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Expression of the *Pseudomonas chlororaphis* strain PA23 Rsm system is under control of GacA, RpoS, PsrA, quorum sensing and the stringent response

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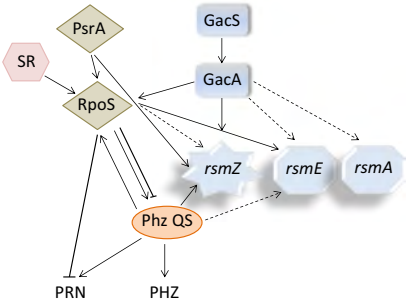
HIGHLIGHTS

- The *Pseudomonas chororaphis* PA23 Rsm system consists of RsmZ, RsmA and RsmE.
- RsmZ is controlled by GacA, RpoS, PsrA, the stringent response and quorum sensing.
- RsmA is positively controlled by GacA and negatively controlled by the stringent response.
- RsmE is activated by GacA, RpoS, PsrA, the stringent response and quorum sensing.

GRAPHICAL ABSTRACT



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ABSTRACT

Pseudomonas chlororaphis strain PA23 is a plant-beneficial bacterium able to suppress disease caused by the pathogenic fungus *Sclerotinia sclerotiorum*. A number of compounds are believed to contribute to fungal antagonism including the antibiotics pyrrolnitrin and phenazine together with degradative enzymes. Overseeing production of these metabolites is a complex regulatory network that includes the GacS–GacA two-component system. In other bacteria, a second network, called Rsm, works in concert with Gac. The aim of the current study was to identify components of the Rsm system in PA23 and elucidate factors controlling their expression. Towards this end, genes encoding repressor proteins (RsmA and RsmE) and a regulatory RNA (RsmZ) were isolated. Through transcriptional fusion analysis, we discovered that *rsmZ* is positively regulated by GacA, PsrA, RpoS, the stringent response (SR) and the PhzI/PhzR quorum-sensing (QS) system. An upstream activating sequence (UAS) corresponding to the GacA recognition sequence was identified in the *rsmZ* promoter. Moreover, a PtrA box and a Phz box were present, suggesting that all three regulators activate *rsmZ* directly. *rsmE* was positively regulated by GacA, PsrA, RpoS, the SR and QS in PA23, but with the exception of RpoS, regulation was indirectly mediated. *rsmA* was negatively regulated by the SR and positively regulated by GacA. A UAS was not found in the promoter region of either *rsmA* or *rsmE,* suggesting indirect control by GacA. Identification of RsmZ, RsmA and RsmE and the discovery that these regulators are tightly controlled add to the increasingly complex network overseeing biocontrol in *P. chlororaphis* PA23.

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1. Introduction

The GacS/GacA two-component regulatory system controls expression of secondary metabolites in a number of different bacterial species (Heeb and Haas, 2001). Entwined within the Gac system is a second regulatory network called Rsm (Regulator of secondary metabolism). The Gac/Rsm circuitry has been well characterized in several bacteria including *Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Erwinia carotovora* (Dubey et al., 2003; Hyytiäinen et al., 2001; Lapouge et al., 2008). In all cases, the Gac/Rsm system serves to modulate the expression of secondary metabolites and extracellular enzymes during the transition from low- to high-population density states (Heeb and Haas, 2001; Bejerano-Sagie and Xavier, 2007). In *P. fluorescens* CHA0, the Rsm system is comprised of two RNA binding proteins, RsmA and E, and three small regulatory RNAs, RsmZ, RsmX, and RsmY (Lapouge et al. 2008). The RsmA and E proteins function as posttranscriptional repressors by binding to the ribosome-binding site (RBS) in the target mRNA. Repression can be alleviated by the action of RsmZ, RsmY and RsmX, which bind multiple copies of RsmA/E, rendering the RBS of target genes accessible to the translational machinery (Lapouge et al., 2008).

Pseudomonas chlororaphis strain PA23 is a biocontrol bacterium capable of protecting canola from Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Fernando et al., 2007; Savchuk and Fernando, 2004). Strain PA23 produces a number of compounds, including phenazines (PHZ), pyrrolnitrin (PRN), hydrogen cyanide (HCN), proteases, lipases and siderophores that likely contribute to its biological control potential (Poritsanos et al., 2006; Zhang et al., 2006). In particular, we have demonstrated that PRN is essential for *S. sclerotiorum* antagonism; whereas, PHZ plays a more minor role (Selin et al., 2010). Multiple regulatory elements oversee production of these metabolites including the GacS/GacA two-component system, the stationary phase sigma factor RpoS, a positive activator of *rpoS* transcription, called PsrA, and the PhzI/PhzR quorum-sensing (QS) system (Poritsanos et al., 2006; Manuel et al., 2012; Selin et al., 2012; Selin, 2012). A global regulatory system that enables bacteria to cope with nutrient deprivation, known as the stringent response (SR), represses production of PRN and therefore PA23-mediated fungal antagonism (Manuel et al., 2012). Adding to this complexity, is the fact that substantial cross-regulation occurs between the regulators themselves. In PA23, RpoS is positively controlled by both QS and the SR; at the same time, RpoS and the SR activate *phzI* and repress *phzR* expression (Selin et al., 2012). Similar interactions between regulatory elements and the Rsm system have been reported in *P. fluorescens* CHA0 and *P. aeruginosa*, where the expression of the Rsm sRNAs is positively regulated by GacA (Kay et al., 2005, 2006). Activation of the regulatory RNAs is believed to occur via GacA binding to an upstream promoter element termed the GacA-box ($TGTAAGN_6CTTACA$) (Valverde et al., 2003; Kay et al., 2005; Lenz et al., 2005; Kulkarni et al., 2006). A recent study in *P. fluorescens* CHA0 revealed that, in addition to GacA, PsrA positively stimulates *rsmZ* transcription (Humair et al., 2010). Conversely in *P. fluorescens* 2P24, PsrA had no effect on *rsmZ* transcription (Wu et al., 2012). As more details regarding secondary metabolite regulation are revealed, one theme continues to emerge. Biocontrol pseudomonads may have regulatory features in common, but differences in the way they govern expression of exoproducts as well as interactions between the regulators themselves are readily apparent.

The aim of the current study was to identify members of the Rsm system in *P. chlororaphis* PA23 and to elucidate factors controlling their expression. Towards this end, genes encoding repressor proteins (RsmA and RsmE) and a regulatory RNA (RsmZ) were isolated. In addition, the impact of GacA, PsrA, RpoS, the SR and the Phz QS system on the expression of *rsmZ*, *rsmA* and *rsmE* was elucidated through reporter fusion analysis.

2. Methods

2.1. Bacterial strains and growth conditions

All bacterial strains, plasmids and primers used in this study are outlined in Table 1. *E. coli* and *P. chlororaphis* PA23 strains were routinely cultured on Lennox Luria Bertani (LB) agar (Difco Laboratories, Detroit, MI) at 37 and 28 °C, respectively. Media were supplemented with antibiotics from Research Products International Corp. (MT. Prospect, IL) as required: piperacillin (30 μg/ml), gentamicin (Gm; 20 μg/ml), tetracycline (Tc; 15 μg/ml), rifampicin (Rif; 100 μg/ml) for PA23 and ampicillin (Amp; 100 μg/ml); Gm (20 μg/ml), Tc (15 μg/ml) and chloramphenicol (Chl; 25 μg/ml) for *E. coli*. For β-galactosidase analysis, cultures were grown in M9 supplemented with 1 mM $MgSO_4$ and 0.2% glucose.

