Regulatory Mechanisms Underlying Biological Control Activity of *Pseudomonas chlororaphis* PA23.

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Abstract

Biological control is an intriguing alternative to the use of chemical pesticides as it represents a safer, more environmentally friendly approach to managing plant pathogens. *Pseudomonas chlororaphis* strain PA23 was isolated from soybean root tips and it was found to be an excellent antagonist of sclerotinia stem rot. Our studies have shown that pyrrolnitrin (PRN) is the key metabolite required for *S. sclerotiorum* inhibition, while phenazine (PHZ) is important for biofilm establishment. For this reason, research efforts were directed towards elucidating the mechanisms governing PA23-mediated antibiotic production. To determine how these compounds were regulated, QS-deficient strains and an *rpoS* mutant were generated. The QS-deficient strains no longer inhibited the fungal pathogen *S. sclerotiorum* in vitro and exhibited reduced PRN, PHZ and protease production. Analysis of transcriptional fusions revealed that RpoS has a positive and negative effect on *phzI* and *phzR*, respectively. In a reciprocal manner, RpoS is positively regulated by QS. Characterization of a *phzRrpoS* double mutant showed reduced antifungal activity as well as PRN and PHZ production, similar to the QS-deficient strains. Furthermore, *phzR* but not *rpoS* was able to complement the *phzRrpoS* double mutant for the aforementioned traits, indicating that the Phz QS system is a central regulator of PA23-mediated antagonism.

GacS/GacA, PsrA, RpoS and the PhzI/PhzR QS are members of a complex regulatory hierarchy that influence secondary metabolite production in PA23. An additional system, termed Rsm, was identified, adding yet another layer of complexity to the regulatory network. The Rsm system in PA23 appears to be comprised of a single small non-coding regulatory RNA termed RsmZ, and two RNA binding proteins RsmA

and RsmE. We discovered that the expression of *rsmZ*, *rsmA* and *rsmE* all require GacA. In addition, both PsrA and QS were shown to positively regulate *rsmZ* transcription. For *rsmE*, GacA may indirectly regulate expression through PsrA, RpoS and QS, as all three regulators control *rsmE* transcription. Furthermore, we believe that the positive effects of PsrA and QS on *rsmE* transcription are likely mediated through RpoS as only RpoS show direct activation of *rsmE* in an *E. coli* background.

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List of Abbreviations

AF	Antifungal
AHL	Acylhomoserine lactone
AI	Autoinducer
Amp	Ampicillin
2,4-DAPG	2,4-Diacetylphloroglucinol
Gac	Global activator of cyanide
Gm	Gentamicin
HCN	Hydrogen cyanide
HPLC	High Performance Liquid Chromatography
ISR	Induced systemic response
LB	Luria Bertani
LP	Lipopeptide
NRPS	Nonribosomal peptide synthetase
2-OH-PHZ	2-hydroxy-phenzine
PCA	Phenazine-1-carboxylic acid
PCN	Phenazine-1-carboxamide
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PGPR	Plant growth promoting rhizobacteria
PHZ	Phenazine
Pip	Piperacillin
PLT	Pyoluteorin

PRN	Pyrrolnitrin
PsrA	*Pseudomonas* sigma regulator A
QS	Quorum sensing
Rif	Rifampicin
RNAP	RNA Polymerase
Rsm	Regulator of secondary metabolism
SAR	Systemic acquired resistance
SSR	Sclerotia stem rot
Tc	Tetracycline
Tn	Transposon

Chapter 1
Literature Review

1.1 Biological control in *Pseudomonas* species

1.1.1 Biological control

Plant pests (harmful insects, parasitic weeds and pathogens) are important biotic agents that cause serious damage to agricultural crops. Currently, plant diseases are controlled by agricultural practices and the use of chemical pesticides (Agrios, 1988; Cook, 1993). However, the increased use of chemicals in agriculture has been a subject for public concern due to the potential harmful effects on the environment, their undesirable effects on non-target organisms, and the development of resistant pathogens (Agrios, 1988; Cook, 1993; Heydari, 2007). These factors, together with consumer demand for pesticide-free food has led to a search for alternative and safer methods for disease management.

Biological control can be defined as the control of one organism by another organism, and it is considered be a viable alternative for treatment of plant diseases (Cook, 1993; Baker, 1987). In some cases biological control is the only option available to protect plants against pathogens (Cook, 1993). This phenomenon was recognized through the discovery of naturally disease-suppressive soils, in which plants grown in these soils were free from disease even in the presence of the pathogen. Interestingly, the suppressive nature of the soil was transferable to soils that exhibited less than 10% suppression (Haas et al. 2002) but was diminished upon treatment with antibiotics (Shipton, 1973; Cook and Rovira, 1976; Scher and Baker, 1980; Stutz et al. 1986). These characteristics were found to be attributable to the presence of antagonistic soil-associated microorganisms (Schneider, 1982; Schippers et al. 1987; Haas and Défago, 2005).

1.1.2 Plant growth promoting bacteria

Plant growth promoting bacteria (PGPB) have been shown to stimulate plant growth and improve soil and plant health (Glick, 1995; Rovira, 1965). These bacteria are associated with several plant species and are commonly present in many different environments (Compant et al. 2005). The most widely studied group of PGPB are plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). A number of PGPR's have been identified; however, those predominantly studied as potential biocontrol agents include species from the genera *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia* and *Agrobacterium* (Weller, 1988; Lugtenburg et al. 2001). Research suggests that these organisms are capable of protecting a host plant through i) effective and competitive colonization; ii) stimulation of plant defenses such as the induced systemic response (ISR) or systemic acquired resistance (SAR); and iii) specific pathogen-antagonistic interactions such as antibiosis (Kloepper and Schroth,1987; Haas and Keel, 2002; Bloemberg and Lugtenberg, 2001). The plant immune responses ISR/ASR have been reviewed elsewhere and will not be discussed here (Thomashow, 1996; Compant et al. 2005; Van Loon, et al. 1998; Toyoda et al. 1988; Utsumi et al. 1991; Schnider-Keel et al. 2000). Instead, this review will focus on pathogen-antagonistic interactions, specifically the mechanisms underlying biocontrol in fluorescent *Pseudomonas* species.

1.1.3 The genus *Pseudomonas*

Bacteria belonging to the genus *Pseudomonas* are classified as gram-negative rod shaped aerobes that are metabolically versatile. They have one or several polar flagella, providing motility (Madigan and Martinko, 2005; Haas and Défago, 2005; Palleroni, 2008). *Pseudomonas* species are ubiquitous in the environment and are widespread in the rhizosphere and phyllosphere. This group has been well studied because they are readily isolated from the environment, they utilize a wide range of substrates, and they are easy to culture and manipulate genetically (Whipps, 1997). Members of the genus *Pseudomonas* are classified within the gammaproteobacteria and include xenobiotic degraders, pathogens of plants, animals and humans, PGPB and strains that exhibit antagonistic activity against oomycete and fungal pathogens (Madigan and Martinko, 2005). This review will focus on pseudomonads exhibiting biocontrol activity.

1.1.4 Biocontrol activity of *Pseudomonas* species.

Some pseudomonads make excellent agricultural biocontrol agents. These bacteria possess diverse mechanisms whereby they inhibit phytopathogens and mediate crop protection, including the production of a wide range of antagonistic compounds (Lugtenberg et al. 1996; Dowling and O'Gara, 1994; Dunlap et al. 1996; Gutterson, 1990). Support for the role of antagonistic metabolites in biological control has come mainly from studies that have shown a correlation between bacterial inhibition of pathogens *in vitro* and disease suppression in the soil. The first experiment demonstrating that a *Pseudomonas* antibiotic was able to suppress plant disease was conducted by Thomashow and Weller (1988), where phenazine-1-carboxylic acid (PCA)

was identified as the essential biocontrol factor. These researchers demonstrated that a PCA-negative mutant of *P. fluorescens* Pf-5 was unable to suppress take-all disease caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* on wheat (Thomashow and Weller, 1988). Subsequently, a second research article was published describing the biocontrol activity of *Pseudomonas fluorescens* CHA0 (Voisard et al. 1989). Both of these strains have emerged as model biocontrol organisms and a number of antibiotics produced by these strains as well as other isolates have been identified.

1.1.5 Biocontrol compounds produced by *Pseudomonas* species

Effective biocontrol strains of *Pseudomonas* produce diffusible and/or volatile antibiotics that inhibit pathogens in vitro (Bender et al. 1999; Défago and Haas, 1990; Haas et al. 2000; Haas et al. 2002; Keel and Défago, 1997). The antibiotics are grouped into six classes of compounds and as shown in FIGURE 1.1 include: i) phenazines (PHZ); ii) pyoluteorin (PLT); iii) 2,4-diacetyphloroglucinol (2,4-DAPG); iv) pyrrolnitrin (PRN); v) lipopeptides and vi) hydrogen cyanide (HCN) (Haas and Keel, 2003).

1.1.5.1 Phenazines

Phenazines comprise a large group of nitrogen-containing heterocyclic pigments with broad-spectrum activity towards bacteria, fungi, plant and animal tissue (Smirnov and Kiprianova, 1990; Laursen and Nielson, 2004). The mechanism of action of PHZ is poorly understood; however, it is assumed that the antibiotic can diffuse across or insert into the membrane and act as a reducing agent. This results in the uncoupling of

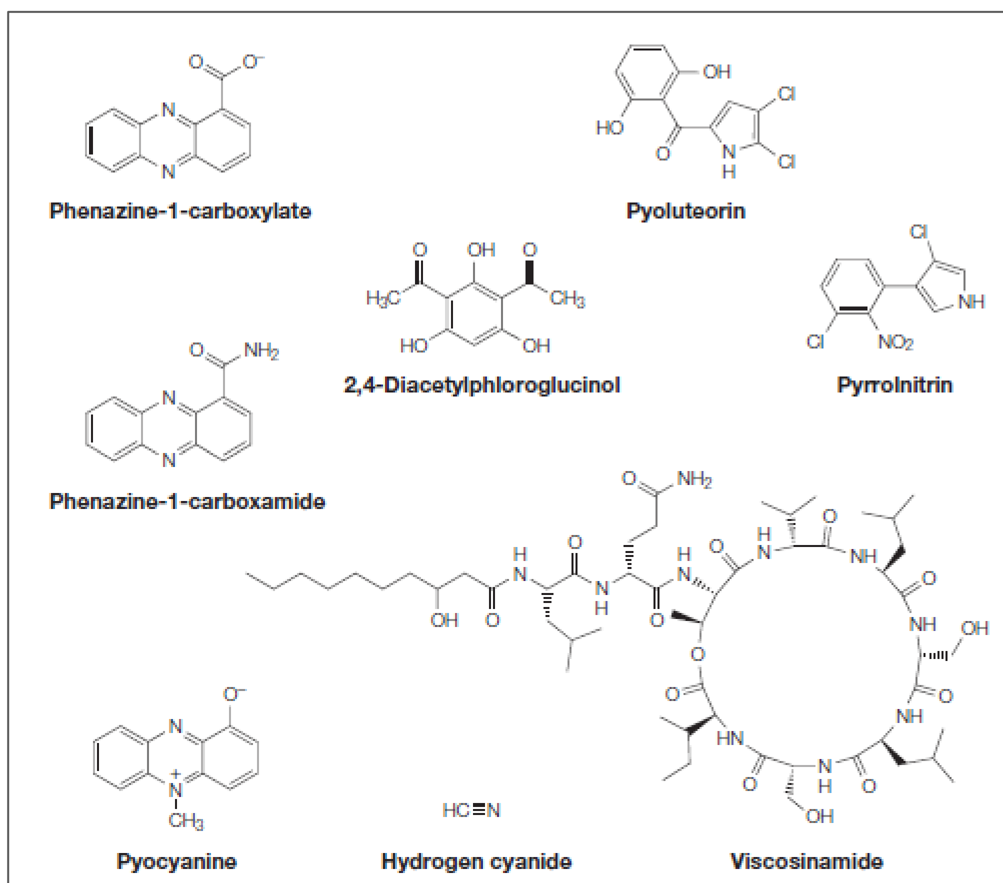

Figure 1.1: Antibiotics produced by fluorescent pseudomonads (Haas and Défago, 2005).

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oxidative phosphorylation and the generation of intracellular superoxide radicals and hydrogen peroxide, which are toxic to many organisms (Turner and Messenger, 1986; Hassett et al. 1992; Mahajan et al. 1999). The most commonly identified derivatives produced by *Pseudomonas* biocontrol strains are pyocyanin (PYO), PCA, phenazine-1-carboxamide (PCN), and a number of hydroxy-phenazines (Turner and Messenger, 1986). Both PCA and PCN have been shown to play a vital role in biological control in *P. fluorescens* Pf-5 (Thomashow and Weller, 1988), and *Pseudomonas chlororaphis* strains 30-84 (Pierson et al. 1995) and PCL1391 (Chin-A-Woeng et al. 1998). In addition to biocontrol activity, PHZ has been shown to play a role in ecological fitness (Mazzola et al. 1992), biofilm development (Maddula et al. 2008) and as well as intracellular signalling and cellular energy generation (Pierson III and Pierson, 2010).

