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

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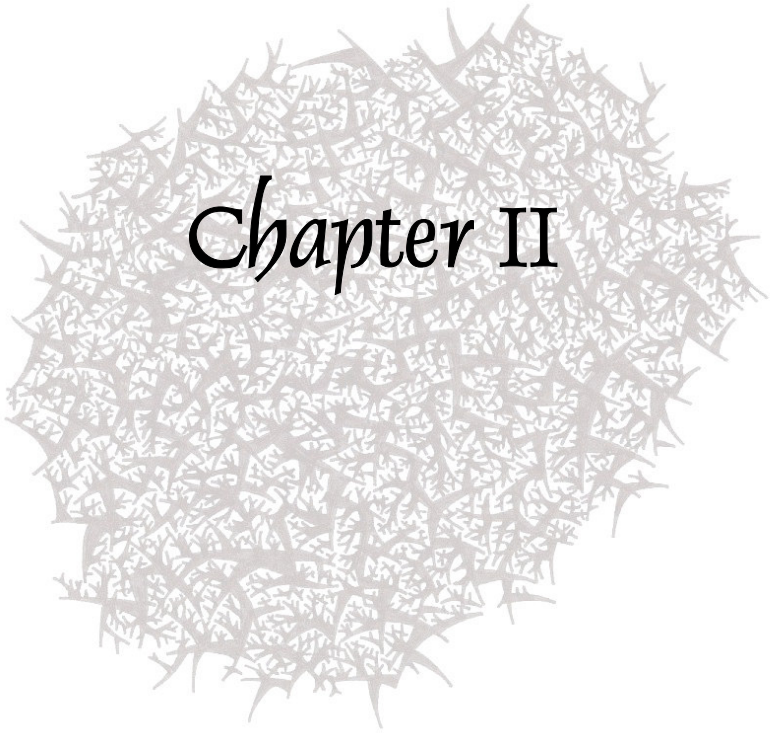

Chapter II

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

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ABSTRACT

Production of the secondary metabolite phenazine-1-carboxamide (PCN) by *Pseudomonas chlororaphis* PCL1391 is crucial for biocontrol activity against the phytopathogen *Fusarium oxysporum* f. sp. *radicis lycopersici* on tomato roots. Regulation of PCN production involves the two-component signaling system GacS/GacA, the quorum-sensing system PhzI/PhzR, and the regulator PsrA. Here we report that a functional *rpoS* is required for optimal PCN and *N*-hexanoyl-L-homoserine lactone (C_6-HSL) production. Constitutive expression of *rpoS* is able to complement partially the defect of a *psrA* mutant for PCN and *N*-acyl-homoserine lactone (*N*-AHL) production. Western blotting shows that *rpoS* is regulated by *gacS*. All together, these results suggest the existence of a cascade consisting of *gacS/gacA* upstream of *psrA* and *rpoS*, which influence expression of *phzI/phzR*. Overproduction of *phzR* complements the effects on PCN and 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 (PCA) and C_6-HSL produced by *rpoS* and *psrA* mutants harboring a constitutively expressed *phzR* indicate an even more complex network of interactions, probably involving other genes.

INTRODUCTION

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

The *phzI* gene is responsible for the synthesis of autoinducers, of which *N*-hexanoyl-L-homoserine lactone (C_6-HSL) is the main product (Chin-A-Woeng *et al.,* 2001b). C_6-HSL is supposed 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 initiating 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 *P. aureofaciens* 30-84 (Pierson III *et al.,* 1994).

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

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

Here we describe *rpoS* in *P. chlororaphis* PCL1391 and its role in the synthesis of PCN. A significant number of PCL1391 derivatives were constructed that are affected in the expression of the following genes: *psrA*, *rpoS* and *phzR*. Quantification of PCN and C_6-HSL showed that the *phz* operon is regulated by a cascade involving GacS, PsrA, RpoS and PhzI/PhzR.

