

**An Investigation of the Impact of the Stringent Response on the Growth Inhibition
of *Sclerotinia sclerotiorum* by the Biocontrol Pseudomonads *Pseudomonas* sp. DF41
and *Pseudomonas chlororaphis* PA23**

By

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Abstract

The stringent response (SR) is a global regulatory mechanism that allows bacteria to survive starvation. The plant surface is one environment where a fluctuation in nutrient availability is experienced. Because both *Pseudomonas sp.* DF41 and *Pseudomonas chlororaphis* PA23 are able to protect canola from the fungal pathogen *Sclerotinia sclerotiorum* when applied as a foliar spray, we sought to investigate the impact of this response on the antifungal activities of these two biocontrol strains.

The SR exerts its effects on gene transcription through production of the alarmone (p)ppGpp. Metabolism of (p)ppGpp is governed by two enzymes; RelA acts as a synthetase, while SpoT can function as either a synthetase or a hydrolase. To investigate how the SR affects the ability of strains DF41 and PA23 to inhibit the fungal pathogen, *relA* and *relAspoT* mutants were generated through allelic exchange. Strain DF41 *relA* and *relAspoT* mutants were found to exhibit increased antifungal activity due to enhanced lipopeptide (LP) antibiotic production. Addition of *relA*, but not *spoT* in trans restored the mutant phenotype to that of the parent. The influence of the SR on the regulatory mechanisms governing strain DF41 biocontrol was also investigated. It was determined that *relA* forms part of the Gac regulon while RpoS is under SR control. In fact, addition of *rpoS* in trans restored protease activity to wild-type levels, but did not attenuate antifungal activity.

The SR mutants PA23*relA* and PA23*relAspoT*, also exhibited increased growth inhibition of *S. sclerotiorum in vitro* compared to the wild type. Both mutants showed enhanced production of the antifungal factors pyrrolnitrin, lipase and protease and were

complemented by the addition of *relA* but not *spoT*. Herein, the SR was found to regulate that Gac system, QS, and RpoS. The presence of *gacS* or *rpoS* in multicopy restored the mutant phenotype to that of the wild type.

In summary, these findings suggest that the SR negatively influences the biocontrol activities of strains DF41 and PA23. It is evident that the SR is merely one mechanism by which DF41 and PA23-mediated antagonism is regulated.

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List of abbreviations.

AHL	Acylhomoserine lactone
AI	Autoinducer
Amp	Ampicillin
Dab	2,4-diamino butyric acid
2,4-DAPG	2,4-Diacetylphloroglucinol
Dhb	2-amino-butenic acid
Gm	Gentamicin
HCN	Hydrogen cyanide
HPLC	High Performance Liquid Chromatography
ISR	Induced Systemic Response
LB	Luria Bertani
LP	Lipopeptide
MOPS	Morpholinepropanesulfonic acid
NRPS	Nonribosomal Peptide Synthetase
NSERC	Natural Sciences and Engineering Research Council
2-OH-PHZ	2-hydroxy-phenazine
PCA	Phenazine-1-carboxylic acid
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PGPR	Plant Growth Promoting Rhizobacteria
PHZ	Phenazine

Pip	Piperacillin
PLT	Pyoluteorin
Poly P	Inorganic polyphosphate
ppGpp	Guanosine tetrphosphate
pppGpp	Guanosine pentaphosphate
PRN	Pyrrolnitrin
QS	Quorum Sensing
Rif	Rifampicin
RNAP	RNA Polymerase
Rsm	Regulator of Secondary Metabolism
RSH	Rel Spo Homolog
SAR	Systemic Acquired Resistance
SR	Stringent response
SSR	Sclerotia stem rot
Tc	Tetracycline
TLC	Thin Layer Chromatography
Tn	Transposon

Chapter 1.
Literature Review.

1.1 The biocontrol activity of pseudomonads

1.1.1 Biocontrol

Difficulty in managing soil-borne diseases has led to the destruction of millions of crop acres each year resulting in significant losses in revenue across the globe. Current methods of disease control include crop rotation, breeding for resistant plant varieties, and the use of pesticides. However, these methods are not always effective. Furthermore, there exists concern over the impact of chemicals on the environment and human health. For these reasons, there is an urgent need to develop of alternative strategies for disease management.

An environmentally friendly approach to managing soil-borne diseases termed biocontrol has been observed for over 70 years. Biocontrol is defined as “the control of one organism by another” (Beirne 1967; Mazzola et al. 1992) and was developed upon discovery of natural disease-suppressive soils. In these soil types, plants are protected from disease even in the presence of the pathogen (Schneider 1982; Schippers et al. 1987). This is in contrast to conducive soils, which allow for disease development. Although rare in nature, these soils have been reported in the Salinas Valley (California, USA), the Chateaufort region (France), the Canary Islands and Broye Valley (Switzerland) (Haas and Défago 2005). Because suppressive soils lose their inhibitory effects when treated with antibiotics (Shipton et al. 1973; Cook and Rovira 1976; Scher and Baker 1980; Stutz et al. 1986) and can transfer their protective nature to conducive soils (Haas et al 2002), rhizosphere-associated bacteria are believed to be responsible for these traits.

1.1.2 Plant growth promoting rhizobacteria

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that stimulate plant growth and/or reduce the incidence of disease (Kloepper and Schroth 1987). Although other bacteria with similar properties have been found on the plant surface (phyllosphere), in the zone surrounding the germinating seed (spermophere), and in the flower (anthosphere) (Hallman et al. 1997), the PGPRs competitively colonize plant roots. Notable biocontrol PGPRs belong to the *Bacillus*, *Streptomyces*, *Burkholderia*, and *Pseudomonas* spp. (Weller 1988; Lugtenberg et al. 2001). It was initially believed that in order for PGPRs to be effective, they must be present at sufficient numbers on the plant surface to compete with the fungal pathogen for nutrients. Because PGPRs represent less 1% of culturable aerobic rhizobacteria in nature, competition for nutrients cannot be their only mode of action (Haas and Défago 2005). Instead, research suggests that these organisms exert their protective effects on plants through i) antibiosis; ii) induction of plant immune responses such as the induced systemic response (ISR) and systemic acquired resistance (SAR); and iii) specific pathogen-antagonist interactions (Kloepper and Schroth 1987; Haas and Défago 2005). The ISR is elicited in response to interaction with non-pathogenic bacteria whereas necrosis from pathogenic species activates the SAR (Thomashow 1996). The ISR/SAR and the impact of the pathogen on the biocontrol agent have been reviewed elsewhere and will not be discussed here (Thomashow 1996; Compant et al. 2005; Van Loon 2006; Toyoda et al. 1988; Utsumi et al. 1991; Schnider-Keel et al. 2000). Rather, the remainder of this review will focus on mechanisms important for the biocontrol activity of pseudomonads.

1.1.3 Antifungal factors influencing disease suppression

The protective nature of biocontrol pseudomonads has been attributed to the production and the concerted efforts of secondary metabolites, which are factors not associated with primary growth (Fig. 1.1). A brief description of some of the metabolites mediating antagonism is discussed below with emphasis on non-volatile antibiotics.

1.1.3.1. Phenazine antibiotics

Phenazines (PHZ) are a diverse group of heterocyclic, nitrogen-containing pigments with broad-spectrum activity against fungi and bacteria (Mavrodi et al. 2006). Their antibiotic properties are attributed to their ability to inhibit electron transport and cause lipid and macromolecule damage through the formation of hydroxyl radicals in the presence of ferripyochelin (Britigan et al. 1992). Moreover, under neutral pH conditions, reduced PHZs have been found to solubilize iron from insoluble sources (Hernandez et al. 2004). Not all strains of fluorescent *Pseudomonas* spp. are phenazine producers; however, those that do typically synthesize two or more of these molecules (Mavrodi et al. 2006). Biosynthesis of PHZs occurs through an offshoot of the shikimic acid pathway, which is also involved in siderophore, tyrosine, and phenylalanine biosynthesis (McDonald et al. 2001; Vandenende et al. 2004). At the branch point of this pathway, enzymes encoded by the *phzABCDEFG* operon modify chorismic acid (Mavrodi et al. 1998; Delaney et al. 2001). Additional genes required for the synthesis of PHZ derivatives

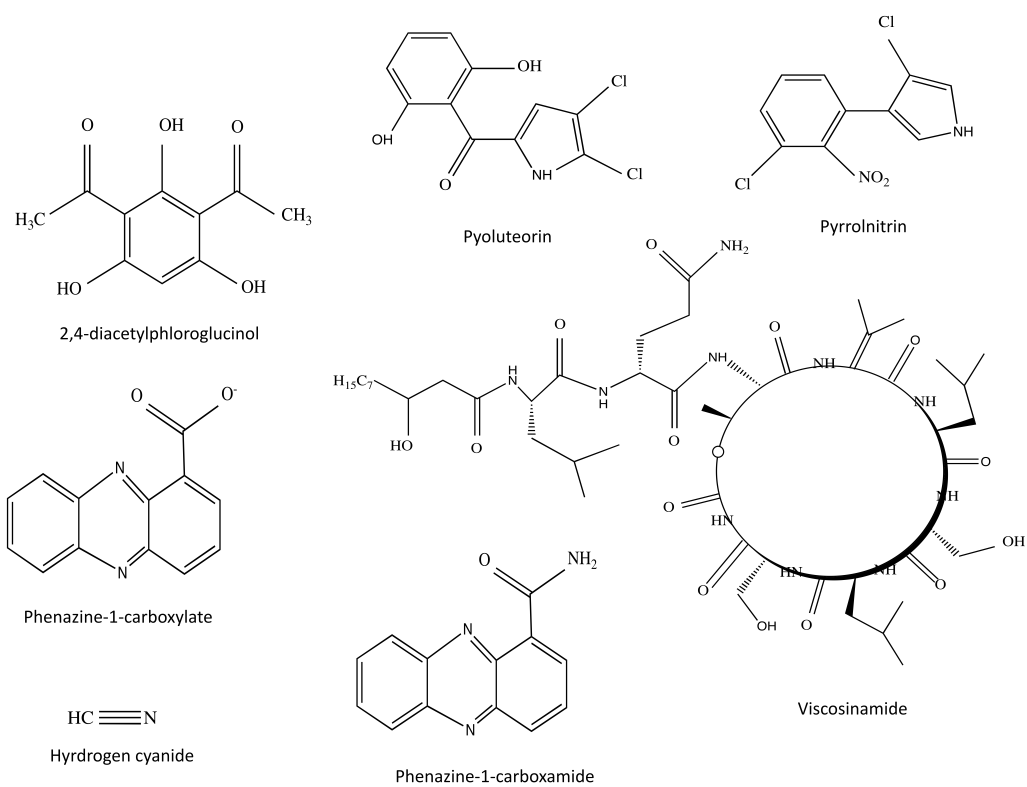


Figure 1.1: Antibiotics produced by fluorescent pseudomonads.

include *phzM*, *phzS*, *phzO*, and *phzH*, which may or may not be located near the core operon (Chin et al. 2001).

1.1.3.2. Pyrrolnitrin

The broad spectrum antibiotic, pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chloro-phenyl) pyrrole; PRN] (Fig. 1.1) was initially isolated from *Pseudomonas (Burkholderia) pyrrocinia* (Ligon et al. 2000) and found to be effective against a variety of fungal pathogens including *Rhizoctonia solani*, *Botrytis cinerea*, *Verticillium dahlia*, and *Sclerotinia sclerotiorum* (Howell and Stipanovic 1979; Hill et al. 1994). The antibiotic action of PRN results from inhibition of the fungal respiratory chain (Tripathi and Gottlieb 1969). A derivative of tryptophan, PRN is synthesized from enzymes encoded by the *prnABCD* operon (Hammer et al. 1997). To date, no pathway-specific regulators of PRN have been identified.

1.1.3.3. Pyoluteorin

Pyoluteorin (PLT) is an aromatic polyketide antibiotic that is produced by certain strains of *Pseudomonas* spp. including *Pseudomonas fluorescens* Pf-5, *Pseudomonas fluorescens* CHA0, and *Pseudomonas* sp. MI8. Although its mode of action is unknown, PLT is quite effective at reducing damping-off disease caused by *Pythium ultimum* on cress (Kraus and Loper 1992; Maurhofer et al. 1992; Maurhofer et al. 1994). Synthesis of PLT involves the modification of proline or a related molecule by enzymes encoded by the *pltLABCDEFG* operon (Nowak-Thompson et

al. 1997; Nowak-Thompson et al. 1999). Located upstream and divergently transcribed from this gene cluster is PltR, a LysR-type transcriptional regulator that positively effects *pltLABCDEFG* transcription (Nowak-Thompson et al. 1999). Furthermore, PLT was found to repress the production of other antibiotics, including PRN and 2,4-diacetyl phloroglucinol (2,4-DAPG) in *P. fluorescens* Pf-5 (Schnider-Keel et al. 2000; Brodhagen et al. 2003; Brohagen et al. 2004).

1.1.3.4. 2, 4-diacetyl phloroglucinol

2,4-DAPG is polyketide antibiotic that possesses broad-spectrum activity against plants, pathogenic fungi, helminthes, viruses, and bacteria (Keel et al. 1992; Duffy and Defago 1997; Thomashow and Weller 1996; de Souza et al. 2003). This metabolite was found to be important in the *P. fluorescens* CHA0-mediated growth inhibition of *Gaeumannomyces graminis* var. *tritici* on wheat and *Thielaviopsis basicola* on tobacco (Keel et al. 1990; Thomashow 1996). Similar to the other antibiotics previously discussed, the genes involved in the biosynthesis of 2,4-DAPG are arranged as an operon (*phlABCD*). Flanking *phlABCD* are two genes, *phlE* and *phlF*, whose products are involved in the export of the antibiotic and inhibition of 2,4-DAPG transcription, respectively (Bangera and Thomashow 1999). Located downstream of *phlF* lies *phlH*, a TetR-like regulator inactivation of which significantly reduces 2,4-DAPG-expression in the aforementioned biocontrol strain (Haas and Keel 2003).

1.1.3.5. Lipopeptides

Lipopeptides (LPs) are a diverse class of bioactive molecules associated with antagonism of enveloped viruses, mycoplasmas, trypanosomes, bacteria, fungi, and oomycetes (Raaijmakers et al. 2006). For plant-associated *Pseudomonas* spp., LPs have been implicated in motility, biofilm formation, and virulence (Raaijmakers et al. 2006). These molecules are composed of an oligopeptide linked to a fatty acid tail and exert their antibiotic activity by forming pores in the cell membrane causing cell lysis (Hutchinson et al. 1995; Hildebrand et al. 1998; Lindow and Brandl 2003). Synthesis of LPs is quite unique in that it involves multifunctional enzymes termed non-ribosomal peptide synthetases (NRPSs) (Maraheil et al. 1997). These synthetases are arranged as modules that allow for the incorporation of one amino acid into the peptide head. The modules are further subdivided into distinct domains including: i) an adenylation domain, which is responsible for amino acid activation; ii) a thiolation domain for thioesterification of the activated amino acid; and iii) a condensation domain where peptide bond formation between the amino acids occurs (Fig. 1.2). The newly formed linear peptide is frequently cyclised by a thioesterase domain. Because peptide synthesis by NRPS complexes is less specific compared to ribosomes, unusual amino acids may be incorporated into the peptide head (Doekel and Maraheil 2001). For a review of genes involved in the regulation of LP expression, please refer to Raaijmakers et al. 2006.

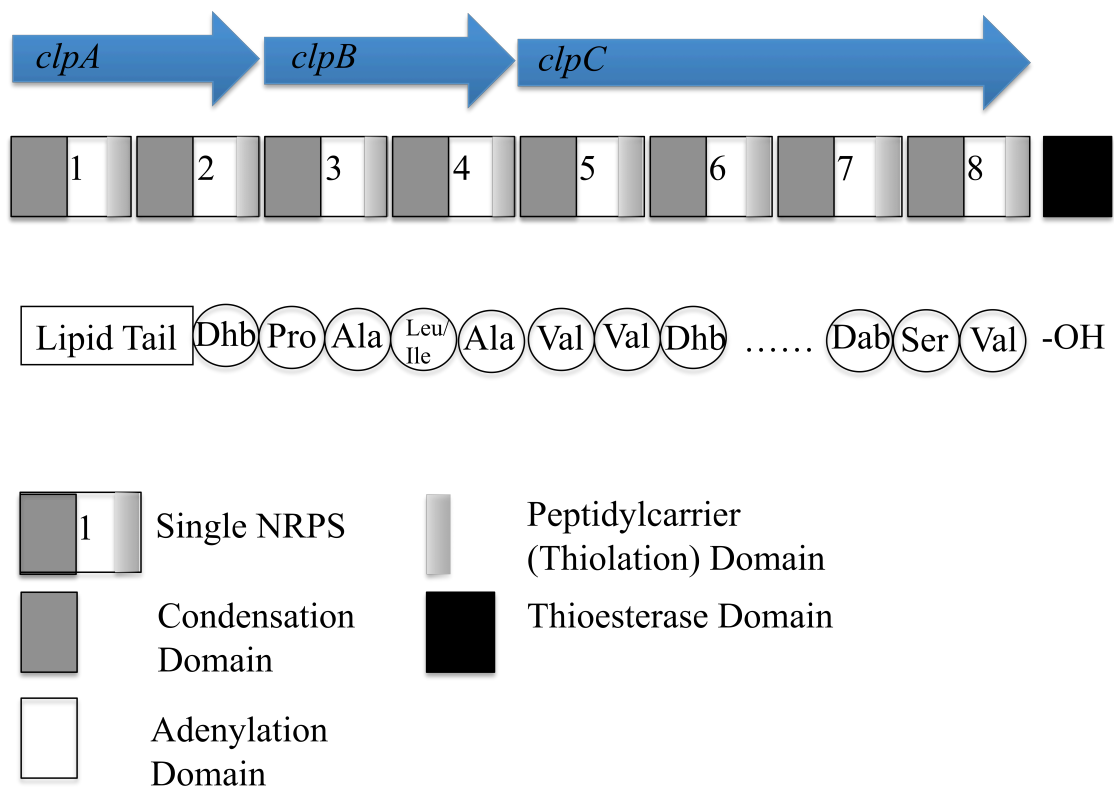


Figure 1.2. Schematic of the NRPS module cluster required for the synthesis of the peptide moiety of the CLP antibiotic. The genes *clpA* and *clpB* encode two modules while *clpC* codes for four modules. The partial peptide sequence shown above is that of *Pseudomonas* sp. DF41 where Dab and Dhb represent the non-proteinogenic amino acids 2,4-diaminobutyric acid and 2-amino-butenic acid.

1.1.3.6. *Hydrogen cyanide*

The volatile antibiotic hydrogen cyanide (HCN) is synthesized from glycine under microaerophilic conditions by the products of the *hcnABC* gene cluster (Voisard et al. 1989; Laville et al. 1998). Important for *P. fluorescens* CHA0-mediated biocontrol of *T. basicola*, antagonism is exerted by the production of cyanide from the ionization of HCN in water (Voisard et al. 1989; Blumer and Haas 2000). The resultant molecule is a potent inhibitor of metalloproteases (Blumer and Haas 2000).

1.1.3.7. *Siderophores*

Siderophores are another class of AF metabolites produced by fluorescent pseudomonads that chelate iron from the environment thereby inhibiting growth of fungal pathogens. *Pseudomonas* spp. produce a variety siderophores including pyoverdine, salicylic acid, and enantio-pyochelin (Ahl et al. 1986; Meyer et al. 1992; Youard et al. 2007). These molecules merely serve a supporting in role in the induction of biocontrol. A derivative of *P. fluorescens* 2-79, for example, only able to synthesize siderophores conferred little protection against take-all disease (Thomashow and Weller 1990).

1.1.3.8 *Cell-wall degrading enzymes*

In addition to antibiotics and siderophores, biocontrol pseudomonads produce extracellular cell-wall degrading enzymes that contribute to biocontrol. These include protease, alkaline protease, phospholipase C, lipase, hemi-cellulase, and

endochitinase, which all target components of the fungal cell wall (Nielsen et al. 1998; Thrane et al. 2001; Saito et al. 1992; Youard et al. 2007; Siddiqui et al. 2005).

1.1.4. Transcriptional and post-transcriptional regulation of secondary metabolites

1.1.4.1. Sigma factor-mediated regulation

Secondary metabolites are synthesized at the onset of stationary phase when nutrient levels wane. To facilitate a rapid response to environmental changes, bacteria utilize sigma factors to direct the core RNAP to a subset of genes. Sigma factors RpoD (σ^{70}), RpoS (σ^{38}), and RpoN (σ^{58}) have all been implicated in the regulation of AF activity. In *P. fluorescens* CHA0, over-expression of the housekeeping sigma factor RpoD was shown to enhance PLT and 2,4-DAPG-production (Maurhofer et al. 1992; Schnider et al. 1995). However, not all of these sigma factors are positive regulators of biocontrol. In fact, the positive effect may be antibiotic-specific. For instance, in *P. fluorescens* Pf-5, an *rpoS* mutation resulted in enhanced production of PLT and 2,4-DAPG (Sarniguet et al. 1995) while the same mutation in *P. chlororaphis* PCL1391 reduced phenazine-1-carboxamide (PCA) expression (Girard et al. 2006). Similarly, in *P. fluorescens* CHA0, mutation of *rpoN*, encoding the sigma factor that responds to nitrogen limitation, resulted in enhanced *phlA* transcription and 2,4-DAPG production, while both *plt* expression and PLT levels were reduced (Péchy-Tarr et al. 2005). For genes that show upregulation in the absence of RpoS or RpoN, it is believed that the housekeeping sigma factor can more effectively compete for the core RNAP and enhance the transcription of RpoD-

regulated genes. Therefore, a balance between RpoD and the alternative sigma factors appears to be important for maintaining optimal antibiotic levels.

1.1.4.2. *PsrA*

The TetR family regulator, PsrA (*Pseudomonas* sigma regulator), controls *rpoS* expression by binding to a palindromic sequence in the promoter region of this gene (Chatterjee et al. 2007; Chin-A-Woeng et al. 2005; Kojic and Venturi 2001; Kojic et al. 2002). Additionally, PsrA exhibits negative autoregulation (Kojic et al. 2002). Identified in a number of pseudomonads including *Pseudomonas aeruginosa*, *Pseudomonas putida*, *P. chlororaphis*, *P. fluorescens* and *Pseudomonas syringae* pv. tomato strain DC3000, PsrA has been linked to virulence and biocontrol (Chatterjee et al. 2007; Chin-A-Woeng et al. 2005; Humair et al. 2010; Kojic and Venturi 2001; Kojic et al. 2002). With regards to the latter phenotype, regulation by PsrA appears to be strongly influenced by growth conditions. In *P. chlororaphis* PCL1391 grown in minimal media, PsrA was found to positively regulate PCN production (Girard et al. 2006); whereas in rich media, PCN was repressed by this regulator (Chin-A-Woeng et al. 2005).

1.1.4.3. *Quorum Sensing*

Another regulatory mechanism controlling antibiotic production is quorum sensing (QS). This system coordinates gene transcription in response to population density through the production of acyl-homoserine lactone (AHL) molecules. AHLs either passively diffuse across the cell envelope or they can be effluxed out of cells

(Pearson et al. 1999). In both situations, AHLs increase in number as the cell population grows. Once a threshold level is achieved, AHLs bind to their cognate transcriptional regulator enabling activation or repression of target genes. More specifically, the R-protein-AHL complex binds to palindromic sequences located upstream of the QS-controlled gene. In *P. fluorescens* 2-79, *P. chlororaphis* 30-84, and *P. chlororaphis* PCL1391, PHZ biosynthesis is regulated by the PhzI/PhzR system (Chin-A-Woeng et al. 2005; Khan et al. 2005; Mavrodi et al. 2006). Genes encoding the AHL synthase (*phzI*) and the regulator (*phzR*) are located upstream of the *phz* biosynthetic operon (Mavrodi et al. 2006). In *P. chlororaphis* 30-84, a second system termed CsaI/CsaR has been identified that regulates protease production and cell surface properties, but not PHZ biosynthesis (Zhang and Pierson 2001). Unlike in *P. aeruginosa* where the two QS-systems LasI/LasR and RhII/RhlR interact (Pesci and Iglewski 1997), PhzI/PhzR and CsaI/CsaR appear to function independently (Zhang and Pierson 2001).

1.1.4.4. Post-transcriptional control by the Gac/Rsm system

The expression of secondary metabolites is also regulated at a post-transcriptional level through the GacA-GacS two-component system, which is present in a number of Gram-negative bacteria (Heeb and Haas 2001). The *gacS* gene encodes a sensor kinase while *gacA* codes for a response regulator. In strains belonging to *P. fluorescens* and *P. chlororaphis*, mutations in either *gacA* or *gacS* result in the loss of antagonism due to the inability to produce antibiotics and extracellular enzymes (Duffy and Défago 2000; Heeb and Haas, 2002; Poritsanos et

al. 2006; Whistler et al. 1998). GacS and GacA have also been shown affect motility and colony morphology (Schmidli-Sacherer et al. 1997).

