR1-MCS5; Km ^r , Gm ^r	(Girard <i>et al.</i> , 2006)
PCL2008	Derivative of PCL1391; <i>pip</i> ::pMP7451; Km ^r	This study
PCL2011	Derivative of PCL1114 containing pBBR1-MCS5; Km ^r , Gm ^r	This study
PCL2012	Derivative of PCL1114 containing pMP7455; Km ^r , Gm ^r	This study
PCL2013	Derivative of PCL1114 containing pMP7447; Km ^r , Gm ^r	This study
PCL2019	Derivative of PCL1391 containing pMP7455; Gm ^r	This study
PCL2035	Derivative of PCL1104 containing pMP7455; Km ^r , Gm ^r	This study
PCL2036	Derivative of PCL1114 containing pMP7420; Km ^r , Gm ^r	This study
PCL2038	Derivative of PCL1111 containing pMP7455; Km ^r , Gm ^r	This study
PCL2040	Derivative of PCL1954 containing pMP7455; Km ^r , Gm ^r	This study
PCL2045	Derivative of PCL1111 containing pMP7465; Km ^r , Gm ^r	(Girard <i>et al.</i> , 2006)
PCL2046	Derivative of PCL1114 containing pMP7465; Km ^r , Gm ^r	This study
PCL2085	Derivative of PCL1114 containing pMP7487; Km ^r , Gm ^r	This study
PCL2086	Derivative of PCL1104 containing pMP7487; Km ^r , Gm ^r	This study
PCL2087	Derivative of PCL1111 containing pMP7487; Km ^r , Gm ^r	This study

	Km ^r , Gm ^r	
<i>Chromobacterium violaceum</i>		
CV026	Double mini-Tn5 mutant from <i>C. violaceum</i> ATCC 31532, AHL biosensor; Km ^r	(Milton <i>et al.</i> , 1997)
<i>Escherichia coli</i>		
DH5α	<i>Escherichia coli</i> ; <i>supE44 ΔlacU169(Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi1 relA1</i>	(Hanahan, 1983)
Plasmids		
pRL1063a	Plasmid harboring promoterless Tn5 <i>luxAB</i> transposon; Km ^r	(Wolk <i>et al.</i> , 1991)
pRK2013	Helper plasmid for tri-parental mating	(Ditta <i>et al.</i> , 1980)
pIC20H	General purpose cloning vector; Cb ^r	(Marsh <i>et al.</i> , 1984)
pGEM-T easy	Plasmid designed for direct ligation of PCR fragments	Promega
pBBR1MCS-5	General purpose cloning vector; Gm ^r	(Kovach <i>et al.</i> , 1995)
pMP5285	Suicide vector for <i>Pseudomonas</i> spp. Used for homologous recombination; Km ^r , Cb ^r	(Kuiper <i>et al.</i> , 2001)
pMP7420	pBBR1MCS-5 containing the <i>rpoS</i> gene of PCL1391 downstream of the <i>P_{tac}</i> promoter, obtained by <i>EcoRI</i> digestion of pMP7424; Gm ^r	(Girard <i>et al.</i> , 2006)
pMP7444	pRL1063a containing <i>pip</i> and flanking regions, Km ^r	This study
pMP7447	pBBR1MCS-5 containing the <i>phzR</i> gene of PCL1391 under control of the <i>P_{tac}</i> promoter, inserted between the <i>XhoI</i> and <i>EcoRI</i> sites; Gm ^r	(Girard <i>et al.</i> , 2006)
pMP7451	pMP5285 containing an internal 350 bp PCR fragment of <i>pip</i> ; Km ^r	This study
pMP7455	pBBR1MCS-5 containing the <i>pip</i> gene of PCL1391 under control of the <i>P_{tac}</i> promoter; Gm ^r	This study
pMP7465	pBBR1MCS-5 containing the <i>psrA</i> gene of PCL1391 under control of the <i>P_{tac}</i> promoter; Gm ^r	(Girard <i>et al.</i> , 2006)
pMP7487	pBBR1MCS-5 containing the <i>pip</i> gene of PCL1381 under control of its own promoter; Gm ^r	This study

For measuring the production of chitinase, plates were poured with 2% agar dissolved in 0.05 M sodium acetate and Cm-Chitin-RBV solution (Loewe Biochemica GmbH, Sauerlach, Germany) following recommendations of the manufacturer. A volume of 200 µl of supernatant of 3-day old LC cultures was applied in wells made in the agar plates. After overnight incubation at 28°C, the distance between the edge of the well and the edge of the halo was measured.

In order to test protease production, bacteria were tested as described previously (Chin-A-Woeng *et al.*, 1998), except that the concentration of milk was increased to 10% in MVB1 agar plates.

The test for siderophore production was performed on solid medium as described previously (Schwyn & Neilands, 1987).

The production of HCN was measured as described previously (Castric, 1975). Whatman 3MM paper was soaked into a chloroform solution containing copper(II) ethyl acetoacetate (5 mg/ml) and 4,4'-methylene-bis-(N,N-dimethylanilin) (5 mg/ml), and subsequently dried and stored in the dark. A piece of this paper was placed in the lid of a Petri dish in which bacteria had been plated on MVB1-agar (1%). The Petri dishes were sealed with parafilm and incubated overnight at 28°C. Production of HCN by the bacteria was shown by blue coloration of the paper.

To test antibiotic resistance, various dilutions (10^4 , 10^5 , 10^6 and 10^7 fold) of overnight cultures were spotted in duplicate on 1.8% agar LC plates containing various concentrations of the following antibiotics: tetracyclin (20, 40, 80 and 160 µg/ml), carbenicillin (200, 400, 800 and 1,600 µg/ml), rifampicin (2, 4, 8 and 16 µg/ml) and gentamicin (7.5, 15, 30 and 60 µg/ml). Growth of bacteria was judged after two days of incubation at 28°C.

Recombinant DNA techniques

General DNA techniques were performed as described previously (Sambrook & Russel, 2001). Polymerase chain reactions were carried out with Super Taq enzyme (Enzyme Technologies Ltd, UK). Only for the production of *pip* under control of the P_{tac} or P_{pip} promoter, PCRs were performed using Phusion from Finnzymes (Bioké, Leiden, The Netherlands). Primers were synthesized by Isogen Life Science (Maarssen, The Netherlands). Restriction enzymes were purchased from New England BioLabs Inc. (Westburg) and ligase from Promega (Leiden, The Netherlands).

Construction of plasmids and PCL1391 mutant strains

In order to construct a suicide plasmid for disruption of *pip* by single homologous recombination, an internal *pip* fragment of 350 bp was obtained by PCR on PCL1391 chromosomal DNA with the primers oMP814 (5'-ATATATGAATTCCCGGCGCTCGGGTGGATGCC-3') and oMP815 (5'-ATATATGAATTCTCTCGCCCAGGGCATGGAGG-3'). The PCR fragment was cloned into the *EcoRI* site of the vector pMP5285. The obtained suicide vector was named pMP7451 and transferred to PCL1391 by triparental mating using the helper plasmid pRK2013. The resulting mutant was named PCL2008.

In order to constitutively express *pip*, a plasmid was constructed harboring *pip* under control of the constitutive P_{tac} promoter. Two primers were designed according to the *pip* sequence obtained from pMP7444: oMP816 (5'-ATATATGAATTCCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGA TAACAATTTTCACACAGGAAACAGCTAAATGACAATGACCACAGAACTCTCCGTAGTG CCC-3'), which contained the P_{tac} promoter and oMP817 (5'-ATATATGAATTCAGGATGCGGTTGAAACCCTGTGCCGCG-3'). These primers were used for PCR on chromosomal DNA of PCL1391. The obtained fragment was cloned in the *EcoRI* site of pBBR1MCS-5. The resulting vector was named pMP7455 and was introduced into PCL1114 by triparental mating to obtain PCL2012. The cloning vector pBBR1MCS-5 was introduced into PCL1114 in order to obtain the control strain PCL2011.

For over-expression of *phzR*, pMP7447 (Girard *et al.*, 2006) was introduced into PCL1114 to obtain PCL2013. For over-expression of *rpoS*, pMP7420 (Girard *et al.*, 2006) was transferred to PCL1114 to obtain PCL2036. For over-expression of *psrA*, pMP7465 (Girard *et al.*, 2006) was introduced into PCL1114 to obtain PCL2046. In order to study the effect of over-expression of *pip* on *phzR* expression, pMP7455 was introduced into PCL1104 (Chin-A-Woeng *et al.*, 2001), which resulted in PCL2035.

The *pip* gene was also constitutively expressed in wild-type, *psrA* and *rpoS* mutant backgrounds. Therefore the plasmid pMP7455 was introduced into PCL1391, PCL1111 (Chin-A-Woeng *et al.*, 2005) and PCL1954 (Girard *et al.*, 2006) by triparental mating and the resulting strains were named PCL2019, PCL2038 and PCL2040, respectively.

Primers oMP1045 (5'-ATATATGAATTCGAGGTCAGCCGGGCCAAGGAG-3') and oMP817 (5'-ATATATGAATTCAGGATGCGGTTGAAACCCTGTGCCGCC-3') were used for PCR on chromosomal DNA of PCL1391 with Phusion enzyme (Finnzymes) to obtain *pip* with 424 nucleotides of the sequence upstream of its start codon. The 1.1 kb product was cloned in the *EcoRI* site of pBBR1MCS-5. The orientation of the insert was verified by PCR and a clone was selected in which *pip* and the β -galactosidase gene of pBBR1MCS-5 have opposite directions of transcription. This plasmid was named pMP7487 and verified by sequencing for the lack of possible mutations introduced by PCR. It was introduced into strains PCL1114, PCL1104 and PCL1111 (*pip*, *phzR* and *psrA* mutants, respectively) to obtain strains PCL2085, PCL2086 and PCL2087, respectively.

Extraction and analysis of phenazine and *N*-acyl-homoserine lactones

Phenazine extraction was carried out on supernatants of 10 ml liquid MVB1 cell cultures at regular time points during growth and/or after overnight growth as described previously (van Rij *et al.*, 2004). For *N*-AHL extraction, supernatants from 50 ml MVB1 cultures were harvested at OD₆₂₀ 3.0 and mixed with 0.7 volume of dichloromethane and shaken for 45 min after which the organic phase was collected. The extract was dried using a rotary evaporator. The dried residue was redissolved in 25 µl of acetonitrile and spotted on RP-C18 TLC plates (Merck, Darmstadt, Germany). The TLC plates were developed in methanol-water (60:40, v:v). For detection of *N*-AHLs, the TLC was overlaid with 0.8% agar LC 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 analyzed for the appearance of violet spots, indicating the presence of *N*-AHLs.

Expression analysis of bioluminescent Tn5*luxAB* reporter strains

Expression of *pip* was monitored in various derivatives making use of the *luxAB* reporter genes of the Tn5 derivative in PCL1114. Expression was determined by quantification of bioluminescence during culturing. Cells from overnight MVB1 cultures were washed with fresh medium and diluted to OD₆₂₀ 0.1 in 10 ml fresh MVB1 medium. During growth, OD₆₂₀ was measured at regular intervals, and 100 µl samples were taken in duplicate to quantify luminescence. A volume of 100 µl of *N*-decyl-aldehyde substrate solution [0.2% *N*-decyl-aldehyde (Sigma, St. Louis, MO, USA) in a 2% bovine serum albumin solution] was added. After 5 min of incubation at room temperature, bioluminescence was determined with a MicroBeta 1450 TriLux luminescence counter (Wallac, Turku, Finland) and normalized to the luminescence per OD₆₂₀ unit.

Western blot analysis

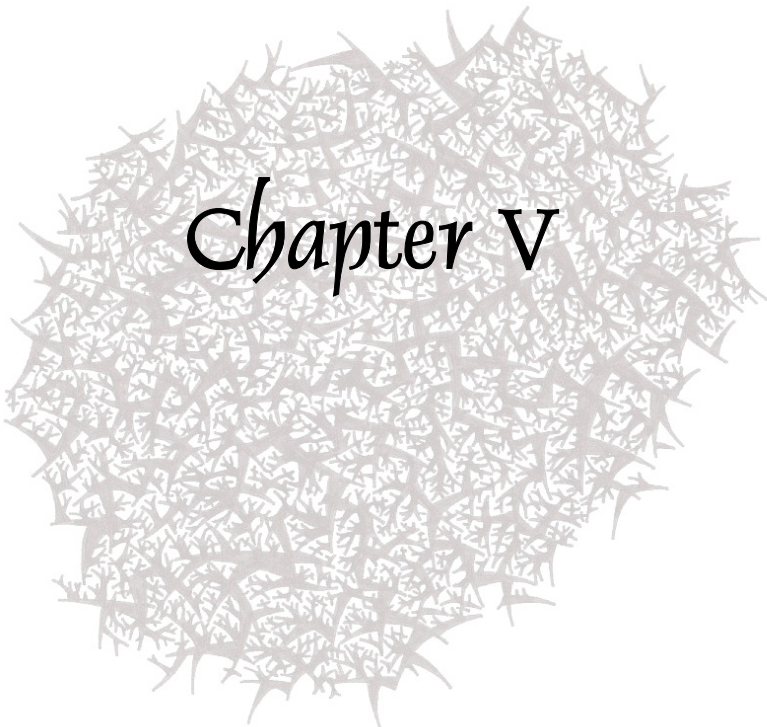
Cells were grown after inoculation of 10 ml MVB1 with an overnight culture washed with fresh medium and subsequently diluted to OD₆₂₀ of 0.1. Cells were harvested at OD₆₂₀ 1.0 or 2.2 (cultures are diluted 10-fold before OD₆₂₀ measurement) in volumes corrected for equal cell amounts. Cell pellets were resuspended in 200 µl of cracking buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 2 mM EDTA, 10% glycerol, 0.01% bromophenol blue, 1% β-mercaptoethanol) and boiled for 3 minutes. The samples were subsequently loaded on a 10% SDS-

polyacrylamide gel and proteins were separated and blotted following a standard Western blot procedure (Ausubel *et al.*, 1997). A dried aliquot of rabbit antibodies against RpoS was kindly provided by Prof. K. Tanaka (Tokyo, Japan). This sample was resuspended in 100 μ l PBS, diluted 1000-fold and allowed to react with the blot. The blots were subsequently incubated with peroxidase-labeled goat anti-rabbit antiserum (Amersham Biosciences, Roosendaal, The Netherlands). Finally, blots were incubated in a luminal solution (250 μ M sodium luminol (Sigma), 0.1 M Tris-HCl, pH 8.6, 0.01% H_2O_2) mixed with 60 μ l enhancer solution [67 μ M p-hydroxy coumaric acid (Sigma) in DMSO]. Hybridizing protein bands were detected using a Super R-X photographic film (Fujifilm, Düsseldorf, Germany).

ACKNOWLEDGEMENTS

The authors thank Daan van den Broek and Thomas Chin-A-Woeng for screening the transposon bank and isolating PCL1114, Prof. K. Tanaka (Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) for providing the anti-RpoS rabbit serum and Prof. B. J. J. Lugtenberg for critical reading of the manuscript.

This project was financially supported by the FW6 EU R&D project QRLT-2002-00914 (acronym “Pseudomics”). The work was financially supported by the BioScience Initiative from Leiden University.



Chapter V

**The transcriptional regulator Pip is involved
in the repression of phenazine synthesis
under stress conditions in
Pseudomonas chlororaphis PCL1391**

Geneviève Girard, Sharief Barends, Sébastien Rigali, E. Tjeerd
van Rij and Guido V. Bloemberg

Submitted

ABSTRACT

Pseudomonas chlororaphis PCL1391 is used as a model organism to study the regulation of secondary metabolism. This rhizobacterium synthesizes an antifungal metabolite named phenazine-1-carboxamide (PCN). PCN synthesis is not only regulated by environmental factors, but also by a complex network of regulatory proteins. Previous work unraveled a cascade of genes upstream of the *phzABCDEFGH* biosynthetic operon, responsible for the synthesis of PCN. However, no study was so far conducted to understand if environmental factors influence PCN synthesis *via* one of the genes of the known regulatory cascade. Here we show that the minimum inhibitory concentration (MIC) of several stress factors is lower for PCN synthesis than for growth of *P. chlororaphis* PCL1391. Subsequently, we tested if the constitutive expression of one of these cascade regulators could restore PCN synthesis in the presence of these stress factors. The results show that the inhibitory effect of the tested stress factors on PCN synthesis involves the Pip transcriptional regulator. For most of the tested stress factors, we propose that Pip is inhibited in response to stress, resulting in switching off PCN synthesis to favor stress resistance.

INTRODUCTION

Among Gram-negative bacteria, pseudomonads are known to produce a wide variety of secondary metabolites, including simple organic molecules such as hydrogen cyanide, enzymes such as chitinases and exoproteases, and antibiotics such as 2,4-diacetyl-phloroglucinol (Phl) and various phenazines (Chin-A-Woeng *et al.*, 2003; Haas & Defago, 2005; Raaijmakers *et al.*, 2002). A lot of these metabolites are involved in direct attack of other microorganisms. In contrast to primary metabolites, secondary metabolites are not essential for growth and reproduction. Many secondary metabolites play a role in interactions between *Pseudomonas* species and other organisms, particularly in pathogenesis and biocontrol. For example pyocyanin produced by *P. aeruginosa* is suggested to be involved in lung infection of cystic fibrosis patients (Lau *et al.*, 2004) whereas Phl and hydrogen cyanide (HCN) produced by *P. fluorescens* protect tobacco plants from black root rot (Laville *et al.*, 1992). The study of the regulation of secondary metabolism is therefore of interest for medicine, agriculture and industry.

Regulatory cascades in *Pseudomonas* species involve various genes. In most of the species, the GacS/GacA two-component system is a master regulator of many traits of secondary metabolism, such as production of HCN and Phl (Laville *et al.*, 1992), production of exoprotease and phospholipase C (Sacherer *et al.*, 1994), and production of phenazine (Reimmann *et al.*, 1997). The GacS sensor is inserted in the membrane that, supposedly after binding an unknown signal, activates the GacA transcriptional regulator by phosphorylation (Heeb *et al.*, 2002; Zuber *et al.*, 2003). Direct targets of GacA are so far unknown. In many *Pseudomonas* species, quorum-sensing also regulates secondary metabolism. The quorum-sensing involves a LuxI homologue synthesizing *N*-acyl-homoserine lactone (*N*-AHL) signal molecules, which are able to traffic across membranes. Their extracellular concentration reflects the number of bacteria present in a (semi-) closed environment. *N*-AHLs bind to a LuxR homologue, thereby activating it. Activated LuxR-like proteins function as transcriptional regulators. *N*-AHLs enable bacteria to sense the density of their population and to induce specific (sets of) genes (Fuqua *et al.*, 2001; Miller & Bassler, 2001).

For the study of the regulation of secondary metabolism, our group uses *P. chlororaphis* PCL1391, a soil bacterium that colonizes tomato roots, as a model. In the rhizosphere it produces phenazine-1-carboxamide (PCN), an antifungal metabolite that inhibits growth of the causative fungal agent of tomato foot and root rot, *Fusarium oxysporum* f. sp. *radicis lycopersici*. The regulation of the synthesis of

PCN is complex (Fig. 1), involving the influence of environmental factors (van Rij *et al.*, 2004) as well as of several genes. The regulatory genes include the GacS/GacA two-component system upstream of the TetR homologue PsrA and the sigma factor RpoS (Girard *et al.*, 2006). Recently, another putative TetR homologue, Pip, was shown to regulate PCN synthesis downstream of PsrA and RpoS (Chapter 4). All these genes are involved in inducing expression of the *phz* operon via the quorum-sensing genes *phzI* and *phzR*. It was shown that a constitutively expressed quorum-sensing system is sufficient for synthesis of PCN in all the mutants of the mentioned regulatory genes (Girard *et al.*, 2006) (Chapters 2 and 4).

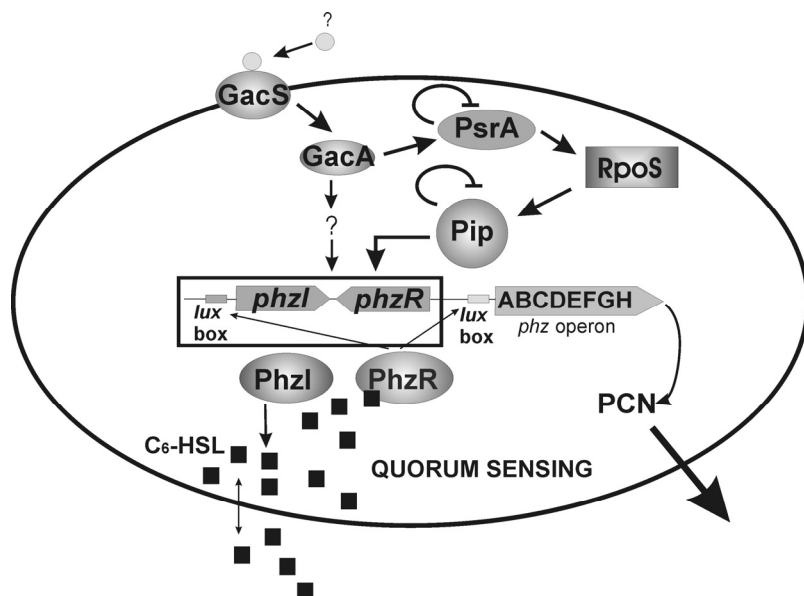


Figure 1. Schematic model showing the role of Pip in the genetic cascade regulating PCN synthesis in *P. chlororaphis* PCL1391. The regulatory cascade of PCN starts with the sensing of a so far unidentified environmental signal by GacS and subsequent activation of GacA. The TetR-homologue PsrA regulates *rpoS* probably by binding to its promoter. The alternative sigma factor RpoS positively regulates *pip*, the product of which stimulates expression the quorum-sensing system *phzI*/*phzR*. Both *pip* and *psrA* exhibit negative autoregulation. PhzI is responsible for the production of C₆-HSL, which is supposed in turn to bind to PhzR. The PhzR-C₆-HSL complex binds to *lux* boxes in the promoter sequences of *phzI* and the *phz* operon. Subsequently *phzI* is upregulated and expression of the *phz* operon is switched on, which finally results in the synthesis of phenazine-1-carboxamide (PCN).

Following the previous observation that various stresses inhibit PCN and *N*-AHL synthesis (van Rij *et al.*, 2004; van Rij *et al.*, 2005), we designed experiments to elucidate the molecular mechanisms that explain the repression of the *phz* operon by several stress factors. We show that stress caused by the addition of antibiotics has similar effects. Subsequently, the influence of stress on genes of the PCN

regulatory cascade was studied. Results are consistent with the notion that in response to several stress factors, Pip is repressed, which in turn inhibits the quorum-sensing system and the *phz* operon. Therefore, Pip may be an essential target for the switch between primary and secondary metabolism.