2.2. Nucleic acid manipulation

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

2.3. Generation of pCR-rsmZOE, pCR-rsmA, pCR-rsmEOE

To identify the presence of *rsmZ* and *rsmA* in strain PA23, PCR primers (RsmZ-F/RsmZ-R and RsmA-F/RsmA-R) were designed using the *rsmZ* and *rsmA* sequences from *P. fluorescens* CHA0 (accession Nos. AF245440 and AF136151, respectively) and *P. aeruginosa* PAO1 (accession No. AE004091). The *P. fluorescens* CHA0 *rsmE* sequence (accession No. AY547575) was used to design primers (RsmE-F/RsmE-R) to amplify this same allele from PA23. The resulting PCR products for *rsmZ* (400-bp), *rsmA* (1-kb) and *rsmE* (600-bp) were cloned into pCR2.1, generating pCR-*rsmZ*OE, pCR-*rsmA*, and pCR-*rsmE*OE, respectively.

2.4. Generation of pCR-gacA and pCR-psrA

PCR was used to amplify the 1.2-kb PA23 *gacA* gene (1.2-kb) using primers GacA-F/GacA-R designed from the *P. chlororaphis* 30-84 *gacA* sequence (accession No. AF115381). The amplicon was cloned into pCR2.1 generating pCR-*gacA*. To generate pCR-*psrA*, a 950-bp fragment was amplified using primers PsrA-F and PsrA-R that were designed from the *psrA* gene sequence from *P. chlororaphis* PCL1391 (accession No. AF502251). The PCR product was then cloned into pCR2.1.

2.5. Analysis of gacA, psrA, rsmZ, rsmA, and rsmE sequences

Plasmids pCR-*gacA*, pCR-*psrA*, pCR-*rsmZ*OE, pCR-*rsmA*, and pCR-*rsmE*OE were sequenced using the M13 forward and reverse primers. Sequencing was performed at the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Ontario). The sequences were analyzed with BLASTN and BLASTX databases and submitted to Genbank. The nucleotide accession numbers for *gacA*, *psrA*, *rsmZ*, *rsmA*, and *rsmE* are JF513195, JQ911919, JQ971980, JF705879, and JF705878, respectively.

Table 1
Bacterial Strains and plasmids.

Strain/plasmid/primer	Relevant genotype or phenotype	Source or reference
Strain		
P. chlororaphis		
PA23	PHZ$^+$Rif^R wild type (soybean plant isolate)	Savchuk and Fernando (2004)
PA23*phzR*	Gm^R marker inserted into the *phzR* gene	Selin et al. (2012)
PA23*rpoS*	PA23 with pKNOCK-Tc vector inserted into the *rpoS* gene	Manuel et al. (2012)
PA23-6863	PA23 carrying pME6863 (AI$^-$)	Selin et al. (2012)
PA23*gacA*	Gm^R marker inserted into the *gacA* gene	This study
PA23*psrA*	PA23 with pKNOCK-Tc vector inserted into the *psrA* gene	This study
PA23*relA*	PA23 with the pKNOCK-Gm vector inserted into the *relA* gene	Manuel et al. (2012)
PA23*relAspoT*	PA23*relA* with a Tet^R cassette inserted into the *spoT* gene	Manuel et al. (2012)
P. aeruginosa		
QSC105	Strain carries pEAL01 plasmid (*lasB-lacZ* transcriptional fusion)	Ling et al. (2009)
E. coli		
DH5α	*supE44 ΔU169 (Φ80lacZΔM15)hadR17 recA1 endA1 gyrA96 thi-1relA1*	Gibco
DH5 λpir	DH5 λpir lysogen of DH5α	House et al. (2004)
C. violaceum CV026	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31532, autoinducer biosensor	Latifi et al. (1996)
Plasmids		
pCR2.1	Cloning vector for PCR products	Invitrogen
pCR-*rsmZ*OE	400-bp region containing promoter, and entire *rsmZ* gene cloned into pCR2.1	This study
pCR-*rsmA*	1-kb fragment comprising the entire *rsmA* gene cloned into pCR2.1	This study
pCR-*rsmE*OE	600-bp fragment with promoter and entire *rsmE* gene cloned into pCR2.1	This study
pCR-*gacA*	1.2-kb fragment containing the *gacA* gene cloned into pCR2.1	This study
pCR-*psrA*	950-bp PCR product containing *psrA* cloned into pCR2.1	This study
*gacA*pCR2.1	1-kb *gacA* deletion fragment in pCR2.1	This study
pEX18Ap	Suicide plasmid Gm^R	Hoang et al. (1998)
*gacA*pEX18Ap	1-kb *gacA* fragment in pEX18Ap	This study
gacA-gentpEX18Ap	*gacA*pEX18Ap with Gm^R cassette inserted into *gacA*	This study
pCR-*psrA*-int	450-bp internal portion of *psrA* cloned into pCR2.1	This study
pKNOCK-Tc	Suicide vector for insertional mutagenesis; R6K ori RP4 oriT Tc^R	Alexeyev (1999)
pKNOCK-*psrA*	450-bp internal fragment cloned into pKNOCK-Tc	This study
pBAD24	Expression vector containing pBAD promoter and *araC* regulatory gene	Guzman et al. (1995)
pBAD-ACYC	2.0-kb fragment containing *araC*, pBAD, MCS, and RBS from pBAD24 cloned into pACYC-184	This study
pACYC-184	Multicopy cloning vector compatible with ColE1-based plasmids	Chang and Cohen (1978)
pCR2.1-*rpoS*-1.3	1.3-kb fragment containing *rpoS* cloned into pCR2.1	Poritsanos et al. (2006)
pCR-*rsmZ*-1.5	1.5-kb fragment with *rsmZ* cloned into pCR2.1	This study
pLP170	*lacZ* transcriptional fusion vector	Preston et al. (1997)
pRSMZ-lacZ	1.5-kb fragment with *rsmZ* promoter cloned into pLP170	This study
pCR-rsmE-p	400-bp fragment containing *rsmE* promoter region cloned into pCR2.1	This study
pRSME-lacZ	*rsmE* promoter in pLP170	This study
pCR-*rsmA*-p	600-bp fragment containing the *rsmA* promoter cloned into pCR2.1	This study
pRSMA-lacZ	600-bp fragment comprising the *rsmA* promoter cloned into pLP170	This study
pME3066	1.65-kb *Bam*HI-*Bgl*II fragment containing *gacA* from *P. fluorescens* CHA0 cloned in pLAFR3, Tc^R	Laville et al., 1992
pME6032	Shuttle vector, Tc^R	Heeb et al. (2002)
pME6032-*phzR*	1.68-kb fragment of *phzR* in pME6032	Selin et al. (2012)
Primers		
RsmZ-F	5′-gtgaaaagccccgacatgtt-3′	This study
RsmZ-R	5′-tttatgacccgcccacattt-3′	This study
RsmA-F	5′-ccgacttcaccttcacgggt-3′	This study
RsmA-R	5′-cggttcgtagccgagtactct-3′	This study
RsmE-F	5′-atgctgatactcacccgcaaa-3′	This study
RsmE-R	5′-ttgacttcgtagagcccct-3′	This study
GacA-F	5′-ttaagacaggaaaggggaacgc-3′	This study
GacA-R	5′-aggtggaaaagaaaagcactggg-3′	This study
psrA-F	5′-gcgtggctggctacaggaa-3′	This study
psrA-R	5′-agtccattgccggcggcaaa-3′	This study
gacAPl	5′-gcatgaattcgttaagacaggaaagggaacg-3′	This study
gacAP2	5′-gcatgagctcattcctcccctgactcgg-3′	This study
gacAP3	5′-gcatgagctctgaagaagatccattgggca-3′	This study
gacAP4	5′-gcatggatccggaaagaaaagcactgggat-3′	This study
psrA-lF	5′-ggcggcggtggtgaattatcattt-3′	This study
psrA-RF	5′-gtattgaccccgaagtcggt-3′	This study
psrA-pstI-rev	5′-aactgcagcgaggaatggcaccatca-3′	This study
rpoS-int-forward	5′-aaagaagtgccggaggtttga-3′	This study
rsmZ -BamHI-rev	5′-ctaggatcccgtcgacacagggttgatatt-3′	This study
RsmA-trans-fwd	5′-gaagatcttcggaaaacacatgcccgtgaaat-3′	This study
RsmA-trans-rev	5′-ggggtacccctacaccgatacgcacttgatt-3′	This study
RsmE-trans-fwd	5′-ggggtaccccaagtctgaggtggcgatttt-3′	This study
RsmE-trans-rev	5′-tcccccgggggattaggtgttcgcgtttgtcc-3′	This study