Perhaps the best-studied Gac system is that of *P. fluorescens* CHA0 where it controls expression of 2,4-DAPG, PLT, PRN, HCN, and exoprotease (Haas and Keel 2003). In this organism, GacA and GacS mediate biocontrol through interaction with a second system termed Rsm (regulator of secondary metabolism). The Rsm system consists of two RNA-binding proteins, RsmA and RsmE, and three small untranslated RNA molecules, RsmX, RsmY, and RsmZ (Lapouge et al. 2008). RsmA and RsmE function to repress translation of target mRNA through interaction with the ANGGA motif in the ribosome-binding site. Activation of the Gac system occurs when an unknown environmental signal stimulates GacS autophosphorylation, followed by phosphotransfer to GacA (Heeb et al. 2004). Binding of the phosphorylated-form of GacA to a conserved sequence (TGAAGN6-CTTACA) located upstream of the *rsmX*, *rsmY*, and *rsmZ* genes allows for their expression (Lapouge et al. 2008). RsmX,Y and Z are able to bind to and sequester RsmA and RsmE, thereby alleviating the translational repression of biocontrol factors (Reimmann et al. 2005; Heeb et al. 2002; Kay et al. 2005).

The aforementioned regulatory systems do not function independent of one another. In fact, in *Pseudomonas* sp. MIS18, *P. fluorescens* CHA0, and *P. chlororaphis* PCL1391, both RpoS and the PhzI/PhzR QS system form part of the Gac regulon (Ge et al. 2006; Heeb et al. 2004; Girard et al. 2006; Chin-A-Woeng et al. 2005). Additionally, depending on the growth condition and the strain in question, the effect of RpoS on the PHZ QS system may be either stimulatory or inhibitory

(Girard et al. 2006; Chin-A-Woeng et al. 2005). From these examples, it is quite evident that regulation of biocontrol is complex, involving a multilayered process. This complexity is further complicated by the role of the environment on secondary metabolite production.

1.1.5. The plant and soil environment

In the rhizosphere, organisms are starved of nitrogen and oxygen, but often have an abundance of carbon from root exudates (Haas and Défago 2005). These conditions favor secondary metabolism allowing for slow growth rates and the development of high cell densities (Haas and Défago 2005). The phyllosphere represents a harsher environment where bacteria are subjected to stresses that may impact their antagonistic potential. Here, cells experience fluctuations in temperature and nutrient availability, moisture or dessication, as well as exposure to UV radiation and free radicals (Lindlow and Brandl 2003). In *P. chlororaphis* PCL1391, temperature and nutrient abundance have been found to influence PCN production; therefore, the prevailing conditions would be expected to impact antibiotic synthesis in the environment (van Rij et al. 2004). The ability of antagonistic bacteria to persist under stressful conditions may tip the balance between disease suppression and disease occurrence.

1.2. The Stringent Response

Microbial adaptation to environmental stress is crucial for survival. Bacteria cope with their environment by activation of stress responses such as the stringent

response (SR). Induction of the SR was initially observed in amino acid-starved *E. coli*, which were found to accumulate ppGpp and pppGpp and at the same time, reduce stable RNA synthesis (Cashel and Gallant 1969; Sands and Roberts 1952). Since its discovery over 40 years ago, the SR definition has grown to encompass all responses that increase production of this hyperphosphorylated GTP molecule.

1.2.1. (p)ppGpp metabolism

The hallmark of the SR is the production of ppGpp and pppGpp, which are collectively referred to as (p)ppGpp. In Gram-negative bacteria, synthesis of the (p)ppGpp occurs by the ribosome-bound pppGpp synthetase, RelA, that catalyzes the transfer of a pyrophosphate from ATP to GTP (Cashel et al. 1996). ppGpp is generated when a phosphate group is subsequently liberated from pppGpp by the guanosine pentaphosphatase (Cashel et al. 1996). Amino acid deprivation triggers RelA-mediated (p)ppGpp synthesis when an uncharged tRNA molecule bound to the “A” site of the ribosome effectively stalls protein synthesis. Once the alarmone (p)ppGpp has been synthesized, RelA dissociates and is free to engage with another stalled ribosome for (p)ppGpp production (Wendrich et al. 2002). Accumulation of the alarmone has been associated with a decrease in growth rate (Cashel et al. 1996). Therefore, it is imperative that proper levels of this molecule be maintained. When no longer needed, (p)ppGpp is hydrolyzed to GTP/GDP and a pyrophosphate by a bi-functional (p)ppGpp hydrolase, called SpoT (Cahsel et al. 1996). However, certain circumstances allow for SpoT-mediated alarmone production such as carbon, iron, phosphate, or fatty acid limitation (Hernandez and Bremmer 1999; Vinella et al.

2005; Seyfzadhey et al. 1993). In the absence of fatty acids, interaction an uncharged acyl carrier protein (ACP) with SpoT shifts its activity from hydrolysis to synthesis (Battesti and Bouveret, 2006). The SR, therefore, is activated in response to several different nutritional deficiencies.

In contrast to Gram-negative bacteria, Gram-positive bacteria possess a single Rel Spo homolog (RSH) with both synthetic and hydrolytic abilities (Potrykus and Cashel 2008). These functions are housed in overlapping domains in the N terminus of the RSH, as observed for Rel*Mtb* of *Mycobacterium tuberculosis* (Avarbock et al. 2005) and RelSeq of *Streptococcus equismilis* (Hogg et al. 2004). It has been suggested that physical interaction of the C-terminal domain with the N-terminal portion may act as a switch to turn the synthase activity of RelSeq on and its hydrolase activity off (Merchold et al. 2002). However, in Rel*Mtb*, the C-terminal domain is involved in the oligomerization of Rel*Mtb* molecules, which reduces their synthetic and hydrolytic activity (Avarbock et al. 2005). In addition to RSH, small truncated RelA-like proteins with weak (p)ppGpp-synthetic abilities were identified in *Streptococcus mutans* (Lemos et al. 2007) and *Bacillus subtilis* (Nanamiya et al. 2008).

1.2.2. (p)ppGpp, a global regulator

1.2.2.1. SR-affected promoters

Much of the regulation by (p)ppGpp is believed to occur at the transcriptional level. Certain promoter characteristics determine whether the alarmone has a repressive or inductive effect on gene transcription. More specifically, features of the

discriminator region, which lies between the TATA-box (-10 box) and the transcriptional start site, and the length of the linker region (between the -35 and -10 sequences) are believed to be involved in (p)ppGpp-mediated regulation. Based on shared characteristics of affected promoters, positively regulated genes have AT-rich discriminators and long linkers (greater than the consensus 17 bp) while SR-inhibited promoters have GC-rich discriminators and short linker regions (16-bp linker) (Potrykus and Cashel 2008).

1.2.2.2. (p)ppGpp targets the RNAP

Part of the novelty of (p)ppGpp as a transcriptional regulator stems from the fact that it exerts its effects by associating with the RNA polymerase (RNAP) as opposed to the DNA. This notion was initially postulated from the discovery that spontaneous mutations in the *rpoB*, *rpoC*, and *rpoD* genes restored prototrophy in *E. coli* CF1693, a (p)ppGpp⁰ derivative of MG1655 that is unable to grow in the absence of amino acids (Cashel et al. 1996; Murphy and Cashel 2003). Evidence of a direct association came from subsequent studies examining the ability of ppGpp to crosslink with the β - and β' -subunits (Chatterji et al. 1998; Touloukhonov II et al. 2001) and ppGpp co-crystallization with the RNAP of *Thermus thermophilus* (Artismovitch et al. 2004). In the latter study, the alarmone was positioned in the secondary channel of the RNAP near the catalytic centre where it is able to interact with both β - and β' -subunits. However, a consensus regarding the exact points of contact between ppGpp and these residues has not been established.

1.2.2.3. DksA, a co-regulator of the SR

DksA is a molecule that acts as a co-regulator of the SR (Paul et al. 2004; Paul et al. 2005). Because of its structural similarity to the well-characterized transcriptional elongation factors, GreA and GreB, it has been suggested that DksA inserts into the secondary channel of the RNAP to stabilize the ppGpp/RNAP interaction (Perederina et al. 2004). In the absence of accessory proteins, DksA was found to bind DNA with low affinity (Perederina et al. 2004). Although considered a co-factor, this regulator and (p)ppGpp appear to function independent of one another. When overexpressed, DksA is able to compensate for a lack of ppGpp with respect to amino acid auxotrophy, cell-cell aggregation, motility, filamentation, stationary-phase morphology, and RpoS accumulation (Magnusson et al. 2007). Transcriptome analysis revealed that not all of the (p)ppGpp-regulated genes are under DksA control (Aberg et al. 2009). In fact, those involved in chemotaxis and flagellum biosynthesis are differentially regulated by the two factors. Altogether, these findings suggest that DksA functions downstream of RelA and may possess distinct regulatory functions.

1.2.3. Direct and indirect effects of (p)ppGpp on gene expression

1.2.3.1. Direct Regulation

One of the best-characterized phenotypes of the SR is the inhibition of rRNA synthesis. Several models explaining this negative regulation have been proposed that involve (p)ppGpp-mediated inhibition of transcription initiation or elongation. In one model, (p)ppGpp and DksA together and independently decrease the stability of open-promoter complex formation by RNAP (Barker et al. 2000; Paul et al. 2004).

Association of the alarmone with the cytosine of the non-template strand further shortens the half-life of this complex (Artsimovitch et al. 2004). Although attractive in its simplicity, this model makes the assumption that only intrinsically unstable promoters are inhibited by (p)ppGpp/DksA while those that are stable are activated. However, an unstable promoter is not a requirement for negative regulation as the stable λ pR promoter is inhibited by ppGpp (Potrykus et al. 2002). Other explanations for the negative effect include the ability of the alarmone to decrease the rate at which the initial phosphodiester bond is formed, thereby stalling the RNAP and preventing it from clearing the promoter (Potrykus et al. 2002), as well as its ability to compete with NTPs for the active site of the RNAP (Jores and Wagner 2003). Moreover (p)ppGpp may increase pausing during transcriptional elongation (Kingston et al. 1998). The occurrence and potential synergistic action of all of these mechanisms is plausible and may be required to elicit the overall negative effect.

Little is known about the direct activation of (p)ppGpp-regulated promoters due to difficulty in distinguishing between the direct and indirect effects of interconnected processes. However, evidence of (p)ppGpp-mediated activation was found using a defined in vitro system where addition of only the alarmone and DksA to the RNAP machinery allowed for the activation of the λ paQ promoter and the promoters of several amino acid biosynthetic genes (Krasny and Gourse 2004; Potrykus et al. 2004). Aside from the hypothesis that intrinsically stable promoters are (p)ppGpp activated, it has been suggested that these factors increase the rate of an isomerization step leading to the formation of the open promoter complex (Paul et al. 2005).

1.2.3.2. Indirect effects of (p)ppGpp – sigma competition model

In addition to the direct effects of (p)ppGpp on gene regulation, the alarmone may passively influence transcription by modulating the availability of core RNAP. During rapid growth, 60-70% of the transcriptional machinery in *E. coli* is sequestered at the RpoD-dependent rRNA promoters (Bremer and Dennis 1996). Therefore, when (p)ppGpp levels are low, less RNAP is available for use by the alternative sigma factors. During periods of starvation, the pool of core RNAP is increased by the liberation of the transcriptional machinery from SR-negatively controlled promoters. It was observed that in the presence of (p)ppGpp, RpoS, RpoH, and RpoN were better able to out-compete RpoD for the core polymerase (Jishage et al. 2002; Laurie et al. 2003). Furthermore, the alternative sigma factors lost their dependency on ppGpp when RpoD was removed by mutation or depleted, indicating that the SR is not directly involved in transcription of genes that are dependent on alternative sigma factors. Additionally, (p)ppGpp and DksA have been demonstrated to not only induce *rpoS* expression (Brown et al. 2002; Cashel et al. 1996; Potrykus and Cashel 2008), but they also increase the stability of the RpoS protein. This is accomplished through the (p)ppGpp-mediated transcriptional induction of *iraP*, the gene product of which prevents targeting of RpoS by the ClpXP protease (Bougdour and Gottesman 2007). So by increasing in concentration, RpoS becomes a more effective competitor.

1.2.4. The SR and virulence

The SR has been implicated in the pathogenicity of several bacteria. For example, *relA* mutants of *Vibrio cholera* exhibit reduced virulence factor production (Haralalka et al. 2003) and in *Legionella pneumophila*, accumulation of (p)ppGpp resulted in a switch from the avirulent replicative form to the virulent form (Hammer et al. 2002). Furthermore, by use of a *Drosophila melanogaster* host model, Erickson et al. (2004) demonstrated that a *relA* mutant of *P. aeruginosa* PAO1 exhibited decreased killing compared to the wild type. Moreover, this phenomenon is not restricted to human pathogens. In the plant pathogen *Erwinia carotovora*, a mutation in the *relA* gene reduced pectate lyase and protease production (Wang et al. 2006). This suggests that the impact of the SR on virulence is wide spread.

1.3. Biological control of *Sclerotinia sclerotiorum*

1.3.1. *Sclerotinia sclerotiorum*

The ascomycete pathogen, *S. sclerotiorum* (Lib.) de Bary is able to infect over 400 species of plants (Purdy 1979) including canola, an economically important crop in the Canadian prairies (Manitoba Agriculture 2002). This pathogen is the causative agent of canola and sunflower stem rot (Nelson, 1998) and is responsible for the loss of 5-100% of canola crops in years that are favorable for disease development (Manitoba Agriculture 2002) making control of this organism vital.

Management of this fungal pathogen is hampered by its unique life cycle (Fig. 1.3). In infected soils, *S. sclerotiorum* persists as hard, irregular shaped, overwintering bodies termed sclerotia. In this form, the pathogen may survive for up to

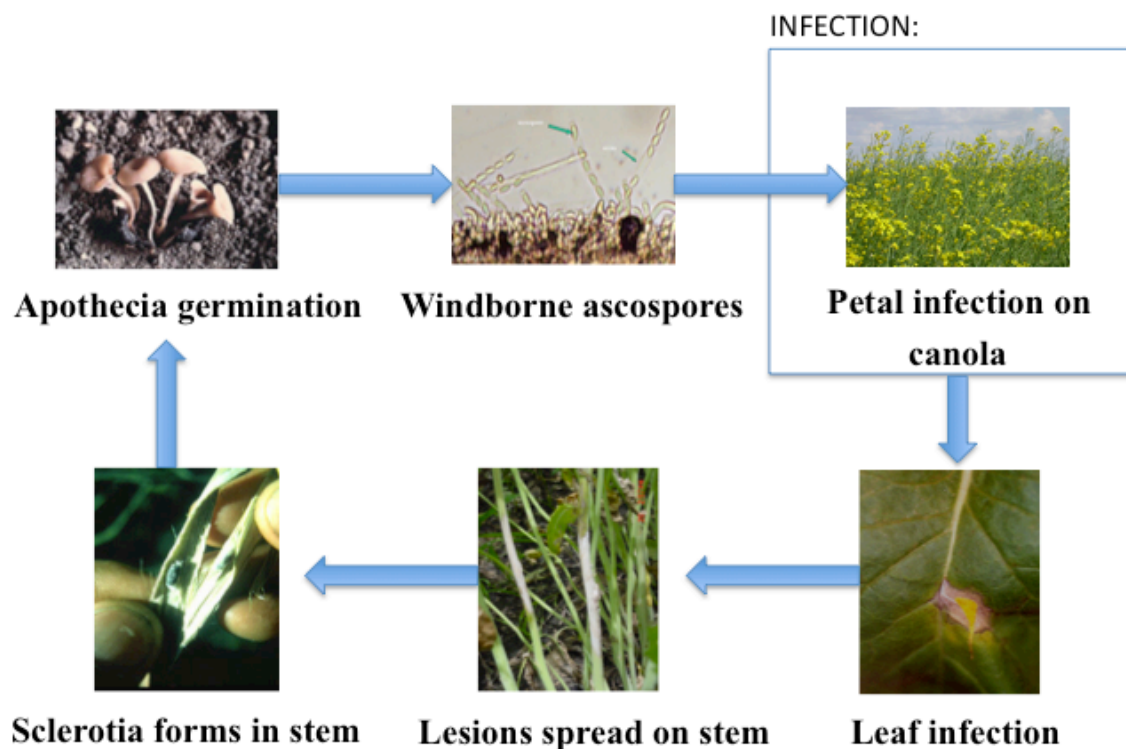


Figure 1.3. Life cycle of *S. sclerotiorum*. In infected soils, sclerotia germinate to into apothecia that release ascospores. Once windborne, these spores have the potential infect neighbouring crops. Canola is most susceptible to infection by *S. sclerotiorum* during the flower stage. When the contaminated petals comes into contact with other portions of the plant, the disease progresses on the leaf and stem ultimately causing stem bleaching and hollowing.

four years (Manitoba Agriculture 2002). During favorable conditions, sclerotia germinate into mushroom-like structures termed apothecia that release spores (Purdy 1979). Once airborne, these spores can travel up to one kilometer and potentially infect neighboring crops (Venette 1998). Canola is most susceptible to *S. sclerotiorum* infection at the flowering stage when ascospores land on the senescing flowers (Purdy 1979; Turkington and Morrall 1993). If infected petals come in contact with other portions of the plant, disease may progress to the stem causing it to whiten and hollow (Purdy 1979). *S. sclerotiorum* virulence has been attributed to the production of oxalic acid, a tissue-damaging agent that sequesters calcium from the middle lamellae (Bateman and Beer 1965; Godoy et al. 1990). This molecule is also known to neutralize the plant's oxidative burst (Cessna et al. 2000). Furthermore, the production of cell-wall degrading enzymes such as cellulases, hemi-cellulases, pectinases (Riou et al. 1991), proteases (Pousserau et al. 2001; Girard et al. 2004) and endopolygalacturonases (Cotton et al. 2003) allow access to the internal portions of the plant.

1.3.2. *Pseudomonas* species DF41

Pseudomonas species DF41 was isolated from canola (cv Cresor) root tips in Manitoba, Canada (Savchuk and Fernando 2004). This bacterium demonstrates excellent growth inhibition of *S. sclerotiniorum* in vitro and in planta (Savchuk and Fernando 2004). DF41 was found to prevent *S. sclerotiniorum* ascospore germination and elongation and branching of germ tubes (Savchuk and Fernando 2004). When co-

inoculated with the pathogen onto canola petals, DF41 is able to degrade ascospores by 24h (Savchuk and Fernando 2004). Several secondary metabolites that contribute to DF41 biocontrol include HCN, protease, and a novel LP molecule (Berry et al. 2010). The latter has been shown to be essential for DF41 biocontrol as an LP-deficient mutant, DF41-1278, exhibited diminished *S. sclerotiorum* growth inhibition (Berry et al. 2010).

1.3.2.1. Regulation of LP production

1.3.2.1.1 Gac System

The phenotypic screening of a transposon (Tn) library of DF41 for mutants with altered AF activity led to the isolation of DF41-469. This mutant had a Tn5 insertion in *gacS*, which rendered it unable to inhibit *S. sclerotiorum* growth (Berry 2010). Characterization of DF41-469 revealed a total loss of LP, protease and HCN production and reduced autoinducer (AI) and alginate production (Berry 2010). Because DF41-469 and the wild type display similar rates of colonization, the inability to inhibit *S. sclerotiorum* was mainly attributed to a lack of LP production. Aside from secondary metabolite production, motility was found to be under GacS control as DF41-469 exhibited increased swimming motility but was unable to swarm. Biofilm formation, on the other hand, remained unchanged.

1.3.2.1.2. Rsm Regulation

Because the Gac system is essential for DF41 biocontrol, it is not surprising that the Rsm system plays a role as well. The non-coding RNA, RsmZ was identified

in DF41 through PCR amplification (Berry 2010). Over expression of the *rsmZ* gene of *P. fluorescens* CHA0 in DF41-469 partially restored AF and protease activity, but fully complemented the *gacS* mutation with respect to AI and HCN production and motility (Berry 2010). Furthermore, in the *gacS* mutant, *rsmZ* transcription was significantly reduced, indicating that *rsmZ* is GacS controlled (Berry 2010). As is the case with other pseudomonads, it appears that in DF41 RsmZ functions downstream of GacS.

1.3.2.1.3. Regulation of *RpoS*

In DF41-469 *rpoS* transcription was reduced, indicating that RpoS is under GacS control (Berry 2010). Because GacS mediates its effects through RsmZ, the impact of this noncoding RNA on the transcription of *rpoS* was examined. Contrary to what was expected, multiple copies of *rsmZ* in trans in DF41 reduced *rpoS* transcription in DF41 (Berry 2010). The conflicting effects of RsmZ on RpoS suggest complexity in the regulation RpoS in DF41.

1.3.2.1.4. Quorum Sensing

It was discovered that DF41 employs QS as part of its lifestyle when this strain was found to produce AI signals (Berry et al. 2010). PCR amplification allowed isolation of an AI synthase gene, called *pdfI*, which confirmed the presence of a QS system in DF41 (Berry 2010). However, failed attempts to generate an AI-deficient strain through allelic exchange led to the supposition that DF41 may possess more than one QS system. As an alternative approach to understand how QS affects

biocontrol, plasmid pME6863, which harbors the *B. subtilis aiiA* gene was mobilized into DF41 resulting in AI degradation. It was discovered that the loss of AI did not impact DF41 biocontrol. In fact, the only phenotype that was altered was motility, which was negatively regulated by QS (Berry 2010). Furthermore, in DF41 and DF41-469, AI degradation increased *rpoS* and *rsmZ* transcription at 24 and 48 hours of growth (Berry 2010). As is typically the case with QS systems, *pdfI* transcription is autoregulated; its expression is reduced in the DF41 AI⁻ strain and enhanced upon addition of exogenous AHL (Berry 2010).

1.3.3. *Pseudomonas chlororaphis* PA23

Another antagonist of *S. sclerotiorum* is the soybean rhizosphere isolate *Pseudomonas chlororaphis* PA23 (Savchuk and Fernando 2004). When applied to canola as a foliar spray, PA23 reduces the incidence and severity of *S. sclerotiorum*-mediated stem rot (Fig. 1.4; Savchuk and Fernando 2004, Zhang 2004). PA23 biocontrol has been attributed to the collaborative effect of several secondary metabolites including PRN, PCA, 2-hydroxyphenazine (2-OH-PHZ), HCN, lipase and protease (Poritsanos et al 2006). However, PRN was found to have the greatest impact on antagonism as a PRN⁻ strain of PA23 showed a considerably reduced ability to inhibit *S. sclerotiorum* growth on canola compared to the wild type (Selin et al. 2010). Furthermore, PA23-63, a PHZ⁻ strain harboring a Tn insertion in the *phzE* gene, generated 2-fold more PRN and exhibited enhanced AF activity in vitro compared to



Figure 1.4. Disease suppression by *P. chlororaphis* PA23 in planta. Canola plants were sprayed with PA23 or derivatives thereof prior to the application of *S. sclerotiorum* ascospores (Pots #1-3), ascospores only (Pot #4), or received no application of either organism (Pot #5). Note that Pot #2 was treated with a derivative of PA23 that lacks biocontrol activity while the complemented form of this organism was utilized in Pot #3.

PA23, indicating that PHZs are not essential for PA23 biocontrol of *S. sclerotiorum* (Selin et al. 2010).

1.3.3.1. Gac system

Similar to DF41, Tn5 mutagenesis followed by phenotypic screening allowed for the isolation of a *gacS* mutant of PA23 termed PA23-314 (Poritsanos et al. 2006). This mutant failed to inhibit the growth of the pathogen on canola primarily due to an inability to produce antibiotics and degradative enzymes (Poritsanos et al. 2006). Not only was PA23-314 unable to produce AI, but it also exhibited reduced RpoS expression, indicating that in PA23 both RpoS and QS are under Gac control (Poritsanos et al. 2006). As previously discussed, Gac exerts its effects through the Rsm system. RsmZ, and the inhibitory proteins RsmA and RsmE have been identified in PA23 (Selin and de Kievit, unpublished) and their role in biocontrol is currently being elucidated.

1.3.3.2. Quorum Sensing

Upstream of the PA23 PHZ biosynthetic operon sits *phzI* and *phzR*, encoding an AI synthase and a transcriptional regulator, respectively (Selin and de Kievit, unpublished). In order to determine the impact of the PhzI/PhzR QS system on biocontrol, QS-deficient strains were generated through mutation of the *phzR* gene and by introduction of a plasmid-borne copy of *aiiA* into PA23 (Selin and de Kievit, unpublished). As mentioned earlier, *aiiA* encodes an AHL lactonase enzyme that degrades AI molecules. Both QS-deficient strains lacked fungal antagonism and

were unable to produce PRN and PHZ; however, lipase was not affected (Selin and de Kievit, unpublished). Protease expression differed slightly between the two strains as PA23*phzR* exhibited reduced activity while the strain harboring the *aiiA* gene was rendered protease deficient (Selin and de Kievit, unpublished). These results clearly indicate that expression of AF metabolites is under QS control in this bacterium.

1.3.3.3. *RpoS*

Because RpoS has been linked to antibiotic production in biocontrol pseudomonads, an investigation of its role in PA23 antagonism was undertaken. A PA23 *rpoS* mutant was generated, called PA23*rpoS* that exhibited enhanced growth inhibition of *S. sclerotiorum* in vitro (Selin and de Kievit, unpublished). The observed increase in antifungal activity likely resulted from elevated production of the AF factors PRN, protease, and lipase by the mutant strain compared to the wild type (Selin and de Kievit, unpublished). However, PHZ biosynthesis remained unchanged (Selin and de Kievit, unpublished). RpoS was found to negatively affect *prnA* transcription but did not alter that of *phzA* (Selin and de Kievit, unpublished). The biosynthetic genes for protease and lipase have not been identified in PA23; consequently, transcriptional analysis was not performed. Altogether, these findings indicate that other sigma factors such as RpoD or RpoN likely control the expression of PRN, protease, and lipase further highlighting the importance of the balance among sigma factors. With respect to QS, PA23*rpoS* exhibited reduced AI production and *phzI* transcription while the transcription of *phzR* was enhanced (Selin

and de Kievit, unpublished). This suggests that under certain situations, PhzI and PhzR may be independently regulated.