Phenazine biosynthesis is derived from the shikimic acid pathway, a pathway which also participates in the synthesis of siderophores and the aromatic amino acids tyrosine and phenylalanine (McDonald et al. 2001; Vandenende et al. 2004). A conserved seven gene operon, *phzABCDEFG,* has been identified in various *Pseudomonas* species that directs the synthesis of PCA (Chin-A-Woeng et al. 2003). Five of the proteins, PhzA, PhzD, PhzC and PhzF and PhzG are required for PHZ biosynthesis, while *phzB* appears to be a duplication of *phzA*. The *phzC* gene encodes a 3-deoxy-D-arabinose-heptulosonate-7-phosphate synthase, which is the enzyme responsible for catalyzing the first step of the shikimic acid pathway, allowing redistribution of intermediates from primary metabolism into PHZ synthesis (Mavrodi et al. 2006). Although the core PHZ operon is conserved, individual species can differ in the range of compounds that are synthesized and as such vary in their biological activity. This type of diversity results

from PCA modification by specific enzymes (Byng and Turner, 1977; Messenger and Turner, 1983). The genes required for these modifications may be linked to the core operon or located elsewhere on the chromosome (Delaney et al. 2001; Chin-A-Woeng, 1998; Mavrodi et al. 2001). For example, in *P. chlororaphis* PCL1391, *phzH* lies downstream of *phzG* and encodes the enzyme, PhzH, which is responsible for the conversion of PCA to PCN (Chin-A-Woeng et al. 2001). In *P. chlororaphis* 30-84, *phzO* is also located downstream of the core biosynthetic cluster and encodes the enzyme that directs the synthesis of 2-hydroxyphenazine carboxylic acid (2-OH-PCA) (Delaney et al. 2001).

1.1.5.2 Pyrrolnitrin

The antibiotic pyrrolnitrin [3-chloro-4-(2′-nitro-3′-chlorophenyl) pyrrole (PRN)] (Figure 1.1) is a tryptophan-derived antifungal compound produced by many pseudomonads (Hammer et al. 1997). The broad spectrum antifungal activity of PRN is attributed to its ability to inhibit the respiratory electron transport system of fungi (Hammer et al. 1997). Pyrrolnitrin production has been implicated in the biological control of fungal plant pathogens by several *Pseudomonas* strains (Howell and Stipanovic, 1979; Hill et al. 1994). Both *P. fluorescens* strains CHA0 and Pf-5 have been shown to produce PRN, although it is not the key biocontrol factor for these strains (Sarniguet et al. 1995; Haas and Keel, 2003). Conceivably, the strongest evidence for the role of PRN in effective disease suppression resulted from a study by Hill and colleagues (1994), where PRN mutants of *P. fluorescens* BL915 lost their ability to suppress cotton damping-off caused by *Rhizoctonia solani*, but regained biocontrol activity after

reintroduction of the PRN biosynthetic cluster. The biosynthetic operon that encodes the pathway for the conversion of tryptophan into PRN is composed of four genes (*prnABCD*). Introduction of the entire PRN cluster into *E. coli* confers the ability to produce PRN, demonstrating that only these four genes are necessary for PRN biosynthesis *in vivo* (Hammer et al. 1997).

1.1.5.3 Pyoluteorin

Pyoluteorin is an antibiotic that is primarily known for its suppression of seedling damping off disease caused by *Pythium ultimum* (Howell and Stipanovic, 1980; Maurofer et al. 1994). Pyoluteroin is composed of a resorcinol ring attached to a dichlorinated pyrrole moiety, derived through polyketide biosynthesis, (Cuppels et al. 1986; Nowak-Thompson et al. 1997). This antibiotic is produced by *P. fluorescens* Pf-5, *P. fluorescens* CHA0, and *Pseudomonas aeruginosa* M18 as well as other bacteria. The biosynthetic gene cluster contains eight structural genes required for PLT synthesis (*pltLABCDEG*) and *pltM*, which encodes an enzyme required for the biochemical transformation of acetate and proline precursors to PLT (Brodhagen et al. 2004). The *pltR* gene, which lies upstream of *pltLABCDEFG* encodes a LysR-type transcriptional activator required for the transcription of the PLT biosynthetic genes (Nowak-Thompson et al. 1997). The *pltIJKNO* operon was also identified and shown to be important in transport and self-resistance to PLT (Brodhagen et al. 2004). A second regulatory gene, *pltZ*, was found upstream of *pltHIJKNO*. This gene codes for the protein PltZ, a TetR-type regulator that inhibits PLT expression in a pathway-specific manner (Huang et al. 2004).

1.1.5.4 2,4-diacetylphloroglucinol

2,4-diacetylphloroglucinol (2,4-DAPG) is a phenolic compound with broad spectrum antifungal, antibacterial, antihelminthic and phytotoxic activity (Dowling et al. 1994; Keel et al. 1992; Thomashow and Weller, 1995). This metabolite was shown to be important in the inhibition of *G. graminis* var. *tritici* on wheat and the inhibition of *Thielaviopsis basicola* on tobacco by *P. fluorescens* CHA0 (Keel et al. 1990; Thomashow, 1996). The genes required for 2,4-DAPG biosynthesis are arranged in an operon (*phlABCD*), similar to other antibiotics previously discussed. The gene products within the operon have been shown to generate 2,4-DAPG from monoacetylphloroglucinol (MAPG) via the activity of an acetyltransferase (Bangera and Thomashow, 1999). The *phlE* and *phlF* genes are located downstream of the *phlABCD* operon, and encode enzymes that are involved in the export of the antibiotic and inhibition of 2,4-DAPG transcription, respectively. Downstream of *phlE* and *phlF*, lies *phlH*, a gene that encodes a TetR-like regulator, which has been shown to significantly reduce 2,4-DAPG expression in *P. fluorescens* CHA0 (Haas and Keel, 2003).

1.1.5.5 Lipopeptides

Lipopeptides (LP) are a diverse group of bioactive molecules that exhibit antimicrobial, cytotoxic and surfactant properties (Raaijmakers et al. 2006). For plant-associated *Pseudomonas* spp, LP production contributes to antimicrobial activity, biofilm formation and motility (Raaijmakers et al. 2006). The structure of an LP consists of a fatty acid tail joined to a cyclic oligopeptide head. There is, however, considerable diversity within these compounds due to differences in the length and composition of the

peptide ring structure (Raaijmaker et al. 2006). The antifungal activity of these molecules is attributable to their ability to form pores in the cell membrane, which causes cell lysis (Hutchison et al. 1995; Hildebrand et al. 1998; Lindow and Brandl, 2003). The synthesis of these molecules is unique in that it involves multifunctional enzymes termed non-ribosomal peptide synthetases (NRPSs) (Maraheil et al. 1997). These NRPSs are arranged as modules, each acting as a building block for the incorporation of one amino acid into the peptide head. The modules are further subdivided such that each contains: i) an adenylation domain responsible for amino acid activation; ii) a thiolation domain for thioesterification of the activated amino acid; and iii) a condensation domain where peptide bonds between amino acids are formed. These domains create a linear peptide that is cleaved and cyclised via the action of a thioesterase domain (Raaijmakers et al. 2006). The complex nature of NRPS-mediated peptide synthesis in comparison to ribosomal peptide synthesis, usually results in the incorporation of unusual amino acids 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).

1.1.5.6 Hydrogen cyanide

Hydrogen cyanide (HCN) is a volatile compound produced by fluorescent pseudomonads, the proteobacteria *Chromobacterium violaceum* and some cyanobacteria (Castric, 1981; Knowles and Bunch, 1986). Hydrogen cyanide is a potent inhibitor of cytochrome *c* oxidase and several other metalloenzymes (Blumer and Haas, 2000) and has been shown to be the key metabolite that provides biocontrol in *P. fluorescens* CHA0 against tobacco black root rot caused by *T. basicola* (Voisard et al. 1989; Laville et al.

1998). The genes required for the production of HCN are arranged as an operon (*hcnABC*), and are responsible for the production of HCN synthase, a membrane-bound flavoenzyme (Askland and Morrison, 1983). Due to its instability and difficulties with purification, the subunit composition and cofactor requirements of this enzyme are unknown (Wissing and Anderson, 1981; Blumer and Haas, 2000). Many biocontrol pseudomonads have been shown to produce HCN; however, the HCN operon has only been identified in *P. fluoresecens* CHA0 and *P. aeruginosa* PAO1 (Blumer and Haas, 2000).

1.1.5.7 Siderophores

Siderophores are high-affinity iron (III) chelators that transport iron into bacterial cells (Leong, 1986; Neilands, 1981). Fluorescent pseudomonads, when grown under low-iron conditions, produce a yellow-green siderophore that when bound to iron is specifically recognized and taken up by membrane-receptor proteins (Buyer and Leong, 1986; Hohnadel and Meyer, 1988; Magazin et al. 1986). Often efficient production of siderophores is combined with the ability to take up related siderophores from other organisms (Koster et al. 1993; Raaijmakers et al. 1995). The first work demonstrating the importance of siderophores in biological control was in 1980 by Kloepper and colleagues (1980), where they were able to convert disease-conducive soils to disease-suppressive soils by inoculating with either *Pseudomonas* strain B10 or its purified siderophore (Kloepper et al. 1980). Subsequently, siderophores have been shown to suppress growth of *F. oxysporum* (Elad and Baker, 1985; Scher, 1986), *G. graminis* var. *tritici* (Cook and Weller, 1987; Weller, 1988) and *Pythium* spp. (Becker and Cook, 1988; Loper, 1988).

The ability of siderophores to sequester the limited supply of iron (III) within the rhizosphere essentially limits iron availability to pathogens which leads to suppression of their growth (Schroth and Hancock, 1981).

1.1.5.8 Cell wall degrading enzymes

Some bacteria are capable of producing lytic enzymes such as chitinases, cellulases, lipases and proteases. Some of these enzymes are involved in the breakdown of fungal cell walls by degrading glucans and chitins, resulting in the destruction of pathogen structures or propagules (Chin-A-Woeng et al. 2003). In *Pseudomonas stutzeri*, extracellular chitinases and laminarinase were shown to digest and lyse mycelia of *Fusarium solani*. Research has also shown that lytic enzymes can act synergistically with other fungal compounds to inhibit pathogens (Di Pietro et al. 1993; Lorito et al. 1994; Duffy et al. 1996; Fogliano et al. 2002).

1.1.5.9 Additional bacterial traits contributing to biocontrol activity

Although antibiosis is one of the major factors that determines biological control, other characteristics may provide an advantage to the bacteria, thereby indirectly impacting their biocontrol activity. One of these contributing traits is motility, which allows bacteria to migrate from areas that are non-favourable to those that are growth promoting. Bacterial movement is achieved by flagellar (swimming) and swarming motility. While swimming in liquid environments is achieved by individual cells recognizing chemical signals that generate adaptive chemotactic responses, swarming is characterized by multicellular movement of bacteria migrating across a semi-solid

surface (Harshey, 2003). This type of locomotion is dependent on the ability of the cells to differentiate into specialized swarm cells, which are longer and more flagellated than planktonic cells (Henrichsen, 1972; Harshey, 2003). Although conflicting results have been reported for *P. fluorescens* (De Weger et al. 1987; Scher et al. 1988; Lugtenberg et al. 2001), studies conducted with other plant-associated microorganism have shown that motility facilitates plant colonization (Van de Broek et al. 1998; Catlow et al. 1990).

A second trait that may enhance persistance within the plant environment is biofilm formation. Evidence has shown that bacteria in most environments exist as attached communities known as biofilms (Costerton et al. 1995). These bacterial communities are afforded protection from environmental stresses and dessication which contributes to long-term survival of the bacteria on the plant host (Danhorn and Fuqua, 2007).

1.1.6 *Pseudomonas chlororaphis* strain PA23

Pseudomonas chlororaphis strain PA23 is a biocontrol agent initially isolated from soybean root tips. In both greenhouse and field studies, this bacterium is able to protect canola from the devastating effects of stem rot caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Fernando et al., 2007; Savchuk and Fernando, 2004; Zhang, 2004). The antifungal activity of this particular strain can be attributed to the production of secondary metabolites that include the antibiotics PHZ, PRN and HCN, as well as proteases, lipases and siderophores (Poritsanos et al. 2006).

1.1.7 *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary, an ascomycete fungus, is a devastating pathogen that is capable of infecting over 400 species of plants worldwide (Boland and Hall 1994), including canola (Manitoba Agriculture, 2002). This pathogen causes canola stem rot (Nelson, 1998) and results in 5-100% loss of canola crops in years that are favorable for disease development (Manitoba Agriculture, 2002) making control of this pathogen essential.