RESULTS

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

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

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

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

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

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

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

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

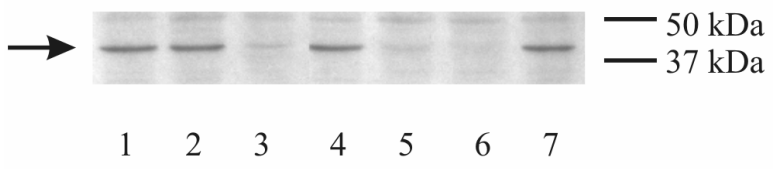

Figure 1. Western blot analysis of RpoS production in *P. chlororaphis* PCL1391 and derivative strains. Lane 1: PCL1391. Lane 2: PCL1103 (*phzI::*Tn5*luxAB*). Lane 3: PCL1111 (*psrA::*Tn5*luxAB*). Lane 4: PCL1119 (*phzB::*Tn5*luxAB*). Lane 5: PCL1123 (*gacS::*Tn5*luxAB*). Lane 6: PCL1954 (*rpoS*::pMP7418). Lane 7: PCL1955 (*rpoS*::pMP7418 + P_{tac} *rpoS*). On the right side of the blot two markers are shown. The arrow on the left indicates the position of RpoS.

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

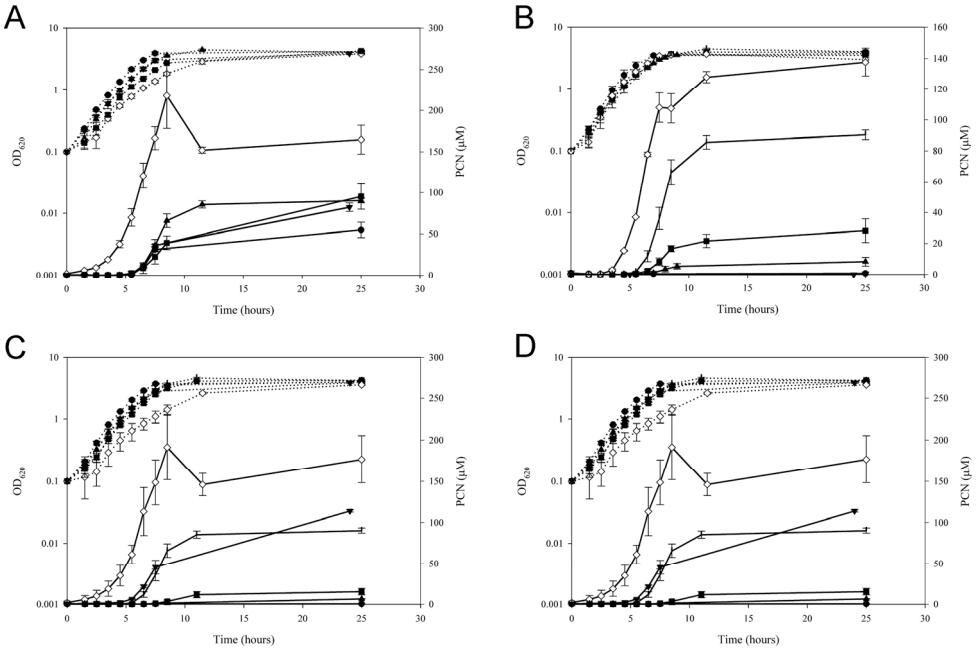

Figure 2. PCN production by *P. chlororaphis* PCL1391 and derivative strains.
Extractions were made from at least three independent cultures in 10 ml MVB1 during time and the PCN concentration was determined by HPLC. On each graph, the absorbance is plotted following the left axis (dotted lines) and the PCN concentration is plotted following the right axe (plain lines). For easier reading, the same symbols were used in the different panels for the following constructs: original strain (●), derivative containing pBBR1MCS-5 (▲), derivative containing P_{tac} *rpoS* (■), derivative containing P_{tac} *phzR* (◊), derivative containing P_{tac} *psrA* (▼). For panels B, C, and D, the values for PCL1960 (PCL1391 + pBBR1MCS-5) are plotted as a control (|).
Panel A. Wild-type derivatives. Panel B. *rpoS* derivatives. Panel C. *psrA* derivatives. Panel D. *gacS* derivatives.