1.3.4. The impact of the SR on biocontrol

Perhaps the most well studied bacteria with regards to the impact of the SR on antibiotic production are the *Streptomyces* spp. (Chakraborty and Bibb 1997; Gomez-Escribano et al 2008; Hesketh et al 2001; Hoyt and Jones 2004; Jin et al. 2004; Jin et al. 2004; Martinex-Costa et al. 1996). Depending on the strain of *Streptomyces*, the antibiotics produced and the growth media employed, the SR has been found to either positively (Chakraborty and Bibb 1997; Hesketh et al 2001; Hoyt and Jones 2004; Jin et al. 2004; Jin et al. 2004; Martinex-Costa et al. 1996) or negatively regulate antibiotic expression (Gomez-Escribano et al. 2008). For instance, mutation of the *relA* gene of *Streptomyces clavuligerus* resulted in elevated levels of clavulanic acid and cephamycin C (Gomez-Escribano et al. 2008). Moreover, transcription of the clavulanic acid and cephamycin C biosynthetic genes was increased substantially in the *relA*-deficient strain (Gomez-Escribano et al. 2008). However, in *Streptomyces coelicolor* ATCC27064, production of the latter antibiotic was abolished in the *relA* mutant compared to the wild type when grown in phosphate-limiting media (Jin et al. 2004). Therefore, the SR appears to have an unpredictable effect on antibiotic production. With the exception of *Pseudomonas* sp. MIS38, very little is known about the impact of the SR on fungal antagonism by pseudomonads. In MIS38, a transposon insertion in *spoT* lead to enhanced (p)ppGpp production and reduced expression of the antibiotic arthrofactin (Washio et al. 2010).

Thus in this strain, the SR negatively regulates arthrofactin production. Clearly more work is needed to determine the effect of the SR on antagonistic traits of other pseudomonads. Biocontrol bacteria are often forced to survive under nutrient-depleted conditions and as such, understanding the impact of the SR on secondary metabolism is critical since this directly impacts antagonism.

1.4. Thesis objectives

The devastating effects of *S. sclerotiorum* on canola may be attenuated when the biocontrol strains *Pseudomonas* sp. DF41 and *P. chlororaphis* PA23 are applied as a foliar spray. As previously discussed, antagonism demonstrated by these strains is attributed to the production of secondary metabolites. A link between the SR and antibiotic production exists in *Streptomyces*; however, very little is known about the role of the SR in biological control by pseudomonads. Therefore, we sought to evaluate the impact of the SR on the antagonistic potential of DF41 and PA23. The main objectives of this thesis are as follows:

1. To determine how the SR affects DF41 and PA23 antifungal activity.
2. To elucidate the impact of the SR on secondary metabolite production by these two bacterial strains.
3. To decipher where the SR falls within the regulatory hierarchy of DF41 and PA23 biocontrol.

Chapter 2.

The Stringent Response Represses *Pseudomonas* sp. DF41 Antifungal Activity

Applied and Environmental Microbiology

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2.2 Introduction

A number of bacteria are able to antagonize the effects of fungal pathogens through a process known as biological control. *Pseudomonas* sp. DF41 is one such bacterium that has demonstrated excellent antifungal activity against *Sclerotinia sclerotiorum* (Lib.) de Bary in both greenhouse and field assays (Berry et al. 2010, Savchuk and Fernando 2004). Strain DF41 produces several secondary metabolites that are believed to contribute to biocontrol including hydrogen cyanide, protease, and a novel lipopeptide (LP) molecule (Berry 2010, Berry et al. 2010). LP production has been shown to be essential for strain DF41 biocontrol as an LP-deficient mutant, DF41-1278, demonstrated greatly reduced *S. sclerotiorum* inhibition (Berry 2010, Berry et al. 2010). LP synthesis is somewhat unique in that it does not involve ribosome-generated proteins; instead these compounds are synthesized on large, multimodular enzymes termed NRPS (Marahiel et al. 1997).

Several regulators have been found to govern secondary metabolite production in biocontrol strains of *Pseudomonas*. For example, the Gac two-component signal transduction system comprised of the sensor kinase GacS and its cognate response regulator GacA is essential for biocontrol. A mutation in either *gacS* or *gacA* results in a loss of biocontrol activity in several pseudomonads, including strain DF41 (Berry et al. 2010, Heeb and Haas 2001). The alternative stationary-phase sigma factor, RpoS, has also been implicated in secondary metabolite production; however, regulation by RpoS appears to differ among species of pseudomonads. For example, an *rpoS* mutant of *Pseudomonas chlororaphis* PCL1391 exhibited decreased production of phenazine-1-carboxamide compared to

the wild type (Girard et al. 2006), whereas in *Pseudomonas fluorescens* Pf-5, an *rpoS* mutation resulted in enhanced PLT and 2,4-DAPG expression (Sarniguet et al. 1995). Several other global and pathway-specific regulators have been identified that influence biocontrol properties of pseudomonads (Haas and Défago 2005).

In addition to these regulatory elements, environmental conditions can have a significant impact on bacterial metabolism. When bacteria such as strain DF41 colonize the plant environment, they often experience dramatic fluctuations in many environmental conditions including nutrient availability. One means by which bacteria are able survive starvation is through induction of a global stress mechanism known as the SR. During the SR, cells accumulate the nucleotides ppGpp and pppGpp, collectively referred to as (p)ppGpp. In γ and β proteobacteria, (p)ppGpp accumulation is controlled by two enzymes known as RelA and SpoT (Potrykus and Cashel 2008 and references therein). RelA is a synthetase that generates (p)ppGpp when available amino acids are in limiting amounts. SpoT is a bifunctional enzyme that can act as either a hydrolase or a synthetase depending on the conditions present (Potrykus and Cashel 2008). (p)ppGpp exerts its influence on cell physiology by binding the RNAP near the catalytic site. This leads to increased transcription of certain genes, for example those involved in amino acid biosynthesis, and decreased transcription of others, for instance tRNA and rRNA genes (Potrykus and Cashel 2008). Accordingly, the SR enables bacteria to alter their gene expression to favor activities that promote survival under nutrient-limiting conditions.

Although the SR has been shown to affect antibiotic production in *Streptomyces* species (Chakraborty and Bibb 1997, Gomez-Escribano et al. 2008,

Hesketh et al. 2001, Hoyt and Jones 1999, Jin et al. 2004), there is a paucity of knowledge regarding how this global stress response influences biocontrol traits in pseudomonads. The purpose of this study was to investigate the impact of the SR on strain DF41 antifungal compound production. In addition, we examined whether there was a link between the SR and other known regulators of biocontrol, including the GacS/GacA two-component regulatory system and RpoS.

2.3. Materials and Methods

2.3.1. Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 2.1. *Escherichia coli* strains were cultured on Lennox Luria Bertani (LB) agar (Difco Laboratories, Detroit, MI). *Pseudomonas sp.* DF41 and its derivatives were routinely cultured at 28°C on King's B agar plates supplemented with 2% glycerol (King et al. 1954). (p)ppGpp analysis was performed using cells grown in morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich Canada, Oakville, ON) media as described by Cashel (1994) supplemented with 1% casamino acids (Difco) and 400 µg/ml of DL-serine hydroxymate (Sigma). For LP analysis, cells were grown in M9 minimal media (Difco) supplemented with 1% casamino acids, 1 mM MgSO₄, and 0.2% glycerol. For β-galactosidase assays, strains were grown in M9 media supplemented with 1 mM MgSO₄ and 0.2% glucose. *S. sclerotiorum* was maintained on Potato Dextrose Agar (PDA; Difco). As required, media were supplemented with the following antibiotics from Research Products International Corp. (Mt. Prospect, IL): gentamicin (Gm; 20 µg/ml), tetracycline (Tc; 15 µg/ml), piperacillin (Pip; 100 µg/ml), rifampicin (Rif; 100 µg/ml) for strain DF41, and ampicillin (Amp; 100 µg/ml), Gm (15 µg/ml), Tc (15 µg/ml) for *E. coli*.

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

TABLE 2.1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype or relevant phenotype ^a	Source or reference
Strains		
<i>P. aeruginosa</i> PAO1	Wild type	Holloway et al. 1979
<i>Pseudomonas</i> sp. DF41	Rif ^R ; Wild type (canola root tip isolate)	Savchuk and Fernando 2004
DF41 <i>relA</i>	DF41 with a Gm ^R cassette inserted into the <i>relA</i> gene	This study
DF41 <i>relAspoT</i>	DF41 <i>relA</i> with a Tet ^R cassette inserted into the <i>spoT</i> gene	This study
DF41-469	Rif ^R ; <i>gacS</i> ::Tn5-1063 genomic insertion	Berry et al. 2010
DF41-1278	Rif ^R ; <i>lp</i> ::Tn5-1063 genomic insertion	Berry et al. 2010
1278 <i>relA</i>	DF41-1278 with a Gm ^R cassette inserted into the <i>relA</i> gene	This study
1278 <i>relAspoT</i>	1278 <i>relA</i> with a Tet ^R cassette inserted into the <i>spoT</i> gene	This study
<i>E. coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hadR17 recA1 endA1 gyrA96 thi-1relA1</i>	Hanahan 1983
Plasmids		
pLP170	Promoterless <i>lacZ</i> transcriptional fusion	Poritsanos et al. 2006
pRPOS- <i>lacZ</i>	<i>rpoS</i> promoter in pLP170	This study
pSW205	Promoterless <i>lacZ</i> translational fusion	Gambello et al. 1991
pSW <i>relA</i>	<i>relA</i> promoter containing 72 nucleotides upstream of ATG and the ribosome binding site cloned into pSW205	This study
pCR2.1	TA cloning vector Amp ^R	Invitrogen
pCR <i>relA</i> -41	<i>relA</i> in pCR2.1	This study
pCR <i>spoT</i>	<i>spoT</i> in pCR2.1	This study
pEX18Ap	Suicide plasmid Amp ^R	Hoang et al. 1998
pEX <i>relA</i>	<i>relA</i> in pEX18Ap	This study
pEX <i>relA</i> -800	pEX18Ap with an 800 bp deletion in <i>relA</i>	This study
pEX <i>relA</i> -Gent	pEX <i>relA</i> -800 Gent ^R	This study
pEX <i>spoT</i>	<i>spoT</i> in pEX18Ap	This study
pEX <i>spoT</i> -Tet	pEX <i>spoT</i> with Tet ^R inserted into <i>spoT</i>	This study

pUCP22	Broad-host-range vector Amp ^R Gent ^R	West et al. 1994
pUCPrelA	<i>relA</i> in pUCP22	This study
pUCPspoT	<i>spoT</i> in pUCP22	This study
pUCP22- <i>rpoS</i>	<i>rpoS</i> in pUCP22	Poritsanos et al. 2006
pUCP23- <i>gacS</i>	<i>gacS</i> in pUCP23	Poritsanos et al. 2006
pME- <i>rpoS</i>	<i>rpoS</i> in pME6010	This study
pME6010	pVS1-p15A shuttle cloning vector Tet ^R	Heeb et al. 2000
pME3219	pME6010 containing an <i>hcnA-lacZ</i> translational fusion	Laville et al. 1998
pRK600	Contains <i>tra</i> genes for mobilization; Chl ^R	Finan et al. 1986
pUCGm	Source of Gm ^R cassette	Schweizer 1993
pFTC1	Source of Tet ^R cassette	Choi et al. 2002
Primers ^b		
relA-FOR	5'-accgtggtaaagggtaggcaag-3'	This study
relA-REV	5'-gggaaatcccctgctctatg-3'	This study
relAtransl-FRW	5'- ggaattc ccgctttttcaagccgat-3'	This study
relAtransl-REV	5'-gaggatctcggcgatctcca-3'	This study
spot-FOR	5'-gcgtcaccgttgaagactg-3'	This study
spot-REV	5'-ttactcgaggacgacgatgg-3'	This study
rpoSF	5'-tacgtcagtgttacggcca-3'	This study
rsmZR	5'-tatgaccgcccacattttt-3'	This study
P170fecorpoS	5'-tgt gaattc gggagggaca-3'	This study
P170rxbarpoS	5'-agt ttaga atcaccacttcccattgctt-3'	This study

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

^b Items in bold represent restriction sites.

2.3.3. Sequence analysis and nucleotide accession numbers. PCR products were sequenced at the Centre for Applied Genomics at The Hospital for Sick Children (Toronto, Ontario) and the sequences were analyzed with Blastn and Blastx databases.

The GenBank accession numbers for the sequences of the DF41 *rpoS*, *relA* and *spoT* genes are EU595545.1, HQ615419, and HQ615420, respectively.

2.3.4. Generating *relA* and *relAspoT* mutants of strains DF41 and DF41-1278.

All primers used for the construction of mutant strains are listed in Table 1. Strain DF41*relA* was generated as follows. The *relA* gene of strain DF41 was amplified using primers relA-FOR and relA-REV. The resulting 2.4-kb fragment was then cloned into pCR2.1-TOPO (Invitrogen) to generate pCR*relA*-41. To liberate the insert, pCR*relA*-41 was digested with *Bam*HI and *Eco*RI. The 2.4-kb fragment was subcloned into the same sites of suicide vector pEX18Ap (Hoang et al. 1998). Next, an 865-bp *Sal*I fragment containing the Gm^R cassette from pUCGm (Schweizer 1993) was cloned into the *Sal*I site of pEX*relA* to create pEX*relA*-Gent. Allelic exchange through tri-parental mating between the donor [*E. coli* DH5 α (pEX*relA*-Gent)], helper [*E. coli* DH5 α (pRK600)] and recipient [either DF41 or DF41-1278] was used to replace the wild type *relA* with the mutated copy of the gene. Transconjugants were screened on LB agar supplemented with 100 μ g/ml Rif and 20 μ g/ml Gm. Bacteria that had undergone a double crossover event were selected on LB agar containing 20% sucrose and 20 μ g/ml Gm. Verification of the mutation was achieved by PCR using the same primers. For creation of the DF41*relAspoT* mutant, a 2.7-kb fragment

containing the *spoT* gene was PCR amplified using primers *spoT*-FOR and *spoT*-REV. The resulting PCR product was subsequently cloned into pCR2.1-TOPO to generate pCR*spoT*. The *spoT* gene was then subcloned into pEX18Ap via *SacI* and *XbaI* sites. The resulting plasmid, pEX*spoT*, was digested with *SmaI* and ligated with a 2.0-kb *SmaI* fragment containing the Tc^R marker from pFTC1 (Choi et al. 2005) to generate pEX*spoT*-Tet. Triparental mating was performed using *E. coli* DH5 α (pEX*spoT*-Tet), *E. coli* DH5 α (pRK600) and either strains DF41*relA* or 1278*relA*. Transconjugants were screened on LB agar supplemented with 15 μ g/ml Tc and 100 μ g/ml of Rif. Sucrose plates containing Tc were used to identify bacteria that had undergone a double cross-over event. To confirm the insertion of the Tc marker into the *spoT* gene, Southern blot analysis was performed. Southern hybridization was performed using a *spoT*-DIG labelled probe, which was generated by PCR amplification of the *spoT* gene using primers *spoT*-FOR and *spoT*-REV. For Southern blot analysis, genomic DNA isolated from DF41*relA* and 1278*relA* were digested with *Bam*HI restriction enzymes and separated on an agarose gel. The DNA was depurinated by treating the gel with 0.25M HCl for 10 min, followed by a neutralization treatment for 45 min using a solution of 1.5M NaCl and 1.0M Tris-HCl (pH 7.5). Alkaline capillary transfer in 10X SSC allowed for the migration of genomic DNA from the agarose gel to Hybond-NTM nitrocellulose membrane (Amersham Biosciences). After rinsing in 5X SSC for 10 min with gentle agitation, the nucleic acids were crosslinked to the membrane by exposure to UV light. A 50 mL aliquot of prehybridization solution was added to the membrane, which was allowed to equilibrate overnight at 65°C in a ProBlot hybridization oven (Mandel,

Canada). The blot was then hybridized at the same temperature for 24 h with a *spoT*-DIG DNA labelled probe. Any unbound probe was removed by two 15 min washes with a solution of 2X SSC and 0.1% SDS at room temperature, followed by two 30 min washes with 0.5X SSC and 0.1% SDS at 65°C. The membrane was incubated with washing buffer for 5 min followed by blocking solution for 30 min. A solution containing anti-DIG polyclonal antibody conjugated to alkaline phosphatase (diluted 1:10, 000 in 1X blocking solution) was added to the blot for 60 min. The membrane was then rinsed three times with water, incubated with washing buffer for 15 min, and rinsed three more times with water before equilibration in 10 mL of detection buffer for 5 min. Antibodies were detected using chemiluminescence detection reagent CDP-Star® (Roche Diagnostics, Mannheim, Germany) and blots were developed overnight using Kodak X-Omat Blue film (Fisher).

2.3.5. Construction of plasmids. All primers are listed in Table 1. To generate the *relA* over-expression plasmid pUCP*relA*, primers relAtranslFRW and relA-REV were used to amplify a 2.9-kb fragment. The PCR product was cloned into pCR2.1-TOPO to yield pCR*relA*-41. pCR*relA*-41 was then digested with *EcoRI*, and the 2.8-kb fragment containing the *relA* gene was cloned into pUCP22 thus placing the gene under the control of the plasmid-borne lac promoter. The *spoT* over-expression vector pUCP*spoT* was created by first amplifying the *spoT* gene from strain DF41 using primers spoT-FOR and spoT-REV. The 2.8-kb fragment was cloned into pCR2.1-TOPO to generate pCR*spoT* which was subsequently digested with *XbaI*. The linearized plasmid was treated with Klenow DNA polymerase (Invitrogen), and

digested again with *Bam*HI. The 2.8-kb insert was cloned into the *Bam*HI and *Sma*I sites of pUCP22 such that the transcription of *spoT* is dependent on the lac promoter. To create an *rpoS-lacZ* transcriptional fusion, the *rpoS* gene was amplified from DF41 genomic DNA using primers rpoSF and rsmZR. The resulting 1.7-kb fragment was cloned into the pCR2.1-TOPO vector resulting in pCR2.1-*rpoS*. Using pCR2.1-*rpoS* as the template, a 1.1-kb fragment was amplified using primers P170fecorpoS and P170rxbarpoS which contain *Eco*RI and *Xba*I sites, respectively. The PCR product was digested with *Eco*RI and *Xba*I and cloned into the same sites of pLP170 (Preston et al. 1997), generating pRPOS-*lacZ*. For the *relA-lacZ* translational fusion pSW*relA*, a 644-bp fragment containing 72 nucleotides upstream of the ATG translational start site was PCR amplified from strain DF41 genomic DNA using primers relAtranslFRW and relAtranslREV. The product was digested with *Eco*R1 and *Sma*I and cloned into the same sites of pSW205 (Gambello and Iglewski 1991). To create pME-*rpoS*, containing *rpoS* constitutively expressed from the kanamycin promoter, a 1.3-kb *Kpn*I-*Hind*III fragment was isolated from pUCP22-*rpoS* and ligated into the same sites of pME6010 (Heeb et al. 2000).

2.3.6. (p)ppGpp and *relA* expression analysis. Determination of (p)ppGpp levels was performed as described by Cashel (1994) with the following modifications. Cells were grown overnight in MOPS minimal medium (Cashel 1994) at 28°C followed by a 1/100 dilution of the culture in MOPS phosphate-free minimal medium containing 1 mg/ml of casamino acids and 100 µCi /ml of ³²P (Perkin Elmer, Waltham, MA). Three 200-µl aliquots of culture were added to wells of a polystyrene microtitre plate

(Costar; Corning Incorporated, Corning, NY) and grown at 28°C for an additional 8 hours. DL-serine hydroxymate was added to each well at a concentration of 400 µg/ml and allowed to incubate for 2 hours. To ensure that an equivalent amount of cells for each strain are to be extracted, on a parallel plate, PA23 and its derivatives were grown as described above with the following modifications. MOPS phosphate-free minimal media was supplemented with 40 µM of KH₂PO₄ in replace of ³²P and five 200 µl aliquots of culture were added to the wells of the microtitre plate. After growth with serine hydroxymate, these aliquots were then pooled to determine their cellular densities at a wavelength of 600 nm. To ensure that an equivalent number of cells were extracted for each strain, DF41 and its derivatives were grown on a parallel plate as described above, but excluding the ³²P. All of the cultures showed an equivalent turbidity (OD₆₀₀). Thus, the three radiolabeled 200-µl aliquots were pooled for each strain and nucleotides were extracted with an equal volume of cold 13 M formic acid. A 20-µl aliquot of the nucleotide sample was separated on polyethyleneimine-cellulose chromatography sheets (Sigma) using 1.5M KH₂PO₄ as the solvent, which was allowed to ascend the plate for 2.5 hours. The spots were visualized by autoradiography. For the (p)ppGpp analysis, *Pseudomonas aeruginosa* strain PAO1 was included as a positive control and the experiments were repeated five times. Expression of a *relA*–*lacZ* translational fusion was monitored in strains DF41, DF41*relA*, DF41*relAspoT* and the *gacS* mutant strain DF41-469. Strains harboring pSW*relA* were grown for 4, 8, 16, and 24 hours and then assayed for β-galactosidase activity (Miller 1972).

2.3.7. Antifungal assays. To assess the ability of strain DF41 and its derivatives to inhibit fungal growth in vitro, radial diffusion assays were performed according to Poritsanos et al. (Poritsanos et al. 2006). Five replicates for each strain were analyzed and the experiments were repeated three times.

2.3.8. HPLC analysis. LP was extracted from cultures and analyzed by HPLC as described by Berry et al. with the following modifications (Berry et al. 2010). To determine the efficiency of the extraction, surfactin (Sigma) was used as an internal control. Cell free supernatants were spiked with a 500- μ l aliquot of surfactin (1 mg/ml stock concentration), which was extracted with the LP. The chromatograms obtained for each strain were normalized using the surfactin peak height. We have previously shown that the peak at 28 minutes corresponds to the DF41 LP (Berry et al. 2010); consequently, the amount of LP present in the extracts was determined by measuring the height of the 28-minute peak.

2.3.9. Bioluminescence. Five-ml cultures were grown in M9 minimal media + 1 mM MgSO_4 + 0.2% glucose and assayed for OD_{600} to determine the number of cells present. The cultures were centrifuged and resuspended in an equal volume of 1 x phosphate buffered saline (PBS). A 1 ml-aliquot of the cell suspension was mixed with 1 ml of 2 x 523 media supplemented with 10 mg/L of sodium citrate (Kado and Keskett 1970). A 2.5- μ l aliquot of a 10% *n*-decanal solution was added to each tube, which was mixed for 15 seconds with a vortex mixer, and measured for bioluminescence 90 seconds later using a BG-P luminometer (GEM Biomedical Inc.,

Hamden, CT). Bioluminescence was expressed as relative light units (RLU) by dividing the total bioluminescence signal by the OD₆₀₀ for each respective strain.

2.3.10. HCN analysis. Qualitative determination of hydrogen cyanide production was performed using Cyantesmo paper (Machery-Nagel GmbH & Co., Duren, Germany). Plasmid pME3219, harboring an *hcnA-lacZ* translational fusion (Laville et al. 1998), was mobilized into strains DF41, DF41*relA* and DF41*relAspoT*. Strains were grown for 24 hours and then assayed for β -galactosidase activity. Samples were analyzed in triplicate and experiments were repeated three times.

2.3.11. Protease production. Herein, cultures were grown in M9 minimal media supplemented with 1 mM MgSO₄, 0.2% glucose, and 1.5% skim milk (Difco) for 5 days at 28°C to induce protease production. A 200- μ l aliquot of cell-free supernatant was analyzed for the activity of this enzyme in a 0.65% solution of casein according to Cupp-Enyard (2008). 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 colored chromophore (Cupp-Enyard 2008). This chromophore is measured spectrophotometrically at a wavelength of 660 nm. To determine the amount of tyrosine liberated, a standard curve was generated using pure tyrosine at the following concentrations (μ Mole): 0.055, 0.111, 0.221, .0442, and 0.553. Each strain was assayed in triplicate and experiments were performed three times.

2.3.12. *rpoS* expression in strains DF41, DF41*relA* and DF41*relAspoT*.

Expression of the *rpoS* gene was monitored using pRPOS-*lacZ*. Cultures of strains DF41, DF41*relA*, and DF41*relAspoT* harboring pRPOS-*lacZ* were grown for 4, 8, 16, 24, and 48 hours and then measured for β -galactosidase activity.

