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# Stringent response mutants of *Pseudomonas chlororaphis* PA23 exhibit enhanced antifungal activity against *Sclerotinia sclerotiorum in vitro*

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The stringent response (SR) is a regulatory mechanism that enables bacteria to adapt to nutrient stress through the production of the alarmone (p)ppGpp. The aim of the current study was to understand how the SR affects the antifungal (AF) activity of *Pseudomonas chlororaphis* PA23. Two SR mutants were generated, PA23*relA* and PA23*relAspoT*, that no longer produced (p)ppGpp. Both mutants exhibited increased inhibition of *Sclerotinia sclerotiorum in vitro* and elevated pyrrolnitrin (PRN), lipase and protease production. Phenazine (PHZ) levels, on the other hand, remained unchanged. Through transcriptional fusion analysis we discovered that *prnA–lacZ* (PRN) activity was increased in the SR mutants, whereas *phzA–lacZ* (PHZ) activity was equal to that of the wild-type. We also examined how the sigma factor RpoS impacts PA23-mediated antagonism. Similar to the SR mutants, an *rpoS* mutant of PA23, called PA23*rpoS*, exhibited enhanced AF activity *in vitro* and increased expression of PRN, protease and lipase. However, PHZ production and expression of *phzA–lacZ* were dramatically reduced. Consistent with what has been reported for other bacteria, the SR exerted positive control over *rpoS* expression. In addition, providing *rpoS in trans* restored the SR phenotype to that of the wild-type. Collectively, our findings indicate that this global stress response impacts production of PA23 AF compounds via regulation of *rpoS* transcription and has an overall negative influence on *S. sclerotiorum* antagonism.

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## INTRODUCTION

When applied as a foliar spray, *Pseudomonas chlororaphis* PA23 is able to protect canola from sclerotinia stem rot caused by the fungal pathogen *Sclerotinia sclerotiorum* (Lib.) (Savchuk & Fernando, 2004). Because the phyllosphere is a nutrient-poor environment, PA23 must employ strategies to cope with low carbon and energy availability. The stringent response (SR) is a mechanism that enables bacteria to alter their metabolism in response to nutrient deprivation, shifting from a reproductive mode to one that promotes survival. This adaptation is accomplished through intracellular accumulation of the nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively known as (p)ppGpp. In Gram-negative bacteria, (p)ppGpp is synthesized by a RelA synthetase, while hydrolysis of this metabolite is mediated by a second enzyme called SpoT (Potrykus & Cashel, 2008). SpoT is considered bifunctional since it is capable of (p)ppGpp synthesis under specific environmental conditions (Potrykus & Cashel, 2008, and references therein). (p)ppGpp binds to RNA polymerase near its catalytic site, leading to increased transcription of certain genes, for instance those involved in amino acid biosynthesis (Potrykus & Cashel, 2008). At the same time, the conformation change induced by (p)ppGpp binding causes decreased transcription at other loci, as observed with the tRNA and rRNA genes (Potrykus & Cashel, 2008). In this manner, the SR enables bacteria to adapt to nutrient deprivation by mediating global changes in gene expression.

As with many biocontrol pseudomonads, PA23 attributes its antifungal (AF) activity to secondary metabolite production (Poritsanos *et al.*, 2006; Selin *et al.*, 2010). These metabolites are synthesized at the onset of stationary phase and are regulated at both the transcriptional and post-transcriptional level. The Gac two-component system is situated at the top of the regulatory cascade and

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Abbreviations: AF, antifungal; HCN, hydrogen cyanide; (p)ppGpp, guanosine tetraphosphate and guanosine pentaphosphate; PRN, pyrrolnitrin; PHZ, phenazine; SR, stringent response.

The GenBank/EBML/DDBJ accession numbers for the sequences of the PA23 *relA*, *spoT* and *rpoS* genes are JF705880, JF705881 and DQ525801, respectively.

comprises the sensor kinase GacS and its cognate response regulator GacA (Heeb & Haas, 2001). In several pseudomonads, including PA23, a mutation in either *gacA* or *gacS* leads to a lack of fungal inhibition (Heeb & Haas, 2001; Poritsanos *et al.*, 2006). The alternative sigma factor RpoS has also been implicated in secondary metabolite production; however, regulation by RpoS varies depending on the producing organism and the antibiotic in question. For example, an *rpoS* mutation in *Pseudomonas fluorescens* Pf-5 resulted in enhanced production of the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol but abolished pyrrolnitrin (PRN) expression (Sarniguet *et al.*, 1995; Whistler *et al.*, 1998), while the same mutation in *P. chlororaphis* PCL1391 caused a decrease in phenazine-1-carboxamide production compared with the wild type (Girard *et al.*, 2006a). In addition, several global and pathway-specific regulators have been identified that play a role in regulating biocontrol traits of *Pseudomonas* species (Haas & Défago, 2005).

PA23 produces an arsenal of factors, including PRN, phenazine (PHZ), hydrogen cyanide (HCN), lipase and protease, that likely contribute to fungal antagonism (Poritsanos *et al.*, 2006). In a previous study, it was discovered that PRN plays a more important role than PHZ in controlling *S. sclerotiorum* infection on canola (Selin *et al.*, 2010). With the exception of *Pseudomonas* sp. MIS38 (Washio *et al.*, 2010), there is little known about the effect of the SR on the AF activity of pseudomonads. Then again, restricted growth due to nutrient limitation has long been known to favour secondary metabolism possibly as a means to stay competitive in the environment (Haas & Défago, 2005). Because biocontrol bacteria are often forced to survive under nutrient-depleted conditions, understanding the impact of the SR on secondary metabolism is essential since this directly impacts fungal inhibition. The focus of the current study was to elucidate how the SR and the stationary phase sigma factor RpoS affect PA23 antagonism of *S. sclerotiorum*, as well as production of antibiotics and degradative enzymes. Furthermore, we sought to determine if there is a link between the SR and RpoS in this bacterium.