Management of *S. sclerotiorum* is difficult due to its unique life cycle (Fig 1.2). In soils, this pathogen persists as hard, irregular shaped, over-wintering bodies called sclerotia. In this form, the pathogen is able to survive for up to 8 years within the soil (Adams and Ayers, 1979). When conditions are favorable, sclerotia germinate into mushroom-like structures termed apothecia, which release ascospores into the environment (Purdy, 1979). The ascospores are carried through the air travelling up to one kilometer with the potential to infect neighbouring plants (Venette, 1998). Canola is susceptible to *S. sclerotiorum* infection at the flowering stage when ascospores land on the maturing flowers (Purdy, 1979; Turkington and Morall, 1993). When the infected petals come into contact with other portions of the plant, the disease spreads to the stem, causing it to whiten and hollow (Purdy, 1979). *S. sclerotiorum* virulence has been attributed to the production of oxalic acid, an agent that causes tissue damage by requisitioning calcium from the middle of the lamellae (Bateman and Beer, 1965; Godoy et al. 1990). In addition, the pathogen gains access to the internal portions of the plant through the production of cell-wall degrading enzymes such as cellulases, hemi-

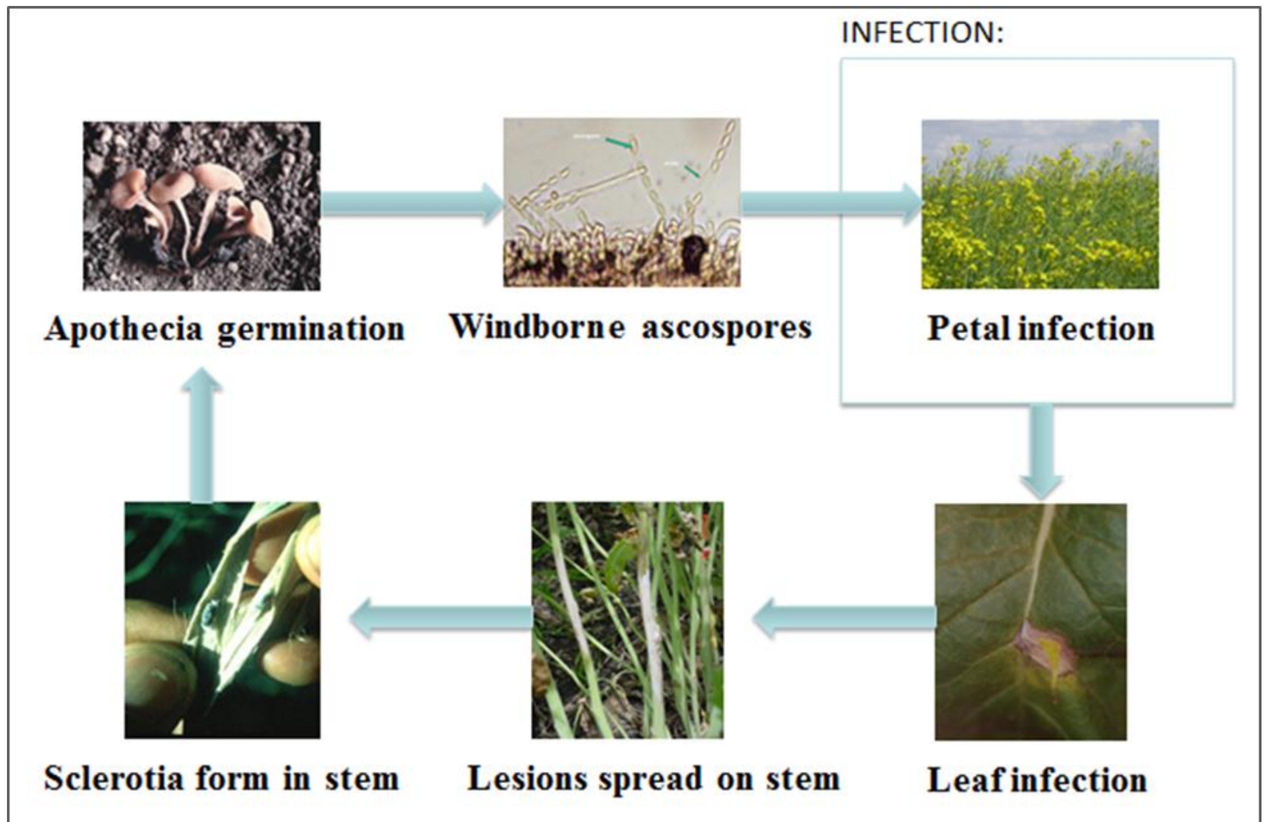

Figure 1.2: Lifecycle of *Sclerotinia sclerotiorum*. In infected soils, sclerotia germinate into a apothecia that release ascospores. Once windborne, the spores can infect neighbouring crops. Canola is susceptible to infection caused by *S. sclerotiorum* during the flower stage. When the contaminated petals come into contact with other portions of the plant, the disease progresses to the leaves and stems which ultimately leads to hollowing and bleaching of the stem.

cellulases, pectinases (Riou et al. 1991), proteases (Pousserau et al. 2001; Girard et al. 2004) and endopolygalacturonases (Cotton et al. 2003).

1.2 Regulatory elements governing biological control activity

Regulation of secondary metabolites is complex and occurs at the transcriptional and posttranscriptional level involving sigma factors, quorum sensing (QS) and two-component signal transduction systems. Together, these individual elements combine to form a complex regulatory hierarchy overseeing expression of biocontrol factors in *Pseudomonas* strains.

1.2.1 Sigma factors

Initiation of transcription is one of the key points at which bacteria regulate expression of their genes. This form of regulation involves the RNA polymerase (RNAP) catalytic core ($\alpha_2\beta\beta'$) and a protein termed the sigma (σ) factor (Wösten, 1998). The sigma factor associates with the RNAP core forming the holoenzyme and it directs RNAP to specific promoter sites (Gross et al. 1998). Most bacteria synthesize an assortment of σ factors which allows them to respond to changes in the environment (Ishihama, 2000). In *P. aeruginosa* and *P. putida* there are at least 20 sigma factors produced in response to environmental changes (Stover et al. 2000; Martinez-Bueno et al. 2002). These proteins include RpoD (σ^{70}) for housekeeping, RpoS (σ^{38}) for stress and stationary phase, RpoE for oxidative and osmotic stress, RpoF for flagella synthesis, and RpoN for motility and catabolism of various carbon and nitrogen sources (Haas and Keel, 2003). In *P. fluorescens*, RpoD, RpoN and RpoS have all been implicated in the regulation of

biocontrol factors (Schnider et al. 1995; Péchy-Tarr et al. 2005; Whistler et al. 1998). RpoS is discussed in more detail in the following section.

1.2.1.1 RpoS

The RpoS (σ^{38}/ σ^{S}) sigma factor in proteobacteria regulates genes essential for stationary phase and response to stress. *Pseudomonas* spp. have been shown to develop stress tolerance and increase their production of secondary metabolites upon entry into stationary phase (Givskov et al. 1994; Klotz and Hutcheson, 1992; Sarniguet et al. 1995). In *P. fluorescens*, an *rpoS* mutation results in increased sensitivity to oxidative stress and an altered pattern of secondary metabolite production (Sarniguet *et al*. 1995; Heeb et al. 2005). Studies have demonstrated that the regulatory effects of RpoS are different depending on the strain and antibiotic in question. For instance in *P. fluorescens* strain Pf-5, a mutation in *rpoS* results in enhanced production of PLT and 2,4-DAPG, and a decrease in PRN levels (Sarniguet et al. 1995; Heeb et al. 2005), whereas the same mutation in *P. fluorescens* CHA0, results in enhanced levels of PLT, but reduced expression of both 2,4-DAPG and PRN (Haas and Keel, 2003). Evidently, the synthesis of PRN in both strains appears to be directly regulated by RpoS, while the regulatory effect of RpoS on PLT expression is strain dependent. Interestingly, it has been suggested that the enhanced levels of PLT and 2,4-DAPG in *P. fluorescens* Pf-5 exhibited by an *rpoS* mutant may simply be due to competition between sigma factors for the limited amount of core RNAP. The competition model assumes that when RpoS levels are reduced, another sigma factor, like RpoD, can bind more efficiently to the core RNAP, redirecting the holoenzyme to the promoters that it recognizes (Farewell et al. 1998).

Therefore in *P. fluorescens* Pf-5, the production of PLT and 2,4 DAPG appear to be positively regulated by RpoD, and negatively regulated by RpoS; the latter is thought to occur indirectly via the competition mechanism.

1.2.2 PsrA

In *E. coli*, the expression of RpoS is regulated at the level of transcription, translation and protein stability (Hengge-Aronis, 2002). In *Pseudomonas*, RpoS appears to be predominantly regulated at the transcriptional level by PsrA (Pseudomonas sigma regulator). The PsrA protein belongs to the TetR family of regulators, and was identified in *P. putida*, *P. aeruginosa* and *P. chlororaphis* PCL1391 (Kojic and Venturi, 2001; Girard et al. 2006), as a positive regulator of RpoS. A *psrA* mutant in both *P. putida* and *P. aeruginosa* results in decreased *rpoS* promoter activity and a decrease in protein accumulation in stationary phase (Kojic and Venturi, 2001), indicating that PsrA is required for adequate expression of RpoS. DNA-binding analysis revealed that PsrA binds to the *rpoS* promoter at nucleotide sequence -35 to -59 with respect to the transcriptional start site. The sequence is conserved in *P. aeruginosa*, *P. putida* and *P. chlororaphis* and contains the palindromic motif C/GAACN$_{2\text{-}4}$GTTG/C, referred to as the PsrA binding box (Kojic et al. 2005; Kojic et al. 2002; Girard et al. 2006). PsrA has also been shown to negatively regulate its own transcription by binding to a similar sequence within the *psrA* promoter (Kojic et al. 2002). Interestingly, the regulatory role of PsrA in the production of secondary metabolites appears to be dependent on the culture medium. For example, in *P. chlororaphis* PCL1391, PsrA was shown to repress PCN and autoinducer (AI) production when cultures were grown in rich media. However, when

cultures were grown in minimal media, PsrA was shown to positively regulate these compounds (Chin-A-Woeng et al. 2005; Girard et al. 2006), indicating that there are other factors that determine PsrA regulation of secondary metabolites.

1.2.3 Quorum sensing (QS)

Pseudomonads regulate secondary metabolite synthesis through a cell-cell communication system known as QS. Quorum sensing is a mechanism by which the synthesis and accumulation of small diffusible signalling molecules enables bacteria to modulate gene expression in response to their population density (Fuqua et al. 2001). In Gram-negative bacteria the most common signalling molecules utilized are *N*-acyl homoserine lactones (AHL) (Venturi, 2006). These AHLs are generated by an autoinducer synthase enzyme, the product of a *luxI*-type gene (Bassler, 2002). The AHL molecules passively diffuse through the cell envelope, increasing in concentration as the bacterial population increases (Mavrodi et al. 2006). After a threshold level of AHL has been reached, it binds to a transcriptional activator of the LuxR family, which triggers a conformational change leading to promoter recognition and activation of target genes (Bassler, 2002). The PhzI/PhzR QS system controls expression of the PHZ biosynthetic operon in *P. chlororaphis* strains 30-84 and PCL1391, and *P. fluorescens* strain 2-79 (Chin-A-Woeng et al., 2001; Khan et al., 2005; Mavrodi et al., 1998; Wood and Pierson, 1996). In each case, the autoinducer synthase and the transcriptional activator genes *phzI* and *phzR*, respectively, are adjacent to one another with the *phzR* gene located immediately upstream of but in the opposite orientation to the *phz* biosynthetic operon (Chin-A-Woeng et al. 2001; Khan et al. 2005; Mavrodi et al. 1998; Wood and Pierson et

al. 1996). The autoinducer synthase enzyme, PhzI, in these strains produces more than one type of AHL; however, it appears that C6-HSL (strains 30-84 and PCL1391) and 3-OH-C6-HSL (strain 2-79) are the most abundant and efficient activators of the PhzR transcriptional regulator. Once PhzR is activated, the PhzR-AHL complex is assumed to bind to the *phz* box promoter element (ACTACAAGATCTGGTAGT), located in the *phzR-phzA* intergenic region, inducing transcription of the *phz* operon (Chin-A-Woeng et al. 2001). In *P. aeruginosa*, the regulation of PHZ production is more complex and involves at least two QS circuits, *las* and *rhl*, that are organized in a hierarchical cascade that utilize 3-oxo-C12-HSL and C4-HSL as cognate signals respectively. In *P. chlororaphis* 30-84 a second QS system, termed Csa (cell surface alterations), has also been identified. This particular QS system affects cell-surface properties and protease production but is not required for PHZ biosynthesis (Zhang and Pierson III, 2001). Unlike the Rhl and Las QS systems in *P. aeruginosa*, the Csa QS system is not organized in a tiered cascade with PhzI/PhzR, as both QS systems function independent of one another (Zhang and Pierson III, 2001).

QS has been shown to regulate an extensive range of physiological phenotypes, including bioluminescence, spore formation, Ti plasmid conjugation, biofilm formation and motility (Dunny and Winans, 1999). In *P. aeruginosa*, QS mutants are not only affected in their ability to produce antibiotics, and virulence factors, they also exhibit altered biofilm structures (Davies et al. 1998; de Kievit et al. 2001) and are reduced in their ability to swarm (Shrout et al. 2006). A recent study conducted by Lee et al. (2010), demonstrated that heterologous *luxI* expression in *P. putida* KT2440, a strain missing the *luxI*-like gene but carrying a *luxR*-type homologue within its genome, results

in reduced biofilm formation and enhanced motility. These studies illustrate a link between QS and the physiological traits of biofilm formation and motility; however, it has been suggested by others that this link may be dependent on the nutritional conditions under which the experiment was conducted (Shrout et al. 2006; Eberl et al. 1996).

1.2.3.1 Regulation of QS systems

Population density is not the only factor that can affect QS regulation. Other elements such as GacS/GacA, RpoS and PsrA may positively or negatively stimulate QS (Venturi, 2006), as discussed in the following sections.