2.4. Results

2.4.1. Generation of strain DF41 *relA* and *relAspoT* mutants and (p)ppGpp

analysis. To determine the impact of the SR on the biocontrol activity strain DF41, *relA* and *relAspoT* mutants were created. Primers designed from the *relA* and *spoT* sequences of *Pseudomonas aeruginosa* PAO1 were used to amplify these genes from strain DF41 genomic DNA. Sequence analysis revealed the DF41 alleles to be 99% identical to the *relA* and *spoT* genes of *P. aeruginosa* PAO1 (AE004091.2), 85% identical to those of *P. fluorescens* Pf0-1 (CP000094.2) and 84% identical to the aforementioned genes of *P. fluorescens* Pf-5 (CP000076.1). Through allelic exchange, strain DF41 *relA* and *relAspoT* mutants were generated. Double cross-over mutations were confirmed by PCR (Fig. 2.1) and Southern blot analysis (Fig. 2.2). We were unable to isolate a *spoT* single null mutant indicating that the absence of SpoT in a *relA*⁺ background might be lethal, similar to what has been reported for other bacteria (Potrykus and Cashel 2008, Xiao et al. 1991).

Next, we analyzed (p)ppGpp levels in the aforementioned strains. In the presence of DL-serine hydroxymate which induces the SR, no (p)ppGpp was detected in the *relA* and *relAspoT* mutants unlike in strain DF41 which produced both nucleotides (Fig. 2.3A). The presence of pUC*PreLA*, a vector harboring the strain DF41 *relA* gene, restored (p)ppGpp production in both mutants (Fig. 2.3A, lanes 5 & 6). The presence of *spoT* on plasmid pUC*PspoT*; however, did not alter (p)ppGpp production in the mutants (Fig. 2.3A).

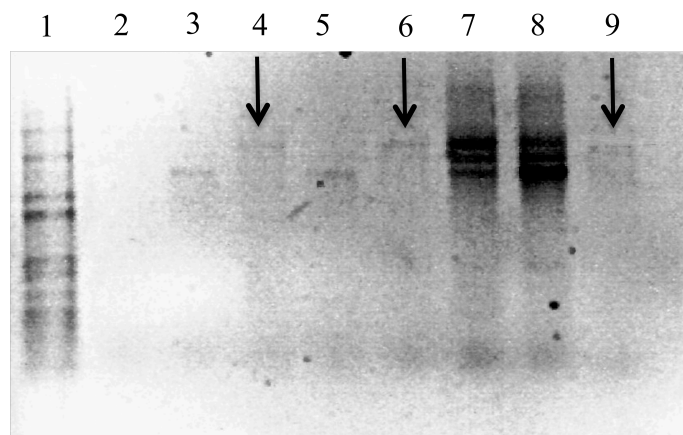


Figure 2.1. PCR confirming the insertion of the Gm^R marker into the *relA* genes of DF41 and DF41-1278. Primers *relA*-FOR and *relA*-REV were used to amplify a 2.4 kb fragment containing the *relA* gene. Lanes are as follows: 1, 1kb+ DNA ladder (Invitrogen); 2, water control; 3, pEX*relA*; 4, pEX*relA*-Gm; 5-6, putative DF41*relA* mutants; and 7-9, putative DF41-1278*relA* mutants. Arrows indicate the mutated allele. Mutants in lanes 6, and 9 appear to have the wild-type gene replaced with the mutated version and were chosen for further characterization.

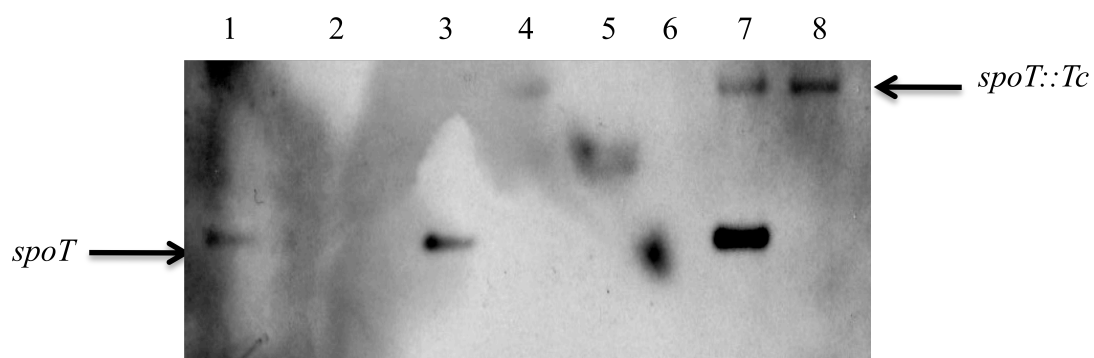


Figure 2.2. Southern blot analysis confirming the insertion of the Tc^R marker into the *spoT* genes of DF41*relA* and DF41-1278*relA*. Genomic DNA was digested with *Bam*HI and probed with a DIG-labelled *spoT* probe. Lanes are as follows: 1, DF4*relA* 1; 2 and 6, empty lanes; 3-5, putative DF41*relAspoT* mutants; and 7-8, putative DF41-1278*relAspoT* mutants. Arrow indicates the wild type and mutated versions of the *spoT* gene. Mutants in lanes 4 and 8 were chosen for further characterization because they appear to have the wild-type gene replaced with the mutated version.

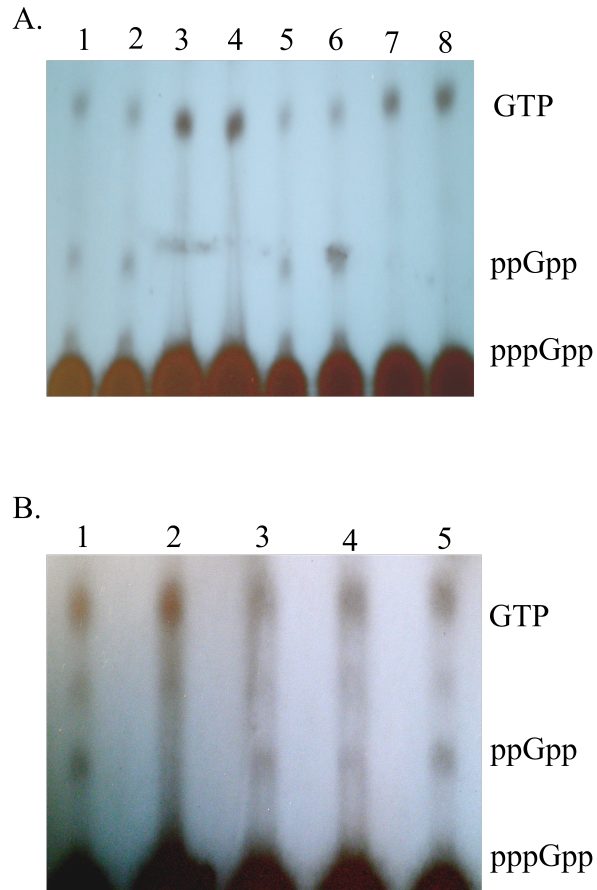


Figure. 2.3. (p)ppGpp analysis of *Pseudomonas* sp. DF41 and its SR derivatives after serine hydroxymate induction. Cells were labeled with ^{32}P and nucleotides were extracted and separated by thin layer chromatography. (A) *Pseudomonas aeruginosa* strain PAO1 (lane 1); *Pseudomonas* sp. DF41 (lane 2); DF41*relA* (lane 3); DF41*relAspoT* (lane 4); DF41*relA* (pUC*PrelA*) (lane 5); DF41*relAspoT* (pUC*PrelA*) (lane 6); DF41*relA* (pUC*PspoT*) (lane 7); and DF41*relAspoT* (pUC*PspoT*) (lane 8). (B) DF41 (lane 1), DF41-469 (*gacS*⁻) (lane 2), DF41-469 (pUCP23-*gacS*) (lane 3), DF41-469 (pUC*PrelA*) (lane 4), and PAO1 (lane 5).

2.4.2. The SR affects strain DF41 antifungal activity. When we assessed the ability of the mutants to inhibit *S. sclerotiorum in vitro*, we observed a 1.5-fold and a 1.8-fold increase in antifungal activity for strains DF41*relA* and DF41*relAspoT*, respectively, compared to the parent (Table 2.2). The presence of pUC*PreLA* was able to restore the antifungal activity of both mutants to that of the wild type (Table 2.2). Production of many secondary metabolites begins at the transition between the logarithmic and stationary phase, also known as the idiophase. As such, it is important to note there were no differences in growth rate between the wild type and the SR mutants (Fig. 2.6A).

2.4.3. Secondary metabolite production by strain DF41 and its SR mutant derivatives. Because LP production is essential for strain DF41 biocontrol (Berry 2010, Berry et al. 2010), we investigated whether the ppGpp-deficient strains exhibited elevated LP expression. A derivative of strain DF41, termed DF41-1278, is LP deficient due to a Tn5-1063 (*luxAB*) insertion in the NRPS locus responsible for LP synthesis (Berry 2010, Berry et al. 2010). Consequently, it is possible to monitor transcription of the NRPS genes in strain DF41-1278 using a bioluminescence assay (Berry 2010). Two SR mutants of strain DF41-1278 were created, designated 1278*relA* and 1278*relAspoT*. After 12 hours of growth, NRPS transcription in strains 1278*relA* and 1278*relAspoT* was elevated 5.6-fold and a 6.1-fold, respectively over that of the wild type (Fig. 2.4A). Addition of *relA* in trans restored transcription close to wild-type levels (Fig. 2.4A). By 24 h all strains exhibited equal levels of transcription (Fig. 2.4B). Next, we determined the level of LP present in strains

TABLE 2.2. Antifungal activity of *Pseudomonas* sp. DF41 and its derivatives after 5 days of growth.

Strains	Zone of fungal growth inhibition (mm) ^a
DF41 (pUCP22)	4.58 (1.0)
DF41 <i>relA</i> (pUCP22)	7.25 (0.5) ^b
DF41 <i>relAspoT</i> (pUCP22)	8.30 (1.5) ^c
DF41 <i>relA</i> (pUCP <i>relA</i>)	4.75 (1.2) ^{d,e}
DF41 <i>relAspoT</i> (pUCP <i>relA</i>)	5.50 (1.2) ^{d,f}
DF41 <i>relA</i> (pUCP <i>spoT</i>)	6.89 (1.5) ^{c,g}
DF41 <i>relAspoT</i> (pUCP <i>spoT</i>)	6.61 (1.7) ^{c,h}
DF41 <i>relA</i> (pUCP23- <i>gacS</i>)	6.90 (0.6) ^{c,g}
DF41 <i>relAspoT</i> (pUCP23- <i>gacS</i>)	7.27 (0.6) ^{g,i}
DF41 <i>relA</i> (pUCP22- <i>rpoS</i>)	6.85 (0.5) ^{g,i}
DF41 <i>relAspoT</i> (pUCP22- <i>rpoS</i>)	8.50 (2.0) ^{b,h}

^a Mean (standard deviation) obtained from five replicates.

^b Significantly different from wild type ($p < 0.005$).

^c Significantly different from wild type ($p < 0.05$).

^d Not significantly different from the wild type.

^e Significantly different from PA23*relA* ($p < 0.05$).

^f Significantly different from PA23*relAspoT* ($p < 0.05$).

^g Not significantly different from PA23*relA*.

^h Not significantly different from PA23*relAspoT*.

ⁱ Significantly different from wild type ($p < 0.001$).

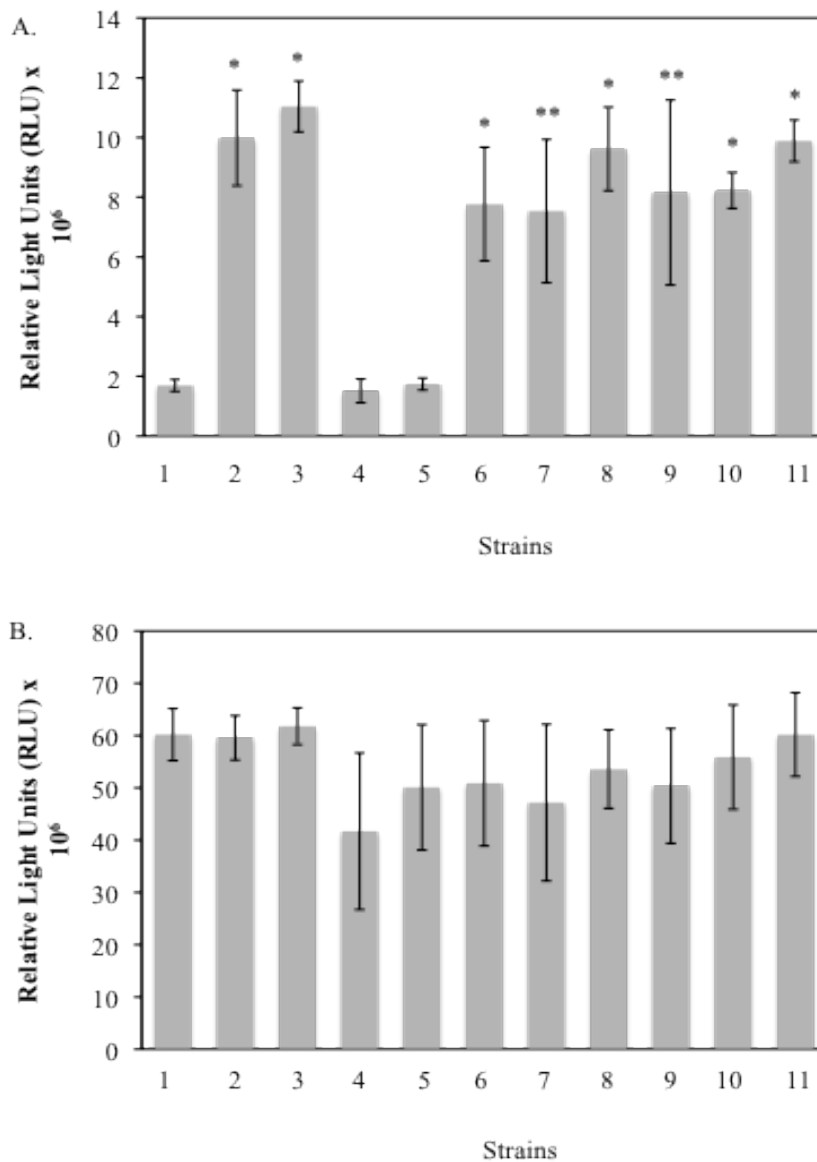


Figure 2.4. Transcription of the NRPS genes in *Pseudomonas* sp. DF41-1278 and its derivatives after (A) 12 hours and (B) 24 hours of growth in M9 minimal media supplemented with 1 mM MgSO₄ and 0.2% glucose. Column 1, DF41-1278 (pUCP22); column 2, 1278*relA* (pUCP22); column 3, 1278*relAspoT* (pUCP22); column 4, 1278*relA* (pUCP*relA*), column 5, 1278*relAspoT* (pUCP*relA*), column 6, 1278*relA* (pUCP*spoT*), column 7, 1278*relAspoT* (pUCP*spoT*); column 8, 1278*relA* (pUCP23-*gacS*); column 9, 1278*relAspoT* (pUCP23-*gacS*); column 10, 1278*relA* (pUCP22-*rpoS*); and column 11, 1278*relAspoT* (pUCP22-*rpoS*). For strains that differ significantly from the wild type, columns are labeled with * ($p < 0.005$) or ** ($p < 0.05$).

DF41, DF41*relA*, and DF41*relAspoT* culture supernatants through HPLC analysis. A single peak eluting at 28 min (Fig. 2.5) was observed for all strains except for strain DF41-1278 (Fig. 2.5B). We have demonstrated previously that this 28-minute peak corresponds to the DF41 LP molecule (Berry et al. 2010). In the SR mutants, the 28-minute peak was 2.0- to 1.5-fold greater in size (Fig. 2.5C & D) compared to the wild type (Fig. 2.5A). When *relA* was expressed in trans, LP production by the SR mutants was markedly reduced (Fig. 2.5E & F).

Besides LP molecules, strain DF41 liberates the volatile antibiotic hydrogen cyanide (HCN) (Berry 2010, Berry et al. 2010). Using cyantesmo paper we determined that the wild type and the SR mutants all produced HCN (Table 2.3). Furthermore, no differences in *hcnA-lacZ* expression were observed between strains DF41, DF41*relA* and DF41*relAspoT* (Table 2.3).

Quantitative analysis of protease production revealed strains DF41*relA* and DF41*relAspoT* produced 2-fold more protease than the wild type (Table 2.4). Addition of pUC*PreA* restored the protease activity of the SR mutants to wild-type levels (Table 2.4).

2.4.4. *relA* expression and (p)ppGpp production are reduced in a strain DF41 *gacS* mutant. To ascertain whether a link exists between the Gac system and the SR, (p)ppGpp production was assessed in a *gacS* mutant of strain DF41 called DF41-469. As observed in Figure 2.3B, (p)ppGpp levels were markedly reduced in the *gacS* mutant. Production of these nucleotides was restored to wild-type levels by the addition of either

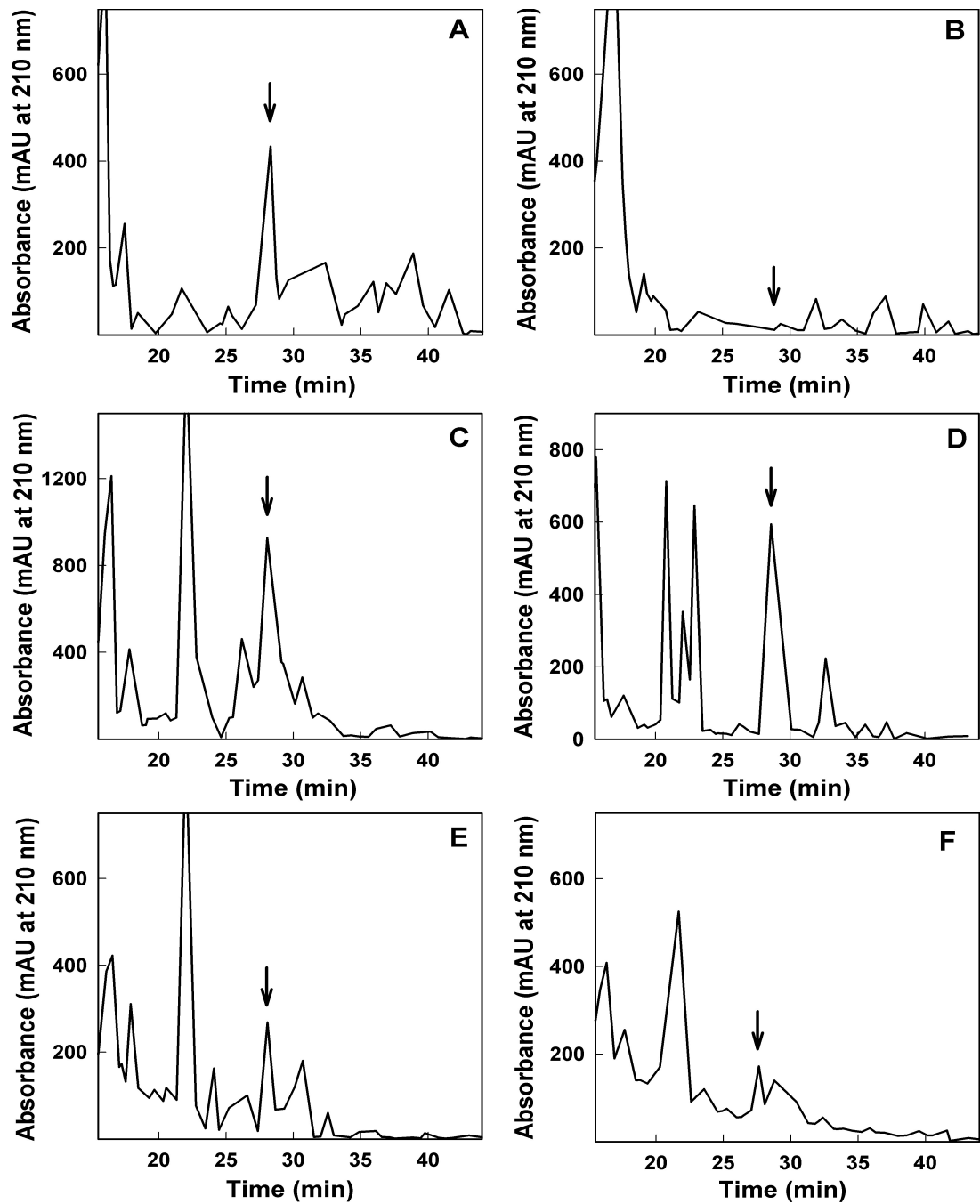


Figure 2.5. HPLC analysis of strain DF41, the stringent response mutants, and strains harbouring pUCPrelA. LP was extracted from cell-free supernatants of 4-day old cultures grown in M9 minimal media supplemented with 1% casamino acids, 2 mM MgSO₄, and 0.2% glycerol. Strains are as follows: (A) DF41(pUCP22), (B) DF41-1278 (pUCP22), (C) DF41relA (pUCP22), (D) DF41relAspoT (pUCP22), (E) DF41relA (pUCPrelA), and (F) DF41relAspoT (pUCPrelA).

TABLE 2.3. HCN activity of *Pseudomonas* sp. DF41 and its derivatives.

Strains ^a	HCN activity ^b	β -galactosidase Activity (Miller Units) ^c
DF41	+	888 (166)
DF41 <i>relA</i>	+	1163 (124)
DF41 <i>relAspoT</i>	+	1152 (113)

^a Strains used for β -galactosidase determination harbor the *hcnA-lacZ* fusion pME3219.

^b Determined using Cyantesmo paper.

^c Values are the mean and standard deviation from three replicates.

TABLE 2.4. Protease activity of *Pseudomonas* sp. DF41 and derivatives harboring the *relA*-overexpression plasmid pUCP*relA*, the *spoT*-overexpression plasmid pUCP*spoT*, the *gacS*-overexpression plasmid pUCP23-*gacS*, and the *rpoS*-overexpression plasmid pUCP22-*rpoS*.

Strains	Protease Activity (Units of enzyme/ml) ^a
DF41 (pUCP22)	0.752 (0.04)
DF41 <i>relA</i> (pUCP22)	1.480 (0.01) ^b
DF41 <i>relAspoT</i> (pUCP22)	1.487 (0.02) ^b
DF41 <i>relA</i> (pUCP <i>relA</i>)	0.882 (0.10) ^{c,d}
DF41 <i>relAspoT</i> (pUCP <i>relA</i>)	0.914 (0.20) ^{c,e}
DF41 <i>relA</i> (pUCP <i>spoT</i>)	1.246 (0.06) ^{f,g}
DF41 <i>relAspoT</i> (pUCP <i>spoT</i>)	1.312 (0.02) ^{b,h}
DF41 <i>relA</i> (pUCP23- <i>gacS</i>)	1.176 (0.08) ^{f,g}
DF41 <i>relAspoT</i> (pUCP23- <i>gacS</i>)	1.269 (0.08) ^{f,h}
DF41 <i>relA</i> (pME- <i>rpoS</i>)	0.677 (0.10) ^{c,d}
DF41 <i>relAspoT</i> (pME- <i>rpoS</i>)	0.735 (0.02) ^{c,e}

^a Mean (standard deviation) from five replicates.

^b Significantly different from wild type ($p < 0.005$).

^c Not significantly different from the wild type.

^d Significantly different from PA23*relA* ($p < 0.005$).

^e Significantly different from PA23*relAspoT* ($p < 0.005$).

^f Significantly different from wild type ($p < 0.01$).

^g Significantly different from PA23*relA* ($p < 0.05$).

^h Significantly different from PA23*relAspoT* ($p < 0.05$).

gacS or *relA* in trans. Next, we generated a *relA*-translational fusion and monitored its activity in strain DF41 and DF41-469 (*gacS*). At 16 h, a 1.7-fold reduction in *relA* expression was observed in the *gacS* mutant compared to the wild type (Fig. 2.6B). Addition of pUCP23-*gacS* in trans was unable to complement the DF41*relA* and DF41*relAspoT* mutants for any of the aforementioned phenotypes, including LP transcription (Fig. 2.4), antifungal (Table 2.2) and protease activity (Table 2.4).

2.4.5. The stringent response is required for maximal *relA* expression.

Expression of a *relA* translational fusion was monitored in strains DF41, DF41*relA* and DF41*relAspoT*. In strain DF41, *relA* expression reached its maximum at 16 h followed by a sharp decline by 24 hours (Fig. 2.6B). In the SR mutants, *relA* expression peaked at 16 h; however, expression was reduced 1.6-fold in these strains. Thus it appears that in strain DF41, the SR is required for maximal *relA* expression.