2.6. Construction of PA23gacA

To create a *gacA* mutant of PA23, a copy of *gacA* missing an internal 150-bp fragment was first generated through PCR. Four sets of primers were designed from the PA23 *gacA* sequence. The 5′ end of *gacA* was amplified using primers gacAP1 and gacAP2 and the 3′ end was amplified using gacAP3 and gacAP4. The two PCR products were digested with *Sac*I and cloned into pCR2.1 to

yield *gacA*pCR2.1. The 1-kb insert was then excised with *Eco*RI and *Bam*HI and subcloned into the same sites of pEX18Ap (*gacA*pEX18-Ap). An 850-bp Gm^R cassette from pUCGm was inserted into the *Sac*I site yielding *gacA*-gentpEX18Ap. Triparental mating between *E. coli* DH5α (*gacA*-gentpEX18Ap), *E. coli* DH5α (pRK600) and PA23 was performed and Pseudomonas Isolation Agar (PIA; Difco) supplemented with Gm (20 μg/ml) was used to screen for transconjugants. Streaking onto LA supplemented with sucrose (10%) and Gm enabled identification of bacteria that had undergone a double-cross over event. PCR analysis was used to verify that the *gacA* gene had been replaced with a mutated copy of the allele.

2.7. Construction of PA23psrA

A 450-bp internal portion of the PA23 *psrA* gene was PCR amplified using primers PsrA-IF and PsrA-IR. The amplicon was cloned into pCR2.1, generating pCR*psrA*-int. The 450-bp fragment was then excised using *Eco*RI and subcloned into the same site of the pKNOCK-Tc vector, creating pKNOCK-*psrA*. The resulting construct was mobilized into PA23 and transconjugants were selected on PIA supplemented with Tc (50 μg/ml). Insertion was verified by sequence analysis of a *Bam*HI rescue clone isolated following previously described methods (Lewenza et al., 1999).

2.8. Generation of pBAD-psrA and pBAD-rpoS

For co-expression of *rpoS* and *psrA* with the transcriptional fusions, pBAD-*rpoS* and pBAD-*psrA* were generated. To construct the pBAD-ACYC backbone vector, pBAD24 was digested with *Pvu*I, the ends were made flush with Klenow followed by digestion with *Cla*I. The resulting 2.3-kb fragment containing the *araC* gene and pBAD promoter was subsequently cloned into the *Xba*I (blunt ended) and *Cla*I sites of pACYC184 generating pBAD-ACYC. To generate pBAD-*rpoS*, a 1.3-kb fragment containing *rpoS* was excised as a *Pst*I/*Kpn*I fragment from pCR2.1-*rpoS*-1.3. The insert was subcloned into the *Pst*I and *Kpn*I sites of pBAD-ACYC to generate pBAD-*rpoS*. To create pBAD-*psrA*, a 950-bp fragment was PCR amplified with psrA-F and psrA-pstI-rev. The PCR product was digested with *Pst*I and cloned directly into the *Sma*I-*Pst*I sites of pBAD-ACYC.

2.9. Generation of rsmZ-, rsmA-, and rsmE-lacZ transcriptional fusions

To construct the *rsmZ-lacZ* fusion, primers rpoS-int-forward and rsmZ-BamHI-rev were used to PCR amplify a 1.5-kb fragment that was cloned into pCR2.1 (pCR-*rsmZ*-1.5). The fragment containing the *rsmZ* promoter was excised with *Eco*RI/*Bam*HI and subcloned into the same sites of pLP170, generating pRSMZ-*lacZ*. The *rsmE-lacZ* fusion was created by amplifying a 400-bp fragment containing the *rsmE* promoter using primers RsmE-trans-fwd and RsmE-trans-rev. The PCR product was cloned into pCR2.1 (pCR-*rsmE*-p) and subcloned as a *Hin*dIII/*Eco*RI fragment into the same sites of pLP170, creating pRSME-*lacZ*. To generate the *rsmA-lacZ* transcriptional fusion, a 600-bp fragment was amplified using primers RsmA-trans-fwd and RsmA-trans-rev and cloned into pCR2.1 (pCR-*rsmA*-p). The 600-bp fragment was excised with *Eco*RI and *Hin*dIII and subcloned into the same sites of pLP170, resulting in pRSMA-*lacZ*.

2.10. Analysis of transcriptional fusions

The activities of *rsmZ*-, *rsmA*- and *rsmE-lacZ* transcriptional fusions were determined in PA23 and derivative strains. Bacteria carrying the *lacZ*-fusion plasmids were grown for 4, 8, 16, 24, and 36 h in M9 (0.2% glucose, 1 mM $MgSO_4$) prior to analysis of β-galactosidase activity (Miller, 1972). The activity of *rsmZ-lacZ* was also measured in *E. coli*, PA23, PA23*gacA* and PA23*phzR* harboring pME6032-*phzR*, supplemented with 1 μM purified C6-AHL (Sigma). To monitor the direct effects of PsrA (pBAD-*psrA*) and RpoS (pBAD-*rpoS*) on gene expression, the activity of *rsmZ*-, *rsmA*-, and *rsmE-lacZ* was measured in an *E. coli* background. Samples were analyzed in triplicate and the experiment was repeated three times.

2.11. Antifungal assays

To assess the ability of PA23 and its derivatives to inhibit the growth of *S. sclerotiorum* in vitro, radial diffusion assays were performed following the method of Poritsanos et al. (2006). Six replicates were analyzed for each strain and experiments were repeated three times.

2.12. Exoproduct analysis

Quantification of PRN and PHZ was performed according to Selin et al. (2012). Quantitative protease assays were carried out on PA23 and its derivatives as described by Manuel et al. (2011). All experiments were repeated at least twice.

2.13. Statistical analysis

For the gene expression analysis, the mean of three replicates accompanied by standard errors were plotted to provide additional statistical information, as suggested by Morse and Thompson (1981). To statistically separate the mean expression of each strain at each time point (hours), the data was subjected to ANOVA (Analysis of Variance) and the means were separated using Fisher's protected LSD test at the 0.05 level of significance (Gomez and Gomez, 1984). The statistical computer package, SAS version 9.2, was used to perform the analyses. In order to prevent symbol overload on the graphs the means that were not statistically different were designated using an asterisk. An unpaired Student's *t* test was used for statistical analysis of PHZ, PRN, protease production, antifungal activity and the effects of RpoS and PsrA on *rsmZ*, *rsmA*, and *rsmE* expression in *E. coli*.