RESULTS

Influence of antibiotic stress on PCN production by *P. chlororaphis* PCL1391

Previously, it was shown that supplementing the growth medium of *P. chlororaphis* PCL1391 with certain concentrations of fusaric acid and NaCl resulted in an inhibition of PCN synthesis whereas the overall growth was not significantly influenced (van Rij *et al.*, 2004; van Rij *et al.*, 2005). In this study the influence of antibiotic stress on PCN synthesis was analyzed by testing sub-lethal concentrations of antibiotics. Colony forming units (CFUs) and PCN production of overnight cultures were measured after applying a concentration range of NaCl, kanamycin and rifampicin (Fig. 2). PCN synthesis was inhibited when the growth medium was supplemented with more than 10 mM NaCl, 0.5 µg/ml kanamycin or 0.2 µg/ml rifampicin, while cells could grow in MVB1 medium containing up to 333 mM NaCl, 5 µg/ml kanamycin or 2 µg/ml rifampicin. These results show that the minimum inhibitory concentration (MIC) of the stress factors tested is about 10-fold lower for PCN synthesis than the MIC for growth of the cells. This is not true with all stress factors, since the MIC of gentamicin for PCN synthesis is the same as for growth (3 µg/ml) (data not shown).

Stress and PCN regulatory genes

In previous work (Chapters 2 and 4), a cascade of genes regulating the *phz* operon was characterized (Fig. 1). Addition of salt or fusaric acid to cultures of PCL1391 resulted in a strong decrease of PCN and *N*-AHL synthesis (van Rij *et al.*, 2004). PCN-inhibitory concentrations of the stress factors were chosen for the next experiments. The absence of detectable *N*-AHL was verified at 333 mM NaCl (Fig. 3, panel A). Addition of 5 µg/ml kanamycin or 2 µg/ml rifampicin in the culture medium also resulted in inhibition of *N*-AHL synthesis (Fig. 3, panels A and D).

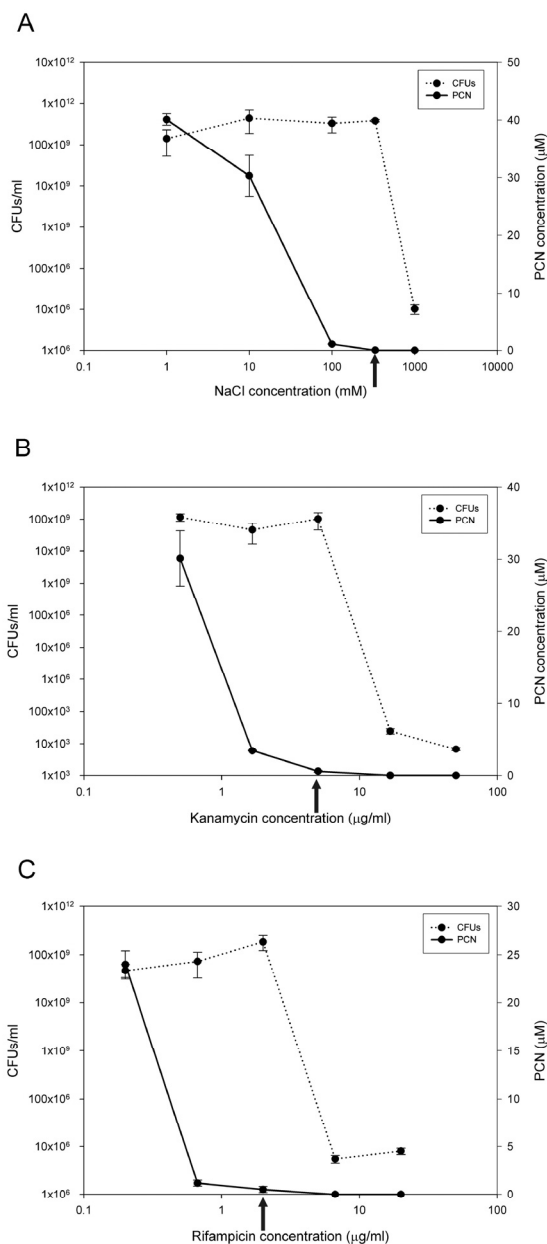


Figure 2. Growth and PCN synthesis of *P. chlororaphis* PCL1391 after overnight incubation under various stress conditions.

Cells of *P. chlororaphis* PCL1391 were grown in 10 ml MVB1 medium supplemented with various concentrations of NaCl (Panel A), kanamycin (Panel B) and rifampicin (Panel C). Concentrations of PCN and CFUs of cultures grown for 20h were determined.

The experiment was repeated twice. Means and standard deviations of duplicate experiments with the same culture are plotted.

Arrows indicate the concentrations chosen for subsequent experiments shown in Figure 3 and Table 1.

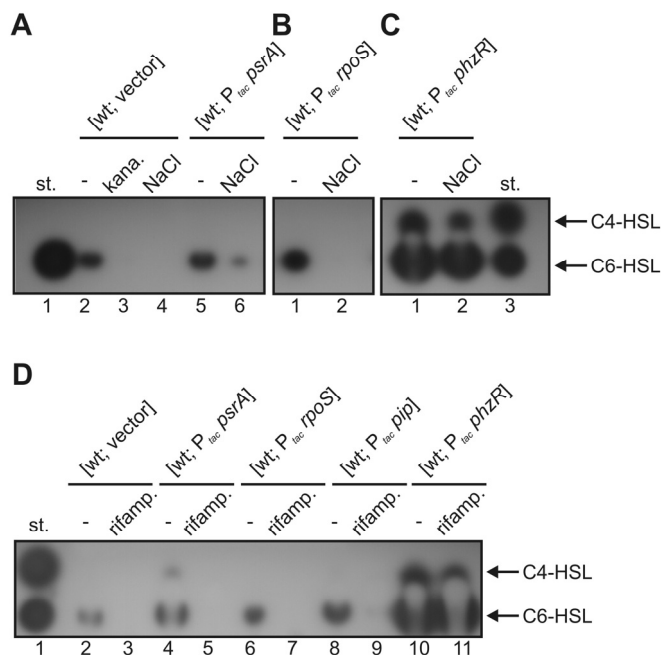


Figure 3. C18-reverse phase TLC analysis of N-AHL produced by various derivatives of strain PCL1391. Various derivatives were grown in MVB1 alone or in the presence of various stress factors as indicated on the figure. Standards: on panel A (2.5 nmol of synthetic C₆-HSL [lane 1]) and on panel D (2.5 nmol C₆-HSL [bottom lane 1] and 2 nmol C₄-HSL [top lane 1]).

In order to test whether the constitutive expression of some PCN regulatory genes could restore PCN production under stress conditions, PCN production of several PCL1391 derivative strains was measured with and without inhibiting concentrations of fusaric acid (1 mM), kanamycin (5 µg/ml), NaCl (333 mM) and rifampicin (2 µg/ml) (Table 1). Constitutive expression of *phzR* was able to restore PCN production under all conditions tested. However, constitutive expression of neither *pip*, nor *rpoS*, nor *psrA* was able to fully restore PCN production under any of the tested stress conditions. Interestingly, the applied concentrations of fusaric acid, kanamycin and NaCl inhibit the growth of PCL2019, which constitutively expresses *pip* (Table 1). Although the cultures of PCL2019 reached a very low OD₆₂₀ after overnight growth (less than 1.0), PCN production was easily detectable. Calculations show indeed that PCL2019 produces 3000.10⁻¹¹, 2000.10⁻¹¹ and 2400.10⁻¹¹ nmol/CFU of PCN in MVB1 non supplemented, supplemented with 1mM of fusaric acid or with 5 µg/ml kanamycin, respectively, whereas in the same

Table 1. CFUs and PCN production in overnight cultures of various *P. chlororaphis* PCL1391 derivative strains in the presence of various stress factors.

strains in the presence of various stress factors.

Supple- ments to MVB1		Empty vector‡	P _{tac} <i>psrA</i>	P _{tac} <i>rpoS</i>	P _{tac} <i>pip</i>	P _{tac} <i>phzR</i>	P _{pip} <i>pip</i>
None	CFUs (%)*	100	100	100	100	100	100
	PCN pro- duction /OD ₆₂₀ (μM)	69±16	101± 24	64±9	117±6	239 ±58	9±3
Fusaric acid (1 mM)	CFUs (%)	108±8	41±1	45±2	0.74 ±0.05	57±16	160± 9
	PCN pro- duction /OD ₆₂₀ (μM)	3±0.8	13±9	1±0.9	2±0.4	74±4	102±1
Kanamy- cin (5 μg/ml)	CFUs (%)	60±12	37±1	67±3	0.08 ±0.01	46±15	91±2
	PCN pro- duction /OD ₆₂₀ (μM)	0.7±0.3	0.8±0.3	0.4 ±0.2	2.9±2.7	96±9	67±0.1
NaCl (333 mM)	CFUs (%)	460±89	79±25	26±4	0.14 ±0.02	282 ±11	436±63
	PCN pro- duction /OD ₆₂₀ (μM)	0.2±0.0	0.8±0.5	0.1 ±0.0	23±0.1	103±3	5±0.2
Rifampi- cin (2 μg/ml)	CFUs (%)	104±18	26±6	75±32	52±5	43±2	120±3
	PCN pro- duction /OD ₆₂₀ (μM)	0.1 ±0.01	0.04 ±0.01	0.1 ±0.01	3.8±0.1	165 ±53	30±1

*CFUs were counted after 20 hours of growth at 28°C. The number of CFUs for each strain was normalized to 1 under no stress conditions. The real numbers in CFUs/ml are $2.68 \cdot 10^{10}$ for PCL1960, $3.27 \cdot 10^{10}$ for PCL2044, $3.93 \cdot 10^{10}$ for PCL1958, $5.02 \cdot 10^{10}$ for PCL1993, $1.66 \cdot 10^{10}$ for PCL2019 and $1.56 \cdot 10^{10}$ for PCL2089.

‡Various strains used in this table: PCL1960 (empty vector), PCL2044 (P_{tac} *psrA*), PCL1958 (P_{tac} *rpoS*), PCL2019 (P_{tac} *pip*), PCL1993 (P_{tac} *phzR*) and PCL2089 (P_{pip} *pip*).

conditions PCL1960 produces 260.10^{-11} , 11.10^{-11} and 5.10^{-11} nmol/CFU of PCN, respectively.

The synthesis of *N*-AHLs by various derivative strains was analyzed after addition of 333 mM NaCl or 2 µg/ml rifampicin. Strains constitutively expressing *rpoS* or *psrA* were not able to restore *N*-AHL production to a wild-type level (Fig. 3, panels B, C and D). In the presence of rifampicin, PCL2019 constitutively expressing *pip* produced relatively small amounts of *N*-AHL, as compared to no stress conditions (Fig. 3, panel D), but higher amounts than the wild-type.

Since constitutive expression of *pip* inhibits growth under stress conditions, we tested the effect of *pip* expression under the control of its own promoter in a low-copy plasmid under stress conditions. Growth of PCL2089 (wt + *P_{pip}* *pip*) was not inhibited as compared to PCL2019 (wt + *P_{tac}* *pip*) (Table 1). In addition, on the contrary to PCL1960 (wt with empty vector) PCL2089 showed high PCN production under every condition tested, except at 333 mM NaCl (Table 1). However, PCN production at 100 mM NaCl was restored in PCL2089 (85.1 ± 1.6 µM) compared to PCL1960 (0.83 ± 0.05 µM) harboring the empty cloning vector. These results indicate Pip as a target of stress factors.

Role of Pip under stress conditions

To test the effect of stress on *pip* further, a classical survival test to high osmolarity (see Experimental Procedures) as reported in studies of *rpoS* (Ramos-González & Molin, 1998; Sarniguet *et al.*, 1995; Suh *et al.*, 1999) was performed. Wild-type and the *pip* mutant showed similar survival rate, whereas the *rpoS* mutant had a decreased survival rate (Fig. 4).

The effect of various stresses on production of the Pip protein was also tested. Since an antiserum is not available for Pip, a plasmid (pMP7488) was constructed expressing a His-tagged Pip under the control of the *pip* promoter. The *pip* mutant PCL1114 was transformed with pMP7488 to obtain PCL2088. PCL2088 showed the same phenotype as PCL2085 (data not shown), meaning that the His-tag did not affect the functioning of Pip. PCL2088 was grown in MVB1 under different stress conditions, including 333 mM NaCl, 2 µg/ml rifampicin and 1 mM fusaric acid. RpoS and Pip were detected with an anti-RpoS serum and an anti-His antibody, respectively (Fig. 5). PCL2085, the *pip* mutant derivative expressing *in trans* non-His-tagged *pip* under the control of its own promoter, was used as a negative control. The results show that RpoS is present under all conditions (Fig. 5, panel A), but in increased amounts in the presence of fusaric acid (Fig. 5, panel A,

compare lanes 3 and 1) and NaCl (Fig. 5, panel A, compare lanes 4 and 1) and in slightly increased amounts in the presence of rifampicin (Fig. 5, panel A, compare lanes 2 and 1). These amounts are modestly increased but in a reproducible manner. RpoS amounts detected in strains producing Pip with or without His-tag are comparable (data not shown).

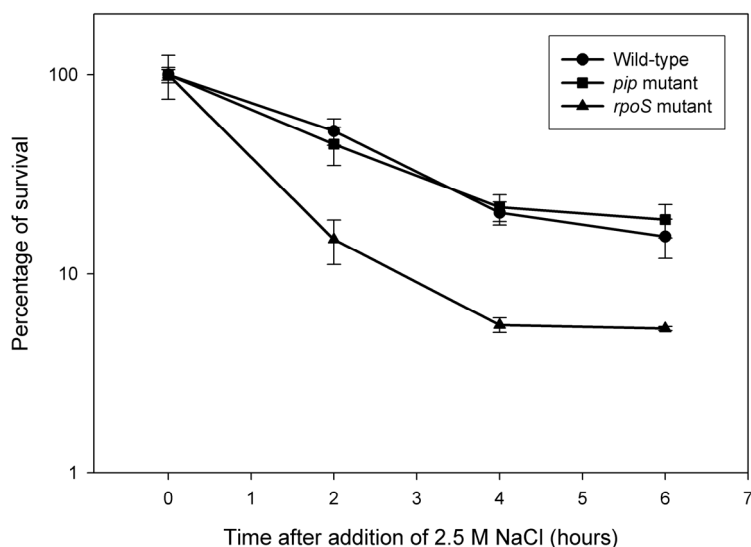


Figure 4. Resistance of strain *P. chlororaphis* PCL1391 and mutant strains to salt stress.

Cells from 3 ml of overnight cultures of wild-type strain PCL1391, *pip* transposon mutant PCL1114 and *rpoS* recombinant mutant PCL1954 were washed and resuspended in 10 ml fresh MVB1 medium. CFUs were determined at time 0, 2, 4 and 6 hours after addition of 2.5 M NaCl. Survival was determined as relative viable counts. Viable counts at time zero were normalized to 100%. The experiment was repeated twice. Means and standard deviations of duplicate experiments with the same culture are plotted.

A strain was constructed that expresses P_{tac} *pip-6his* under P_{tac} promoter and Pip-6His was purified in high amounts through a nickel column. Hybridization with the anti-His antibody resulted in a very intense band after detection (Fig. 5, panel B, lane 6). Bacteria grown under fusaric acid and NaCl stress showed decreased amounts of Pip (Fig. 5, panel B, compare lanes 4-5 and 2). However, rifampicin stress did not really affect the production of Pip (Fig. 5, panel B, compare lanes 3 and 2). To test the effect of kanamycin, (*pip* + P_{pip} *pip-6his*) could not be used because it is Km^r . (wt + P_{pip} *pip-6his*) was used instead and showed a strong decrease in Pip amounts (Fig. 5, panel C, compare lanes 3 and 2).

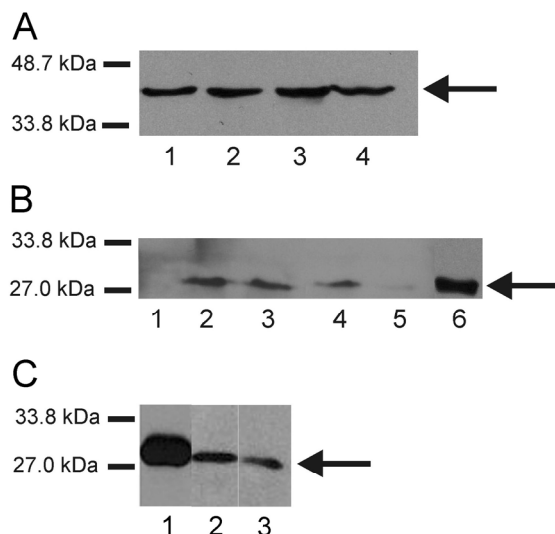


Figure 5. Western blot analysis of RpoS and Pip production in *P. chlororaphis* PCL1391 and derivative strains.

Panel A: analysis of RpoS production in PCL2088 (*pip* + P_{pip} *pip-6his*) grown without stress (lane 1), with 2 μ g/ml rifampicin (lane 2), with 1 mM fusaric acid (lane 3) and with 333 mM NaCl (lane 4). An arrow indicates RpoS.

Panel B: analysis of Pip production in PCL2085 (*pip* + P_{pip} *pip*) (lane 1) and PCL2088 (*pip* + P_{pip} *pip-6his*) (lanes 2-5) grown without stress (lanes 2), with 2 μ g/ml rifampicin (lane 3), with 1 mM fusaric acid (lane 4) and with 333 mM NaCl (lane 5). Lane 6: purified Pip-6His. An arrow indicates Pip.

Panel C: analysis of Pip production in (*wt*; P_{pip} *pip-6his*) at OD₆₂₀ 1.0 without stress (lane 2) or grown with 5 μ g/ml kanamycin (lane 3). Lane 1: purified Pip-6His.

Resistance to stress *versus* secondary metabolism

The results described above indicate that stress inhibits the synthesis of *N*-AHL and PCN via Pip. This model implies that inhibition of *N*-AHL and PCN synthesis would give a real advantage for the growth of bacteria under stress conditions. Wild-type and *phzI* mutant showed similar growth with fusaric acid, NaCl and rifampicin (Fig. 6). However, if a vector expressing P_{tac} *phzR* was present in the strains, the strain with *phzI* background clearly grew better than the strain with wild-type background (Fig. 6) under all stress conditions tested. This result shows that when PCN is off [*phzI*; P_{tac} *phzR*], cells do grow in the presence of different stress factors, whereas when cells are forced to produce PCN [*wt*; P_{tac} *phzR*], they show reduce growth under stress. The experiment could not be performed with kanamycin, since the *phzI* mutant is Km^r. So, as the model predicts, growth under stress significantly improves when PCN synthesis is blocked.

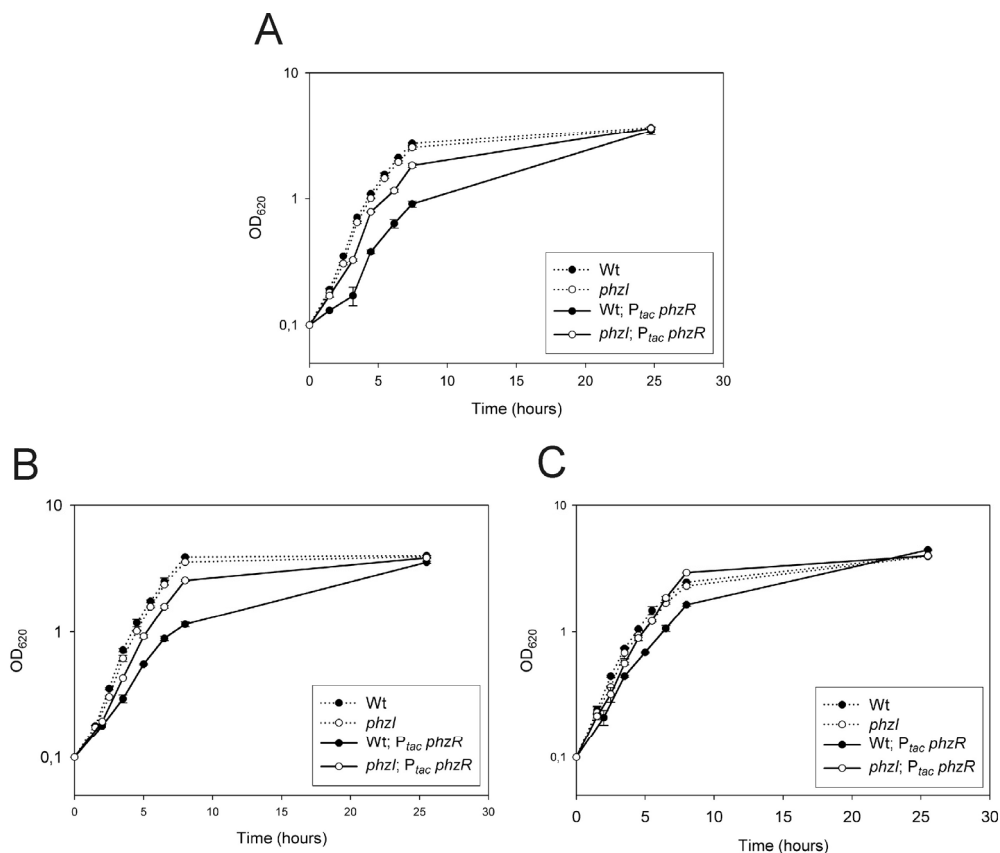


Figure 6. Growth of several *P. chlororaphis* PCL1391 derivatives under various stress conditions. The strains used are wild-type background (●) or *phzI* (○) background with empty vector (plain lines) or vector containing *P_{tac} phzR* (dotted lines). Cell cultures were grown in 10 ml MVB1 medium supplemented with 1 mM fusaric acid (panel A), 100 mM NaCl (panel B) or 2 µg/ml rifampicin (panel C) and samples were taken at regular time intervals for measuring OD₆₂₀.

Searching for the direct target of Pip

Because of the homology between Pip and AcrR/TetR, we considered the hypothesis that Pip might directly regulate the expression of a gene encoding an efflux pump. Results described above could support that this pump would secrete PCN or even more likely *N*-AHL. To test this hypothesis, the effect of PCN and *N*-AHL on *pip* transcription was measured, since it was shown that the expression of *acrR* and *tetR* are under the regulation of the molecules secreted by their target pumps. Results show that neither PCN, nor *N*-AHL regulates the activity of *P_{pip}* (Fig. 7, panels A and B). The same results were obtained with an intact *pip* gene *in trans* by using the strain (*pip*::Tn5*luxAB* + *P_{pip} pip*). Importantly, the tested concentrations of PCN and *N*-AHL were sufficient to inhibit *phzB* (Fig. 7, panel C) and stimulate *phzI*

(Fig. 7, panel D), respectively. These results indicate that the transcription of *pip* is not influenced by levels of *N*-AHL or PCN.