2.4.6. RpoS overexpression complements for *rpoS* transcription and protease activity in the SR mutants. In strain DF41, *rpoS* was found to be under GacS control (Berry et al. 2010). To determine the impact of the SR on the transcription of this sigma factor, β -galactosidase activity of the *rpoS-lacZ* transcriptional fusion was monitored in strain DF41 and its derivatives. At 24 h, *rpoS* transcription was reduced by almost 1.5-fold in the ppGpp-deficient strains compared to the wild type (Fig. 2.7A). To determine the effect of constitutively expressed *rpoS* on transcription of this sigma factor and the NRPS genes in the SR mutants, pME-*rpoS* was mobilized into strains DF41, DF41*relA*,

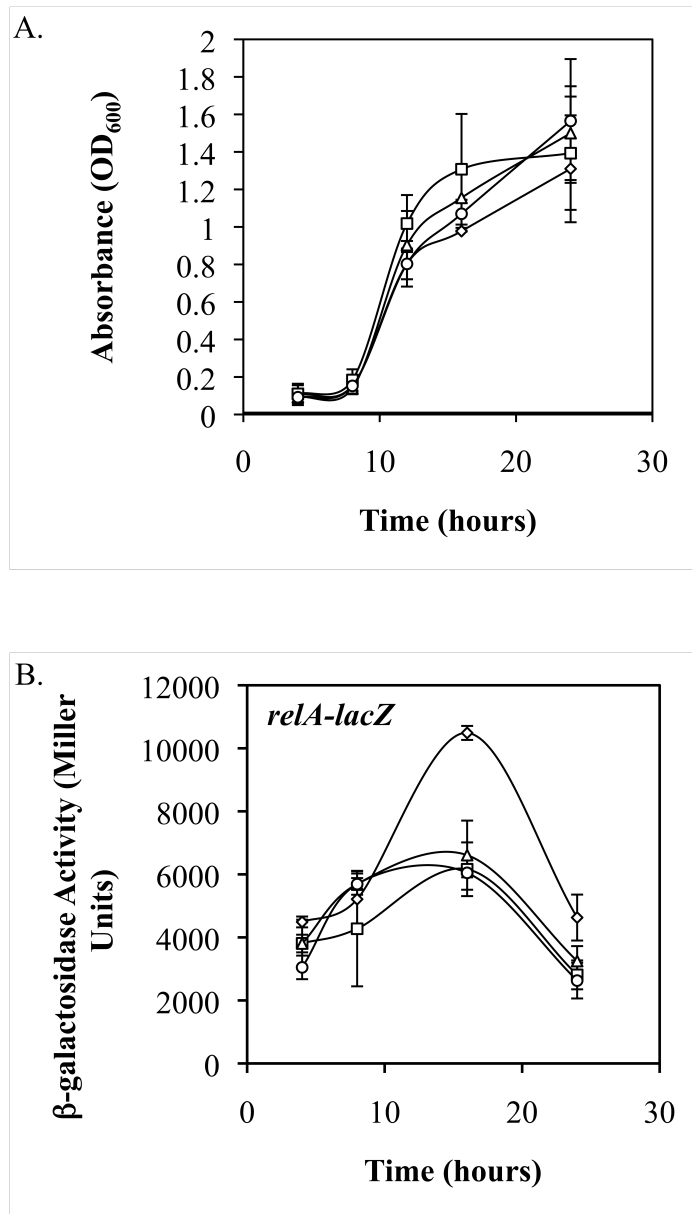
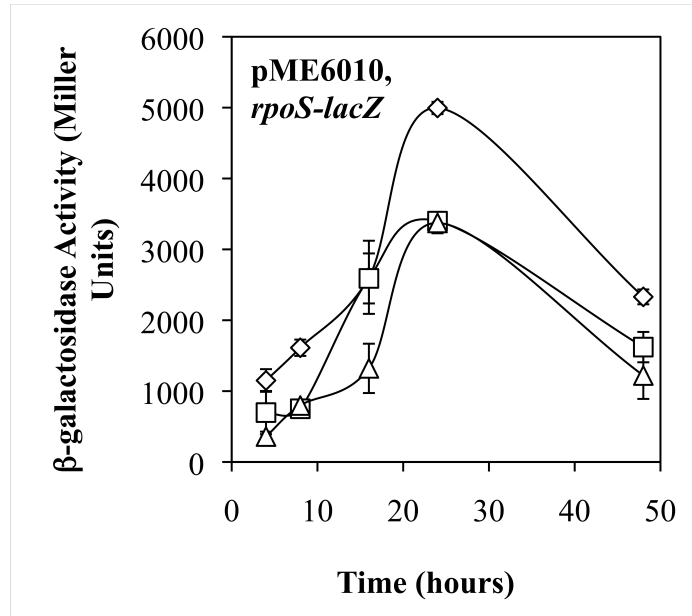


Figure 2.6. The ability of GacS and the stringent response to regulate *relA* translation. The growth analysis of each strain is depicted in panel A while translational analysis of *relA* is shown in panel B. Strains used are as follows: *Pseudomonas* sp. DF41 (diamond), DF41*relA* (square), DF41*relAspoT* (triangle), and DF41-469 (circle). All strains analyzed harbor the *relA* translation fusion pSW*relA*. Strains were grown in M9 minimal media supplemented with 1 mM MgSO₄ and 0.2% glucose. Note that *relA* expression is markedly reduced in both the SR mutants and the *gacS* mutant.

A.



B.

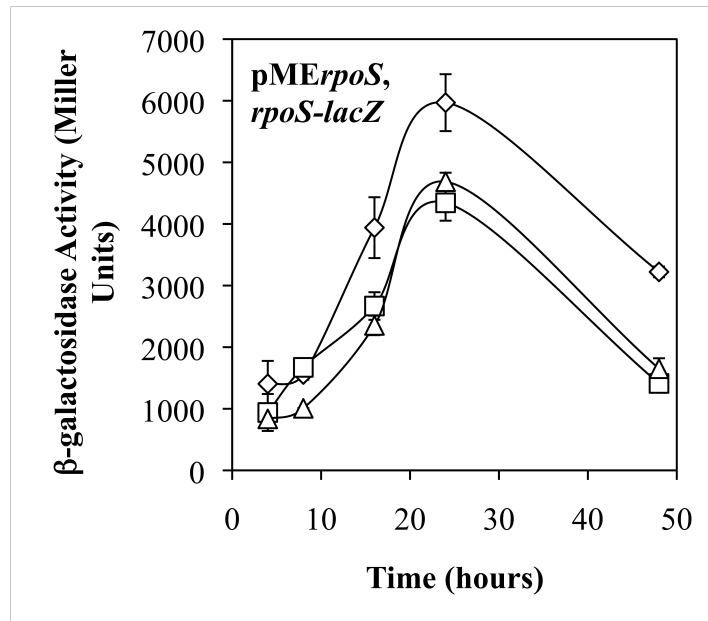


Figure 2.7. Transcription of *rpoS* from pPROS-lacZ in strain DF41 and its derivatives. Strains used are as follows: *Pseudomonas* sp. DF41 (diamond), DF41*relA* (square), and DF41*relAspoT* (triangle) carrying the *rpoS-lacZ* reporter plasmid pRPOS-lacZ. In panel A, strains harbor pME6010 while in panel B, strains contain pME*rpoS*. Strains were grown in M9-minimal media supplemented with 1 mM MgSO₄ and 0.2% glucose.

and DF41*relAspoT*, all of which carry the *lacZ* fusion plasmids. As shown in Figure 2.6B, the presence of *rpoS* in trans in the SR mutants was able to restore *rpoS-lacZ* expression close to wild-type levels. To see if constitutively expressed *rpoS* would alter phenotypic traits displayed by the SR mutants, pME-*rpoS* was introduced in trans into the mutants and LP production and protease activity was measured. No difference in antifungal activity and LP transcription (Fig. 2.4) was observed (Table 2.2); however, protease activity exhibited by the ppGpp-deficient strains was restored to wild-type levels upon the addition of *rpoS* in trans (Table 2.4).

2.5. Discussion

The SR is a global regulatory mechanism that enables bacteria to alter a broad range of physiological traits including growth rate, motility, persistence, biofilm formation, virulence and secondary metabolite production in response to nutrient limitation (Potrykus and Cashel 2008). In light of this, we were interested to learn how the SR affects the antifungal activity of DF41. This is particularly important because when DF41 is used as a biocontrol agent against *Sclerotinia* stem rot of canola, it is applied as a foliar spray (Berry et al. 2010, Savchuk and Fernando 2004). The aerial surfaces of the plant represent a harsh environment for bacteria as they must endure exposure to high and low temperatures, UV radiation, and desiccation (Lindow and Brandl 2003). Additionally, nutrients are scarce and so bacteria on the plant surface are hypothesized to undergo the SR (Lindow and Brandl 2003).

Characterization of strain DF41 *relA* and *relAspoT* SR mutants revealed a lack of (p)ppGpp production (Fig. 2.3) and enhanced inhibition of *S. sclerotiorum* (Table 2.2), indicating that the SR negatively regulates strain DF41 biocontrol. We next addressed whether the SR affects LP expression. HPLC analysis of culture extracts showed that LP levels were elevated between 1.5- and 2-fold in the strain DF41 *relA* and *relAspoT* mutants compared to strain DF41 (Fig. 2.4). Moreover at 12 h, transcription of the NRPS biosynthetic genes was increased over 5 times in the SR mutant background relative to wild type (Fig. 2.4). These findings suggest that in the absence of (p)ppGpp, the NRPS genes are induced earlier leading to enhanced LP production. A similar finding was reported for *Streptomyces clavuligerus*, in which a *relA* mutant produced elevated levels of the antibiotics clavulanic acid and

cephamycin C (Gomez-Escribano et al. 2008). Moreover, transcription of the clavulanic acid and cephamycin C biosynthetic genes was increased substantially in the *relA*-deficient strain (Gomez-Escribano et al. 2008). With respect to SR control over antibiotic production, the *Streptomyces* are probably the most well studied group of bacteria (Chakraborty and Bibb 1997, Gomez-Escribano et al. 2008, Hesketh et al. 2001, Hoyt and Jones 1999, Jin et al. 2004). Depending on the strain of *Streptomyces* in question, the antibiotics produced and the growth media employed, the SR has been found to either positively (Chakraborty and Bibb 1997, Hesketh et al. 2001, Hoyt and Jones 1999, Jin et al. 2004) or negatively regulate antibiotic production (Gomez-Escribano et al. 2008). Hence for *Streptomyces* spp., the SR appears to have a variable effect on antibiotic production. In biocontrol strains of *Pseudomonas*, little is known about SR control over secondary metabolite production and fungal antagonism. One exception, however, is *Pseudomonas* sp. MIS38. Therein, a transposon insertion in *spoT* resulted in elevated (p)ppGpp and reduced production of the LP arthrofactin (Washio et al. 2010), which is consistent with the inverse relationship between (p)ppGpp levels and LP expression observed for strain DF41.

In pseudomonads, regulation of biocontrol factors is governed by a complex network that functions at both the transcriptional and the post-transcriptional level. At the top of the hierarchy, the GacS/GacA two-component signal transduction system is essential for biological control in *Pseudomonas* spp. (Haas and Défago 2005, Heeb and Haas 2001). Deletion of either component typically leads to a complete loss of antagonistic activity (Heeb and Haas 2001), which was found to be the case for strain DF41 (Berry et al. 2010). Because the SR inhibits expression of

factors that are also under Gac control, we were interested in determining whether a connection exists between the two systems in strain DF41. Herein, we observed a 1.7-fold decrease in *relA* expression in the *gacS*-minus background (Fig. 2.5). (p)ppGpp analysis also revealed a dramatic reduction in the levels of this nucleotide in the *gacS* mutant, which was restored upon the addition of *gacS* in trans (Fig. 2.3B). These findings indicate that the expression of *relA* is influenced by the Gac system, although the molecular mechanism(s) underlying this regulation have yet to be uncovered. In other pseudomonads, the Rsm system forms part of the Gac regulatory cascade (Haas and D  fago 2005). After Gac activation, a series of regulatory RNAs are produced that antagonize the effects of RsmA-like proteins, which bind to and block translation of biocontrol mRNAs (Haas and D  fago 2005). The only Rsm component that has been identified in strain DF41 thus far is a homolog of RsmZ, which is a regulatory RNA (de Kievit and Berry, personal communication). The presence of RsmZ suggests that cognate repressor protein(s) and additional regulatory RNAs likely exist; some or all of which may play a role in *relA* regulation.

During the SR, RNAP binding to (p)ppGpp and additional effectors, such as DksA, leads to activation and repression of different subsets of genes (Potrykus and Cashel 2008). For genes that are activated, increased expression is believed to occur by both direct and indirect mechanisms (Potrykus and Cashel 2008 and references therein). The direct effect involves RNAP in complex with (p)ppGpp increasing transcription from a given promoter. Indirect effects arise because (p)ppGpp facilitates RNAP binding to alternative sigma factors, leading to induction of these regulons. In this study, the sigma factor *rpoS* was found to be upregulated during the

SR while the NRPS genes were repressed. To better understand how the SR affects transcription of *rpoS* and the NRPS genes, we expressed *rpoS* constitutively from the kanamycin promoter in the SR mutants. The presence of plasmid pME-*rpoS* in the *relA* and *relAspoT* mutants increased *rpoS* transcription to near wild type levels (Fig. 2.7B). In strain DF41, *rpoS* is positively autoregulated, as evidenced by the increased transcription brought about by pME-*rpoS* (Fig. 2.7B). It is possible that in the SR mutants, the elevated levels of plasmid-encoded RpoS enabled it to bind RNAP in the absence of (p)ppGpp, resulting in increased *rpoS* transcription. On the other hand, addition of *rpoS* had no effect on NRPS gene transcription. In this situation, (p)ppGpp may affect transcription from these promoters directly by decreasing NRPS transcription. Alternatively, the SR may exert its effects indirectly through yet-to-be identified regulator of NRPS gene expression. Clearly, a great deal of work remains in order for us to understand exactly how the SR affects expression of these and other genes in strain DF41. When we examined whether the presence of *rpoS* in trans altered the SR mutant phenotype, the only trait that was affected was protease activity, which was restored to wild-type levels (Table 2.4). Protease-encoding gene(s) have not yet been identified in strain DF41; however, the results of the present study suggest that expression of these genes is under RpoS control.

In summary, we demonstrate that the SR negatively affects the antifungal activity of strain DF41 in vitro. Whether SR mutants would demonstrate enhanced biocontrol in planta has not been established. When an organism encounters a stress, several stress mechanisms may be induced to mount an appropriate response. For instance, bacteria frequently produce long chain polymers of phosphate residues

termed polyPs when starved for nutrients (Rao et al. 2009). These molecules are synthesized by polyphosphate kinase, the product of the *ppk* gene while their hydrolysis to Pi residues depends upon the *ppx*-encoded exopolyphosphatase (Rao et al. 2009). It was reported that in *Streptomyces lividans*, expression of the antibiotics actinorhodin and undecylprodigiosin was enhanced in the *ppk* mutant under phosphate-limiting conditions presumably as a result of reduced endogenous Pi levels (6). Furthermore, Pi-mediated inhibition is believed to occur independent of (p)ppGpp as *relA* transcription in the polyP-deficient strain was similar to wild type (6). Taken together, these findings suggest that the regulation of antibiotic production is complex and requires multiple pathways to respond appropriately to the prevailing conditions. Synthesis of antifungal factors including LP and protease is an energetically costly process. When nutrients are scarce, the SR would enable strain DF41 to shift resources from secondary metabolite production to activities required for coping with starvation. Because biocontrol bacteria are often forced to survive under nutrient-depleted conditions, it is essential to understand the impact of the SR on secondary metabolism as this directly impacts antagonism.

2.6. Acknowledgements

I would like to thank Chrystal Berry for her help with the HPLC analysis and for generating strain DF41-1278 as well as the *rpoS-lacZ* reporter plasmid pRPOS-lacZ, and Carrie Selin for her assistance in generating the following plasmids: pUCP22-*rpoS*, pUCP22-*gacS*, and pME-*rpoS*. In addition, I am grateful to Dr. Dieter Haas for providing plasmid pME3219. And finally, I would like to thank Dr. Fernando for critical reading of the manuscript and Dr. de Kievit for help writing this paper.

Chapter 3.

Stringent Response Mutants of *Pseudomonas chlororaphis* PA23 Exhibit

Enhanced Antifungal Activity against *Sclerotinia sclerotiorum* *in vitro*.

Microbiology

(Submitted)

3.2. Introduction

When applied as a foliar spray, *Pseudomonas chlororaphis* PA23 is able to protect canola from sclerotinia stem rot (SSR) caused by the fungal pathogen *Sclerotinia sclerotiorum* (Lib.) (Savchuk and Fernando 2004). Because the phyllosphere is a nutrient-poor environment, PA23 may employ strategies to cope with low carbon and energy availability. The SR is a mechanism that enables bacteria to alter 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 ppGpp and 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 and Cashel 2008). SpoT is considered bifunctional since it is capable of (p)ppGpp synthesis under specific environmental conditions (Potrykus and Cashel 2008, and references therein). (p)ppGpp binds to RNAP near its catalytic site, leading to increased transcription of certain genes, for instance those involved in amino acid biosynthesis (Potrykus and 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 and 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 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 is comprised of the sensor kinase GacS and its cognate response regulator GacA (Heeb and Haas 2001). In several pseudomonads, including PA23, a mutation in either *gacA* or *gacS* leads to a lack of fungal inhibition (Heeb and Haas 2001, Poritsanos et al. 2006). Another regulatory mechanism governing expression of antifungal compound is QS. Quorum sensing enables bacteria to regulate gene expression according to population density through the production of AHL molecules (Venturi 2006). In a variety of pseudomonads, PHZ biosynthesis is controlled by the PhzI/PhzR QS system (Chin-A-Woeng et al. 2003, Mavrodi et al. 2006). In addition, the alternative sigma factor RpoS has 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 PLT and 2,4-DAPG (Sarniguet et al. 1995) while the same mutation in *P. chlororaphis* PCL1391 caused a decrease in PCN production compared to the wild type (Girard et al. 2006).

PA23 produces an arsenal of factors including PRN, PHZ, 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 biocontrol activity of pseudomonads. What's more, the connection

between the SR and the regulatory mechanisms involved in biocontrol by *Pseudomonas* species has not been explored. The focus of the current study was to elucidate how the SR affects PA23 antagonism of *S. sclerotiorum*, as well as production of antibiotics and degradative enzymes by this bacterium. Furthermore, we sought to determine if there is any link between the SR and other regulators of biocontrol including the GacS-GacA two-component system, QS and the stationary phase sigma factor RpoS.

3.3. Materials and Methods

3.3.1. Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 3.1. *Escherichia coli* and *P. chlororaphis* PA23 strains were cultured on LB agar (Difco Laboratories, Detroit, MI) at 37°C and 28°C, respectively. *S. sclerotiorum* was maintained on PDA (Difco). As required, media were supplemented with the following antibiotics from Research Products International Corp. (Mt. Prospect, IL): Gm (20 µg/ml), Tc (15 µg/ml), Pip (30 µg/ml), Rif (100 µg/ml) for PA23 and Amp (100 µg/ml), Gm (15 µg/ml), Tc (15 µg/ml) for *E. coli*.

3.3.2. Nucleic acid manipulation. Standard techniques for purification, cloning and other DNA manipulations were used (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.

3.3.3. 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 analyzed with Blastn and Blastx databases. The GenBank accession numbers for the sequences of the PA23 *gacA*, *relA*, and *spoT* genes are JF513195, JF705880, and JF705881, respectively.

TABLE 3.1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype or relevant phenotype ^a	Source or reference
Strains		
<i>P. aeruginosa</i> PAO1	Wild type	Holloway et al. 1979
<i>P. chlororaphis</i> PA23	Rif ^R ; Wild type (soybean root tip isolate)	Savchuk and Fernando 2004
PA23 <i>relA</i>	PA23 with the pKNOCK-Gm vector inserted into the <i>relA</i> gene	This study
PA23 <i>relAspoT</i>	PA23 <i>relA</i> with a Tet ^R cassette inserted into the <i>spoT</i> gene	This study
PA23-314	Rif ^R ; <i>gacS</i> ::Tn5-OT182 genomic insertion	Poritsanos et al. 2006
PA23 <i>gacA</i>	Gm ^R marker inserted into the <i>gacA</i> gene in PA23	This study
<i>E. coli</i> DH5α	<i>supE44 ΔlacU169(φ80lacZΔM15) hadR17 recA1 endA1 gyrA96 thi-1relA1</i>	Hanahan 1983
DH5α λpir	λpir lysogen of DH5α	House et al. 2004
Plasmids		
pCR2.1	TA cloning vector Amp ^R	Invitrogen
pCR <i>relA</i> -int	650 bp internal fragment of <i>relA</i> in pCR2.1	This study
pKNOCK-Gm	Suicide vector for insertional mutagenesis; R6K ori RP4 oriT Gm ^R	Alexeyev 1999
pKNOCK- <i>relA</i>	Internal fragment (650 bp) of <i>relA</i> in pKNOCK-Gm	This study
pRK600	Contains <i>tra</i> genes for mobilization; Chl ^R	Finan et al. 1986
pEX18Ap	Suicide plasmid Gm ^R	Hoang et al. 1998
pEX <i>spoT</i>	<i>spoT</i> from PA23 in pEX18Ap	This study
pFTC1	Source of Tet ^R cassette	Choi et al. 2005
pEX <i>spoT</i> -Tet	pEX <i>spoT</i> with Tet ^R inserted into <i>spoT</i>	This study
<i>gacA</i> pCR2.1	1- kb fragment containing the <i>gacA</i> gene in pCR2.1	This study
<i>gacA</i> pEX18Ap	1- kb <i>gacA</i> fragment in pEX18Ap	This study
pUCGm	Source of Gm ^R cassette	Schweizer 1993
<i>gacA</i> -gentpEX18Ap	<i>gacA</i> pEX18Ap with Gm ^R inserted into <i>gacA</i>	This study
pCR <i>relA</i> -23	2.8 kb fragment containing the <i>relA</i> gene in pCR2.1	This study
pUCP22	Broad-host-range vector Amp ^R Gent ^R	West et al. 1994
pUCP22- <i>relA</i>	<i>relA</i> in pUCP22	This study

pCR <i>spoT</i> -23	<i>spoT</i> from <i>P.chlororaphis</i> PA23 in pCR2.1	This study
pUCP22- <i>spoT</i>	<i>spoT</i> in pUCP22	This study
pUCP22- <i>rpoS</i> pME3066	<i>rpoS</i> in pUCP22 contains <i>gacA</i> of <i>P. fluorescens</i> CHA0 Tet ^R	Poritsanos et al. 2006 Laville et al. 1992
pCR <i>gacA</i> up pLP <i>gacA</i> pLP170	<i>gacA</i> promoter in pCR2.1 <i>gacA</i> promoter in pLP170 <i>lacZ</i> transcriptional fusion vector	This study This study Preston et al. 1997
pUCP23- <i>gacS</i>	<i>gacS</i> in pUCP23	Poritsanos et al. 2006
pCR <i>gacS</i> up pLP <i>gacS</i> <i>phzI</i> -pCR2.1	<i>gacS</i> promoter in pCR2.1 <i>gacS</i> promoter in pLP170 1.3 kb fragment containing <i>phzI</i> in pCR2.1	This study This study This study
pPHZI- <i>lacZ</i>	674-bp fragment containing <i>phzI</i> promoter in pLP170	This study
pCR- <i>phzAR</i>	1.68 kb fragment containing <i>phzR</i> in pCR2.1	This study
pPHZR- <i>lacZ</i>	1.1-kb fragment containing <i>phzR</i> promoter in pLP170	This study
pSW205	Promotorless <i>lacZ</i> translational fusion	Gambello and Iglewski 1991
pSW <i>relA</i> -23	650-bp fragment containing the promoter and first 70 codons of <i>relA</i> in pSW205	This study
pPHZA- <i>lacZ</i> pPRNA- <i>lacZ</i> pRPOS- <i>lacZ</i>	<i>phzA</i> promoter in pLP170 <i>prnA</i> promoter in pLP170 <i>rpoS</i> promoter in pLP170	Selin et al. 2010 Selin et al. 2010 Poritsanos et al. 2006
pME3219 Primers ^b	<i>hcnA-lacZ</i> translational fusion	Laville et al. 1998
relA-KpnIFOR	5'- ggggtaccc atctggcgcaagatgcag-3'	This study
relA-BamHIREV	5'- cgggatccc gttgaaaccagtggacga-3'	This study
spoT-FOR	5'-gcgtcaccgttgaaactg-3'	This study
spoT-REV	5'-ttactcgaggacgacgatgg-3'	This study
23relA-REV	5'-gaagatcgctccactggttaa-3'	This study
spoT-23:newFRW	5'-ggaaatcgtaacaagatcga-3'	This study
spoT-23:newREV	5'-ttggtgttccagttctacgc-3'	This study
gacAP1	5'-gcat gaattc gttaagacaggaaaggaacg-3'	This study
gacAP2	5'-gcat gagctc attcctcccctgactcgg-3'	This study
gacAP3	5'-gcat gagctc tgaagaagatccattgggca-3'	This study

gacAP4	5'-gcat ggatcc ggaagaaaagcactgggat-3'	This study
nGacAtrans-FRW	5'-gaagatcttctaagacaggaaaggaacgc-3'	This study
nGacAtrans-REV	5'-cca agctt gggactgcaggccatcgatgt-3'	This study
newGacStrans-FRW	5'-ggggtac cccgga ttcattagcttctgcaa-3'	This study
newGacSR-trans	5'-c gggatcc ccgattcccaaatggtgagcaca-3'	This study
phzI-FWD	5'-tacgactgcctggaccaaact-3'	This study
phzI-REV	5'-gcattcaaaaaggacgac-3'	This study
phzAR-F	5'-aatcctgcatccaactc-3'	This study
phzAR-R	5'-aagttgttcgaaggggttca-3'	This study

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

^b Items in bold represent restriction sites

3.3.4. Generating PA23 *relA*, *relAspoT*, and *gacA* mutants.

All primers and plasmids utilized for the construction of mutant strains are listed in Table 3.1.

