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

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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. Regulation of PCN production involves the two-component signalling system GacS/GacA, the quorum-sensing system PhzI/PhzR and the regulator PsrA. This paper reports that a functional *rpoS* is required for optimal PCN and *N*-hexanoyl-L-homoserine lactone (C_6-HSL) production. Constitutive expression of *rpoS* is able to complement partially the defect of a *psrA* mutant for PCN and *N*-acylhomoserine lactone production. Western blotting shows that *rpoS* is regulated by *gacS*. Altogether, 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 C_6-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 and C_6-HSL produced by *rpoS* and *psrA* mutants harbouring a constitutively expressed *phzR* indicate an even more complex network of interactions, probably involving other genes. Preliminary microarray analyses of the transcriptomics of the *rpoS* and *psrA* mutants support the model of regulation described in this study and allow identification of new genes that might be involved in secondary metabolism.

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 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 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 (C_6-HSL) is the main product (Chin-A-Woeng *et al.*, 2001b). C_6-HSL is believed to bind to PhzR, thereby activating it. Subsequently, the PhzR–C_6-HSL complex probably binds to the *lux* (or *phz*) box upstream of the *phz* biosynthetic operon, which results in initiation of the transcription of the *phz* operon. The PhzR–C_6-HSL complex also upregulates *phzI* via a second *lux* box. A similar regulation of phenazine synthesis by quorum sensing was shown in *Pseudomonas aureofaciens* 30-84 (Pierson *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 FixJ family. In *Pseudomonas* species, GacS and GacA are global regulators of secondary metabolism, since they are situated upstream of many regulatory cascades and seem to function as master regulators. 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 2,4-diacetylphloroglucinol (Phl) in *Pseudomonas fluorescens* CHAO (Laville *et al.*, 1992), of enzymes like exoprotease and phospholipase C in

Abbreviations: C_6-HSL, *N*-hexanoyl-L-homoserine lactone; *N*-AHL, *N*-acylhomoserine lactone; PCA, phenazine-1-carboxylic acid; PCN, phenazine-1-carboxamide.

P. fluorescens CHAO (Sacherer *et al.*, 1994) and of various phenazines in *P. aureofaciens* 30-84 and *Pseudomonas 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*-acylhomoserine lactone (*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 *P. chlororaphis* PCL1391 (Chin-A-Woeng *et al.*, 2005). The *psrA* gene of *Pseudomonas 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 *psrA*, *rpoS* and *phzR* genes. Quantification of PCN and C_6-HSL, as well as preliminary microarray analyses, showed that the *phz* operon is regulated by a cascade involving GacS, PsrA, RpoS and PhzI/PhzR. In addition, the microarray survey allowed us to identify new genes of the *psrA*/*rpoS* regulon that might be involved in secondary metabolism.

METHODS

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 r.p.m. on a Janke und Kunkel shaker KS501D (IKA Labortechnik). *E. coli* strains were grown at 37 °C in Luria–Bertani medium (Sambrook & Russell, 2001) under vigorous aeration. LC medium, used for some experiments as indicated in the text, contained 10 g Bactotryptone (Difco) l^{-1}, 5 g yeast extract l^{-1}, 137 mM NaCl, 51 mM $MgSO_4$, 5 mM Tris. Media were solidified with 1·8 % Bacto agar (Difco). When appropriate, growth media were supplemented with kanamycin (50 μg ml^{-1}), carbenicillin (200 μg ml^{-1}) and gentamicin (10 μg ml^{-1} for *Escherichia coli* and 30 μg ml^{-1} for *P. chlororaphis*), and X-Gal (40 μl ml^{-1}). To follow growth, the optical density of liquid cultures was measured at 620 nm.

Construction of vectors and PCL1391 mutant strains. PCRs were in general carried out with Super Taq enzyme (Enzyme Technologies). However, for the production of genes under P_{tac} promoter control for complementations, PCRs were done with Proof Start (Qiagen). Primers were synthesized by Isogen Life Science. Restriction enzymes were purchased from New England BioLabs and ligase from Promega. 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 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.

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 into pGEM-T easy (Promega) to obtain pMP7425. An *Eco*RI digestion of pMP7425 produced an *Eco*RI internal fragment of *rpoS* which was cloned into the suicide plasmid pMP5285, resulting in pMP7418. This vector was transformed into PCL1391 for single homologous recombination by triparental mating using pRK2043 as helper. The *rpoS* mutant of PCL1391 that was obtained was checked by Southern blotting and by PCR with oMP686 primer (annealing on pMP5285 close to the multi-cloning site) 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 PCR fragment obtained 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 *Eco*RI digestion and ligated into pBBR1MCS-5 to produce pM7420, which was transformed 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 pBBR1MCS-5 into PCL1391 and PCL1954, respectively. pMP7420 and pBBR1MCS-5 were also transformed into the *psrA* mutant PCL1111 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 *Xho*I and *Eco*RI and ligated into *Xho*I/*Eco*RI-digested pBBR1MCS-5 to obtain pMP7447, which was validated by sequencing and its ability to restore a wild-type PCN