3. Results

3.1. Identification of rsmZ, rsmA, and rsmE in PA23

Using primers based on conserved sequences from other *Pseudomonas* strains, the *rsmZ, rsmA* and *rsmE* genes from strain PA23 were isolated. Sequence analysis revealed that the *rsmZ* gene demonstrated the highest identity (98%) with *rsmZ* from *P. chlororaphis* strains O6 (accession No. NZ_AH0T01000019) and 30-84 (accession No. AHJ01000004). In non-*chlororaphis* strains, the PA23 *rsmZ* gene shared the greatest identity with *P. fluorescens* strains CHA0 (96%; accession No. AF245440) and Pf0-1 (91%; accession No. CP000094). The PA23 *rsmA* gene is 99% and 100% identical to that of *P. chlororaphis* strains 30-84 (accession No. AHHJ01000011) and O6 (NZ_AH0101000008), respectively. A high degree of identity was also observed with the *rsmA* genes of *P. fluorescens* strains Pf-5 (96%; accession No. NC004129), Pf0-1 (93%; accession No. CP000094), and CHA0 (92%; accession No. AF136151). Finally, the PA23 *rsmE* gene was found to exhibit 98% and 99% identity with the *rsmE* genes of *P. chlororaphis* strains 30-84 (accession No. AHHJ0100004) and 06 (accession No. NZ_AH0T0100027), respectively. A high degree of identity (88%) with the *rsmE* alleles of *P. fluorescens* strains CHA0 (accession No AY547575) and Pf-5 (accession No. CP000076) was also observed.

3.2. Phenotypic characterization of gacA and psrA mutants of strain PA23

Studies have shown that in *P. fluorescens* CHA0, GacA is required for transcription of *rsmX, Y* and *Z* (Heeb et al., 2002; Kay et al., 2005; Valverde et al., 2003). Moreover PsrA activates *rsmZ* expression (Humair et al., 2010). To determine how these regulators affect the Rsm circuitry in PA23, it was first necessary to generate *gacA* and *psrA* mutants. Phenotypic characterization revealed that the PA23 *gacA* mutant no longer exhibited fungal antagonism and showed a marked reduction in exoproducts (Table 2), consistent with what has been reported for other biocontrol pseudomonads (Heeb and Haas, 2001). This phenotype is very similar to that of a PA23 *gacS* mutant (Poritsanos et al., 2006). The *psrA* mutant on the other hand exhibited enhanced antifungal activity and increased protease and PRN production, but reduced PHZ (Table 2). Complementation with plasmids pME3066 (*gacA*) and pUCP22-*psrA* (*psrA*) restored the wild-type phenotype to the *gacA* and *psrA* mutants, respectively (Table 2).

3.3. Transcription of rsmZ, rsmA, and rsmE depends upon GacA

To investigate the regulatory effects of the Gac system on *rsmZ*, *rsmA* and *rsmE* expression, transcriptional fusions were analyzed for β-galactosidase activity in both PA23*gacA* and PA23. As shown in Fig. 1A, *rsmZ* expression was significantly reduced in the *gacA* mutant compared to the wild type, indicating that GacA is required for optimal expression of *rsmZ*. In *P. fluorescens* CHA0, GacA-mediated control of *rsmZ* expression is reportedly through a palindromic upstream activating sequence (UAS) within the promoter region (Heeb et al., 2002). This region is predicted to be bound by GacA in its phosphorylated state (Heeb et al., 2002). Analysis of the PA23 *rsmZ* promoter region revealed a conserved UAS with the sequence TGTAAGCAAAGGCTTACT (Fig. 2A and B). This sequence matched 15 of the 18 nucleotides found within the UAS of *P. fluorescens* CHA0 (Humair et al., 2010) and 16 of 18 nucleotides in the *P. aeruginosa* UAS (Brencic et al., 2009) (Fig. 2B). Taken together, these results suggest that GacA positively influences *rsmZ* expression and it may do so through binding to this UAS element.

As illustrated in Fig. 1B and C, the expression of both *rsmA-lacZ* and *rsmE-lacZ* were reduced in the *gacA* mutant compared the wild type; therefore, a functional Gac system is required to obtain maximum expression of both genes. A UAS consensus was not located upstream in the *rsmA* and *rsmE* promoter regions (data not shown), indicating that GacA may control expressions of these genes in an indirect manner.

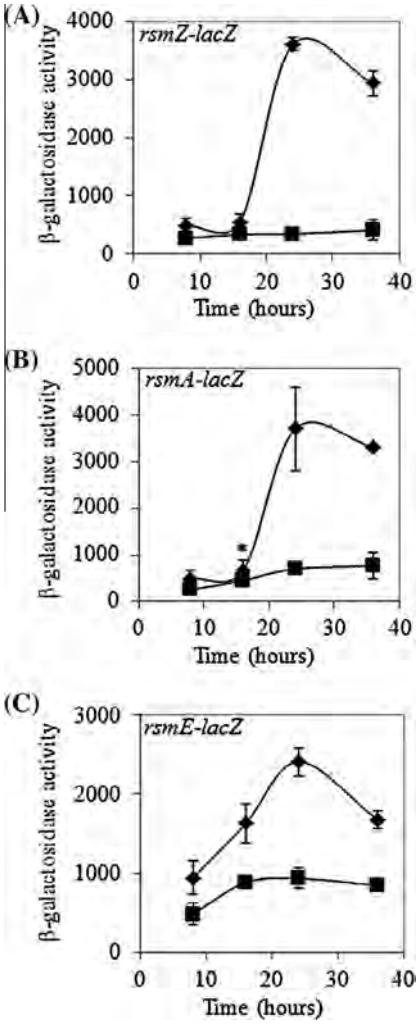

Fig. 1. Expression analysis of (A) *rsmZ-lacZ* (B) *rsmA-lacZ* and (C) *rsmE-lacZ* in PA23 (closed diamonds) and PA23*gacA* (closed squares). Bacteria were grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose. Each value is the mean from three different cultures ± standard error. Experiments were performed three times; one representative data set is shown. Values are statistically different from wild type ($p < 0.05$) unless indicated with an asterisk.

Table 2
Phenotypic characteristics of PA23 and its derivatives.

Strain	Extracellular Metabolite Activity			
	Antifungal[a] (mm)	Protease[b] (Units of enzyme ml^{-1})	Total PHZ[b] (ng ml^{-1})	PRN[c] (μg)
PA23 (pUCP22)	7.9 (0.8)	0.6 (0.06)	82.4 (0.6)	15.6 (3.5)
PA23*gacA* (pME6010)	0[d]	0.07 (0.01) [d]	4.7 (0.5)[d]	n.d.
PA23*gacA* (pME3066)	7.6 (1.0)[e]	0.5 (0.3)[e]	90.5 (0.8)[e]	10.3 (3.7)[e]
PA23*psrA* (pUCP22)	10.5 (1.5)[f]	0.9 (0.1)[f]	23.0 (1.6)[f]	23.0 (1.2)[f]
PA23*psrA* (pUCP22-*psrA*)	7.3 (0.7)[e]	0.6 (0.1)[e]	79.0 (2.0)[e]	17.4 (1.0)[e]

n.d.: not detected.
[a] Mean (standard deviation) obtained from six replicates.
[b] Mean (standard deviation) obtained from a triplicate set.
[c] Mean (standard deviation) obtained from a duplicate set.
[d] Significantly different from wild type ($p < 0.001$).
[e] Not significantly different from wild type.
[f] Significantly different from wild type ($p < 0.05$).