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 *KpnI* and *BamHI* and cloned into the same sites of pKNOCK-Gm, creating pKNOCK-*relA*. Allelic exchange was performed through tri-parental mating among *E. coli* DH5 α λ pir (pKNOCK-*relA*), *E. coli* DH5 α (pRK600) and PA23. Transconjugants were screened on LB agar supplemented with Rif (100 μ g/ml) and Gm (20 μ g/ml). To verify the insertion into the PA23 *relA* gene, the pKNOCK-Gm vector was rescued from the PA23 genome by digestion with *HincII*. Linearized genomic fragments were subsequently recircularized with T4 DNA ligase (Invitrogen), transformed into *E. coli* DH5 α λ pir, and screened on LB agar + 20 μ g/ml of 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 PCR amplified 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 *SacI/XbaI* digestion and cloned into the same sites of pEX18Ap, creating pEX*spoT*. A 2.0-kb *SmaI* fragment containing the Tc^R marker from pFTC1 was cloned into a *SmaI* site within the *spoT* gene, forming pEX*spoT*-Tet. Triparental mating was performed using *E. coli* DH5 α (pEX*spoT*-Tet), *E. coli* DH5 α (pRK600) and PA23, and transconjugants were screened on LB agar plates supplemented with Tc (15

µg/ml) and Rif (100 µg/ml). Bacteria that had undergone a double-cross over event were identified by streaking onto LA containing sucrose (10%) and Tc. To verify that the *spoT* gene had been replaced with the mutated allele, southern hybridization was performed. A *spoT*-DIG labelled probe was generated by PCR amplification of the *spoT* gene using primers mentioned above. Genomic DNA isolated from PA23*relA* was digested with *Bam*HI and *Hind*III restriction enzymes and separated on an agarose gel. Depurination of the DNA was carried out by treating the gel with 0.25M HCl for 10 min, followed by a neutralization treatment for 45 min using a solution of 1.5M NaCl and 1.0M Tris-HCl (pH 7.5). In order for the genomic DNA to migrate from the agarose gel to the Hybond-N™ nitrocellulose membrane (Amersham Biosciences), alkaline capillary transfer in 10X SSC was performed. The blot was then rinsed in 5X SSC for 10 min with gentle agitation and exposed to UV light. A 50 mL aliquot of prehybridization solution was added to the membrane and allowed to equilibrate overnight at 65°C in a ProBlot hybridization oven (Mandel, Canada). The blot was then hybridized at the same temperature for 24 h with the *spoT*-DIG DNA labelled probe and any unbound probe was removed by two 15 min washes with a solution of 2X SSC and 0.1% SDS at room temperature. Two 30 min washes with 0.5X SSC and 0.1% SDS at 65°C were also performed. The membrane was incubated with washing buffer for 5 min followed by blocking solution for 30 min. A solution containing anti-DIG polyclonal antibody conjugated to alkaline phosphatase (diluted 1:10, 000 in 1X blocking solution) was added to the blot for 60 min. The membrane was then rinsed three times with water, incubated with washing buffer for 15 min, and rinsed three more times with water before equilibration in 10

mL of detection buffer for 5 min. Antibodies were detected using chemiluminescence detection reagent CDP-Star® (Roche Diagnostics, Mannheim, Germany) and blots were developed overnight using Kodak X-Omat Blue film (Fisher). To create a *gacA* mutant of PA23, a copy of *gacA* missing an internal 150-bp fragment was first generated through PCR. 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 *SacI* and cloned into the pCR2.1 to yield *gacApCR2.1*. The 1-kb insert was then excised with *EcoRI* and *BamHI* and subcloned into the same sites of pEX18Ap (*gacApEX18Ap*). An 850-bp Gm^R cassette from pUCGm was inserted into the *SacI* site yielding *gacA-gentpEX18Ap*. Triparental mating between *E. coli* DH5α (*gacA-gentpEX18Ap*), *E. coli* DH5α (pRK600) and PA23 was performed. Pseudomonas Isolation Agar (PIA; Difco) supplemented with Gm (20 µg/ml) enabled screening of 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 successfully been replaced with mutated copy of the allele.

3.3.5. Plasmid construction. For complementing the SR mutants, pUCP22-*relA* was generated as follows. The PA23 *relA* gene was PCR amplified 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 *EcoRI* and *BamHI* 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 made flush with Klenow (Invitrogen) and then subject to *Bam*HI digestion. The 2.8-kb fragment containing *spoT* was ligated into the *Bam*HI- *Sma*I sites of pUCP22 generating pUCP22-*spoT*.

3.3.6. Construction of *lacZ* fusions. To construct a *gacA-lacZ* transcriptional fusion, the promoter region of *gacA* was PCR amplified using primers nGacAtrans-FRW and nGacAtrans-REV. The 565-bp product was cloned into pCR2.1 (pCR $gacA$ up). pCR $gacA$ up was digested with *Hind*III and *Sma*I and the insert was subcloned into the same sites of pLP170, creating pLP $gacA$. To generate the *gacS-lacZ* fusion pLP $gacS$, the *gacS* promoter region was PCR amplified using primers newGacStrans-FRW and newGacSR-trans-REV. The 480-bp product was cloned into pCR2.1 (pCR $gacS$ up), and then excised with *Sma*I and *Bam*HI and cloned into the same sites of pLP170. The *phzI-lacZ* fusion was constructed by PCR amplifying the *phzI* promoter using primers phzI-FRW and phzI-REV. The 674-bp PCR product was cloned into pCR2.1 (*phzI*-pCR2.1), and then subcloned as a *Hinc*II-*Eco*RI fragment 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*, which was cloned into pCR2.1 (pCR-*phzAR*). A 1.1-kb fragment was excised from the resulting plasmid by *Eco*RI-*Eco*RV digestion and subcloned into the *Eco*RI-*Sma*I sites of pLP170 creating pPHZR-*lacZ*. The *relA-lacZ* translational fusion pSW $relA$ -23 was

generated by PCR amplification of a 650-bp fragment containing the promoter and first 70 codons of the PA23 *relA* gene using primers relAtransl-FRW and 23newrelA-REV. The product was digested with *Eco*R1 and cloned into the *Eco*RI and *Sma*I sites of pSW205.

3.3.7. (p)ppGpp and *relA* expression analysis. Determination of (p)ppGpp levels was performed as described by Cashel (1994) with the following modifications. Cells were grown overnight in morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich Canada, Oakville, ON) media at 28°C followed by a 1/100 dilution of the culture in MOPS phosphate-free minimal medium containing 1 mg/ml of casamino acids (Difco) and 100 µCi /ml of ³²P (Perkin Elmer, Waltham, MA). Three 200-µl aliquots of each strain were added to wells of a polystyrene microtitre plate (Costar; Corning Incorporated, Corning, NY) and grown at 28°C for an additional 8 hours. Four hundred µg/ml of DL-serine hydroxymate (Sigma) was then added to each well and allowed to incubate for 2 hours. 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 at least twice. Expression of pSW*relA*-23 was monitored in PA23, PA23*relA*, PA23*relAspoT*, PA23*gacA*, and PA23-314. Strains harboring the plasmid were grown for 4, 8, 12, 16, and 24 hours and then assayed for β-galactosidase activity (Miller 1972).

3.3.8. 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 analyzed for each strain and the experiments were repeated three times.

3.3.9. Quantitative analysis of PHZ. Overnight cultures of PA23 and the SR mutants were grown in M9 minimal media supplemented with 1 mM MgSO₄ and 0.2% glucose and subject to PHZ extraction as described by Selin et al. (2010). Samples were analyzed in triplicate and the experiment was repeated twice.

3.3.10. HPLC analysis of PRN. The amount of PRN produced by PA23 and its derivatives were quantified by HPLC as described by Selin et al. (2010) with the following modifications. Strains were grown in 100 ml of M9 minimal media + 1mM MgSO₄ + 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 analyzed by UV absorption at 225 nm using a Varian 335 diode array detector. Samples were analyzed in duplicate and the experiment was repeated twice.

3.3.11. HCN analysis. Qualitative determination of HCN production was performed using Cyantesmo paper (Machery-Nagel GmbH & Co., Duren, Germany). The pME3219 plasmid, which contains an *hcnA-lacZ* translational fusion, was mobilized into PA23, PA23*relA* and PA23*relAspoT* and monitored for β-galactosidase activity

after 4, 8, 12, 16, and 24 hours of growth. Samples were analyzed in triplicate and the experiments were repeated three times.

3.3.12. Protease production. Cultures were grown in M9 minimal media supplemented with 1 mM MgSO₄, 0.2% glucose, and 1.5% skim milk (Difco) for 5 days at 28°C to induce protease production. A 200-μl aliquot of cell-free supernatant was analyzed for the activity of this enzyme 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 colored chromophore (Cupp-Enyard 2008). This chromophore is measured spectrophotometrically at a wavelength of 660 nm. To determine the amount of tyrosine liberated, a standard curve was generated using pure tyrosine at the following concentrations (μMole): 0.055, 0.111, 0.221, .0442, and 0.553. Each strain was assayed in triplicate and experiments were performed three times.

3.3.13. Lipase production. Lipase assays were performed according to Winkler and Stuckmann (1979). Briefly, strains were grown in M9 minimal media (Difco) supplemented with 1 mM MgSO₄, 0.2% glucose, and 0.2% Tween 20 (Fisher Scientific) at 28°C for 24 hours with aeration. After an additional 96 hours of static growth, cell-free supernatant was analyzed for lipase activity. A 200-μl aliquot of supernatant was diluted with 2.4 ml of a solution consisting of 30 mg of p-nitrophenylpalmitate (Sigma) dissolved in 10 ml of isopropanol and 90 ml of 0.5 M Sorensen phosphate buffer supplemented with 207 mg of sodium deoxycholate

(Sigma) and 100 mg of gum arabic (Sigma). After incubation with the supernatant for 30 minutes at 28°C, the absorbance was measured at a wavelength of 410 nm. One enzyme unit was defined as the liberation of 1 nmol of p-nitrophenyl/ml/min.

3.3.14. Total AI analysis. Total autoinducer production was monitored using *P. aeruginosa* QSC105 (pEAL01) (Ling et al. 2001). Strains were grown in 10 ml of M9 minimal media for 18 hours at 28°C. Cultures were centrifuged and supernatants extracted with ethyl acetate as described by Pesci et al. (1999). Briefly, extracts were dried in test tubes under a stream of nitrogen gas. An overnight culture of *P. aeruginosa* QSC105 (pEAL01) was diluted to a final OD₆₀₀ of 0.1 and 1-ml aliquots were transferred to tubes containing dried extracts. These cultures were grown for 18 hours at 37°C with vigorous shaking before β -galactosidase activity was determined.

3.3.15. Analysis of transcriptional fusions. The activity of *prnA*, *phzA*, *phzI*, *phzR*, *rpoS*, *gacA*, and *gacS* transcriptional fusions was monitored in PA23 and the SR mutants. Strains harboring the pLP170 derivatives were grown for 4, 8, 12, 16, and 24 hours prior to analysis of β -galactosidase activity (Miller 1972).

3.3.16. RpoS expression. In order to assess RpoS protein levels, sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis were performed. Strains were grown in 3 ml of M9 minimal media at 28°C. After 24 hours growth, the cultures had reached an OD_{600nm} of approximately 1.3 and were subsequently diluted to an OD of 1.0 to ensure that an equal number of cells were

analyzed. Cells were pelleted, resuspended in sample buffer (63 mmol/L Tris-HCl 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 μ g 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 raised against *E. coli* (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, Laval, PQ). RpoS was quantified with a Fluorochem 2000 Phosphoimager using Fluorochem Stand- Alone software, version 2.0. The Western analysis was repeated twice with similar findings.

3.3.17. Statistical Analysis.

An unpaired Student's *t* test was used for statistical analysis of antifungal, protease, and lipase activity as well as AI levels.

3.4. Results

3.4.1. 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 (Fig. 3.1). 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 (Fig. 3.2). We were unable to generate a PA23 *spoT* single mutant, leading us to believe that in a *relA*⁺ background, *spoT* may be required for cell viability.

To determine if disruption of the aforementioned genes impacted (p)ppGpp production, bacteria were grown in the presence of DL-serine hydroxymate, a potent inducer of the SR. As shown in Figure 3.3, PA23 was able to produce both ppGpp and pppGpp (lane 2); however, (p)ppGpp was undetectable in the SR mutants (lanes 3 & 4). Production of these nucleotides was restored in PA23*relA* and PA23*relAspoT* when the *relA* gene was added *in trans* (Fig 3.3, lanes 5 & 6). The presence of *spoT* in multicopy, however, did not restore (p)ppGpp production in the SR mutants (Fig. 3.3).

Rescue clone containing the PA23 *relA* gene

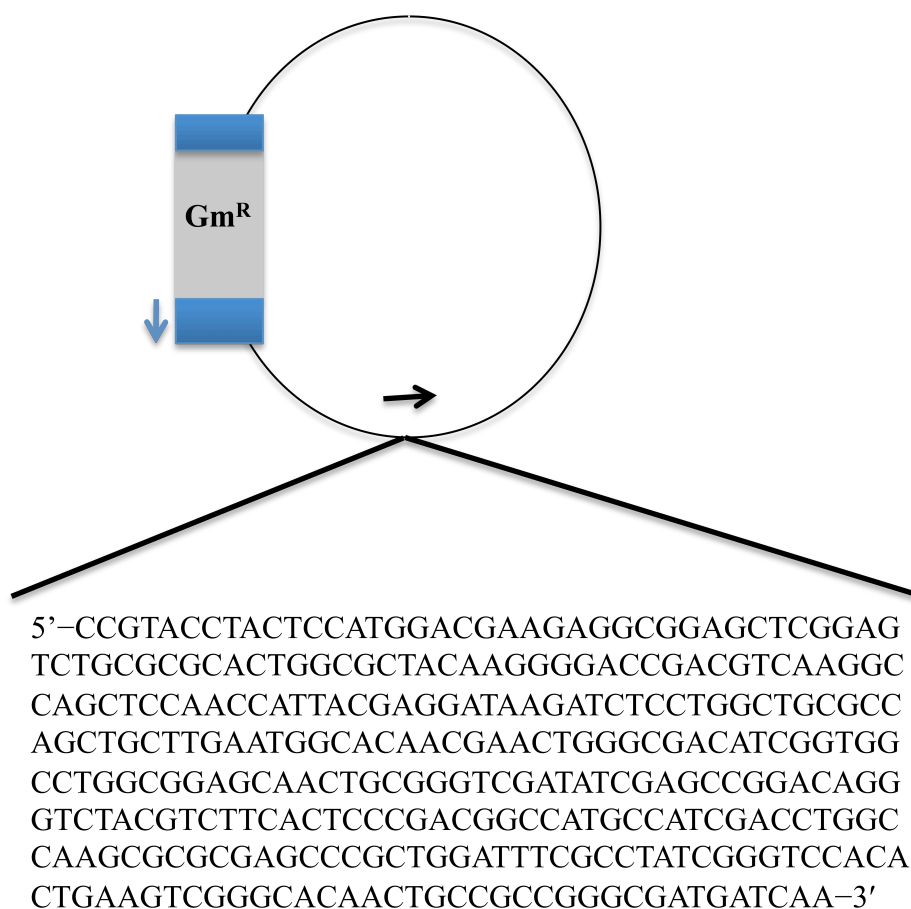


Figure 3.1. Confirmation of pKNOCK-Gm insertion into the *relA* gene of *Pseudomonas chlororaphis* PA23. The pKNOCK-Gm vector was rescued from the PA23 genome by digestion with *Hinc*II and sequenced using primer *relA*-KpnIFOR, which is indicated by the blue arrow. The sequence corresponds to nucleotides 1075 to 1392 of the PA23 *relA* gene.

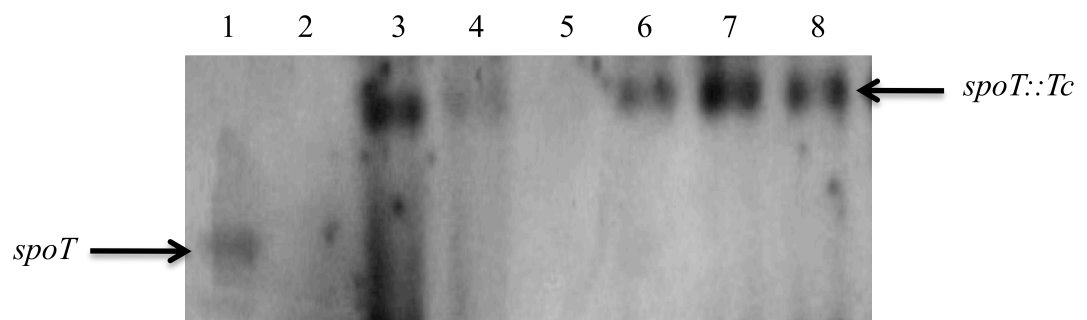
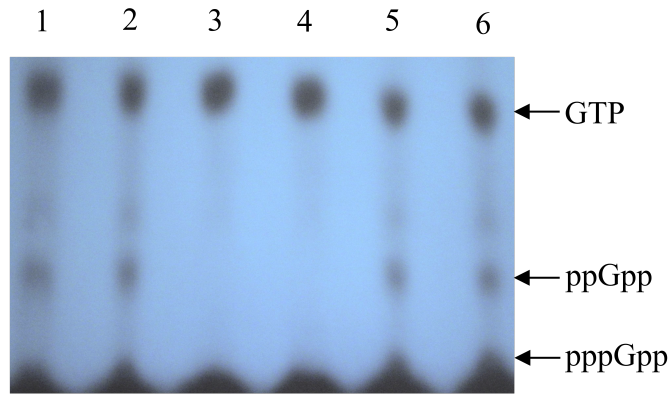


Figure 3.2. Southern blot analysis confirming the insertion of the Tc^R marker into the *spoT* gene of PA23relA. Genomic DNA was digested with *Bam*HI and *Hind*III enzymes and probed with a *spoT* DIG-labelled probe. Lanes are as follows: 1, PA23relA; 2, empty well; and 3-8, putative PA23relA*spoT* mutants. Mutants in lanes 3, 6, 7, and 8 appear to have the wild-type *spoT* gene replaced with the mutated version of the gene. The mutant in lane 3 was chosen for further analysis.

A.



B.

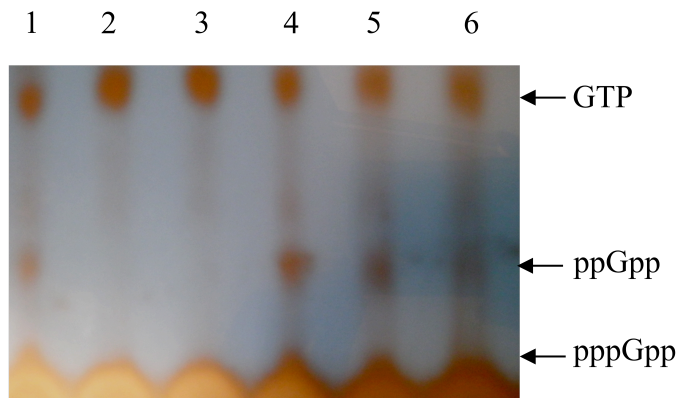


Figure 3.3. (p)ppGpp analysis of *Pseudomonas chlororaphis* PA23 and its SR derivatives after induction with serine hydroxymate. Cells were labeled with ^{32}P and separated by thin layer chromatography. Strains analyzed in panel A are as follows: Lane 1: PAO1; Lane 2: PA23; Lane 3: PA23*relA*; Lane 4: PA23*relAspoT*; Lane 5: PA23*relA* (pUCP22-*relA*); and Lane 6: PA23*relAspoT* (pUCP22-*relA*). Strains analyzed in panel B are as follows: Lane 1: PAO1; Lane 2: PA23*relA* (pUCP22-*spoT*); Lane 3: PA23*relAspoT* (pUCP22-*spoT*); Lane 4: PA23-314; Lane 5: PA23*gacA*; and Lane 6: PA23.

3.4.2. The SR affects PA23-mediated biocontrol 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 to the wild type (Table 3.2). The presence of pUCP22-*relA* in the SR mutants restored the AF activity to that of the wild type (Table 3.2). Conversely, pUCP22-*spoT* did not alter fungal antagonism by the mutants (Table 3.2.).

PA23 produces a number of AF factors including PRN, PHZ, and HCN. Of these antibiotics, 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 biocontrol activity demonstrated by the ppGpp-deficient strains was the result of increased antibiotic production. Expression of *phzA-lacZ* (PHZ) and *prnA-lacZ* (PRN) transcriptional fusions and an *hcnA-lacZ* (HCN) translational fusion were monitored in PA23, PA23*relA*, and PA23*relAspoT*. As shown in Figure 3.4, *phzA-lacZ* and *hcnA-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*. When we looked at PHZ, PRN and HCN production, we observed the same trend. There were no differences in terms of total PHZ produced (PA23, 25.05 ± 1.13 µg/ml; PA23*relA*, 26.54 ± 1.43 µg/ml; PA23*relAspoT*, 24.53 ± 0.26 µg/ml) and all three strains tested positive for HCN production (Table 3.3). In contrast, the total amount PRN in the cell-free supernatants of the SR mutants was 2.5-fold greater than PA23 (Table 3.4). Mobilization of pUCP22-*relA* into PA23*relA* and PA23*relAspoT* reduced PRN production to wild-type levels (Table 3.4).

TABLE 3.2. Phenotypic characterization of *P. chlororaphis* PA23 and derivatives harboring *relA* (pUCP22-*relA*), *spoT* (pUCP22-*spoT*), *gacA* (pME3066), *gacS* (pUCP23-*gacS*), and *rpoS* (pUCP22-*rpoS*).

Strains	Extracellular Metabolite Activity		
	Antifungal (cm) ^a	Protease (Units of enzyme/ml) ^a	Lipase (Units of enzyme/ml/min) ^a
PA23 (pUCP22)	1.5 (0.1)	0.68 (0.10)	1.49 (0.02)
PA23 <i>relA</i> (pUCP22)	2.0 (0.2) ^b	1.19 (0.05) ^c	1.87 (0.06) ^c
PA23 <i>relAspoT</i> (pUCP22)	2.0 (0.2) ^b	1.55 (0.17) ^b	2.02 (0.16) ^c
PA23 <i>relA</i> (pUCP22- <i>relA</i>)	1.4 (0.1) ^{d,e}	0.58 (0.13) ^{d,i}	1.39 (0.32) ^{d,e}
PA23 <i>relAspoT</i> (pUCP22- <i>relA</i>)	1.3 (0.2) ^{d,f}	0.54 (0.17) ^{d,j}	1.48 (0.07) ^{d,l}
PA23 <i>relA</i> (pUCP22- <i>spoT</i>)	1.9 (0.2) ^{b,g}	1.02 (0.14) ^{c,g}	1.92 (0.06) ^{c,g}
PA23 <i>relAspoT</i> (pUCP22- <i>spoT</i>)	2.1 (0.1) ^{c,h}	1.27 (0.12) ^{c,h}	2.17 (0.20) ^{c,h}
PA23 <i>relA</i> (pME3066)	2.5 (0.3) ^{b,g}	1.81 (0.67) ^{c,g}	1.83 (0.10) ^{c,g}
PA23 <i>relAspoT</i> (pME3066)	2.3 (0.1) ^{b,h}	1.34 (0.20) ^{c,h}	1.91 (0.10) ^{b,h}
PA23 <i>relA</i> (pUCP23- <i>gacS</i>)	1.4 (0.1) ^{d,e}	0.79 (0.20) ^{d,j}	1.48 (0.13) ^{d,i}
PA23 <i>relAspoT</i> (pUCP23- <i>gacS</i>)	1.2 (0.2) ^{d,f}	0.86 (0.05) ^{d,f}	1.58 (0.04) ^{d,f}
PA23 <i>relA</i> (pUCP22- <i>rpoS</i>)	1.4 (0.2) ^{d,e}	0.67 (0.02) ^{d,k}	1.31 (0.23) ^{d,e}
PA23 <i>relAspoT</i> (pUCP22- <i>rpoS</i>)	1.4 (0.1) ^{d,f}	0.85 (0.10) ^{d,j}	1.16 (0.30) ^{d,f}

^a Mean (standard deviation) obtained from five replicates.

^b Significantly different from wild type ($p < 0.01$).

^c Significantly different from wild type ($p < 0.05$).

^d Not significantly different from wild type.

^e Significantly different from PA23*relA* ($p < 0.05$).

^f Significantly different from PA23*relAspoT* ($p < 0.05$).

^g Not significantly different from PA23*relA*.

^h Not significantly different from PA23*relAspoT*.

ⁱ Significantly different from PA23*relA* ($p < 0.01$).

^j Significantly different from PA23*relAspoT* ($p < 0.01$).

^k Significantly different from PA23*relA* ($p < 0.005$).

^l Significantly different from PA23*relAspoT* ($p < 0.005$).

