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The PhzI/PhzR quorum-sensing system is required for pyrrolnitrin and phenazine production, and exhibits cross-regulation with RpoS in *Pseudomonas chlororaphis* PA23

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The aim of the current study was to determine how quorum sensing (QS) affects the production of secondary metabolites in *Pseudomonas chlororaphis* strain PA23. A *phzR* mutant (PA23*phzR*) and an *N*-acylhomoserine lactone (AHL)-deficient strain (PA23-6863) were generated that no longer inhibited the fungal pathogen *Sclerotinia sclerotiorum in vitro*. Both strains exhibited reduced pyrrolnitrin (PRN), phenazine (PHZ) and protease production. Moreover, *phzA–lacZ* and *prnA–lacZ* transcription was significantly reduced in PA23*phzR* and PA23-6863. As the majority of secondary metabolites are produced at the onset of stationary phase, we investigated whether cross-regulation occurs between QS and RpoS. Analysis of transcriptional fusions revealed that RpoS has a positive and negative effect on *phzI* and *phzR*, respectively. In a reciprocal manner, RpoS is positively regulated by QS. Characterization of a *phzRrpoS* double mutant showed reduced antifungal activity as well as PRN and PHZ production, similar to the QS-deficient strains. Furthermore, *phzR* but not *rpoS* was able to complement the *phzRrpoS* double mutant for the aforementioned traits, indicating that the Phz QS system is a central regulator of PA23-mediated antagonism. Finally, we discovered that QS and RpoS have opposing effects on PA23 biofilm formation. While both QS-deficient strains produced little biofilm, the *rpoS* mutant showed enhanced biofilm production compared with PA23. Collectively, our findings indicate that QS controls diverse aspects of PA23 physiology, including secondary metabolism, RpoS and biofilm formation. As such, QS is expected to play a crucial role in PA23 biocontrol and persistence in the environment.

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INTRODUCTION

When applied to planting material or soil, certain pseudomonads are able to inhibit fungal pathogens via the production of secondary metabolites. These metabolites include antibiotics, degradative enzymes and siderophores (Haas & Défago, 2005). *Pseudomonas chlororaphis* strain PA23 has shown excellent biocontrol of sclerotinia stem rot of canola caused by the fungal pathogen *Sclerotinia sclerotiorum* (Fernando *et al.*, 2007; Savchuk & Dilantha Fernando, 2004). Strain PA23 produces a number of secondary metabolites, including phenazine (PHZ), pyrrolnitrin (PRN), HCN, proteases, lipases and siderophores, some of which have been shown to contribute to antagonism (Poritsanos *et al.*, 2006; Zhang *et al.*, 2006; Selin *et al.*, 2010).

Abbreviations: AHL, *N*-acylhomoserine lactone; C_6-AHL, *N*-hexanoyl-DL-homoserinelactone; PHZ, phenazine; PRN, pyrrolnitrin; QS, quorum-sensing.

The GenBank/EMBL/DDBJ accession number for the *phzI* and *phzR* gene sequence of *P. chlororaphis* is JN593239.

Regulation of antifungal compound production is complex, involving several elements arranged as a regulatory cascade. At the top of the hierarchy sits the Gac two-component system, comprised of the sensor kinase GacS and its cognate response regulator GacA (Heeb & Haas, 2001). In many pseudomonads, including PA23, a mutation in *gacS* or *gacA* results in a loss of antifungal activity (Heeb & Haas, 2001; Poritsanos *et al.*, 2006). The stationary phase sigma factor RpoS has also been implicated in secondary metabolite production. Regulation by RpoS, however, varies depending on the producing organism and the antibiotic in question. For example, an *rpoS* mutant of *Pseudomonas fluorescens* strain Pf-5 exhibits decreased PRN production and increased levels of 2,4-diacetylphloroglucinol and pyoluteorin (Sarniguet *et al.*, 1995), whereas the same mutation in *P. chlororaphis* strain PCL1391 reduces PHZ production (Girard *et al.*, 2006). In an earlier study, it was discovered that in PA23, PHZ is positively regulated, while PRN and protease are repressed by RpoS (Manuel *et al.*, 2012). For many pseudomonads, production of exoproducts is under

quorum-sensing (QS) control. QS enables bacteria to alter their transcription profile in response to population density through the production of small diffusible signals (Bassler, 2002). In Gram-negative bacteria the most common signalling molecules utilized are *N*-acylhomoserine lactones (AHLs) (Venturi, 2006). These AHLs are generated by an autoinducer synthase, the product of a *luxI*-type gene (Bassler, 2002). After a threshold level of AHL accumulates, it binds to and activates a cognate LuxR-type protein, enabling it to induce expression of target genes (Bassler, 2002). The PhzI/PhzR QS system controls expression of the PHZ biosynthetic operon in *P. chlororaphis* strains 30-84 and PCL1391, and *P. fluorescens* strain 2-79 (Chin-A-Woeng *et al.*, 2001; Khan *et al.*, 2005; Mavrodi *et al.*, 1998; Wood & Pierson, 1996). In each case, the QS genes are located immediately upstream of the *phz* biosynthetic operon. In *P. chlororaphis* 30-84 a second QS system has been identified, called Csa, which affects cell-surface properties and protease production but is not required for PHZ biosynthesis (Zhang & Pierson, 2001). Besides those listed above, several global and pathway-specific regulators have been found to govern expression of *Pseudomonas* secondary metabolites (Haas & Défago, 2005).

Closer inspection of the network overseeing secondary metabolite production has revealed that the regulators themselves may be subject to cross-regulation. In *Pseudomonas aeruginosa*, for example, RpoS and QS exert modest effects on each other. QS induces *rpoS* transcription twofold, whereas RpoS has both a positive (*lasR* and *rhlR*) and negative effect (*rhlI*) on QS genes (Schuster *et al.*, 2004). In *P. chlororaphis* PCL1391, RpoS levels remain unchanged in a QS mutant; however, AHL production was found to be positively regulated by this sigma factor (Girard *et al.*, 2006). As a final example, two of the three *Pseudomonas putida* QS genes, namely *ppuI* and *rsaL*, are repressed by RpoS. Thus, the interconnectivity between QS and RpoS is readily apparent in terms of the metabolites that they control and their cross-regulation. However, the nature of this regulation is variable, observed as positive, negative or non-existent. So for each bacterial strain in question, details regarding how these systems function must be uncovered anew.

The aim of the current study was to investigate how QS affects expression of antifungal compounds produced by PA23. We discovered that both PHZ and PRN are under QS control. While the former was not a surprise, to the best of our knowledge this is the first report of PRN being QS-regulated in a *Pseudomonas* species. Furthermore, we show that QS and RpoS exhibit cross-regulation. RpoS activates and represses *phzI* and *phzR* expression, respectively. QS, on the other hand, positively controls *rpoS* transcription. Finally, it was discovered that both of these global regulators affect the ability of strain PA23 to form biofilms.

METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *P. chlororaphis* PA23 strains were cultured on Lennox Luria–Bertani (LB) agar (Difco Laboratories) at 37 and 28 °C, respectively. *S. sclerotiorum* was maintained on Potato Dextrose Agar (PDA; Difco). Media were supplemented with the following antibiotics from Research Products International, as required: piperacillin (30 μg ml^{-1}), gentamicin (Gm; 20 μg ml^{-1}), tetracycline (Tc; 15 μg ml^{-1}), rifampicin (Rif; 100 μg ml^{-1}) for PA23; and ampicillin (Amp; 100 μg ml^{-1}), Gm (20 μg ml^{-1}), Tc (15 μg ml^{-1}) and chloramphenicol (Chl; 25 μg ml^{-1}) for *E. coli*.

Nucleic acid manipulation. Standard techniques for purification, cloning and other DNA manipulations were used (Sambrook *et al.*, 1989). PCR was performed following standard conditions suggested by Invitrogen Life Technologies data sheets supplied with their *Taq* polymerase.

Sequence analysis. The sequence of the *phzI* and *phzR* genes was determined through a primer walking strategy using a Tn*5 Xho*I rescue clone previously isolated in our laboratory (Selin *et al.*, 2010). This plasmid contains a portion of the *phz* biosynthetic locus (*phzABCDE*) and approximately 17 kb of upstream DNA. Sequencing was performed at the University of Calgary Core DNA Services facility, and sequences were analysed with BLASTN and BLASTX databases.

Generation of PA23*phzR*, PA23*rpoS*, PA23*phzRrpoS* and PA23-6863. All primers and plasmids utilized for the construction of mutant strains are listed in Table 1. To generate PA23*phzR*, a copy of *phzR* missing an internal 66 bp fragment was generated through PCR. To accomplish this, the 5′ end of *phzR* was amplified using primers phzRP1 and phzRP2, and the 3′ end was amplified using phzRP3 and phzRP4. The two PCR products were digested with *Sac*I and cloned into pCR2.1 to yield *phzR*1.4pCR2.1. The 1.4 kb insert was subsequently digested with *Eco*RI and *Bam*HI and subcloned into the same sites of pEX18Ap (*phzR*pEX18Ap). An 850 bp Gm^R cassette was excised from pUCGm and inserted into the *Sac*I site to yield *phzR*-gentpEX18Ap. Triparental mating between *E. coli* DH5α (*phzR*-gentpEX18Ap), *E. coli* DH5α (pRK600) and PA23 was performed. Pseudomonas Isolation Agar (PIA; Difco) + Gm (20 μg ml^{-1}) was used to screen for transconjugants. To select for bacteria that had undergone a double cross-over event, colonies were streaked onto LA supplemented with sucrose (10 %) and Gm (20 μg ml^{-1}). PCR analysis was used to verify that *phzR* had been successfully replaced with a mutated copy of the allele (data not shown). The *rpoS* mutant PA23*rpoS* was created by first excising an internal portion of the *rpoS* gene from PCR2.1-*rpoS* using the enzymes *Bam*HI and *Eco*RV. The resulting 400 bp fragment was then subcloned into the same sites of pKNOCK-Tc, generating pKNOCK-*rpoS*. Triparental mating of *E. coli* DH5α λpir (pKNOCK-*rpoS*), *E. coli* DH5α (pRK600) and PA23 was performed, and transconjugants obtained were screened on LB agar supplemented with Rif and Tc. PA23*phzRrpoS* was created by insertion of plasmid pKNOCK-*rpoS* into the *rpoS* gene of PA23*phzR*. To verify disruption of the *rpoS* gene, the pKNOCK-Tc vector was rescued from the PA23*rpoS* and PA23*phzRrpoS* genomes by digestion with *Bgl*II. Linearized genomic fragments were recircularized with T4 DNA ligase, transformed into *E. coli* DH5α λpir, and screened on LB agar supplemented with 15 μg Tc ml^{-1}. Sequencing of the rescue clones, using primers RpoS RC-fwd and RpoS RC-rev, verified that an *rpoS* insertion had occurred in both strains. A quorum-quenching approach enabled us to generate an AHL-deficient strain. Plasmid pME6863, which contains the AHL lactonase gene (*aiiA*), was mobilized in PA23, generating PA23-6863. The presence of the lactonase enzyme renders bacteria AHL-deficient due to hydrolysis of the lactone ring.

Plasmid construction. To complement PA23*phzR* and PA23*phzRrpoS*, pUCP23-*phzR* was generated as follows. A 1.68 kb *Hin*dIII–*Xba*I fragment was excised from pCR-*phzAR* and subcloned into the same sites of pUCP23, creating pUCP23-*phzR*. To generate

Table 1. Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Relevant genotype, phenotype or sequence	Reference or source
Strains		
P. chlororaphis		
PA23	Phz$^+$ Rif^R wild-type (soybean isolate)	Savchuk & Dilantha Fernando (2004)
PA23*phzR*	PA23 with Gm^R marker inserted into *phzR*	This study
PA23-6863	PA23 carrying pME6863	This study
PA23*rpoS*	PA23 with pKNOCK-Tc vector inserted into *rpoS*	This study
PA23*rpoSphzR*	PA23*rpoS* with a Gm^R marker inserted into *phzR*	This study
P. aeruginosa		
QSC105	Strain carrying pEAL01 (*lasB–lacZ* transcriptional fusion), Carb^R	Ling *et al.* (2009)
E. coli		
DH5α	*supE44* Δ*U169* (*ϕ*80*lacZ*ΔM15)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco
DH5α λpir	λpir lysogen of DH5α	House *et al.* (2004)
Chromobacterium violaceum		
CVO26	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31532, autoinducer biosensor	Latifi *et al.* (1996)
Plasmids		
pME6863	pME6000 carrying the *aiiA* gene from *Bacillus* sp. A24 under the constitutive P_{lac} promoter	Reimmann *et al.* (2002)
pCR2.1	TA cloning vector, Amp^R	Invitrogen
pUCGm	Source of Gm^R cassette	Schweizer (1993)
pEX18Ap	Suicide plasmid, Amp^R	Hoang *et al.* (1998)
*phzR*1.4pCR2.1	1.4 kb *phzR* fragment in pCR2.1	This study
*phzR*pEX18Ap	*phzR* from PA23 in pEX18Ap	This study
phzR-gentpEX18Ap	Gm^R marker inserted into the *Sac*I site of *phzR*pEX18Ap	
pRK600	Contains *tra* genes for mobilization, Chl^R	Finan *et al.* (1986)
pCR2.1-*rpoS*	1 kb fragment containing the *rpoS* gene in pCR2.1	Poritsanos *et al.* (2006)
pKNOCK-Tc	Suicide vector for insertional mutagenesis; R6K ori RP4 oriT Tc^R	Alexeyev (1999)
pKNOCK-*rpoS*	400 bp internal fragment from *rpoS* in pKNOCK-Tc	This study
pUCP23	Broad-host-range vector, Amp^R Gm^R	West *et al.* (1994)
pUCP23-*phzR*	*phzR* in pUCP23	This study
pME6032	Shuttle vector, Tc^R	Heeb *et al.* (2002)
pME6032-*phzR*	*phzR* in pME6032	This study
pUCP22	Broad-host-range vector, Amp^R Gm^R	West *et al.* (1994)
pUCP22-*rpoS*	*rpoS* in pUCP22	Poritsanos *et al.* (2006)
phzI-pCR2.1	1.3 kb fragment containing *phzI* in pCR2.1	This study
pLP170	*lacZ* transcriptional fusion vector	Preston *et al.* (1997)
pPHZI-*lacZ*	674 bp fragment containing the *phzI* promoter in pLP170	This study
pCR-*phzAR*	1.68 kb fragment containing *phzR* and 5′ end of *phzA* in pCR2.1	This study
pPHZR-*lacZ*	1.1 kb fragment containing *phzR* promoter in pLP170	This study
pPHZA-*lacZ*	*phzA* promoter in pLP170	Selin *et al.* (2010)
pPRNA-*lacZ*	*prnA* promoter in pLP170	Selin *et al.* (2010)
pRPOS-*lacZ*	*rpoS* promoter in pLP170	Poritsanos *et al.* (2006)
Primers		
phzRP1	5′-gcatgaattcaattggcgatgccgttgtt-3′	This study
phzRP2	5′-gcatgagctccacggtggtgaagcacagcaaa-3′	This study
phzRP3	5′-gcatgagctcatataagtcctggggccgcat-3′	This study
phzRP4	5′-gcatggatcccgatgtactgttcgacggt-3′	This study
phzI-FRW	5′-tacgactgcctggaccaaact-3′	This study
phzI-REV	5′-aatcctgccatccaactc-3′	This study
phzAR-F	5′-aatcctgccatccaactc-3′	This study
phzAR-R	5′-aaggttgttcgaaggggttca-3′	This study
RpoS RC-fwd	5′-gatatcgccactgattcgatc-3′	This study
RpoS RC-rev	5′-ggatccaccaggtggcata-3′	This study

