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Role of the phenazine-inducing protein Pip in stress resistance of *Pseudomonas chlororaphis*

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The triggering of antibiotic production by various environmental stress molecules can be interpreted as bacteria's response to obtain increased fitness to putative danger, whereas the opposite situation – inhibition of antibiotic production – is more complicated to understand. Phenazines enable *Pseudomonas* species to eliminate competitors for rhizosphere colonization and are typical virulence factors used for model studies. In the present work, we have investigated the negative effect of subinhibitory concentrations of NaCl, fusaric acid and two antibiotics on quorum-sensing-controlled phenazine production by *Pseudomonas chlororaphis*. The selected stress factors inhibit phenazine synthesis despite sufficient cell density. Subsequently, we have identified connections between known genes of the phenazine-inducing cascade, including PsrA (*Pseudomonas* sigma regulator), RpoS (alternative sigma factor), Pip (phenazine inducing protein) and PhzI/PhzR (quorum-sensing system). Under all tested conditions, overexpression of Pip or PhzR restored phenazine production while overexpression of PsrA or RpoS did not. This forced restoration of phenazine production in strains overexpressing regulatory genes *pip* and *phzR* significantly impairs growth and stress resistance; this is particularly severe with *pip* overexpression. We suggest a novel physiological explanation for the inhibition of phenazine virulence factors in pseudomonas species responding to toxic compounds. We propose that switching off phenazine-1-carboxamide (PCN) synthesis by attenuating *pip* expression would favour processes required for survival. In our model, this 'decision' point for promoting PCN production or stress resistance is located downstream of *rpoS* and just above *pip*. However, a test with the stress factor rifampicin shows no significant inhibition of Pip production, suggesting that stress factors may also target other and so far unknown protagonists of the PCN signalling cascade.

INTRODUCTION

Pseudomonads are known to be able to produce various phenazines (Chin-A-Woeng *et al.*, 2003; Haas & Defago, 2005; Raaijmakers *et al.*, 2002). For over five decades, studies have focused on the toxic role of phenazines, both in the rhizosphere and the human host. Indeed phenazine-1-carboxamide (PCN) of *Pseudomonas chlororaphis* inhibits the growth of the fungal agent of tomato foot and root rot, *Fusarium oxysporum* f. sp. *radicis lycopersici* (Chin-A-Woeng *et al.*, 1998). The phenazine pyocyanin produced by *Pseudomonas aeruginosa* is suggested to be involved in lung infection of cystic fibrosis patients (Caldwell *et al.*, 2009). However, recent evidence indicates that pyocyanin also acts as a signal molecule in *P. aeruginosa* (Dietrich *et al.*, 2006) and may probably even interfere with central metabolism (Dietrich *et al.*, 2008; Price-Whelan *et al.*, 2007). Therefore, the function of phenazines still appears to be mysterious in its multiplicity and complexity (Price-Whelan *et al.*, 2006).

PCN enables *P. chlororaphis* to eliminate competitors for nutrients and niche colonization in the rhizosphere. It is a typical virulence factor that is used for model studies. As depicted in Fig. 1, PCN production by the *phz* operon is a quorum-sensing-dependent process interspersed with multiple checkpoints controlled by regulatory proteins. Regulation of PCN synthesis involves the two-component system GacS/GacA, known as a master regulator of many secondary metabolism traits (Laville *et al.*, 1992;

Abbreviations: *N*-AHL, *N*-acetyl homoserine lactone, PCN: phenazine-1-carboxamide.

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A supplementary table of the strains and plasmids used in this study is available with the online version of this paper.

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Reimmann *et al.*, 1997; Sacherer *et al.*, 1994). GacS/GacA probably responds to environmental signals (Heeb *et al.*, 2002; Zuber *et al.*, 2003). In strain PCL1391, GacS/GacA regulates the alternative sigma factor RpoS (Girard *et al.*, 2006a), which is downstream of PsrA and upstream of Pip. PsrA and Pip are both TetR homologues (Girard *et al.*, 2006b). All of these genes are involved in inducing the *phz* operon via the quorum sensing genes *phzI* and *phzR* (Fig. 1). The latter two are essential and sufficient for synthesis of PCN (Girard *et al.*, 2006a). The regulator PhzI, a LuxI homologue, synthesizes *N*-acylhomoserine lactone (*N*-AHL) molecules, which traffic across membranes. Their extracellular concentration reflects the number of bacteria present in a (semi-) closed volume and is a primary signal for quorum sensing. *N*-AHLs bind to PhzR, a LuxR homologue, thereby activating it. Activated LuxR-like proteins function as transcriptional regulators; *N*-AHLs thus activate the first step of a cascade that enables bacteria to sense the density of their population (Fuqua *et al.*, 2001; Miller & Bassler, 2001).

It has recently been proposed that various toxic molecules in the rhizosphere, including phenazines, may act not only as toxins but also as signals and regulators (Davies *et al.*, 2006; Fajardo & Martinez, 2008). In particular, at subinhibitory concentrations, many antibiotics appear to influence the expression of virulence factor genes, such as those for phenazine biosynthesis and regulation in pseudomonads (Cummins *et al.*, 2009; Liang *et al.*, 2008; Linares *et al.*, 2006; Mitova *et al.*, 2008; Shen *et al.*, 2008; Skindersoe *et al.*, 2008). The observations indicate that the effects of antibiotics may vary depending on the target bacterial species and the kind of antibiotic (Fajardo & Martinez, 2008). The physiological meaning of these results needs further clarification. So far, an interpretation was only suggested for the effect of subinhibitory antibiotic concentrations on the triggering of virulence factor expression. Sensing such foreign toxic molecules would allow pseudomonas species to obtain increased fitness in a competitive environment (Cummins *et al.*, 2009; Shen *et al.*, 2008).