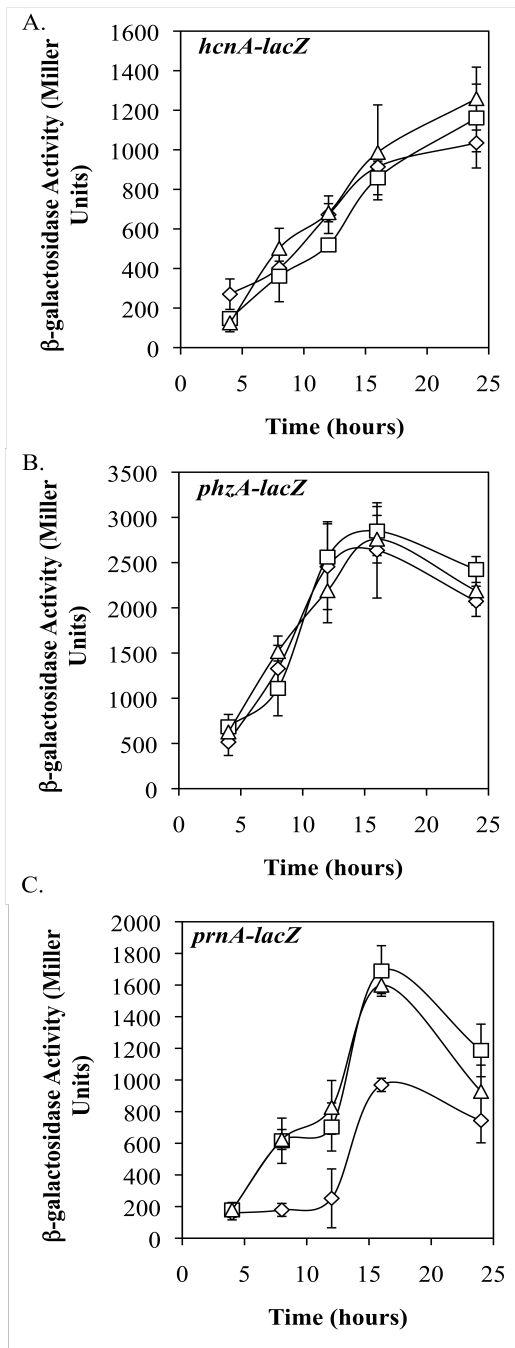


Figure 3.4. The impact of the SR on *hcnA*, *phzA* and *prnA* expression. Beta-galactosidase activity associated with strains harboring either an *hcnA-lacZ* translational fusion (panel A), a *phzA-lacZ* transcriptional fusion (panel B) or a *prnA-lacZ* transcriptional fusion (panel C) are shown. Bacterial strains are as follows: *P. chlororaphis* PA23 (diamond), PA23relA (square), and PA23relAspoT (triangle). Bacteria were grown in M9 minimal media supplemented with 1 mM MgSO₄ and 0.2% glucose.

TABLE 3.3. HCN activity of *Pseudomonas chlororaphis* PA23 and the SR mutants.

Strains	HCN activity ^a
PA23	+
PA23 <i>relA</i>	+
PA23 <i>relAspoT</i>	+

^a Determined using Cyantesmo paper.

TABLE 3.4. Pyrrolnitrin production by *P. chlororaphis* PA23 and derivatives harboring *relA* (pUCP22-*relA*), *spoT* (pUCP22-*spoT*), *gacA* (pME3066), *gacS* (pUCP23-*gacS*), and *rpoS* (pUCP22-*rpoS*).

Strains	Pyrrolnitrin (μg) ^a
PA23 (pUCP22)	1.80 (0.50)
PA23 <i>relA</i> (pUCP22)	4.49 (1.16) ^b
PA23 <i>relAspoT</i> (pUCP22)	5.04 (0.90) ^b
PA23 <i>relA</i> (pUCP22- <i>relA</i>)	1.83 (0.09) ^c
PA23 <i>relAspoT</i> (pUCP22- <i>relA</i>)	1.54 (0.20) ^c
PA23 <i>relA</i> (pUCP22- <i>spoT</i>)	5.56 (0.30) ^b
PA23 <i>relAspoT</i> (pUCP22- <i>spoT</i>)	6.31 (0.87) ^b
PA23 <i>relA</i> (pME3066)	3.00 (0.07) ^b
PA23 <i>relAspoT</i> (pME3066)	4.21 (0.52) ^b
PA23 <i>relA</i> (pUCP23- <i>gacS</i>)	1.03 (0.43) ^c
PA23 <i>relAspoT</i> (pUCP23- <i>gacS</i>)	1.66 (0.10) ^c
PA23 <i>relA</i> (pUCP22- <i>rpoS</i>)	1.86 (0.40) ^c
PA23 <i>relAspoT</i> (pUCP22- <i>rpoS</i>)	1.61 (0.10) ^c

^a Mean (standard deviation) of PRN extracted from 100-ml culture volume.

^b Significantly different from wild type.

^c Not significantly different from wild type.

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 3.2). Similarly, protease activity was elevated 1.75 fold and 2.3 fold in the single and double mutants, respectively (Table 3.2). Addition of pUCP22-*relA* restored lipase and protease activity to wild-type levels (Table 3.2).

3.4.3. RelA is negatively regulated by (p)ppGpp. When expression of a *relA-lacZ* translational fusion was monitored in PA23 and the SR mutants, all three strains showed maximal *relA* expression at stationary phase (16 h of growth) followed by a sharp decline in expression at 24 h. However at 16 h, PA23*relA* and PA23*relAspoT* exhibited a 2-fold or greater increase in *relA* expression compared to the wild type (Fig. 3.5). Thus it appears that the SR negatively regulates *relA* in PA23.

3.4.4. The Gac system is not required for *relA* expression or (p)ppGpp synthesis, rather the SR regulates *gacS* and *gacA*. To explore whether *relA* is under control of the Gac system, a *relA-lacZ* translational fusion was mobilized into *gacA* and *gacS* mutants of PA23. No differences in *relA-lacZ* activity were observed between PA23*gacA* (2997 $\pm$ 229 Miller Units), the *gacS* mutant PA23-314 (3141 $\pm$ 392 Miller Units), and the wild type (2801 $\pm$ 156 Miller Units) after 16 hours of growth. (p)ppGpp levels were also unaffected by the *gacA* and *gacS* mutations (Fig. 3.3). However, when we investigated whether the SR controls *gacA* and *gacS*, we discovered that expression of these genes was altered in the SR mutants.

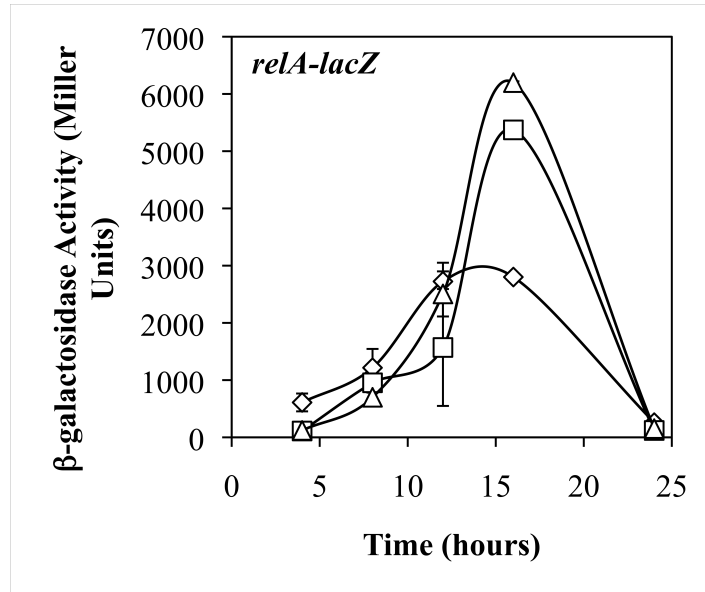


Figure 3.5. RelA exhibits negative autoregulation. Expression of *relA* was monitored in PA23 (diamond), PA23*relA* (square), and PA23*relAspoT* (triangle). Strains were grown in M9 minimal media supplemented with 1 mM MgSO₄ and 0.2% glucose.

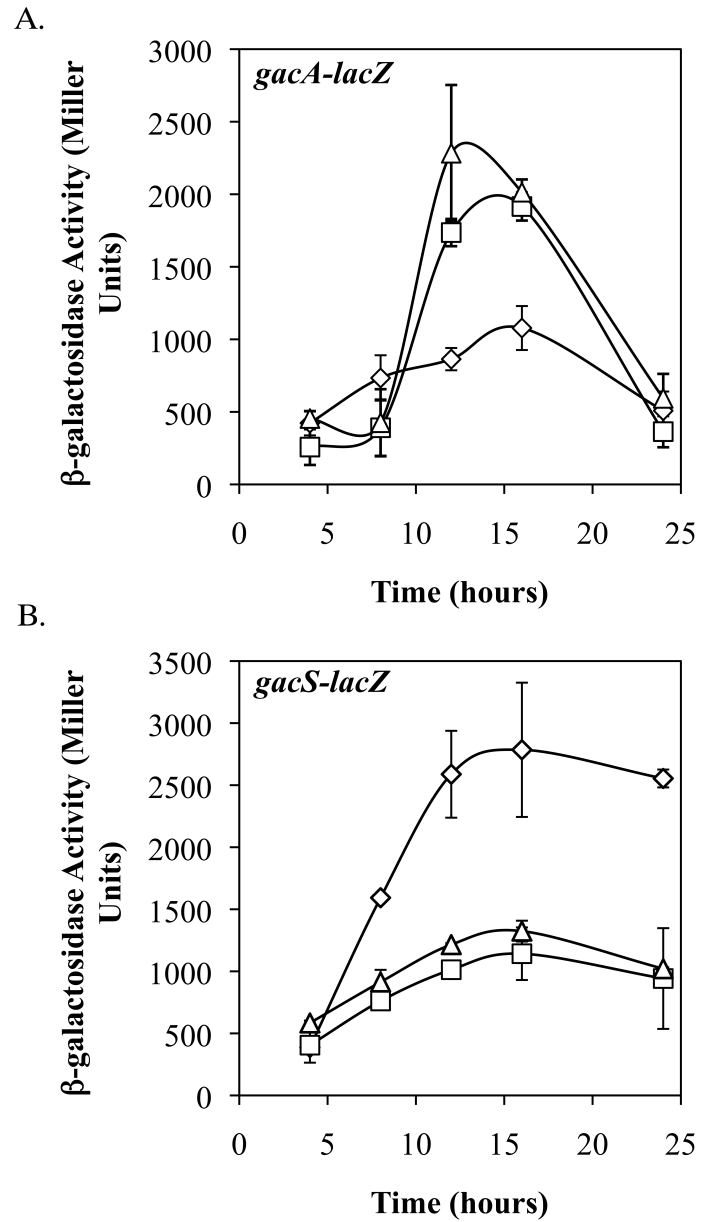


Figure 3.6. Transcription of *gacA* (A) and *gacS* (B) is regulated by the SR. Strains used are as follows: *P. chlororaphis* PA23 (diamond), PA23*relA* (square), and PA23*relAspoT* (triangle). Strains were grown in M9-minimal media supplemented with 1 mM MgSO₄ and 0.2% glucose.

Transcription of *gacA* was elevated 2 fold in the SR mutants at 12 and 16 h (Fig. 3.6A). The opposite trend was observed for *gacS* where transcription levels were dramatically reduced between 8 and 24 h of growth (Fig. 3.6B). Next, plasmids containing either *gacS* or *gacA* were mobilized into the mutants to see if one or both genes could complement PA23*relA* and PA23*relAspoT*. We discovered that pUCP*gacS* restored the mutant phenotype to that of the wild type (Tables 2.2 & 2.3). Conversely, pME3066, a low-copy number plasmid harboring the *gacA* gene of *P. fluorescens* CHA0 (Laville et al. 1998) was unable to complement the SR mutants (Tables 2.2 & 2.3).

3.4.5. The SR affects transcription of *phzR* and *phzI*, and production of AI. To establish if a connection exists between the SR and the Phz QS system, we assayed the activity of *phzI-lacZ* and *phzR-lacZ* transcriptional fusions. Mutations in the *relA* and *relAspoT* genes resulted in a ≥ 2 -fold decrease in *phzI* transcription between 12 and 24 h of growth (Fig. 3.7A). Transcription of *phzR*, on the other hand, was elevated in the SR mutant backgrounds (Fig. 3.7B). The total amount of AI present in the supernatants of PA23, PA23*relA* and PA23*relAspoT* was determined using *P. aeruginosa* QSC105 (pEAL01), a strain capable of detecting a broad range of AHLs (Ling et al., 2009). AI production was down in both SR mutants (Fig. 3.7C), consistent with the *phzI* transcriptional data. As expected, addition of pUCP*relA* restored AI levels back to wild type (Fig. 3.7C).

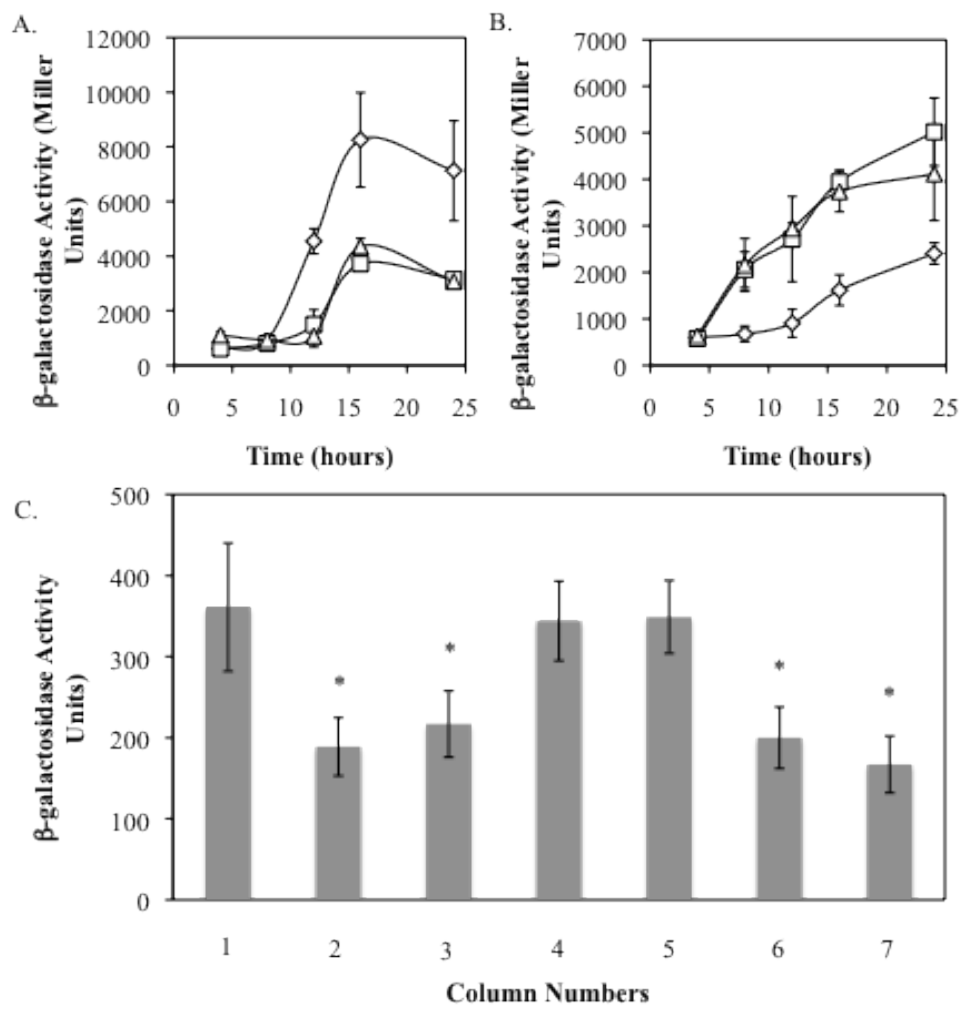


Figure 3.7. The impact of the SR on the QS system. Transcription of *phzI* (A) and *phzR* (B) were monitored in PA23 (diamond), PA23relA (square), and PA23relAspoT (triangle). (C) Total AI activity extracted from culture supernatants of PA23 and its derivatives using reporter strain *P. aeruginosa* QSC105 (pEAL01.) Strains are as follows: column 1, PA23 (pUCP22); column 2, PA23relA (pUCP22); column 3, PA23relAspoT (pUCP22); column 4, PA23relA (pUCP22-relA); column 5, PA23relAspoT (pUCP22-relA); column 6, PA23relA (pUCP22-spoT); and column 7, PA23relAspoT (pUCP22-spoT). For strains that differ significantly from the wild type, columns are labeled with * ($p < 0.05$). All strains utilized in these studies were grown in M9 minimal media supplemented with 1 mM MgSO₄ and 0.2% glucose.

3.4.6. 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, Erikson et al. 2004, Potrykus and Cashel 2008). To determine if *rpoS* forms part of the PA23 SR regulon, expression of an *rpoS-lacZ* transcriptional fusion was monitored in the SR mutants. As shown in Fig 3.8, *rpoS* transcription was dramatically reduced in the mutants during both the logarithmic and stationary phases of growth. Furthermore, there was markedly less RpoS protein produced by the SR mutants (Fig. 3.9). As illustrated in Fig. 3.9, addition of *relA*, *rpoS*, or *gacS* in trans restored RpoS production by the SR mutants to that of the wild type or higher. To address whether decreased *rpoS* expression was responsible for the phenotypic changes observed for the SR mutants, strains harboring pUCP22-*rpoS* were examined for production of biocontrol factors. The presence of the plasmid-borne *rpoS* fully complemented the *relA* and *relAspoT* mutants with respect to antifungal, protease and lipase activity (Table 3.2) and PRN production (Table 3.3). Therefore, it appears that the effects of the SR on PA23 biocontrol are primarily mediated through RpoS.

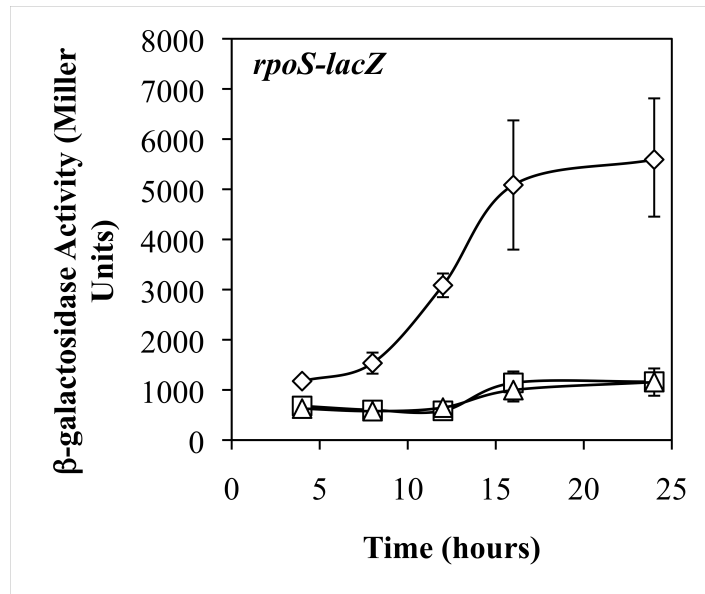


Figure 3.8. Transcription of *rpoS* in PA23 and its derivatives. Strains used are as follows: PA23 (diamond), PA23*relA* (square), and PA23*relAspoT* (triangle). Strains were grown in M9 minimal media supplemented with 1 mM MgSO₄ and 0.2% glucose.

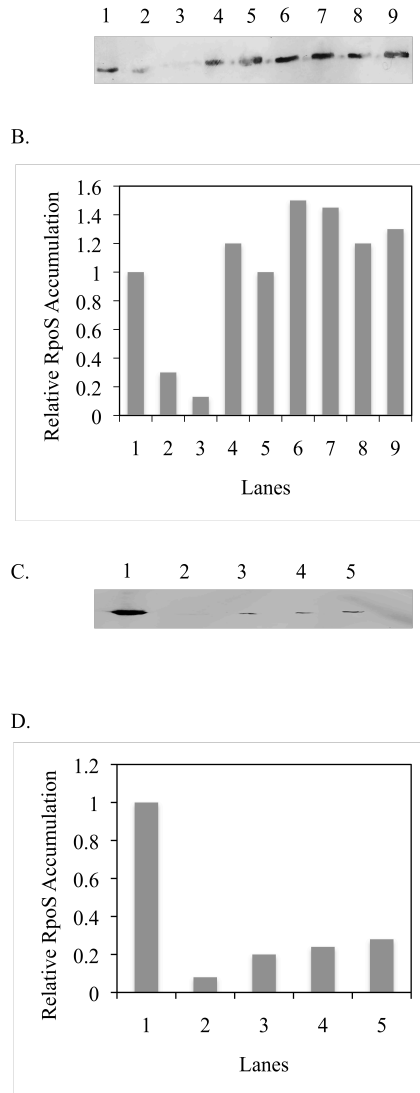


Figure 3.9. Relative accumulation of RpoS protein in *Pseudomonas chlororaphis* PA23 and its derivatives. (A) and (C) RpoS-specific antiserum that was raised against *E. coli*, was used to visualize σ^S from Western blot of protein extracted from cultures grown to stationary phase. Each lane contains 100 mg of protein. Lanes for panel A are as follows: 1, PA23 (pUCP22); 2, PA23*relA* (pUCP22); 3, PA23*relAspoT* (pUCP22); 4, PA23*relA* (pUCP*relA*); 5, PA23*relAspoT* (pUCP*relA*); 6, PA23*relA* (pUCP22-*rpoS*); 7, PA23*relAspoT* (pUCP22-*rpoS*); 8, PA23*relA* (pUCP22-*gacS*); and 9, PA23*relAspoT* (pUCP23-*gacS*). Lanes for panel C are as follows: 1, PA23 (pUCP22); 2, PA23*relA* (pUCP22-*spoT*); 3, PA23*relAspoT* (pUCP22-*spoT*); 4, PA23*relA* (pME3066); and 5, PA23*relAspoT* (pME3066). (B) and (D) The RpoS content in each lane was estimated by analyzing Western blots with a phosphoimager. The Western blot analysis was performed twice and a representative data set is shown. Note: the presence of *relA* (lanes 4 and 5) is able to restore RpoS expression to wild-type levels in the SR mutants (Panel A). On the other hand, *spoT* (lanes 4 and 5) do not affect RpoS production in the SR mutants (Panel B).

3.5. Discussion

The SR is a regulatory mechanism that enables bacteria to adapt to nutrient deprivation through global changes in gene expression (Potrykus and Cashel 2008). A myriad of physiological traits are altered during the SR including growth rate, biofilm formation, motility, and secondary metabolite production (Potrykus and Cashel 2008). When applied as a foliar spray, *P. chlororaphis* strain PA23 is an effective biocontrol agent capable of inhibiting SSR of canola (Savchuk and Fernando 2004, Poritsanos et al. 2006). Because nutrients are scarce in the phyllosphere (Lindow and Brandle 2003), we were interested in understanding how the SR affects the biocontrol properties of this bacterium.

PA23 *relA* and *relAspoT* derivatives were created that showed reduced (p)ppGpp accumulation and enhanced antifungal activity under nutrient-limiting conditions (Table 2). Thus it appears that the SR negatively affects the antagonistic potential of this organism. PA23 produces a number of compounds that contribute to biocontrol, including diffusible antibiotics (PRN, PHZ), HCN, and degradative enzymes (lipase, and protease) (Poritsanos et al. 2006). We previously reported that PRN plays a more important role than PHZ in inhibiting *S. sclerotiorum* (Selin et al. 2010). The enhanced fungal antagonism led us to speculate that the SR mutants were producing increased amounts one or more of these metabolites. This was found to be the case as lipase, protease, and PRN were all elevated in the SR mutants (Tables 2.2 & 2.3); PHZ on the other hand was unaffected. 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. 2.4). 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 by PA23 remained unchanged, we conclude that the SR impacts not all of the antifungal compounds produced by this bacterium. Similarly in *Streptomyces* spp, the SR differentially affects antibiotic production. Depending upon the strain in question, the antibiotics produced and the growth media 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* ATCC27064 abolished cephamycin C production (Jin et al. 2004). This same mutation in *Streptomyces coelicolor* strain A3(2) had little effect on actinorhordin and undecylprodigiosin expression (Sun et al. 2001). From these examples and our own findings, it is evident that a great deal of variability exists with respect to how this global stress response impacts production of antibiotics.

In pseudomonads, a complex regulatory network governs expression of biocontrol factors. It was of interest, therefore, to determine whether the SR has any effect on these other regulatory systems. At the top of the regulatory hierarchy is the GacA/GacS two-component system. Expression of both *gacA* and *gacS* was

impacted by the SR, but in an opposing fashion with *gacS* being positively regulated and *gacA* being negatively regulated by the SR (Fig. 3.6). Considering *gacA* transcription was already elevated in the SR mutants, we were not surprised to find that *gacA* provided in trans had no discernible effect on the SR mutants. *gacS* transcription on the other hand, was decreased and addition of pUCP23-*gacS* to the SR-deficient strains restored the mutant phenotype to that of the wild type (Tables 2.2 & 2.3). These findings indicate that in PA23, GacS functions downstream of RelA. To the best of our knowledge, this is the first report of Gac forming part of the SR regulon in *Pseudomonas* spp. However in *Legionella pneumophila*, two orthologs of GacS and GacA, called LetA and LetS, were needed for activation of the RelA-controlled transmission phenotype (Hammer et al. 2002). It was discovered that *letA* and *letS* mutants were unable to differentiate into the virulent form, and these researchers concluded that (p)ppGpp accumulation in response to nutrient starvation triggers the LetA/S-dependent differentiation process (Hammer et al. 2002). Hammer and colleagues proposed that (p)ppGpp might be responsible for direct activation of the GacS-GacA phosphorelay cascade. As the nature of the signaling molecule to which GacS responds has not yet been discovered, the idea that (p)ppGpp might be directly or indirectly involved in Gac signaling is intriguing. Current efforts are ongoing in our laboratory to explore this possibility.