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
P. chlororaphis		
PCL1391	Wild-type, producing PCN; biocontrol strain of tomato foot and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici*	Chin-A-Woeng *et al.* (1998)
PCL1392	Derivative of PCL1391 tagged with *lacZ* with wild-type colonizing ability, Km^r	Chin-A-Woeng *et al.* (2000)
PCL1103	Derivative of PCL1391 in which a promoterless Tn*5luxAB* is inserted in *phzI*, Km^r	Chin-A-Woeng *et al.* (2001b)
PCL1104	Derivative of PCL1391 in which a promoterless Tn*5luxAB* is inserted in *phzR*, Km^r	Chin-A-Woeng *et al.* (2001b)
PCL1111	Derivative of PCL1391 in which a promoterless Tn*5luxAB* is inserted in *psrA*, Km^r	Chin-A-Woeng *et al.* (2005)
PCL1123	Derivative of PCL1391 in which a promoterless Tn*5luxAB* is inserted in *gacS*, Km^r	Chin-A-Woeng *et al.* (2005)
PCL1954	Derivative of PCL1391, *rpoS*::pMP7418, Km^r	This study
PCL1955	Derivative of PCL1954, *rpoS*::pMP7418, containing pMP7420, Km^r Gm^r	This study
PCL1957	Derivative of PCL1954, *rpoS*::pMP7418, containing pBBR1MCS-5, Km^r Gm^r	This study
PCL1958	Derivative of PCL1391 containing pMP7420, Gm^r	This study
PCL1960	Derivative of PCL1391 containing pBBR1MCS-5, Gm^r	This study
PCL1961	Derivative of PCL1111 containing pMP7420, Km^r Gm^r	This study
PCL1962	Derivative of PCL1111 containing pBBR1MCS-5, 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 pBBR1MCS-5, Gm^r	This study
PCL2004	Derivative of PCL1123 containing pBBR1MCS-5, Gm^r	This study
PCL2009	Derivative of PCL1391 mutated in a putative transcriptional regulator by recombination of pMP7452	This study
PCL2010	Derivative of PCL1123 P_{tac} *rpoS*, 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
PCL2050	Derivative of PCL1391 mutated in a putative GGDEF/EAL regulator by recombination of pMP7467	This study
PCL2052	Derivative of PCL1391 mutated in a hypothetical protein by recombination of pMP7470	This study
C. violaceum		
CV026	Double mini-Tn*5* mutant from *C. violaceum* ATCC 31532, AHL biosensor	Milton *et al.* (1997)
E. coli		
DH5α	*supE44* Δ*lacU169*(ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi1 relA1*	Hanahan (1983)
Plasmids		
pRK2013	Helper plasmid for triparental mating	Ditta *et al.* (1980)
pGEM-Teasy	Plasmid designed for direct ligation of PCR fragments	Promega
pBBR1MCS-5	Cloning vector, Gm^r	Kovach *et al.* (1995)
pMP4030 (previously pMP6007)	pBluescript containing a 4·5 kb chromosomal fragment of strain PCL1391 with the *phzI* and *phzR* genes and the first part of the *phzA* gene	Chin-A-Woeng *et al.* (2001b)
pMP5285	Suicide plasmid for *Pseudomonas* spp.; used for homologous recombination, Km^r Cb^r	Kuiper *et al.* (2001)
pMP7418	pMP5285 containing a 0·5 kb fragment of *rpoS* from pMP7425 by *Eco*RI digestion	This study
pMP7420	pBBR1MCS-5 containing the *rpoS* gene of PCL1391 downstream of the P_{tac} promoter, obtained by *Eco*RI digestion of pMP7424	This study

Table 1. cont.

Strain or plasmid	Description	Reference or source
pMP7424	pGEM-T containing *rpoS* of PCL1391 downstream of the P_{tac} promoter, obtained by PCR	This study
pMP7425	pGEM-T containing a 0·5 kb PCR product of the central part of *rpoS* of PCL1391	This study
pMP7447	pBBR1MCS-5 containing *phzR* of PCL1391 downstream of the P_{tac} promoter, inserted between the *Xho*I and *Eco*RI sites	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	This study
pMP7465	pBBR1MCS-5 containing *psrA* of PCL1391 downstream of the P_{tac} promoter, inserted in the *Eco*RI site	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	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	This study

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 pBBR1MCS-5. pMP7447 was also transformed 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 *Eco*RI and ligated into the *Eco*RI site of pBBR1MCS-5, to obtain pMP7465. pMP7465 was validated by sequencing. pMP7465 was subsequently transformed into PCL1391, PCL1111, PCL1123 and PCL1954 to obtain PCL2044, PCL2045, PCL2047 and PCL2048, respectively.

Three mutants were constructed in genes selected by microarray analyses. The genes chosen were a putative transcriptional regulator gene found in microarray clone 76_G2, a putative GGDEF/EAL regulator found in microarray clone 42_G8 and a hypothetical protein found in microarray clones 76_G2 and 47_F5. Primers oMP810 and oMP811, oMP972 and oMP973, oMP977 and oMP978 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, 0·4 kb for the putative GGDEF/EAL regulator gene and 0·5 kb for the hypothetical protein gene, respectively. The PCR products obtained were cloned in the *Eco*RI site of pMP5285, resulting in pMP7452, pMP7467 and pMP7470, respectively. These vectors were transformed into PCL1391 to obtain PCL2009, PCL2050 and PCL2052, respectively. The mutations were verified by PCR and/or sequencing.

Extraction and analysis of phenazine and *N*-AHL. 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 liquid MVB1 cultures in 500 ml Erlenmeyer flasks were mixed with 0·7 vol. dichloromethane, and shaken for 1 h, 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 acetonitrile and spotted on C18 TLC plates (Merck). As a control, 0·5 µl synthetic C_6-HSL (5 µM) (Fluka) was spotted on the TLC plate. The plates were developed in methanol/water (60 : 40, v/v). For detection of *N*-AHL, 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^{-1}). 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 OD_{620} 0·1. Cells were harvested at OD_{620} 1·0 or 2·2 in volumes of culture corrected for their differences in OD_{620} to obtain similar amounts of cells. Cell pellets were suspended in 200 µl cracking buffer (50 mM Tris/HCl pH 6·8, 1 % SDS, 2 mM EDTA, 10 %, v/v, glycerol, 0·01 % bromophenol blue, 1 % β-mercaptoethanol) and boiled for 3 min. The samples were subsequently loaded on a 10 % SDS-PAGE gel and proteins were separated and transferred on to a blot following a standard Western blot procedure (Ausubel *et al.*, 1997). A dry aliquot of RpoS antibodies was kindly provided by Professor K. Tanaka (Tokyo, Japan). The pellet was suspended in 100 µl PBS and diluted 1000-fold for reaction with immobilized protein, as recommended. Peroxidase-labelled goat anti-rabbit antiserum (Amersham Biosciences) was subsequently incubated with the blots. Finally, blots were incubated in luminal solution [250 µM sodium luminol (Sigma), 0·1 M Tris/HCl, pH 8·6, 0·01 % H_2O_2] mixed with 60 µl enhancer solution [67 µM *p*-hydroxycoumaric acid (Sigma) in DMSO]. Hybridizing protein bands were visualized on Super R-X photographic film (Fujifilm) after chemiluminescence detection.