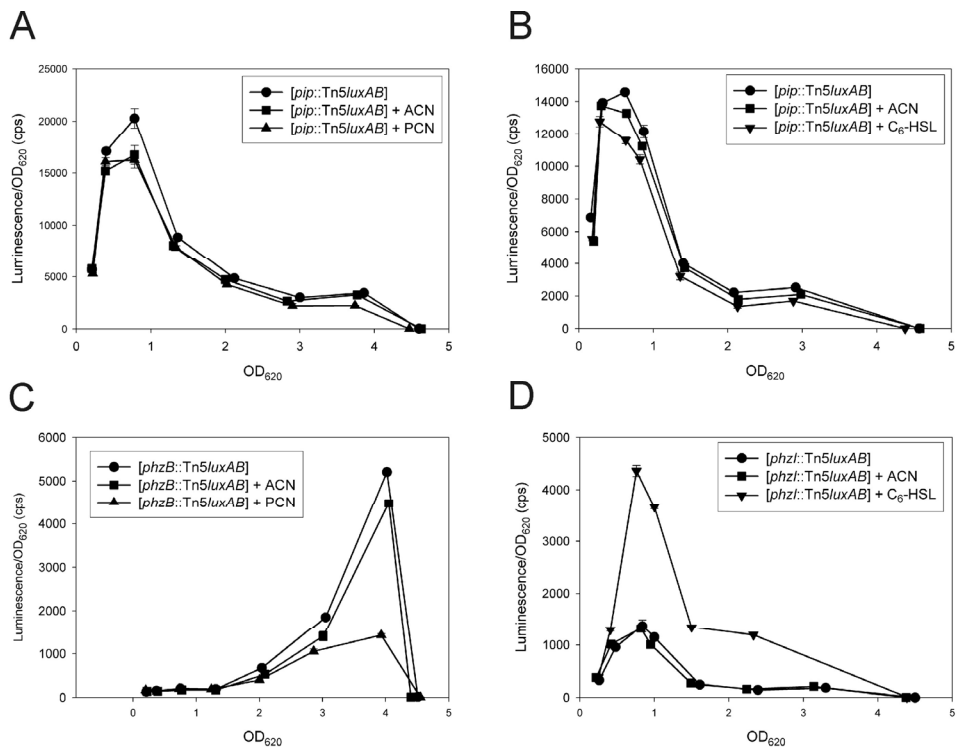


Figure 7. Influence of *C*₆-HSL and PCN on *P*_{*pip*} activity. Cell cultures were grown in 10 ml MVB1 medium supplemented or not (●) with acetonitrile (ACN, ■), 2 μM PCN (▲) or 5 μM *C*₆-HSL (▼). Samples were taken at regular time intervals. Activity of the *luxAB* reporter was determined by quantifying bioluminescence. Measurements were performed in duplicate and averages are plotted. The bars represent the standard deviation and some are too small to be visible. The strains used in these experiments are PCL1114 (*pip*::Tn5*luxAB*) (panels A and B), PCL1119 (*phzB*::Tn5*luxAB*) (panel C) and PCL1103 (*phzI*::Tn5*luxAB*) (panel D).

Since Pip shows homologies with DNA-binding transcriptional regulators and autoregulates its own expression, we searched for a Pip-specific *cis*-acting element within its upstream region. To precisely determine the exact location of the Pip binding site within its upstream region, we made an *in silico* search of conserved motifs in several upstream region of *pip* orthologues. A 47-nt sequence (with the consensus CGCCATCGCGCTTCCTTCGCTGGGCGGCGCGCCCCATAATC GCCCG) was proposed as the best conserved motif among orthologous *pip* upstream regions. The putative -35 and -10 boxes previously suggested (Chapter 4) are included in this sequence. A 120-bp PCR product of the promoter region of *pip* containing the conserved sequence was obtained and labeled with [³²P] (see Experimental

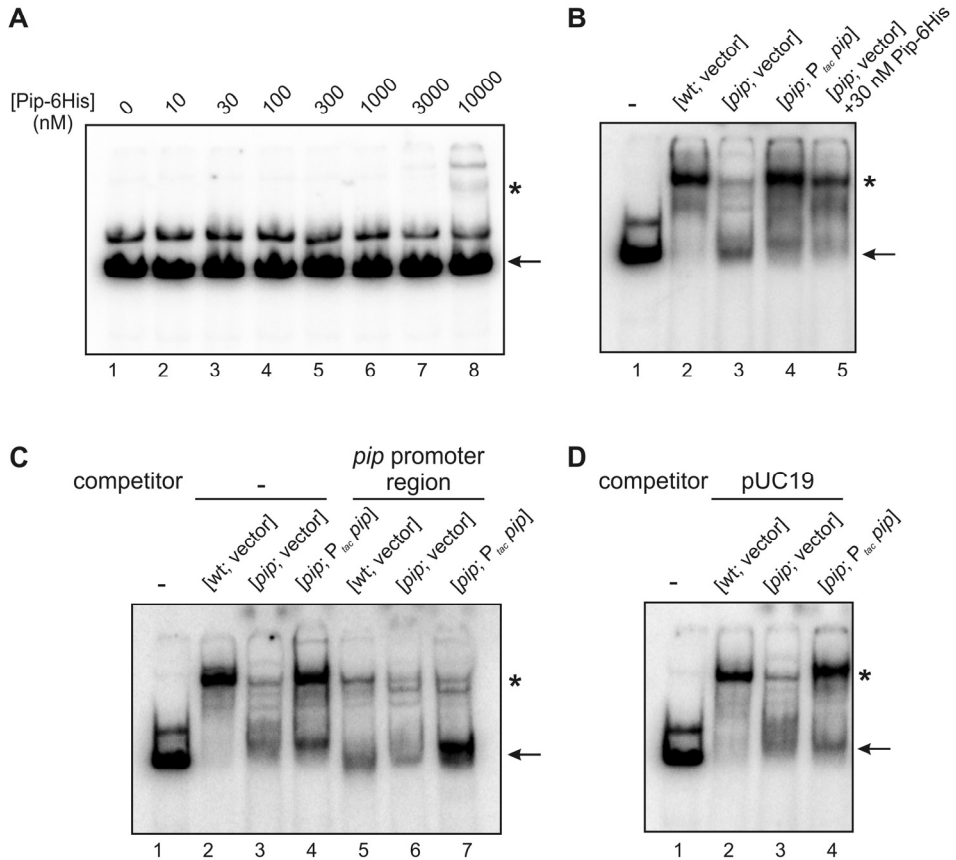


Figure 8. Binding of Pip to the promoter region of *pip*.

One ng of [³²P]-labeled DNA fragment of 140 bp, corresponding to the *pip* promoter region, was used as probe for bandshift assays with either purified Pip protein (Panel A), with cell-free extracts from different strains (Panels B, C and D) or a combination of both (Panel B). Competition assays were performed with 50 ng of *pip* promoter region (Panel C) and with 50 ng of pUC19 plasmid (Panel D). Samples were separated on 8% native PAGE at 120 V during 20 min, and bands were visualized by phosphor imaging. The arrow indicates the position of the free probe and the asterisk that of the complex.

Procedures). Purified Pip-6His did not seem to be able to bind and shift the labeled DNA (Fig. 8, panel A). However, S30 fractions of the wild-type and of (*pip*; P_{tac} *pip*) were able to bind the [³²P]-labeled PCR product as shown by band retardation, whereas the S30 fraction of the *pip* mutant was not (Fig. 8, panel B, lanes 2, 4 and 3, respectively). Interestingly, the addition of purified Pip-6His in the S30 fraction of the *pip* mutant resulted in the shifting of the DNA (Fig. 8, panel B, lane 5). Competition with the unlabeled PCR product inhibited the shift, whereas competition with unlabeled pUC19 plasmid did not (Fig. 8, panels C and D, respectively). These results show that Pip is able to specifically recognize and bind a

DNA sequence including in the 120 bp upstream of the start codon. However, an additional element present in S30 fractions obtained from *P. chlororaphis* cultures is apparently necessary for Pip DNA-binding activity.

Table 2. Results of the *in-silico* research was performed in the *P. aeruginosa* PAO1 genome using the Predict Regulon Server

Score	Position	Site	Gene	Function
9.7759 5	-78	CGGCAGGTGCTCTTCC TACAGCCGGGCGGGCC CCGCATAATCGGCCG	PA0243	probable transcriptional regulator
8.2913 9	-83	GGTCGCGGACGGTTCC CAGGCGGGCCCATCCC CCGCATAATTGGCGC	<i>epd</i> /PA05 51	D-erythrose 4- phosphate dehydrogenase conserved
8.2794	-65	CGTCCCGACGCGTGGC CCCGCCCTCCTGTCTC CGGCATAATCGCCCG	PA1189	hypothetical protein conserved
8.1658	-84	CGCTTTCCGGGTTTGCC GCCACTTTCGTGCGTCA CCATAATCATCCC	PA4969	hypothetical protein
8.1234 9	-228	AGCCGACGCCTCTTCC CTCTCCTGCCGTTCTG TTCCTGGTCGGCTG	PA5406	hypothetical protein
8.0918 8	-282	GGCTCGCCACTGTTCC ATCTGTTTCAGCGGGGC GACCATGCTCGGCGC	PA3493	conserved hypothetical protein
8.0672	-75	CGCCCGGCGGGTTCCC CCCTGGCCGGCGCGGT CTGTATACTGGCCGG	PA5130	conserved hypothetical protein
8.0190 6	-197	GGACACGGCTCCTTCG GTACCCGCATGGGCGA CTACATCATCGCCTA	PA2289	conserved hypothetical protein
8.0014 8	-39	GGCCATTTCCGTTTCCA GCCCCAGCAGCGAGA ACCGTCATGCCCGG	PA0446	conserved hypothetical protein

Since a conserved region upstream of *pip* was shown to be recognized by Pip (Fig. 8), we hypothesized that similar conserved patterns could be present upstream of potential Pip target genes in genomes of pseudomonads. We generated a position weight matrix from the set of the seven conserved sequences upstream of *pip* homologues in other *Pseudomonas* species (see Experimental Procedures). No sequences with significant scores (similar to those obtained by the 47 nt motifs upstream *pip* homologues) were recovered from a very restrictive search in the partially sequenced (66%) genome of strain PCL1391. Identical results were obtained from *P. aeruginosa* PAO1 and *P. fluorescens* Pf-5 genome scans. As an alternative, the same *in-silico* approach was performed using a less restrictive program (Predict Regulon, see Experimental Procedures). This program predicted in

P. aeruginosa PAO1 eight sequences that contain patterns similar to the one observed upstream of Pip (Table 2). The best scoring hit is located 83 nt upstream the *epd* gene coding for the D-erythrose 4-phosphate dehydrogenase that connects the pentose phosphate pathway to the vitamin B6 metabolism. The other seven potential target genes all encode hypothetical proteins with unknown function.

DISCUSSION

Several stress factors inhibit PCN synthesis

Complex regulatory cascades allow the cell to modulate its response to various environmental conditions. PCN production was shown to be influenced by many environmental factors, including temperature, pH, iron, NaCl and fusaric acid (van Rij *et al.*, 2004; van Rij *et al.*, 2005). However, the link between environmental and genetic factors has not been explored in detail in *P. chlororaphis* PCL1391.

In order to elucidate the effect of stress on PCN production, several relevant stress factors were selected. NaCl is a common abiotic molecule of the soil and is a classical stress factor (Munns, 2005). Antibiotics are produced by many microorganisms, for example by *Streptomyces* species (Cundliffe, 1989), which is abundantly present in the soil. Rifampicin and kanamycin were chosen because they act differently, rifampicin at the transcriptional level by interfering with the β subunit of the RNA polymerase, whereas the kanamycin inhibits translation by binding to the ribosome. Finally, fusaric acid is a phytotoxin produced by *Fusarium oxysporum* (Bacon *et al.*, 1996), a competitor of *P. chlororaphis* in the rhizosphere (Bolwerk *et al.*, 2003; Chin-A-Woeng *et al.*, 2000; de Weert *et al.*, 2004). The effect of increasing concentrations of the selected stress compounds in *P. chlororaphis* cultures was tested. Increasing the concentrations of the two antibiotics and NaCl resulted in a decreased PCN production before growth was affected (Fig. 2). A similar effect was reported for fusaric acid (van Rij *et al.*, 2005). In case of gentamicin, the MIC for growth was identical to the MIC for PCN synthesis (data not shown), indicating that the observed effects of NaCl, fusaric acid, rifampicin and kanamycin cannot be generalized for every stress factor.

Pip is involved in the response to stress factors

It can be hypothesized that stress affects PCN synthesis *via* one or several genes of the previously described regulatory cascade of PCN synthesis (Fig. 1). Interestingly, productions of *N*-AHL and PCN were well correlated under stress conditions tested, since fusaric acid (van Rij *et al.*, 2005), NaCl, kanamycin and

rifampicin stress (Fig. 3) inhibited *N*-AHL production at concentrations inhibitory for PCN production. Moreover, constitutive expression of the quorum-sensing gene *phzR* restored production of *N*-AHL (Fig. 3) and PCN (Table 1) under all the stresses tested. These results show that stress inhibits PCN synthesis *via* the PhzI/PhzR quorum-sensing system.

To test whether quorum-sensing is inhibited by stress *via* known genes of the regulatory cascade, several of these genes were constitutively expressed under the stress conditions chosen. The inability of constitutively expressed *psrA* or *rpoS* to restore wild-type levels of PCN and *N*-AHL (Table 1 and Fig. 3) indicates that stress acts between *rpoS* and *phzI/phzR* or on a so far unknown second cascade regulating quorum-sensing and the *phz* operon. A candidate regulatory gene between *rpoS* and *phzI/phzR* is *pip*. Surprisingly, constitutive expression of *pip* strongly inhibited the growth of PCL1391 under all tested stress conditions (Table 1), except rifampicin. In addition, Pip production is reduced, probably indirectly, by PCN-inhibitory concentrations of NaCl, kanamycin and fusaric acid (Fig. 5, panel B). These results can be interpreted as follows (Fig. 9, panel B). (i) A reduced Pip production would allow the cell to save the energy usually spent to synthesize *N*-AHL and PCN and focus on stress resistance. (ii) Additionally, the regulatory cascade for PCN is then interrupted just downstream of *rpoS*, leaving the RpoS amounts unaffected and able to direct stress resistance, which is one of its primary roles (Jorgensen *et al.*, 1999; Ramos-González and Molin, 1998; Sarniguet *et al.*, 1995; Suh *et al.*, 1999). When *pip* is expressed constitutively, the regulatory cascade would be forced towards *N*-AHL and PCN synthesis, to the detriment of stress resistance and therefore of survival. This would explain that, although growth is strongly impaired, PCN is detected in amounts (Table 1 and see PCN concentrations in nmol/CFU in the Results section) that wild-type would not reach at this OD₆₂₀ value (van Rij *et al.*, 2004). The impaired survival to stress of PCL2019 expressing *pip* constitutively could also be explained by the fact that over-produced *pip* seems to inhibit *psrA* transcription (Chapter 4, p. 103, Fig. 4, panel C). This would result in a decreased production of RpoS, explaining the toxic effect of constitutive *pip* transcription in the cells.

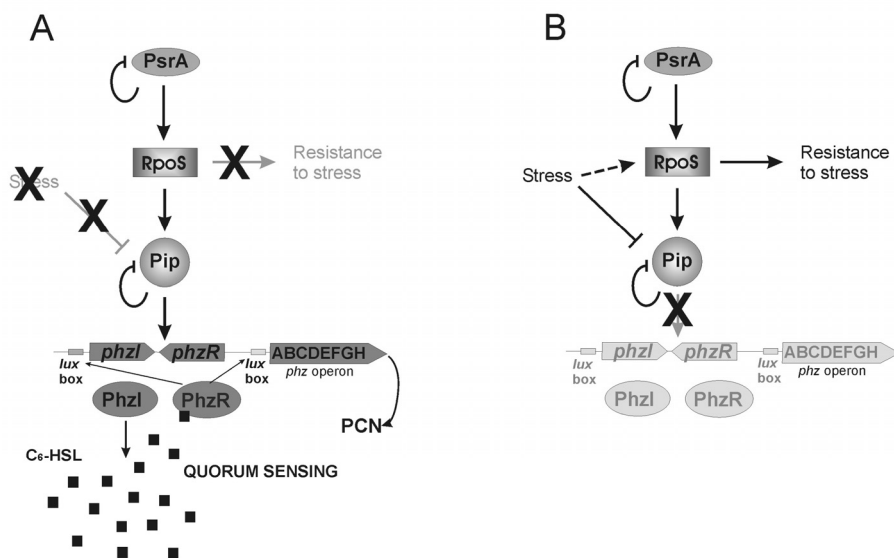


Figure 9. Schematic model showing the role of Pip in the genetic cascade regulating PCN synthesis in *P. chlororaphis* PCL1391 and in the presence of several stress factors.

Panel A. Under no particular stress, the regulatory cascade of PCN starts with the sensing of a so far unidentified environmental signal by GacS and subsequent activation of GacA. The TetR-homologue *PsrA* regulates *rpoS* probably by binding to its promoter. The alternative sigma factor *RpoS* positively regulates *pip*, the product of which stimulates expression of the quorum-sensing system *phzI/phzR*. Both *pip* and *psrA* exhibit negative autoregulation. *PhzI* is responsible for the production of C₆-HSL, which is supposed in turn to bind to *PhzR*. The *PhzR*-C₆-HSL complex binds to *lux* boxes in the promoter sequences of *phzI* and the *phz* operon. Subsequently *phzI* is upregulated and expression of the *phz* operon is switched on, which finally results in the synthesis of phenazine-1-carboxamide (PCN). Possibly *Pip* represses the genes involved in resistance to stress.

Panel B. In the presence of certain stress factors, such as NaCl, fusaric acid or kanamycin, *pip* expression is repressed in an unknown manner. The decrease in *Pip* concentrations results in the repression of *N*-AHL and PCN production, which allows the cell to save energy for its resistance to stress, via *RpoS*. Stress factors can stimulate *rpoS* expression (dashed line), but the inhibition of *pip* expression by stress factors overrules its stimulation by *RpoS*.

Validation of the proposed model

Several experiments were performed to test this model (Fig. 9) involving *Pip* as a switch between secondary metabolism, e.g. production of PCN, and primary metabolism, e.g. stress resistance. A first consequence of the model would be that a mutation in *pip* would not affect the resistance of PCL1391 to stress, since the cascades regulating PCN synthesis and stress resistance branch downstream of *rpoS* and just above *pip*. Therefore the resistance to increased osmolarity of a *pip* mutant was compared to that of the wild type and an *rpoS* mutant. Results (Fig. 4) show as expected that the wild-type survives better than the *rpoS* mutant PCL1954. A similar result was obtained in *P. putida* (Ramos-González & Molin, 1998). The *pip* mutant PCL1114 is as resistant to high osmolarity as the wild-type. This indicates

that the stress resistance mechanisms are not affected in the *pip* mutant, which also supports the model.

Amounts of Pip are decreased by NaCl, fusaric acid and kanamycin stress, indicating that stress inhibits *pip* expression (Fig. 5 panels B and C). On the contrary, the amounts of RpoS are stimulated by stress (Fig. 5 panel A). These data also support the model suggesting that some stress factors interrupt the PCN regulatory cascade downstream of *rpoS*, whereas RpoS is unaffected, or even stimulated, so that it can activate stress resistance genes. However, this model cannot be generalized to all stress conditions, since *pip* expression is not affected by addition of rifampicin in the culture. It is very likely that other factors explain the inhibition of PCN synthesis by rifampicin. However *pip* must be involved, since its expression in multi-copies restores PCN synthesis under rifampicin stress as well as with other stresses tested. It would be of interest to understand what other factors are involved in the repression of PCN synthesis by rifampicin.

If the growth inhibition of PCL2019 (wt + P_{tac} *pip*) in the presence of stress factors is due to the fact that a high overproduction of Pip under stress conditions is toxic, a derivative containing *pip* under its own promoter (PCL2089) is expected to grow like the wild-type with empty vector (PCL1960) under stress. The results confirm this hypothesis (Table 1). In addition to being able to grow under stress, PCL2089 (wt + P_{pip} *pip*) also produces elevated amounts of PCN (Table 1). Clearly, the regulation of *pip* is a key in the effect of various stresses on strain PCL1391. It cannot be easily explained why the presence of *pip* in multi-copy is sufficient to restore PCN production. It seems that amounts of Pip in the cell are very important for the balance between PCN production and growth/survival under stress conditions. Very high amounts of Pip could also have an inhibitory effect on stress resistance, *via* inhibition of *psrA* expression (Chapter 4). An explanation for the PCN production under stress by PCL2089 (wt + P_{pip} *pip*) could be the following: the regulator inhibiting *pip* expression would be titrated out because of the higher number of *pip* copies, therefore enough Pip would be produced to induce PCN synthesis, but not enough to inhibit stress resistance *via* *psrA*.

In order to validate further the model, it was necessary to verify that decreasing the production of *N*-AHL and PCN indeed represents an advantage for growth in the presence of the stress factors. A *phzI* mutant does not produce any *N*-AHL or PCN and shows similar growth as compared to the wt in the presence of fusaric acid, NaCl or rifampicin (Fig. 6). This supports our model, which predicts that the presence of these concentrations of stress factors inhibit production of *N*-

AHL and PCN in the wt, giving it therefore the same phenotype as a *phzI* mutant. A constitutive *phzR* gene was introduced in the wt and the *phzI* mutant, resulting into PCL1993 and PCL2083, respectively. PCL1993 showed slower growth under stress conditions than PCL2083 (Fig. 6). Both these strains over-produce PhzR, but only PCL1993 is able to produce *N*-AHL and PCN. These results demonstrate that the production of these metabolites is a handicap for growth of *P. chlororaphis* under stress conditions, further supporting our model.