pME6032-*phzR*, a 1.68 kb *Eco*RI fragment was excised from pUCP23-*phzR* and subcloned into the same sites of pME6032. The *phzI–lacZ* transcriptional fusion was constructed by PCR-amplifying the *phzI* promoter using primers phzI-FRW and phzI-REV. The 1.3 kb PCR product was cloned into pCR2.1 (*phzI*-pCR2.1), and then excised as a *Hinc*II–*Eco*RI fragment and subcloned into the *Sma*I–*Eco*RI sites of pLP170, generating pPHZI-*lacZ*. The *phzR–lacZ* fusion was constructed using primers phzAR-F and phzAR-R to amplify a 1.68 kb fragment containing the entire *phzR* gene and the 5′ end of *phzA*. This fragment was cloned into pCR2.1, creating pCR-*phzAR*. The *phzR* promoter was excised from pCR-*phzAR* as a 1.1 kb *Eco*RI–*Eco*RV fragment and ligated into the *Eco*RI–*Sma*I sites of pLP170, generating pPHZR-*lacZ*.

Antifungal assays. To assess the ability of PA23 and its derivatives to inhibit the growth of *S. sclerotiorum in vitro*, a radial diffusion assay was performed as described by Poritsanos *et al.* (2006). Five replicates were analysed for each strain and the experiments were repeated three times.

Quantitative analysis of PHZ. Overnight cultures grown in M9 minimal medium supplemented with 1 mM $MgSO_4$ and 0.2 % glucose were subject to PHZ extraction according to Selin *et al.* (2010). Samples were analysed in triplicate and the experiment was repeated twice.

HPLC analysis of PRN. The amount of PRN produced by PA23 and its derivatives was quantified by HPLC as described by Selin *et al.* (2010), with the following modifications. Strains were grown in 100 ml M9 minimal medium + 1 mM $MgSO_4$ + 0.2 % glucose for 5 days. Toluene was added to the culture supernatants as an internal control. Peaks corresponding to the toluene and PRN were analysed by UV absorption at 225 nm using a Varian 335 diode array detector. Samples were analysed in triplicate and the experiment was repeated twice.

HCN analysis. Qualitative determination of HCN production was performed using Cyantesmo paper (Machery-Nagel). Experiments were repeated three times.

Protease production. Quantitative protease assays were carried out on PA23 and its derivatives as described previously (Manuel *et al.*, 2011). Each strain was analysed in triplicate and experiments were done on three separate occasions.

Motility analysis. Flagellar (swimming) motility was monitored according to Poritsanos *et al.* (2006). For the assays, five replicates were analysed and the experiment was repeated three times.

Biofilm development. A highly reproducible 96-well plate assay (O'Toole & Kolter, 1998) was employed to assess the ability of PA23, PA23*phzR*, PA23-6863, PA23*rpoS* and PA23*phzRrpoS* to form biofilms.

AHL analysis. Total autoinducer was monitored according to Ling *et al.* (2009), with the following modifications. Cell-free supernatants of cultures grown in 10 ml M9 minimal medium for 18 h at 28 °C were extracted with ethyl acetate. The extract was concentrated to a final volume of 1 ml, and a 3 µl aliquot was added to test tubes and dried under a stream of nitrogen gas. An overnight culture of *P. aeruginosa* QSC105 (pEAL01) grown in PTSB (Ohman *et al.*, 1980) supplemented with carbenicillin (200 µg ml^{-1}) was diluted to a final OD_{600} of 0.1, and 1 ml aliquots were added to tubes containing dried extracts. The cultures were grown for 18 h at 37 °C with vigorous shaking and then analysed for β-galactosidase activity (Miller, 1972). Samples were analysed in triplicate and the experiments were repeated three times.

Analysis of transcriptional fusions. The activity of *prnA–*, *phzA–*, *phzR–*, *phzI–* and *rpoS–lacZ* transcriptional fusions was determined in PA23, PA23*phzR*, PA23-6863, PA23*rpoS* and PA23*rpoSphzR*. Strains carrying the *lacZ* fusion plasmids were grown for 4, 8, 16, 24 and 36 h in M9 minimal medium supplemented with 1 mM $MgSO_4$ and 0.2 % glucose prior to analysis of β-galactosidase activity (Miller, 1972). The activity of *prnA–lacZ* was also measured in *E. coli* harbouring pME6032-*phzR*, in the presence and absence of 1 µm purified *N*-hexanoyl-DL-homoserinelactone (C_6-AHL; Sigma) in M9 minimal medium (1 mM $MgSO_4$, 0.2 % glucose, 0.4 % Casamino acids). *E. coli* cultures were grown for 24 h prior to analysis of β-galactosidase activity.

RpoS expression. To determine RpoS protein levels, Western blot analysis was performed using an RpoS-specific polyclonal antibody as described previously (Poritsanos *et al.*, 2006). The Western analysis was carried out twice with similar findings.

Statistical analysis. An unpaired Student's *t* test was used for statistical analysis of PHZ, PRN and protease production, antifungal activity and swimming motility.

