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

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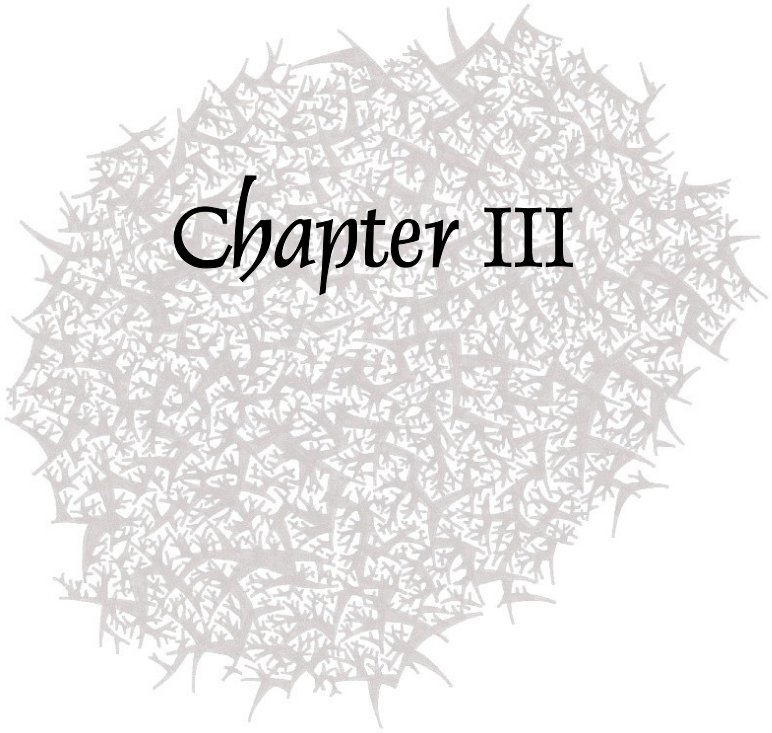

Chapter III

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

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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 of 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 *phzABCDEFGH* 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 transriptome 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 PsrA/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 $5x10^{10}$ 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.

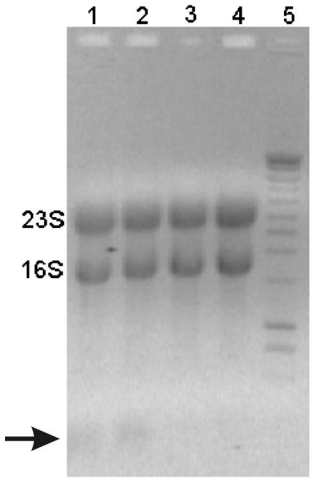

Figure 1. Total RNA extracted from *P. chlororaphis* PCL1391.
The RNA was extracted from PCL1391 cells and separated on 1.2% agarose gel following recommendations of QIAGEN (for details see Experimental Procedures section). For preparations loaded in lanes 1 and 2, the RNA extraction was stopped after 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.

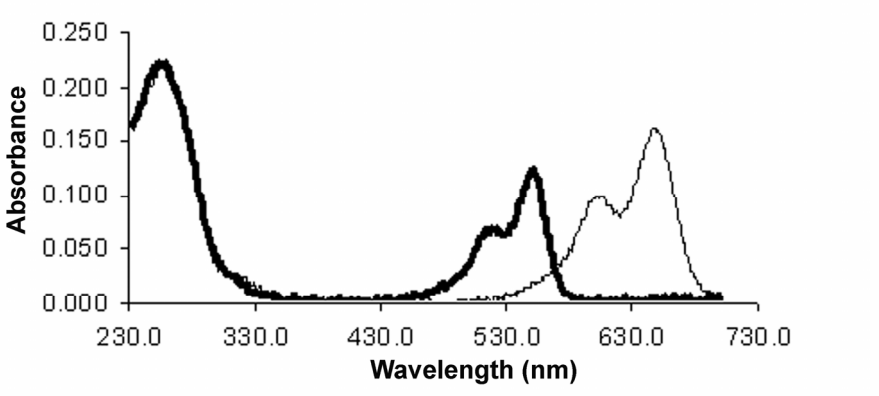

Figure 2. Photospectrometric analysis of dye label incorporation in Cy-cDNA.
After labeling of cDNA with Cy3 or Cy5, samples were analyzed in a photospectrometer and the spectrum determined. The thick line represents the absorption spectrum of Cy3 labeled cDNA. The thin line represents the absorption 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).