Implications of the model for Pip and RpoS

Interestingly, the Pip-homologue AcrR (Chapter 4) was also described to be regulated by stress (Ma *et al.*, 1996). Several traits are common to AcrR and Pip and it is possible that they both belong to a sub-family of AcrR/TetR homologues that would play a role in stress resistance. Many systems involved in stress resistance and homologous to AcrRAB were found in *E. coli* (Ma *et al.*, 1994; Miller & Sulavik, 1996) and in *P. aeruginosa* (Evans *et al.*, 2001). It is likely that many other factors are involved in the general stress resistance by *P. chlororaphis*. A hypothesis could be that Pip regulates an efflux pump for PCN or *N*-AHL. Results show that the expression of *pip* is not under the influence of these metabolites (Fig. 7), in contrast to *tetR* or *acrR* that are regulated by the molecules secreted by the pump they regulate (Ma *et al.*, 1995; Hillen and Berens, 1994). It is possible that Pip does not act directly on an efflux pump, but that it has evolved from this family of efflux pump regulators. Although a factor present in the cell is necessary for Pip to bind DNA, it cannot be either *N*-AHL or PCN, since both are absent in a *pip* background (Fig. 8, panel B, lane 5). These results do not support that *pip* is directly regulating a pump for *N*-AHL or PCN. *In-silico* analysis did not retrieve an obvious primary target for Pip. It is possible that (i) a putative Pip target gene is within the genome region that still needs to be sequenced (only 66% has been sequenced), (ii) the conserved motif upstream of *pip* does not contain the pattern sequence bound by Pip, or (iii) the method/program is not appropriated to identify similar patterns. It is also possible that the motif recognized by Pip is not very conserved, like for other known transcriptional regulators, for example LasR in *P. aeruginosa* (Schuster *et al.*, 2004). A large amount of additional experiments will be required to precisely identify the primary target of Pip.

Our model is also interesting in the perspective of previous work that showed the lower importance of RpoS in the regulation of PCN synthesis in more complex medium (Girard *et al.*, 2006). In the presence of many nutrients, RpoS

could only play a limited role in the regulation of PCN synthesis, whereas in nutrient-limited conditions, reflecting a higher level of stress, RpoS would become an important regulator of PCN synthesis. This would allow the cells to switch off completely PCN synthesis downstream of RpoS *via* Pip in case of even higher level of stress.

Concluding remarks

The discovery of *pip* (Chapter 4) was an important step in unraveling the regulatory mechanism of PCN regulation. In the present chapter, we also tried to understand in a broader way the physiological role of Pip. The model proposed in Fig. 9 is a first step in describing at least part of the cellular events induced by several common stress factors, but cannot be generalized to general stress encountered by *P. chlororaphis* in the rhizosphere without further studies. It also gives novel insights on how cells could favor stress resistance by switching off secondary metabolite production. Future work should focus on the exact mechanism resulting in the inhibition of *pip* expression. It is likely that at least one regulatory protein targets *pip* at the transcriptional and/or translational level. This protein would be regulated by stress factors, maybe *via* RpoS, the stress sigma factor. Future work could also include a broader analysis of genes regulated by *pip*, using the microarray developed for strain PCL1391 (Girard *et al.*, 2006).

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Pseudomonas* strains were cultured at 28°C in liquid MVB1 (van Rij *et al.*, 2004) and shaken at 195 rpm on a Janke und Kunkel shaker KS501D (IKA Labortechnik, Staufen, Germany). *Escherichia coli* strains were grown at 37°C in LC medium (Girard *et al.*, 2006) under vigorous aeration. Media were solidified with 1.8% Bacto agar (Difco, Detroit, MI, USA). When appropriate, growth media were supplemented with kanamycin (50 µg/ml), carbenicillin (200 µg/ml), gentamicin (30 µg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 µg/ml) or hexanoyl-homoserine lactone (C₆-HSL) (5 µM) (Fluka, Sigma-Aldrich, Zwijndrecht, the Netherlands). For growth the absorbance of liquid cultures was measured at 620 nm.

Table 2. Bacterial strains and plasmids.

Bacterial strains and plasmids	Description	Reference or source
<i>Pseudomonas chlororaphis</i>		
PCL1391	Wild-type <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide; biocontrol strain of tomato foot and root rot caused by <i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	(Chin-A-Woeng <i>et al.</i> , 1998)
PCL1114	Derivative of PCL1391 in which a promoterless Tn5luxAB has been inserted in <i>pip</i> , Km ^r	Chapter 4
PCL1954	Derivative of PCL1391; <i>rpoS</i> ::pMP7418	(Girard <i>et al.</i> , 2006)
PCL1958	Derivative of PCL1391 containing pMP7420; Gm ^r	(Girard <i>et al.</i> , 2006)
PCL1960	Derivative of PCL1391 containing pBBR1MCS-5; Gm ^r	(Girard <i>et al.</i> , 2006)
PCL1993	Derivative of PCL1391 containing pMP7447; Gm ^r	(Girard <i>et al.</i> , 2006)
PCL2019	Derivative of PCL1391 containing pMP7455; Gm ^r	Chapter 4
PCL2044	Derivative of PCL1391 containing pMP7465; Gm ^r	(Girard <i>et al.</i> , 2006)
PCL2082	Derivative of PCL1103 containing pBBR1MCS-5; Km ^r , Gm ^r	This study
PCL2083	Derivative of PCL1103 containing pMP7447; Km ^r , Gm ^r	This study
PCL2085	Derivative of PCL1114 containing pMP7487; Km ^r , Gm ^r	Chapter 4
PCL2088	Derivative of PCL1114 containing pMP7488; Km ^r , Gm ^r	This study
PCL2089	Derivative of PCL1391 containing pMP7487; Gm ^r	This study
<i>Chromobacterium violaceum</i>		
CV026	Double mini-Tn5 mutant from <i>C. violaceum</i> ATCC 31532, AHL biosensor; Km ^r	(Milton <i>et al.</i> , 1997)
<i>Escherichia coli</i>		
DH5α	<i>Escherichia coli</i> ; <i>supE44</i> Δ <i>lacU</i> 169(Φ80 <i>lacZ</i> Δ <i>M</i> 15) <i>hsdR</i> 17 <i>recA</i> 1 <i>endA</i> 1 <i>gyrA</i> 96 <i>thi</i> 1 <i>relA</i> 1	(Hanahan, 1983)
Plasmids		
pRK2013	Helper plasmid for tri-parental mating; Km ^r	(Ditta <i>et al.</i> , 1980)
pBBR1MCS-5	General purpose cloning vector; Gm ^r	(Kovach <i>et al.</i> , 1995)
pMP7420	pBBR1MCS-5 containing the <i>rpoS</i> gene of PCL1391 downstream of the P _{tac} promoter, obtained by <i>Eco</i> RI digestion of pMP7424; Gm ^r	(Girard <i>et al.</i> , 2006)
pMP7447	pBBR1MCS-5 containing the <i>phzR</i> gene of PCL1391 downstream of the P _{tac} promoter, inserted between the <i>Xho</i> I and <i>Eco</i> RI sites; Gm ^r	(Girard <i>et al.</i> , 2006)
pMP7455	pBBR1MCS-5 containing the <i>pip</i> gene of PCL1391 under control of the P _{tac} promoter; Gm ^r	Chapter 4

pMP7465	pBBR1MCS-5 containing the <i>psrA</i> gene of PCL1391 under control of the P_{tac} promoter; Gm ^r	(Girard <i>et al.</i> , 2006)
pMP7487	pBBR1MCS-5 containing the <i>pip</i> gene of PCL1391 under control of its own promoter; Gm ^r	Chapter 4
pMP7488	pBBR1MCS-5 containing the <i>pip</i> gene of PCL1391 with a His-tag and under control of its own promoter; Gm ^r	This study

Recombinant DNA techniques

General DNA techniques were performed as described previously (Sambrook & Russel, 2001). Polymerase chain reactions were carried out with Super Taq enzyme (Enzyme Technologies Ltd, UK). Only for the production of *pip* under control of the P_{pip} promoter, PCRs were performed using Phusion from Finnzymes (Bioké, Leiden, The Netherlands). Primers were synthesized by Isogen Life Science (Maarssen, The Netherlands). Restriction enzymes were purchased from New England BioLabs Inc. (Westburg) and ligase from Promega (Leiden, The Netherlands).

Construction of plasmids and PCL1391 mutant strains

Primers oMP1044 (5'-ATATATCTCGAGTTATCAATGGTGATGGTGATGGTGGGCCTGCAAGTAACGCAGCACC GACTCGC-3') and oMP1045 (5'-ATATATGAATTCGAGGTCAGCCGGGCCAAGGAG-3') were used for PCR on chromosomal genome with Phusion enzyme (Finnzymes) to obtain the *pip* gene under its own promoter and with a tag of 6 histidines at the C-terminus. The 1.1 kb product was cloned in the *EcoRI* and *XhoI* sites of pBBR1MCS-5 resulting in opposite directions of transcription of *pip* and the β -galactosidase gene of pBBR1MCS-5. This plasmid was named pMP7488 and verified by sequencing. It was transferred to PCL1114 (*pip* mutant) to obtain PCL2088.

The vector pMP7487 (Chapter 4) containing the *pip* gene under its own promoter and no his-tag was introduced by triparental mating into PCL1391 to obtain PCL2089.

Extraction and analysis for phenazine and *N*-acyl-homoserine lactones

Phenazine extraction was carried out on 10 ml liquid MVB1 cultures at regular time points during growth and/or after overnight growth of bacterial strains as described previously (van Rij *et al.*, 2004).

For *N*-AHL extraction, supernatants from 50 ml MVB1 cultures were harvested at OD₆₂₀ 3.0 and mixed with 0.7 volume of dichloromethane, shaken for 45 min after which the organic phase was collected. The extract was dried using a rotary evaporator. The dried residue was redissolved in 25 µl of acetonitrile and spotted on RP-C18 TLC plates (Merck, Darmstadt, Germany). The plates were developed in methanol-water (60:40, v:v). For detection of *N*-AHL's, the TLC was overlaid with 0.8% agar LC 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 analyzed for appearance of violet spots.

Western blot analysis

Cells were grown after inoculation of 10 ml MVB1 with an overnight culture washed with fresh medium and subsequently diluted to OD₆₂₀ of 0.1. Cells were harvested at OD₆₂₀ 1.0 or 2.2 (samples from the cultures were diluted 10-times for OD₆₂₀ measurement) in volumes corrected for equal cell amounts. Cell pellets were resuspended in 200 µl of cracking buffer (50 mM Tris-HCl, pH 6.8-1% SDS-2 mM EDTA-10% glycerol-0.01% bromophenol blue-1% β-mercaptoethanol) and boiled for 3 minutes. The samples were subsequently loaded on a 12% SDS-polyacrylamide gel and proteins were separated and blotted following a standard Western blot procedure (Ausubel *et al.*, 1997). A dried aliquot of antibodies against RpoS was kindly provided by Prof. K. Tanaka (Tokyo, Japan). This sample was resuspended in 100 µl PBS, diluted 1000-fold and allowed to react with the blot. For detection of Pip, an Anti HIS-PROBE (G-18) (Santa Cruz Biotechnology, Tebu-Bio, Heerhugowaard, The Netherlands) was used. The blots were subsequently incubated with peroxidase-labeled goat anti-rabbit antiserum (Amersham Biosciences). Finally, blots were incubated in a luminal solution (250 µM sodium luminol (Sigma)-0.1 M Tris-HCl, pH 8.6-0.01% H₂O₂) mixed with 60 µl enhancer solution (67 µM p-hydroxy coumaric acid (Sigma) in DMSO). Hybridizing protein bands were visualized on Super R-X photographic film (Fujifilm, Düsseldorf, Germany) after chemiluminescence detection.

Shift assays

A PCR product of the conserved promoter region of *pip* was obtained using the primers oMP1116 (5'-CCAAGTTGTAGGAGTTTCGTAAC-3') and oMP1117 (5'-

TGTGGTCATTGTCATTCTGGG-3') with pMP7444 as template and the Phusion DNA polymerase (Finnzymes). After purification on Qiaquick columns (Qiagen), the PCR product was labeled with [γ - ^{32}]ATP using polynucleotide kinase (Fermentas, St. Leon-Rot, Germany) and purified over MicroSpin S-200 HR columns (GE Healthcare, Roosendaal, The Netherlands).

Cellular extracts of several *P. chlororaphis* derivatives (as indicated) were produced using the following method. 50 ml of fresh MVB1 was inoculated with washed cells from overnight cultures at an initial OD₆₂₀ of 0.1. Cells were harvested by spinning down cultures at an OD₆₂₀ of 1.0 for 15 minutes at 3,000 rpm at 4°C. The pellets were resuspended in 2 ml B-PER bacterial protein extraction reagent (Pierce, Perbio Science, Etten-Leur, The Netherlands) and gently shaken at room temperature for 15 min. The samples were centrifuged at 25,000 rpm for 30 minutes at 4°C in a Centrikon T-2070 ultra centrifuge (Kontron Instruments, Beun-De Ronde, Abcoude, The Netherlands). Supernatants were collected and frozen at -80°C in 10% glycerol for later use in binding reactions.

Reactions were performed in 10 μ l final volume, containing 50 mM Tris-HCl (pH 7.6), 60 mM NH₄Cl, 7 mM MgCl₂, 0.9 ng of [^{32} P]-labeled PCR product (1 nM) and purified Pip-6His protein and/or S30 extracts as indicated. A 1000-fold excess of genomic DNA was present in the samples to avoid non-specific DNA-protein interactions. After 20 min incubation at room temperature samples were complemented with 10% glycerol and loaded on an 8% PAGE in 20 mM Tris-borate (pH 7.6) and run in the same buffer. Radioactivity was visualized by phosphor imaging (Bio-Rad, Veenendaal, The Netherlands).

Computational prediction of Pip target genes

Search for conserved motifs in the upstream region of *pip* orthologues of *P. aeruginosa* PAO1, *P. chlororaphis* PCL1391, *P. fluorescens* Pf-5, *P. fluorescens* PfO1, *P. putida* KT2440, *P. syringae* B728a, and *P. syringae* pv. *tomato* str. DC3000, was performed using the MEME program (Bailey and Elkan, 1994) (available at <http://meme.sdsc.edu/meme/meme.html>). A 47-nt sequence (with the consensus CGCCATCGCGGCTTCCTTCGCTGGGCGGCGCGCCCCATAATCG CCCG) was proposed as the best conserved motif amongst orthologous *pip* upstream regions. In order to identify similar conserved patterns and therefore potential Pip target genes in genomes of pseudomonads, we generated a position weight matrix from the set of the seven conserved sequences (Table 4) deduced from the MEME program using the Target Explorer web tool (Sosinsky *et al.*, 2003) (available at

http://trantor.bioc.columbia.edu/Target_Explorer/). The maximum score that a 47-nt sequence could obtain with the “Pip” scoring matrix is 39.44 bits and the minimum score is - 95.04 bits. We used the generated matrix to scan the partial genome of *P. chlororaphis* PCL1391 (about 66% sequenced), and scoring of potential binding sites is based on program PATSER (Hertz and Stormo, 1999). The cut-off score was fixed to 10 bits, 10.23 bits below the minimum score for the training set of sequences (20.23 bits for Pip of *P. putida* KT2440) in order to allow identification of sequences with several mismatches versus the consensus described above. No sequences with significant scores (similar to those obtained by the 47-nt motifs upstream *pip* genes) were recovered. Identical results were obtained from *P. aeruginosa* PAO1 and *P. fluorescens* Pf-5 genome scans. As an alternative, the same *in-silico* approach was performed using the Predict Regulon Server (Yellaboina *et al.*, 2004) (available at <http://210.212.212.6/cgi-bin/regsites/predictregulonv1.pl>). This program, although similar to Target Explorer, is less restrictive but predictions contain much more false positive hits than with the former one.

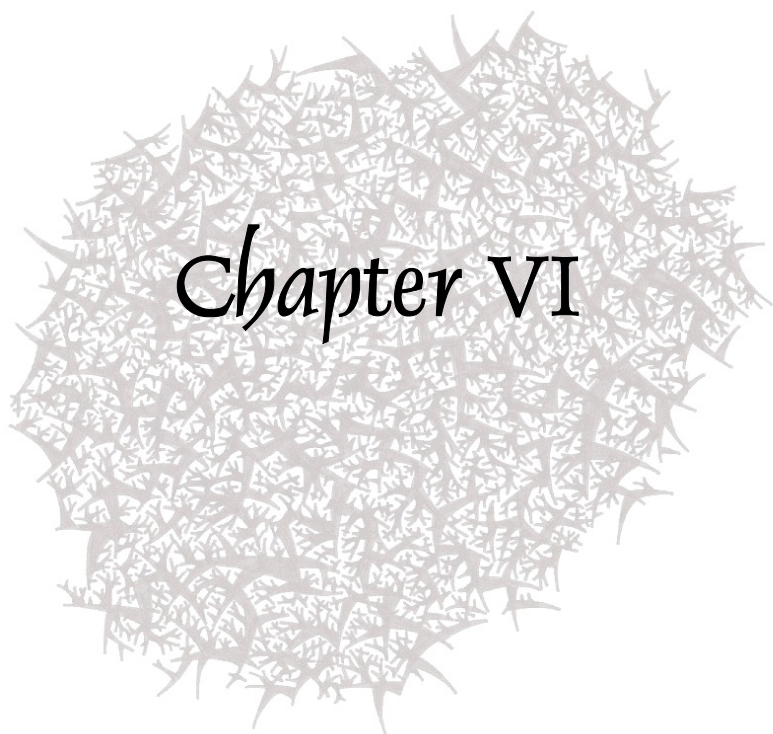
Table 4. Alignment matrix used for the *in-silico* search for a conserved region upstream of Pip

pipPAO1	CGGCAGGTGCTCTTCCTACAGCCGGGCGGG CCCCGCATAATCGGCCG	score=22.85
pipPCL1391	CGCCGTCCTGCTTTTCCTTCGCTGCGCGACGC GGCCCATAATCGCCCCG	score=33.92
pipPf-5	CGCCATCGCGCTTTCTTTTCGCAGGGTGACGC GGCCCATAATCGCCCCG	score=35.06
pipPFO1	CGGCCGCGCGGCTTCCTTCACTGGGGCGCG CGGCCATAATCGCCAG	score=35.04
pipKT2440	AGGCATGATGGCTACCCGACCAACAGCGTGC TTCCCTGCATGGCCGC	score=20.23
PipB728a	CGCCCTCGCGGCTTCCCTCGGTTGCCGGTCG GCTTCATACTCCGCGC	score=30.14
pipDC3000	AGCCCTCGCAGCTTCCCTCCATTCACGGTCA GCTTCATACTCCGCGC	score=30.62

ACKNOWLEDGEMENTS

The authors thank Prof. K. Tanaka for (Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) for providing the anti-RpoS rabbit serum.

This project was financially supported by the FW6 EU R&D project QRLT-2002-00914 (acronym “Pseudomics”) and by the BioScience Initiative from Leiden University.



Chapter VI

Summary and discussion

Geneviève Girard and Guido V. Bloemberg

INTRODUCTION

As reviewed in Chapter 1, a high diversity of secondary metabolites is produced by *Pseudomonas* species and elucidating the corresponding mechanisms of regulation is relevant for medicine, agriculture and industry. One model strain used for such studies is *Pseudomonas chlororaphis* strain PCL1391, which originates from the rhizosphere of tomato plants. *P. chlororaphis* PCL1391 secretes many enzymes and secondary metabolites, including exoproteases, lipase, hydrogen cyanide (HCN), phenazine-1-carboxamide (PCN) and its precursor, phenazine-1-carboxylic acid (PCA) (Chin-A-Woeng *et al.*, 1998).

Regulation of PCN synthesis: previous work

Fusarium oxysporum f. sp. *radicis lycopersici* is the causing agent of tomato foot and root rot and a competitor of *P. chlororaphis* in the rhizosphere (Bolwerk *et al.*, 2003; Chin-A-Woeng *et al.*, 2000; de Weert *et al.*, 2004). PCN production, combined with efficient root colonization to deliver PCN in the rhizosphere, is a crucial trait for the biocontrol ability of strain PCL1391 (Chin-A-Woeng *et al.*, 1998). Previous work showed that an operon of 8 genes, *phzABCDEFGH*, is responsible for the synthesis of PCA and PCN (Chin-A-Woeng *et al.*, 1998).

Several regulators of the *phz* operon were also characterized (Chin-A-Woeng *et al.*, 2001; Chin-A-Woeng *et al.*, 2005). The GacS/GacA system is composed of a GacS sensor protein and a GacA transcriptional activator. GacS is inserted in the membrane and supposedly responds to a so far unknown environmental signal by autophosphorylation (Zuber *et al.*, 2003). The phosphate residue is subsequently transmitted to the GacA regulator. Activated GacA regulates the transcription of many genes. However its direct targets are unknown. The GacS/GacA system positively regulates PCN synthesis. Expression of *psrA* encoding a TetR homologue was shown to be dependent on GacS/GacA (Chin-A-Woeng *et al.*, 2005). The PhzI/PhzR quorum-sensing system is another important regulator of the *phz* operon. PhzI synthesizes several *N*-acyl homoserine lactones (*N*-AHL), of which C₆-HSL was shown to be the activator of PhzR in the regulation of the *phz* operon (Chin-A-Woeng *et al.*, 2001). C₆-HSL probably binds to PhzR. The C₆-HSL-PhzR complex would be the active form of the transcriptional regulator PhzR and would bind to *lux* boxes in the promoter of *phzI* and in the promoter of the *phzABCDEFGH* operon. These events result in the self-activation of the quorum-sensing system and in the stimulation of PCN synthesis at the onset of the stationary phase. In all previous studies (Chin-A-Woeng *et al.*, 2001; Chin-A-Woeng *et al.*, 2005; van Rij *et*

al., 2004; van Rij *et al.*, 2005) and the chapters of this thesis, *N*-AHL and PCN amounts were shown to be correlated in *P. chlororaphis* PCL1391, indicating that regulation of the quorum-sensing system determines regulation of PCN synthesis.

Aim of this thesis

Studies on regulation of secondary metabolites synthesis in bacterial species (Chapter 1) indicate that additional genes could be involved in the regulation of PCN synthesis in strain PCL1391 and that their interactions might be very complex. Besides, several environmental factors were shown to influence the synthesis of PCN by *P. chlororaphis* PCL1391 (van Rij *et al.*, 2004; van Rij *et al.*, 2005). However, the detailed molecular mechanisms through which environmental factors affect the regulation of the *phz* operon have not been studied yet. The major goal of the studies presented in this thesis has been to elucidate the complex mechanisms regulating PCN production in *P. chlororaphis* strain PCL1391. An overview of the obtained model is presented in Figure 1.