RESULTS

Generation and phenotypic characterization of PA23 QS-deficient strains

For bacteria that harbour a copy of the *phz* biosynthetic operon, the *phzI*/*phzR* QS locus is typically located just upstream. Sequencing of a plasmid containing part of the PA23 *phz* operon (*phzABCDE*) revealed the presence of *phzI* and *phzR* homologues upstream of *phzA* (data not shown). This QS locus showed the highest degree of identity with *phzI* and *phzR* of *P. chlororaphis* strains O6 (99 %; accession no. AY927995.1), 30-84 (94 %; accession nos L33724 and EF62944.1) and PCL1391 (94 %; accession no. AF19615). The genes are organized with *phzR* immediately upstream of and divergently transcribed from *phzA*. Similarly, *phzI* is upstream of *phzR* but oriented in the opposite direction (Fig. 1a). To investigate the effect of QS on PA23 secondary metabolite production, a *phzR* mutant was created through allelic exchange. Replacement of the wild-type copy of *phzR* with the mutated allele was confirmed through PCR (data not shown). A quorum-quenching approach was taken to generate an AHL-deficient derivative of PA23. The *aiiA* AHL lactonase gene on plasmid pME6863 was mobilized into PA23, creating PA23-6863.

When PA23*phzR* and PA23-6863 were tested for their ability to inhibit *S. sclerotiorum in vitro*, both strains exhibited a complete loss of fungal antagonism (Table 2). Mobilization of pUCP23-*phzR* into the *phzR* mutant restored antifungal activity to near wild-type levels (Table 2). We have previously shown that PHZ production imparts an orange phenotype to PA23, whereas PRN is the primary antibiotic responsible for *S. sclerotiorum* antagonism (Selin *et al.*, 2010). The reduced pigmentation and lack of antifungal activity exhibited by the QS mutants suggested that decreased levels of PHZ and PRN were being produced. Quantitative analysis revealed that PHZ levels were down five- and sevenfold in PA23*phzR* and PA23-6863, respectively. This was not unexpected, as the *phz* biosynthetic operon is immediately downstream of *phzI* and *phzR*, and PHZs are QS-regulated in other bacterial strains (Mavrodi *et al.*, 2006). QS control of PRN production (Table 2), on the

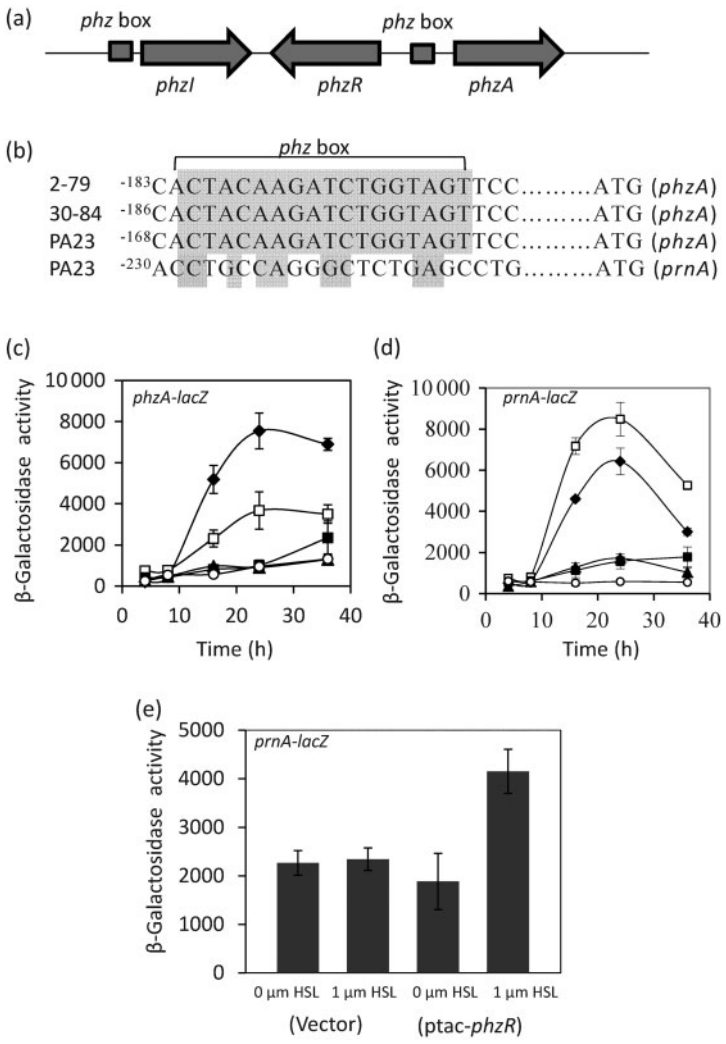

Fig. 1. Chromosomal localization of the *phzI* and *phzR* QS genes and analysis of their effect on *phzA* and *prnA* expression in PA23. (a) The *phzI* and *phzR* genes are located upstream of the PHZ operon. The first gene of the operon, *phzA*, is illustrated along with the location of the *phz* boxes. (b) Identification of the *phz* box sequences in the *phzA* and *prnA* promoter regions of *P. chlororaphis* PA23. Alignments of the *phz* box sequences found in *P. fluorescens* 2-79, *P. chlororaphis* 30-84 and *P. chlororaphis* PA23 are illustrated. The sequences and relative positions of the *phz* boxes and the ATG start codons of *phzA* and *prnA* are indicated. The conserved nucleotides within the *phz* boxes are shaded in grey. Nucleotide sequences of the *phzA* promoter regions of *P. fluorescens* 2-79 and *P. chlororaphis* 30-84 were obtained from GenBank as accession numbers L48616 and AF007801, respectively. (c, d) Effect of QS on *phzA* (c) and *prnA* (d) expression in PA23. Bacterial strains are as follows: *P. chlororaphis* PA23 (◆), PA23*phzR* (▲), PA23*phzRrpoS* (■), PA23-6863 (○) and PA23*rpoS* (□). Bacteria were grown in M9 minimal medium supplemented with 1 mM $MgSO_4$ and 0.2 % glucose. (e) Impact of QS on the expression of *prnA–lacZ* in *E. coli*. *E. coli* harbouring the *prnA–lacZ* fusion together with either *phzR*-pME6032 or pME6032 (empty vector) was grown in the presence and absence of C_6-HSL (HSL) in M9 minimal medium (1 mM $MgSO_4$, 0.2 % glucose, 0.4 % Casamino acids). Cultures were grown for 24 h prior to measuring β-galactosidase activity (Miller units). Values shown the means of three replicates; error bars, SD.

other hand, has not been reported for other *Pseudomonas* spp. Because PA23 produces a number of other secondary metabolites that may contribute to fungal antagonism, including HCN, protease and lipase, we investigated whether these compounds were QS-regulated. HCN and lipase production was not affected by the lack of PhzR or AHL (data not shown). However, protease activity was reduced twofold and sixfold in PA23*phzR* and PA23-6863, respectively (Table 2). Mobilization of the *phzR* gene into PA23*phzR* increased protease production but, for reasons unknown, only partial complementation was achieved (Table 2).