3.4. Predicted secondary structure of rsmZ

Regulatory RNAs, such as *rsmZ,* form unique stem-loop secondary structures which are important for their function (Haas and Défago, 2005). Analysis of the PA23 *rsmZ* sequence using the program Mfold (Zuker, 2003) predicted that this RNA adopts a flower-like secondary conformation with five stem-loop structures, all of which contain AGG(g)A-motifs (Fig. 2C).

3.5. PsrA and RpoS positively regulate rsmZ and rsmE transcription, but are not required for expression of rsmA

To determine if PsrA and/or RpoS regulate the expression of *rsmZ*, *rsmE* and *rsmA*, the transcriptional activity of the aforementioned genes was measured in PA23*psrA* and PA23*rpoS*. As shown in Fig. 3A, the expression levels of *rsmZ* at 24 h in PA23*psrA* and PA23*rpoS* were reduced approximately 3.6- and 2.1-fold, respectively, compared to the wild type. Similarly, *rsmE* expression was reduced 1.5-fold in the *psrA* mutant and 2.3-fold in the *rpoS* mutant

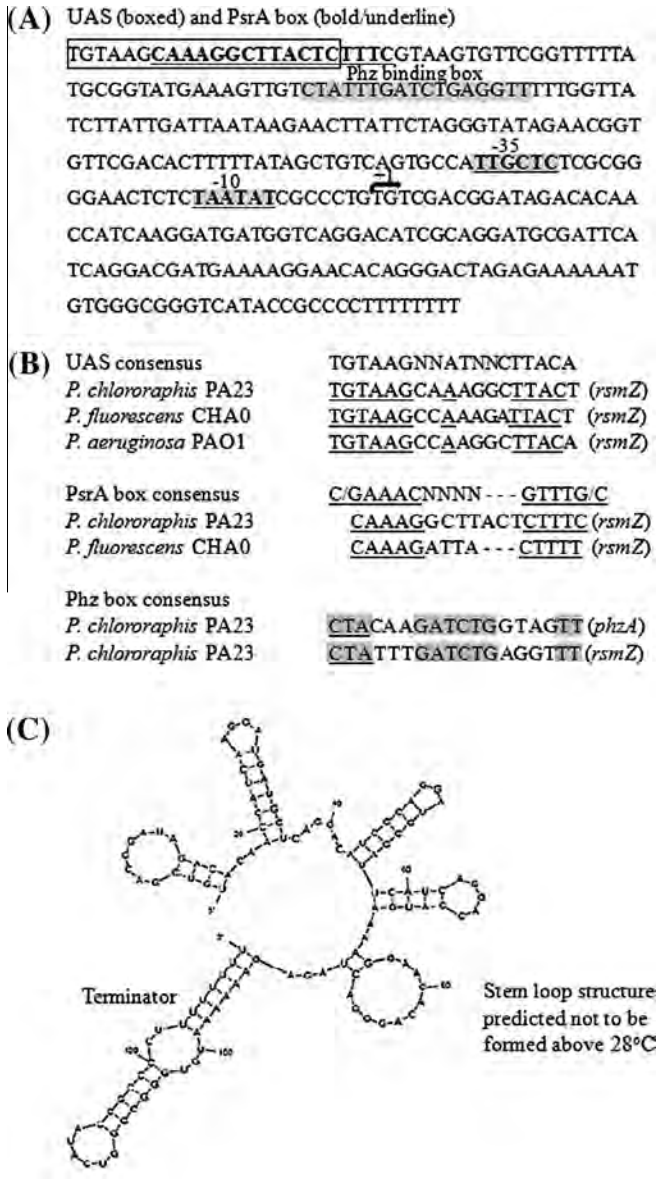

Fig. 2. Analysis of the PA23 *rsmZ* gene. (A) The PA23 *rsmZ* sequence determined through sequence analysis; the upstream activating sequence (UAS), the PsrA binding box and the Phz binding box are all clearly defined. For orientation, the transcriptional + 1 site as well as the putative −10 and −35 sites are highlighted as reported for *P. fluorescens* CHA0 (Heeb et al., 2002); (B) alignment of the UAS, PsrA and Phz box consensus sequences of PA23 and other pseudomonads; (C) the predicted secondary structure of RsmZ from *P. chlororaphis* PA23 determined using the Mfold program (Zuker, 2003).

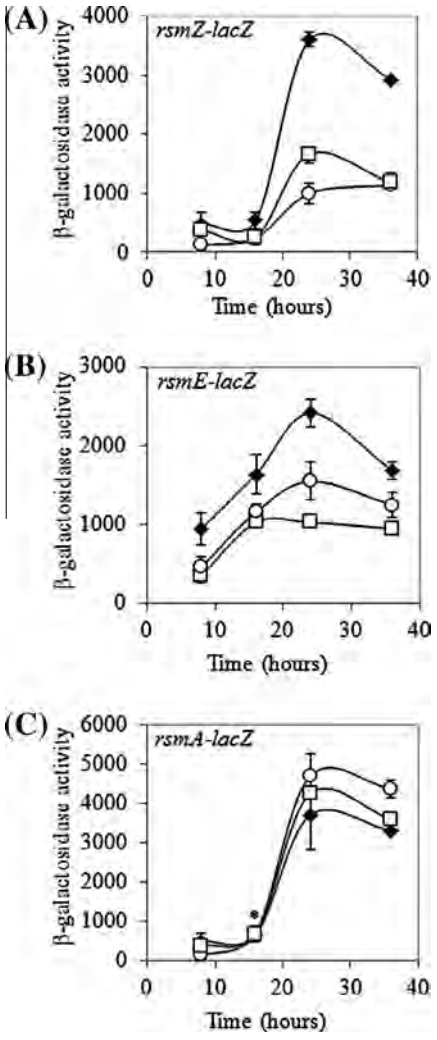

Fig. 3. Expression analysis of (A) *rsmZ-lacZ* (B) *rsmA-lacZ* and (C) *rsmE-lacZ* in PA23 (closed diamonds), PA23*psrA* (open circles), and PA23*rpoS* (open squares). Bacteria were grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose. Each value is the mean from three different cultures ± standard error. Experiments were performed three times; one representative data set is shown. Values are statistically different from wild type ($p < 0.05$) unless indicated with an asterisk.

(Fig. 3B). Transcription of *rsmA* was slightly increased in the *rpoS* and the *psrA* mutant at 24 and 36 h (Fig. 3C). These results suggest that *rsmZ* and *rsmE* transcription is positively influenced by PsrA and RpoS; whereas these regulators have a modest repressive effect on *rsmA* in PA23.

Next, we sought to determine if the positive effects of PsrA and RpoS on *rsmZ* and *rsmE* transcription were direct. Towards this end, *rsmZ* and *rsmE-lacZ* transcriptional fusions were co-expressed with either pBAD-*psrA* or pBAD-*rpoS* in an *E. coli* background. The presence of pBAD-*psrA* led to a 2.8-fold increase in *rsmZ-lacZ* expression compared to that observed for *E. coli* carrying the empty vector (pBAD24; Table 3). Conversely, no direct effect on *rsmZ* transcription was observed in the presence of RpoS (pBAD-*rpoS*) (Table 3). The opposite was observed when *rpoS* (pBAD-*rpoS*) and *psrA* (pBAD-*psrA*) were co-expressed with the *rsmE-lacZ* fusion. In this case, a 2-fold increase in the transcription of *rsmE* was observed in the presence of pBAD-*rpoS*, while pBAD-*psrA* had no effect (Table 3). Collectively, these findings indicate that PsrA has a direct effect on *rsmZ* expression, while *rsmE* transcription is directly controlled by RpoS. No change in *rsmA* expression was observed upon the addition of pBAD-*rpoS* or pBAD-*psrA* (Table 3).