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

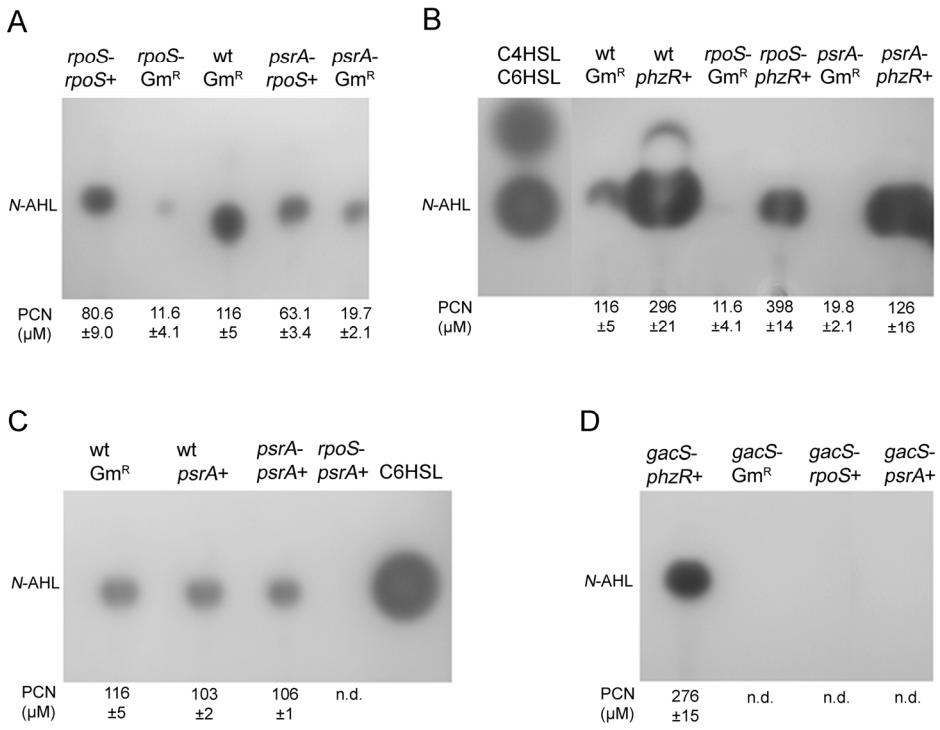

Figure 3. C18-reverse phase TLC analysis of *N*-AHLs produced by *P. chlororaphis* strain PCL1391 derivatives in 50 ml MVB1.
On each panel a group of PCL1391 derivatives is analyzed. The strains are described above the TLC image: wt indicates a wild-type derivative, *psrA*- a PCL1111 derivative and *rpoS*-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

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

The observations that the amounts of PCN and 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 to PCL1954 (*rpoS*-) The resulting strain PCL1986 showed complementation for PCN production of the *rpoS* mutation, as it produced 1.5-fold

higher PCN than PCL1391 harboring empty pBBR1-MCS5 (Fig. 2, panel B) and showed an increased C_6-HSL production (Fig. 3, panel B).

Regulation of RpoS synthesis by genes involved in PCN synthesis

The effect of mutations in *gacS*, *psrA*, *phzI* or *phzB* of PCL1391 on the production of RpoS protein was tested by Western blot analysis. The experiments were performed in MVB1 medium and samples for RpoS analysis were harvested during logarithmic phase (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 Figure 1. RpoS amounts were severely reduced as a result of mutations in *psrA* (PCL1111, lane 3) and *gacS* (PCL1123, lane 5). Mutations in *phzI* (PCL1103, lane 2) and in *phzB* (PCL1119, lane 4) did not affect the production of RpoS.

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

A *psrA* mutant of PCL1391 showed low production of PCN and *N*-AHL when grown in MVB1 medium as compared to PCL1391 (decrease of 99%) (Fig. 2, panel C and Fig. 3, panel A). It was shown that *psrA* regulates *rpoS* in other *Pseudomonas* species (Kojic & Venturi, 2001), probably *via* binding to the promoter of the *rpoS* gene at a PsrA-binding box. Therefore, an attempt was made to complement the *psrA* mutant PCL1111 with constitutively expressed *rpoS*. For this purpose, the vector pMP7420 (P_{tac} *rpoS)* was transformed into PCL1111, which resulted into PCL1961. PCL1961 showed increased PCN and *N*-AHL levels compared to the *psrA* mutant and produced up to 55% of the amount of PCN produced by the wild-type (Fig. 2, panel C and Fig. 3, panel A). PCL 2048, the *rpoS* mutant over-expressing *psrA*, was unable to produce PCN (Fig. 2, panel B) or *N*-AHL (Fig. 3, panel C). As a control, we transformed pMP7465 (pBBR1MCS-5 harboring P_{tac} *psrA*) into PCL1111 (*psrA*). The resulting strain, PCL2045, showed restored levels of PCN and C_6-HSL (Fig. 2, panel C, and Fig. 3, panel C). Constitutive expression of *phzR* also restored production of PCN and *N*-AHL in a *psrA* background (strain PCL1996, Fig. 2, panel C and Fig. 3, panel B).

