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

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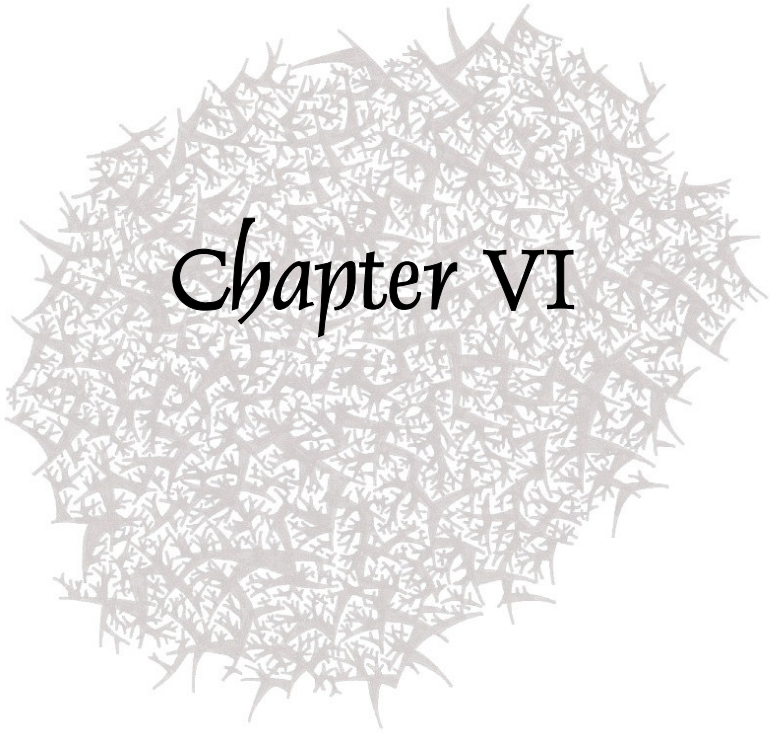

Chapter VI

Summary and discussion

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INTRODUCTION

As reviewed in Chapter 1, a high diversity of secondary of 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 C_6-HSL was shown to be the activator of PhzR in the regulation of the *phz* operon (Chin-A-Woeng *et al.,* 2001). C_6-HSL probably binds to PhzR. The C_6-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.

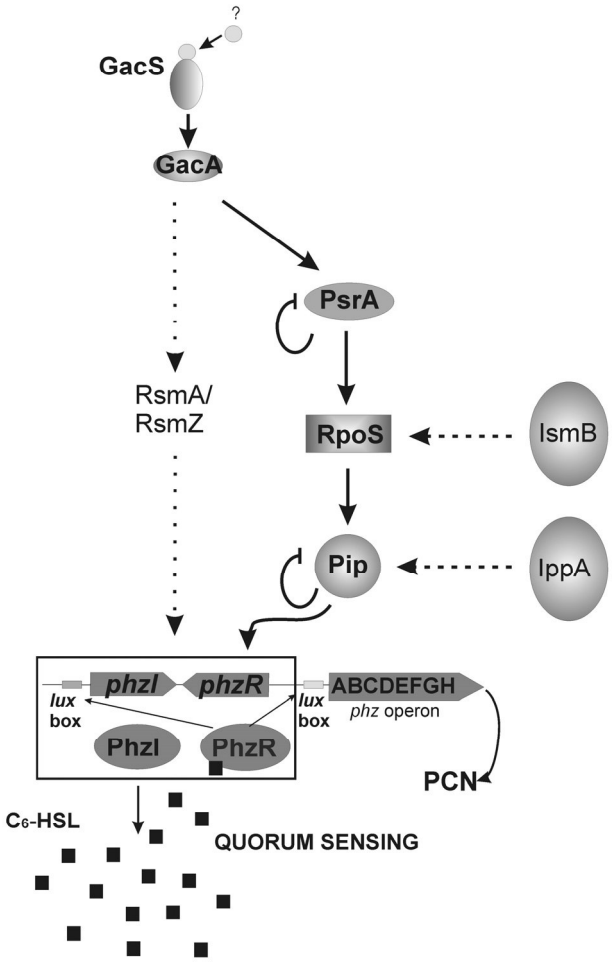

Figure 1. Hypothetical regulatory network for PCN synthesis in *P. chlororaphis* PCL1391.
The triangular arrow heads indicate a positive regulation, whereas the flat ones indicate a negative regulation. Results of this thesis show the existence of the main cascade drawn in the center (solid lines). Other 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; Reimmann *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 *rhlI*, 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.