For *P. putida* and *P. aeruginosa*, PsrA has been shown to bind to a palindromic sequence, termed the PsrA box located within the pro-

Table 3
The effect of RpoS and PsrA on *rsmZ, rsmA* and *rsmE* expression in an *E. coli* background.

Construct	pBAD-ACYC[a]	pBAD-*psrA*[a]	pBAD-*rpoS*[a]
	β-galactosidase activity (Miller units)[b]		
pRSMZ-*lacZ*	1526 (52)	4352 (503)[c]	1635 (352)[c]
pRSMA-*lacZ*	3369 (695)	3068 (40)[d]	3132 (177)[d]
pRSME-*lacZ*	1675 (76)	1542 (104)[d]	3354 (289)[e]

[a] Expression from the pBAD promoter was induced using 0.2% arabinose.
[b] Mean (standard deviation) obtained from a triplicate set.
[c] Significantly different from strain carrying empty vector ($p < 0.0001$).
[d] Not significantly different from strain carrying empty vector.
[e] Significantly different from strain carrying empty vector ($p < 0.001$).

moter regions of the *rpoS* and *psrA* genes (Kojic and Venturi, 2001; Kojic et al., 2002). A conserved PsrA-binding sequence was identified within the *rsmZ* promoter region (Fig. 2A and B); however, no such sequence was found upstream of *rsmE* (data not shown). These findings are consistent with the idea that PsrA directly activates *rsmZ*; whereas its effects on *rsmE* are indirect.

3.6. The stringent response controls expression of rsmZ, rsmA and rsmE

Expression of *rsmZ*, *rsmE* and *rsmA* was analyzed in PA23*relA* and PA23*relAspoT*, two mutants that lack (p)ppGpp production and as a result are unable to undergo the SR (Manuel et al., 2012). We discovered that *rsmZ* and *rsmE* were positively regulated by the SR, since transcriptional fusions for both genes showed markedly decreased activity in the SR mutants (Fig. 4A and B). Conversely, the SR exerts a negative impact on *rsmA* expression (Fig. 4C).

3.7. The Phz QS system regulates rsmZ transcription

To determine if QS had an impact on the transcription of *rsmZ* in PA23, the *rsmZ-lacZ* transcriptional fusion was monitored in PA23,

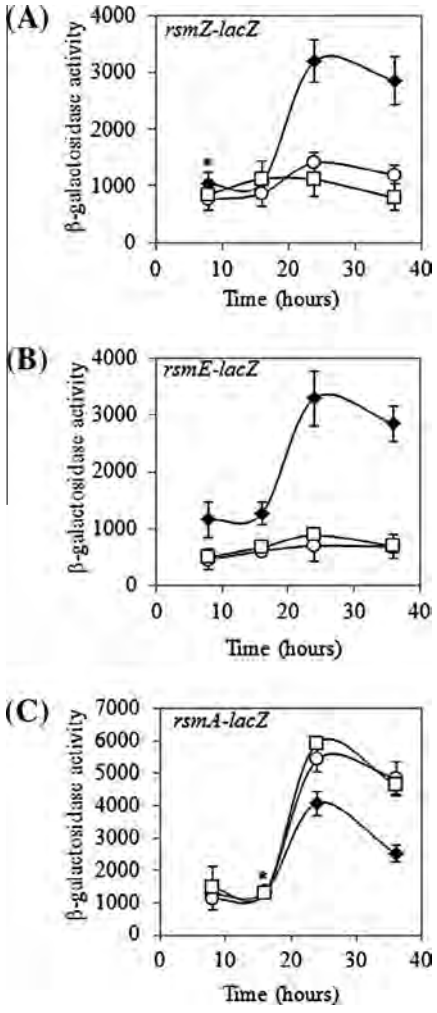

Fig. 4. Expression analysis of (A) *rsmZ-lacZ* (B) *rsmA-lacZ* and (C) *rsmE-lacZ* in PA23 (closed diamonds), PA23*relA* (open circles), and PA23*relAspoT* (open squares). Bacteria were grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose. Each value is the mean from three different cultures ± standard error. Experiments were performed three times; one representative data set is shown. Values are statistically different from wild type ($p < 0.05$) unless indicated with an asterisk.

PA23*phzR*, and PA23-6863. At 16, 24 and 36 h, the expression of *rsmZ* was dramatically reduced in the QS-deficient strains compared to the wild type (Fig. 5A), suggesting that QS positively regulates this gene. Analysis of the *rsmZ-lacZ* transcriptional fusion in *E. coli* revealed that in the presence of PhzR (pME6032-*phzR*) and autoinducer, *rsmZ* expression increased 3-fold (Fig. 5B). No induction of *rsmZ* transcription was observed for *E. coli* carrying either the empty vector (± C6-HSL) or pME6032-*phzR* in the absence of autoinducer (Fig 5B). Thus it appears that *rsmZ* transcription is directly activated by the Phz QS system.

The transcriptional activator, PhzR, when bound to C6-HSL, is believed to activate the transcription of the *phz* operon by binding to the Phz box located within the *phzA* promoter (Chin-A-Woeng et al., 2001). A Phz box sequence has been identified in the *phzA* and *prnA* promoter regions of PA23, which are believed to be directly regulated by PhzR (Selin et al., 2012). Examination of the *rsmZ* promoter revealed a potential Phz box 131-bp upstream of the putative + 1 transcriptional start site (Fig. 2A). This sequence matches 11 of the 18 nucleotides found within the Phz box of the PA23 *phzA* promoter (Fig. 2B) further supporting the notion that PhzR-C6-HSL exerts direct control over *rsmZ* transcription.

3.8. PhzR-C6-HSL is able to partially restore rsmZ expression in a gacA mutant

In *P. chlororaphis* PCL1391, constitutive expression of *phzR* in the presence of autoinducer is able to complement a *gacS* mutant, indicating that a functional QS system alone is sufficient for PHZ expression. Thus, we were interested to determine whether PhzR together with C6-HSL would restore *rsmZ* expression in the *gacA* mutant. As shown in Supplemental Figure 1, in the presence of PhzR (pME6032-*phzR*) and autoinducer, *rsmZ* transcription in PA23*gacA* was enhanced 2.5-fold but does not reach wild-type levels. As expected, there was no induction of *rsmZ* transcription in PA23*gacA* carrying the empty vector (± C6-HSL) or in PA23*gacA* in the absence of autoinducer.

3.9. The Phz QS system indirectly activates rsmE transcription, but is not required for rsmA expression

To determine if QS regulates transcription of *rsmA* and/or *rsmE*, the expression of the *rsmA*- and *rsmE-lacZ* transcriptional fusions were monitored in PA23, PA23*phzR* and PA23-6863. The results indicate that the expression of *rsmA* is not activated by QS, as *rsmA* transcription remained at wild-type levels or slightly higher in the QS-deficient strains (Fig. 5C). Expression of *rsmE* on the other hand is modestly upregulated by QS (Fig. 5E). To discern whether the Phz QS system directly activates *rsmE*, transcription of the *rsmE-lacZ* fusion was analyzed in *E. coli* expressing pME6032-*phzR* in the presence and absence of autoinducer. As shown in Fig. 5F, the expression of *rsmE* was not affected by the addition of PhzR and C6-HSL, suggesting an indirect relationship between QS and *rsmE* transcription. A Phz box consensus sequence was not detected in the *rsmE* promoter region (data not shown), supporting the idea that the positive effect of the Phz QS on *rsmE* expression is likely mediated through other regulatory element(s). There was no change in *rsmA* expression in the presence and absence of PhzR-C6-HSL (Fig. 5D).