We also examined whether biofilm development and motility were altered in the QS-deficient strains. As shown in Fig. 2, biofilm formation was reduced over fivefold in PA23*phzR* and PA23-6863. The presence of pUCP23-*phzR* in PA23*phzR* increased the adherent biomass close to that of PA23. Taken together, these findings indicate that a functional PHZ QS system facilitates establishment of PA23 biofilms. Flagellar motility is important for not only biocontrol (Haas & Défago, 2005) but also the early stages of biofilm formation (Davey & O'Toole, 2000). Therefore, we examined whether there were any differences in swimming motility between PA23 and the PhzR- and AHL-deficient strains. We discovered that PA23*phzR* was as motile as PA23; PA23-6863 on the other hand exhibited decreased motility at both 24 and 48 h (Table 3). As both QS-deficient strains exhibited diminished biofilm formation, but only PA23-6863 showed altered motility, other factors must be responsible for the impaired biofilm development.

QS regulates *phzA* and *prnA* expression

Expression of *phzA*– and *prnA–lacZ* transcriptional fusions were analysed in PA23 and the QS-deficient strains. The transcription of both genes was markedly reduced in PA23*phzR* and PA23-6863 (Fig. 1c, d), indicating that QS

Table 2. Phenotypic characteristics of PA23 and its derivatives

Strain	Extracellular metabolite activity			
	Antifungal activity* (mm)	Protease† (units of enzyme ml^{-1})	Total PHZ† ($\mu g\ ml^{-1}$)	PRN†‡ (μg)
PA23(pUCP22)	8.6 (0.7)	0.56 (0.04)	79.4 (0.5)	16.9 (1.5)
PA23-6863	0§	0.09 (0.01)§	11.5 (0.9)§	0§
PA23*phzR*(pUCP22)	0§	0.30 (0.03)§	14.1 (0.3)§	0§
PA23*phzR*(pUCP23-*phzR*)	7.0 (0.9)‖	0.40 (0.01)¶	68.5 (1.8)¶	14.8 (1.4)
PA23*phzRrpoS*(pUCP22)	0§	1.15 (0.04)§	17.7 (0.4)§	0§
PA23phzRrpoS(pUCP22-*rpoS*)	0§	0.36 (0.01)¶	16.0 (1.2)§	0§
PA23*phzRrpoS*(pUCP23-*phzR*)	6.7 (1.1)¶	0.87 (0.02)§	63.9 (0.5)§	14.5 (2.3)#

*Mean (SD) obtained from six replicates.
†Mean (SD) obtained from a triplicate set.
‡Mean (SD) amount of PRN extracted from 100 ml culture volume.
§Significantly different from wild-type ($P<0.001$).
‖Significantly different from wild-type ($P<0.05$).
¶Significantly different from wild-type ($P<0.01$).
#Not significantly different from wild-type.

positively regulates *phzA* and *prnA* expression. Next, we searched for the presence of a phz box element upstream of the aforementioned genes. A sequence was identified upstream of *phzA* (168 bp from the ATG start) that was a 100 % match (18/18 nt) with the phz box found in other *P. chlororaphis* strains (Fig. 1b). Similarly, we found an 18 bp region (54 bp upstream of the *phzI* ATG start) that is 100 % identical to the phz box consensus upstream of *phzI* (Chin-A-Woeng *et al.*, 2001; data not shown). Inspection of the *prnA* promoter region revealed a sequence that has 9/18 nt in common with the phz box consensus of *phzA* (Fig. 1b). To determine whether QS has a direct effect on *prn* expression, the activity of a *prnA–lacZ* transcriptional fusion was monitored in *E. coli* in the presence and absence of pME6032-*phzR*. This plasmid carries *phzR* under the control of the *tac* promoter. As shown in Fig. 1(e), no difference in *prnA–lacZ* activity was observed between cells carrying pME6032-*phzR* versus those carrying the empty vector. However, in the presence of PhzR and 1 μM C_6-HSL, *prnA–lacZ* activity increased twofold (Fig. 1e), suggesting that QS exerts a direct effect on *prnA* transcription. As expected, no change in transcription was observed in the presence of C_6-HSL alone (Fig. 1e).

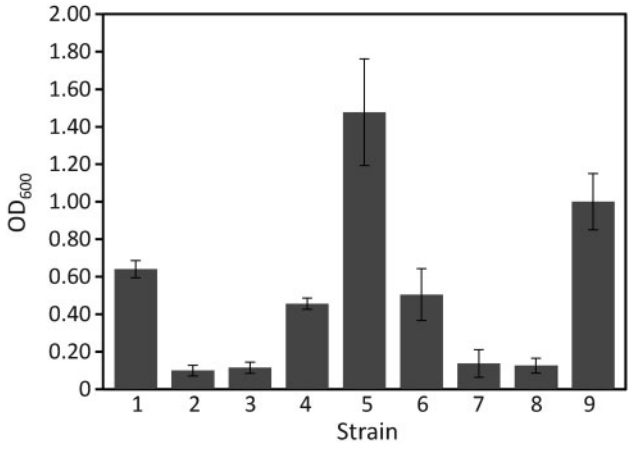

Fig. 2. Biofilm formation in PA23 and its derivatives. Bacterial strains are as follows: 1, PA23(pUCP22); 2, PA23-6863(pUCP22); 3, PA23*phzR*(pUCP22); 4, PA23*phzR*(pUCP23-*phzR*); 5, PA23*rpoS*(pUCP22); 6, PA23*rpoS*(pUCP22-*rpoS*); 7, PA23*rpoSphzR* (pUCP22); 8, PA23*phzRrpoS*(pUCP22-*rpoS*); 9, PA23*rpoSphzR* (pUCP23-*phzR*). Cultures were grown in 96-well microtitre plates containing M9 medium (1 mM $MgSO_4$ and 0.2 % glucose) for 24 h at 28 °C. Biofilm formation, indicated by crystal violet staining, was measured by A_{600}. Values shown are the means of five replicates; error bars, SD.

The Phz QS system is subject to positive autoregulation

QS systems are typically arranged as an autoinduction circuit with both the I- and R-genes subject to positive autoregulation. To determine whether the same holds true for PA23, the activity of *phzI–lacZ* and *phzR–lacZ* transcriptional fusions was analysed in PA23, PA23*phzR* and PA23-6863. Expression of *phzI* in both PA23*phzR* and PA23-6863 remained at low levels throughout growth, unlike PA23, in which *phzI* expression peaked at 24 h (Fig. 3a). The same trend was observed for *phzR* transcription (Fig. 3b). Thus, it appears that the expression of the Phz QS genes requires both the AHL signalling molecule and PhzR. Next, we analysed the amount of AHL present in culture extracts using *P. aeruginosa* QSC105 (pEAL01), a strain capable of detecting a broad range of AHLs (Ling *et al.*, 2009). Levels of AHL were reduced two- and eightfold in

Table 3. Flagellar motility of *P. chlororaphis* PA23 and derivative strains

Strain	Motility* (mm)	
	24 h	48 h
PA23(pUCP22)	22.3 (2.3)	31.3 (3.6)
PA23-6863(pUCP22)	11.5 (1.3)†	22.5 (1.5)‡
PA23*phzR*(pUCP22)	22.8 (1.4)§	26.5 (1.7)§
PA23*phzR*(pUCP23-*phzR*)	27.2 (2.1)§	39.7 (4.8)§
PA23*phzRrpoS*(pUCP22)	39.0 (3.1)‖	56.3 (7.5)‖
PA23*phzRrpoS*(pUCP23-*phzR*)	31.3 (3.5)‡	52.5 (7.8)‡
PA23*phzRrpoS*(pUCP22-*rpoS*)	25.3 (4.3)§	32.0 (2.8)§
PA23*rpoS*(pUCP22)	34.8 (0.3)†	56.7 (5.9)†
PA23*rpoS*(pUCP22-*rpoS*)	31.3 (2.1)‖	48.3 (1.5)‖

*Mean (SD) from triplicates.
†Significantly different from wild-type ($P<0.001$).
‡Significantly different from wild-type ($P<0.05$).
§Not significantly different from wild-type.
‖Significantly different from wild-type ($P<0.01$).

the *phzR* mutant and PA23-6863, respectively. When strain PA23*phzR* was complemented with *phzR in trans*, AHL levels exceeded those of PA23 (Fig. 4).