1.2.3.1.1 Positive regulation of QS systems

Positive regulation of AHL signal production by the GacS/GacA two-component transduction system has been demonstrated in *P. aeruginosa* (Reimmann et al. 1997), *P. syringae* (Chatterjee et al. 2003), *P. fluorescens* (Wei and Zhang, 2006) and *P. putida* (Bertani and Venturi, 2004). In *P. chlororaphis* PCL1391, both PsrA and RpoS along with GacS/GacA have been shown to be essential for C6-HSL and PCN synthesis (Girard et al. 2006). Additionally, another positive transcriptional regulator, termed Pip (phenazine inducing protein), was identified in this strain (Girard et al. 2006). The Pip transcriptional activator acted downstream of PsrA and RpoS and stimulated the *phz* operon by modifying PhzI and PhzR levels (Girard et al. 2006).

1.2.3.1.2 Negative Regulation of QS systems

A number of negative regulatory elements of QS systems in *Pseudomonas* have been identified. For instance, in *P. aeruginosa*, RsmA was shown to repress both *lasI*

and *rhlI* expression, thereby negatively regulating the production of pyocyanin (Heurlier et al. 2004). Similarly, another protein, termed RsaL, was shown to directly bind to the *lasI* promoter in *P. aeruginosa*, resulting in the repression of *lasI* and a reduction in autoinducer production (Rampioni et al. 2006).

1.2.3.1.3 Regulation of QS systems by environmental factors

Environmental factors can significantly impact the QS machinery in many fluorescent *Pseudomonas* spp. Although these strains differ in how they respond to specific conditions, there seems to be an overall trend whereby the various carbon and nitrogen sources significantly alter the yields of QS-controlled antibiotics (Mavrodi et al. 2006). A study by van Rij et al. (2004) revealed that PCN production in *P. chlororaphis* PCL1391 was affected by pH, oxygen tension, nitrogen source, and temperature. In addition, low magnesium concentrations favored PCN production, while ammonium and ferric ions inhibited its synthesis. In each case, changes in AHL levels mirrored those of PCN; consequently, the observed effects on antibiotic production were thought to be mediated through QS. These findings indicate that an intricate regulatory network exists for modulating the QS machinery, allowing *Pseudomonas* to adjust antibiotic levels in response to various environmental cues.

1.2.4 The GacS-GacA two-component regulatory system

The Gac two-component regulatory system has been identified in a diverse number of bacteria including *Pseudomonas*, *Erwinia*, *Vibrio*, *Escherichia*, *Legionella* and *Salmonella* (Heeb and Haas, 2001). Studies on the Gac system have been primarily focused on pseudomonads, particularly *P. aeruginosa* because of its role in virulence,

quorum sensing and biofilm formation (Parkins et al. 2001; Goodman et al. 2004; Kay et al. 2006; Ventre et al. 2006) and *P. fluorescens* due to its role in the regulation of biological factors (Haas and Défago, 2005). Mutations within *gacS* or *gacA* significantly impair secondary metabolite production by *P. fluorescens* strains CHA0, and Pf-5, and *P. chlororaphis* strains PCL1391 and 30-84 (Duffy and Défago, 2000; Corbell and Loper, 1995; Chin-A-Woeng, 2000; Chancey et al. 1999). These metabolites include 2,4 DAPG, PLT, PRN, HCN, and PHZ compounds as well secreted enzymes such as proteases, phospholipases and chitinases (Duffy and Défago, 2000; Corbell and Loper, 1995; Chin-A-Woeng, 2000; Chancey et al. 1999).

1.2.4.1 GacS and GacA; protein and function

The Gac (global activator) two-component regulatory system consists of a membrane bound sensor kinase, GacS, and its cognate response regulator GacA (Heeb and Haas, 2001). Evidence that these two proteins were functional pairs within the transduction system was first obtained genetically in *P. syringae* pv. *syringae* (Rich et al. 1994) and later confirmed in several other bacteria (Aarons et al. 2000; Altier et al. 2000; Bull et al. 2000; Ligon et al. 2000; Pernestig et al. 2001; Whistler et al. 1998). Perhaps, the best characterized Gac system is that of *P. fluorescens* CHA0 (Zuber et al. 2003). The GacS protein of *P. fluorescens* CHA0 has two transmembrane segments that are separated by a periplasmic loop and consists of three phosphotransfer domains each with a conserved residue essential for phosphorelay (Zuber et al. 2003). The GacA protein has two essential features: i) an active site in the N-terminal receiver domain (Asp-54) and ii) a C- terminal DNA-binding domain with a helix-turn helix motif. The phosphorelay

mechanism depicted in Fig 1.3 is assumed to function as follows: in response to an unknown signal, GacS undergoes a conformational change that is accompanied by autophosphorylation at a conserved histidine residue (Robinson et al. 2000). The phosphoryl group is subsequently relayed to the conserved amino acid residues His-294 (H) in the transmitter domain, Asp-717 (D) in the receiver domain and the His-863 (H) in the carboxy-terminal secondary transmitter domain (Zuber et al. 2003). Transfer then proceeds to a conserved aspartate in the receiver domain of the response regulator GacA (Zuber et al. 2003). Once activated, GacA initiates transcription of one or several non-coding regulatory RNAs which are part of a downstream regulatory system, known as Rsm (regulator of secondary metabolism). As stated above, for many Pseudomonads regulatory elements like RpoS and QS systems control production of secondary metabolites (Ge et al. 2006; Girard et al. 2006; Heeb et al. 2005). It appears that the expression of both *rpoS* and the *phzI/phzR* QS genes are subject to positive regulation by the GacS/GacA system (Heeb et al. 2005; Girard et al. 2006). These findings suggest that secondary metabolite and extracellular enzyme production is governed by a complex regulatory cascade, with the GacS/GacA pair at the top of the hierarchy. Additional complexity is added to the puzzle by the fact that the activity of the GacS/GacA system can be modulated by accessory regulators called RetS (regulator of exopolysaccharide and type III secretion) and LadS (lost adherence). A recent study in *P. aeruginosa* demonstrated that both RetS and LadS physically interact with GacS (Goodman et al. 2009; Workentine et al. 2009).

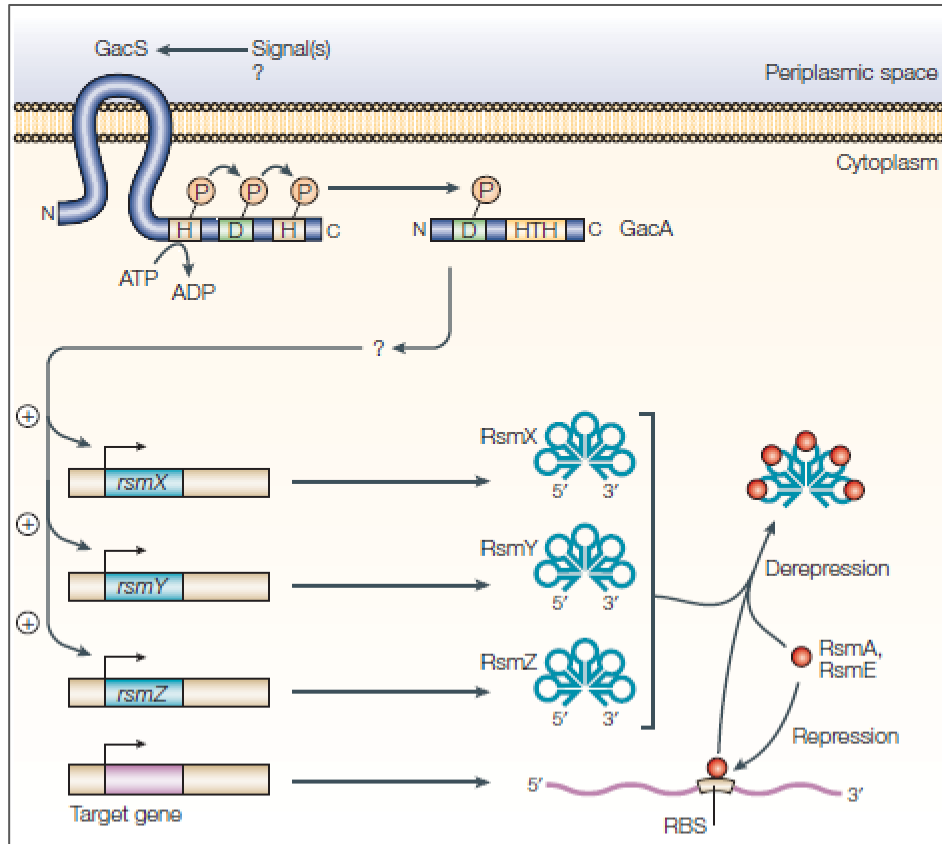

Figure 1.3: The Gac/Rsm regulatory pathway in *P. fluorescens* CHA0. As stated in the text, GacS undergoes a conformation change in response to an unknown signal followed by autophosphorylation at a conserved histidine residue. The phosphate is then transferred via a phosphorelay mechanism to GacA which, upon activation, induces the transcription of regulatory RNAs. The regulatory RNAs then bind to multiple copies of RsmA/RsmE, alleviating translational repression of target genes (Haas and Défago 2005).

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Although the exact mechanism of how LadS interacts with GacS is currently not known, the interaction between GacS and RetS was elucidated by Goodman and colleagues (2009). It was demonstrated that RetS directly interacts with GacS by forming non-productive GacS:RetS heterodimers. The formation of the RetS/GacS complex directly interferes with GacS phosphorylation because it prevents the formation of GacS homodimers necessary for phosphorelay activity (Goodman et al. 2009).

1.2.4.2 The Gac/Rsm regulatory system

The Gac/Rsm system has been identified in *E. coli*, *P, fluorescens*, *P. aeruginosa* and *Erwinia carotovora* (Mulcahy et al. 2008; Dubey et al. 2003; Hyytiäninen et al. 2001; Heeb and Haas, 2005). In all cases, the Gac/Rsm circuitry serves to modulate expression of secondary metabolites and extracellular enzymes during the transition from low to high density states (Bejerano-Sagie and Xavier, 2007). The well characterized *P. fluorescens* CHA0 Rsm system, depicted in Fig 1.3, is comprised of two RNA binding proteins RsmA/E and three small regulatory RNAs, RsmZ, RsmX, and RsmY (Lapouge et al. 2008). The RsmA/E proteins function as posttranscriptional repressors by binding to a specific ribosome-binding sequence in the target mRNA and blocking translation. Translational repression can be alleviated by the action of RsmZ, RsmY and RsmX, which bind multiple copies of RsmA/E, rendering the ribosome binding site (RBS) of target genes inaccessible to the translational machinery (Haas and Keel, 2003).

Phosphorylated GacA is suspected to activate transcription of the regulatory RNAs by binding an upstream element termed the GacA-box (TGTAAGN$_6$CTTACA) (Valverde et al. 2003; Kay et al. 2005; Lenz et al. 2005; Kulkarni et al. 2006), although

this remains to be demonstrated *in vitro*. The products of the *rsmZ*, *rsmX* and *rsmY* genes of *P. fluorescens* CHA0 are small untranslated RNAs that are between 119 and 118 nucleotides in length (Valverde and Haas, 2008). The RNA molecules fold into flower-like (Fig 1.3) secondary structures and within each of the stem loops there is a conserved ANGGA motif, similar to the sequence of a ribosome binding site (Aarons et al. 2000; Heeb et al. 2002). The multiple GGA motifs are common to all Rsm sRNAs and allows them to bind multiple copies of RsmA/RsmE with high affinity (Babitzke and Romeo, 2007; Lapouge et al. 2008; Sonnleitner and Haas, 2011). Expression of the sRNAs relies on a functional GacS/GacA system (Heeb et al. 2002; Valverde et al. 2003). These sRNAs are required for the biocontrol activity of strain CHA0, as an *rsmZrsmYrsmX* triple mutant demonstrated a phenotype similar to that of a *gacS* or *gacA* mutant, in which secondary metabolite production was abolished (Kay et al. 2005).

The RsmA and RsmE proteins are highly similar in function; however, the expression levels of the two proteins differ from one another during cell growth (Reimmann et al. 2005). RsmA levels remain fairly constant throughout growth while RsmE is expressed at higher levels during stationary phase (Reimmann et al. 2005). The overall function of RsmE is similar to RsmA. Both proteins are required for effective translational repression of GacS/GacA controlled target genes, as it was shown that a *gacS* defect was only alleviated by the deletion of both *rsmA* and *rsmE* (Reimmann et al. 2005). It also appears that RsmA may have an effect on other regulators within the cascade as a study by Heeb et al. (2005) demonstrated that RsmA negatively regulates the stationary phase sigma factor, RpoS. Interestingly, positive regulation of RpoS by RsmA was shown in *Pseudomonas aeruginosa* M18 (Ge et al. 2007), while the opposite trend

was observed in *Erwinia carotovora* where RpoS positively regulated the expression of RsmA (Mukherjee et al. 1998).