## 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) at 37 and 28 °C, respectively. *S. sclerotiorum* was maintained on Potato Dextrose Agar (PDA; Difco). As required, media were supplemented with the following antibiotics from Research Products International: gentamicin (Gm; 20 μg $ml^{-1}$), tetracycline (Tc; 15 μg $ml^{-1}$), piperacillin (30 μg $ml^{-1}$), rifampicin (Rif; 100 μg $ml^{-1}$) for PA23, and ampicillin (Amp; 100 μg $ml^{-1}$), Gm (15 μg $ml^{-1}$), Tc (15 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 and nucleotide accession numbers.** Sequencing was performed at the Centre for Applied Genomics in The Hospital for Sick Children (Toronto, Ontario). Sequence information was analysed with BLASTN and BLASTX databases. The GenBank accession numbers for the sequences of the PA23 *relA*, *spoT* and *rpoS* genes are JF705880, JF705881 and DQ525801, respectively.

**Generating PA23 *relA*, *relAspoT* and *rpoS* mutants.** All primers and plasmids utilized for the construction of mutant strains are listed in Table 1. PA23*relA* was generated as follows. An internal portion of the *relA* gene from PA23 was amplified using primers relA-KpnIFOR and relA-BamHIREV. The resulting 650 bp fragment was cloned into pCR2.1 (Invitrogen) generating pCR*relA*-int. The *relA* insert was removed by digestion with *Kpn*I and *Bam*HI and cloned into the same sites of pKNOCK-Gm, creating pKNOCK-*relA*. Allelic exchange was performed through triparental mating among *E. coli* DH5α λpir (pKNOCK-*relA*), *E. coli* DH5α (pRK600) and PA23. Transconjugants were screened on LB agar supplemented with Rif and Gm. To verify the insertion into the PA23 *relA* gene, the pKNOCK-Gm vector was rescued from the PA23 genome by digestion with *Hinc*II. Linearized genomic fragments were subsequently recircularized with T4 DNA ligase (Invitrogen), transformed into *E. coli* DH5α λpir, and screened on LB agar + Gm. Sequencing of the DNA flanking the pKNOCK-Gm vector confirmed that *relA* had been interrupted. To generate PA23*relAspoT*, a 2.7 kb fragment containing the *spoT* gene of strain PA23 was amplified by PCR using primers spoT-FOR and spoT-REV. The PCR product was cloned into pCR2.1, generating pCR*spoT*-23. Next, the insert was removed through *Sac*I/*Xba*I digestion and cloned into the same sites of pEX18Ap, creating pEX*spoT*. A 2.0 kb *Sma*I fragment containing the $Tc^R$ marker from pFTC1 was cloned into a *Sma*I site within the *spoT* gene, forming pEX*spoT*-Tet. Triparental mating was performed using *E. coli* DH5α (pEX*spoT*-Tet), *E. coli* DH5α (pRK600) and PA23, and transconjugants were screened on LB agar plates supplemented with Tc and Rif. Bacteria that had undergone a double cross-over event were identified by streaking onto LB agar containing sucrose (10 %) and Tc. Southern blot and PCR analysis were used to verify that the *spoT* gene had successfully been replaced with a mutated copy of the alleles. 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 the transconjugants obtained were screened on LB agar supplemented with Rif and Tc. To verify disruption of the *rpoS* gene, the pKNOCK-Tc vector was rescued from the PA23 genome 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 Tc. Sequencing of the rescue clone, using primers RpoS RC-fwd and RpoS RC-rev, verified that an insertion had occurred in *rpoS*.

**Plasmid construction.** For complementing the SR mutants, pUCP22-*relA* and pUCP22-*spoT* were created such that the *relA* and *spoT* genes were under control of the *lac* promoter on pUCP22. The PA23 *relA* gene was amplified by PCR using primers relAtranslFRW and 23relAREV and the 2.8 kb product was cloned into pCR2.1. The resulting plasmid, pCR*relA*-23, was digested with *Eco*RI and *Bam*HI and the insert containing *relA* was subcloned into the same sites of pUCP22, creating pUCP22-*relA*. A 2.8 kb PCR product containing the PA23 *spoT* gene was amplified using primers spoT-23 : newFRW and spoT-23 : newREV and cloned into pCR2.1. The resulting plasmid, pCR*spoT*-23, was digested with *Xba*I, the ends were blunted with Klenow (Invitrogen) and then subject to *Bam*HI

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

Amp, ampicillin; Chl, chloramphenicol; Gm, gentamicin; Rif, rifampicin; Tet, tetracycline.