In the present work, we have investigated how some stress factors may, in contrast, inhibit the production of virulence factors by pseudomonas species and which regulator(s) is targeted by adverse environmental conditions. Stress factors are defined as agents that negatively interfere with physiological performance of an organism, here *P. chlororaphis*. This study only considers stress compounds and not competing organisms; the range of effects studied is limited to the inhibition of PCN biosynthesis that has no effect on growth. PCN production was previously shown to be influenced by many environmental factors, including temperature, pH, iron, NaCl and fusaric acid (van Rij *et al.*, 2004, 2005). Here, we have tried to understand the molecular link between external stress agents and the decrease in PCN synthesis. A selection of the agents that inhibit PCN synthesis, as well as some antibiotics, was applied to cultures of *P. chlororaphis* strain PCL1391. By evaluating which genes were affected and how, we have built a model involving Pip as a key regulator and proposed a novel explanation for the inhibitory effect that some stress factors have on phenazine production.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Supplementary Table S1 (available with the online version of this paper). *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). *Escherichia coli* strains were

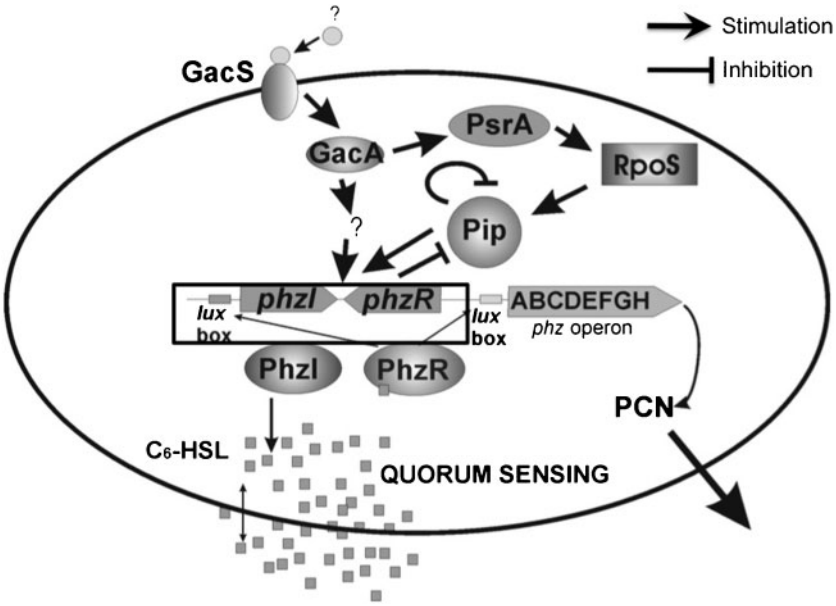

Fig. 1. Schematic model showing the role of Pip in the genetic cascade regulating PCN synthesis in *P. chlororaphis* PCL1391 (Girard *et al.*, 2006b). See text for details.

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grown at 37 °C in LC medium (Girard *et al.*, 2006a) under vigorous aeration. 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}), gentamicin (30 μg ml^{-1}), X-Gal (40 μg ml^{-1}) or hexanoyl-homoserine lactone (C_6-HSL) (5 μM) (Fluka, Sigma-Aldrich). To measure growth, the optical density of liquid cultures was measured at 620 nm.

Recombinant DNA techniques. General DNA techniques were performed as described previously (Sambrook & Russell, 2001). PCRs were carried out with Super *Taq* enzyme (Enzyme Technologies). In order to produce *pip* under the control of the P_{pip} promoter, PCRs were performed using Phusion from Finnzymes. Primers were synthesized by Isogen Life Science). Restriction enzymes were purchased from New England BioLabs and ligase from Promega. When necessary, DNA was verified by sequencing (Baseclear).

Construction of plasmids and PCL1391 mutant strains. Primers oMP1044 (5′-ATATATCTCGAGTTATCAATGGTGATGGTGATGG-TGGGCCTGCAAGTAACGCAGCACCGACTCGC-3′) and oMP1045 (5′-ATATATGAATTCGAGGTCAGCCGGGCCAAGGAG-3′) were used for PCR amplification of chromosomal genome with Phusion enzyme (Finnzymes) to obtain the *pip* gene under its own promoter and with 6× His tag at the C terminus. The 1.1 kb product was cloned in the *Eco*RI and *Xho*I sites of pBBR1MCS-5 resulting in opposite directions of transcription for *pip* and the *β*-galactosidase gene of pBBR1MCS-5. This plasmid was named pMP7488 and verified by sequencing. It was transferred to PCL1114 (*pip* mutant) to obtain PCL2088.

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

For *N*-AHL extraction, supernatants from 50 ml MVB1 cultures were harvested at OD_{620} 3.0 and mixed with 0.7 vols dichloromethane and shaken for 45 min, after which the organic phase was collected. The extract was dried using a rotary evaporator. The dried residue was redissolved in 25 μl acetonitrile and spotted on RP-C18 TLC plates (Merck). The plates were developed in methanol–water (60 : 40, v/v). To detect *N*-AHLs, the TLC plate was overlaid with 0.8 % agar LC containing a 10-fold-diluted overnight culture of the *Chromobacterium violaceum* indicator strain CV026 (Milton *et al.*, 1997) and kanamycin (50 μg ml^{-1}). After incubation for 48 h at 28 °C, chromatograms were analysed for the appearance of violet spots.

Western blot analysis. An aliquot of MVB1 overnight culture (10 ml) was washed with fresh medium, subsequently diluted to OD_{620} of 0.1 and used to inoculate a fresh culture. Cells were harvested at OD_{620} 1.0 or 2.2 (samples from the cultures were diluted 10 times for OD_{620} measurement) in volumes corrected for equal cell amounts. Cell pellets were resuspended in 200 μl 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 min. The samples were subsequently loaded on a 12 % SDS-polyacrylamide gel and proteins were separated and blotted following a standard Western blot procedure (Ausubel *et al.*, 1997). A dried aliquot of antibodies against RpoS was kindly provided by Professor K. Tanaka (Tokyo, Japan). This sample was resuspended in 100 μl PBS, diluted 1000-fold and allowed to react with the blot. To detect Pip, an anti-His-probe (G-18) (Santa Cruz Biotechnology) was used. The blots were subsequently incubated with peroxidase-labelled goat anti-rabbit antiserum (GE Healthcare). Finally, blots were incubated in a luminol 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. Semiquantitative measurement of the protein was performed using ImageJ1.41o software (National Institutes of Health). Experiments were performed at least twice.

RESULTS AND DISCUSSION

Identification of stress conditions specifically inhibiting the PCN biosynthesis signalling pathway

For this study, we needed to select stresses that directly switched off the PCN signalling pathway to the detriment of those interrupting PCN production as an indirect consequence of impaired growth. We therefore first assessed the effect of several stress factors on both growth and PCN production. NaCl is a common soil abiotic molecule and a classical stress factor when above a certain concentration (Munns, 2005). In addition to this, antibiotics are produced by many soil-dwelling microorganisms (Chater, 2006). Rifampicin (produced by *Amycolatopsis rifamycinica*) and kanamycin (produced by *Streptomyces kanamyceticus*) were chosen because they act differently: rifampicin at the transcriptional level by interfering with the *β*-subunit of the RNA polymerase and kanamycin at the translational level by binding to the ribosome. Several results relevant for this study and concerning fusaric acid, a phytotoxin produced by the fungus *F. oxysporum* (Bacon *et al.*, 1996), were previously obtained by our group (van Rij *et al.*, 2005). We therefore included this factor in this study and used it in some additional experiments.