RESULTS PRESENTED IN THIS THESIS

Chapter 2

PsrA is a transcriptional regulator that probably directly binds to the promoter of *rpoS* in *P. putida* and *P. aeruginosa* (Kojic *et al.*, 2002). RpoS is an alternative sigma factor that regulates general gene expression in case of stress conditions and during stationary phase of growth (Jorgensen *et al.*, 1999; Ramos-González & Molin, 1998; Sarniguet *et al.*, 1995; Suh *et al.*, 1999). Since PCN synthesis occurs at the onset of the stationary phase (van Rij *et al.*, 2004) and is regulated by PsrA (Chin-A-Woeng *et al.*, 2005), it seemed logical to test if RpoS regulates PCN synthesis. An *rpoS* mutant was constructed and exhibited strongly reduced levels of PCN in MVB1 synthetic medium. Similarly, a *psrA* mutant was impaired in the production of PCN in MVB1 medium. Epistatic studies combined with quantifications of PCN and *N*-AHL synthesized in MVB1 cultures showed that RpoS regulates the *phz* operon downstream of PsrA and upstream of the PhzI/PhzR quorum-sensing system. Interestingly, RpoS was shown to be of less influence on the production of PCN when strain PCL1391 was grown in complex (nutrient-rich) media. Additionally some results indicate that RpoS could also regulate the conversion of PCA into PCN. This observation nuances the notion that quorum-sensing only determines the synthesis of PCN. Results of this chapter also suggest that a secondary cascade, in parallel to PsrA/RpoS but also downstream of

GacS/GacA and upstream of PhzI/PhzR, regulates the expression of the *phz* operon. This is indicated by dotted lines in Figure 1.

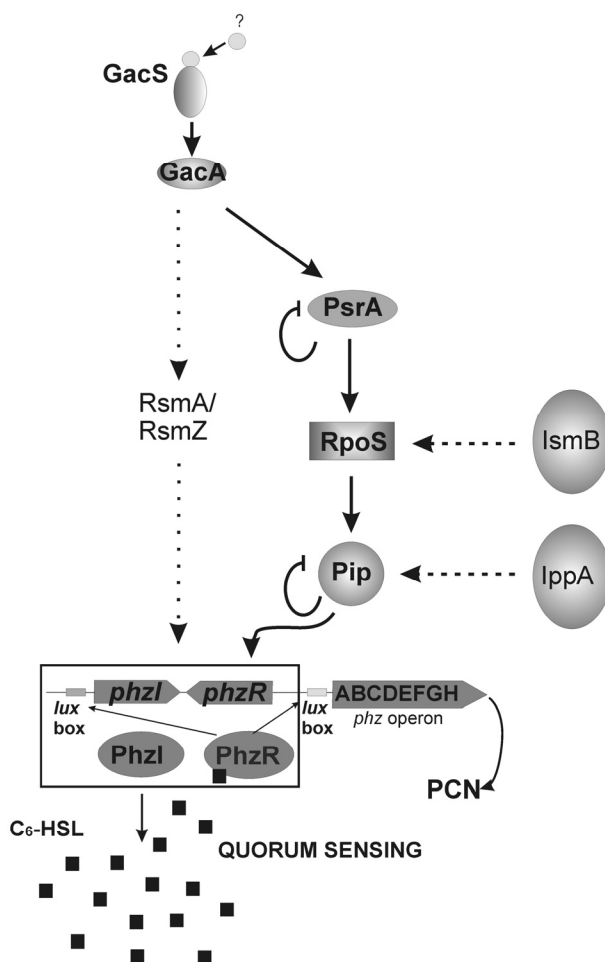


Figure 1. Hypothetical regulatory network for PCN synthesis in *P. chlororaphis* PCL1391.

The triangular arrow heads indicate a positive regulation, whereas the flat ones indicate a negative regulation. Results of this thesis show the existence of the main cascade drawn in the center (solid lines). Other work from our group indicates that IsmB regulates PCN synthesis upstream of RpoS (van Rij *et al.*, *in preparation*), but not downstream of PsrA, and that IppA regulates the *phz* operon upstream of Pip (dashed lines) (van Rij *et al.*, *in preparation*). Finally, it was shown in Chapter 2 that another important cascade must exist downstream of GacA. It can be hypothesized that this cascade involves the small RNAs and proteins of the Rsm family of regulators (dotted lines). Several observations (see text for details) indicate that this second cascade would actually also be an important intermediate between GacS/GacA and PhzI/PhzR, while the PsrA/RpoS/Pip cascade, fine-tuned by IsmB and IppA, would be of increasing importance with increasing stress conditions.

Chapter 3

In parallel to studies using random mutagenesis, a high-throughput method was developed for the identification of large numbers of genes involved in regulatory processes, of the secondary metabolism in particular. A library of random fragments of the PCL1391 chromosomal genome was constructed. This library was used to build a “home-made” microarray for *P. chlororaphis* PCL1391. Several protocols were tested for isolation of mRNA, synthesis of fluorescent-labeled cDNA and hybridization on the microarray. The most efficient procedure involves a phenol/chloroform extraction of total RNA, followed by column purification. Total RNA is used as a template for the synthesis of fluorescent Cy-DNA using an indirect labeling, *via* an amino-allyl-cDNA intermediate. The microarray was tested by the analysis of the transcriptomes for the *psrA* and *rpoS* mutant strains as compared with the wild-type strain. Results validate the model of phenazine regulation (Chapter 2) and microarray analyses led to the identification of several new genes that might be involved in regulating secondary metabolism, several of which could play a role in fine-tuning PCN synthesis.

Chapter 4

A transposon mutant of strain PCL1391 was isolated that is decreased in its PCN production. Analysis of the genome of this mutant showed that the transposon is inserted in a putative transcriptional regulator, which was named Pip (phenazine inducing protein). Pip shows overall homology to the AcrR regulator and partial homology (at the N-terminus) to the TetR regulator. Homologues of *pip* were identified in many bacterial species, of which the function is unknown. Expression studies and analysis of PCN and *N*-AHL production in various strains mutated in *pip* and in the known regulators of PCN show that Pip regulates the *phz* operon downstream of PsrA and RpoS and upstream of the PhzI/PhzR quorum-sensing system. This is the first time that a phenotype is described for a mutation in *pip*. Although both AcrR and TetR regulate the production of an efflux pump, such a pump could not be detected on basis of analysis of the genome sequence surrounding *pip*.

Chapter 5

Several environmental factors influence the synthesis of PCN by *P. chlororaphis* PCL1391 (van Rij *et al.*, 2004). Some of them are related to stress and were shown to inhibit the *phz* operon, such as NaCl and the phytotoxin fusaric acid.

In Chapter 5 it is shown that the minimum inhibitory concentration (MIC) of not only NaCl and fusaric acid, but also the antibiotics rifampicin and kanamycin, is lower for *N*-AHL and PCN synthesis than for growth of *P. chlororaphis* PCL1391. After testing if the over-expression of PCN regulatory genes could restore PCN synthesis under these stress factors, and measuring the production of Pip under several stress conditions, the following conclusions were drawn. (i) The selected stress factors inhibit the *phz* operon by repressing the quorum-sensing system PhzI/PhzR. (ii) Pip is involved in the repression of PCN synthesis by all the stress factors tested. (iii) The inhibition of PCN synthesis by fusaric acid, kanamycin and NaCl could be explained by a reduction in Pip amounts in the cell. (iv) In the case of stress by rifampicin, other factors additional to Pip must be involved.

The present results support that in the presence of several stress factors, *pip* expression is (probably indirectly) repressed, which results in inhibition of PCN synthesis (and possibly other yet unidentified Pip-dependent processes) and saves energy in the cell to favor stress resistance. Fitting with results of Chapter 2, it can be hypothesized that RpoS regulates PCN synthesis mostly in nutrient-limiting conditions and switches to stress resistance when necessary, after activity of the *phz* operon is lowered by the decrease of Pip amounts. To our knowledge it is the first time that such a switch between secondary and primary metabolism depending on stress is described.

CONCLUSIONS

A new model for the regulation of PCN synthesis

All the studies were conducted in synthetic MVB1 medium, which seemed more relevant than the previously used complex King's B medium, since the rhizosphere is known to be nutrient-limiting. In this MVB1 medium, GacS/GacA is still the master regulator at the start of the regulatory cascade for PCN synthesis, as in KB medium. At least two cascades must branch downstream of this two-component regulator (Chapter 2). One cascade is still unidentified (see below). The second one consists of the sequence PsrA, RpoS and Pip, all positively regulating the PhzI/PhzR quorum-sensing system. Pip is characterized in this thesis (Chapter 4) as a new regulator of PCN synthesis. Interestingly, constitutively expressed *phzR* could restore PCN production in all the mutants tested, including the *gacS* mutant (Chapters 2 and 4). This indicates that all cascades downstream of GacA probably converge (just) upstream of PhzI/PhzR. Some observations in Chapter 2 led to the hypothesis that *phzR* might also be post-transcriptionally regulated. Finally, this

thesis broadens the study of PCN regulation by elucidating the link between several environmental factors and the genetic regulators of PCN production.

Taken together, the results of this thesis suggest that the cascade studied through the four experimental chapters is not the only GacS/GacA intermediate in the regulation of PCN synthesis. This is supported by the fact that constitutive expression of *psrA*, *rpoS* or *pip* is not able to restore PCN synthesis in a *gacS* mutant, although constitutive expression of *phzR* is able to. Besides, RpoS was shown to play a more limited role in nutrient-rich medium and Pip seems to be important under some stress conditions. These observations could mean that an unknown main cascade, possibly including homologues of the Rsm family of regulators (see below) would function as another important intermediate between GacS/GacA and PhzI/PhzR. Also downstream of GacS/GacA and upstream of PhzI/PhzR, the cascade PsrA/RpoS/Pip would represent a second branch, of which importance would increase with nutrient-poorer conditions and increased stress (Fig. 1). This brings a novel aspect to the model for the regulation of PCN synthesis, which should be tested in the future (see below).

General relevance for the regulation of secondary metabolism

Although it was already known that RpoS can play a role in regulating secondary metabolism, the studies and results described in this thesis bring new insights into the diversity of interactions between RpoS and other regulators. For example, a stimulation of quorum-sensing by RpoS is not common to most other *Pseudomonas* species. The identification of Pip as a regulator of secondary metabolism is very relevant for the *Pseudomonas* research field, since highly conserved homologues of Pip were found in many other *Pseudomonas* species. Finally, a broader physiological function for Pip is proposed: besides regulating PCN synthesis, it is hypothesized that Pip has a more general role for the functioning of the cell under stress conditions. The presented model provides novel insights on how cells could favor stress resistance by switching off secondary metabolite production, even if other proteins than Pip homologues could have such a function in other species.

Technical improvements

In general, an important proportion of the technical problems encountered during the work with strain PCL1391 derived from the high resistance of this strain to a wide spectrum of antibiotics. The quality of the work presented in this thesis

could have been improved if several other techniques had been used. Firstly another directed mutagenesis technique could have allowed the construction of mutants in smaller genes. For efficient single homologous recombination, the technique used during our studies, a minimum of 300 bp of the target gene is required to be cloned in the suicide vector. The recombination event leaves two truncated copies of the target gene in the genome, which means that the 3' end of the insert in the suicide vector must be relatively far before the end of the target gene, so that the truncation of the C-terminus affects the function of the gene product. Therefore single homologous recombination in mutagenesis can be used only to target genes of at least 500-600 bp. If another method had been set up, for example involving double homologous recombination as described previously in *P. fluorescens* (Heeb *et al.*, 2002), the study of PCN regulation could have been broadened to small genes such as *rsmA*, which encodes a central intermediate of the GacS/GacA system in many *Pseudomonas* species (see Chapter 1 and below).

Secondly, regulatory interactions between the various genes characterized in this work could have been studied more thoroughly if a proper vector for promoter fusions could have been found or constructed. Unfortunately this was not possible. A common problem encountered with most of the available vectors was that strain PCL1391, like many *Pseudomonas* species (Poole, 2004), is resistant to the most commonly used antibiotics (carbenicillin, tetracyclin, chloramphenicol and syringomycin) for plasmid selection. Only two markers were found to be usable in strain PCL1391, namely kanamycin and gentamicin resistance. Since most of the strains used in the studies were kanamycin-resistant (due to a Tn5 transposon insertion), the only option was to use a gentamicin-resistance-marked vector. The vector pML103 (Gm^R) containing a promoterless *lacZ* (Labes *et al.*, 1990) was tested but unfortunately cloning failed. Several attempts were made to build a vector, particularly by cloning the promoterless *luxAB* genes from pRL1063a (Wolk *et al.*, 1991) into pBBR1MCS-5 (Gm^R, used in Chapters 2 to 5 for complementation studies). However this pBBR1MCS-5 derivative showed constitutive expression of *luxAB*. Despite the cloning of a tryptophane terminator upstream of *luxAB*, the expression of these genes could not be shut off. Finally, a pME6031 (Tc^R) (Heeb *et al.*, 2000) derivative containing a promoterless *luxAB* (Dubern, Thesis) was also used as a cloning template: its Tc resistance cassette was replaced by a Gm resistance cassette. This cloning was successful and the construct transformed into PCL1391 showed a very low background of expression. However, the PCL1391 derivative

containing this plasmid showed very slow growth, which is not compatible with promoter studies.

FUTURE PROSPECTS

The various results obtained through the chapters establish a branch of the regulation of PCN synthesis downstream of the GacS/GacA two-component system (Fig. 1). Thorough experimental analyses assessed this cascade, of which the most interesting gene is probably *pip*, because it is novel and original in its function. Other work showed that other novel proteins are involved in the regulation of PCN synthesis (van Rij *et al.*, *in preparation*). One of them, IppA, probably regulates PhzI/PhzR via Pip, but is not in the main cascade, because it is not downstream of RpoS (Fig. 1). Another gene involved in regulation of PCN synthesis, named *ismB*, is part of an operon and its precise role in the regulatory network is still unclear (van Rij *et al.*, *in preparation*). Interestingly, the *ippA* and *ismB* mutants show a PCN-phenotype dependent on medium conditions (van Rij *et al.*, *in preparation*), which fits well with the model discussed above (see also Fig. 1).

A second regulatory branch downstream of GacS/GacA is likely present upstream of the PhzI/PhzR system (Chapter 2). This is indicated by the inability of a constitutive expression of *psrA*, *rpoS* and *pip* genes to restore PCN and *N*-AHL synthesis in a *gacS* background, although a constitutive *phzR* is able to. Even more strikingly, a constitutively expressed *phzI* gene was unable to restore PCN production in a *gacS* background, although *N*-AHL was synthesized in high amounts (*unpublished data*). This result supports the hypothesis postulated in the discussion of Chapter 2, that *phzR* could be regulated at post-transcriptional level, maybe by the RsmA small protein. It is possible that genes of the Rsm family of small proteins and small RNAs form an important branch of regulation downstream of GacS/GacA (Fig. 1). These genes are involved in the regulation of many secondary metabolites in *P. fluorescens* and *P. aeruginosa* (Chapter 1), where they act as the crucial intermediary regulators of the GacS/GacA system (Haas & Defago, 2005; Heeb & Haas, 2001; Kay *et al.*, 2005; Reimann *et al.*, 2005). Besides, an *rsmA* homologue was sequenced from the genome of *P. chlororaphis* PCL1391 (Chapter 2). However, no *rsmZ* homologue encoding a small RNA could be characterized downstream of *rpoS* where it is usually found in other genomes (Chapter 2). Extended studies are needed to evaluate the importance of *rsm* genes in strain PCL1391 for PCN synthesis and regulation of other traits.

In the same field of post-transcriptional regulation by small RNAs, Hfq-related sRNAs appear more and more as central regulators of the cell metabolism (Gottesman, 2004; Valentin-Hansen *et al.*, 2004). Most studies have been performed in *E. coli* and led to the characterization of several Hfq-dependent sRNAs involved in the regulation of iron metabolism (Masse *et al.*, 2003a; Masse & Gottesman, 2002), regulation of *rpoS* (Repoila *et al.*, 2003), and of many other genes (Masse *et al.*, 2003b). The role of Hfq in regulation of cell events in *Pseudomonas* sp. was only recently evaluated (Sonnleitner *et al.*, 2002; Sonnleitner *et al.*, 2003). In *P. aeruginosa* PAO1, functional homologues of sRNAs of *E. coli* were shown to be involved in iron homeostasis (Wilderman *et al.*, 2004). Therefore, it is also very likely that a group of sRNAs and Hfq play an important role in the regulation of secondary metabolism in strain PCL1391, even if data for *Pseudomonas* species are very preliminary. It was recently shown that Hfq regulates *rhII*, a quorum-sensing gene of *P. aeruginosa* PAO1, and stabilizes the sRNA RsmY (Sonnleitner *et al.*, 2006). This suggests that Hfq and small RNAs could very well play a role in the regulation of PCN synthesis in strain PCL1391.

Sigma factors are also important regulators of secondary metabolism (Chapter 1). Particularly in *Pseudomonas* sp. many sigma factors are encoded in the genome: up to twenty are predicted to exist in *P. aeruginosa* PAO1 (Haas & Keel, 2003; Stover *et al.*, 2000) and thirty-two in *P. fluorescens* Pf-5 (Paulsen *et al.*, 2005), whereas only seven are present in *E. coli* (Gruber & Gross, 2003). The most thoroughly studied sigma factor is σ^S , encoded by *rpoS*. This protein was shown to regulate the production of many secondary metabolites in various strains (Sarniguet *et al.*, 1995; Schuster *et al.*, 2004; Suh *et al.*, 1999). A few other sigma factors were shown to play a role in regulation of secondary metabolism. PvdS is an alternative sigma factor that directs the cell response to changes in iron concentration in the environment (Vasil & Ochsner, 1999). RpoN, another alternative sigma factor which is more closely linked to the production of secondary metabolites, was shown to regulate the production of antifungal compounds in *P. fluorescens* (Péchy-Tarr *et al.*, 2005) and to interact with GacA and quorum-sensing in *P. aeruginosa* (Heurlier *et al.*, 2003). In *P. chlororaphis* strain PCL1391 the sigma factor σ^S was shown to regulate PCN synthesis (Chapters 2 and 3). It is possible that other alternative sigma factors also play a role in the regulation of the *phz* operon. Identification of regulators in *P. chlororaphis* PCL1391 will be facilitated by the genome sequencing project that was initiated recently.

REFERENCES

- Abbas, A., Morrissey, J. P., Marquez, P. C., Sheehan, M. M., Delany, I. R., & O'Gara, F. (2002). Characterization of interactions between the transcriptional repressor PhlF and its binding site at the *phlA* promoter in *Pseudomonas fluorescens* F113. *J Bacteriol* **184**, 3008-3016.
- Albus, A. M., Pesci, E. C., Runyen-Janecky, L. J., West, S. E., & Iglewski, B. H. (1997). Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**, 3928-3935.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1997). Current protocols in molecular biology.
- Bacon, C. W., Porter, J. K., Norred, W. P., & Leslie, J. F. (1996). Production of fusaric acid by *Fusarium* species. *Appl Environ Microbiol* **62**, 4039-4043.
- Bailey, T. L. & Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology*, 28-36. Menlo Park, CA, AAAI Press.
- Bakker, P. A., Glandorf, D. C., Viebahn, M., Ouwens, T. W., Smit, E., Leeflang, P., Wernars, K., Thomashow, L. S., Thomas-Oates, J. E., & van Loon, L. C. (2002). Effects of *Pseudomonas putida* modified to produce phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol on the microflora of field grown wheat. *Antonie Van Leeuwenhoek* **81**, 617-624.
- Bassler, B. L. (1999). How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr Opin Microbiol* **2**, 582-587.
- Beck, C. F., Mutzel, R., Barbe, J., & Muller, W. (1982). A multifunctional gene (*tetR*) controls Tn10-encoded tetracycline resistance. *J Bacteriol* **150**, 633-642.
- Bertani, I. & Venturi, V. (2004). Regulation of the *N*-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase RpoS sigma factor and the global regulator GacA. *Appl Environ Microbiol* **70**, 5493-5502.
- Blattner, F. R., Plunkett, G., III, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., & Shao, Y. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453-1474.
- Blumer, C. & Haas, D. (2000a). Iron regulation of the *hcnABC* genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator GacA in *Pseudomonas fluorescens* CHAO. *Microbiology* **146** (Pt 10), 2417-2424.
- Blumer, C. & Haas, D. (2000b). Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Arch Microbiol* **173**, 170-177.

- Blumer, C., Heeb, S., Pessi, G., & Haas, D. (1999).** Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci U S A* **96**, 14073-14078.
- Bolwerk, A., Lagopodi, A. L., Wijffes, A. H., Lamers, G. E., Chin, A. W. T., Lugtenberg, B. J. J., & Bloemberg, G. V. (2003).** Interactions in the tomato rhizosphere of two *Pseudomonas* biocontrol strains with the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol Plant Microbe Interact* **16**, 983-993.
- Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C., Gaasterland, T., Glenisson, P., Holstege, F. C., Kim, I. F., Markowitz, V., Matese, J. C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S., Stewart, J., Taylor, R., Vilo, J., & Vingron, M. (2001).** Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat Genet* **29**, 365-371.
- Brint, J. M. & Ohman, D. E. (1995).** Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhIR-RhII, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J Bacteriol* **177**, 7155-7163.
- Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L., Dodson, R. J., Deboy, R. T., Durkin, A. S., Kolonay, J. F., Madupu, R., Daugherty, S., Brinkac, L., Beanan, M. J., Haft, D. H., Nelson, W. C., Davidsen, T., Zafar, N., Zhou, L., Liu, J., Yuan, Q., Khouri, H., Fedorova, N., Tran, B., Russell, D., Berry, K., Utterback, T., Van Aken, S. E., Feldblyum, T. V., D'Ascenzo, M., Deng, W. L., Ramos, A. R., Alfano, J. R., Cartinhour, S., Chatterjee, A. K., Delaney, T. P., Lazarowitz, S. G., Martin, G. B., Schneider, D. J., Tang, X., Bender, C. L., White, O., Fraser, C. M., & Collmer, A. (2003).** The complete genome sequence of the Arabidopsis and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *Proc Natl Acad Sci U S A* **100**, 10181-10186.
- Castric, P. A. (1975).** Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. *Can J Microbiol* **21**, 613-618.
- Challis, G. L. & Hopwood, D. A. (2003).** Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc Natl Acad Sci U S A* **100 Suppl 2**, 14555-14561.
- Chancey, S. T., Wood, D. W., & Pierson, L. S., III (1999).** Two-component transcriptional regulation of *N*-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol* **65**, 2294-2299.
- Chatterjee, A., Cui, Y., Yang, H., Collmer, A., Alfano, J. R., & Chatterjee, A. K. (2003).** GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. *tomato* DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol Plant Microbe Interact* **16**, 1106-1117.
- Chin-A-Woeng, T. F. C., Bloemberg, G. V., & Lugtenberg, B. J. J. (2003a).** Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytologist* **157**, 503-523.