| Strain, plasmid or primer | Genotype or relevant phenotype* | Source or reference |
|---|---|---|
| **Strains** | | |
| *P. aeruginosa* PAO1 | Wild-type | Holloway *et al.* (1979) |
| *P. chlororaphis* | | |
| PA23 | $Rif^R$; wild-type (soybean root tip isolate) | Savchuk & Fernando (2004) |
| PA23*relA* | PA23 with the pKNOCK-Gm vector inserted into the *relA* gene | This study |
| PA23*relAspoT* | PA23*relA* with a $Tet^R$ cassette inserted into the *spoT* gene | This study |
| PA23*rpoS* | PA23 with the pKNOCK-Tc vector inserted into the *rpoS* gene | This study |
| *E. coli* | | |
| DH5α | *supE44* Δ*lacU169*(*ϕ80lacZ*ΔM15) *hadR17 recA1 endA1 gyrA96 thi-1 relA1* | Gibco |
| DH5α λpir | λpir lysogen of DH5α | House *et al.* (2004) |
| **Plasmids** | | |
| pCR2.1 | TA cloning vector; $Amp^R$ | Invitrogen |
| pCR*relA*-int | 650 bp internal fragment of *relA* in pCR2.1 | This study |
| pKNOCK-Gm | Suicide vector for insertional mutagenesis; R6K *ori* RP4 *oriT*, $Gm^R$ | Alexeyev (1999) |
| pKNOCK-*relA* | Internal fragment (650 bp) of *relA* in pKNOCK-Gm | This study |
| pRK600 | Contains *tra* genes for mobilization; $Chl^R$ | Finan *et al.* (1986) |
| pEX18Ap | Suicide plasmid; $Gm^R$ | Hoang *et al.* (1998) |
| pEX*spoT* | *spoT* from PA23 in pEX18Ap | This study |
| pFTC1 | Source of $Tet^R$ cassette | Choi *et al.* (2005) |
| pEX*spoT*-Tet | pEX*spoT* with $Tet^R$ inserted into *spoT* | This study |
| pCR2.1-*rpoS* | 1.01 kb fragment containing the *rpoS* gene in pCR2.1 | Poritsanos *et al.* (1996) |
| pKNOCK-Tc | Suicide vector for insertional mutagenesis; R6K *ori* RP4 *oriT* $Tc^R$ | Alexeyev (1999) |
| pKNOCK-*rpoS* | 400 bp internal fragment of *rpoS* in pKNOCK-Tc | This study |
| pCR*relA*-23 | 2.8 kb fragment containing the *relA* gene in pCR2.1 | This study |
| pUCP22 | Broad-host-range vector; $Amp^R$ $Gm^R$ | West *et al.* (1994) |
| pUCP22-*relA* | *relA* in pUCP22 | This study |
| pCR*spoT*-23 | *spoT* from *P. chlororaphis* PA23 in pCR2.1 | This study |
| pUCP22-*spoT* | *spoT* in pUCP22 | This study |
| pUCP22-*rpoS* | *rpoS* in pUCP22 | Poritsanos *et al.* (2006) |
| pLP170 | *lacZ* transcriptional fusion vector | Preston *et al.* (1997) |
| pSW205 | Promoterless *lacZ* translational fusion | Gambello *et al.* (1993) |
| pSW*relA*-23 | 650 bp fragment containing the promoter and first 70 codons of *relA* in pSW205 | 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** | | |
| relA-KpnIFOR | 5′-ggggtaccccatctggcgcaagatgcag-3′ | This study |
| relA-BamHIREV | 5′-cgggatcccgttgaaccagtggacga-3′ | This study |
| spoT-FOR | 5′-gcgtcaccgttgaagactg-3′ | This study |
| spoT-REV | 5′-ttactcgaggacgacgatgg-3′ | This study |
| 23relA-REV | 5′-gaagatcgtccactggttcaa-3′ | This study |
| spoT-23 : newFRW | 5′-ggaaatcgtcaacaagatcga-3′ | This study |
| spoT-23 : newREV | 5′-ttggtgttccagttctacgc-3′ | This study |
| RpoS RC-fwd | 5′-gatatcgccactgattcgatc-3′ | This study |
| RpoS RC-rev | 5′-ggatccaccaggtggcata-3′ | This study |

digestion. The 2.8 kb fragment containing *spoT* was ligated into the *Bam*HI–*Sma*I sites of pUCP22 generating pUCP22-*spoT*. For the *relA*–*lacZ* translational fusion, a 650 bp fragment containing the promoter and the first 70 codons of the PA23 *relA* gene was amplified by PCR using primers relAtransl-FRW and 23newrelA-REV. The product was digested with *Eco*RI and cloned into the *Eco*RI and *Sma*I sites of pSW205 creating the plasmid-borne *relA*–*lacZ* translational fusion pSW*relA*-23.

**(p)ppGpp and *relA* expression analysis.** (p)ppGpp levels were determined as described by Cashel (1994) with the following modifications. Cells were grown overnight in morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich) media at 28 °C followed by a 1 : 100 dilution of the culture in MOPS phosphate-free minimal medium containing 1 mg casamino acids $ml^{-1}$ (Difco) and 100 μCi $^{32}P$ $ml^{-1}$ (Perkin Elmer). Three 200 μl aliquots of each strain were added to wells of a polystyrene microtitre plate (Costar; Corning) and

grown at 28 °C for an additional 8 h. DL-Serine hydroxymate (400 μg $ml^{-1}$; Sigma) was then added to each well and incubated for 2 h. Nucleotides were extracted with an equal volume of cold 13 M formic acid, separated on polyethyleneimine-cellulose chromatography sheets (Sigma) and visualized by autoradiography. The (p)ppGpp analysis was repeated three times. Expression of pSW*relA*-23 was monitored in PA23, PA23*relA* and PA23*relAspoT*. Strains harbouring the plasmid were grown for 4, 8, 12, 16 and 24 h and then assayed for β-galactosidase activity (Miller, 1972).

**Antifungal assays.** To assess the ability of PA23 and its derivatives to inhibit fungal growth *in vitro*, radial diffusion assays were performed according to Poritsanos *et al.* (2006). Five replicates were analysed for each strain and the experiments were repeated three times.

**Quantitative analysis of PHZ.** Overnight cultures of PA23 and the SR mutants were grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2 % glucose, and subjected to PHZ extraction as described by 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 media + 1 mM $MgSO_4$ + 0.2 % glucose for 5 days. Toluene was added to the culture supernatants as an internal control. Peaks corresponding to toluene and PRN were analysed by UV absorption at 225 nm using a Varian 335 diode array detector. Samples were analysed in duplicate and the experiment was repeated twice.

**HCN analysis.** Qualitative determination of HCN production was performed using Cyantesmo paper (Machery-Nagel). Samples were analysed in triplicate and the experiments were repeated three times.