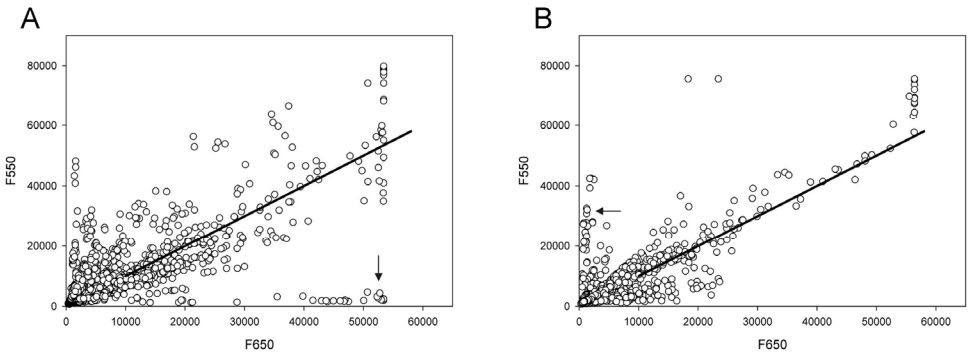

Figure 3. Plot of the intensities of spots of two microarrays.
Each spot of the microarrays is plotted for its intensity in the green channel (F550, absorption measured at 550 nm) and in the red channel (F650, absorption measured at 650 nm). On both graphs the normalization line (F650=F550) is indicated.
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 (*psrA*) §	Change of expression in PCL1954 (*rpoS*) §	Gene homology and/or accession number #	Bacterium corresponding to the gene homology ‡	Predicted function #†
phz genes					
2_A5	20±6	13±3	*phzR* AAF17494	*Pseudomonas chlororaphis*	Transcriptional activator
93_G11	6±2	8±2	*phzI* AAF17493	*Pseudomonas chlororaphis*	Autoinducer synthase
24_C5	3±0.7	6±0.8	*phzI* AAF17493 and *phzR* AAF17494	*Pseudomonas chlororaphis*	Autoinducer synthase and transcriptional activator
4_D1	21±8	12±0.8	*phzB/C* AAF17496 and AAF17497	*Pseudomonas chlororaphis*	Biosynthetic genes for PCN
97_D1	29±5	18±3	*phzD* AAF17498	*Pseudomonas chlororaphis*	Biosynthetic gene for PCN
119_D12	10±3	7±1	*phzE* AAF17499	*Pseudomonas chlororaphis*	Biosynthetic gene for PCN
126_G12	14±4	9±2	*phzH* AAF17502	*Pseudomonas chlororaphis*	Biosynthetic gene for PCN
Membrane protein genes					
2_C4	3.6±0.4	10±3	ZP_00262806	*Pseudomonas fluorescens*	Autotransporter adhesin
13_B1	3.8±0.5	9±2	ZP_00262806	*Pseudomonas fluorescens*	Autotransporter adhesin
36_A12	3±0.4	7±2	ZP_00262806	*Pseudomonas fluorescens*	Autotransporter adhesin
38_G7	3.3±0.3	8±2	ZP_00262806	*Pseudomonas fluorescens*	Autotransporter adhesin
105_A12	3.5±0.2	8±2	ZP_00262806	*Pseudomonas fluorescens*	Autotransporter adhesin
115_A2	3±0.4	5±1	ZP_00263190 and NP_745085	*Pseudomonas fluorescens* and *Pseudomonas putida*	Integral membrane protein (1-59/223) and conserved hypothetical protein (169-253/261)
4_H7	4.7±0.7	2±0.3	*nlpD* AAP97085	*Pseudomonas chlororaphis*	Lipoprotein (86-265/294)
Primary metabolism genes					
4_C1	4±1	4±2	NP_762166	*Vibrio*	Deoxycytidyla