QS positively regulates *rpoS* expression

To determine whether QS has an impact on *rpoS* expression, an *rpoS–lacZ* fusion was monitored in PA23, PA23*phzR* and PA23-6863. At 24 h, a 1.5-fold and a 3.5-fold decrease in *rpoS* transcription was demonstrated by PA23*phzR* and PA23-6863, respectively (Fig. 5a). We also examined the total amount of RpoS protein present through Western blot analysis. Protein levels were significantly reduced in PA23*phzR* compared with the wild-type, while PA23-6863 did not produce any detectable RpoS (Fig. 5b). Taken together, both the transcriptional fusion data and Western blot analysis indicate that RpoS is positively regulated by QS.

RpoS regulates *phzI* and *phzR*

Since QS positively regulates *rpoS* expression, we were interested to see if there was reciprocal cross-regulation of QS by RpoS. Therefore, expression of *phzI–lacZ* and *phzR–lacZ* was measured in PA23 and PA23*rpoS*. As shown in Fig. 3, *phzI* transcription was reduced over threefold at 24 h of growth in PA23*rpoS* (Fig. 3a). The opposite was observed for *phzR*, where transcription levels were elevated in the *rpoS* single mutant (Fig. 3b). Collectively these results suggest that under minimal conditions, transcription of *phzI* but not *phzR* is dependent on RpoS. Quantitative analysis revealed reduced levels of AHL in PA23*rpoS* culture extracts (Fig. 4), which is consistent with the *phzI* transcriptional fusion data.

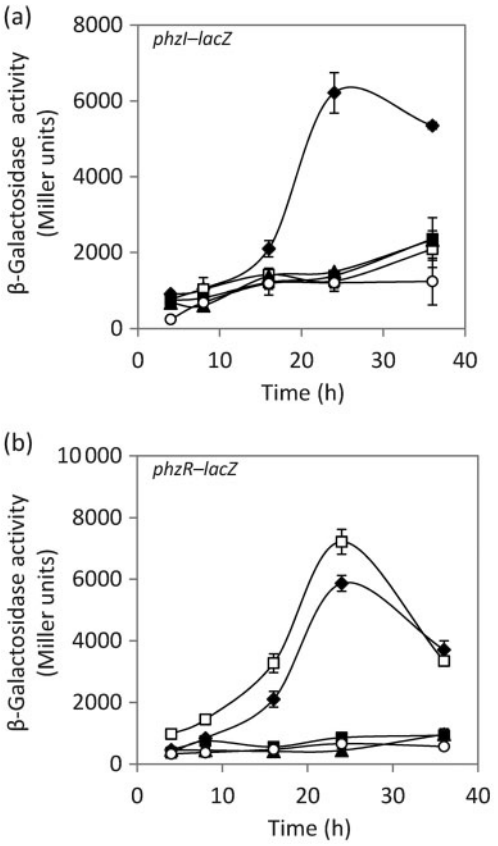

Fig. 3. Effects of QS and RpoS on *phzI* (a) and *phzR* (b) transcription. Bacterial strains are as follows: *P. chlororaphis* PA23 (◆), PA23*phzR* (▲), PA23-6863 (○), PA23*phzRrpoS* (■) and PA23*rpoS* (□). Bacteria were grown in M9 minimal medium supplemented with 1 mM $MgSO_4$ and 0.2 % glucose. Values shown are the means of three replicates; error bars, SD.

RpoS controls PA23 antifungal activity indirectly through the Phz QS system

Next we addressed whether constitutively expressed *phzR* and/or *rpoS* would be able to complement PA23*phzRrpoS*. When pUCP23-*phzR* was mobilized into the *phzRrpoS* double mutant, antifungal activity and antibiotic production were restored to wild-type levels (Table 2). Conversely, PA23*phzRrpoS* harbouring pUCP22-*rpoS* was virtually indistinguishable from PA23*phzR* (Table 2). Regulation of protease production was found to be unusual. In an earlier study, we discovered that RpoS has a repressive effect on protease production (Manuel *et al.*, 2012). Thus, it was not surprising that the double mutant harbouring pUCP22-*rpoS* produced less protease than the mutant carrying the empty vector (Table 2). As protease production is positively regulated by QS, we expected to see an increase in protease levels in the *rpoSphzR* double mutant harbouring pUCP23-*phzR*. As illustrated in Table 2, this was the case.

Next we examined the ability of PA23*phzRrpoS* and PA23*rpoS* to form biofilms and translocate via flagellar

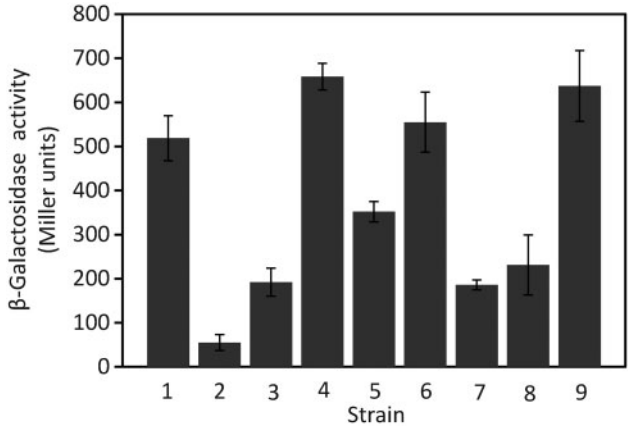

Fig. 4. *β*-Galactosidase activity of *lasB–lacZ* in *P. aeruginosa* spiked with autoinducer extracts from PA23 and derivative strains. Extracts were analysed from the following bacteria: 1, PA23 (pUCP22); 2, PA23-6863(pUCP22); 3, PA23*phzR*(pUCP22); 4, PA23*phzR*(pUCP23-*phzR*); 5, PA23*rpoS*(pUCP22); 6, PA23 *rpoS*(pUCP22-*rpoS*); 7, PA23*rpoSphzR*(pUCP22); 8, PA23*phz RrpoS*(pUCP22-*rpoS*); 9, PA23*rpoSphzR*(pUCP23-*phzR*). Cell-free supernatants of bacterial cultures grown in M9 minimal medium (1 mM $MgSO_4$, 0.2 % glucose) were extracted using ethyl acetate. The concentrated AHL preparations were added to cultures of *P. aeruginosa* QSC105 (pEAL01), which were grown for 18 h prior to *β*-galactosidase analysis. Assays were carried out three times in triplicate and a representative dataset is shown. Values shown are the means of three replicates; error bars, SD.