**Protease production.** Cultures were grown in M9 minimal media supplemented with 1 mM $MgSO_4$, 0.2 % glucose and 1.5 % skimmed milk (Difco) for 5 days at 28 °C to induce protease production. A 200 μl aliquot of cell-free supernatant was analysed for protease activity in a 0.65 % solution of casein at 28 °C for 10 min. Tyrosine, which is released upon the hydrolysis of casein by the protease enzyme, is able to react with the Folin–Ciocalteu reagent (Sigma) to produce a blue-coloured chromophore (Cupp-Enyard, 2008). This chromophore was measured spectrophotometrically at 660 nm. To determine the amount of tyrosine liberated, a standard curve was generated using pure tyrosine at the following concentrations (μM): 0.055, 0.111, 0.221, 0.0442 and 0.553. Each strain was assayed in triplicate and experiments were performed three times.

**Lipase production.** Lipase assays were performed according to Winkler & Stuckmann (1979). Briefly, strains were grown in M9 minimal media (Difco) supplemented with 1 mM $MgSO_4$, 0.2 % glucose and 0.2 % Tween 20 (Fisher Scientific) at 28 °C for 24 h with aeration. After an additional 96 h of static growth, cell-free supernatant was analysed for lipase activity. A 200 μl aliquot of supernatant was diluted with 2.4 ml of a solution consisting of 30 mg *p*-nitrophenylpalmitate (Sigma) dissolved in 10 ml 2-propanol and 90 ml 0.5 M Sorensen phosphate buffer supplemented with 207 mg sodium deoxycholate (Sigma) and 100 mg gum arabic (Sigma). After incubation with the supernatant for 30 min at 28 °C, the absorbance was measured at 410 nm. One enzyme unit was defined as the liberation of 1 nmol *p*-nitrophenyl $ml^{-1}$ $min^{-1}$. Each strain was assayed in triplicate and experiments were performed three times.

**Analysis of transcriptional fusions.** The activity of the plasmid-borne *prnA*–, *phzA*–, and *rpoS–lacZ* transcriptional fusions was monitored in PA23 and the SR mutants. Strains harbouring the pLP170 derivatives were grown for 4, 8, 12, 16 and 24 h prior to analysis of β-galactosidase activity (Miller, 1972).

**RpoS expression.** In order to assess RpoS protein levels, SDS-PAGE and Western blot analysis were performed. Strains were grown in 3 ml M9 minimal media supplemented with 0.2 % glucose and 1 mM $MgSO_4$ at 28 °C. After 24 h growth, the cultures had reached $OD_{600}$ ~1.3 and were subsequently diluted to $OD_{600}$ 1.0 to ensure that an equal number of cells were analysed. Cells were pelleted, resuspended in sample buffer [63 mmol Tris/HCl $l^{-1}$ pH 6.8, 2 % SDS, 25 % (v/v) glycerol, 0.01 % bromophenol blue] containing 1 % β-mercaptoethanol and then boiled for 3 min. A 100 μg protein sample, determined by the Bradford assay (Bradford, 1976), was loaded and separated on a 12 % SDS-PAGE gel. Blots were incubated with RpoS-specific antibodies (Institute of Molecular and Cellular Bio-sciences, University of Tokyo, Tokyo, Japan), followed by alkaline phosphatase-conjugated goat anti-rabbit antiserum (Sigma). Antibodies were detected using the colorimetric detection reagent NBT/BCIP (Roche Diagnostics). RpoS was quantified with a Fluorochem 2000 Phosphoimager using Fluorochem Stand-Alone software, version 2.0. The Western blot analysis was repeated twice with similar findings.

**Statistical analysis.** An unpaired Student's *t*-test was used for statistical analysis of AF, protease and lipase activity.

## RESULTS

### PA23 SR mutant generation and (p)ppGpp analysis

To assess the impact of the SR on AF activity, *relA* and *relAspoT* mutants of strain PA23 were generated. Primers designed from the *P. aeruginosa* PAO1 *relA* and *spoT* sequences enabled us to amplify these genes from PA23. The PA23*relA* and *spoT* genes were 95 and 92 % identical to those of *P. fluorescens* Pf0-1 (CP000094.2) and *P. fluorescens* Pf-5 (CP000076.1), respectively. The first mutant, PA23*relA*, was created through plasmid insertion. Rescue cloning and sequence analysis of the DNA flanking the pKNOCK-Gm vector confirmed disruption of the wild-type allele (data not shown). To generate a *relAspoT* double mutant, the *spoT* gene of PA23*relA* was mutated through allelic exchange. PCR and Southern blot analysis verified that in PA23*relAspoT*, *spoT* had been replaced with a mutated copy of the allele (data not shown). We were unable to generate a PA23 *spoT* single mutant, leading us to believe that in a $relA^+$ background, *spoT* is required for cell viability, similar to reports for other bacteria (Potrykus & Cashel, 2008).

To determine if disruption of the aforementioned genes affected (p)ppGpp production, bacteria were grown in the presence of DL-serine hydroxymate, a potent inducer of the SR. As shown in Fig. 1, PA23 was able to produce both ppGpp and pppGpp (lane 2); however, (p)ppGpp was undetectable in the SR mutants (lanes 3 and 4). Production of these nucleotides was restored in PA23*relA* and PA23*relAspoT* when the *relA* gene was added *in trans* (Fig. 1, lanes 5 and 6). The presence of *spoT* in multicopy,

however, did not restore (p)ppGpp production in the SR mutants (data not shown).