When grown at 28 °C in liquid MVB1 (van Rij *et al.*, 2004), *P. chlororaphis* PCL1391 produced up to 37 ± 1.6 μM PCN. Increasing the concentration of NaCl, kanamycin and rifampicin (at 10 mM, 0.5 and 0.2 μg ml^{-1}, respectively) resulted in decreased PCN production before growth was affected (Fig. 2). A similar effect was reported for fusaric acid (van Rij *et al.*, 2004, 2005). For subsequent experiments, the highest subinhibitory concentrations of NaCl, kanamycin and rifampicin (333 mM, 5 μg ml^{-1} and 2 μg ml^{-1}, respectively) were chosen as appropriate conditions to assess if and how environmental stresses target protagonists of the PCN production regulatory cascade. For fusaric acid, the previously used (van Rij *et al.*, 2005) concentration of 1 mM was chosen.

Constitutive expression of PhzR and Pip regulators restore PCN production under stress conditions

While growth of *P. chlororaphis*, as reflected by c.f.u. values after overnight incubation, is not affected under the chosen conditions, we tested whether stress factors directly perturb the quorum sensing system. Indeed, no *N*-AHL could be detected in the supernatant of strain PCL1391 cultures

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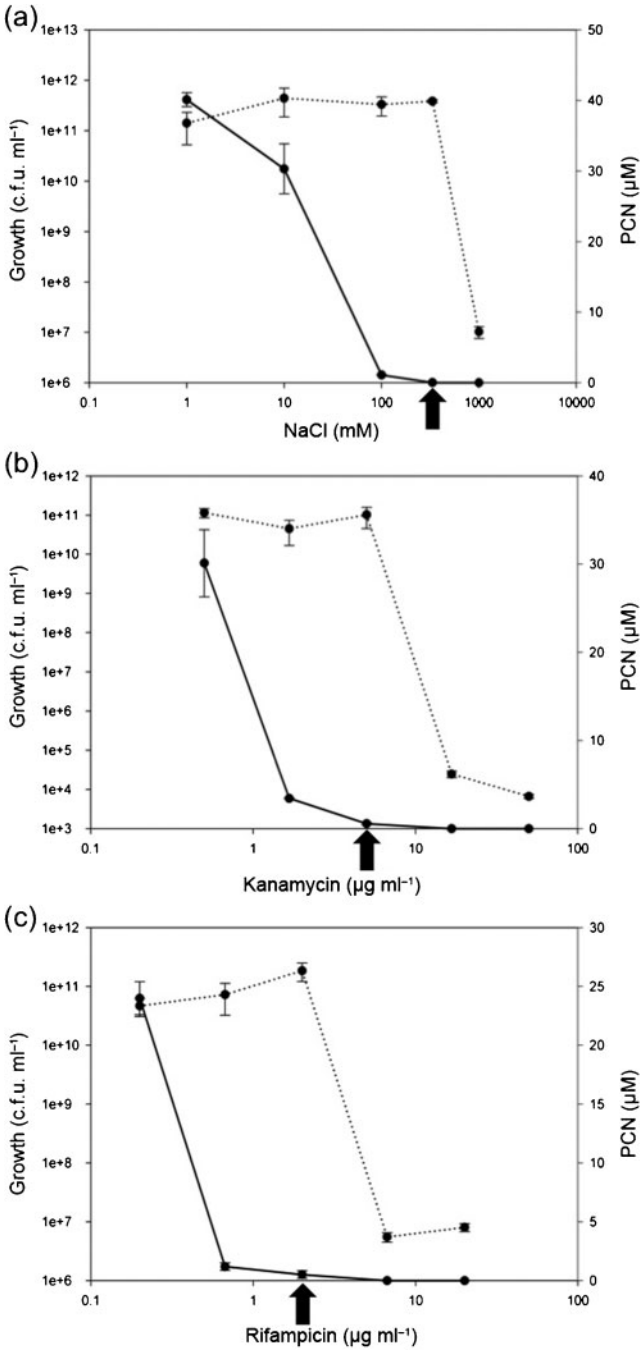

Fig. 2. Growth and PCN synthesis of *P. chlororaphis* PCL1391 after overnight incubation under various stress conditions. Cells of *P. chlororaphis* PCL1391 were grown in 10 ml MVB1 medium supplemented with various concentrations of NaCl (a), kanamycin (b) and rifampicin (c). PCN concentration (solid lines) and growth (dashed lines) of cultures grown for 20 h were determined. The experiment was performed three times independently and means ± SD of triplicate experiments from the same culture are plotted. Arrows indicate the concentrations chosen for subsequent experiments shown in Fig. 3 and Table 1.

supplemented with 333 mM NaCl, 5 μg kanamycin ml^{-1} or 2 μg rifampicin ml^{-1} (Fig. 3a, lanes 4 and 3; Fig. 3d, lane 3), although the minimum cell density required for PCN production was more than reached. Similar results were previously observed for fusaric acid (van Rij *et al.*, 2005). Interestingly, constitutive expression of *phzR* was able to restore *N*-AHL production under all conditions tested (Fig. 3c, lanes 1 and 2; Fig. 3d, lanes 10 and 11; data not shown for fusaric acid and kanamycin). As expected, PCN production was also restored in the strain forced to produce *N*-AHL by the constitutive expression of *phzR* (Table 1). These results suggest that stress inhibits PCN synthesis by preventing the signal, via the PhzI/PhzR quorum sensing system, that cell density is sufficient.