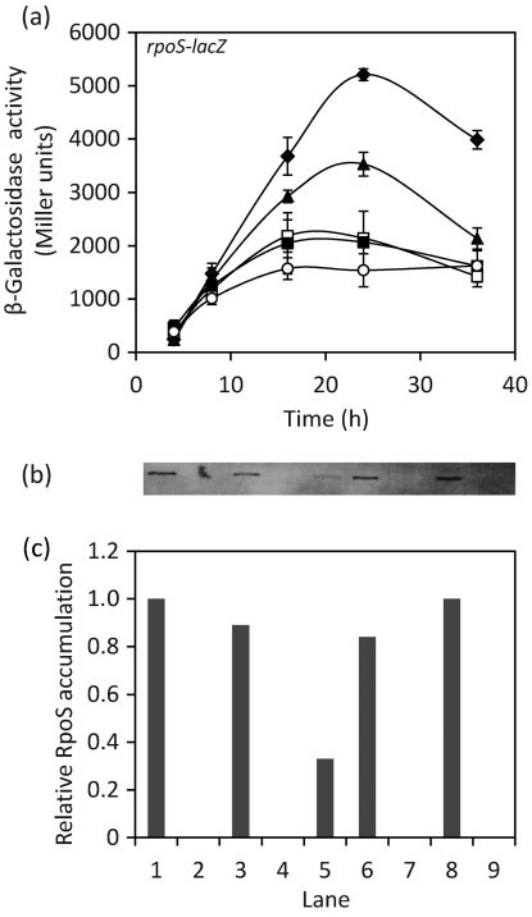

Fig. 5. Analysis of *rpoS–lacZ* activity and relative accumulation of RpoS protein in *P. chlororaphis* PA23 and its derivatives. (a) Strains used are as follows: PA23 (◆), PA23*phzR* (▲), PA23-6863 (○), PA23*phzRrpoS* (■) and PA23*rpoS* (□). All strains harboured the *rpoS–lacZ* reporter plasmid pRPOS-*lacZ*. Values shown are the means of three replicates; error bars, SD. (b) RpoS-specific antiserum was used to visualize RpoS from Western blots of protein extracted from cultures grown to stationary phase. Each lane contains 100 μg protein. Lanes: 1, PA23(pUCP22); 2, PA23*rpoS*(pUCP22); 3, PA23*rpoS*(pUCP22-*rpoS*); 4, PA23-6863; 5, PA23*phzR*(pUCP22); 6, PA23*phzR*(pUCP23-*phzR*); 7, PA23*phzRrpoS*(pUCP22); 8, PA23*phzRrpoS*(pUCP22-*rpoS*); 9, PA23*phzRrpoS*(pUCP23-*phzR*). (c) RpoS content in each lane was estimated by analysing Western blots with a phosphoimager. The Western blot analysis was performed twice and a representative dataset is shown.

motility. Biofilm analysis revealed that PA23*phzRrpoS* closely resembles the QS-deficient strains, producing significantly less adherent biomass (Fig. 2). Constitutively expressed *phzR* restored PA23*phzRrpoS* biofilm formation to wild-type levels. Compared with PA23 and the QS-deficient strains, the *rpoS* single mutant showed increased biofilm formation. Addition of pUCP22-*rpoS* had no effect on PA23*phzRrpoS*; however, for PA23*rpoS*, it reduced biofilm formation (Fig. 2). When the aforementioned strains were assessed for flagellar motility, the double mutant showed increased swim zones, much like the *rpoS* single mutant (Table 3).

Lastly, we examined the activity of the *phzA*–, *prnA*–, *phzI*– and *phzR–lacZ* transcriptional fusions in PA23*phzRrpoS*. No differences in expression were observed between PA23*phzRrpoS* and PA23*phzR* (Figs 1c, d and 3).

DISCUSSION

The aim of the current study was to discover how QS affects production of antifungal compounds, biofilm formation and motility in *P. chlororaphis* strain PA23. As the majority of secondary metabolites are produced at the onset of stationary phase, we were also interested to learn whether cross-regulation occurs between QS and the stationary-phase sigma factor RpoS. A *phzR* mutant (PA23*phzR*) and an AHL-deficient strain (PA23-6863) were created that no longer inhibited *S. sclerotiorum* (Table 2), indicating that QS is required for fungal antagonism. A number of metabolites are thought to contribute to PA23 biocontrol, including diffusible antibiotics (PHZ, PRN), HCN and extracellular enzymes (Poritsanos *et al.*, 2006). We hypothesized that the lack of antifungal activity exhibited by the QS-deficient derivatives was due to diminished production of one or more of these compounds. The observed reduction in PHZ

levels was expected, because PHZs are positively regulated by QS in other *P. chlororaphis* strains (Chin-A-Woeng *et al.*, 2001; Pierson *et al.*, 1994; Wood & Pierson, 1996). Conversely, the markedly lower amounts of PRN indicated that this antibiotic was also under QS control, which has not been reported in *P. chlororaphis* or other *Pseudomonas* spp. Studies of PRN regulation have lagged behind those of other antibiotics in biocontrol pseudomonads. In *P. chlororaphis* for example, PHZs are essential for biocontrol; consequently, this antibiotic has been the focus of most investigations (Chin-A-Woeng et al., 1998; Pierson & Thomashow, 1992; Spencer *et al.*, 2003). Furthermore, while *P. fluorescens* strains Pf-5 and CHA0 both produce PRN, neither strain has a QS system (Haas & Keel, 2003).

LuxR-type transcriptional regulators bind to distinct elements upstream of target genes called 'lux boxes' or in the case of the *phz* biosynthetic operon, 'phz boxes'. Upstream of *phzI* and *phzA*, we found sequences that were 100 % identical to phz boxes present in the promoter regions of homologous genes from other pseudomonads. In addition, a less conserved (9/18 nt) phz box lies upstream of *prnA*. To further establish that QS has a direct effect on *prnA*, we monitored *prnA–lacZ* activity in *E. coli* in the presence and absence of PhzR–C_6-HSL. A similar approach has been used to demonstrate direct gene activation by the *P. aeruginosa* Las and Rhl QS systems (de Kievit *et al.*, 1999; Pesci *et al.*, 1997). In the *E. coli* background, *prnA–lacZ* activity increased twofold in the presence of pME6032-*phzR* and exogenous C_6-HSL (Fig. 1e). These finding suggest that QS activates the *prn* operon directly; however, DNA binding assays are required to confirm a direct interaction between PhzR–C_6-HSL and the *prn* promoter region. Although this is believed to be the first report of PRN being under QS control in a pseudomonad, it has been observed in *Serratia plymuthica* and certain *Burkholderia* spp. (Liu *et al.*, 2007; Schmidt *et al.*, 2009). QS-deficient strains of *S. plymuthica* and *B. lata* fail to produce PRN and no longer exhibit antagonistic activity against the fungal pathogen in question (Liu *et al.*, 2007; Schmidt *et al.*, 2009). While Liu and coworkers did not search for lux box sequences in *S. plymuthica*, a survey of the *prnABCD* promoter regions in members of the *Burkholderia cepacia* complex (Bcc) revealed the presence of a 'cep box' in some but not all cases (Schmidt *et al.*, 2009). These findings led the authors to conclude that QS may not be required for PRN expression in all members of the Bcc (Schmidt *et al.*, 2009).