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

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

DISCUSSION

psrA and *rpoS* control PCN production in strain PCL1391

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

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

Our data confirm the key role of *gacS* for PCN synthesis (Fig. 2, panel D). Constitutive *rpoS* expression did not restore PCN synthesis in a *gacS* mutant (PCL2010), which indicates that next to *rpoS* in the regulatory cascade, other factors affected by *gacS* are necessary for PCN production (Fig. 4). However, constitutive expression of the *phzR* gene restores PCN and *N*-AHL synthesis in a *gacS* mutant (PCL1998). This could be surprising considering that in *P. aureofaciens* 30-84, which is closely related to *P. chlororaphis* PCL1391, GacS/GacA affect mostly the transcription of *phzI* and not that of *phzR* (Chancey *et al.,* 1999). Additionally, phenazine synthesis is regulated in a comparable way in both strains by PhzI/PhzR/C_6-HSL and GacS/GacA (Chancey *et al.,* 1999; Pierson III *et al.,* 1994;

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

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

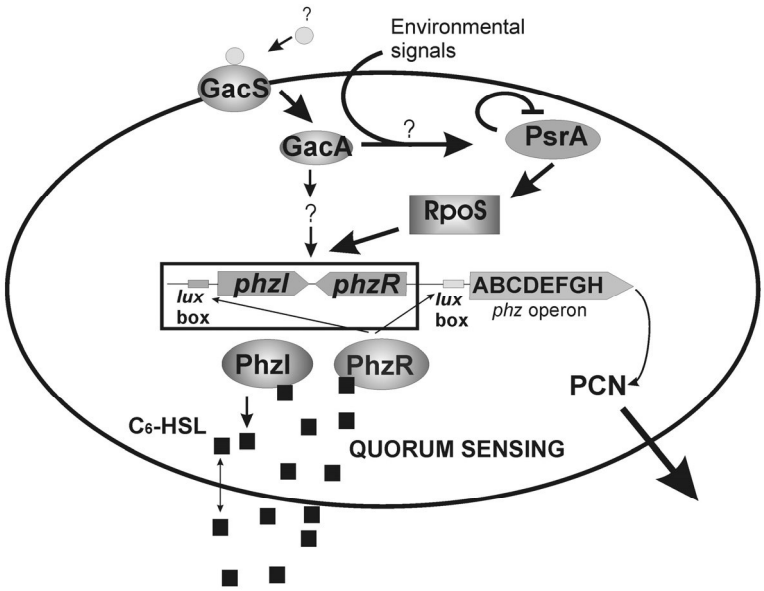

Figure 4. Model for the regulatory cascade governing PCN production in *P. chlororaphis* PCL1391 in MVB1 medium. Upstream in the cascade, the sensor GacS is activated by a putative environmental factor. Subsequently, GacS stimulates its cognate kinase GacA. GacA activates a cascade of genes including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

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

Under various growth conditions the amounts of 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; Chin-A-Woeng *et al.,* 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. 2, panels B and C) show that *rpoS* stimulates PCN production via *phzI*/*phzR*. Conversely, *phzI* does not regulate *rpoS* expression (Fig. 1). A role of *rpoS* in antibiotic production has been reported (Sarniguet *et al.,* 1995; Suh *et al.,* 1999), but not for regulating PCN production. The inhibitory effect of RpoS on quorum sensing and pyocyanin in *P. aeruginosa* (Whiteley *et al.,* 2000) or of PsrA and RpoS on quorum-sensing in *P. putida* WCS358 (Bertani & Venturi, 2004) is the opposite of what we observed for strain PCL1391.

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

Western blot analysis showed that a mutation in *phzB* does not affect the RpoS level, which suggests that there is no feedback from PCN production on *rpoS* expression. A defect in the regulatory genes *psrA* or *gacS* results in a severe decrease of the amounts of RpoS. Similar observations would result in an excess of PhzR mRNA that would overcome negative post-transcriptional regulation. The PhzR protein produced in turn would bind the low amounts of C_6-HSL resulting from leakage of the *phzI* promoter and restart the positive regulatory loop of PhzI/PhzR by binding to the *lux* box upstream of *phzI*. It would be of great interest to test if a constitutive expression of *phzR* could restore phenazine production in a *gacS* mutant of strain 30-84.