Regulatory genes upstream of PhzR within the PCN induction cascade were also overexpressed to test their ability to restore *N*-AHL and PCN production. Constitutively expressed *psrA* or *rpoS* were unable to restore wild-type levels of PCN (not shown) or *N*-AHL (Fig. 3a, lanes 5 and 6; Fig. 3b, lanes 1 and 2; Fig. 3d, lanes 4–7; data not shown for kanamycin and fusaric acid). When we forced the constitutive expression of *pip* under stress conditions, the most striking phenomenon was a strong inhibition of growth, except in the presence of rifampicin (Table 1). Under further analysis of the data, we could measure some PCN production (Table 1), which, in spite of its very low level, was remarkably higher than expected for such low growth (van Rij *et al.*, 2004). Interestingly, calculations show that strain PCL2019 (overexpressing *pip*) produced 2.3×10^{-9}, 5.9×10^{-9}, 3.7×10^{-8} and 7.2×10^{-8} μM PCN $c.f.u.^{-1}$ in MVB1 medium, non-supplemented, medium supplemented with 1 mM fusaric acid, 333 mM NaCl or 5 μg kanamycin ml^{-1}, respectively, whereas in the same conditions, strain PCL1960 (empty vector, parental strain) produced 2.6×10^{-9}, 1.1×10^{-10}, 1.6×10^{-12} and 4.3×10^{-11} μM PCN $c.f.u.^{-1}$, respectively. Under all stress conditions, with the exception of rifampicin (Fig. 3d, lanes 8 and 9), overexpression of *pip* was able to restore levels of *N*-AHL (not shown) relative to the inability to restore PCN production (1.5×10^{-10} μM PCN $c.f.u.^{-1}$ for P_{tac} *pip* with rifampicin). This suggests that the role of Pip in inducing PCN production directly targets the quorum sensing system. In addition to PhzR, Pip is therefore another regulator that is able to re-establish PCN production under stress conditions, except that constitutive overexpression of PhzR does not affect growth as that of Pip does (see below).

Forced restoration of PCN production impairs stress resistance

A plausible explanation for a direct interruption of PCN synthesis under adverse environmental conditions is that *P. chlororaphis* would focus maximal energy on stress resistance. We further supported this idea by showing that increasing the production of *N*-AHL and PCN does represent a disadvantage for growth in the presence of

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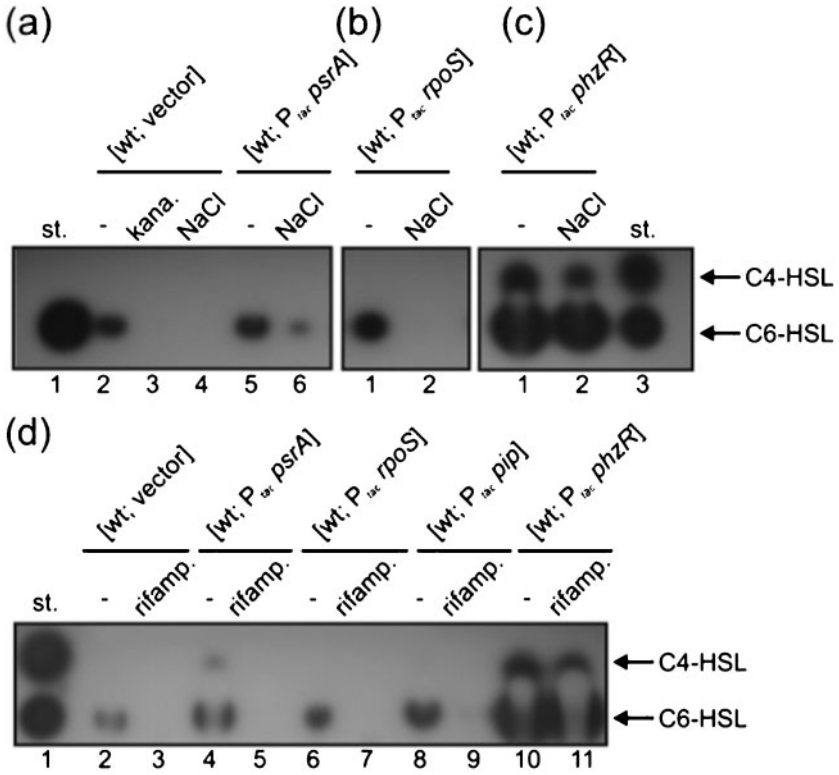

Fig. 3. C18-reverse phase TLC analysis of *N*-AHL produced by various derivatives of strain PCL1391. Strains were grown in MVB1 alone or in the presence of various stress factors as indicated on the figure. Standards (st.) were 2.5 nmol synthetic C_6-HSL (a), and 2.5 nmol C_6-HSL (bottom) and 2 nmol C_4-HSL (top) (c, d). wt, Wild-type.

stress factors. Indeed, cell growth was significantly slower when production of *N*-AHL and PCN was forced by constitutive expression of *phzR* (wild-type + P_{tac} *phzR*) in the presence of fusaric acid or rifampicin (Table 1). This effect is not related to the overproduction of PhzR itself, since the constitutive expression of *phzR* in a *phzI* mutant background (strain PCL2083, unable to produce *N*-AHL and hence PCN) resulted in a much less significant effect on growth than expression in a wild-type background (strain PCL1993) (Fig. 4). Both strains overproduce *phzR*, but only the wild-type background is forced to produce *N*-AHL and PCN. These results therefore suggest that production of these metabolites is a handicap when *P. chlororaphis* faces stress conditions.

In the case of forced PCN production by constitutive expression of *pip*, the effect on growth appeared even more drastic than with constitutive expression of *phzR*. Except with rifampicin – the stress condition in which Pip does not restore PCN production – cultures of strain PCL2019 (overexpressing *pip*) reached a very low OD_{620} after overnight growth ($OD_{620}<1$). We supposed that the growth inhibition of PCL2019 (wild-type + P_{tac} *pip*) in the presence of stress factors is due to the fact that a

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

Growth (c.f.u.) was measured after 20 h of growth at 28 °C. The c.f.u. for each strain was normalized to 1 under no stress conditions. Values given are percentages. The real numbers (c.f.u. ml^{-1}) are 2.7×10^{10} for (wild-type + empty vector), 5.0×10^{10} for (wild-type + P_{tac} *pip*), 1.7×10^{10} for (wild-type + P_{tac} *phzR*) and 1.6×10^{10} for PCL2089 (wild-type + P_{pip} *pip*). PCN, PCN production (μM). Values are mean ± SD.

	No stress		Fusaric acid (1 mM)		Kanamycin (5 μg ml^{-1})		NaCl (333 mM)		Rifampicin (2 μg ml^{-1})	
	c.f.u.	PCN	c.f.u.	PCN	c.f.u.	PCN	c.f.u.	PCN	c.f.u.	PCN
Empty vector*	100	68.8 ± 16.1	108 ± 8	3.1 ± 0.8	60 ± 12	0.7 ± 0.3	460 ± 89	0.2 ± 0.0	104 ± 18	0.1 ± 0.0
P_{tac} *pip*	100	116.9 ± 6.1	0.74 ± 0.05	2.2 ± 0.4	0.08 ± 0.01	2.9 ± 2.7	0.14 ± 0.02	2.6 ± 0.1	52 ± 5	3.8 ± 0.1
P_{tac} *phzR*	100	239 ± 58	57 ± 16	73.6 ± 4.2	46 ± 15	96.4 ± 9.3	282 ± 11	103 ± 2.7	43 ± 2	165 ± 53
P_{pip} *pip*	100	93.2 ± 2.8	160 ± 8.7	102.2 ± 0.6	91 + 1.8	67.2 ± 0.0	436 ± 63	4.5 ± 0.2	120 ± 3	30.0 ± 1.4

*The various empty vector strains used in this table all have a wild-type background.