				vulnificus	te deaminase (139-514/622
4_G11	4±2	7±4	NP_901074	*Chromobacterium violaceum*	Aminotransferase (4-268/367)
36_F1	0.034±0.01	1.1±0.1	ZP_00262398.1	*Pseudomonas fluorescens*	Acyl-CoA dehydrogenase
38_B12	0.016±0.01	1±0.07	ZP_00262398.1	*Pseudomonas fluorescens*	Acyl-CoA dehydrogenase
116_B10	0.025±0.01	1±0.08	ZP_00262398.1	*Pseudomonas fluorescens*	Acyl-CoA dehydrogenase
Intermediate metabolism genes					
2_B3	3±0.7	7±1	*phaC2* BAB78721	*Pseudomonas chlororaphis*	PHA-synthase 2 (225-560/560)
41_G2	3±0.7	7±2	*phaC2* BAB78721	*Pseudomonas chlororaphis*	PHA-synthase 2 (1-376/560)
53_F2	3±1	5±0.8	*phaG* BAB32432	*Pseudomonas* 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	*Pseudomonas* sp 61-3	Clone identical to 53_F2
71_A4	2±0.3	4±0.6	Clone identical to 53_F2	*Pseudomonas* sp 61-3	Clone identical to 53_F2
74_B4	3±0.6	5±1	Clone identical to 53_F2	*Pseudomonas* sp 61-3	Clone identical to 53_F2
74_E7	3±0.5	5±2	Clone identical to 53_F2	*Pseudomonas* sp 61-3	Clone identical to 53_F2
93_D8	2.5±0.5	6±2	*phaG* BAB32432	*Pseudomonas* sp 61-3	3-hydroxyacyl-acyl carrier protein CoA transferase (124-294/294)
Secondary metabolism genes					
11_G8	4±0.5	6±1	*chic* NP_250990	*Pseudomonas aeruginosa*	Chitinase (293-373/483)

121_H3	3±0.3	6±0.8	*chiC* NP_250990	*Pseudomonas aeruginosa*	Chitinase (165-479/483)
100_B7	3±0.4	7±0.6	NP_746359	*Pseudomonas putida*	Pyoverdine synthase? (665-987/4317)
86_H8	6±4	10±0.8	NP_901071 and NP_901070	*Chromobacterium violaceum*	Probable dihydrorhizobitoxine desaturase (248-353/369) and probable 5'—methylthioadenosine phosphorylase (31-186/302)
Regulatory genes					
65_B7	5±0.7	3±0.3	*rpoS* AAP97086 and *nlpD* AAP97085	*Pseudomonas chlororaphis*	RNA polymerase sigma factor (1-155/334) and lipoprotein (96-294/294)
98_B2	6±0.5	3±0.4	*rpoS* (AAP97086)	*Pseudomonas chlororaphis*	RNA polymerase sigma factor (40-334/334)
42_G8	4±1	7±2	ZP_00264029	*Pseudomonas fluorescens*	GGDEF/EAL domains containing regulator (260-617/624)
76_G2	3±0.9	4±1	ZP_00263882 and ZP_00263883	*Pseudomonas fluorescens*	Hypothetical protein (378-454/454) and ATP-dependent transcriptionnal regulator (1-236/911)
Hypothetical protein genes					
72_D12	7±2	40±12	ZP_00262803, ZP_00262802 and ZP_00262796	*Pseudomonas fluorescens*	Microcystin-dependent protein (1-191/191), hypothetical protein (1-

					103/103) and histone acetyltrans-ferase (71-161/163)
119_A8	7±1	32±5	ZP_00262803 and ZP_00262802	*Pseudomonas fluorescens*	Microcystin-dependent protein (1-191/191) and hypothetical protein (1-92/103)
1_B9	3±0.6	5±1	ZP_00266535	*Pseudomonas fluorescens*	Hypothetical protein (58-170/170)
3_F11	3±0.9	4±0.4	ZP_00128106	*Pseudomonas syringae* pv. *syringae*	Hypothetical protein (31-170/170)
11_E2	3±0.5	5±0.5	ZP_00263881.1 and ZP_00263880	*Pseudomonas fluorescens*	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	*Pseudomonas fluorescens*	Clone identical to 11_E2
12_G9	4±0.8	6±2	PP2941 NP_745085	*Pseudomonas putida*	Conserved hypothetical protein (187-253/261)
41_A12	6±2	12±2	ZP_00262372 and ZP_00262371	*Pseudomonas fluorescens*	Uncharacte-rized 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	*Pseudomonas fluorescens*	Hypothetical protein (524-629/629) and hypothetical protein (1-335/454)
72_H6	5±1	7±2	Clone	*Pseudomonas*	Clone