- Chin-A-Woeng, T. F. C., Bloemberg, G. V., Mulders, I. H., Dekkers, L. C., & Lugtenberg, B. J. J. (2000).** Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant Microbe Interact* **13**, 1340-1345.
- Chin-A-Woeng, T. F. C., Bloemberg, G. V., van der Bij, A. J., van der Drift, K. M. G. M., Schripsema, J., Kroon, B., Scheffer, R. J., Keel, C., Bakker, P. A. H. M., Tichy, H. V., de Bruijn, F. J., Thomas-Oates, J. E., & Lugtenberg, B. J. J. (1998).** Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol Plant-Microbe Interact* **11**, 1069-1077.
- Chin-A-Woeng, T. F. C., Lugtenberg, B. J. J., & Bloemberg, G. V. (2003b).** Mechanisms of biological control of phytopathogenic fungi by *Pseudomonas* spp. 173-225.
- Chin-A-Woeng, T. F. C., Thomas-Oates, J. E., Lugtenberg, B. J. J., & Bloemberg, G. V. (2001a).** Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. *Mol Plant Microbe Interact* **14**, 1006-1015.
- Chin-A-Woeng, T. F. C., van den Broek, D., de Voer, G., van der Drift, K. M., Tuinman, S., Thomas-Oates, J. E., Lugtenberg, B. J. J., & Bloemberg, G. V. (2001b).** Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium. *Mol Plant Microbe Interact* **14**, 969-979.
- Chin-A-Woeng, T. F. C., van den Broek, D., Lugtenberg, B. J. J., & Bloemberg, G. V. (2005).** The *Pseudomonas chlororaphis* PCL1391 sigma regulator *psrA* represses the production of the antifungal metabolite phenazine-1-carboxamide. *Mol Plant-Microbe Interact* **18**, 244-253.
- Chugani, S. A., Whiteley, M., Lee, K. M., D'Argenio, D., Manoil, C., & Greenberg, E. P. (2001).** QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **98**, 2752-2757.
- Compant, S., Duffy, B., Nowak, J., Clement, C., & Barka, E. A. (2005).** Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl Environ Microbiol* **71**, 4951-4959.
- Cornelis, P. & Matthijs, S. (2002).** Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. *Environ Microbiol* **4**, 787-798.
- Costerton, J. W. (2001).** Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends Microbiol* **9**, 50-52.
- Cullinane, M., Baysse, C., Morrissey, J. P., & O'Gara, F. (2005).** Identification of two lysophosphatidic acid acyltransferase genes with overlapping function in *Pseudomonas fluorescens*. *Microbiology* **151**, 3071-3080.

- Cundliffe, E. (1989).** How antibiotic-producing organisms avoid suicide. *Annu Rev Microbiol* **43**, 207-233.
- de Weert, S., Kuiper, I., Lagendijk, E. L., Lamers, G. E., & Lugtenberg, B. J. J. (2004).** Role of chemotaxis toward fusaric acid in colonization of hyphae of *Fusarium oxysporum* f. sp. *radicis-lycopersici* by *Pseudomonas fluorescens* WCS365. *Mol Plant Microbe Interact* **17**, 1185-1191.
- Denhardt, D. T. (1966).** A membrane-filter technique for the detection of complementary DNA. *Biochem Biophys Res Commun* **23**, 641-
- Deziel, E., Comeau, Y., & Villemur, R. (2001).** Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpilated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J Bacteriol* **183**, 1195-1204.
- Diggle, S. P., Winzer, K., Chhabra, S. R., Worrall, K. E., Camara, M., & Williams, P. (2003).** The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates *rhl*-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* **50**, 29-43.
- Ditta, G., Stanfield, S., Corbin, D., & Helinski, D. R. (1980).** Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci U S A* **77**, 7347-7351.
- Dong, Y. H., Gusti, A. R., Zhang, Q., Xu, J. L., & Zhang, L. H. (2002).** Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. *Appl Environ Microbiol* **68**, 1754-1759.
- Dubern, J. F., Lagendijk, E. L., Lugtenberg, B. J. J., & Bloemberg, G. V. (2005).** The heat shock genes *dnaK*, *dnaJ*, and *grpE* are involved in regulation of putisolvin biosynthesis in *Pseudomonas putida* PCL1445. *J Bacteriol* **187**, 5967-5976.
- Duffy, B. & Defago, G. (1997).** Zinc improves biocontrol of fusarium crown and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic synthesis. *Phytopathology* **87**, 1250-1257.
- Duffy, B. K. & Defago, G. (1999).** Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl Environ Microbiol* **65**, 2429-2438.
- Erickson, D. L., Lines, J. L., Pesci, E. C., Venturi, V., & Storey, D. G. (2004).** *Pseudomonas aeruginosa* *relA* contributes to virulence in *Drosophila melanogaster*. *Infect Immun* **72**, 5638-5645.
- Evans, K., Adewoye, L., & Poole, K. (2001).** MexR repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic region. *J Bacteriol* **183**, 807-812.

- Evans, K., Passador, L., Srikumar, R., Tsang, E., Nezezon, J., & Poole, K. (1998). Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **180**, 5443-5447.
- Fischer, H. M. (1994). Genetic regulation of nitrogen fixation in rhizobia. *Microbiol Rev* **58**, 352-386.
- Folders, J., Algra, J., Roelofs, M. S., van Loon, L. C., Tommassen, J., & Bitter, W. (2001). Characterization of *Pseudomonas aeruginosa* chitinase, a gradually secreted protein. *J Bacteriol* **183**, 7044-7052.
- Fujita, M., Tanaka, K., Takahashi, H., & Amemura, A. (1994). Transcription of the principal sigma-factor genes, *rpoD* and *rpoS*, in *Pseudomonas aeruginosa* is controlled according to the growth phase. *Mol Microbiol* **13**, 1071-1077.
- Fuqua, C., Parsek, M. R., & Greenberg, E. P. (2001). Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* **35**, 439-468.
- Fuqua, C., Winans, S. C., & Greenberg, E. P. (1996). Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol* **50**, 727-751.
- Gallagher, L. A. & Manoil, C. (2001). *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J Bacteriol* **183**, 6207-6214.
- Galperin, M. Y., Nikolskaya, A. N., & Koonin, E. V. (2001). Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* **203**, 11-21.
- Gambello, M. J., Kaye, S., & Iglewski, B. H. (1993). LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. *Infect Immun* **61**, 1180-1184.
- Girard, G., van Rij, E. T., Lugtenberg, B. J. J., & Bloemberg, G. V. (2006). Roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL1391. *Microbiology* **152**, 43-58.
- Gottesman, S. (2004). The small RNA regulators of *Escherichia coli*: roles and mechanisms. *Annu Rev Microbiol* **58**, 303-328.
- Gould, T. A., Schweizer, H. P., & Churchill, M. E. (2004). Structure of the *Pseudomonas aeruginosa* acyl-homoserinelactone synthase LasI. *Mol Microbiol* **53**, 1135-1146.
- Grkovic, S., Brown, M. H., & Skurray, R. A. (2002). Regulation of bacterial drug export systems. *Microbiol Mol Biol Rev* **66**, 671-701, table.
- Gruber, T. M. & Gross, C. A. (2003). Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu Rev Microbiol* **57**, 441-466.

- Gutierrez, P., Li, Y., Osborne, M. J., Pomerantseva, E., Liu, Q., & Gehring, K. (2005).** Solution structure of the carbon storage regulator protein CsrA from *Escherichia coli*. *J Bacteriol* **187**, 3496-3501.
- Haas, D. & Defago, G. (2005).** Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* **3**, 307-319.
- Haas, D. & Keel, C. (2003).** Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annu Rev Phytopathol*
- Hanahan, D. (1983).** Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557-580.
- Heeb, S., Blumer, C., & Haas, D. (2002).** Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHAO. *J Bacteriol* **184**, 1046-1056.
- Heeb, S. & Haas, D. (2001).** Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. *Mol Plant Microbe Interact* **14**, 1351-1363.
- Heeb, S., Itoh, Y., Nishijyo, T., Schnider, U., Keel, C., Wade, J., Walsh, U., O'Gara, F., & Haas, D. (2000).** Small, stable shuttle vectors based on the minimal pVS1 replicon for use in Gram-negative, plant-associated bacteria. *Mol Plant Microbe Interact* **13**, 232-237.
- Heeb, S., Valverde, C., Gigot-Bonnefoy, C., & Haas, D. (2005).** Role of the stress sigma factor RpoS in GacA/RsmA-controlled secondary metabolism and resistance to oxidative stress in *Pseudomonas fluorescens* CHAO. *FEMS Microbiol Lett* **243**, 251-258.
- Hengge-Aronis, R. (1993).** Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. *Cell* **72**, 165-168.
- Hengge-Aronis, R. (2002a).** Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev* **66**, 373-95, table.
- Hengge-Aronis, R. (2002b).** Stationary phase gene regulation: what makes an *Escherichia coli* promoter sigmaS-selective? *Curr Opin Microbiol* **5**, 591-595.
- Henne, A., Bruggemann, H., Raasch, C., Wiezer, A., Hartsch, T., Liesegang, H., Johann, A., Lienard, T., Gohl, O., Martinez-Arias, R., Jacobi, C., Starkuviene, V., Schlenczeck, S., Dencker, S., Huber, R., Klenk, H. P., Kramer, W., Merkl, R., Gottschalk, G., & Fritz, H. J. (2004).** The genome sequence of the extreme thermophile *Thermus thermophilus*. *Nat Biotechnol* **22**, 547-553.

- Hentzer, M., Wu, H., Andersen, J. B., Riedel, K., Rasmussen, T. B., Bagge, N., Kumar, N., Schembri, M. A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J. W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Hoiby, N., & Givskov, M. (2003). Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J* **22**, 3803-3815.
- Hertz, G. Z. & Stormo, G. D. (1999). Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. *Bioinformatics* **15**, 563-577.
- Heurlier, K., Denervaud, V., Pessi, G., Reimmann, C., & Haas, D. (2003). Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**, 2227-2235.
- Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P., & Haas, D. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **186**, 2936-2945.
- Hillen, W. & Berens, C. (1994). Mechanisms underlying expression of Tn10 encoded tetracycline resistance. *Annu Rev Microbiol* **48**, 345-369.
- Hogan, D. A., Vik, A., & Kolter, R. (2004). A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Mol Microbiol* **54**, 1212-1223.
- Isackson, P. J. & Bertrand, K. P. (1985). Dominant negative mutations in the Tn10 tet repressor: evidence for use of the conserved helix-turn-helix motif in DNA binding. *Proc Natl Acad Sci U S A* **82**, 6226-6230.
- Jorgensen, F., Bally, M., Chapon-Herve, V., Michel, G., Lazdunski, A., Williams, P., & Stewart, G. S. (1999). RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. *Microbiology* **145** (Pt 4), 835-844.
- Juhas, M., Wiehlmann, L., Huber, B., Jordan, D., Lauber, J., Salunkhe, P., Limpert, A. S., von Gotz, F., Steinmetz, I., Eberl, L., & Tummli, B. (2004). Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiology* **150**, 831-841.
- Kang, B. R., Cho, B. H., Anderson, A. J., & Kim, Y. C. (2004). The global regulator GacS of a biocontrol bacterium *Pseudomonas chlororaphis* O6 regulates transcription from the *rpoS* gene encoding a stationary-phase sigma factor and affects survival in oxidative stress. *Gene* **325**, 137-143.
- Kaufmann, G. F., Sartorio, R., Lee, S. H., Rogers, C. J., Meijler, M. M., Moss, J. A., Clapham, B., Brogan, A. P., Dickerson, T. J., & Janda, K. D. (2005). Revisiting quorum sensing: Discovery of additional chemical and biological functions for 3-oxo-N-acylhomoserine lactones. *Proc Natl Acad Sci U S A* **102**, 309-314.
- Kay, E., Dubuis, C., & Haas, D. (2005). Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHAO. *Proc Natl Acad Sci U S A* **102**, 17136-17141.

- Keel, C., Voisard, C., Berling, C. H., Kahr, G., & Defago, G. (1989). Iron sufficiency, a prerequisite for suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHAO under gnotobiotic conditions. *Phytopathology* **79**, 584-589.
- Kim, E. J., Wang, W., Deckwer, W. D., & Zeng, A. P. (2005). Expression of the quorum-sensing regulatory protein LasR is strongly affected by iron and oxygen concentrations in cultures of *Pseudomonas aeruginosa* irrespective of cell density. *Microbiology* **151**, 1127-1138.
- King, E. O., Ward, M. K., & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* **44**, 301-307.
- Kiratisin, P., Tucker, K. D., & Passador, L. (2002). LasR, a transcriptional activator of *Pseudomonas aeruginosa* virulence genes, functions as a multimer. *J Bacteriol* **184**, 4912-4919.
- Kojic, M., Aguilar, C., & Venturi, V. (2002). TetR family member PsrA directly binds the *Pseudomonas rpoS* and *psrA* promoters. *J Bacteriol* **184**, 2324-2330.
- Kojic, M., Degrassi, G., & Venturi, V. (1999). Cloning and characterisation of the *rpoS* gene from plant growth-promoting *Pseudomonas putida* WCS358: RpoS is not involved in siderophore and homoserine lactone production. *Biochim Biophys Acta* **1489**, 413-420.
- Kojic, M., Jovcic, B., Vindigni, A., Odreman, F., & Venturi, V. (2005). Novel target genes of PsrA transcriptional regulator of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **246**, 175-181.
- Kojic, M. & Venturi, V. (2001). Regulation of *rpoS* gene expression in *Pseudomonas*: involvement of a TetR family regulator. *J Bacteriol* **183**, 3712-3720.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175-176.
- Kuiper, I., Bloemberg, G. V., Noreen, S., Thomas-Oates, J. E., & Lugtenberg, B. J. J. (2001). Increased uptake of putrescine in the rhizosphere inhibits competitive root colonization by *Pseudomonas fluorescens* strain WCS365. *Mol Plant Microbe Interact* **14**, 1096-1104.
- Kuiper, I., Kravchenko, L. V., Bloemberg, G. V., & Lugtenberg, B. J. J. (2002). *Pseudomonas putida* strain PCL1444, selected for efficient root colonization and naphthalene degradation, effectively utilizes root exudate components. *Mol Plant Microbe Interact* **15**, 734-741.
- Labes, M., Puhler, A., & Simon, R. (1990). A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. *Gene* **89**, 37-46.

- Lamb, J. R., Patel, H., Montminy, T., Wagner, V. E., & Iglewski, B. H. (2003).** Functional domains of the RhlR transcriptional regulator of *Pseudomonas aeruginosa*. *J Bacteriol* **185**, 7129-7139.
- Lange, R. & Hengge-Aronis, R. (1991).** Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol Microbiol* **5**, 49-59.
- Latifi, A., Foglino, M., Tanaka, K., Williams, P., & Lazdunski, A. (1996).** A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol* **21**, 1137-1146.
- Latifi, A., Winson, M. K., Foglino, M., Bycroft, B. W., Stewart, G. S., Lazdunski, A., & Williams, P. (1995).** Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* **17**, 333-343.
- Lau, G. W., Hassett, D. J., Ran, H., & Kong, F. (2004).** The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol Med* **10**, 599-606.
- Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G., & Haas, D. (1992).** Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc Natl Acad Sci U S A* **89**, 1562-1566.
- Leadbetter, J. R. & Greenberg, E. P. (2000).** Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. *J Bacteriol* **182**, 6921-6926.
- Ledgham, F., Ventre, I., Soscia, C., Foglino, M., Sturgis, J. N., & Lazdunski, A. (2003).** Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhIR. *Mol Microbiol* **48**, 199-210.
- Lonetto, M., Gribskov, M., & Gross, C. A. (1992).** The sigma 70 family: sequence conservation and evolutionary relationships. *J Bacteriol* **174**, 3843-3849.
- Loper, J. E. & Henkels, M. D. (1997).** Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. *Appl Environ Microbiol* **63**, 99-105.
- Loper, J. E. & Henkels, M. D. (1999).** Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. *Appl Environ Microbiol* **65**, 5357-5363.
- Lugtenberg, B. J. J. & Bloemberg, G. V. (2003).** Like in the rhizosphere.
- Lugtenberg, B. J. J., Chin-A-Woeng, T. F. C., & Bloemberg, G. V. (2002).** Microbe-plant interactions: principles and mechanisms. *Antonie Van Leeuwenhoek* **81**, 373-383.

- Lugtenberg, B. J. J., Kravchenko, L. V., & Simons, M. (1999).** Tomato seed and root exudate sugars: composition, utilization by *Pseudomonas* biocontrol strains and role in rhizosphere colonization. *Environ Microbiol* **1**, 439-446.
- Ma, D., Alberti, M., Lynch, C., Nikaido, H., & Hearst, J. E. (1996).** The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. *Mol Microbiol* **19**, 101-112.
- Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., & Hearst, J. E. (1995).** Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. *Mol Microbiol* **16**, 45-55.
- Ma, D., Cook, D. N., Hearst, J. E., & Nikaido, H. (1994).** Efflux pumps and drug resistance in Gram-negative bacteria. *Trends Microbiol* **2**, 489-493.
- Marsh, J. L., Erfle, M., & Wykes, E. J. (1984).** The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene* **32**, 481-485.
- Mashburn, L. M. & Whiteley, M. (2005).** Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* **437**, 422-425.
- Masse, E., Escorcia, F. E., & Gottesman, S. (2003a).** Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev* **17**, 2374-2383.
- Masse, E. & Gottesman, S. (2002).** A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc Natl Acad Sci U S A* **99**, 4620-4625.
- Masse, E., Majdalani, N., & Gottesman, S. (2003b).** Regulatory roles for small RNAs in bacteria. *Curr Opin Microbiol* **6**, 120-124.
- Medina, G., Juarez, K., Valderrama, B., & Soberon-Chavez, G. (2003).** Mechanism of *Pseudomonas aeruginosa* RhlR transcriptional regulation of the *rhlAB* promoter. *J Bacteriol* **185**, 5976-5983.
- Mikulecky, P. J., Kaw, M. K., Brescia, C. C., Takach, J. C., Sledjeski, D. D., & Feig, A. L. (2004).** *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs. *Nat Struct Mol Biol* **11**, 1206-1214.
- Miller, M. B. & Bassler, B. L. (2001).** Quorum sensing in bacteria. *Annu Rev Microbiol* **55**, 165-199.
- Miller, P. F. & Sulavik, M. C. (1996).** Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in *Escherichia coli*. *Mol Microbiol* **21**, 441-448.
- Milton, D. L., Hardman, A., Camara, M., Chhabra, S. R., Bycroft, B. W., Stewart, G. S., & Williams, P. (1997).** Quorum sensing in *Vibrio anguillarum*: characterization of the

- vanI/vanR* locus and identification of the autoinducer N-(3-oxodecanoyl)-L-homoserine lactone. *J Bacteriol* **179**, 3004-3012.
- Moller, T., Franch, T., Udesen, C., Gerdes, K., & Valentin-Hansen, P. (2002).** Spot 42 RNA mediates discoordinate expression of the *E. coli* galactose operon. *Genes Dev* **16**, 1696-1706.
- Moran, G. R. (2005).** 4-Hydroxyphenylpyruvate dioxygenase. *Arch Biochem Biophys* **433**, 117-128.
- Munns, R. (2005).** Genes and salt tolerance: bringing them together. *New Phytol* **167**, 645-663.
- Nishikawa, T., Ogawa, K., Kohda, R., Zhixiong, W., Miyasaka, H., Umeda, F., Maeda, I., Kawase, M., & Yagi, K. (2002).** Cloning and molecular analysis of poly(3-hydroxyalkanoate) biosynthesis genes in *Pseudomonas aureofaciens*. *Curr Microbiol* **44**, 132-135.
- Nystrom, T. (2004).** Growth versus maintenance: a trade-off dictated by RNA polymerase availability and sigma factor competition? *Mol Microbiol* **54**, 855-862.
- O'Sullivan, D. J. & O'Gara, F. (1992).** Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiol Rev* **56**, 662-676.
- Ochsner, U. A. & Reiser, J. (1995).** Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**, 6424-6428.
- Ojangu, E. L., Tover, A., Teras, R., & Kivisaar, M. (2000).** Effects of combination of different -10 hexamers and downstream sequences on stationary-phase-specific sigma factor sigma(S)-dependent transcription in *Pseudomonas putida*. *J Bacteriol* **182**, 6707-6713.
- Okusu, H., Ma, D., & Nikaido, H. (1996).** AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *J Bacteriol* **178**, 306-308.
- Olliver, A., Valle, M., Chaslus-Dancla, E., & Cloeckaert, A. (2004).** Role of an *acrR* mutation in multidrug resistance of in vitro-selected fluoroquinolone-resistant mutants of *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol Lett* **238**, 267-272.
- Ownley, B. H., Duffy, B. K., & Weller, D. M. (2003).** Identification and manipulation of soil properties to improve the biological control performance of phenazine-producing *Pseudomonas fluorescens*. *Appl Environ Microbiol* **69**, 3333-3343.
- Paulsen, I. T., Press, C. M., Ravel, J., Kobayashi, D. Y., Myers, G. S., Mavrodi, D. V., Deboy, R. T., Seshadri, R., Ren, Q., Madupu, R., Dodson, R. J., Durkin, A. S., Brinkac, L. M., Daugherty, S. C., Sullivan, S. A., Rosovitz, M. J., Gwinn, M. L., Zhou, L., Schneider, D. J., Cartinhour, S. W., Nelson, W. C., Weidman, J.,**

- Watkins, K., Tran, K., Khouri, H., Pierson, E. A., Pierson, L. S., Thomashow, L. S., & Loper, J. E. (2005). Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nat Biotechnol* **23**, 873-878.
- Pearson, J. P., Pesci, E. C., & Iglewski, B. H. (1997). Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* **179**, 5756-5767.
- Péchy-Tarr, M., Bottiglieri, M., Mathys, S., Bang Lejbolle, K., Schnider-Keel, U., Maurhofer, M., & Keel, C. (2005). RpoN (sigma 54) controls production of antifungal compounds and biocontrol activity in *Pseudomonas fluorescens* CHAO. *Mol Plant-Microbe Interact* **18**, 260-272.
- Pesci, E. C., Pearson, J. P., Seed, P. C., & Iglewski, B. H. (1997). Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**, 3127-3132.
- Pessi, G. & Haas, D. (2000). Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 6940-6949.
- Pessi, G. & Haas, D. (2001). Dual control of hydrogen cyanide biosynthesis by the global activator GacA in *Pseudomonas aeruginosa* PAO1. *FEMS Microbiol Lett* **200**, 73-78.
- Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T., Camara, M., Haas, D., & Williams, P. (2001). The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6676-6683.
- Pierson, L. S., III, Keppenne, V. D., & Wood, D. W. (1994). Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *J Bacteriol* **176**, 3966-3974.
- Pocard, J. A., Smith, L. T., Smith, G. M., & Le Rudulier, D. (1994). A prominent role for glucosylglycerol in the adaptation of *Pseudomonas mendocina* SKB70 to osmotic stress. *J Bacteriol* **176**, 6877-6884.
- Pohl, E., Haller, J. C., Mijovilovich, A., Meyer-Klaucke, W., Garman, E., & Vasil, M. L. (2003). Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator. *Mol Microbiol* **47**, 903-915.
- Poole, K. (2004). Efflux-mediated multiresistance in Gram-negative bacteria. *Clin Microbiol Infect* **10**, 12-26.
- Qi, Q., Rehm, B. H., & Steinbuchel, A. (1997). Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene *phaC2* from *Pseudomonas aeruginosa*: comparison of PhaC1 and PhaC2. *FEMS Microbiol Lett* **157**, 155-162.
- Raaijmakers, J. M., Vlami, M., & de Souza, J. T. (2002). Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek* **81**, 537-547.