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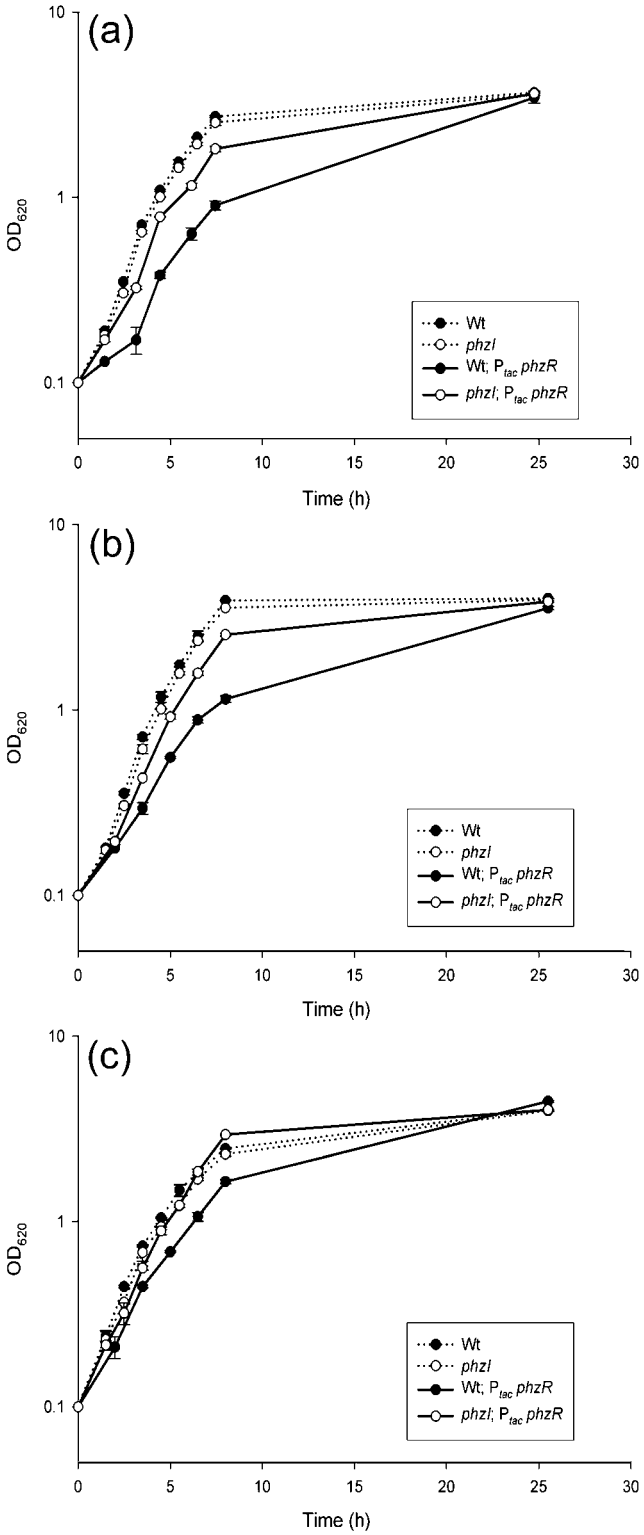

Fig. 4. Growth of *P. chlororaphis* PCL1391 derivatives under various stress conditions. The strains used were wild-type background (●) or *phzI* (○) background with empty vector (dotted lines) or vector containing P_{tac} *phzR* (solid lines). Cell cultures were grown in 10 ml MVB1 medium supplemented with 1 mM fusaric acid (a), 100 mM NaCl (b) or 2 μg rifampicin ml^{-1} (c) and samples were taken at regular time intervals to measure OD_{620}. Experiments were performed at least three times independently; data shown are means ± SD of triplicate experiments. These experiments could not be performed for kanamycin, since the *phzI* mutant is kanamycin resistant.

non-adjustable overproduction of Pip under stress conditions is toxic. Following this idea, a derivative containing *pip* expressed under its own promoter was expected to grow the same as the wild-type under stress, as Pip autoregulates its own expression (Girard *et al.*, 2006b). Indeed, strain wild-type + P_{pip} *pip* not only grows as the strain wild-type + empty vector but also produces elevated amounts of PCN (Table 1), except under NaCl stress (see below). For the overexpression of both *phzR* and of *pip*, production of *N*-AHL and PCN is forced, but the inhibitory effect on growth is far more dramatic in the case of P_{tac} *pip*. While slower growth due to constitutive expression of *phzR* could be attributed to the forced production of *N*-AHL and PCN, in the case of constitutive *pip* expression there could be an even higher PCN expression, due to the lack of autoregulation of *pip* expression, and subsequently poisonous levels of PCN inhibiting growth (see also below). Furthermore, it is known that self-resistance of pseudomonas to phenazines, which are redox-active components, is mediated by the quorum-sensing-dependent production of catalase and superoxide dismutase (Hassett *et al.*, 1999). In P_{tac} *pip* strains struggling for growth, it can be hypothesized that the quorum is not reached and its associated mechanisms of defence are not performing optimally. This inappropriate self-protection under stress conditions combined with early and high PCN production could significantly contribute to the severely impaired growth of P_{tac} *pip* strains. In addition to that, the Pip protein may also have another unknown effect (direct or indirect).

Relationships between the amount of RpoS/Pip and stress

To further understand the relationship of Pip to the chosen PCN inhibitory factors, the resistance to osmotic stress of a *pip* null mutant was compared with that of the wild-type and of an *rpoS* mutant. Results (Fig. 5) of this classical test for *rpoS* studies (Ramos-Gonzalez & Molin, 1998; Sarniguet *et al.*, 1995; Suh *et al.*, 1999) show that the wild-type and the *pip* mutant have a similar survival rate, whereas the *rpoS* mutant has a decreased survival rate. This indicates that Pip seems to be involved in the activation of PCN production only and not in that of stress resistance.