			identical to 47_F5	*fluorescens*	identical to 47_F5
93_H9	4±0.4	5±0.6	Clone identical to 47_F5	*Pseudomonas fluorescens*	Clone identical to 47_F5
112_C1	4±0.4	6±0.5	Clone identical to 47_F5	*Pseudomonas fluorescens*	Clone identical to 47_F5
59_C10	4±0.5	4±0.6	NP_929618	*Photorhabdus luminescens* subsp. *laumondii*	Hypothetical protein (2-93/93)
65_H9	4±1	8±2	ZP_00267318 and ZP_00266917	*Pseudomonas fluorescens*	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	*Photorhabdus luminescens* subsp. *laumondii*	Hypothetical protein (12-281/325)
81_G2	4±1	7±2	ZP_00128106	*Pseudomonas syringae* pv. *syringae*	Hypothetical protein (58-170/170)
101_H9	4±0.4	4±1	NP_929618	*Photorhabdus luminescens* subsp. *laumondii*	Hypothetical protein (2-70/93)
114_H5	3±0.4	5±0.7	ZP_00263821	*Pseudomonas fluorescens*	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* PfO1, *P. putida* KT2440, *P. aeruginosa* PAO1, *P. syringae* pv. *syringae* B728a, *V. vulnificus* CMCP6, *C. violaceum* ATCC 12472 and *Photorhabdus 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±28 µM) and PCL2052 (435±14 µM) appeared to be 2-fold increased compared to PCL1391 (237±9 µM).

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±4.1 µM PCN whereas PCL1391 produced 51.8±2.5 µM. Synthesis of C_6-HSL of both strains was visualized by TLC analysis (Fig. 4).

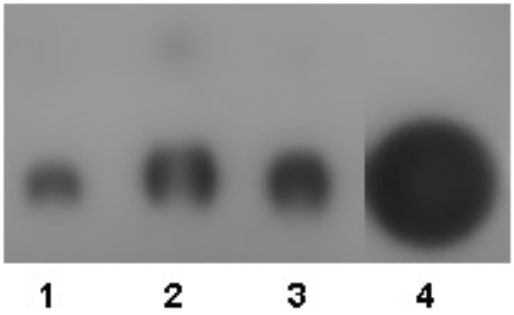

Figure 4. C18-reverse phase TLC analysis of *N*-AHLs produced by *P. chlororaphis* PCL1391 derivatives. Lane 1: PCL1391. Lanes : PCL1981. Lane 3: PCL1982. Lane 4: 2.5 nmol synthetic C_6-HSL

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 is it 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 (Folders *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 µg/ml), carbenicillin (200 µg/ml), gentamicin (10 µg/ml for *E. coli* and 30 µg/ml for *P. chlororaphis*), or 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.

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 (Maarssen, 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
Pseudomonas chlororaphis		
PCL1391	Wild-type *Pseudomonas chlororaphis*, producing phenazine-1-carboxamide and biocontrol strain of tomato foot and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici*	(Chin-A-Woeng *et al.,* 1998)
PCL1111	Derivative of PCL1391 in which a promoterless Tn5*luxAB* is inserted in *psrA*; Km^r	(Chin-A-Woeng *et al.,* 2005)
PCL1123	Derivative of PCL1391 in which a promoterless Tn5*luxAB* is inserted in *gacS*; Km^r	(Chin-A-Woeng *et al.,* 2005)
PCL1954	Derivative of PCL1391, *rpoS*::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
Escherichia coli		
DH5α	*Escherichia coli*; *supE44* Δ*lac*U169(Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi1 relA1*	(Hanahan, 1983)
Plasmids		
pRK2013	Helper plasmid for tri-parental mating; Km^r	(Ditta *et al.,* 1980)
pGEM-T easy	Plasmid designed for direct ligation of PCR fragments	Promega
pMP5285	Suicide plasmid for *Pseudomonas* spp. Used for homologous recombination; Km^r	(Kuiper *et al.,* 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 *Eco*RI 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'-ATATATGAATTCCTCGGTATTTCGCTACGGTTCGG3'
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 *Sau*3AI (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 *Xho*I site of pIC20H after partial filling with Klenow DNA polymerase (New England BioLabs Inc.). The resulting plasmids were transformed into *E. coli* DH5α.