Additional layers of complexity underlying the Gac/Rsm posttranscriptional regulatory mechanism is observed in *P. fluorescens* CHA0, where *rsmE* expression is subject to positive regulation by GacA and negative regulation by RsmA and RsmE (Reimann et al. 2005). Furthermore, both RsmA and RsmE have been shown to stabilize the regulatory RNAs RsmZ and RsmY potentially protecting the RNA molecules from degradation by cellular RNases (Reimmann et al. 2005). Interestingly, Tekeuchi et al. (2009) demonstrated that the expression of these small regulatory RNAs is subject to positive regulation by Krebs cycle intermediates, specifically 2-oxoglutarate, succinate and fumarate. These results suggest that the Rsm system is dependent upon a number of factors for optimal expression of both the regulatory RNAs and the RNA binding proteins. Furthermore, although the Gac/Rsm system is conserved in many gram-negative bacteria, fine-tuning of the cascade and the target genes regulated vary greatly.

1.3 Thesis Objectives

As discussed above, biocontrol strains provide an attractive alternative to chemical pesticides for control of plant diseases caused by fungal pathogens. Several *Pseudomonas* species produce secondary metabolites which are antagonistic towards other microorganisms (Haas and Keel, 2003). *P. chlororaphis* strain PA23 has demonstrated excellent biocontrol activity against *S. sclerotiorum* in vivo. The antifungal activity has been attributed to production of secondary metabolites which include the antibiotics PHZ and PRN (Zhang et al. 2006) as well HCN, proteases and siderophores.

Clearly, the regulatory cascade overseeing expression of antifungal compounds in other *Pseudomonas* spp. is complex, involving the Gac/Rsm system, PsrA, RpoS and QS. However, not much is known about the regulatory systems governing expression of biocontrol traits in PA23. Therefore, we sought to determine how secondary metabolites were being regulated in *P. chlororaphis* PA23. The main objectives of this thesis are as follows:

1) To determine if both PHZ and PRN contribute to PA23-mediated fungal antagonism.

2) To identify the regulatory role of the stationary phase sigma factor, RpoS, in the biocontrol of PA23.

3) To elucidate how QS affects the production of antifungal metabolites, including PHZ and PRN.

4) To identify components of the Rsm system in PA23.

5) To determine the regulatory effects of GacS, PsrA, RpoS and QS on the Rsm system.

CHAPTER 2

Materials and Methods

2.0 Materials and Methods

2.1 Bacterial strains and growth conditions.

All bacterial strains, plasmids and primers used in this study are outlined in Table 2.1. *E. coli* and *P. chlororaphis* PA23 strains were routinely cultured on Lennox Luria Bertani (LB) agar (Difco Laboratories, Detroit, MI) at 37°C and 28°C, respectively. *S. sclerotiorum* was maintained on Potato Dextrose Agar (PDA; Difco). Media were supplemented with the following antibiotics from Research Products International Corp. (MT. Prospect, IL) as required: piperacillin (30 µg/ml), gentamicin (Gm; 20 µg/ml), tetracycline (Tc; 15 µg/ml), rifampicin (Rif; 100 µg/ml) for PA23 and ampicillin (Amp; 100 µg/ml), Gm (20 µg/ml), Tc (15 µg/ml) and chloramphenicol (Chl; 25 µg/ml) for *E. coli*. For β-galactosidase analysis, cultures were grown in either peptone tryptic soy broth (PTSB) (Ohman et al. 1980) or M9 supplemented with 1mM $MgS0_4$ and 0.2% glucose.

2.2 Nucleic acid manipulation.

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

2.3 Sequence analysis and nucleotide accession numbers.

2.3.1 Sequence of the *prnABCD*, *phzE* and *phzI* and *phzR* genes.

The sequence of the *prnABCD* operon on plasmid pTOPO-*prn* was determined using a combination of universal primers and primer walking. Sequence analysis of the

Tn5 *Xho*I rescue clones was performed using oligonucleotide primer Tn*5*-ON82 (Table 2.1), which anneals to the 5′ end of Tn*5*-OT182. The sequence of the *phzI* and *phzR* genes was determined through a primer walking strategy using a Tn*5 Xho*I rescue clone previously isolated in our lab (Selin et al. 2010). This plasmid contains a portion of the *phz* biosynthetic locus (*phzABCDE*) and approximately 17 kb of upstream DNA. Sequencing was performed at the University of Calgary Core DNA Services facility and sequences were analyzed with BLASTN and BLASTX databases. The Genbank accession numbers are as follows: *prnABCD* (Genbank accession No. EU188755), *phzE* (Genbank accession No. AF915615) and *phzI* and *phzR* (Genbank accession No. JN593239).

2.3.2 Analysis of *psrA*, *rsmZ, rsmA,* and *rsmE* sequences.

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

Table 2.1 Bacterial Strains and plasmids

Strain/plasmid/primer	Relevant genotype or phenotype*	Source or reference
Strain		
P. chlororaphis PA23	$PHZ^{+}Rif^{R}$ wild type (soybean plant isolate)	Savchuk and Fernando (2004)
PA23-63	PHZ^{-} Rif^{R} *phzE*::Tn*5*-OT182 genomic fusion	This study
PA23-8	PRN^{-} Rif^{R} *prnBC* deletion mutant	This study
PA23-63-1	PHZ^{-} PRN^{-} Rif^{R} *phzE*::Tn*5*-OT182 genomic fusion; *prnBC* deletion mutant	This study
PA23*phzR*	Gm^{R} marker inserted into the *phzR* gene	This study
PA23*rpoS/phzR*	PA23*rpoS* with a Gm^{R} marker inserted into the *phzR* gene	This study
PA23*rpoS*	PA23 with pKNOCK-Tc vector inserted into the *rpoS* gene	This study
PA23-6863	PA23 carrying pME6863 (AI^{-})	This study
PA23*gacA*	Gm^{R} marker inserted into the *gacA* gene	Manuel et al. (2011)
PA23*psrA*	PA23 with pKNOCK-Tc vector inserted into the *psrA* gene	This study
P. aeruginosa QSC105	Strain carries pEAL01 plasmid (*lasB-lacZ* transcriptional fusion), $Carb^{R}$	Ling et al. (2009)
E.coli		
DH5α	*supE44* Δ*U169* (*Φ80lacZ*Δ*M15)hadR17 recA1 endA1 gyrA96 thi-1relA1*	Gibco
DH5 λpir	DH5 λpir lysogen of DH5α	House et al. (2004)
C. violaceum CV026	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31532, autoinducer biosensor	Latifi et al. (1995)

Plasmids		
pCR2.1	Cloning vector for PCR products	Invitrogen
pTOPO-*prn*	pCR2.1 containing *prnABCD* genes	This study
pEX18Ap	Suicide plasmid, Amp^R	Hoang et al. (1998)
pUCGm	Source of Gm^R cassette	Schweizer (1993)
pME6863	pME6000 carrying the *aiiA* gene from *Bacillus* sp. A24 under the constitutive P_{lac} promoter	Reimmann et al (2002)
*phzR*1.4PCR2.1	1.4-kb fragment containing *phzR* cloned into pCR2.1	This study
*phzR*pEX18Ap	*phzR* from PA23 in pEX18Ap	This study
*phzR*gentpEX18Ap	Gm^R marker inserted into the *Sac*I sites of *phzR*pEX18Ap	This study
pRK600	Contains *tra* genes for mobilization; Chl^R	Finan et al. (1986)
pCR2.1-*rpoS*	1-kb fragment containing the *rpoS* gene cloned into pCR2.1	Poritsanos et al. (2006)
pKNOCK-Tc	Suicide vector for insertional mutagenesis; R6K ori RP4 oriT Tc^R	Alexeyev (1999)
pKnock-*rpoS*	400-bp internal fragment of *rpoS* in pKnock; Tc^R	Manuel et al. (2011)
pCR-*psrA*-int	450-bp internal portion of *psrA* in pCR2.1	This study
pKNOCK-*psrA*	450-bp internal fragment of *psrA* cloned into pKNOCK-Tc	This study
pCR-*phzAR*	1.68-kb fragment containing *phzA/phzR* promoter region	This study
pUCP23	Broad-host-range vector Amp^RGm^R	This study
pUCP23-*phzR*	1.68-kb fragment containing *phzR* in pUCP23	This study
pME6032	Shuttle vector, Tc^R	Heeb et al. (2002)

pME6032-*phzR*	1.68-kb fragment of *phzR* in pME6032	This study
pUCP22-*rpoS*	1.3-kb fragment of *rpoS* cloned in pUCP22	Poritsanos et al. (2006)
pCR-*psrA*	950-bp PCR product containig *psrA* cloned into pCR2.1	This study
pCR-*rsmZ*	160-bp fragment comprising *rsmZ* cloned into pCR2.1	This study
pCR-*rsmA*	190-bp fragment containing *rsmA* cloned into pCR2.1	This study
pCR-*rsmE*	190-bp PCR product containing *rsmE* cloned into pCR2.1	This study
pCR-*rsmZ*OE	400-bp region containing promoter, and entire *rsmZ* gene cloned into pCR2.1	This study
pCR-*rsmE*OE	600-bp fragment with promoter and entire *rsmE* gene cloned into pCR2.1	This study
pCR2.1-*rpoS*-1.3	1.3-kb fragment containing the entire *rpoS* gene cloned into pCR2.1	This study
pACYC-BAD	2.0-kb fragment containing *araC*, pBAD, MCS, and RBS from pBAD24 cloned into pACYC-184	This study
pBAD-*rpoS*	1.3-kb *rpoS* fragment cloned into pACYC	This study
pBAD-*psrA*	950-bp *psrA* fragment cloned into pACYC	This study
phzI-pCR2.1	1.3-kb fragment containing *phzI* in pCR2.1	This study
pLP170	*lacZ* transcriptional fusion vector	Preston et al. (1997)
pPHZI-*lacZ*	674-bp fragment containing *phzI* promoter in pLP170	This study
pCR-*phzAR*	1.68-kb fragment containing *phzR* and 5' end of *phzA* in pCR2.1	This study
pPHZR-*lacZ*	1.1-kb fragment containing *phzR* promoter in pLP170	This study

pPHZA-*lacZ*	1.1-kb fragment containing *phzA* promoter in pLP170	This study
pPRNA-*lacZ*	1.5-kb fragment containing *prnA* promoter in pLP170	This study
pRPOS-*lacZ*	1.1-kb fragment containing *rpoS* promoter in pLP170	Poritsanos et al. (2006)
pPSRA-*lacZ*	850-bp fragment containing *psrA* promoter in pLP170	This study
pCR-*rsmZ*-1.5	1.5-kb fragment with *rsmZ* cloned into pCR2.1	This study
pRSMZ-*lacZ*	1.5-kb fragment with *rsmZ* promoter cloned into pLP170	This study
pCR-*rsmE*-p	400-bp fragment containing *rsmE* promoter region cloned into pCR2.1	This study
pRSME-*lacZ*	*rsmE* promoter in pLP170	This study
pCR-*rsmA*-p	600-bp fragment comprising the *rsmA* promoter cloned into pCR2.1	This study
pRSMA-*lacZ*	*rsmA* promoter cloned into pLP170	This study
Primers		
prnF	5′-ggtccgtgttatgaacaagccgatcaa-3′	This study
prnR	5′-tctagatgaacgtgacgccgctcg-3′	This study
phzAR-F	5′-aatcctgccatccaactc-3′	This study
phzAR-R	5′-aagttgttcgaaggggttca-3′	This study
prnA-F	5′-ccaatgcccttgcatttgag-3′	This study
prnA-R	5′-tgccggttcgcgagccaga-3′	This study
Tn5-ON82	5′-gatcctggaaaacgggaaagg-3′	Poritsanos et al. (2006)
phzRP1	5′-gcatgaattcaattggcgatgccgttgtt-3′	This study
phzRP2	5′-gcatgagctccacggtggtgaagcacagcaaa-3′	This study
phzRP3	5′-gcatgagctcatataagtcctggggccgcat-3′	This study
phzRP4	5′-gcatggatcccgatgtactgttcgacggt-3′	This study
phzI-FRW	5′-tacgactgcctggaccaaact-3′	This study
phzI-REV	5′-aatcctgccatccaactc-3′	This study
phzAR-F	5′-aatcctgccatccaactc-3′	This study
phzAR-R	5′-aaggttgttcgaaggggttca-3′	This study
RpoS RC-fwd	5′--gatatcgccactgattcgatc-3′	This study
RpoS RC-rev	5′-ggatccaccaggtggcata-3′	This study

psrA-IF	5′-ggcggcggtggtgaattatcattt-3′	This study
psrA-RF	5′-gtattgaccccgaagtcggt-3′	This study
psrA-pstI-rev	5′-aactgcagcgaggaatggcaccatca-3′	This study
psrA-BamHI	5′-ccggatccggtgacgccggtttca-3′	This study
rsmZ-F	5′-tgccggggaactctctaatat-3′	This study
rsmZ-R	5′-tatgacccgcccacattt-3′	This study
rsmA-F	5′-tatgctgattctgactcgtcg-3′	This study
rsmA-R	5′-ttatggcttggttcttcgtc-3′	This study
rsmE-F	5′-atgctgatactcacccgcaaa-3′	This study
rsmE-R	5′-ttcagggggtttcgcgttt-3′	This study
RsmZ-F	5′-gtgaaaagccccgacatgtt-3′	This study
RsmZ-R	5′-tttatgacccgcccacattt-3′	This study
RsmE-F	5′-atgctgatactcacccgcaaa-3′	This study
RsmE-R	5′-ttgacttcgtagagcccct-3′	This study
rsmZ-BamHI-rev	5′-ctaggatcccgtcgacacagggttgatatt-3′	This study
rpoS-int-forward	5′-aaagaagtgccggaggtttga-3'	This study