Another regulatory mechanism, known as QS has been shown to play a role in the antifungal activity of various pseudomonads (Heeb and Haas 2001, Haas and D  fago 2005, Mavrodi et al. 2006). Links between the SR and QS have been demonstrated in *P. aeruginosa* PAO1 where the SR positively affects AHL

production (Van Delden et al. 2001, Erickson et al. 2004). In a *relA* mutant of PAO1, a slight delay in 3-oxo-C₁₂-HSL and C₄-HSL production was observed (Erickson et al. 2004). Similarly, PAO1 overexpressing the *relA* gene of *E. coli* showed a premature induction of autoinducer expression (Van Delden et al. 2001). In the present study, the PA23 SR mutants exhibited reduced *phzI* transcription, while *phzR* transcription was elevated (Fig 3.7A & 3.7B). These findings indicate that the SR differentially regulates PhzI and PhzR, which, much like GacA and GacS, seems counterintuitive since they function as a pair. However in *P. aeruginosa* strain PAO1, *lasI* and *rhlI* expression were found to correlate poorly with *lasR* and *rhlR* expression, respectively, under 42 different test parameters (Duan and Surette 2007). It was concluded that under many conditions the *P. aeruginosa* I- and R-genes are independently regulated (Duan and Surette 2007). We observed reduced AHL levels in the PA23*relA* and PA23*relAspoT* supernatants (Fig 3.7C), consistent with our *phzI* expression data. It was somewhat surprising, therefore, to find that PHZ production was unchanged in the SR mutants. A possible explanation for this discrepancy is that under low-nutrient conditions, PHZ expression is controlled by factors outside of QS that are not affected by the SR. In support of this idea, when *P. chlororaphis* strain 30-84 was grown in minimal media, a repressor called RpeA decreased production of this antibiotic independent of the PHZ QS system (Whistler and Pierson 2003). Collectively, these findings suggest that PHZ expression is complex and controlled not only by regulatory elements but also environmental conditions.

In *E. coli* and *P. aeruginosa*, the SR positively regulates expression of RpoS (Van Delden et al. 2001, Erickson et al. 2004, Potrykis and Cashel, 2008). In much

the same way, we observed a 5-fold decrease in *rpoS* transcription in the SR mutants compared to PA23 (Fig. 3.8). 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. 2006, Park et al. 2011). Although we have not yet determined how RpoS affects PA23 biocontrol, the 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 antifungal, protease and lipase activity were restored to that of the wild type (Tables 2.2 and 2.3). Moreover, the SR mutants containing multicopy *relA* or *gacS* exhibited RpoS levels equal to or higher than PA23 (Fig. 3.9). It is not surprising that *relA* was able to complement the SR mutants in terms of RpoS and antifungal factor production. The elevated RpoS exhibited by the mutants harboring pUCP23-*gacS*, conversely, explains why *gacS* reduces PRN, protease and lipase down to parental levels. Collectively, these findings indicate that the effect of the SR on PA23 biocontrol is mediated primarily through RpoS. Reduced RpoS levels are expected to increase the pool of available core RNAP for the sigma factor(s) governing PRN, protease and lipase transcription. Alternatively, RpoS may be required for expression of a repressor of these antifungal factors. Regardless of the mechanism we believe that in the SR mutants, reduced RpoS leads to elevated PRN, lipase and protease activity, which ultimately increases fungal antagonism in vitro. Whether these strains would exhibit enhanced biocontrol in planta has yet to be established. Decreased RpoS levels would likely render the SR mutants 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. Clearly more work is required to understand how the SR affects PA23 colonization and biocontrol in different environments.

In summary, we have established that the SR exerts a negative effect on PA23 biocontrol in vitro, mediated by increased RpoS expression. Because manufacture of antifungal factors is energetically costly, it is logical for bacteria to reduce production when nutrients are scarce. Future work will be directed at understanding how the SR affects PA23 persistence and biocontrol of *S. sclerotiorum* in the canola phyllosphere.

3.6. Acknowledgements

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Chapter 4.

Major Conclusions and Future Directions.

4.1 Major conclusions and future directions

Fluctuation in the availability of nutrients is a salient characteristic of the plant surface (Lindow and Brandl 2003). For bacteria to persist in this environment, they must induce sophisticated coping strategies. The SR is one such mechanism that regulates the transition from active growth to stress survival during periods of nutrient limitation. When applied as a foliar spray, both *Pseudomonas* sp. DF41 and *P. chlororaphis* PA23 are able to protect canola from the fungal pathogen *S. sclerotiorum*. As such, we sought to investigate the influence of the SR on the antifungal activities of these two biocontrol strains.

Through allelic exchange DF41 and PA23 *relA* and *relAspoT* mutants were generated that were no longer producing (p)ppGpp. Characterization of DF41*relA* and DF41*relAspoT* revealed enhanced growth inhibition of *S. sclerotiorum* in vitro as a consequence of early induction of the NRPS genes and elevated LP production. Over-expression of *relA* and not *spoT* in the SR mutants was able to restore the mutant phenotype to that of the wild type suggesting that *spoT* may not have a significant role in the regulation of secondary metabolites. Moreover, a *spoT* mutant could not be generated suggesting that in this scenario, elevated amounts of (p)ppGpp produced by this strain may be lethal. However, for reasons unknown, the viability of the *relAspoT* mutant was not compromised when *relA* was present in multicopy. A *spoT* mutant has been reported in *Pseudomonas* sp. MIS38 (Washio et al. 2010); therefore, other means instead of traditional allelic exchange may be necessary to generate DF41*spoT*. In several bacteria, (p)ppGpp functions as a global regulator of gene expression (Potrykus and Cashel 2008). Another possible explanation for the

elevated amounts of LP found in the supernatants of the SR mutants compared to the wild type is that genes involved LP efflux may be SR regulated. Licciarello and coworkers (2009) described the importance of antibiotic transport on *Pseudomonas corrugata* virulence (Licciarello et al. 2009). Therein, secretion of the LPs corpeptin A and corpeptin B was found to be mediated by RfiA, a transcriptional regulator of a multidrug efflux system located downstream of the *rfiA* gene (Licciardello et al. 2009). Mutation of this gene rendered *P. corrugata* incapable of infecting tomato plants due to an inability to secrete the aforementioned antibiotics (Licciardello et al. 2009). In strain DF41, an *rfiA* homolog has been identified downstream of the AI-synthase gene *pdfI* (Berry 2010). It would be of interest to determine if the SR inhibits the LP efflux through regulation of *rfiA*. The impact of the *rfiA* on DF41-mediated antagonism of *S. sclerotiorum* is currently under investigation.

Consistent with other biocontrol pseudomonads, strain DF41 requires the two-component regulatory system, GacA/GacS for the expression of its antifungal factors (Berry et al. 2010). More specifically, a mutation in the *gacS* gene inhibited the synthesis of a number of important traits including the production of LP and protease, and swarming motility (Berry et al. 2010). Herein, the *gacS* mutant DF41-469 exhibited reduced *relA* expression and (p)ppGpp synthesis. These results indicate that in strain DF41, the SR forms part of the Gac regulon. Gac is known to exert its effects through an auxiliary system termed Rsm (Haas and D  fago 2005). Essentially, the translation of target mRNA is inhibited by RsmA and RsmE and this repression is lifted by the Gac-regulated small RNA molecules that sequester these proteins (Haas and D  fago 2005). One could speculate, therefore, that in strain

DF41-469 RsmA and RsmE might continuously repress the translation of *relA* due to the absence of small non-coding RNAs. An RsmZ homolog has been identified in strain DF41 and appears to function downstream of GacS since over-expression of the former in the *gacS* mutant increased AF, protease, and HCN activity, and autoinducer production (Berry 2010). Therefore, this strain should be assessed for *relA* translation and (p)ppGpp production. Homologs of RsmA and RsmE, and the non-coding RNAs RsmX and RsmY have yet to be found in strain DF41. The identification of these molecules would aid in establishing where the SR lies in the Gac/Rsm cascade. To date, there are no reports in the literature linking the SR to Rsm-mediated post-transcriptional regulation and vice versa.

Although QS has been demonstrated to influence the biocontrol activity of other pseudomonads (Haas and Défago 2005), it is not required for DF41-mediated antagonism of *S. sclerotiorum* (Berry 2010). Instead, the role of this system may be to influence the expression of other regulatory factors such as *rpoS* and *rsmZ* (Berry 2010). In this study, *relA* translation was unaffected by the presence of pME6863, a plasmid harboring the gene *aiiA* encoding an AHL-degrading enzyme (Manuel and de Kievit, unpublished data). Thus it appears that the SR is not QS regulated. In strains DF41*relA* and DF41*relA**spoT*, transcription of *pdfI* was reduced yet total amounts of AHLs between the wild type and the SR mutants remained equivalent (Manuel and de Kievit, unpublished data). These results indicate that in the SR mutants, the expression of a second, yet-to-be identified QS system might be enhanced. The presence of a second system has been previously suggested from the observation that in a *pdfI* mutant of DF41, residual amounts of AHLs were observed (Berry and d

Kievit, unpublished data). Therefore, it would be of benefit to identify the genes involved in the second QS system and establish whether they are indeed SR regulated.

(p)ppGpp-mediated negative control is believed to occur when the alarmone directly binds to the RNAP and/or when the pool of available core RNAP is increased for use by alternative sigma factors (Potrykus and Cashel 2008). In the latter situation, genes that depend on the housekeeping sigma factor for their transcription are inhibited. Mutation of the *relA* and *relAspoT* genes reduced *rpoS* transcription indicating that the SR positively regulates this sigma factor. However, when *rpoS* was over-expressed in the mutant strains, LP production was unaffected while protease activity was restored to wild-type levels. These findings suggest that the SR has a direct influence on antibiotic expression while protease production is indirectly regulated through RpoS. Alternatively, the presence of an RpoS-dependent negative regulator may function to inhibit the synthesis of protease. In *P. fluorescens* CHA0, the repressor proteins RsmA and RsmE were found to prevent the translation of *aprA*, which encodes exoprotease (Reimmann et al. 2005). Therefore, to clarify the role of RpoS on protease production in strain DF41, isolation *rsmA*, *rsmE*, and *aprA* homologs would be beneficial.

Similar to strain DF41, the (p)ppGpp-minus strains PA23*relA* and PA23*relAspoT* exhibited enhanced antifungal activity against *S. sclerotiorum* in vitro. This phenotype has been attributed to elevated *prnA* transcription and PRN production demonstrated by the SR mutants. Furthermore, mutation of the *relA* and *relAspoT* genes also increased lipase and protease activity indicating that the SR

negatively regulates these aforementioned biocontrol factors. Because the genes encoding the lipase and protease enzymes have not been identified in strain PA23, it is difficult to determine if this stress response exerts its effect on these factors at a transcriptional level. Isolation of genes encoding these enzymes is required to clarify how the SR affects these traits.

In a previous study, GacS was found to be necessary for strain PA23-mediated biocontrol of *S. sclerotiorum* (Poritsanos et al. 2005). As such, we were interested in determining whether a link exists between the Gac system and the SR. Interestingly, *relA* translation and (p)ppGpp production were unaffected by a *gacA* or *gacS* mutation. However, the SR was found to exhibit reciprocal control over *gacA* and *gacS*; positively regulating *gacS* and negatively regulating *gacA*. Moreover, over expression of *gacS* but not *gacA* in PA23*relA* and PA23*relAspoT* restored the mutant phenotype to that of the wild type. Collectively, these findings indicate that the impact of the SR on PA23 antagonism of *S. sclerotiorum* is mediated, at least in part, by GacS. Because GacA and GacS are interconnected with the Rsm system, the potential of the SR to regulate PA23 Rsm components was also evaluated. One unexpected finding was that *rsmZ* transcription was reduced in the SR mutants despite the elevated *gacA* expression in these strains (Manuel and de Kievit, unpublished data). Possible explanations for this observation are that GacA requires GacS for activation of *rsmZ* transcription or that (p)ppGpp may directly control the transcription of this gene. To further clarify this regulation, the effect of *gacS* on *rsmZ* transcription should be assayed in the SR mutants. PA23*relA* and PA23*relAspoT* also exhibited enhanced transcription of *rsmA* while that of *rsmE* was

reduced (Manuel and de Kievit, unpublished data). In fact, multiple copies of *rsmE* but not *rsmA* attenuated the protease activity of the SR mutants (Manuel and de Kievit, unpublished data). Taken together, these results clearly indicate that in strain PA23, the SR regulates the Rsm system.

The PA23 PhzI/PhzR QS system was also found to be part of the SR regulon as the *relA* and *relAspoT* mutations exhibited decreased *phzI* transcription while *phzR* was enhanced. These findings suggest that under the conditions tested, *phzI* and *phzR* are independently regulated. Consistent with the *phzI* transcriptional data, PA23*relA* and PA23*relAspoT* produced less AHLs than the wild type. Although this QS system controls PHZ biosynthesis in strain PA23 (Selin and de Kievit, unpublished data), this antibiotic remained unchanged in the *relA* and *relAspoT* mutants. Therefore, under stringent conditions, PHZ expression may not be QS controlled. Rather, a regulator that is unaffected by the SR may be responsible for the synthesis of the aforementioned antibiotic. In *P. chlororaphis* 30-84, RpeA represses PHZ production when the bacterium is grown in minimal media despite being able to produce AHLs (Whistler and Pierson 2003). It would be intriguing to know if other regulators such as RpeA control PHZ production in strain PA23. Several attempts to identify an *rpeA* homolog through PCR amplification have been unsuccessful (Manuel and de Kievit, unpublished data) indicating that this factor either does not exist in strain PA23 or that its isolation requires an alternative approach. Although PHZ may not be required for PA23-mediated biocontrol of *S. sclerotiorum* (Selin et al. 2010), it may serve other functions that are important to the survival of the bacterium. In a review by Price-Whelan et al. (2006), it was suggested that PHZs may act as signaling

molecules or may assist in maintenance of intracellular redox balance. Essentially, PHZs can be used to alter the bacterium's physiology and/or aid in survival when oxygen is limiting. Therefore, an understanding of the regulation of this antibiotic may give insight as to how this organism may be optimized as a biocontrol agent. Additionally, *P. chlororaphis* 30-84 possesses a second QS system termed CsaI/CsaR, which regulates exoprotease and cell surface properties, but not PHZ expression (Zhang and Pierson 2001). Homologs corresponding to this second system have been identified in strain PA23 (Selin and de Kievit, unpublished data). An understanding of the impact of the SR on CsaI/CsaR expression may provide insight into the regulation of protease production.

Aside from QS, the transcriptional factor RpoS provides another layer of regulation in several biocontrol pseudomonads (Chin-A-Woeng et al. 2005; Girard et al. 2006; Sarniguet et al. 1995). Herein, RpoS was found to mediate the effects of the SR on PA23 biocontrol of *S. sclerotiorum*. Several observations have led us to this postulation. First, transcription of *rpoS* was reduced in the SR mutants indicating that this gene forms part of the SR regulon. Secondly, RpoS protein levels were also diminished in the mutant strains compared to the wild type, but were enhanced by the addition of *gacS*, *relA*, or *rpoS* in trans. Thirdly, when multiple copies of *rpoS* were introduced into PA23*relA* and PA23*relAspoT* in trans, the mutant phenotype was restored to that of the wild type. And finally, when *rpoS* was over-expressed in the SR mutants, *prnA* production was reduced down to wild-type levels. We believe that when RpoS levels are low, transcriptional activity favors genes that do not require RpoS, such as *prnA*. Conversely, when RpoS is in excess, it is able to out-compete

other sigma factors for the core RNAP thus reducing *prnA* transcription. It is also possible that a yet-to-be-identified RpoS-dependent inhibitor of this antibiotic may be reduced in the absence of (p)ppGpp. However, no inhibitors of PRN expression have been identified thus far.

In summary, we demonstrate that the SR negatively affects the antifungal activities of strains DF41 and PA23 by suppressing production of antibiotics that are essential for *S. sclerotiorum* antagonism. In doing so, resources may be allocated to the expression of genes that are required for stress survival. A summary of the regulatory cascade governing DF41 and PA23-mediated antagonism is provided in Figs. 4.1 and 4.2, respectively. These organisms produce a number of secondary metabolites, some of which are important in the growth inhibition of *S. sclerotiorum*. Production of several antibiotics may arm the organism when attacked by predators. As suggested earlier, these antibiotics may serve other functions as well. For instance, PHZs have been suggested to play a role in signaling as well as a terminal electron acceptor in the absence of oxygen (Price-Whelan et al. 2006). Furthermore, LPs were implicated in triggering cannibalism and biofilm formation in *Bacillus subtilis* (Lopez et al. 2009a, b). Therefore, aside from their antibiotic activity, these secondary metabolites may be needed for enhancing the bacterium's survival in the environment. From this study, it is evident that the regulation of secondary metabolites is complex and appears to differ between the two strains.

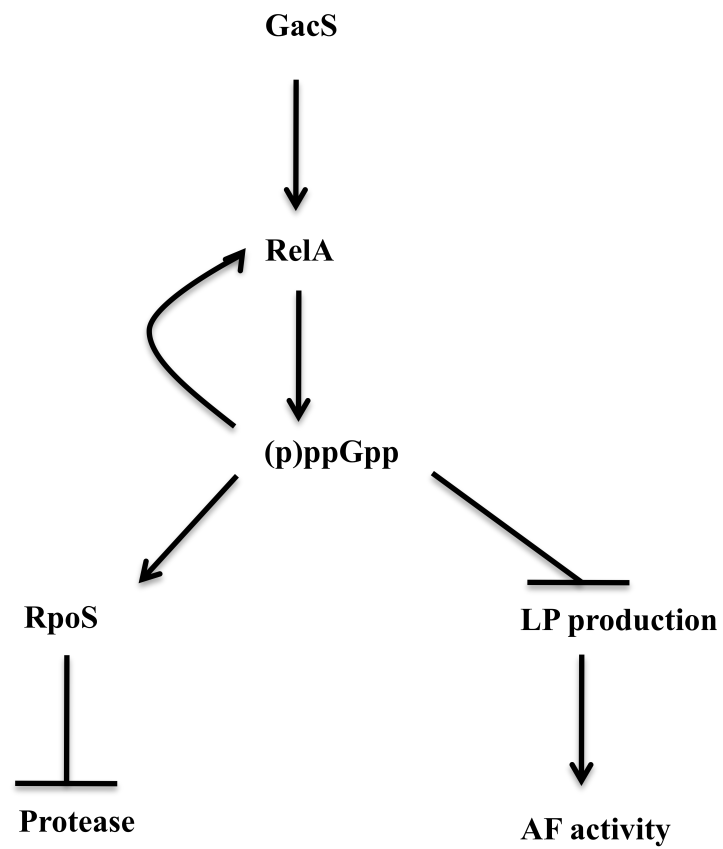


Figure 4.1. Model of the regulatory cascade governing DF41-mediated antagonism of *S. sclerotiorum* in vitro. Arrows are indicative of positive effects while bars represent inhibitory effects.

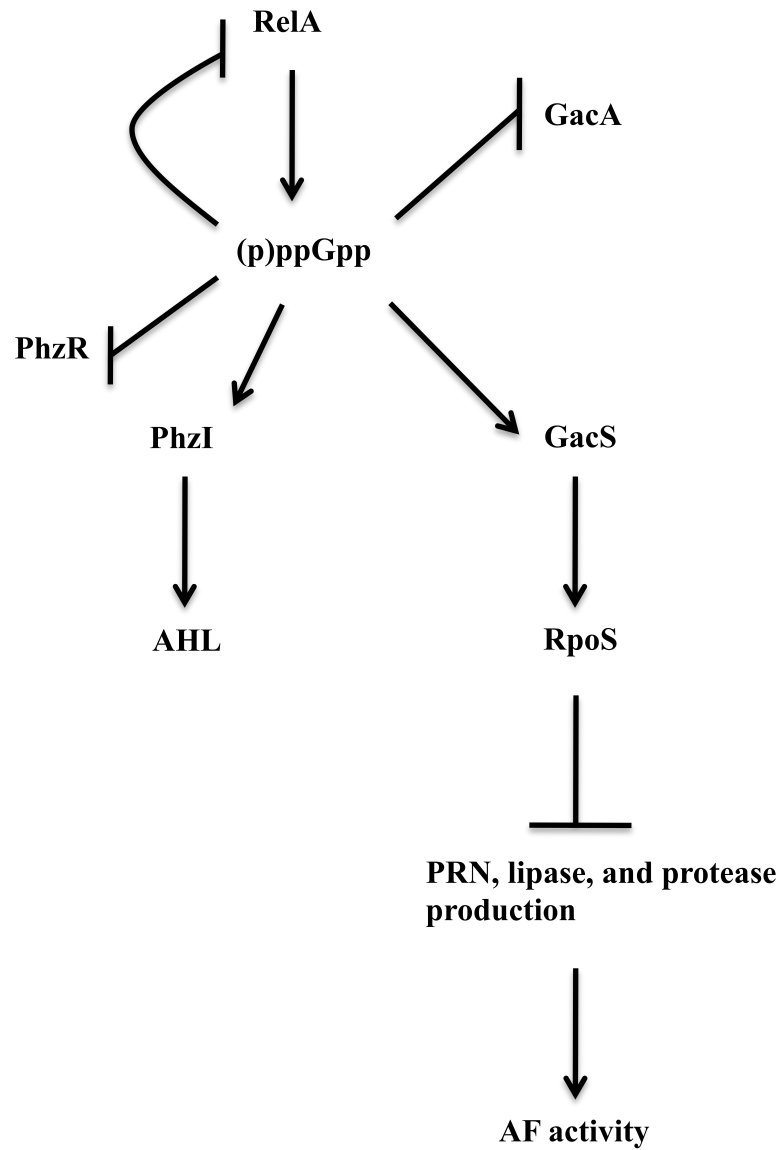


Figure 4.2. Model of the regulatory cascade governing PA23-mediated antagonism of *S. sclerotiorum* in vitro. Arrows indicate a positive influence while bars represent a negative impact.

In the former, the Gac system appears to control the SR while the converse is true for the latter. The reason for this discrepancy may be due to the location from which these organisms were isolated. Savchuck and Fernando (2005) obtained DF41 from the canola rhizosphere and PA23 from the root tip of soybean. Because both plants differ significantly as soybean is a legume while canola is not, the root exudates produced and thus the rhizobacterial composition may not be consistent between the two plants. Bais et al. 2006 suggested that secretion of certain compounds might attract beneficial organisms while deterring association with pathogenic ones. Furthermore, on leguminarious plants, Rhizobia are able to form a symbiotic relationship with the plant due to the bacteria's ability to fix nitrogen (Bais et al. 2006). Therefore, one could surmise that bacteria required for nitrogen fixation would be more abundant in the soybean rhizosphere than that of canola. With these points in mind, DF41 and PA23 may have evolved in such a way that would make them more competitive in the particular environment from which they were initially isolated. It is possible that in comparison to DF41, PA23 might be more responsive to fluctuating nitrogen levels in the soil. When this particular nutrient becomes limited, the SR could fine-tune the expression of the Gac system in order to adapt to the conditions.

Although being the first study to unify the role of the SR with other known regulators of pseudomonad-mediated antagonism, the work presented here is subject to several limitations. The first limitation being that the antagonism exhibited by DF41 and PA23 on *S. sclerotiorum* was assessed in vitro. In planta studies to

determine the degree to which the SR mutants of both strains are able to protect canola from the fungal pathogen were attempted on several occasions. However, usable results could not be obtained as the work was hampered by the presence of aphids, the lack of greenhouse space, and time. Therefore, it is imperative that the greenhouse assays be repeated to determine if the impact of the SR on DF41 and PA23-mediated biocontrol of *S. sclerotiorum* is restricted to the laboratory or whether similar results would be found in the environment. Secondly, the alarmone was found to influence the expression of only a few factors in strains DF41 and PA23 indicating that some but not all biocontrol traits are affected during the SR. We postulate that in these bacteria other factors may have a more prominent role in regulating the growth inhibition of *S. sclerotiorum*. For example, polyPs are long polymers of phosphate residues that are also synthesized during periods of starvation (Rao et al. 2009). The *ppk* gene encodes a polyphosphate kinase that is responsible for the synthesis of polyP from ATP while degradation of the molecule to Pi units is achieved by the *ppx*-encoded enzyme exopolyphosphatase (Rao et al. 2009). It was found that under phosphate-limiting conditions, mutation of the *ppk* gene in *Streptomyces lividans* enhanced production of the antibiotics actinorhodin and red undecylprodigiosin (Chouayekh and Virolle 2002). Chouayekh and Virolle (2002) suggest that the negative effect is the consequence of reduced Pi levels in the mutant strain. Homologs of the genes involved in polyP metabolism have yet to be identified in strains DF41 and PA23. Thirdly, from the results obtained from this study, the application of DF41 and PA23 as a foliar spray may not be optimal for antibiotic production. Because the phyllosphere represents a harsher environment than the

rhizosphere, the SR may inhibit expression of key antibiotics that are required for the inhibition of *S. sclerotiorum*. Instead, it may be more beneficial to amend the rhizosphere with these organisms. Moreover, use of a cocktail of several pseudomonad strains rather than a single-strain treatment may provide an even greater advantage, as a wider range of AF factors would be present. And finally, it is uncertain as to whether the SR mutants can be used in long-term treatments. Although the SR mutants were maintained equally as well as the wild type after 5 days on the canola plant (Manuela and de Kievit, unpublished data), sustainability beyond that time point is unknown. Presumably when starved of amino acids, the mutants would not be able to synthesize these biomolecules, thereby generating proteins with a high degree of misincorporation. As a result, the potency of the AF factors produced may decrease as well the organism's ability to survive in the rhizosphere or on the plant.

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