RNA preparation, cDNA probe generation and microarray processing. The methods followed were described previously (van Rij *et al.*, 2005). Briefly, 12 ml MVB1 medium was inoculated in 100 ml flasks to an OD_{620} of 0·1 from overnight cultures of *P. chlororaphis* PCL1391 or derivative strains. The cultures were shaken at 28 °C at a speed of 195 r.p.m. on a Janke und Kunkel shaker KS501D until the OD_{620} reached a value of 2·0. This optical density corresponds to the moment where PCN starts to be produced (see Fig. 2), which indicates that the genes regulating the *phz* operon are probably expressed. 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). RNA was immediately used for cDNA probe generation

Table 2. Oligonucleotides

Name	Nucleotide sequence (5′–3′)
oMP49	CAGGAAACAGCTATGACCATGATTAC
oMP50	CCCAGTCACGACGTTCTAAAACG
oMP500	CCCAAGCTTCGGTGGACTTCACTGGC
oMP501	CCCAAGCTTGGCACACGTACCTCAAGGCT
oMP582	GGAATTCGGTGAAATAGCCTCCCAACA
oMP583	GGAATTCGGTCATCTTCGATGGTCAGG
oMP604	CCCAAGCTTTCGGCGTAGATCATGGGGGTGTGC
oMP605	CCCAAGCTTGCGCCGGGGCGCCGCCCAAGCATCC
oMP652	GGAATTCGCCCGCCTGCAYCARCARGGSCARTCC
oMP653	GGAATTCAGCAGATGGCTGGCGAAGGAGTGYCG
oMP686	TTAAGTTTATTCTTATCAATATAGG
oMP689	CGCGGATCCGGCTGCTGGAACGCTACACA
oMP690	CGCGGATCCACGGTCGAGCAATATATGCG
oMP768	AAMGAAGBGCCGGAGTTTGAC
oMP769	GTRTCSAGCAGGGTCTTGTCCGA
oMP770	CAGTGGCGATGTCCGTCTCC
oMP771	CTCGACCATGAACCCTCCCC
oMP772	TCCTGCCGTTGAAAACCCCG
oMP773	AAGCAACCTGCGTCTGGTGG
oMP774	CGATGCTTTGCGACAGGTCG
oMP775	ATATATGAATTCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAA-TTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGCTCT-CAGTAAAGAAGTGCCGGAGTTTGACATCGACG
oMP776	ATATATGGATCCGGGATTCCGTGAAGAACCAATAAAAAGCCCC
oMP777	ATATATCTCGAGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAA-TTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGAGTT-AGGGCAGCAGTTGGGATGGG
oMP778	ATATATGAATTCCCCCTCAGATATAGCCCATCGCAACTGCG
oMP783	GGGGTACCGGCGGTGAATTATCATTTCG
oMP784	GGGAATTCGGAATACCTTGCCGTACATG
oMP689	CGCGGATCCGGCTGCTGGAACGCTACACA
oMP690	CGCGGATCCACGGTCGAGCAATATATGCG
oMP810	CAAGAGTTCGCTGGCGGTGG
oMP811	GATTCGTCGTAGGTCAGGCG
oMP836	ATATATGAATTCTCAGAAGAACTCGTCAAGAAGGCG
oMP837	ATATATCTCGAGATGATTGAACAAGATGGATTGCACG
oMP859	ATGGCCCAGTCGGAAACCGTTGAACGC
oMP860	ATATATGAATTCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAA-TTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGCCCA-GTCGGAAACCGTTGAACGC
oMP861	ATATATGAATTCCCGCGCCCACCCGGACGGTCAGGCC
oMP972	ATATATGAATTCCTCGGTATTTCGCTACGGTTCGG
oMP973	ATATATGAATTCCCCAGCCATGGCCGGGCCGG
oMP977	ATATATGAATTCGGGAAACTACAAGATGCCGG
oMP978	ATATATGAATTCTCGAGGGTTTCGTGCACCAG

using the CyScribe post-labelling kit (Amersham Biosciences). After purification, the efficiency of Cy label incorporation into the cDNA and the quality and amounts of labelled cDNA were verified with an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences). Equal amounts of each dye were hybridized on the microarray. A minimum of 45 pmol of each dye was hybridized.

Construction of the chromosomal microarray of *P. chlororaphis* PCL1391 was described previously (van Rij *et al.*, 2005). Before hybridization, the DNA on the microarrays was UV-cross-linked at 250 mJ cm^{-2} (Amersham LifeSciences UV cross-linker). After prehybidization and washing of the slides, the Cy-labelled cDNA was hybridized on the microarrays overnight at 65 °C in a GeneTAC Hybstation (Genomic Solutions). After washing and drying, the slides were scanned in a G2565AA Microarray Scanner (Agilent).

Each experiment was repeated at least four times, including at least two independent experiments and a dye swap. Each experiment

included as 'test' the Cy-labelled cDNA deriving from the RNA of a mutant, and as 'reference' the Cy-labelled cDNA deriving from the RNA of the wild-type.

Microarray data analysis. After scanning, the microarrays were analysed 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 differentially expressed genes: spots were selected if the mean of the ratio of red and green laser intensities was higher than 2 [in GenePixPro: Ratio of Medians (650/550) > 2] or lower than 0·5, but positive [in GenePixPro: Ratio of Medians (650/550) < 0·5 and Ratio of Medians (650/550) > 0]. These values of 2 and 0·5 were arbitrarily chosen to select genes of which the expression is increased or decreased at least twofold in the *rpoS* or *psrA* mutant as compared to the wild-type. 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 [in GenePixPro: (% > 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 additional selection criteria, spots that had intensities lower than the intensity of the λ control in both the red and green channel were eliminated. This eliminates spots where labelled cDNA hybridized non-specifically to the spotted DNA. In order to avoid false positives due to problems of uniformity of the background and/or the feature, all the selected spots were finally controlled directly on the image of the scan.