2.4 Mutant Generation

2.4.1 Construction of PA23*phzR*, PA23*rpoS*, PA23*phzRrpoS* and PA23*psrA*.

To generate PA23*phzR*, a copy of *phzR* missing an internal 66-bp fragment was generated through PCR. To accomplish this, the 5′ end of *phzR* was amplified using primers phzRP1 and phzRP2 and the 3′ end was amplified using primers phzRP3 and phzRP4. The two PCR products were digested with *Sac*I and cloned into pCR2.1 to yield *phzR*1.4pCR2.1. The 1.4-kb insert was subsequently digested with *Eco*RI and *Bam*HI and subcloned into the same sites of pEX18Ap (*phzR*pEX18Ap). An 850-bp Gm^R cassette was excised from pUCGm and inserted into the *Sac*I site to yield *phzR*-gentpEX18Ap. Triparental mating between *E. coli* DH5α (*phzR*-gentpEX18Ap), *E. coli* DH5α (pRK600) and PA23 was performed. Pseudomonas Isolation Agar (PIA; Difco) + Gm (20 μg/ml) was used to screen for transconjugants. To select for bacteria that had undergone a double cross-over event, colonies were streaked onto LA supplemented with sucrose (10%) and Gm (20 μg/ml). PCR analysis was used to verify that *phzR* had been successfully replaced with a mutated copy of the allele. The *rpoS* mutant, PA23*rpoS,* was created by first excising an internal portion of the *rpoS* gene from PCR2.1-*rpoS* using the enzymes *Bam*HI and *Eco*RV. The resulting 400-bp fragment was then subcloned into the same sites of pKNOCK-Tc, generating pKNOCK-*rpoS*. Tri-parental mating of *E. coli* DH5α λpir (pKNOCK-*rpoS*), *E. coli* DH5α (pRK600) and PA23 was performed and transconjugants obtained were screened on LB agar supplemented with Rif and Tc. PA23*phzRrpoS* was created by insertion of plasmid pKNOCK-*rpoS* into the

rpoS gene of PA23*phzR*. To verify disruption of the *rpoS* gene, the pKNOCK-Tc vector was rescued from the PA23*rpoS* and PA23*phzRrpoS* genome by digestion with *Bgl*II. Linearized genomic fragments were recircularized with T4 DNA ligase, transformed into *E. coli* DH5α λpir, and screened on LB-agar supplemented with 20 µg/ml Tc. Sequencing of the rescue clones, using primers RpoS RC-fwd and RpoS RC-rev, verified that an *rpoS* insertion had occurred in both strains. A quorum-quenching approach enabled us to generate an AHL-deficient strain. Plasmid pME6863, which contains the AHL lactonase gene (*aiiA*) from *Bacillus subtilis* (obtained from D. Haas) was mobilized into PA23, generating PA23-6863. The presence of the lactonase enzyme renders bacteria AHL-deficient due to hydrolysis of the lactone ring. PA23*psrA* was generated by PCR amplifying a 450bp internal portion of *psrA* using PsrA-IF and PsrA-IR and cloned into pCR2.1 to generate pCR*psrA*-int. The 450bp fragment was then excised using *Eco*RI and subcloned into the same site of the pKNOCK-Tc vector, creating pKNOCK-*psrA*. The resulting construct was mated into PA23 and the conjugants were selected on PIA supplemented with Tc (50ug/ml). Insertion was verified by sequence analysis of a *Bam*HI rescue clone.

2.5 Plasmid Construction

2.5.1 Creation of pUCP23-*phzR* and pME6032-*phzR*.

To complement PA23*phzR* and PA23*phzRrpoS*, pUCP23-*phzR* was generated as follows. A 1.68-kb *phzR/A* PCR product was amplified using PhzAR-F and PhzAR-R and cloned into pCR2.1 to yield pCR-*phzAR*. For *phzR* complementation, the 1.68kb *Hin*dIII-*Xba*I fragment was excised from pCR-*phzAR* and subcloned into the same sites of

pUCP23, creating pUCP23-*phzR*. To generate pME6032-*phzR*, a 1.68-kb *Eco*R1 fragment was excised from pUCP23-*phzR* and subcloned into the same sites of pME6032.

2.5.2 Generation of pCR-*rsmZ*, pCR-*rsm*ZOE, pCR-*rsmA*, pCR-*rsmE* and pCR-*rsmE*OE.

To identify the presence of the *rsmZ*, *rsmA* and *rsmE* genes in strain PA23, PCR primers (rsmZ-F/rsmZ, rsmA-F/rsmA-R, rsmE-F/rsmE-R) were designed using the *rsmZ*, and *rsmA* sequences found in *P. fluorescens* (accession No AF245440; Heeb and Haas 2002, accession No AF136151; Blumer et al. 1999), *P. aeruginosa* (accession No. AE004091; Stover et al. 2000) and *Pseudomonas entomophila* (accession No. NC_008027; Vodovar et al. 2006). The *rsmE* gene sequence of *P. fluorescens* CHA0 (accession No. AY547575; Reimmann et al. 2005) was used to design primers to amplify the *rsmE* gene in PA23. PCR products were obtained for *rsmZ* (164-bp), *rsmA* (190-bp) and *rsmE* (190) respectively, and were cloned into pCR2.1 topo to generate pCR-*rsmZ*, pCR-*rsmA,* pCR-*rsmE*. All three constructs were verified by sequence analysis.

To obtain sequence upstream and downstream from the *rsmZ* gene, a 400 bp PCR product containing the *rsmZ* gene was amplified using RsmZ-F and RsmZ-R and cloned into pCR2.1 (pCR-*rsmZ*OE). To obtain sequence downstream of the *rsmE* gene, a 600-bp PCR product was generated using the primers RsmE-F and RsmE-R and cloned into pCR2.1 (pCR-*rsmE*OE). Both constructs were analyzed via sequence and BLAST analysis.

2.5.3 Generation of pBAD-*psrA* and pBAD-*rpoS*

To co-express *psrA* and *rpoS* and the *lacZ*-transcriptional fusions in *E.coli*, pBAD-*rpoS* and pBAD-*psrA* were generated as follows: The *rpoS* gene was excised as a 1.3-kb *Nsi*I/*Kpn*I fragment from pCR2.1-*rpoS*-1.3. The insert was subcloned into the *Pst*I and *Kpn*I sites of pBAD-ACYC to generate pBAD-*rpoS*. To create the pBAD-*psrA* plasmid, a 950-bp *psrA* fragment was PCR amplified with psrA-F and psrA-pstI-rev and cloned directly into the *Sma*I-*Pst*I sites of pBAD-ACYC.

2.6 Construction of transcriptional *lacZ* fusions.

2.6.1. Generation of *phzA*- and *prnA-lacZ* fusions.

To monitor PHZ gene expression, a *phzA-lacZ* transcriptional fusion was created using PCR primers phzAR-F and phzAR-R (Table 2.1). The *phzA* promoter region was PCR amplified and cloned into pCR2.1. A 1.1-kb *Hin*dIII and *Eco*RV fragment was removed and cloned into *Hin*dIII – *Sma*I-digested pLP170, creating pPHZA-*lacZ*. To generate a *prn* (PRN) transcriptional fusion, PCR primers prnA-F and prnA-R (Table 2.1) were used to amplify a 1.5-kb fragment corresponding to the *prnA* promoter region of PA23. The PCR product was first cloned into pCR2.1-TOPO, and then excised using *Hin*dIII and *Xba*I and ligated into the same sites of pLP170, creating pPRNA-*lacZ*.

2.6.2 Generation of *phzI*- and *phzR-lacZ* transcriptional fusions.

The *phzI-lacZ* transcriptional fusion was constructed by PCR amplifying the *phzI* promoter using primers phzI-FRW and phzI-REV. The 1.3-kb PCR product was cloned into pCR2.1 (*phzI*-pCR2.1), and then excised as a *Hinc*II-*Eco*RI fragment and subcloned

into the *Sma*I-*Eco*RI sites of pLP170, generating pPHZI-*lacZ*. The *phzR*-*lacZ* fusion was constructed using primers phzAR-F and phzAR-R to amplify a 1.68-kb fragment containing the entire *phzR* gene and the 5′ end of *phzA*. This fragment was cloned into pCR2.1, creating pCR-*phzAR*. The *phzR* promoter was excised from pCR-*phzAR* as a 1.1-kb *Eco*RI-*Eco*RV fragment and ligated into the *Eco*RI-*Sma*I sites of pLP170, generating pPHZR-*lacZ*.

2.6.3 Generation of *psrA*-, *rsmZ*-, *rsmA*-, and *rsmE*-*lacZ* transcriptional fusions.

To generate a *psrA*-*lacZ* transcriptional fusion, primer psrA-BamHI (Table 2.1) and M13 universal forward primer were used to PCR amplify a 950-bp product using pCR-*psrA* as template. The PCR product was digested with *Eco*RI and *Bam*HI and cloned into the same sites of pLP170 creating pPSRA-*lacZ*. The *rsmZ*-*lacZ* fusion was constructed by amplifying a 1.5-kb fragment using rpoS-int-forward and rsmZ-BamHI-rev that was then cloned into pCR2.1 (pCR-*rsmZ*-1.5). The 1.5-kb fragment containing the *rsmZ* promoter was excised with *Eco*RI/*Bam*HI and subcloned into the same sites of pLP170, generating pRSMZ-*lacZ*. The *rsmE*-*lacZ* plasmid was generated by PCR amplifying a 400-bp fragment containing the *rsmE* promoter using the primers RsmE-trans-fwd and RsmE-trans-rev. The PCR product was cloned into pCR2.1 (pCR-*rsmE*-p) and subcloned as a *Hin*dIII/*Eco*RI fragment into the same sites of pLP170, creating pRSME-*lacZ*. To generate the *rsmA*-*lacZ* transcriptional fusion, a 600-bp fragment was amplified using primers RsmA-trans-fwd and RsmA-trans-rev and cloned into pCR2.1 (pCR-*rsmA*-p). The 600-bp fragment was excised with *Eco*RI and *Hin*dIII and subcloned into the same sites of pLP170, generating pRSMA-*lacZ*.

2.7 Analysis of transcriptional fusions.

The activity of *phzA*-, *prnA-, phzR*-, *phzI*-, *psrA*-, *rpoS-, rsmZ-*, *rsmA*- and *rsmE*-*lacZ* transcriptional fusions was determined in PA23, PA23*phzR*, PA23-6863, PA23*rpoS*, PA23*rpoSphzR*, PA23*psrA*, PA23*gacA*, PA23-63, PA23-8, and PA23-63-1. Strains carrying the *lacZ*-fusion plasmids were grown for 4, 8, 16, 24, and 36 hours in either PTSB or M9 (0.2% glucose, 1mM $MgS0_4$) prior to analysis of β-galactosidase activity (Miller, 1972). The activity of *prnA*-*lacZ* and *rsmZ*-*lacZ* were also measured in *E. coli* harboring pME6032-*phzR*, supplemented with 1 µm purified C6-AHL (Sigma). Similarly, the activity of *rsmZ-, rsmA*-, and *rsmE-lacZ* was measured in *E. coli* to monitor direct effects of PsrA (pBAD-*psrA*) and RpoS (pBAD-*rpoS*) on gene expression. *E. coli* cultures were grown for 24 hours prior to analysis of β-galactosidase activity. Samples were analyzed in triplicate and the experiment was repeated three times.

2.8 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 OD600nm of approximately 1.3 and were subsequently diluted to an OD of 1.0 to ensure that an equal number of cells was analyzed. Cells were pelleted, resuspended in sample buffer (63nmol/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 µg sample, determined by the Bradford assay (Bradford, 1976), was loaded and separated on a 12% SDS-PAGE gel. Blots were then 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 blot analysis was repeated twice with similar findings.

2.9 Antifungal Assays

To assess the ability of PA23 and its derivatives to inhibit the growth of *S. sclerotiorum* in vitro, radial diffusion assays were performed. Briefly, 5 µl of an overnight culture was spotted onto PDA or M9 Agar. After 24 hours incubation at 30°C, an agar plug containing fungal mycelia was placed in the center of the plate and incubated at room temperature. After 3 days, the inhibition was assessed by measuring the distance between the edges of the bacterial colony and *S. sclerotiorum*. Five replicates were analyzed for each strain and the experiments were repeated at least three times.

2.10 Quantitative analysis of PHZ

Overnight cultures grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose were subject to PHZ extraction following the method of Chancey et al. (1999). Briefly, 5 ml of benzene and 1 drop of concentrated HCl were added to 5 ml of culture supernatant. The mixture was shaken for 1 hour and the top layer was collected and dried down under air. The dried extracts were suspended in a 1

ml volume of 0.1 M NaOH and quantified with UV-visible spectroscopy according to Maddula et al. (2008). The absorption maxima for PCA and 2-OH-PHZ were measured at 367 nm and 490 nm, respectively. Relative amounts of PCA and 2-OH-PHZ were calculated by dividing the absorption maxima by their standard extinction coefficients (PCA: 3019; 2-OH-PHZ: 7943; Olson et al., 1967). The PHZ quantification was repeated three times.