## The SR affects PA23-mediated antagonism of *S. sclerotiorum* by regulating PRN, lipase and protease activity

Next, we assessed the ability of the SR mutants to antagonize *S. sclerotiorum in vitro*. When grown in M9 minimal media, both PA23*relA* and PA23*relAspoT* exhibited a 1.3-fold increase in AF activity compared with the wild type (Table 2). The presence of pUCP22-*relA* in the SR mutants restored the AF activity to that of the wild type (Table 2). Conversely, pUCP22-*spoT* did not alter fungal antagonism by the mutants (Table 2). As production of secondary metabolites occurs at the transition between the logarithmic and stationary phases of growth, it is important to note that there were no differences in the growth rate among all strains (data not shown).

PA23 produces a number of AF factors including PRN, PHZ and HCN. Of these, PRN was found to be most important for PA23 antagonism of *S. sclerotiorum* (Selin *et al.*, 2010). Therefore, we explored the possibility that the enhanced AF activity demonstrated by the ppGpp-deficient strains was the result of increased antibiotic production. Expression of plasmid-borne *phzA–lacZ* (PHZ) and *prnA–lacZ* (PRN) transcriptional fusions was monitored in PA23, PA23*relA* and PA23*relAspoT*. As shown in Fig. 2(a), *phzA–lacZ* activity was similar for all three strains. Expression of the *prnA* fusion, on the other hand, was elevated in PA23*relA* and PA23*relAspoT* (Fig. 2b). When we looked at PHZ, PRN and HCN production, we observed the same trend. There were no differences in terms of total PHZ produced and all three strains tested positive for HCN production (Table 2). In contrast, the total amount of PRN in the cell-free supernatants of the SR mutants was 2.5-fold greater than in PA23 (Table 2). Mobilization of pUCP22-*relA* into PA23*relA* and PA23*relAspoT* reduced PRN production to wild-type levels (Table 2).

Next, we quantified the amount of lipase and protease in cell-free supernatants of PA23 and the SR mutants. Lipase activity was increased 1.2-fold in PA23*relA* and 1.3-fold in PA23*relAspoT* (Table 2). Similarly, protease activity was elevated 1.75- and 2.3-fold in the single and double mutants, respectively (Table 2). Addition of pUCP22-*relA* restored lipase and protease activity to wild-type levels (Table 2).

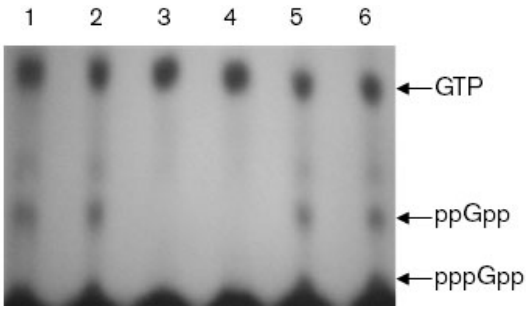


**Fig. 1.** (p)ppGpp analysis of *Pseudomonas chlororaphis* PA23 and its SR derivatives after induction with serine hydroxymate. Cells were labelled with $^{32}P$ and nucleotides were extracted and separated by thin layer chromatography. Lanes: 1, PAO1; 2, PA23; 3, PA23*relA*; 4, PA23*relAspoT*; 5, PA23*relA* (pUCP22-*relA*); 6, PA23*relAspoT* (pUCP22-*relA*).

## The SR exerts its effects on PA23-mediated fungal antagonism through RpoS

In both *E. coli* and *P. aeruginosa*, *rpoS* expression is upregulated during the SR (van Delden *et al.*, 2001; Erickson *et al.*, 2004; Potrykus & Cashel, 2008). To determine if *rpoS* forms part of the PA23 SR regulon, expression of a plasmid-borne *rpoS–lacZ* transcriptional fusion was monitored in the SR mutants. As shown in Fig. 3(a), *rpoS* transcription was dramatically reduced in the mutants during both the exponential and stationary phases of growth. Furthermore, there was markedly less RpoS protein produced by the SR mutants (Fig. 3b, c), and addition of *relA* or *rpoS in trans* restored RpoS levels to those of the wild type. To address whether decreased *rpoS* expression was responsible for the phenotypic changes observed for the SR mutants, strains harbouring pUCP22-*rpoS* were examined for production of AF factors. The presence of *rpoS* constitutively expressed from the *lac* promoter was able to fully complement the *relA* and *relAspoT* mutants with respect to AF, protease and lipase activity and PRN production (Table 2). As expected, no RpoS protein was detected in PA23*rpoS*; however, production of this sigma factor was restored upon addition of pUCP22-*rpoS in trans* (Fig. 3). Therefore, it appears that the effects of the SR on PA23-mediated antagonism are mediated primarily through RpoS.

## RpoS regulates PRN, lipase and protease activity as well as PHZ biosynthesis

In various *Pseudomonas* species, RpoS has been found to be involved in antibiotic production (Sarniguet *et al.*, 1995; Heeb *et al.*, 2005; Ge *et al.*, 2006; Girard *et al.*, 2006a; Park *et al.*, 2011). The fact that *rpoS* transcription was markedly decreased in the SR mutants and the wild-type phenotype was restored by providing *rpoS in trans* led us to investigate the role of RpoS in PA23 antagonism. Generation and characterization of an *rpoS* mutant, PA23*rpoS*, revealed enhanced AF activity and PRN production (Table 2) as well as increased *prnA–lacZ* expression (Fig. 2b), similar to the observations for the SR mutants. Conversely, we found that both *phzA–lacZ* expression (Fig. 2a) and PHZ levels were reduced in the *rpoS* mutant compared with the wild-type (Table 2). No differences in HCN production (Table 2) or growth rate were observed between PA23 and PA23*rpoS*

**Table 2.** Phenotypic characterization of *P. chlororaphis* PA23 and derivatives harbouring *relA* (pUCP22-*relA*), *spoT* (pUCP22-*spoT*) and *rpoS* (pUCP22-*rpoS*)