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

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

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

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

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

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

Construction of vectors and PCL1391 mutant strains

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

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

Table 1. Bacterial strains and plasmids used

Bacterial strains and plasmids	Description	Reference or source
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)
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 Tn5*luxAB* is inserted in *phzI*, Km^r	(Chin-A-Woeng *et al.,* 2001b)
PCL1104	Derivative of PCL1391 in which a promoterless Tn5*luxAB* is inserted in *phzR*; Km^r	(Chin-A-Woeng. *et al.,* 2001b)
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	This study
PCL1955	Derivative of PCL1954, *rpoS*::pMP7418, containing pMP7420; Km^r, Gm^r	This study
PCL1957	Derivative of PCL1954, *rpoS*::pMP7418, containing pBBR1-MCS5; Km^r, Gm^r	This study
PCL1958	Derivative of PCL1391 derivative, containing pMP7420; Gm^r	This study
PCL1960	Derivative of PCL1391 containing pBBR1-MCS5; Gm^r	This study
PCL1961	Derivative of PCL1111, containing pMP7420; Km^r, Gm^r	This study
PCL1962	Derivative of PCL1111, containing pBBR1-MCS5; Km^r, Gm^r	This study
PCL1986	Derivative of PCL1954, containing pMP7447; Km^r, Gm^r	This study
PCL1993	Derivative of PCL1391, containing pMP7447; Gm^r	This study
PCL1996	Derivative of PCL1111, containing pMP7447; Km^r, Gm^r	This study
PCL1998	Derivative of PCL1123, containing pMP7447; Km^r, Gm^r	This study
PCL2000	Derivative of PCL1104, containing pMP7447;Km^r, Gm^r	This study
PCL2001	Derivative of PCL1104, containing pBBR1-MCS5;Gm^r	This study
PCL2004	Derivative of PCL1123, containing pBBR1-MCS5;Gm^r	This study
PCL2010	Derivative of PCL1123, containing pMP7420; Km^r, Gm^r	This study
PCL2044	Derivative of PCL1391 containing pMP7465;Gm^r	This study

PCL2045	Derivative of PCL1111 containing pMP7465; Km^r, Gm^r	This study
PCL2047	Derivative of PCL1123 containing pMP7465; Km^r, Gm^r	This study
PCL2048	Derivative of PCL1954 containing pMP7465; Km^r, Gm^r	This study
Chromobacterium violaceum		
CV026	Double mini-Tn5 mutant from *C. violaceum* ATCC 31532, AHL biosensor; Km^r	Milton *et al.*, 1997
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; Cb^r	Promega
pBBR1MCS-5	Cloning vector; Gm^r	(Kovach *et al.,* 1995)
pMP4030 (previously pMP6007)	pBluescript containing a 4.5 chromosomal fragment of strain PCL1391 with the *phzI* and *phzR* genes and the first part of the *phzA* gene; Cb^r	(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; Km^r	This study
pMP7420	pBBR1MCS-5 containing the *rpoS* gene of PCL1391 downstream of the P_{tac} promoter, obtained by *Eco*RI digestion of pMP7424; Km^r	This study
pMP7424	pGEM-T containing *rpoS* of PCL1391 downstream of the P_{tac} promoter, obtained by PCR; Cb^r	This study
pMP7425	pGEM-T containing a 0.5-kb PCR product of the central part of *rpoS* of PCL1391; Cb^r	This study
pMP7447	pBBR1MCS-5 containing *phzR* of PCL1391 downstream of the P_{tac} promoter, inserted between the *Xho*I and *Eco*RI sites; Gm^r	This study
pMP7465	pBBR1MCS-5 containing *psrA* of PCL1391 downstream of the P_{tac} promoter, inserted in the *Eco*RI site; Gm^r	This study