2.11 HPLC analysis of PRN.

The amount of PRN produced by PA23 and its derivatives was quantified by HPLC as described by Selin et al. (2010) with the following modifications. Strains were grown in 100 ml of M9 minimal media supplemented with 1mM $MgSO_4$ and 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 triplicate and the experiment was repeated twice.

2.12 HCN analysis.

Qualitative determination of HCN production was performed using Cyantesmo paper (Machery-Nagel GmbH & Co, Germany). To monitor expression of the genes encoding hydrogen cyanide, plasmid pME3219 containing an *hcnA-lacZ* translational fusion was transformed into PA23, PA23-63, PA23-8, and PA23-63-1. Cultures were grown in PTSB until they reached stationary phase ($OD_{600\ nm}$= 2.5 - 3.0), at which point *hcnA* expression was assessed using β-galactosidase assays (Miller, 1972). Samples were analyzed in triplicate and experiments were repeated three times.

2.13 Protease production.

Extracellular protease production was measured qualitatively by inoculating 5 µl of overnight culture on a 1.5% agar plate containing 2% skim milk. Proteolysis was indicated by a zone of lysis around the bacterial colony after 24-36 hours of growth at 28°C. To quantitatively measure protease production bacterial cultures were grown in M9 minimal media supplemented with 1mM $MgS0_4$, 0.2% glucose and 1.5% skim milk (Difco) for 5 days at 28°C. A 200-µl aliquot of cell-free supernatant was analyzed for the protease activity in a 0.65% casein solution at 28°C for 10 min. Tyrosine is released upon the hydrolysis of casein by the protease enzyme and reacts with the Folin-Ciocalteu reagent (Sigma) by producing a blue colored chromophore (Cupp-Enyard 2008). This chromophore is measured spectrophotometrically at a wavelength of 660 nm. In order to determine how much tyrosine is being liberated by each sample, a standard curve was generated using pure tyrosine at the following concentrations (µMole): 0.055, 0.111, 0.221, 0.442 and 0.553. Each strain was assayed in triplicate and experiments were performed three times.

2.14 Motility analysis.

Flagellar (swimming) motility was assayed by inoculating 5 µl of an overnight bacterial culture below the surface of a 25 ml 0.3% LB agar plate. Five replicates per strain were analyzed and motility was measured at 24, 48 and 72 hours. Swarming motility was assayed by inoculating bacterial cells onto the surface of a 30 ml 0.5% SWM plate (0.5% Peptone, 0.3 Yeast extract and 0.5 % agar) previously air dried for 2 hours. Results were recorded after incubation for 16-30 hours.

2.15 Analysis of Biofilm development.

A static, 96-well plate assay was used to assess the ability of PA23, and derivative strains to form biofilms (O'Toole and Kolter, 1998). Briefly, overnight cultures grown in M9 minimal casamino acid media + 0.2% glucose were adjusted to an OD of 1.0 and then diluted 1 in 100 in fresh media. One hundred µl aliquots of the diluted culture were inoculated into 96-well plates (Becton-Dickenson, Oakville, ON). After 24 and 48 h, the adherent cell population was quantified by crystal violet (CV) staining and measuring the optical density at a wavelength of 600 nm. Results are averages of eight replicates [± standard deviation (SD)] and representative of five independent experiments. For direct enumeration of the adherent cell population, cultures were prepared as described above except that 1.5 ml-aliquots were added to each well of a 6-well plate (Becton-Dickenson). Biofilms were allowed to form for 24 and 48 h, after which wells were washed four times with PBS to remove planktonic cells. A 1-ml aliquot of PBS was added to each well and the surface-attached cells were removed by a combination of scraping and sonication. Cell suspensions were diluted and viable plate counting was performed. Results are averages of four replicates (± SD) and representative of three independent experiments. An unpaired Student's *t* test was used for statistical analysis of the CV staining and viability counts.

2.16 AHL analysis.

Homoserine lactone production was assessed qualitatively by spotting 5 µl of an overnight culture onto *Chromobacterium violaceum* CV026 indicator plates. The production of C4-, C6- or C8-HSL was indicated by the production of violacein, a purple

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

2.17 Growth Analysis.

Growth rate analysis of PA23, PA23-63 (*phzE*), PA23-8 (*prnBC*), and PA23-63-1 (*phzE; prnBC*) was performed in rich (LB, PTSB, TB) and minimal (M9CA + 0.2% glucose) media using a Bioscreen^{R} C automated turbidometer. Overnight cultures were adjusted to an OD_{600} of 0.1 by diluting with the same media and 100 µL of culture was inoculated into each well of a Bioscreen^{R} C microtiter plate. Control wells contained an equal volume of sterile media. Growth of the cultures at 28°C was monitored every 15 min. over a 45 h period. Samples were analyzed in triplicate and the growth rate analysis was repeated three times.

2.18 Biocontrol under greenhouse conditions.

Strains PA23, PA23-63, PA23-8, and PA23-63-1 were assessed for their efficiency in suppressing stem rot of canola [*Brassica napus* (cv. Westar)] under

greenhouse conditions. *Brassica napus* (cv. Westar) plants were grown in pots (21 cm x 20 cm) at 24/16°C with a 16 h photoperiod. The plants were sprayed at 30% flowering with bacterial strains (2.0 x 10^{8} cfu/ml) suspended in 100 mM phosphate buffer, pH 7.0 with 0.02 % Tween 20 and kept in humidity chamber (24/16°C, 16 h photoperiod). Twenty four hours after bacterial inoculation, canola petals were sprayed with ascospores of *S. sclerotiorum* (8 x 10^{4} spores/ml) suspended in 100 mM phosphate buffer, pH 7.0 containing 0.02 % Tween 20. The pathogen control plants were inoculated with ascospores, while the healthy control plants were sprayed with phosphate buffer. All plants were incubated in a humidity chamber. Fourteen days after inoculation of *Sclerotinia* ascospores, symptom development on stem and leaves was observed and recorded using a 0-7 scale (0: no lesions on the stem; 1: leaf lesion with no stem symptom; 2: 1-20 mm stem lesion; 3: 21-40 mm stem lesion; 4: 41-60 mm stem lesion; 5: 61-80 mm stem lesion; 6: 81-100 mm stem lesion, 7: >100 mm stem lesion or plant death). Based on symptom development, percent leaf incidence by *Sclerotinia* (PLI) and stem rot disease severity (DS) was calculated. Ten plants were used for each treatment. For assessing infection on leaves, the first 10 leaves, from top to bottom, were scored for the presence or absence of the symptom per plant. The plant studies were repeated two times.

$$PLI = \frac{\text{Number of leaves infected with } \textit{Sclerotinia}}{\text{Number of leaves observed}} \times 100$$

$$DS = \frac{\text{Total points for all plants using a 0-7 scale}}{\text{Number of plants observed}}$$

2.19 Statistical analysis.

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

Chapter 3

Phenazines are not Essential for *Pseudomonas chlororaphis* PA23 Biocontrol of *Sclerotinia sclerotiorum* but may Contribute to Ecological Fitness Through Biofilm Formation

3.1 Introduction

Compared to chemical pesticides, biological control represents a safer, more environmentally friendly approach to managing plant pathogens. Fluorescent pseudomonads are present in high numbers in natural soils and various strains have been found to produce metabolites deleterious to fungal pathogens. Inhibitory compounds include antibiotics, for example 2,4-DAPG, PHZs, PRN, PLT, LPs, and HCN, together with hydrolytic enzymes such as proteases, cellulase, β-glucanase and chitinase (Dowling and O'Gara, 1994; Haas and Défago, 2005; Thomashow and Weller, 1995). This potent arsenal of antimicrobial metabolites enables pseudomonads to inhibit a myriad of plant pathogens.

Pseudomonas chlororaphis strain PA23 is a biocontrol agent initially isolated from soybean root tips. In both greenhouse and field studies, this bacterium is able to protect canola from the devastating effects of stem rot caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Fernando et al. 2007; Savchuk and Fernando, 2004; Zhang, 2004). *S. sclerotiorum* is a ubiquitous, soil-borne fungus that infects more than 400 plant hosts causing significant loss of economically important crops (Purdy, 1979). In commercially grown canola cultivars there is no known resistance against *S. sclerotiorum*, making management of this pathogen essential. A well-defined zone of inhibition surrounding PA23 on AF plates suggests antibiotic production is the primary mechanism of pathogen inhibition (Poritsanos et al. 2006; Savchuk and Fernando, 2004). Strain PA23 produces the diffusible antibiotics PCA, 2-OH-PHZ and PRN (Zhang et al. 2006); however the individual contribution of these antibiotics to PA23 biocontrol has yet to be defined.

Many biocontrol agents display excellent disease control in the greenhouse but suffer from inconsistent performance in the field, due in part to variable expression of disease-suppressive factors (Cook, 1993; Haas and Keel, 2003; Walsh et al. 2001). Environmental conditions can have a dramatic impact on antibiotic production and conditions that favor expression of one antibiotic may not be optimal for others. For instance, maximal PHZ production by *P. chlororaphis* strain PCL1391 was found to occur when glucose, L-pyroglutamic acid or glycerol was used as the carbon source (van Rij et al. 2004). Whereas fructose and mannitol favored PRN production by *P. fluorescens* strain CHA0 (Duffy and Défago, 1999). Other factors that influence production of PHZ and PRN include pH, temperature, oxygen availability, and mineral amendment (Duffy and Défago, 1999, Hwang et al. 2002; Slininger and Jackson, 1992; Slininger and Shea-Wilbur, 1995 van Rij et al. 2004). Identifying metabolites that are essential for PA23 biocontrol is an important first step in the development of a successful biocontrol agent. Once this has been established, factors affecting production of key compounds can be determined.

The aim of the present study was to assess the contribution of PHZ and PRN to PA23 inhibition of the fungal pathogen *S. sclerotiorum*. We also examined whether these antibiotics are subject to autoregulatory or co-regulatory control. Finally, the impact of PHZ and PRN on biofilm formation was examined.

3.2 Results

3.2.1 Isolation of a *P. chlororaphis* PHZ-deficient mutant.

To identify genes involved in PA23 biocontrol, Tn mutagenesis was performed. Out of approximately 5000 mutants, one mutant was identified (PA23-63) that was white in color indicative of no PHZ production. Surprisingly this strain exhibited increased AF activity compared to the wild type (Fig.3.1, Table 3.1). Sequence analysis of DNA flanking the Tn insertion in this mutant showed 92% identity to the *phzE* gene of *P. chlororaphi*s PCL1391 [Genbank accession #AF195615]. PhzE is an enzyme involved in PHZ biosynthesis (Chin-A-Woeng, et al. 2003).

3.2.2 Isolation of the *prn* gene cluster and generation of PRN-deficient mutants.

Because no PRN biosynthetic mutants were identified in our Tn screen, they were generated using allelic exchange. To accomplish this, the PA23 *prn* biosynthetic operon was isolated through PCR amplification. Sequence analysis revealed that the *prn* cluster is comprised of four genes, *prnABCD*, exhibiting 93% identity at the nucleotide level to the *prn* operon of *Pseudomonas fluorescens* Pf-5 [Genbank accession #NC004129]. The same *prnABCD* genetic arrangement has been reported for other bacteria including *P. fluorescens*, *Pseudomonas pyrrocinia*, and *Burkholderia cepacia* (Hammer et al. 1997). PRN-deficient derivatives of PA23 and PA23-63, called PA23-8 (PHZ^+, PRN^-) and PA23-63-1 (PHZ^-, PRN^-) respectively, were created as described in Materials and Methods.

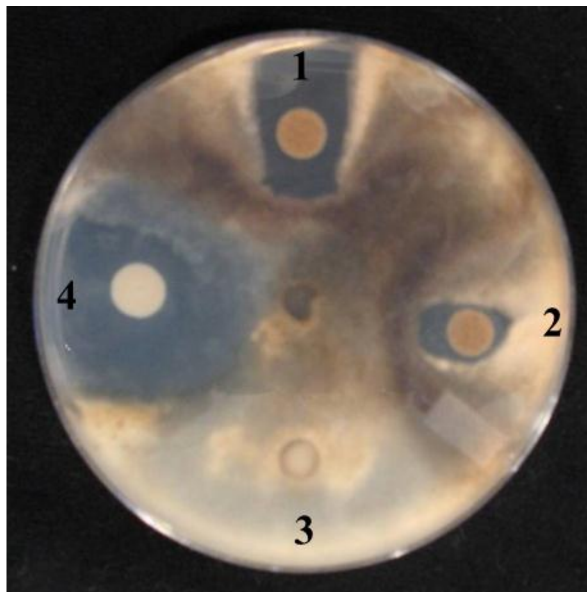

Figure 3.1. Antifungal activity of *Pseudomonas chlororaphis* PA23 and antibiotic-deficient derivatives against *Sclerotinia sclerotiorum*. A 5-µl aliquot of an overnight bacterial culture was spotted onto a potato dextrose agar plate. After 24 h, a fungal plug was placed in the centre of the plate, which was incubated at 24°C until the fungal mycelia reached the periphery of the plate. Sample 1: PA23 (wild type); sample 2: PA23-8 (PRN^-); sample 3: PA23-63-1 (PRN^- PHZ^-); sample 4: PA23-63 (PHZ^-).