ND, not determined. HCN activity was positive for all strains, with no significant difference from the wild-type.

| Strains | Extracellular metabolite activity | | | | |
|---|---|---|---|---|---|
| | AF (mm)* | PRN (μg)† | PHZ (μg $ml^{-1}$) | Protease (units enzyme $ml^{-1}$)‡ | Lipase (units enzyme $ml^{-1}$ $min^{-1}$)‡ |
| PA23 (pUCP22) | 1.5 (0.1) | 1.80 (0.50) | 25.05 (1.13) | 0.68 (0.10) | 1.49 (0.02) |
| PA23*relA* (pUCP22) | 2.0 (0.2)§ | 4.49 (1.16)‖ | 26.54 (1.43) | 1.19 (0.05)¶ | 1.87 (0.06)¶ |
| PA23*relAspoT* (pUCP22) | 2.0 (0.2)§ | 5.04 (0.90)‖ | 24.53 (0.26) | 1.55 (0.17)§ | 2.02 (0.16)¶ |
| PA23*rpoS* (pUCP22) | 2.0 (0.1)# | 2.91 (0.07)‖ | 3.89 (0.57) | 1.51 (0.10)¶ | 3.10 (0.08)§ |
| PA23*rpoS* (pUCP22-*rpoS*) | 1.6 (0.1)** | 1.67 (0.14)** | 29.67 (2.26) | 0.83 (0.10)** | 1.86 (0.30)** |
| PA23*relA* (pUCP22-*relA*) | 1.4 (0.1)** | 1.83 (0.09)** | ND | 0.58 (0.13)** | 1.39 (0.32)** |
| PA23*relAspoT* (pUCP22-*relA*) | 1.3 (0.2)** | 1.54 (0.20)** | ND | 0.54 (0.17)** | 1.48 (0.07)** |
| PA23*relA* (pUCP22-*spoT*) | 1.9 (0.2)§ | 5.56 (0.30)‖ | ND | 1.02 (0.14)¶ | 1.92 (0.06)¶ |
| PA23*relAspoT* (pUCP22-*spoT*) | 2.1 (0.1)¶ | 6.31 (0.87)‖ | ND | 1.27 (0.12)¶ | 2.17 (0.20)¶ |
| PA23*relA* (pUCP22-*rpoS*) | 1.4 (0.2)** | 1.86 (0.40)** | ND | 0.67 (0.02)** | 1.31 (0.23)** |
| PA23*relAspoT* (pUCP22-*rpoS*) | 1.4 (0.1)** | 1.61 (0.10)** | ND | 0.85 (0.10)** | 1.16 (0.30)** |

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

(data not shown). Next, we determined whether the *rpoS* mutation affects extracellular enzyme production. More than a twofold increase in both protease and lipase activity was observed for PA23*rpoS* compared with the wild-type (Table 2). The *rpoS* mutant harbouring pUCP22-*rpoS* exhibited wild-type AF, protease and lipase activity as well as PRN and PHZ production (Table 2). Therefore, with the exception of PHZ expression, the phenotype of the *rpoS* mutant resembles that of the SR mutants.

### RelA is negatively regulated by (p)ppGpp

When expression of a plasmid-borne *relA–lacZ* translational fusion was monitored in PA23 and the SR mutants, all three strains showed maximal *relA* expression at stationary phase (16 h growth) followed by a sharp decline in expression at 24 h. However at 16 h, PA23*relA* and PA23*relAspoT* exhibited a twofold or greater increase in *relA* expression compared with the wild-type (Fig. 4). Thus it appears that the SR negatively regulates *relA* in PA23.

## DISCUSSION

When applied as a foliar spray, *P. chlororaphis* strain PA23 is an effective biocontrol agent capable of inhibiting sclerotinia stem rot of canola (Savchuk & Fernando, 2004; Poritsanos *et al.*, 2006). Because nutrients are scarce in the phyllosphere (Lindow & Brandl, 2003), we were interested in understanding how the SR affects the AF properties of this bacterium. PA23 *relA* and *relAspoT* derivatives were created that showed reduced (p)ppGpp accumulation and enhanced AF activity under nutrient-limiting conditions (Table 2). Thus it appears that the SR negatively affects the antagonistic potential of this organism. We discovered that lipase, protease and PRN production was increased in the SR mutants; conversely PHZ production remained unchanged (Table 2). Consistent with the antibiotic levels, *prnA–lacZ* and *phzA–lacZ* fusion analysis showed elevated expression of *prnA* but not *phzA* in the SR mutant background (Fig. 2). Thus, we believe that the increase in PA23*relA*- and PA23*relAspoT*-mediated antagonism results from elevated production of PRN, and possibly protease and lipase, by these strains.

With the exception of *Pseudomonas* sp. MIS38, very little is known about the impact of the SR on fungal antagonism by pseudomonads. A *spoT* mutant of MIS38 exhibited increased (p)ppGpp and reduced arthrofactin levels, suggesting that the SR negatively regulates production of this antibiotic (Washio *et al.*, 2010), similar to what we observed with PRN. As PHZ production remained unchanged, we conclude that not all of the AF compounds produced by PA23 are regulated by the SR. In a similar fashion, the SR differentially affects antibiotic production