Phenotypic analyses of mutants deriving from microarray analyses. Bacteria were tested for protease production as described by 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 by Deziel *et al.* (2001) was used, in which 1/20 KB-0·3 % agar plates were used for the swimming, and 1/20 KB-0·5 % agar plates were used for 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), following recommendations of the manufacturer. Samples (200 µl) 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 verified.

The production of hydrogen cyanide (HCN) was measured as described by Castric (1975). Whatman 3MM paper was soaked in a chloroform solution containing copper(II) ethyl acetoacetate (5 mg ml^{-1}) and 4,4′-methylene-bis-(*N*,*N*-dimethylaniline) (5 mg ml^{-1}), 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. Production of HCN by the bacteria was indicated by blue colouration of the paper.

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 Methods). Sequence analyses of the *P. chlororaphis* PCL1391 fragments confirmed the presence of an ORF (accession no. 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 % with *rpoS* of *P. fluorescens* PfO1 (accession no. ZP_00266495.1), 93 % with *rpoS* of *P. putida* (Kojic *et al.*, 1999), and 93 % with *rpoS of P. syringae* pv. *tomato* DC3000 (accession no. NP_791390).

A putative Shine–Dalgarno sequence was detected starting 12 nt upstream of the start codon, and a putative rho-independent terminator sequence is present 22 nt 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 no. NP_5 20694.1).

In contrast, *rpoS* of other *Pseudomonas* strains is followed by the small RNA regulator *rsmZ* (regulator of secondary metabolites) and *fdxA* (ferredoxin 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 nt 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 in PCL1954 (for details see Methods). 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. 2a, b). Constitutive

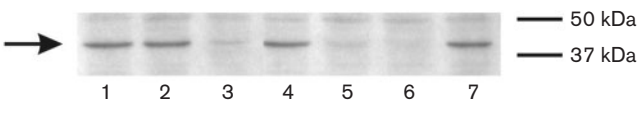

Fig. 1. Western blot analysis of RpoS production in *P. chlororaphis* PCL1391 and derivative strains. Lanes: 1, PCL1391; 2, PCL1103 (*phzI*::Tn*5luxAB*); 3, PCL1111 (*psrA*::Tn*5luxAB*); 4, PCL1119 (*phzB*::Tn*5luxAB*); 5, PCL1123 (*gacS*::Tn*5luxAB*); 6, PCL1954 (*rpoS*::pMP7418); 7, PCL1955 (*rpoS*::pMP7418+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

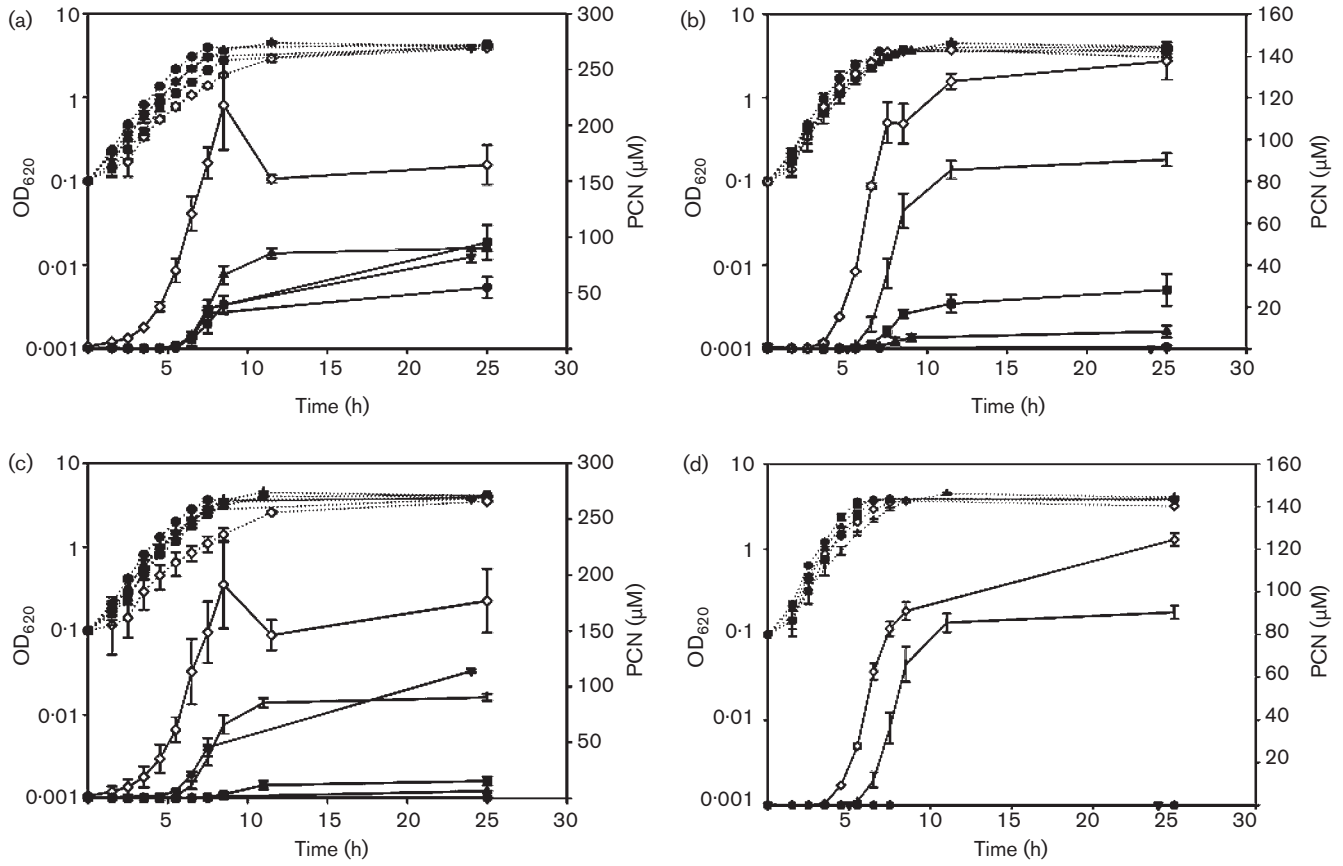

Fig. 2. PCN production by *P. chlororaphis* PCL1391 and derivative strains. Extractions were made at intervals from at least three independent cultures in 10 ml MVB1 and the PCN concentration was determined by HPLC. On each graph, the culture OD_{620} is plotted on the left axis (dotted lines) and the PCN concentration is plotted on the right axis (full lines). For easier reading, the same symbols are 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 (|). (a) Wild-type derivatives; (b) *rpoS* derivatives; (c) *psrA* derivatives; (d) *gacS* derivatives. The error bars represent SD.