- Ramos-González, M. I. & Molin, S. (1998).** Cloning, sequencing, and phenotypic characterization of the *rpoS* gene from *Pseudomonas putida* KT2440. *J Bacteriol* **180**, 3421-3431.
- Rashid, M. H., Rajanna, C., Ali, A., & Karaolis, D. K. (2003).** Identification of genes involved in the switch between the smooth and rugose phenotypes of *Vibrio cholerae*. *FEMS Microbiol Lett* **227**, 113-119.
- Rehm, B. H., Kruger, N., & Steinbuchel, A. (1998).** A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. The PHAG gene from *Pseudomonas putida* KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme a transferase. *J Biol Chem* **273**, 24044-24051.
- Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., & Haas, D. (1997).** The global activator GacA of *Pseudomonas aeruginosa* PAO1 positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* **24**, 309-319.
- Reimmann, C., Valverde, C., Kay, E., & Haas, D. (2005).** Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHAO. *J Bacteriol* **187**, 276-285.
- Repoila, F., Majdalani, N., & Gottesman, S. (2003).** Small non-coding RNAs, co-ordinators of adaptation processes in *Escherichia coli*: the RpoS paradigm. *Mol Microbiol* **48**, 855-861.
- Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H., Hoiby, N., Givskov, M., Molin, S., & Eberl, L. (2001).** *N*-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. *Microbiology* **147**, 3249-3262.
- Rodrigue, A., Quentin, Y., Lazdunski, A., Mejean, V., & Foglino, M. (2000).** Two-component systems in *Pseudomonas aeruginosa*: why so many? *Trends Microbiol* **8**, 498-504.
- Romeo, T. (1998).** Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Mol Microbiol* **29**, 1321-1330.
- Ruby, E. G. (1996).** Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri*-*Euprymna scolopes* light organ symbiosis. *Annu Rev Microbiol* **50**, 591-624.
- Sacherer, P., Defago, G., & Haas, D. (1994).** Extracellular protease and phospholipase C are controlled by the global regulatory gene *gacA* in the biocontrol strain *Pseudomonas fluorescens* CHAO. *FEMS Microbiol Lett* **116**, 155-160.
- Sambrook, J. & Russel, D. (2001).** Molecular cloning: a laboratory manual. **Third edition**,

- Sarniguet, A., Kraus, J., Henkels, M. D., Muehlchen, A. M., & Loper, J. E. (1995).** The sigma factor sigma s affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. *Proc Natl Acad Sci U S A* **92**, 12255-12259.
- Schmidt-Eisenlohr, H., Gast, A., & Baron, C. (2003).** Inactivation of *gacS* does not affect the competitiveness of *Pseudomonas chlororaphis* in the *Arabidopsis thaliana* rhizosphere. *Appl Environ Microbiol* **69**, 1817-1826.
- Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Defago, G., Haas, D., & Keel, C. (2000).** Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHAO and repression by the bacterial metabolites salicylate and pyoluteorin. *J Bacteriol* **182**, 1215-1225.
- Schumacher, M. A., Pearson, R. F., Moller, T., Valentin-Hansen, P., & Brennan, R. G. (2002).** Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J* **21**, 3546-3556.
- Schuster, M., Hawkins, A. C., Harwood, C. S., & Greenberg, E. P. (2004a).** The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* **51**, 973-985.
- Schuster, M., Lostroh, C. P., Ogi, T., & Greenberg, E. P. (2003).** Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* **185**, 2066-2079.
- Schuster, M., Urbanowski, M. L., & Greenberg, E. P. (2004b).** Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci U S A* **101**, 15833-15839.
- Schwyn, B. & Neilands, J. B. (1987).** Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47-56.
- Sonnleitner, E., Hagens, S., Rosenau, F., Wilhelm, S., Habel, A., Jager, K. E., & Blasi, U. (2003).** Reduced virulence of a *hfq* mutant of *Pseudomonas aeruginosa* O1. *Microb Pathog* **35**, 217-228.
- Sonnleitner, E., Moll, I., & Blasi, U. (2002).** Functional replacement of the *Escherichia coli* *hfq* gene by the homologue of *Pseudomonas aeruginosa*. *Microbiology* **148**, 883-891.
- Sonnleitner, E., Schuster, M., Sorger-Domenig, T., Greenberg, E. P., & Blasi, U. (2006).** Hfq-dependent alterations of the transcriptome profile and effects on quorum-sensing in *Pseudomonas aeruginosa*. *Mol Microb* **59**, 1542-1558.
- Sosinsky, A., Bonin, C. P., Mann, R. S., & Honig, B. (2003).** Target Explorer: An automated tool for the identification of new target genes for a specified set of transcription factors. *Nucleic Acids Res.* **31**, 3589-3592.

- Storz, G., Opdyke, J. A., & Zhang, A. (2004).** Controlling mRNA stability and translation with small, noncoding RNAs. *Curr Opin Microbiol* **7**, 140-144.
- Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrenner, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., & Olson, M. V. (2000).** Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959-964.
- Suh, S. J., Silo-Suh, L., Woods, D. E., Hassett, D. J., West, S. E., & Ohman, D. E. (1999).** Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181**, 3890-3897.
- Tanaka, K. & Takahashi, H. (1994).** Cloning, analysis and expression of an *rpoS* homologue gene from *Pseudomonas aeruginosa* PAO1. *Gene* **150**, 81-85.
- Trotochaud, A. E. & Wassarman, K. M. (2005).** A highly conserved 6S RNA structure is required for regulation of transcription. *Nat Struct Mol Biol* **12**, 313-319.
- Valentin-Hansen, P., Eriksen, M., & Udesen, C. (2004).** The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol* **51**, 1525-1533.
- Valverde, C., Heeb, S., Keel, C., & Haas, D. (2003).** RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHAO. *Mol Microbiol* **50**, 1361-1379.
- Valverde, C., Lindell, M., Wagner, E. G., & Haas, D. (2004).** A repeated GGA motif is critical for the activity and stability of the riboregulator RsmY of *Pseudomonas fluorescens*. *J Biol Chem* **279**, 25066-25074.
- Van Delden, C. & Iglewski, B. H. (1998).** Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* **4**, 551-560.
- van den Broek, D., Bloemberg, G. V., & Lugtenberg, B. J. J. (2005).** The role of phenotypic variation in rhizosphere *Pseudomonas* bacteria. *Environ Microbiol* **7**, 1686-1697.
- van Loon, L. C., Bakker, P. A., & Pieterse, C. M. (1998).** Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol* **36**, 453-483.
- van Rij, E. T., Girard, G., Lugtenberg, B. J. J., & Bloemberg, G. V. (2005).** Influence of fusaric acid on phenazine-1-carboxamide synthesis and gene expression of *Pseudomonas chlororaphis* strain PCL1391. *Microbiology* **151**, 2805-2814.
- van Rij, E. T., Wesselink, M., Chin-A-Woeng T.F.C., Bloemberg, G. V., & Lugtenberg, B. J. J. (2004).** Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. *Mol Plant-Microbe Interact* **17**, 557-566.

- Vasil, M. L. & Ochsner, U. A. (1999).** The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol Microbiol* **34**, 399-413.
- Ventre, I., Ledgham, F., Prima, V., Lazdunski, A., Foglino, M., & Sturgis, J. N. (2003).** Dimerization of the quorum sensing regulator RhlR: development of a method using EGFP fluorescence anisotropy. *Mol Microbiol* **48**, 187-198.
- Venturi, V. (2003).** Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? *Mol Microbiol* **49**, 1-9.
- Vytvytska, O., Moll, I., Kaberdin, V. R., von Gabain, A., & Blasi, U. (2000).** Hfq (HF1) stimulates *ompA* mRNA decay by interfering with ribosome binding. *Genes Dev* **14**, 1109-1118.
- Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., & Iglewski, B. H. (2003).** Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* **185**, 2080-2095.
- Wassarman, K. M., Repoila, F., Rosenow, C., Storz, G., & Gottesman, S. (2001).** Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev* **15**, 1637-1651.
- Weaver, V. B. & Kolter, R. (2004).** *Burkholderia* spp. Alter *Pseudomonas aeruginosa* Physiology through Iron Sequestration. *J Bacteriol* **186**, 2376-2384.
- Weller, D. M., Raaijmakers, J. M., Gardener, B. B., & Thomashow, L. S. (2002).** Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu Rev Phytopathol* **40**, 309-348.
- Whistler, C. A., Corbell, N. A., Sarniguet, A., Ream, W., & Loper, J. E. (1998).** The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor sigmaS and the stress response in *Pseudomonas fluorescens* Pf-5. *J Bacteriol* **180**, 6635-6641.
- Whistler, C. A. & Pierson III, L. S. (2003).** Repression of phenazine antibiotic production in *Pseudomonas aureofaciens* strain 30-84 by RpeA. *J Bacteriol* **185**, 3718-3725.
- Whiteley, M. & Greenberg, E. P. (2001).** Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. *J Bacteriol* **183**, 5529-5534.
- Whiteley, M., Parsek, M. R., & Greenberg, E. P. (2000).** Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 4356-4360.
- Wilderman, P. J., Sowa, N. A., FitzGerald, D. J., FitzGerald, P. C., Gottesman, S., Ochsner, U. A., & Vasil, M. L. (2004).** Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. *Proc Natl Acad Sci U S A* **101**, 9792-9797.

- Winson, M. K., Camara, M., Latifi, A., Foglino, M., Chhabra, S. R., Daykin, M., Bally, M., Chapon, V., Salmond, G. P., Bycroft, B. W., & . (1995). Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**, 9427-9431.
- Wolk, C. P., Cai, Y., & Panoff, J. M. (1991). Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. *Proc Natl Acad Sci U S A* **88**, 5355-5359.
- Wood, D. W., Gong, F., Daykin, M. M., Williams, P., & Pierson, L. S., III (1997). *N*-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *J Bacteriol* **179**, 7663-7670.
- Wood, D. W. & Pierson, L. S., III (1996). The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**, 49-53.
- Yates, E. A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S. R., Sockett, R. E., Goldner, M., Dessaux, Y., Camara, M., Smith, H., & Williams, P. (2002). *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect Immun* **70**, 5635-5646.
- Yehuda, Z., Shenker, M., Romheld, V., Marschner, H., Hadar, Y., & Chen, Y. (1996). The role of ligand exchange in the uptake of iron from microbial siderophores by gramineous plants. *Plant Physiol* **112**, 1273-1280.
- Yellaboina, S., Seshadri, J., Kumar, M. S., & Ranjan, A. (2004). PredictRegulon: a web server for the prediction of the regulatory protein binding sites and operons in prokaryote genomes. *Nucleic Acids Res.* **32**, W318-W320.
- Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., Allen, H. L., DeKievit, T. R., Gardner, P. R., Schwab, U., Rowe, J. J., Iglewski, B. H., McDermott, T. R., Mason, R. P., Wozniak, D. J., Hancock, R. E., Parsek, M. R., Noah, T. L., Boucher, R. C., & Hassett, D. J. (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* **3**, 593-603.
- Zhang, A., Wassarman, K. M., Rosenow, C., Tjaden, B. C., Storz, G., & Gottesman, S. (2003). Global analysis of small RNA and mRNA targets of Hfq. *Mol Microbiol* **50**, 1111-1124.
- Zuber, S., Carruthers, F., Keel, C., Mattart, A., Blumer, C., Pessi, G., Gigot-Bonnefoy, C., Schnider-Keel, U., Heeb, S., Reimmann, C., & Haas, D. (2003). GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHAO. *Mol Plant Microbe Interact* **16**, 634-644.



Résumé & Samenvatting

RESUME

Les métabolites secondaires jouent un rôle central dans les interactions entre les bactéries *Pseudomonas* et les autres organismes. Contrairement aux métabolites primaires, les métabolites secondaires ne sont pas essentiels à la croissance ou à la reproduction. Ils ne dérivent pas directement des nutriments, mais des métabolites primaires, et sont en principe produits à l'entrée en phase stationnaire de croissance. Comme résumé dans le chapitre 1, les métabolites secondaires sont produits en grande diversité par les espèces de *Pseudomonas*. En comprendre les mécanismes régulateurs est d'intérêt pour des disciplines aussi différentes que la médecine et l'agriculture, mais aussi pour l'industrie. *Pseudomonas chlororaphis* souche PCL1391 est utilisée comme modèle dans l'étude de la régulation du métabolisme secondaire. Cette souche a été isolée de la rhizosphère (fraction du sol à proximité immédiate des racines des plantes, beaucoup plus riches en nutriments que le reste du sol et favorisant la croissance de nombreux micro-organismes) de plants de tomate. *P. chlororaphis* PCL1391 sécrète des enzymes et de nombreux métabolites secondaires, comme des exoprotéases, des lipases, le cyanure d'hydrogène (HCN), la phenazine-1-carboxamide (PCN) et son précurseur, l'acide phenazine-1-carboxylique (PCA). Ces métabolites sont importants dans la compétition entre *P. chlororaphis* et d'autres micro-organismes pour une meilleure place dans la niche écologique.

Fusarium oxysporum f. sp. *radicis lycopersici* est l'agent causal de la pourriture de la racine et du collet de la tomate. Par conséquent, ce champignon est la cause de pertes économiques importantes pour l'agriculture. Entrant en compétition dans la rhizosphère avec ce champignon, *P. chlororaphis* exerce un contrôle négatif sur sa croissance et protège ainsi les plants de tomate de la maladie. Ce phénomène est appelé biocontrôle. La production de PCN par *P. chlororaphis* est cruciale pour ses capacités de biocontrôle. Des travaux ont précédemment montré qu'un opéron (série de gènes adjacent dont l'ADN est transcrit en un seul ARN messenger commun) de 8 gènes, *phzABCDEFGH*, est responsable de la synthèse de PCA et de PCN.

Plusieurs régulateurs de l'opéron *phz* ont été caractérisés par des travaux précédents dans notre groupe. Le système GacS/GacA (contrôle global des antibiotiques) comprend une protéine sensorielle (GacS) et un activateur transcriptionnel (GacA). GacS est inséré dans la membrane et on suppose qu'il répond à un signal environnemental, jusque maintenant inconnu. GacA est ensuite activé par GacS et régule la transcription de plusieurs gènes. Ses cibles directes

n'ont pas encore été identifiées. Le système GacS/GacA est un régulateur positif de la synthèse de PCN. Il a été montré que l'expression du gène *psrA*, codant pour un autre régulateur de la synthèse de PCN, dépend de GacS/GacA. Le système quorum-sensing PhzI/PhzR est un autre régulateur important de l'opéron *phz*. Brièvement, le "quorum-sensing" est un mode de communication intercellulaire bactérienne permettant aux individus de la population de coordonner un comportement adaptatif aux conditions environnementales quand une certaine densité bactérienne ("quorum") est atteinte. Souvent, cette communication est le fait d'une molécule diffusible appelée *N*-acyl-homosérine lactone (*N*-AHL) qui s'accumule dans le milieu. A forte concentration cette *N*-AHL se fixe sur un régulateur transcriptionnel et modifie l'expression de gènes (induction/répression). Dans le cas de *P. chlororaphis* souche PCL1391, PhzI synthétise les *N*-AHLs, qui se fixent sur PhzR. Ceci induit l'activation de PhzR qui peut alors fonctionner comme régulateur transcriptionnel. Deux cibles connues de PhzR activé sont *phzI* et *phzABCDEFGH*. Cela signifie que le système quorum-sensing peut s'auto-activer, mais aussi stimule la synthèse de PCN. Dans tous les travaux précédents, ainsi que dans les chapitres de cette thèse, les quantités de *N*-AHL et de PCN sont corrélées, indiquant que la régulation du système quorum-sensing détermine la régulation de la synthèse de PCN.

Les études portant sur la régulation du métabolisme secondaire chez les bactéries (passées en revue dans le **chapitre 1**) indiquent que des gènes supplémentaires pourraient être impliqués dans la régulation de la synthèse de PCN, et que leurs interactions pourraient être très complexes. De plus, plusieurs facteurs environnementaux ont été décrits qui influent sur la synthèse de PCN par *P. chlororaphis* PCL1391. Cependant, les mécanismes moléculaires impliqués dans la régulation de l'opéron *phz* par les facteurs environnementaux n'ont pas encore été étudiés en détail. Le but général des études présentées dans cette thèse est d'éclaircir les mécanismes complexes régulant la production de PCN par *P. chlororaphis* PCL1391.

PsrA est un régulateur transcriptionnel qui reconnaît directement le promoteur (région régulatrice d'un gène en amont de ce gène) du gène *rpoS* dans d'autres espèces de *Pseudomonas*. RpoS est un facteur sigma alternatif (un composant essentiel de l'ARN polymérase, elle-même transcrivant l'ADN en ARN lors de l'expression des gènes) qui régule l'expression générale des gènes en cas de stress et pendant la phase de croissance stationnaire. Comme la synthèse de PCN a lieu au début de la phase de croissance stationnaire et qu'elle est régulée par PsrA,

il semblait logique de vérifier si RpoS régule la synthèse de PCN. Pour le **chapitre 2**, un mutant du gène *rpoS* a été construit. Ce mutant présente un niveau extrêmement réduit de production de PCN. De même, un mutant *psrA* est affecté dans sa production de PCN. Ainsi, des mutants présentant une protéine PsrA ou RpoS non fonctionnelle sont affectés dans leur production de PCN. Cela signifie que PsrA et RpoS sont nécessaires pour une synthèse normale de PCN. Des études impliquant l'expression constitutive de certains gènes dans des mutants, combinée à la quantification de PCN et *N*-AHL produit dans le milieu de culture, ont montré l'existence de la cascade régulatrice suivante : PsrA régule RpoS, qui lui-même régule (directement ou indirectement) le système quorum-sensing PhzI/PhzR, lui-même régulant l'opéron *phz*. En outre, on a remarqué que RpoS a une influence réduite sur la production de PCN quand PCL1391 est cultivé en milieu riche par rapport à un milieu pauvre en nutriments. D'autres résultats suggèrent que RpoS pourrait aussi réguler la conversion de PCA en PCN. Cette observation apporte des nuances à l'hypothèse précédente que seulement le quorum-sensing détermine la synthèse de PCN. Enfin, les résultats de ce chapitre suggèrent également qu'une deuxième cascade régulatrice, subordonnée à GacS/GacA et parallèle à PsrA/RpoS, régule aussi le système quorum-sensing et l'opéron *phz*.

En parallèle avec ces expériences utilisant la mutation de gènes dans les bactéries, une méthode à grande échelle a été développée dans le **chapitre 3** afin d'étudier l'expression de milliers de gènes de façon simultanée. Cette méthode, impliquant la fabrication d'une puce à ADN, peut aider à l'identification de grands nombres de gènes jouant un rôle dans les processus régulateurs. Une banque (très nombreux fragments d'ADN provenant de la fragmentation du génome, classés et ordonnés de façon à faciliter leur identification) de fragments du chromosome unique de PCL1391 a été construite. Cette banque a été utilisée pour fabriquer une puce à ADN « maison » pour PCL1391. Brièvement, cette puce se présente sous la forme d'une lame de verre (utilisée habituellement pour la préparation d'échantillons pour le microscope) de quelques centimètres de long, sur laquelle les différents fragments de la banque sont fixés dans des très petites gouttes (quelques nanolitres). Douze mille gouttes sont présentes sur chaque puce, chaque goutte représentant un seul fragment (en de nombreuses copies identiques) du génome de *P. chlororaphis* PCL1391. Cette puce permet de mesurer en une seule expérience la transcription de la plupart des gènes de PCL1391 à un moment donné dans des conditions données. Pour cela, le produit de la transcription des gènes (l'ARN) doit être isolées des bactéries dans les conditions voulues, puis être reverse-transcrit en

ADN. Cet ADN (ADNc) est marqué par fluorescence et est hybridé sur la puce. Les gouttes d'ADN de la puce correspondant aux gènes transcrits s'hybrident à l'ADNc provenant des mêmes gènes, et deviennent donc fluorescentes. Une photo de la puce permet la visualisation de cette fluorescence et ainsi la mesure de la transcription du génôme. Plusieurs protocoles ont été testés pour l'isolation de l'ARN, la synthèse et le marquage fluorescent de l'ADNc, et son hybridation sur la puce. La procédure la plus efficace pour l'extraction d'ARN comprend une extraction phénol/chloroforme et une purification sur colonne. L'ARN ensuite utilisé pour la synthèse d'ADNc fluorescent dans une méthode de marquage indirect. La puce a été testée pour comparer la transcription du génôme (le transcriptome) dans les mutants *psrA* et *rpoS* par rapport à la souche sauvage (non mutée) PCL1391. Les résultats valident le modèle de régulation décrit précédemment (Chapter 2) et les analyses de transcriptome ont conduit à l'identification de plusieurs gènes nouveaux qui pourraient être impliqués dans la régulation du métabolisme secondaire. Certains de ces gènes pourraient jouer un rôle dans la régulation fine de la synthèse de PCN.

Dans le **chapitre 4**, un mutant a été isolé qui présente une synthèse très réduite de PCN. L'analyse du génôme de ce mutant a montré que la mutation était localisée dans un gène codant pour un possible régulateur de transcription, qui a été appelé Pip (protéine inductrice de phénazine). Pip est homologue aux régulateurs de transcription AcrR et TetR. Ces deux régulateurs sont impliqués dans la réponse au stress, particulièrement le stress causé par les antibiotiques. Des homologues de *pip* ont été identifiés dans beaucoup d'autres espèces bactériennes, et leur fonction précise est *a priori* inconnue. Des études d'expression et l'analyse de production de PCN et *N*-AHL par plusieurs mutants ont montré que Pip régule l'opéron *phz* à la suite de *PsrA* et *RpoS* et par l'intermédiaire du système quorum-sensing. Bien que AcrR et TetR régulent tous les deux la production d'une pompe membranaire codée par des gènes voisins de *acrR* et *tetR*, aucune pompe de ce type n'a pu être détectée dans l'ADN voisin du gène *pip*.