4. Discussion

P. chlororaphis strain PA23 is able to inhibit fungal pathogens through the production of an arsenal of secreted compounds (Poritsanos et al., 2006). As with other biocontrol *pseudomonads*, expression of these products is governed by a complex regulatory

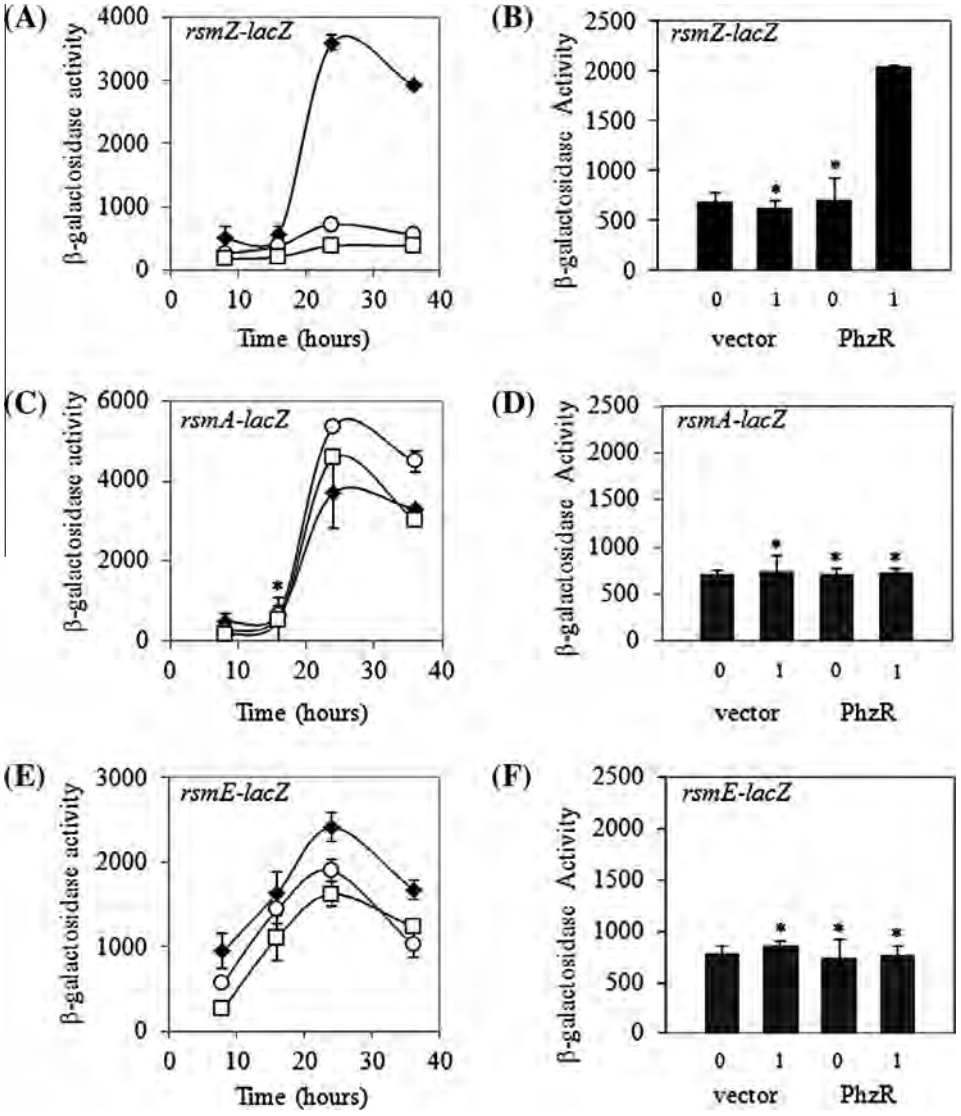

Fig. 5. Quorum sensing control over the Rsm system. The transcriptional activity of (A) *rsmZ-lacZ* (C) *rsmA-lacZ* and (E) *rsmE-lacZ* was measured in PA23 (closed diamonds), PA23*phzR* (open circles), and PA23-6863 (open squares). Panels B, D and F depict the *rsmZ-*, *rsmA-* and *rsmE-lacZ* fusions, respectively, co-expressed in *E. coli* with *phzR*-pME6032 and pME6032 (empty vector) in the presence (1) or absence (0) of C6-HSL. Where indicated, autoinducer was added to the cultures at a concentration of 1 μM. Bacteria were grown for 24 h prior to measuring β-galactosidase activity (Miller units). Each value is the mean from three different cultures ± standard error. Experiments were performed three times; one representative data set is shown. Values are statistically different from wild type ($p < 0.05$) unless indicated with an asterisk.

network (Poritsanos et al., 2006; Manuel et al., 2012; Selin et al., 2012). Many of these regulators are conserved, for example the Gac-Rsm system, PtrA, RpoS and in some cases, homoserine lactone-based QS. However distinctions exist with respect to the impact these regulators have on the expression of a particular product. Adding to this variability is the fact that interactions between the controlling elements within the regulatory network tend to vary between strains. The aim of the current study was to identify members of the PA23 Rsm system and determine how key regulators of fungal antagonism, including GacA, RpoS, PsrA, the SR and QS control their expression.

We discovered that strain PA23 produces the sRNA, RsmZ, together with two translational repressors, RsmA and RsmE. Secondary structure analysis predicts that RsmZ adopts a flower-like conformation with a conserved AGG(g)A motif in the hairpin loops. Similar structures have been predicted for RsmX, Y and Z of *P. fluorescens* strain CHA0 (Haas and Défago, 2005; Heeb et al., 2002). This configuration is hypothesized to facilitate titration of the RsmA and RsmE repressor proteins, thereby alleviating translational repression (Haas and Défago, 2005). Several regulators of *rsmZ* expression were identified indicating that control over this sRNA is complex. The fact that GacA activates RsmZ was not surprising as this has been reported for numerous other bacterial strains (Valverde and Haas, 2008). In *P. aeruginosa,* only two genes were found to be directly controlled by the Gac pathway, namely *rsmY* and *rsmZ* (Brencic et al., 2009). It was concluded that, at least for this bacterium, the multitude of Gac-controlled genes are channelled through RsmY- and RsmZ-dependent modulation of RsmA activity (Brencic et al., 2009). In this same study, MvaT, a member of the H-NS family of DNA binding proteins was found to repress *rsmZ* transcription (Brencic et al., 2009). MvaT preferentially binds to sequences with a high A + T content (Castang et al., 2008), and the 174-bp region between the *P. aeruginosa rsmZ* UAS and transcriptional start site contains an exceptionally high percentage of these nucleotides (55%) (Brencic et al., 2009). Inspection of the region upstream of the PA23 *rsmZ* gene revealed it to be noticeably enriched for A and T residues (61%; Fig. 2A) compared with an overall A + T composition of 34% for other *P. chlororaphis* genomes (Loper et al., 2012). Whether transcriptional silencing of *rsmZ* by H-NS-like proteins occurs in PA23 is currently unknown. Brencic et al.