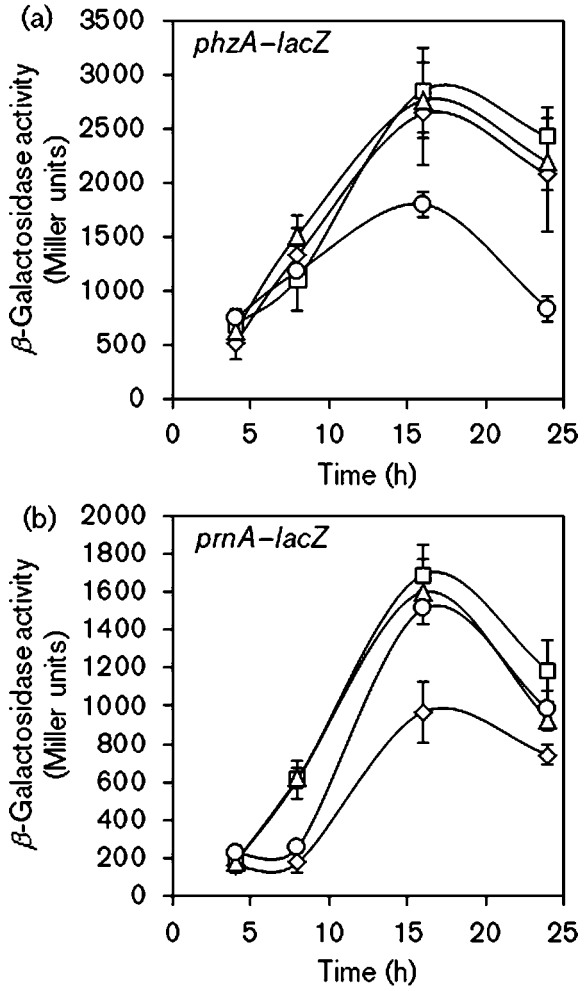


**Fig. 2.** The impact of the stringent response and RpoS on *phzA–lacZ* (a) and *prnA–lacZ* (b) expression. Bacterial strains are as follows: *P. chlororaphis* PA23 (◇), PA23*relA* (□), PA23*relAspoT* (△) and PA23*rpoS* (○). Bacteria were grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2 % glucose. Error bars indicate SD.

by *Streptomyces* species. Depending upon the strain in question, the antibiotics produced and the growth medium employed, the SR was found to influence antibiotic expression positively, negatively or not at all. For instance, a *Streptomyces clavuligerus relA* mutant produced elevated levels of clavulanic acid and cephamycin C (Gomez-Escribano *et al.*, 2008), while a *relA* deficiency in *Streptomyces coelicolor* ATCC 27064 abolished cephamycin C production (Jin *et al.*, 2004). This same mutation in *Streptomyces coelicolor* strain A3(2) had little effect on actinorhodin and undecylprodigiosin expression (Sun *et al.*, 2001). From these examples and our own findings, it is evident that a great deal of variability exists. So far, we have been unable to define a role for SpoT in PA23 secondary metabolism. The phenotype of our *relAspoT* double mutant was remarkably similar to the *relA* mutant, and constitutive expression of *spoT* in PA23*relAspoT* imparted no discernible changes. But we cannot rule out the possibility that under different conditions, SpoT plays a more dominant role in PA23 secondary metabolism.

In *E. coli* and *P. aeruginosa*, the SR positively regulates expression of RpoS (van Delden *et al.*, 2001; Erickson *et al.*, 2004; Potrykus & Cashel, 2008). In much the same way, we observed a fivefold decrease in *rpoS* transcription in the SR mutants compared with PA23 (Fig. 3a). This dramatic decrease in *rpoS* expression led us to speculate that the SR mutant phenotype might result from reduced levels of RpoS. Indeed, when *rpoS* was added *in trans*, PRN production, as well as AF, protease and lipase activity were restored to that of the wild type (Table 2). Moreover, the SR mutants containing multicopy *relA* exhibited RpoS levels equal to or higher than those of PA23 (Fig. 3b, c). Collectively, these findings indicate that the effect of the SR on PA23 AF activity is mediated primarily through RpoS.

RpoS has been implicated in the production of antibiotics in several biocontrol pseudomonads (Sarniguet *et al.*, 1995; Heeb *et al.*, 2005; Ge *et al.*, 2006; Girard *et al.*, 2006a; Park *et al.*, 2011). Depending on the strain in question and the antibiotic produced, RpoS may have a positive (Sarniguet *et al.* 1995; Whistler *et al.*, 1998; Ge *et al.*, 2006; Girard *et al.*, 2006a; Park *et al.*, 2011), negative (Sarniguet *et al.* 1995; Whistler *et al.*, 1998; Heeb *et al.*, 2005; Ge *et al.*, 2006) or no (Heeb *et al.*, 2005) effect on antibiotic expression. To assess the role of this sigma factor in PA23-mediated fungal antagonism, an *rpoS* mutant was generated. As was demonstrated by the SR mutants, PA23*rpoS* exhibited increased growth inhibition of *S. sclerotiorum in vitro* presumably through enhanced production of the same metabolites (Table 2). The discovery that RpoS negatively regulates PRN differs from the situation that has been observed in *P. fluorescens* Pf-5 where an *rpoS* mutation abolished PRN production (Sarniguet *et al.*, 1995; Whistler *et al.*, 1998). At the same time, wild-type and *rpoS*$^{-}$ strains of both PA23 and Pf-5 were found to produce HCN and protease. The reason for the discrepancy in RpoS-mediated control over PRN expression is unclear. As these two bacteria produce a different spectrum of secondary metabolites, it is possible that upregulation of one metabolite causes downregulation of another, which has been reported previously (Schnider-Keel *et al.*, 2000; Baehler *et al.*, 2005). Alternatively, RpoS may be affecting PRN expression indirectly through regulatory elements which differ between these two bacteria.