expression of *rpoS* was established by cloning *rpoS* under control of the *tac* promoter in the vector pBBRMCS-5, resulting in pMP7420 (for details see Methods). 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 to between 35 % (Fig. 2b) and 70 % (Fig. 3a) of that of the control strain PCL1960 (wild-type + pBBR1MCS-5).

The production of C_6-HSL by the *rpoS* mutant was much decreased, but detectable (Fig. 3a). C_6-HSL levels in an *rpoS* mutant background were restored by the constitutive production of RpoS in PCL1955 (Fig. 3a).

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

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

The observations that the amounts of PCN and C_6-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 into the *rpoS* mutant PCL1954. The resulting strain PCL1986 showed complementation for PCN production of the *rpoS* mutation, as it produced 1·5-fold higher PCN than PCL1391 harbouring empty pBBR1MCS-5 (Fig. 2b) and showed increased C_6-HSL production (Fig. 3b).

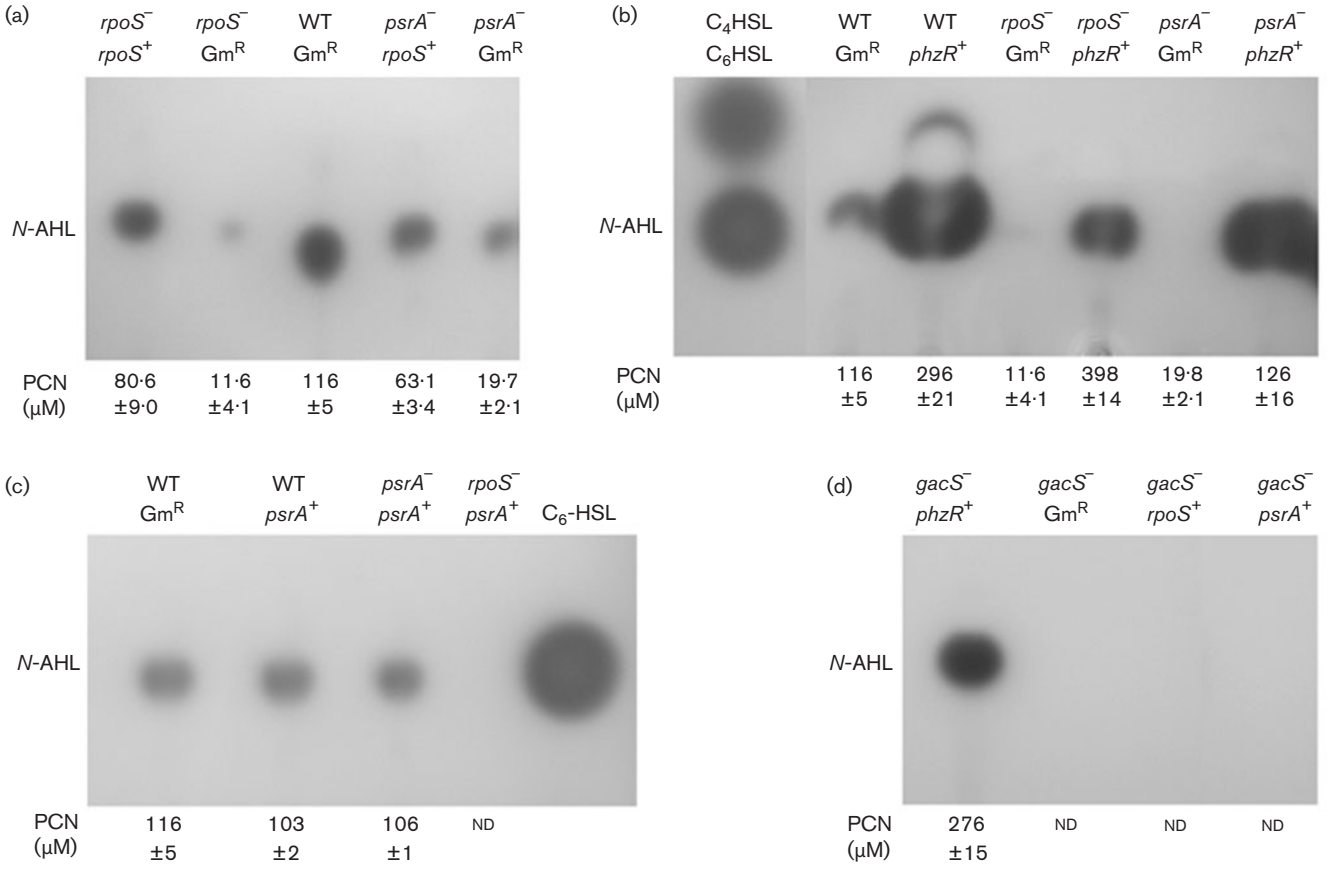

Fig. 3. C18-reverse phase TLC analysis of *N*-AHLs produced by *P. chlororaphis* PCL1391 derivatives in 50 ml MVB1. On each panel a group of PCL1391 derivatives is analysed. The strains are described above the TLC image: WT indicates a wild-type derivative, *psrA*$^-$ a PCL1111 derivative and *rpoS*$^-$ 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 numbers under the TLC image indicate the PCN production after overnight growth in 50 ml MVB1 for each PCL1391 derivative. These numbers are means and standard deviations calculated from extractions made in at least three independent cultures (ND, not detectable). The PCN concentration was determined by HPLC.