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

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

Table 2. Oligonucleotides used

Name	Nucleotide sequence
oMP49	5'-CAGGAAACAGCTATGACCATGATTAC-3'
oMP50	5'-CCCAGTCACGACGTTCTAAAACG-3'
oMP500	5'-CCCAAGCTTCGGTGGACTTCACTGGC-3'
oMP501	5'-CCCAAGCTTGGCACACGTACCTCAAGGCT-3'
oMP582	5'-GGAATTCGGTGAAATAGCCTCCCAACA-3'
oMP583	5'-GGAATTCGGTCATCTTCGATGGTCAGG-3'
oMP604	5'-CCCAAGCTTTCGGCGTAGATCATGGGGGTGTGC-3'
oMP605	5'-CCCAAGCTTGCGCCGGGGCGCCGCCCAAGCATCC-3'
oMP652	5'-GGAATTCGCCCGCCTGCAYCARCARGGSCARTCC-3'
oMP653	5'-GGAATTCAGCAGATGGCTGGCGAAGGAGTGYCG-3'
oMP686	5'-TTAAGTTTATTCTTATCAATATAGG-3'
oMP689	5'-CGCGGATCCGGCTGCTGGAACGCTACACA-3'
oMP690	5'-CGCGGATCCACGGTCGAGCAATATATGCG-3'
oMP768	5'-AAMGAAGBGCCGGAGTTTGAC-3'
oMP769	5'-GTRTCSAGCAGGGTCTTGTCCGA-3'
oMP770	5'-CAGTGGCGATGTCCGTCTCC-3'
oMP771	5'-CTCGACCATGAACCCTCCCC-3'
oMP772	5'-TCCTGCCGTTGAAAACCCCG-3'
oMP773	5'-AAGCAACCTGCGTCTGGTGG -3'
oMP774	5'-CGATGCTTTGCGACAGGTCG -3'
oMP775	5'-ATATATGAATTCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAAT TGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGCTCTCAGT AAAGAAGTGCCGGAGTTTGACATCGACG-3'
oMP776	5'-ATATATGGATCCGGGATTCCGTGAAGAACCAATAAAAAGCCCC-3'
oMP777	5'-ATATATCTCGAGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAA TTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGAGTTAG GGCAGCAGTTGGGATGGG-3'
oMP778	5'-ATATATGAATTCCCCCTCAGATATAGCCCATCGCAACTGCG-3'
oMP783	5'-GGGGTACCGGCGGTGAATTATCATTTCG-3'
oMP784	5'-GGGAATTCGGAATACCTTGCCGTACATG-3'
oMP689	5'-CGCGGATCCGGCTGCTGGAACGCTACACA-3'
oMP690	5'-CGCGGATCCACGGTCGAGCAATATATGCG-3'
oMP836	5'-ATATATGAATTCTCAGAAGAACTCGTCAAGAAGGCG-3'
oMP837	5'-ATATATCTCGAGATGATTGAACAAGATGGATTGCACG-3'
oMP859	5'-ATGGCCCAGTCGGAAACCGTTGAACGC-3'
oMP860	5'-ATATATGAATTCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAAT TGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGGCCCAGTCG GAAACCGTTGAACGC-3'
oMP861	5'-ATATATGAATTCCCGCGCCCACCCGGACGGTCAGGCC-3'

In order to construct an *rpoS* mutant derivative of PCL1391, two primers (oMP773 and oMP774) were used in PCR on the chromosomal DNA of PCL1391 and an internal fragment of 0.5 kb of the *rpoS* gene was obtained. This fragment was ligated in pGEM-T easy (Promega) to obtain pMP7425. An *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 introduced into PCL1391 for single homologous recombination by triparental mating using pRK2043 as helper. The obtained *rpoS* mutant of PCL1391 was checked by Southern blot and by PCR with oMP686 primer annealing on pMP5285 close to the multi-cloning site (MCS) and oMP776 (annealing on the *rpoS* 3' end which is not present in pMP7418) and named PCL1954.

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

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

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

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

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

For extraction of *N*-AHLs, supernatants from 50 ml MVB1 liquid MVB1 cultures in 500 ml Erlenmeyer flasks were mixed with 0.7 volume of dichloromethane, and shaken for one hour, after which the organic phase was collected. Each supernatant was extracted twice and the pooled extracts were dried using a rotary evaporator. The dried residue was dissolved in 25 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) (5 µM) (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.

Western blot analysis

Cells were grown after inoculation of 10 ml MVB1 from an overnight culture diluted to OD_{620} of 0.1. Cells were harvested at OD_{620} 1.0 or 2.2 (cultures are diluted 10-fold before OD_{620} measurement) in volumes of culture corrected for their differences in OD_{620} to obtain similar amounts of cells. Cell pellets were suspended in 200 µl of cracking buffer (50 mM Tris HCl pH 6.8, 1% SDS, 2 mM EDTA, 10% glycerol, 0.01% bromophenol blue, 1% β-mercaptoethanol) and boiled for 3 minutes. The samples were subsequently loaded on a 10% SDS-PAGE gel and proteins were separated and transferred on a blot following a standard Western blot procedure (Ausubel *et al.,* 1997). A dry aliquot of RpoS antibodies was kindly provided by Prof. K. Tanaka (Tokyo, Japan). The pellet was suspended in 100 µl PBS and diluted 1000 times for reaction with immobilized protein, as recommended. Peroxidase-labeled goat anti-rabbit antiserum (Amersham Biosciences) was subsequently incubated with the blots. Finally, blots were incubated in luminal solution (250 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-hydroxy coumaric acid (Sigma) in DMSO). Hybridizing

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

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