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 µl per well. The resulting PCR fragments were precipitated by adding 10 µl of 3 M NaAc and 110 µl 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 OD_{620} 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 µl of lysozyme (1 mg/ml) in TE buffer, subsequently 40 µl of 20% SDS was added and the cells were allowed to lyze for 2 minutes at 65°C. A volume of 30 µl 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 µl H_2O.

The microarrays were prehybridized with 60 µl of prehybridization buffer (60% formamide, 5X Denhardt's and 50 µg/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 Na_3-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 µg/ml herring sperm DNA; all final concentrations). The slides were incubated for 16 hours at 37ºC. After hybridization, the slides were washed 3 times for 10 min in 2X SSC at 37ºC, once 10 min in 2X SSC at 50ºC, 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).

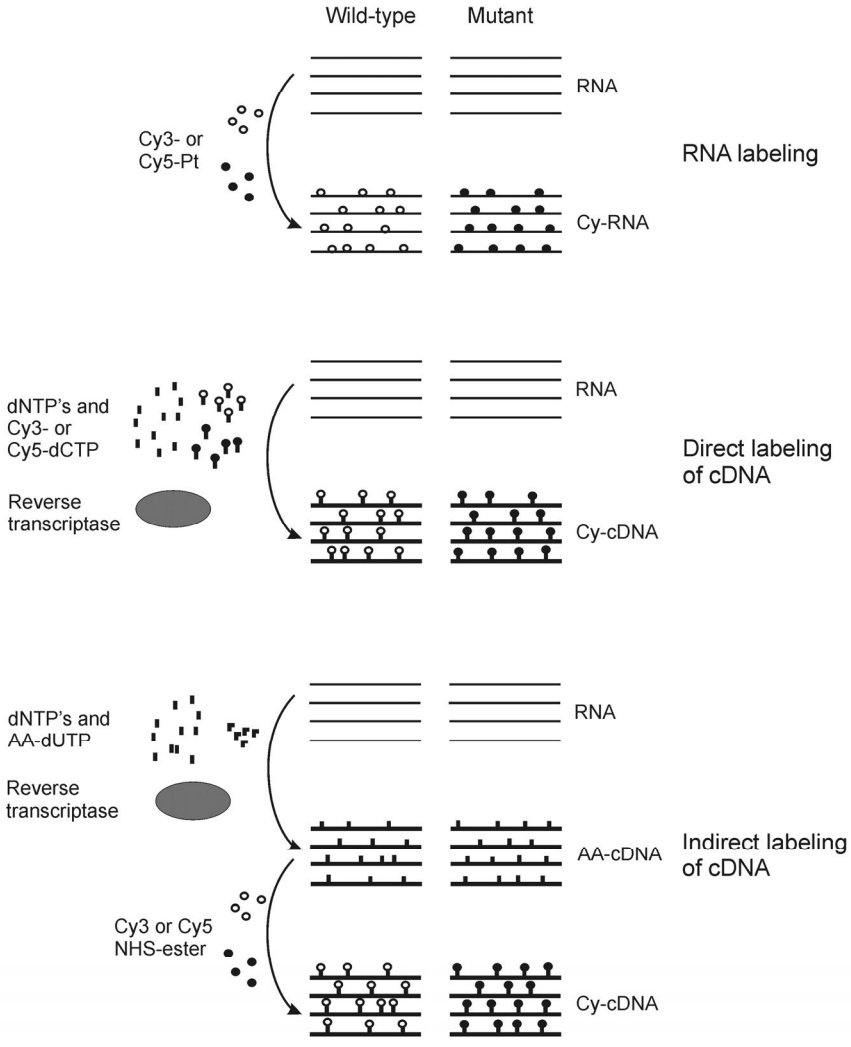

Figure 5. Three methods tested for probe labeling with Cy3 or Cy5 dyes before hybridization on the microarray. For details see Experimental Procedures.

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 H_2O. The amount of cDNA and label was evaluated using a LKB Ultrospec 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 Ultrospec 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 H_2O steam at 50°C and snap-dried on a hot plate. Then DNA was UV-crosslinked at 250 mJoules/cm^2 (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^{0}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 (C_6-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.

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