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 exponential phase (OD_{620} 1·0) and at the beginning of the stationary phase (OD_{620} 2·2). The amounts of RpoS appeared to be similar at the two time points. A blot of the results at OD_{620} 1·0 is shown in Fig. 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.

Relationship 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 %) (Figs 2c and 3a). 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 in 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 (Figs 2c and 3a). PCL 2048, the *rpoS* mutant overexpressing *psrA*, was unable to produce PCN (Fig. 2b) or *N*-AHL (Fig. 3c). As a control, we transformed pMP7465 (pBBR1MCS-5 harbouring P_{tac} *psrA*) into PCL1111 (*psrA*). The resulting strain, PCL2045, showed restored levels of PCN and C_6-HSL (Figs 2c and 3c). Constitutive expression of *phzR* also restored production of PCN and *N*-AHL in a *psrA* background (strain PCL1996, Figs 2c and 3b).

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 (Figs 2d and 3d). Neither constitutive *rpoS* expression, nor constitutive expression of *psrA* (Figs 2d and 3d), was sufficient to compensate for the *gacS* mutation. Only the constitutive *phzR* gene restored PCN and AHL production in a *gacS* mutated background (Figs 2d and 3d). Surprisingly, after overnight growth (see Figure 1 in the supplementary materials available at http://rulbim.leidenuniv.nl/girard/suppl material.htm), high amounts of PCA are present in strains PCL1986 and PCL1998 (*rpoS* and *gacS* mutant, respectively, both overexpressing *phzR*), but not in PCL1993 or PCL1996 (wild-type and *psrA* mutant, respectively, both overexpressing *phzR*).

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* (PCL1111) and *rpoS* (PCL1954) were compared with the gene expression of wild-type PCL1391 on microarrays. As an example for the reader and in order to comply with 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 at http://rulbim.leidenuniv.nl/girard/suppl material.htm. The data filter (see Methods for details of the filtering of the data) selected in total 190 spots for the experiments with the *psrA* mutant, of which 157 had a stronger intensity in the wild-type, and 33 a lower intensity. Two hundred and thirty-four spots were selected from the experiments involving the *rpoS* mutant, of which 211 had a strong 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 at http://rulbim.leidenuniv.nl/girard/suppl material.htm. The analysis of sequences and the variations of expression due to *rpoS* and *psrA* mutations are presented in Table 3. 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 sevenfold in the *rpoS* mutant PCL1954 and around 15-fold in the *psrA* mutant PCL1111 (clones 4_D1, 119_D12 and 126_G12 in Table 3, 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 decreased expression around fivefold in the *psrA* mutant and around sevenfold in the *rpoS* mutant.

Sequencing of 19 selected clones and homology studies identified the presence of hypothetical proteins. 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. Additional RT-PCR experiments should be performed to show which gene or operon is responsible for the ratio measured. For most genes, 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 obviously linked to *rpoS* and *psrA* functions, like an aminotransferase (clone 4_G11), or 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 intermediary and secondary metabolism (see Table 3). 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 analysed (see Methods). The mutants showed wild-type production of HCN, chitinase and exoprotease. They were all able to swim and swarm, although PCL2052 showed decreased swimming ability and PCL2050 seemed to be also affected in its swarming (not shown). The PCN production of PCL2009 (465 ± 28 μM) and PCL2052 (435 ± 14 μM) appeared to be increased twofold compared to PCL1391 (237 ± 9 μM).

DISCUSSION

psrA and *rpoS* control PCN production in *P. chlororaphis* PCL1391

The organization of the *rpoS* gene in strain PCL1391 is comparable to that observed in other pseudomonads (Fujita *et al.*, 1994; Heeb & Haas, 2001; Kojic *et al.*, 1999, 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* spp. *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* in PCL1391. Measurements of the production of PCN and *N*-AHL in various derivatives (Figs 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

Table 3. Genes whose expression was identified as being regulated by *rpoS* and *psrA* by using microarrays