In order to assess which components of the PCN signalling cascade were targeted by the chosen stress factors, we monitored the protein level of RpoS and Pip under the selected stress conditions. The plasmid expressing a His-tagged P_{pip} Pip [pMP7488 (Girard *et al.*, 2006b)] was used together with anti-His antiserum. A *pip* mutant transformed with this vector (strain PCL2088) showed the same

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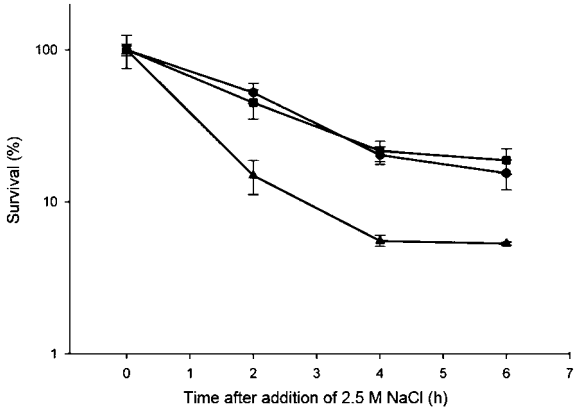

Fig. 5. Resistance of *P. chlororaphis* strain PCL1391 and mutant strains to salt stress. Cells from 3 ml overnight cultures of wild-type strain PCL1391 (●), *pip* transposon mutant PCL1114 (■) and *rpoS* recombinant mutant PCL1954 (▲) were washed and resuspended in 10 ml fresh MVB1 medium. C.f.u. were determined 0, 2, 4 and 6 h after addition of 2.5 M NaCl. Survival was determined as relative viable counts. Viable counts at time zero were normalized to 100 %. The experiment was repeated twice; data shown are means ± SD of duplicate experiments with the same culture.

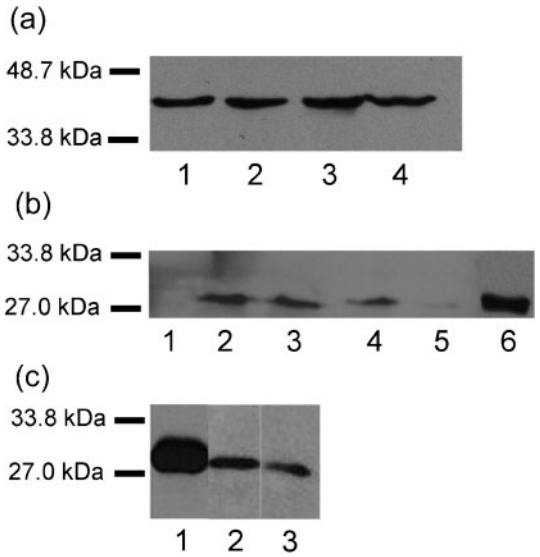

Fig. 6. Western blot analysis of RpoS and Pip production in *P. chlororaphis* PCL1391 and derivative strains. (a) Analysis of RpoS production in PCL2088 (*pip*+P_{pip} *pip-6His*) grown without stress (lane 1), with 2 μg rifampicin ml^{-1} (lane 2), with 1 mM fusaric acid (lane 3) and with 333 mM NaCl (lane 4). (b) Analysis of Pip production in PCL2085 (*pip*+P_{pip} *pip*) (lane 1) and PCL2088 (*pip*+P_{pip} *pip-6His*) (lanes 2–5) grown without stress (lane 2), with 2 μg rifampicin ml^{-1} (lane 3), with 1 mM fusaric acid (lane 4) and with 333 mM NaCl (lane 5). Lane 6: purified Pip-6His. (c) Analysis of Pip production in wild-type, P_{pip} *pip-6His* at OD_{620} 1.0 without stress (lane 2) or grown with 5 μg kanamycin ml^{-1} (lane 3). Lane 1: purified Pip-6His.

phenotype as the strain transformed with non-His-tagged Pip vector (PCL2085; data not shown), indicating that the His-tag did not affect the functioning of Pip. Strain PCL2088 was grown under normal or stress conditions in MVBI medium and RpoS and Pip were detected by Western blot. Results show that equivalent amounts of RpoS were detected under all conditions (Fig. 6a). The amount of RpoS detected in strains producing Pip with or without a His-tag were also comparable (data not shown). Only rifampicin stress did not significantly affect the amount of Pip (~80 % of Pip amounts without stress, Fig. 6b lane 3). Rifampicin stress was also the only condition studied where overproduction of Pip could not restore *N*-AHL (Fig. 3d, lanes 8 and 9) and PCN (Table 1) production. When subjected to NaCl (Fig. 6b, lane 5), fusaric acid (Fig. 6b, lane 4) and kanamycin (Fig. 6c, lane 3) stresses, Pip production was reduced to about 3, 50 and 59 % compared with cultures without stress, respectively.

Proposed model for stress resistance versus PCN production in *P. chlororaphis*

We aimed to understand how stress factors inhibit PCN production at subinhibitory concentrations, and our main results show that: (i) overexpression of *pip* and *phzR* but not of *psrA* or *rpoS* restores PCN production; (ii) constitutive overexpression of *pip* strongly impairs growth under stress conditions; (iii) controlled expression of *pip* restores stress tolerance and maintains PCN production, except in the case of salt stress; (iv) inactivation of *rpoS* but not of *pip* reduces stress resistance; and (v) stresses can reduce production of Pip but not of RpoS. Our data therefore suggest that regulation of PCN synthesis and stress resistance branches downstream of RpoS, and that the cascade of gene expression triggering that ensures stress tolerance does not reach the Pip checkpoint on the route to PCN synthesis.

The compilation of the data described above generated our proposed model for understanding the switch from PCN production to stress resistance in *P. chlororaphis* under stress conditions (Fig. 7). In favourable conditions, the amount of Pip would be sufficient to activate quorum sensing and therefore the *phz* operon. In certain stress conditions, *pip* expression would be repressed, thereby inhibiting *N*-AHL and PCN synthesis in order to focus energy on growth and resistance instead. Indeed, the regulatory cascade for PCN would then be interrupted just downstream of *rpoS*, leaving the RpoS remaining in the same quantity and able to direct stress resistance, one of its primary roles (Jorgensen *et al.*, 1999; Ramos-Gonzalez & Molin, 1998; Sarniguet *et al.*, 1995; Suh *et al.*, 1999).