Where PA23*rpoS* differs from PA23*relA* and PA23*relAspoT* is in the expression of PHZ. While the SR mutants are able to produce PHZ despite having low amounts of RpoS (Fig. 3b, c), the *rpoS* mutant showed an eightfold reduction in PHZ levels and significantly reduced *phzA* transcription (Fig. 2a). Perhaps the residual level of RpoS in the SR mutants enables wild-type PHZ production under the conditions tested. It is important to note that previous

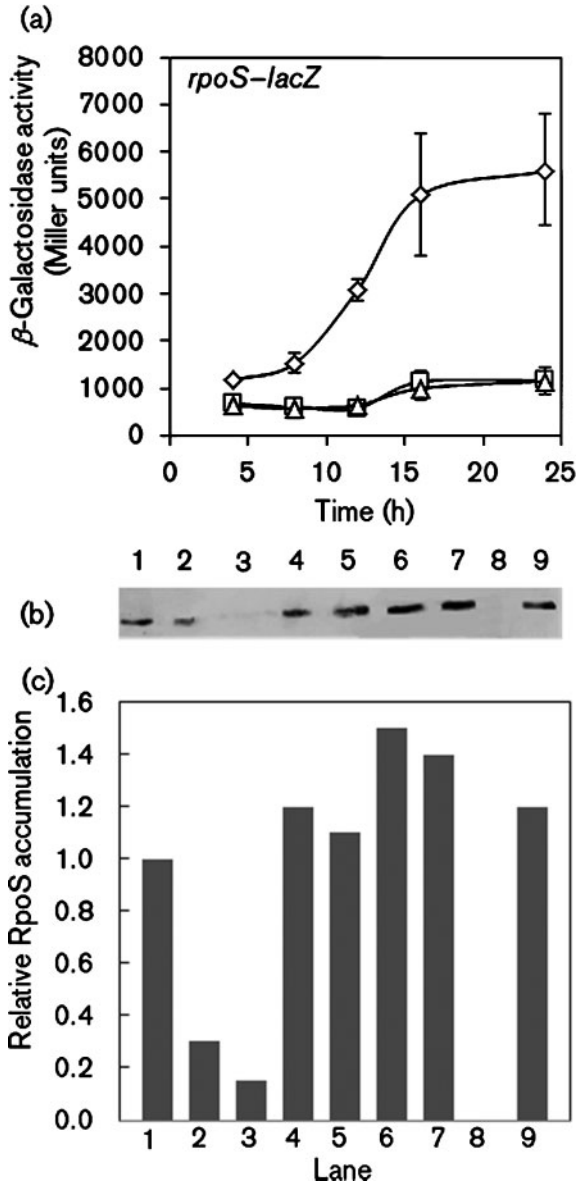


**Fig. 3.** Expression of *rpoS–lacZ* and relative accumulation of RpoS protein in *P. chlororaphis* PA23 and its derivatives. (a) Strains used are as follows: PA23 (◇), PA23*relA* (□) and PA23*relAspoT* (△). All strains harboured the *rpoS–lacZ* reporter plasmid pRPOS-*lacZ*. Error bars indicate SD. (b) RpoS-specific antiserum was used to visualize $\sigma^s$ from Western blots of protein extracted from cultures grown to stationary phase. Each lane contains 100 μg protein. Lanes: 1, PA23 (pUCP22); 2, PA23*relA* (pUCP22); 3, PA23*relAspoT* (pUCP22); 4, PA23*relA* (pUCP22-*relA*); 5, PA23*relAspoT* (pUCP22-*relA*); 6, PA23*relA* (pUCP22-*rpoS*); 7, PA23*relAspoT* (pUCP22-*rpoS*); 8, PA23*rpoS* (pUCP22); 9, PA23*rpoS* (pUCP22-*rpoS*). (c) The 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.

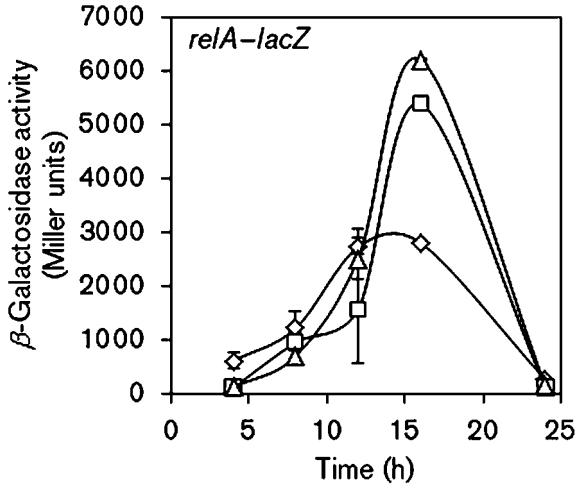


**Fig. 4.** RelA exhibits negative autoregulation. Expression of *relA* was monitored in PA23 (◇), PA23*relA* (□) and PA23*relAspoT* (△). All strains harboured the *relA–lacZ* reporter plasmid pSW*relA*-23. Error bars indicate SD.

studies have shown PHZ regulation in *P. chlororaphis* to be complex. In *P. chlororaphis* 30-84, for example, a repressor called RpeA has been identified that decreases PHZ biosynthesis under minimal media conditions (Whistler & Pierson, 2003). Furthermore, an RpoS-dependent protein called Pip was found to induce phenazine-1-carboxamide expression in *P. chlororaphis* 1391 (Girard *et al.*, 2006b). Homologues of *rpeA* and *pip* have not yet been identified in PA23. If these regulators are present, their characterization may help to clarify the connection between nutrient status and the regulatory network governing PHZ expression in this bacterium.

In summary, we have established that the SR exerts a negative effect on PA23 AF activity *in vitro*, mediated by increased RpoS expression. Because manufacture of AF factors is energetically costly, it is logical for bacteria to reduce production of these compounds when nutrients are scarce. Whether the SR mutants would exhibit enhanced biocontrol *in planta* has yet to be established. Decreased RpoS levels would likely render these strains more sensitive to oxidative stress, heat shock and high osmolarity conditions. Such changes could decrease the environmental fitness of the bacteria, possibly diminishing their biocontrol potential. Future work will be directed at understanding how the SR affects PA23 colonization and antagonism in different environments.

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