(2009) speculated that MvaT-mediated repression is likely antagonized by GacA or other yet-to-be identified transcriptional activators. In the current study, we have identified a number of transcriptional activators that might function in this manner including PsrA, which had a positive effect on *rsmZ* expression. Interestingly, in *P. fluorescens* 2P24, PsrA showed no effect on *rsmZ* expression (Wu et al., 2012). A *P. fluorescens* CHA0 *psrA* mutant, on the other hand, exhibited decreased *rsmZ* transcription, although the effect was less pronounced than in PA23 (Heeb et al., 2002). A possible explanation for this latter discrepancy is the involvement of RpoS. In *P. fluorescens* CHA0, RpoS had no effect on *rsmZ* expression (Humair et al., 2010); whereas RpoS is a positive activator of the PA23 *rsmZ* gene (Fig. 3). Therefore, PsrA likely activates *rsmZ* transcription directly as well as indirectly through RpoS. In addition to GacA, PsrA and RpoS, we discovered that the Phz QS system positively controls *rsmZ*. To the best of our knowledge, this is the first report of RsmZ being positively regulated by an AHL-based QS system.

We also discovered that *rsmZ* expression is positively regulated by the SR, a global regulatory mechanism that enables bacteria to alter their metabolism in response to nutrient deprivation. During the SR, bacteria shift activities from ones that favor reproduction to those that promote survival through accumulation of the alarmone (p)ppGpp (Potrykus and Cashel, 2008). (p)ppGpp binds to RNAP near the catalytic site, leading to increased transcription of certain genes and decreased transcription of others (Potrykus and Cashel, 2008). We have previously demonstrated that PA23 biocontrol is repressed by the SR. PA23 *relA* and *relA/spoT* mutants that cannot undergo the SR due to a lack of (p)ppGpp production exhibited elevated fungal antagonism mediated by increased *prn* transcription and PRN production (Manuel et al., 2012). Because the SR impacts production of PA23 AF compounds through *rpoS* (Manuel et al., 2012), it was not surprising that the pattern of SR control over *rsmZ* (and *rsmA* and *rsmE*) resembled that of RpoS.

Interestingly, Humair et al. (2010) have reported an inability to demonstrate direct binding of GacA to the promoter regions of the RsmX, Y, and Z sRNAs in *P. fluorescens* CHA0. These researchers speculated that auxiliary protein interaction with the "linker region" (between the −180 UAS and the −10) is required for GacA binding. In PA23, GacA, PhzR-C6-HSL, and PsrA positively regulate expression of *rsmZ* and putative binding sites for all three have been identified in the *rsmZ* promoter region. These findings are consistent with the notion that GacA and other regulatory proteins may act in concert at the *rsmZ* promoter to stimulate transcription. At the moment, this is merely speculative; DNA binding assays are required to prove that GacA, PhzR, and PsrA physically interact with the *rsmZ* promoter region. Moreover it remains to be established whether one or more of these proteins in particular facilitate GacA binding.

For the translational repressors RsmA and RsmE markedly different patterns of regulation were observed. GacA activated both *rsmA* and *rsmE*; however, an upstream UAS was not identified in the promoter regions of either gene suggesting the effect of GacA is indirect. Our findings are contrary to an earlier report in which RNAseq analysis revealed no change in *rsmE* and a 2.2-fold increase in *rsmA* expression in a *gacA* mutant of *P. chlororaphis* 30-84 (Wang et al., 2013). One possible explanation for this discrepancy is that the earlier study employed AB minimal media supplemented with a high concentration of Cas-amino acids (CAA; 2%). For PA23-mediated control of sclerotinia stem rot, the bacteria are applied as a foliar spray to the canola plants (Savchuk and Fernando, 2004). Accordingly, we routinely use M9 minimal media lacking CAA to mimic the nutrient-poor environment of the phyllosphere. Kreb's cycle intermediates have been shown to impact *rsmZ* expression in *P. fluorescens* CHA0 (Takeuchi et al., 2009); thus, media composition may affect expression and functioning of the Gac-Rsm circuitry.

Several additional regulators were found to positively control RsmE including QS, PsrA, RpoS and the SR. Two pieces of evidence suggest that with the exception of RpoS, control was indirectly mediated. First, PsrA and PhzR binding sequences were not identified in the *rsmE* promoter region. Second, overexpression of PsrA and PhzR-C6-HSL in an *E. coli* background caused no change in *rsmE* transcription. The only regulator that increased expression in the heterologous background was RpoS. We have previously reported that the Phz QS system positively regulates *rpoS* in PA23 (Selin et al., 2012), and PsrA is an activator of *rpoS* (data not shown). In a similar manner, the SR exerts positive control over *rpoS* in PA23 (Manuel et al., 2012). Thus, we believe that the positive effects of QS, PsrA, and the SR on *rsmE* expression are likely indirectly mediated through RpoS. A similar finding was reported for *P. fluorescens* 2P24, where PsrA indirectly affects *rsmA* expression via activation of RpoS (Wu et al., 2012). Although *rsmA* expression was elevated in both the SR and the *rpoS* mutants, a more pronounced effect was seen in the former, which may reflect the physiological changes ongoing in the SR mutants beyond altered *rpoS* expression (Potrykus and Cashel, 2008). Collectively our findings indicate that RsmA and RsmE are differentially regulated. Such an arrangement would presumably allow more fine-tuned control of exoproduct expression. Factors such as nutrient availability and population density among others could then form part of the input signals controlling expression.

In Fig. 6, we present a model to reflect our current understanding of the regulatory hierarchy governing expression of antifungal compounds in *P. chlororaphis* strain PA23. It is clear that there is a significant amount of cross-regulation between the regulators themselves. Control over the newly identified Rsm components RsmZ, RsmA and RsmE is quite complex, in particular RsmZ and RsmE. Genome sequences for *P. chlororaphis* strains 30-84 and O6 have recently become available, revealing the presence of *rsmX* and *rsmY*, as well as *rsmZ*. Thus it remains to be established whether these RNAs are present in PA23 and if so, whether they are regulated in a similar manner to *rsmZ*. Future studies will be directed at understanding how the Rsm system governs expression

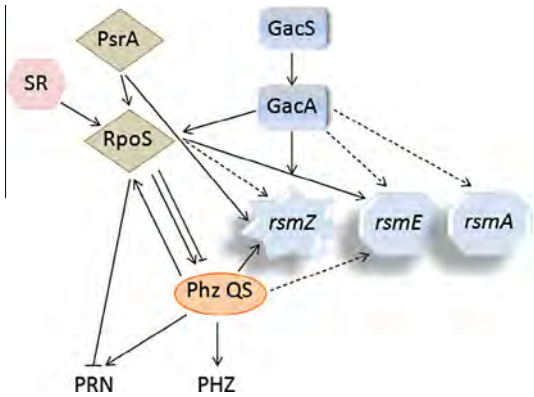

Fig. 6. Model for the regulatory network overseeing production of antifungal factors in *P. chlororaphis* PA23. Evidence for the proposed pathway comes from this work and previous studies (Poritsanos et al., 2006; Manuel et al., 2012; Selin et al., 2012). In response to an unknown signal, the sensor kinase GacS undergoes autophosphorylation and phosphotransfer to the response regulator GacA. Once activated, GacA induces expression of the non-coding RNA RsmZ, the post transcriptional repressors RsmA and RsmE, and the sigma factor RpoS. RpoS is under positive control of PsrA, which is seen in other pseudomonads, and the SR. RpoS exerts a repressive effect on the pyrrolnitrin biosynthetic genes and *phzR*; whereas *phzI* is activated by this sigma factor. Genes that are positively regulated by the Phz QS system include *rpoS* and the phenazine and pyrrolnitrin biosynthetic loci (Selin et al., 2012). Substantial cross talk clearly occurs between the regulatory elements within the cascade. Symbols: ↓, positive effect; ⊥, negative effect; solid lines, direct effect; broken lines, indirect effect.

of secondary metabolites in PA23 and its overall contribution to biocontrol.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2013.10.015.

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