Plusieurs facteurs environnementaux influencent la synthèse de PCN par *P. chlororaphis* PCL1391. Certains de ces facteurs - comme le chlorure de sodium (NaCl) et l'acide fusarique, une toxine produite par le champignon *Fusarium* - sont liés au stress et il a été montré qu'ils inhibent l'expression de l'opéron *phz*. Dans le **chapitre 5**, il est montré que la concentration inhibitoire minimum (MIC) de non seulement NaCl et l'acide fusarique, mais aussi des antibiotiques rifampicine et kanamycine, est inférieure pour la synthèse de *N*-AHL et PCN par rapport à la MIC

pour la croissance de *P. chlororaphis* PCL1391. Ceci signifie que *P. chlororaphis* est capable de pousser à des concentrations relativement élevées de certains facteurs de stress, bien qu'alors la synthèse de PCN et de *N*-AHL soit alors abolie. Après avoir testé si l'expression constitutive de gènes régulateurs pouvait restaurer la synthèse de PCN dans les conditions de stress, et avoir mesuré la production de Pip en présence des différents facteurs de stress, les conclusions suivantes ont été tirées. (i) Les facteurs de stress testés inhibent l'opéron *phz* en réprimant le système de quorum-sensing PhzI/PhzR. (ii) Pip est impliqué dans la répression de synthèse de PCN par tous les facteurs de stress testés. (iii) L'inhibition de la synthèse de PCN par l'acide fusarique, la kanamycine et NaCl est causée par une réduction de la concentration de la protéine Pip dans la cellule. (iv) Dans le cas du stress par la rifampicine, d'autres facteurs en plus de Pip doivent entrer en jeu pour expliquer l'inhibition de la synthèse de PCN. Ces résultats vont dans le sens de l'hypothèse suivante : en présence de certains facteurs de stress, l'expression du gène *pip* est (probablement indirectement) réprimée, d'où une inhibition de la synthèse de PCN (et possiblement d'autres processus dépendant de Pip) et une économie d'énergie au sein de la cellule, qui peut être alors utilisée pour mieux résister au stress. En combinant à ces résultats ceux du deuxième chapitre, on peut supposer que RpoS régule la synthèse de PCN surtout dans des conditions limitées en nutriments et passe à une régulation de la résistance au stress si nécessaire, après que l'activité de l'opéron *phz* est réduite par une diminution de la quantité de Pip. C'est à notre connaissance la première fois qu'un tel système commutateur est décrit qui permet de passer du métabolisme secondaire (synthèse de PCN) au métabolisme primaire (résistance au stress) en cas de stress.

Tous ces résultats permettent d'affiner notre connaissance du réseau de régulateurs de la synthèse de PCN. GacS/GacA serait un régulateur clé en amont du réseau régulateur. Au moins deux cascades de régulateurs partent en aval de GacS/GacA (voir paragraphe suivant). Une des ces cascades comprend PsaA, RpoS et Pip, tous régulant positivement le système quorum-sensing PhzI/PhzR. Pip a été découvert dans cette thèse comme nouveau régulateur de la synthèse de PCN (Chapitre 4). Il est intéressant de remarquer que le gène *phzR* exprimé de façon constitutive est capable de restaurer la production de PCN dans tous les mutants testés, y compris le *gacS* mutant (Chapitres 2 et 4). Cela indique que toutes les cascades (régulant la synthèse de PCN) en aval de GacA convergent probablement juste en amont de PhzI/PhzR. Certaines observations du chapitre 2 ont conduit à l'hypothèse novatrice que *phzR* pourrait aussi être régulé post-

transcriptionnellement. Enfin, cette thèse élargit la connaissance de la régulation de la synthèse de PCN en apportant un éclairage nouveau sur le lien entre plusieurs facteurs environnementaux et les régulateurs génétiques.

Globalement, les résultats de cette thèse suggèrent que la cascade étudiée dans les quatre chapitres expérimentaux n'est pas le seul intermédiaire du système GacS/GacA en ce qui concerne la régulation de la synthèse de PCN. En effet, l'expression constitutive de ni *psrA*, ni *rpoS*, ni *pip* n'est capable de restaurer la synthèse de PCN dans un mutant *gacS*, alors que l'expression constitutive de *phzR* en est capable. En outre, RpoS joue un rôle plus limité dans un milieu riche en nutriments et l'existence de Pip semble prendre de l'importance particulièrement dans des conditions de stress. Ces observations pourraient signifier qu'une cascade inconnue, peut-être constituée d'homologues des régulateurs de la famille Rsm (Chapitre 1), serait un autre intermédiaire important entre GacS/GacA et PhzI/PhzR. Egaleme nt entre GacS/GacA et PhzI/PhzR, la cascade PsrA/RpoS/Pip représenterait une autre branche dont l'importance serait déterminée par les conditions nutritives et de stress. Cela est un nouvel aspect du modèle de régulation de la synthèse de PCN, qui devrait être testé dans de futures recherches.

L'identification de Pip comme régulateur du métabolisme secondaire est importante pour le domaine de recherche de *Pseudomonas* en général, puisque des homologues très conservés de Pip ont été trouvés dans la plupart des autres espèces de *Pseudomonas*. En outre, une large fonction physiologique est proposée pour Pip : en plus de son implication dans la régulation de la synthèse de PCN, nous supposons que Pip a un rôle plus général pour le fonctionnement de la cellule dans des conditions de stress. Le modèle présenté à la fin de cette thèse donne un nouvel aperçu de la façon dont les cellules pourraient favoriser leur résistance au stress en réduisant leur production de métabolites secondaires.

SAMENVATTING

Secundaire metabolieten spelen een centrale rol in de interacties tussen *Pseudomonas* species en andere organismen. In tegenstelling tot primaire metabolieten zijn de secundaire niet essentieel voor groei en reproductie. De secundaire metabolieten zijn niet direct afgeleid van nutriënten maar van primaire metabolieten en worden meestal geproduceerd aan het begin van de stationaire groeifase. Zoals beschreven in **Hoofdstuk 1**, produceren *Pseudomonas* species een scala aan secundaire metabolieten. Het ophelderen van de regulatiemechanismen voor de productie van deze groep metabolieten ligt aan de basis voor een breder begrip in medicijnontwikkeling, landbouw en andere industriële toepassingen. Een modelstam dat gebruikt wordt voor dit soort onderzoek is *Pseudomonas chlororaphis* PCL1391 die is geïsoleerd uit de rhizosfeer (de directe omgeving van de wortels rijker aan nutriënten dan de rest van de grond) van een tomatenplant. *P. chlororaphis* PCL1391 scheidt verscheidene enzymen en vele secundaire metabolieten uit zoals protease, lipase, waterstofcyanide (HCN), phenazine-1-carboxamide (PCN) en zijn precursor phenazine-1-carboxylzuur (PCA).

Het organisme *Fusarium oxysporum* f. sp. *radicis lycopersici* veroorzaakt voet- en wortelrot bij tomaat. Deze schimmel is de oorzaak van oogstverliezen met grote economische gevolgen. *P. chlororaphis* beschermt de tomaat tegen deze ziekte door de schimmeligroei te remmen en zo de infectie te controleren. Dit fenomeen wordt ook wel biocontrole genoemd. PCN productie is een vereiste voor de biocontrole die *P. chlororaphis* PCL1391 uitoefent. Een cluster van acht genen, *phzABCDEFGH*, is verantwoordelijk voor de aanmaak van PCA, en uiteindelijk van PCN.

Verschillende regulatoren die het *phz* cluster reguleren zijn al beschreven. Het GacS/GacA systeem bestaat uit een GacS sensoreiwit en de GacA transcriptie-activator. GacS bevindt zich in het celmembraan en reageert op een nog onbekend signaal van buiten de cel. Vervolgens wordt GacA geactiveerd en reguleert dan de transcriptie van vele genen. Activering van het GacS/GacA koppel induceert de PCN productie. Expressie van een andere regulator van de PCN synthese, PsrA, wordt ook door GacS/GacA gestuurd. Ook het PhzI/PhzR quorum sensing koppel is een belangrijke regulator van de *phz* cluster. PhzI synthetiseert verschillende *N*-acyl homoserine lactonen (*N*-AHL), die vervolgens waarschijnlijk aan PhzR binden. Het complex van *N*-AHL gebonden aan PhzR is dan de actieve vorm van de transcriptie-activator PhzR en bindt aan de zogenaamde *lux* boxen in de promotergebieden van *phzI* en *phzABCDEFGH*. Al deze gebeurtenissen resulteren in de zelfactivering van

het quorum sensing systeem en in de productie van PCN aan het begin van de stationaire groeifase. Zowel in onderzoek door anderen als in dit proefschrift wordt herhaaldelijk duidelijk dat er een correlatie bestaat tussen de hoeveelheden van *N*-AHL en PCN in *P. chlororaphis* PCL1391, hetgeen impliceert dat de regulatie van het quorum sensing systeem uiteindelijk de PCN productie reguleert.

Verscheidene studies aan de synthese van secundaire metabolieten in bacteriën (samengevat in **Hoofdstuk 1**) wijzen er op dat er mogelijk nog andere genen betrokken zijn bij de regulatie van de PCN productie in *P. chlororaphis* PCL1391 en dat de communicatie over en weer tussen die genen wel eens complex zou kunnen zijn. Bovendien blijken ook bepaalde omgevingsfactoren invloed op de PCN synthese te hebben, maar een gedetailleerd moleculair mechanisme van deze regulatie ontbreekt nog. De onderzoeksvraag die ten grondslag ligt aan dit proefschrift is hoe het moleculaire netwerk dat verantwoordelijk is voor de PCN productie door *P. chlororaphis* PCL1391 er op moleculair niveau uit ziet.

Onderzoek aan andere *Pseudomonas* stammen laat zien dat PsrA, een transcriptieregulator, direct aan het promotergebied van *rpoS* bindt. RpoS, ook wel de alternatieve sigmafactor genoemd (als component van het RNA polymerase), reguleert de genexpressie onder strescondities en tijdens de stationaire groeifase. Omdat PCN synthese aan het begin van de stationaire groeifase plaats vindt en onder invloed staat van PsrA, lag het voor de hand om te onderzoeken of RpoS de PCN synthese reguleert. In **Hoofdstuk 2** beschrijf ik dat een *rpoS* mutant die ik gemaakt heb, een sterk gereduceerde PCN synthese vertoont wanneer PCL1391 wordt gekweekt in het synthetische medium MVB1. Een *psrA* mutant vertoonde hetzelfde effect op de PCN productie in hetzelfde medium. De resultaten van deze genetische aanpak, gecombineerd met gekwantificeerde PCN en *N*-AHL niveaus van bacterieculturen gekweekt in MVB1 groeimedium, plaatsen RpoS hiërarchisch onder PsrA en boven het PhzI/PhzR quorum sensing koppel in de regulatiecascade van het *phz* genencluster. Een interessant gegeven dat uit dit onderzoek voortvloeide is dat RpoS minder invloed uitoefent op de PCN productie als de bacterie in een rijk medium wordt gekweekt. Ook impliceren sommige resultaten uit **Hoofdstuk 2** dat RpoS een regulerende rol speelt in de conversie van de precursor PCA naar PCN en nuanceert dus het idee dat slechts quorum sensing de synthese van PCN bepaalt. Ook kan op basis van de resultaten in **Hoofdstuk 2** niet worden uitgesloten dat er een tweede parallelle cascade bestaat die naast PsrA/RpoS, maar via GacS/GacA en PhzI/PhzR, de expressie van het *phz* genencluster reguleert.

In plaats van de gerichte aanpak zoals beschreven in **Hoofdstuk 2** voor het vinden van nieuwe genen die betrokken zijn bij PCN productie heb ik tijdens mijn promotie een hoogproductieve methode ontwikkeld voor de identificatie van een groot aantal genen betrokken bij de regulatie van secundair metabolisme. Deze methode is beschreven in **Hoofdstuk 3**. Van het genoom van de bacterie PCL1391 werd een grote verzameling van willekeurige kleine DNA fragmenten vermenigvuldigd en gebruikt voor de constructie van een zelfgemaakte DNA chip. Met behulp van deze chip kan in één enkel experiment het expressiepatroon van een enorm aantal genen gemeten worden. Verschillende protocollen voor de isolatie van boodschapper-RNA (mRNA), de synthese van fluorescent gelabeld kopie-DNA (cDNA) en hybridisatie op de DNA chip zijn uitvoerig getest en staan beschreven in **Hoofdstuk 3**. De meest efficiënte procedure behelst het opzuiveren van het RNA door middel van een extractie met fenol en chloroform, gevolgd door een chromatografiestap. Het totale geïsoleerde RNA wordt vervolgens gebruikt als matrijs voor de synthese van fluorescent gelabeld kopie-DNA. De DNA chip werd getest met kopie-DNAs gesynthetiseerd op RNA matrijzen geïsoleerd uit de *psrA* en *rpoS* mutanten en vergeleken met kopie-DNA van RNA verkregen uit het wildtype (ongemuteerde) bacterie. De verkregen resultaten zijn in overeenstemming met het model voor PCN synthese zoals beschreven in Hoofdstuk 2. Opmerkelijk genoeg leidde deze aanpak met de DNA-chip tot de identificatie van nieuwe genen die betrokken blijken te zijn bij de verfijning van de regulatie van PCN synthese.

In **Hoofdstuk 4** beschrijf ik de isolatie van een mutant van stam PCL1391 die een sterk gereduceerde PCN productie vertoont. Een gedetailleerde analyse van deze mutant onthulde dat de mutatie zich bevindt in een gen dat voor een mogelijke transcriptieregulator codeert, die ik Pip heb genoemd (voor phenazine inducerend proteïne). Pip vertoont algemene sequentiehomologie met de AcrR regulator en ten dele met de TetR regulator. Beide regulatoren zijn betrokken in de fysiologische respons van bacteriën wanneer ze aan stress, en met name antibioticumstress, worden blootgesteld. Homologen van het *pip* gen zijn in vele andere bacteriesoorten aanwezig, maar de functie van de Pip homologen was tot dusverre nog niet bekend. Analyse van de PCN en *N*-AHL productie niveaus door verschillende stammen die gemuteerd waren in het *pip* gen en in andere genen die de PCN productie reguleren wijzen erop dat Pip zich bevindt ná *PsrA* en *RpoS* maar vóór het *PhzI*/*PhzR* koppel in de cascade van de regulatie van de PCN synthese. Dit is tevens voor het eerst dat een fenotype voor een *pip* mutant beschreven is. Hoewel AcrR en TetR allebei de

aanmaak van een membraanpomp reguleren kon zo een pomp voor Pip niet aannemelijk gemaakt worden.

Verschillende omgevingsfactoren beïnvloeden de synthese van PCN in *P. chlororaphis* PCL139. Sommige zijn stressgerelateerde factoren die de expressie van *phz* genen remmen, zoals natrium chloride (NaCl) en het fytotoxine fuzaarzuur, dat geproduceerd wordt door de schimmel *Fusarium oxysporum*, een concurrent in de natuurlijke habitat van *P. chlororaphis*. In **Hoofdstuk 5** toonde ik aan dat de minimale concentratie voor groeiremming (MIC) van *P. chlororaphis* PCL139 niet alleen voor NaCl en fuzaarzuur, maar ook voor de antibiotica rifampicine en kanamycine hoger is dan voor de remming van de productie van *N*-AHL en PCN. Dit betekent dat *P. chlororaphis* PCL139 onder betrekkelijk hoge concentraties van deze stressfactoren kan groeien, terwijl de PCN synthese dan al stil ligt. Ik heb de verschillende PCN regulatiegenen door de bacterie tot hoge expressie laten komen om te kijken of daarmee de PCN synthese onder de stresscondities hersteld kan worden. De niveaus van PCN productie door deze stammen onder verschillende stresssituaties heb ik vervolgens gemeten en dit leidde tot de volgende resultaten. (i) De geteste stressfactoren remmen de expressie van de *phz* genen via het quorum sensing koppel PhzI/PhzR. (ii) Pip is betrokken bij deze repressie onder alle geteste stresscondities. (iii) De deling in PCN synthese kan voor fuzaarzuur en NaCl, en waarschijnlijk ook voor kanamycine, direct worden gecorreleerd met een daling in het niveau van het Pip eiwit in de bacteriecel. (iv) In het geval van rifampicinstress zijn additionele factoren betrokken bij de regulatie van de *phz* genen. De verkregen resultaten leiden tot de conclusie dat in aanwezigheid van verscheidene stressfactoren de Pip expressie (waarschijnlijk indirect) onderdrukt wordt en aldus leidt tot de remming van PCN synthese (en waarschijnlijk ook van andere nog niet ontdekte Pip-gerelateerde processen). Dit zou een overlevingsstrategie van de cel kunnen zijn om zo energie te besparen die nodig is voor de bescherming tegen stress. In overeenstemming met de resultaten van **Hoofdstuk 2** kan de aanname gemaakt worden dat RpoS vooral de PCN synthese reguleert onder nutriëntarme omstandigheden om zo de PCN synthese stil te leggen via Pip en om te schakelen naar stressresistentie wanneer dat vereist is. Dit is de eerste keer dat een stressafhankelijke, moleculaire schakel tussen primair en secundair metabolisme wordt beschreven.

De resultaten in dit proefschrift vormen de basis voor een nieuwe hypothese betreffende het netwerk dat de productie van PCN reguleert. GacS/GacA staat bovenaan de regulatiecascade voor PCN synthese. Daaronder staan PsrA, RpoS en

Pip die allen de aanmaak van het PhzI/PhzR quorum sensing koppel induceren, waarvan Pip een nieuw ontdekt eiwit is (**Hoofdstuk 4**). Opmerkelijk genoeg kan constitutieve expressie van *phzR* de gereduceerde PCN synthese in alle mutanten herstellen, met inbegrip dat in een *gacS* mutant (**Hoofdstukken 2 en 4**). Dit duidt erop dat alle regulatiecascaden die onder invloed van GacA staan hiërarchisch net boven PhzI/PhzR samenkomen. Sommige observaties uit **Hoofdstuk 2** leiden tot de nieuwe gedachte dat *phzR* na de transcriptie gereguleerd wordt. Ten slotte biedt dit proefschrift een nieuw perspectief in de PCN expressieregulatie door de ontdekking van een moleculaire link tussen de regulatie van de PCN productie door omgevingsfactoren en door genen.

Bovengenoemde resultaten suggereren dat de PsrA/RpoS/Pip cascade (Fig. 1 in Hoofdstuk 6) die beschreven wordt in de vier experimentele hoofdstukken, niet de enige GacS/GacA-afhankelijke weg is in de regulatie van de synthese van PCN. Dit idee wordt ondersteund door het feit dat in een *gacS* mutant de constitutieve expressie van *psrA*, *rpoS* of *pip* niet leidt tot herstel van de PCN synthese, hoewel dit effect wel wordt bereikt door constitutieve expressie van *phzR*. Bovendien bleek dat RpoS een beperkte rol speelt in rijk medium en dat Pip belangrijk lijkt te zijn onder bepaalde stress omstandigheden. Het is mogelijk dat deze waarnemingen betekenen dat een onbekende belangrijke cascade (Fig. 1 in Hoofdstuk 6), die mogelijk homologen bevat van de Rsm regulator familie (zie Hoofdstuk 1), functioneert als een tweede belangrijke weg tussen GacS/GacA en PhzI/PhzR. De eerstgenoemde PsrA/RpoS/Pip cascade zou vooral van belang kunnen zijn onder omstandigheden van voedselbeperking en grotere stress. Dit voegt een nieuw aspect toe aan het model en zou in de toekomst getest moeten worden.

De identificatie van Pip als een regulator van het secundaire metabolisme is een belangrijke stap voorwaarts in het *Pseudomonas* onderzoeksveld, omdat vele andere *Pseudomonas* soorten een homoloog van Pip bezitten. Tevens heb ik resultaten behaald die een bredere rol voor Pip aangeven. Naast een rol in de regulatie van de PCN synthese stel ik dat Pip een meer algemenere rol speelt in de celfysiologie *via* een centrale rol in de resistentie tegen stress. Het voorgestelde model biedt een nieuw antwoord op de vraag welke strategie een bacterieel gebruikt om de resistentie tegen stress een hogere prioriteit te geven dan de synthese van secundaire metabolieten.

CURRICULUM VITAE

Geneviève Girard was born on the 21st of February 1979 in Les Lilas (Seine-St Denis, France). After obtaining the baccalauréat in 1995, she started a 2-year study in the BCPST section (Biologie, Chimie, Physique et Sciences de la Terre/Biology, Chemistry, Physics and Geology) of the Lycée Hoche in Versailles (Yvelines, France). In 1997 she entered the Ecole Normale Supérieure in Cachan (Val de Marne, France). In 1999 she obtained a master degree in Biochemistry at the University Denis Diderot of Paris (Paris 7) and visited the Netherlands for the first time for a training period of 6 months at Genexpress (Biochemistry Laboratory, Leiden University) under the direction of Prof. Kees Pleij. In 2000 she got the agrégation of SVT (Sciences de la Vie et de la Terre/Biology and Geology). In 2001 she was back at the University of Leiden for a training period of 10 months at Genexpress in collaboration with the group of Prof. Herman Spaink (Molecular Cell Biology) and obtained a Master degree in Biology *cum laude*. She did her PhD study in the Microbiology group of Prof. Ben Lugtenberg between September 2001 and February 2006. She is currently working as a molecular virologist at Crucell in Leiden.

SCIENTIFIC WORK

Publications

- Van Meerten D, Girard G, van Duin J. Translational control by delayed RNA folding: identification of the kinetic trap. *RNA* (2001) **7** (3):483-494.
- Girard G, Roussis A, Gulyaev AP, Pleij CW, Spaink HP. Structural motifs in the RNA encoded by the early nodulation gene *enod40* of soybean. *Nucleic Acids Res.* (2003) **31** (17): 5003-5015.
- van Rij T, Girard G, Lugtenberg, BJJ, Bloemberg GV. Influence of fusaric acid on phenazine-1-carboxamide synthesis and gene expression of *Pseudomonas chlororaphis* strain PCL1391. *Microbiology* (2005) **151**: 2805-2814.
- Girard G, van Rij ET, Lugtenberg BJJ, Bloemberg GV. Regulatory roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL1391. *Microbiology* (2006), **152**: 43-58.
- Girard G, Barends S, Rigali S, van Rij ET, Lugtenberg BJJ, Bloemberg GV. Role of the novel transcriptional regulator Pip in phenazine synthesis and stress resistance in *Pseudomonas chlororaphis* PCL1391. *Submitted*.

Oral presentations

- Presentation at the EPS symposium in Amsterdam (10/01/2003): Set up of a micro-array technique to study the genetic regulation of phenazine production in biocontrol strain *Pseudomonas chlororaphis* PCL1391
- Presentation at the ALW Platform Molecular Genetics (28-29/10/2004) in Lunteren: Genetic regulation of the biosynthesis of the biofungicide phenazine-1-carboxamide in *Pseudomonas chlororaphis* PCL1391
- Poster at the 10th international congress on *Pseudomonas* in Marseille (27-31/08/2005): Regulatory roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL1391