In many *Pseudomonas* spp., there is evidence that RpoS and QS are subject to cross-regulation (Bertani & Venturi, 2004; Girard *et al.* 2006; Schuster *et al.*, 2004; Whiteley *et al.*, 2001). Therefore, we sought to determine whether a similar link exists between QS and RpoS. In PA23*rpoS*, *phzI* transcription was reduced, while that of *phzR* was elevated (Fig. 3), indicating that RpoS both activates *phzI* and represses *phzR*. Although differential regulation of 'partners' within a QS network seems counterintuitive, similar findings have been observed in *P. aeruginosa* strain PAO1, where *rhlR* is positively regulated by RpoS, while *rhlI* is repressed (Schuster *et al.*, 2004). Moreover, an extensive study looking at *lasR*/*rhlR* and *lasI*/*rhlI* gene transcription revealed little correlation between the expression profiles of the cognate I- and R-genes under most growth conditions (Duan & Surette, 2007). Approaching cross-regulation from the other side, we found RpoS to be positively controlled by QS in PA23. In other pseudomonads, the involvement of QS in RpoS regulation varies. For instance, in *P. chlororaphis* PCL1391, a *phzI* mutant shows no difference in RpoS levels (Girard *et al.*, 2006), whereas in *P. putida* WCS358 and *P. aeruginosa* PAO1, the QS system positively regulates *rpoS* expression (Bertani & Venturi, 2004; Schuster *et al.*, 2004), similar to what was found here.

To better understand the regulatory roles of QS and RpoS in PA23, a *phzRrpoS* double mutant was created. Characterization of this strain revealed that it resembled PA23*phzR* in terms of antifungal activity, production of PHZ and PRN, and expression of *phzA–*, *prnA–*, *phzI–* and *phzR–lacZ* fusions. What is more, constitutive expression of *phzR* but not *rpoS* was able to complement PA23*phzRrpoS* for both antibiotic expression and antifungal activity (Table 2). These results, together with the fact that RpoS positively regulates *phzI* transcription, suggest that RpoS regulation is mediated at least in part through QS. Girard *et al.* (2006) reported similar findings, wherein constitutively expressed *phzR* was found to complement *rpoS psrA* and *gacS* mutants of *P. chlororaphis* strain PCL1391. The authors concluded that PhzR is a master regulator controlling expression of antifungal metabolites in strain PCL1391 (Girard *et al.*, 2006). Herein, the only difference in secondary metabolite production between the *phzR* mutant and the *phzRrpoS* double mutant was protease levels, which were greatly enhanced in the latter (Table 2). In a previous study, we discovered that RpoS represses protease production (Manuel *et al.*, 2012). Thus, it appears that RpoS-mediated repression of this exoproduct does not involve QS. In *P. chlororaphis* 30-84, protease expression has been found to be regulated by both the PhzI/PhzR and CsaI/CsaR QS systems, as only a double *phzR*/*csaR* or *phzI*/*csaI* knockout abolishes protease production (Zhang & Pierson, 2001). Although we have yet to identify a second QS system in PA23, it should be noted that greatly diminished protease activity was only observed in PA23-6863 (Table 2). The *phzR* mutant conversely demonstrated a modest (1.5-fold) decrease in protease levels (Table 2). The possible involvement of a second QS system and repression by RpoS may help to explain why protease levels were not completely restored in PA23*phzR*(pUCP23-*phzR*). Taken together, the results of this and other studies suggest that the regulatory network overseeing protease expression in *P. chlororaphis* is quite complex.

Bacteria that are able to establish themselves as an adherent biofilm community are afforded protection from assaults that threaten their planktonic counterparts, including desiccation, UV radiation and grazing predators. As such,

biofilm formation may facilitate bacterial persistence in the environment. To date, an in-depth analysis of genes and gene products essential for *P. chlororaphis* biofilm development has not been undertaken. In the current study, the QS-deficient strains were found to produce less biofilm than the wild-type (Fig. 2). In *P. chlororaphis* 30-84, QS is involved in biofilm formation; specifically, the production of PHZ has been found to be critical (Maddula *et al.*, 2008). Similarly, we have shown that a *phzA* mutant produces slightly less biofilm than the wild-type (Selin *et al.*, 2010). In the present study, PA23*rpoS* exhibited increased biofilm formation despite the reduction in PHZ levels (Fig. 2), suggesting that PHZs play only a minor role during the initial attachment and colonization process. Consistent with our findings, an *rpoS* mutant of *P. aeruginosa* produced more biofilm than the PAO1 parent, and the biofilms showed elevated resistance to the antibiotic tobramycin (Whiteley *et al.*, 2001). For many bacteria, flagella are involved in the interaction with surfaces during the early stages of biofilm formation (Davey & O'Toole, 2000). The enhanced flagellar motility exhibited by the PA23 *rpoS* mutant may be related to the increased biomass on the plates. With respect to swimming motility, a mutation in *rpoS* is epistatic to one in *phzR*, as the PA23 *rpoSphzR* double mutant was more motile than either the wild-type or the *phzR* mutant (Table 3). In *E. coli*, an *rpoS* mutant exhibited enhanced motility together with increased FliA-controlled flagellar gene expression (Dong & Schellhorn, 2009). The authors suggested that reduced RpoS may allow other sigma factors access to core RNA polymerase, resulting in elevated expression of motility genes under their control (Dong & Schellhorn, 2009). At present, the sigma factors controlling flagellar gene expression in PA23 are not known. Our findings suggest that sigma factors other than RpoS are involved; consequently, depletion of RpoS enables these factors to compete better for core polymerase binding.

In conclusion, our results provide insight into the regulatory mechanisms governing secondary metabolite production in *P. chlororaphis* strain PA23. We demonstrate that the Phz QS system is required for PA23 antifungal activity, with PRN, PHZ and protease expression all under QS control. In addition, RpoS and QS are subject to cross-regulation. QS induces *rpoS* expression, whereas RpoS exerts positive and negative control over *phzI* and *phzR*, respectively. We also discovered that both global regulators affect PA23 biofilm formation. The Phz QS system positively regulates biofilm development; RpoS, on the other hand, reduces the adherent biomass. Studies in other pseudomonads show that such complexity is not exclusive to PA23 (Bertani & Venturi, 2004; Schuster *et al.*, 2004). Integration of the QS network with other global regulators presumably facilitates sensing and processing of multiple signals, allowing tight control of secondary metabolite production under fluctuating environmental conditions. Future studies will focus on the biocontrol capabilities of these strains and their persistence in the environment.

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