Clone no.*	Change of expression in PCL1111 (*psrA*)†	Change of expression in PCL1954 (*rpoS*)†	Gene homology and/or accession no.‡	Bacterium corresponding to the gene homology§	Predicted function‖
***phz* genes**					
2_A5	20·2±5·69	13·9±3·15	*phzR* (AAF17494)	*P. chlororaphis*	Transcriptional activator
93_G11	6·00±1·95	8·27±2·30	*phzI* (AAF17493)	*P. chlororaphis*	Autoinducer synthase
24_C5	3·76±0·659	6·13±0·803	*phzI* (AAF17493) and *phzR* (AAF17494)	*P. chlororaphis*	Autoinducer synthase and transcriptional activator
4_D1	21·2±8·40	12·2±0·794	*phzB/C* (AAF17496 and AAF17497)	*P. chlororaphis*	Biosynthetic genes for PCN
97_D1	29·1±5·38	18·0±2·59	*phzD* (AAF17498)	*P. chlororaphis*	Biosynthetic gene for PCN
119_D12	10·1±2·97	7·21±1·44	*phzE* (AAF17499)	*P. chlororaphis*	Biosynthetic gene for PCN
126_G12	14·6±4·01	9·22±2·27	*phzH* (AAF17502)	*P. chlororaphis*	Biosynthetic gene for PCN
Membrane protein genes					
2_C4	3·63±0·444	10·9±3·49	ZP_00262806	*P. fluorescens*	Autotransporter adhesin
13_B1	3·77±0·507	9·16±1·57	ZP_00262806	*P. fluorescens*	Autotransporter adhesin
36_A12	3·32±0·386	7·62±1·656	ZP_00262806	*P. fluorescens*	Autotransporter adhesin
38_G7	3·39±0·296	8·04±1·70	ZP_00262806	*P. fluorescens*	Autotransporter adhesin
105_A12	3·57±0·238	7·95±1·72	ZP_00262806	*P. fluorescens*	Autotransporter adhesin
115_A2	3·10±0·427	4·98±1·03	ZP_00263190 and NP_745085	*P. fluorescens* and *P. putida*	Integral membrane protein (1–59/223) and conserved hypothetical protein (169–253/261)
4_H7	4·71±0·669	2·23±0·290	*nlpD* (AAP97085)	*P. chlororaphis*	Lipoprotein (86–265/294)
Primary metabolism genes					
4_C1	4·11±0·695	3·77±1·66	NP_762166	*V. vulnificus*	Deoxycytidylate deaminase (139–514/622)
4_G11	4·93±1·77	7·39±3·58	NP_901074	*C. violaceum*	Aminotransferase (4–268/367)
Intermediary metabolism genes					
2_B3	3·45±0·684	7·36±1·17	*phaC2* (BAB78721)	*P. chlororaphis*	PHA synthase 2 (225–560/560)
41_G2	3·28±0·741	7·19±1·82	*phaC2* (BAB78721)	*P. chlororaphis*	PHA synthase 2 (1–376/560)
53_F2	2·89±1·01	5·05±0·785	*phaG* (BAB32432)	*Pseudomonas* sp. 61-3	3-Hydroxyacyl-acyl carrier protein CoA transferase (131–294/294)
60_E1	2·85±0·924	4·91±0·901	Clone identical to 53_F2	*Pseudomonas* sp. 61-3	Clone identical to 53_F2
71_A4	2·40±0·313	4·29±0·592	Clone identical to 53_F2	*Pseudomonas* sp. 61-3	Clone identical to 53_F2
74_B4	2·75±0·632	5·29±1·01	Clone identical to 53_F2	*Pseudomonas* sp. 61-3	Clone identical to 53_F2
74_E7	2·74±0·548	5·19±1·82	Clone identical to 53_F2	*Pseudomonas* sp. 61-3	Clone identical to 53_F2
93_D8	2·50±0·507	6·26±1·847	*phaG* (BAB32432)	*Pseudomonas* sp. 61-3	3-Hydroxyacyl-acyl carrier protein CoA transferase (124–294/294)
Secondary metabolism genes					
11_G8	4·00±0·500	5·68±1·39	*chiC* (NP_250990)	*P. aeruginosa*	Chitinase (293–373/483)
121_H3	3·15±0·320	6·27±0·768	*chiC* (NP_250990)	*P. aeruginosa*	Chitinase (165–479/483)

Table 3. cont.

Clone no.*	Change of expression in PCL1111 (*psrA*)†	Change of expression in PCL1954 (*rpoS*)†	Gene homology and/or accession no.‡	Bacterium corresponding to the gene homology§	Predicted function‖
100_B7	3·36±0·399	7·41±0·574	NP_746359	*P. putida*	Pyoverdine synthase? (665–987/4317)
86_H8	5·93±3·87	10·4±0·836	NP_901071 and NP_901070	*C. violaceum*	Probable dihydrorhizobitoxine desaturase (248–353/369) and probable 5′-methylthioadenosine phosphorylase (31–186/302)
Regulatory genes					
65_B7	5·20±0·747	2·89±0·321	*rpoS* (AAP97086) and *nlpD* (AAP97085)	*P. chlororaphis*	RNA polymerase sigma factor (1–155/334) and lipoprotein (96–294/294)
98_B2	5·94±0·511	3·06±0·405	*rpoS* (AAP97086)	*P. chlororaphis*	RNA polymerase sigma factor (40–334/334)
42_G8	3·77±0·958	7·22±1·73	ZP_00264029	*P. fluorescens*	GGDEF/EAL domains containing regulator (260–617/624)
76_G2	3·37±0·916	4·38±1·32	ZP_00263882 and ZP_00263883	*P. fluorescens*	Hypothetical protein (378–454/454) and ATP-dependent transcriptional regulator (1–236/911)
Hypothetical protein genes					
72_D12	7·48±2·188	40·1±12·2	ZP_00262803, ZP_00262802 and ZP_00262796	*P. fluorescens*	Microcystin-dependent protein (1–191/191), hypothetical protein (1–103/103) and histone acetyltransferase (71–161/163)
119_A8	7·46±1·16	31·8±5·42	ZP_00262803 and ZP_00262802	*P. fluorescens*	Microcystin-dependent protein (1–191/191) and hypothetical protein (1–92/103)
1_B9	3·28±0·573	5·05±1·05	ZP_00266535	*P. fluorescens*	Hypothetical protein (58–170/170)
3_F11	3·20±0·882	4·33±0·417	ZP_00128106	*P. syringae* pv. *syringae*	Hypothetical protein (31–170/170)
11_E2	3·39±0·469	4·75±0·522	ZP_00263881.1 and ZP_00263880	*P. fluorescens*	Hypothetical protein (1–184/629) and acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II (528–551/560)
21_E2	2·80±0·621	3·96±0·278	Clone identical to 11_E2	*P. fluorescens*	Clone identical to 11_E2
12_G9	3·75±0·831	6·30±1·68	PP2941 (NP_745085)	*P. putida*	Conserved hypothetical protein (187–253/261)
41_A12	5·56±2·02	11·5±1·66	ZP_00262372 and ZP_00262371	*P. fluorescens*	Uncharacterized conserved protein (1–153/423) and putative Ser protein kinase (525–640/640)
47_F5	4·46±0·668	7·59±3·30	ZP_00263881 and ZP_00263882	*P. fluorescens*	Hypothetical protein (524–629/629) and hypothetical protein (1–335/454)
72_H6	4·65±1·17	7·02±2·08	Clone identical to 47_F5	*P. fluorescens*	Clone identical to 47_F5
93_H9	4·11±0·385	5·27±0·557	Clone identical to 47_F5	*P. fluorescens*	Clone identical to 47_F5
112_C1	3·95±0·386	5·77±0·457	Clone identical to 47_F5	*P. fluorescens*	Clone identical to 47_F5
59_C10	3·67±0·508	3·70±0·614	NP_929618	*Ph. luminescens* subsp. *laumondii*	Hypothetical protein (2–93/93)

Table 3. cont.