When *pip* is expressed constitutively, the regulatory cascade would be artificially forced towards *N*-AHLs and PCN synthesis, while Pip would be unable to regulate its own expression. At a very early phase of growth, the amount of

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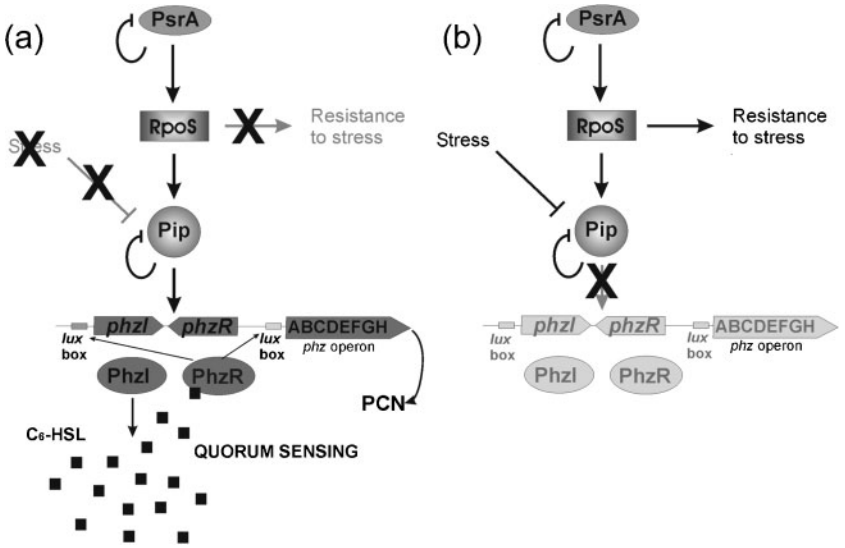

Fig. 7. Schematic model showing the role of Pip in the genetic cascade regulating PCN synthesis in *P. chlororaphis* PCL1391 without stress and in the presence of several stress factors. (a) Under no particular stress, the regulatory cascade of PCN functions as in Fig. 1. Pip possibly represses the genes involved in resistance to stress. (b) However, in the presence of certain stress factors, such as NaCl, fusaric acid or kanamycin, *pip* expression is repressed in an unknown manner. The decrease in Pip concentration results in the repression of *N*-AHL and PCN production, which allows the cell to save energy for its resistance to stress, via RpoS. Stress factors can stimulate *rpoS* expression, but the inhibition of *pip* expression by stress factors overrules its stimulation by RpoS.

PCN would therefore reach unnaturally high levels, which would be toxic for the cells and jeopardize further growth. This would explain that, although growth is strongly impaired, PCN is detected in amounts (Table 1) that the wild-type strain would not be able to reach at this OD_{620} value (van Rij *et al.*, 2004). The impaired survival of strain PCL2019 (wt + P_{tac} *pip*) in response to stress could also partially be explained by the fact that overproduced *pip* inhibits *psrA* transcription (Girard *et al.*, 2006b). This would result in a decreased production of RpoS and subsequent impaired stress resistance, explaining a part of the toxic effect of constitutive *pip* transcription in the cells. In experiments where *phzR* is constitutively overexpressed, *pip* under its own promoter can still be switched off by stress factors, but PCN synthesis occurs because PhzR binds to basal amounts of *N*-AHLs and induces *phzI* expression. However, PhzR is downstream of Pip in the PCN regulatory cascade. Increased amounts of PhzR are therefore expected to have a lower impact than increased amounts of Pip. This could explain why under stress conditions, overexpression of *phzR* does not have a similar negative effect on growth as that of *pip*.

However, complexity arises from several results. Firstly, it is noteworthy that, although *pip* expression is only minimally affected by rifampicin, its expression in multicopy moderately increases PCN production in the presence of rifampicin. Remarkably, rifampicin is the PCN-inhibiting condition that leaves both growth and the amount of Pip unaffected. This suggests that rifampicin targets other yet unidentified regulators to prevent PCN production. Secondly, some of the other stress factors only reduce *pip* expression to about 50 % of the amount of Pip measured under normal conditions. Finally, although Pip clearly seems to be involved in response to salt stress, its controlled overexpression does not restore PCN production, in contrast with what is observed with other stresses. This actually correlates well with the results in Fig. 6, indicating that NaCl is the stress factor repressing Pip expression in the strongest way (~3 % of Pip amounts without stress). In the context of P_{pip} *pip*, the amount of Pip would not be sufficient to restore PCN production. The difference between NaCl and other stress factors could be due to other unknown regulators being involved or simply to the concentrations chosen for the experiments. We cannot exclude that higher concentrations of fusaric acid or kanamycin could indeed reduce Pip production more drastically. Taken together, these observations strengthen the role of Pip in the response of *P. chlororaphis* to stress as well as indicating that other mechanisms are likely to be implicated, both downstream and independently of Pip.

The discovery of the *pip* gene (Girard *et al.*, 2006b) was an important step in unravelling the regulatory mechanisms of PCN production. Here, new results help to understand the physiological role of Pip in a broader way. The model proposed in Fig. 7 is a first step in describing how and why subinhibitory concentrations of some toxic molecules inhibit virulence genes in *Pseudomonas*. It gives novel insights into how cells could favour stress resistance by switching off secondary metabolite production. Our model is interesting in the perspective of previous work that showed the lower importance of RpoS in the regulation of PCN synthesis in more complex medium (Girard *et al.*, 2006a). We propose that in the presence of many nutrients, RpoS could play a limited role in the regulation of PCN synthesis, whereas in nutrient-limited conditions, reflecting a higher level of stress, RpoS would become an important regulator of PCN synthesis. This would allow the cells to completely switch off PCN synthesis downstream of RpoS via Pip in case of even higher levels of stress.

The fact that antibiotics at subinhibitory concentrations can also act as signalling molecules (Fajardo & Martinez, 2008) adds complexity to our study and could explain the various intensities of inhibition of PCN synthesis that we

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observed. Additional data are needed to support and further develop our model. The role of Pip should be determined in light of other relevant stress factors such as starvation, temperature and pH change, desiccation etc. Also, it would be of interest to examine whether the highly conserved Pip homologues found in other pseudomonas species (Girard *et al.*, 2006b) are also involved in stress response. Overall, understanding why and how each of the molecular signals in the rhizosphere triggers or inhibits expression of various genes in micro-organisms remains a fascinating challenge.

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REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1997). *Currents Protocols in Molecular Biology*. New York: Wiley.

Bacon, C. W., Porter, J. K., Norred, W. P. & Leslie, J. F. (1996). Production of fusaric acid by *Fusarium* species. *Appl Environ Microbiol* **62**, 4039–4043.

Caldwell, C. C., Chen, Y., Goetzmann, H. S., Hao, Y., Borchers, M. T., Hassett, D. J., Young, L. R., Mavrodi, D., Thomashow, L. & other authors (2009). *Pseudomonas aeruginosa* exotoxin pyocyanin causes cystic fibrosis airway pathogenesis. *Am J Pathol* **175**, 2473–2488.