Clone no.*	Change of expression in PCL1111 (*psrA*)†	Change of expression in PCL1954 (*rpoS*)†	Gene homology and/or accession no.‡	Bacterium corresponding to the gene homology§	Predicted function‖
65_H9	3·67 ± 1·30	8·22 ± 2·40	ZP_00267318 and ZP_00266917	*P. fluorescens*	RTX toxin and related Ca^{2+}-binding protein (428–468/468) and hypothetical protein (34–248/300)
74_H8	6·88 ± 1·11	25·3 ± 4·08	NP_929844	*Ph. luminescens* subsp. *laumondii*	Hypothetical protein (12–281/325)
81_G2	3·76 ± 1·09	7·07 ± 1·92	ZP_00128106	*P. syringae* pv. *syringae*	Hypothetical protein (58–170/170)
101_H9	3·75 ± 0·396	4·03 ± 1·19	NP_929618	*Ph. luminescens* subsp. *laumondii*	Hypothetical protein (2–70/93)
114_H5	3·04 ± 0·422	4·93 ± 0·653	ZP_00263821	*P. fluorescens*	Hypothetical protein (19–163/390)
119_C6	2·91 ± 0·441	4·18 ± 0·963			No homology

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

†All the spots selected in this table corresponded to genes of which the expression was lower in the mutant than in the wild-type. Thus the ratios represent the intensity of the spots in the wild-type over the intensity in the mutant.

‡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 column (''clone identical to'').

§Genus abbreviations: *C.*, *Chromobacterium*; *P.*, *Pseudomonas*; *Ph.*, *Photorhabdus*; *V.*, *Vibrio*. 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 *Ph. luminescens* subsp. *laumondii* TTO1.

‖In parentheses, the region of the protein encoded on the insert of the clone is indicated (first amino acid–last amino acid/total amino acid length).

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 the poor MVB1 medium, *psrA* was shown to activate PCN and *N*-AHL production in PCL1391. 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 3). Constitutively expressed *rpoS* strongly increased PCN and *N*-AHL production in a *psrA* mutant (strain PCL1961, Figs 2c and 3a). 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 in cultures grown in different volumes of medium (Figs 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, 2003). RpeA was shown to regulate PCN production mostly in minimal 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; this could 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.

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 C_6-HSL present in PCL1391 spent culture medium were shown to be correlated with the amounts of PCN produced (Chin-A-Woeng *et al.*, 2001b, 2003; van Rij *et al.*, 2004). In our study, the correlation of PCN and C_6-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. 2b, 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 on environmental conditions for any one species.

Western blot analysis showed that a mutation in *phzB* does not affect the RpoS level, which suggests that there is no feedback effect 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 were made in other strains (Kojic & Venturi, 2001; Schmidt-Eisenlohr *et al.*, 2003; Whistler *et al.*, 1998).

Our data confirm the key role of *gacS* for PCN synthesis (Fig. 2d). Constitutive *rpoS* expression did not restore PCN synthesis in a *gacS* mutant (PCL2010), which indicates that as well as *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/C_6-HSL and GacS/GacA (Chancey *et al.*, 1999; Pierson *et al.*, 1994; Wood *et al.*, 1997; Wood & Pierson, 1996). The role of PsrA and RpoS in phenazine synthesis has so far not been studied in strain 30-84. The following hypothesis would reconcile the 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* 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 C_6-HSL resulting from leakage of the *phzI* promoter and restart the positive

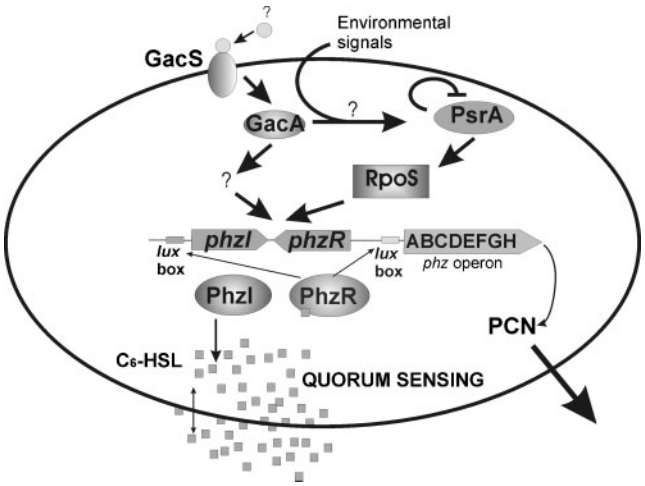

Fig. 4. Model for the regulatory cascade governing PCN production in *P. chlororaphis* PCL1391 in MVB1 medium. Upstream in the cascade, the sensor GacS is activated by a putative environmental factor. Subsequently, GacS stimulates its cognate kinase GacA. GacA activates a cascade of genes including PsrA and RpoS. As well as 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 PCN.

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 C_6-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, overexpression of *phzR* induces high amounts of PCA only in *rpoS* and *gacS* mutants (see Figure 1 in the supplementary material available at http://rulbim.leidenuniv.nl/girard/supplmaterial.htm). Conversely, only PCL1993 and PCL1996 (wild-type and *psrA* mutant, respectively, both overexpressing *phzR*) show a peak of PCN production (Fig. 2a, c). These observations could be explained by precipitation of PCN, indicated by the presence of numerous crystals in overnight cultures observed only in the 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 the *phz* operon in PCL1391. It is remarkable that the distances between the *phz* genes *phzA*, *B*, *C*, *D*, *E*, *F* and *G* do not exceed 15 nt, whereas there are 111 nt 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 nt.

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 of selecting genes which 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 Table 3 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 intermediary and secondary metabolism, such as *phaC2*, *phaG*, *chiC*, pyoverdine synthase and a probable dihydrorhizobitoxine desaturase (Table 3). *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 twofold increase in PCN production. The function of these genes has to our knowledge not yet been characterized in other strains. Our data show that these genes affect 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 signalling 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.

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. 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.

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