Chater, K. F. (2006). *Streptomyces* inside-out: a new perspective on the bacteria that provide us with antibiotics. *Philos Trans R Soc Lond B Biol Sci* **361**, 761–768.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., van der Bij, A. J., van der Drift, K. M. G. M., Schripsema, J., Kroon, B., Scheffer, R. J., Keel, C., Bakker, P. A. H. M. & other authors (1998). Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol Plant Microbe Interact* **11**, 1069–1077.

Chin-A-Woeng, T. F., Bloemberg, G. V. & Lugtenberg, B. J. (2003). Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytol* **157**, 503–523.

Cummins, J., Reen, F. J., Baysse, C., Mooij, M. J. & O'Gara, F. (2009). Subinhibitory concentrations of the cationic antimicrobial peptide colistin induce the pseudomonas quinolone signal in *Pseudomonas aeruginosa*. *Microbiology* **155**, 2826–2837.

Davies, J., Spiegelman, G. B. & Yim, G. (2006). The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* **9**, 445–453.

Dietrich, L. E., Price-Whelan, A., Petersen, A., Whiteley, M. & Newman, D. K. (2006). The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol Microbiol* **61**, 1308–1321.

Dietrich, L. E., Teal, T. K., Price-Whelan, A. & Newman, D. K. (2008). Redox-active antibiotics control gene expression and community behavior in divergent bacteria. *Science* **321**, 1203–1206.

Fajardo, A. & Martinez, J. L. (2008). Antibiotics as signals that trigger specific bacterial responses. *Curr Opin Microbiol* **11**, 161–167.

Fuqua, C., Parsek, M. R. & Greenberg, E. P. (2001). Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* **35**, 439–468.

Girard, G., van Rij, E. T., Lugtenberg, B. J. & Bloemberg, G. V. (2006a). Regulatory roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL1391. *Microbiology* **152**, 43–58.

Girard, G., Barends, S., Rigali, S., van Rij, E. T., Lugtenberg, B. J. & Bloemberg, G. V. (2006b). Pip, a novel activator of phenazine biosynthesis in *Pseudomonas chlororaphis* PCL1391. *J Bacteriol* **188**, 8283–8293.

Haas, D. & Defago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* **3**, 307–319.

Hassett, D. J., Ma, J. F., Elkins, J. G., McDermott, T. R., Ochsner, U. A., West, S. E., Huang, C. T., Fredericks, J., Burnett, S. & other authors (1999). Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Mol Microbiol* **34**, 1082–1093.

Heeb, S., Blumer, C. & Haas, D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* **184**, 1046–1056.

Jorgensen, F., Bally, M., Chapon-Herve, V., Michel, G., Lazdunski, A., Williams, P. & Stewart, G. S. (1999). RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. *Microbiology* **145**, 835–844.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G. & Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc Natl Acad Sci U S A* **89**, 1562–1566.

Liang, H., Li, L., Dong, Z., Surette, M. G. & Duan, K. (2008). The YebC family protein PA0964 negatively regulates the *Pseudomonas aeruginosa* quinolone signal system and pyocyanin production. *J Bacteriol* **190**, 6217–6227.

Linares, J. F., Gustafsson, I., Baquero, F. & Martinez, J. L. (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci U S A* **103**, 19484–19489.

Miller, M. B. & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu Rev Microbiol* **55**, 165–199.

Milton, D. L., Hardman, A., Camara, M., Chhabra, S. R., Bycroft, B. W., Stewart, G. S. & Williams, P. (1997). Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI/vanR* locus and identification of the autoinducer *N*-(3-oxodecanoyl)-L-homoserine lactone. *J Bacteriol* **179**, 3004–3012.

Mitova, M. I., Lang, G., Wiese, J. & Imhoff, J. F. (2008). Subinhibitory concentrations of antibiotics induce phenazine production in a marine *Streptomyces* sp. *J Nat Prod* **71**, 824–827.

Munns, R. (2005). Genes and salt tolerance: bringing them together. *New Phytol* **167**, 645–663.

Price-Whelan, A., Dietrich, L. E. & Newman, D. K. (2006). Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat Chem Biol* **2**, 71–78.

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Price-Whelan, A., Dietrich, L. E. & Newman, D. K. (2007). Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14. *J Bacteriol* **189**, 6372–6381.

Raaijmakers, J. M., Vlami, M. & de Souza, J. T. (2002). Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* **81**, 537–547.

Ramos-Gonzalez, M. I. & Molin, S. (1998). Cloning, sequencing, and phenotypic characterization of the *rpoS* gene from *Pseudomonas putida* KT2440. *J Bacteriol* **180**, 3421–3431.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. & Haas, D. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* **24**, 309–319.

Sacherer, P., Defago, G. & Haas, D. (1994). Extracellular protease and phospholipase C are controlled by the global regulatory gene *gacA* in the biocontrol strain *Pseudomonas fluorescens* CHA0. *FEMS Microbiol Lett* **116**, 155–160.

Sambrook, J. & Russell, D. (2001). *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sarniguet, A., Kraus, J., Henkels, M. D., Muehlchen, A. M. & Loper, J. E. (1995). The sigma factor sigma s affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. *Proc Natl Acad Sci U S A* **92**, 12255–12259.

Shen, L., Shi, Y., Zhang, D., Wei, J., Surette, M. G. & Duan, K. (2008). Modulation of secreted virulence factor genes by subinhibitory concentrations of antibiotics in *Pseudomonas aeruginosa*. *J Microbiol* **46**, 441–447.

Skindersoe, M. E., Alhede, M., Phipps, R., Yang, L., Jensen, P. O., Rasmussen, T. B., Bjarnsholt, T., Tolker-Nielsen, T., Hoiby, N. & other authors (2008). Effects of antibiotics on quorum sensing in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **52**, 3648–3663.

Suh, S. J., Silo-Suh, L., Woods, D. E., Hassett, D. J., West, S. E. & Ohman, D. E. (1999). Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181**, 3890–3897.

van Rij, E. T., Wesselink, M., Chin, A. W. T. F., Bloemberg, G. V. & Lugtenberg, B. J. (2004). Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. *Mol Plant Microbe Interact* **17**, 557–566.

van Rij, E. T., Girard, G., Lugtenberg, B. J. & Bloemberg, G. V. (2005). Influence of fusaric acid on phenazine-1-carboxamide synthesis and gene expression of *Pseudomonas chlororaphis* strain PCL1391. *Microbiology* **151**, 2805–2814.

Zuber, S., Carruthers, F., Keel, C., Mattart, A., Blumer, C., Pessi, G., Gigot-Bonnefoy, C., Schnider-Keel, U., Heeb, S. & other authors (2003). GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. *Mol Plant Microbe Interact* **16**, 634–644.

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