Table 3.1 Phenotypic characterization of *Pseudomonas chlororaphis* PA23 and mutants PA23-8 (PRN^-), PA23-63 (PHZ^-), and PA23-63-1 (PHZ^- PRN^-).

Strain	Extracellular Metabolite Activity				
	Antifungal[*]	Protease[*]	Autoinducer[*]	HCN[†]	Lipase
PA23	5.8 (1.3)	19.3 (1.3)	12.4 (0.5)	+	+
PA23-8 (PRN^-)	3.8 (0.7)[‡]	20.3 (1.5)[‖]	12.2 (0.3)[‖]	+	+
PA23-63 (PHZ^-)	9.9 (0.5)[§]	20.1 (1.4)[‖]	13.3 (1.3)[‖]	+	+
PA23-63-1 (PHZ^- PRN^-)	0[§]	20.1 (1.1)[‖]	12.2 (1.0)[‖]	+	+

[*]Mean (SD) of the zones of activity (mm) obtained from six replicates.
[†]Determined using Cyantesmo paper.
[‡]Significantly different from the wild type ($P < 0.01$)
[§]Significantly different from the wild type ($P < 0.0001$)
[‖]Not significantly different from the wild type

3.2.3 Production of antifungal metabolites in the wild-type and mutant strains.

Strain PA23 produces the compounds PCA and 2-OH-PHZ, resulting in an orange phenotype (Zhang et al. 2006). The loss of orange color together with presence of a *phzE*-Tn insertion in PA23-63 suggested this strain was not producing PHZ. Spectral analysis confirmed that PHZ compounds were not present in culture extracts of this mutant (Table 3.2). The increased AF activity led us to hypothesize that some other AF metabolite(s) was upregulated in PA23-63. Through HPLC analysis, we discovered that PRN production is increased over two fold in the PHZ$^-$ background (Table 3.2). However the reverse was not true; wild-type levels of PHZ were produced by PRN-deficient mutant PA23-8 (Table 3.2). As expected, no PRN was detected in culture supernatants of the *prnBC* mutants PA23-8 and PA23-63-1 (Table 3.2).

In addition to PHZ and PRN, PA23 produces a number of compounds believed to contribute to biocontrol including hydrogen cyanide, protease, lipase, and autoinducer molecules (Poritsanos et al. 2006). Accordingly, PA23, PA23-63 (*phzE*), PA23-8 (*prnBC*) and PA23-63-1 (*phzE;prnBC*) were analyzed for production of these molecules. Using Cyantesmo paper, all three strains demonstrated production of the volatile antibiotic HCN (Table 3.1). Analysis of an *hcnA-lacZ* translational fusion revealed no significant differences in expression between PA23-63 (10,200 ± 230 Miller units), PA23-63-1 (10,481 ± 318 Miller units), PA23-8 (10,889 ± 311 Miller units) and the PA23 wild-type strain (10,545 ± 655 Miller units). Similarly, no differences in protease activity, lipase activity and autoinducer production were detected among the strains (Table 3.2).

Table 3.2 PRN and PHZ production by *Pseudomonas chlororaphis* PA23 and mutants PA23-8 (PRN^-), PA23-63 (PHZ^-), and PA23-63-1 (PHZ^- PRN^-).

	Antibiotic ($\mu g\ ml^{-1}$)			
Strain	PRN*	PCA*	2-OH-PCA*	Total PHZ*
PA23	1.37 (0.31)	28.5 (0.3)	4.9 (0.2)	33.7 (0.6)
PA23-8 (PRN^-)	ND	29.2 (0.4)‡	4.7 (0.7)‡	33.4 (1.0)‡
PA23-63 (PHZ^-)	3.05 (0.14)†	ND	ND	ND
PA23-63-1 (PHZ^- PRN^-)	ND	ND	ND	ND

*Mean (SD) from three replicates.
†Significantly different from the wild type ($P < 0.01$)
‡Not significantly different from the wild type
ND, not detectable.

3.2.4 Expression of *phzA* and *prnA* in the wild type and antibiotic-deficient mutants.

The increased production of PRN in PA23-63 led us to speculate that PHZ might be acting as a repressor of the *prn* operon. Therefore, we generated a *prnA-lacZ* transcriptional fusion (pPRNA-*lacZ*) to monitor expression of the PRN biosynthetic genes. We were particularly interested in two parameters: i) the onset of *prnA* gene expression and ii) the level of transcriptional activity. As illustrated in Figure 3.2A, no difference in either the initiation of *prnA* transcription or the level of expression was observed between PA23 and PA23-63. Similarly, *prnA* expression in PA23-8 and PA23-63-1 was identical to that of the wild type (Fig. 3.2A). These findings indicate that PHZ does not have a repressive effect on *prn* transcription. Moreover the *prn* operon is not subject to autoregulation. When we monitored *phz* gene activity, the expression kinetics of the *phzA-lacZ* fusion were nearly identical in all four strains (Fig. 3.2B). Therefore, we conclude that i) PRN has no impact on *phz* gene expression and ii) PHZ neither stimulates nor represses the PA23 *phz* operon.

3.2.5 Effect of PHZ and PRN production on growth rate and motility.

One of the determining factors for the success of a biocontrol agent in managing plant disease is how quickly it can obtain a sufficient population size to begin production of pathogen-inhibiting compounds. To explore whether antibiotic production represents a significant metabolic burden for the bacteria, growth rate analysis was undertaken. No differences in growth rate between PA23, PA23-63, PA23-8, and PA23-63-1 were detected in either rich or minimal media (data not shown).

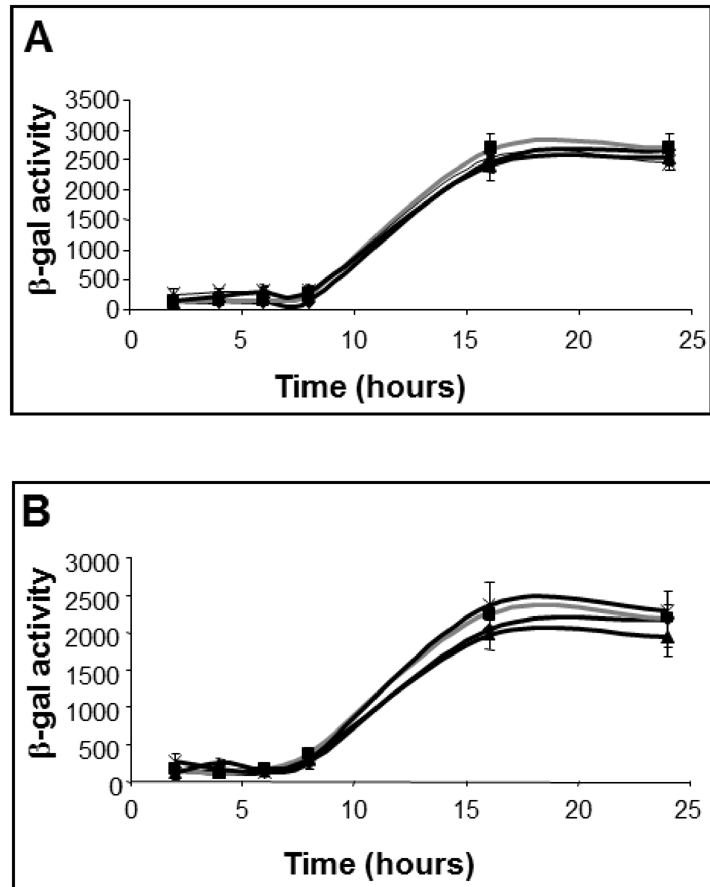

Figure 3.2. Time course of *prnA* and *phzA* expression in *Pseudomonas chlororaphis* PA23 (◆; wild type), PA23-63 (■; PHZ⁻), PA23-8 (▲; PRN⁻) and PA23-63-1 (✕; PRN⁻ PHZ⁻). Cultures containing a *prnA-lacZ* fusion (Panel A) or a *phzA-lacZ* fusion (Panel B) were analyzed for β-galactosidase activity throughout growth as described in Materials and Methods. Note: for some data points, error bars representing SD are too small to show on the graph.

Motility is a trait that can also impact biocontrol (Lugtenberg et al. 2001); accordingly, the influence of PHZ and/or PRN deficiency on bacterial translocation was assessed. We previously reported that strain PA23 is capable of swimming and swarming motility (Poritsanos et al. 2006). Over the course of 72 h, PA23-63 (*phzE)*, PA23-8 (*prnBC*), and PA23-63-1 (*phzE;prnBC*) were able to swim at the same rate as the wild type (Table 3.3), indicating production of these two antibiotics does not affect motility *in vitro*.

3.2.6 The effect of PHZ and PRN production on PA23 biofilm formation.

We employed a highly reproducible 96-well plate assay to assess the ability of PA23, PA23-63, PA23-8, and PA23-63-1 to form biofilms. As illustrated in Figure 3.3A, there was a statistically significant decrease in biofilm production observed for the PHZ- strains, compared to the wild type and mutant PA23-8. Conversely, PRN was shown to have no impact on biofilm formation (Fig. 3.3A). Because CV stains biofilm matrix components as well as bacterial cells, we performed direct enumeration of the adherent cell population. The results of these analyses revealed the same outcome; PHZ but not PRN production leads to increased attached biomass (Fig. 3.3B).

3.2.7 The contribution of PHZ and PRN to PA23 biocontrol of *S. sclerotiorum* in the greenhouse.

The wild-type PA23, PA23-63 (*phzE)*, PA23-8 (*prnBC*), and PA23-63-1 (*phzE;prnBC*) were evaluated for their ability to protect canola from stem rot disease caused by *S. sclerotiorum*. Two parameters were evaluated; 1) incidence of leaf infection and 2) stem rot disease severity.

Table 3.3 Flagellar motility analysis of *Pseudomonas chlororaphis* PA23, and mutants PA23-1 (PRN^-), PA23-63 (PHZ^-) and PA23-63-1 (PRN^-, PHZ^-).

	Swim Zone Diameter (mm)		
Strain	24 h*	48 h*	72 h*
PA23	24.4 (2.3)	35.2 (2.5)	72.1 (2.6)
PA23-8 (PRN^-)	22.5 (2.7)	36.8 (1.8)	70.0 (2.8)
PA23-63 (PHZ^-)	23.8 (2.0)	35.3 (2.6)	70.7 (2.4)
PA23-63-1 (PHZ^-, PRN^-)	23.4 (2.3)	36.8 (2.6)	70.3 (1.8)

* Mean (standard deviation) of swim zones from four replicates.

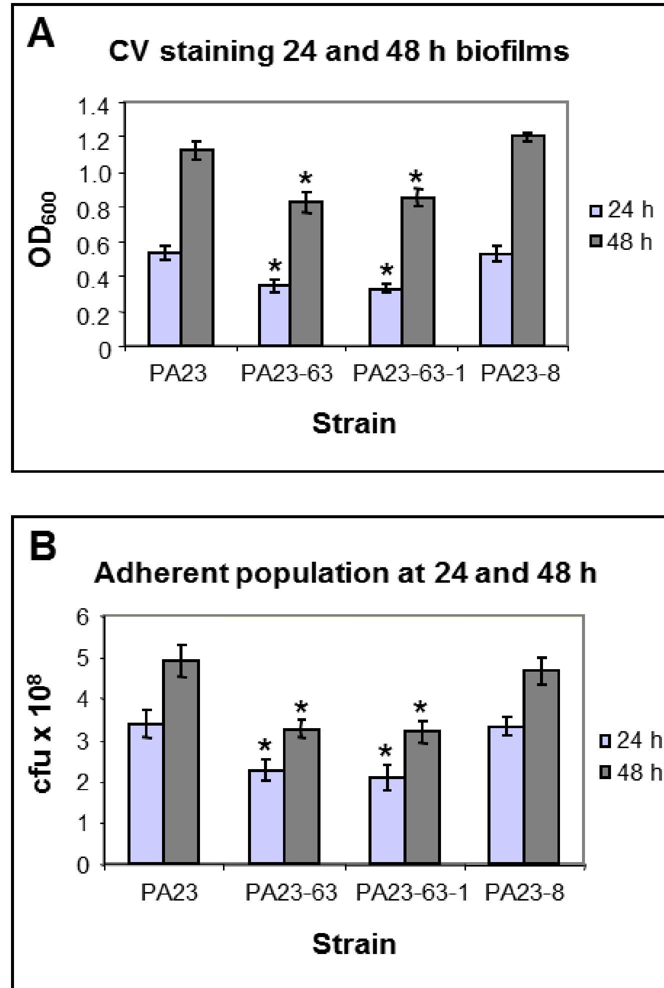

Figure 3.3 Biofilm formation by *Pseudomonas chlororaphis* PA23 (wild type), PA23-63 (PHZ^-), PA23-8 (PRN^-) and PA23-63-1 (PRN^- PHZ^-). Panel A: cultures were grown in 96-well microtitre plates containing M9CA (0.2% glucose) for 24 and 48 h at 28°C. Biofilm formation, indicated by crystal violet (CV) staining, was measured at an absorbance of 600 nm. Panel B: cultures were grown in M9CA (0.2% glucose) in 6-well tissue culture plates. After 24 and 48 h, the adherent cell population was enumerated by viable plate counting. Column means labeled with (*) are significantly different from the wild type ($p < 0.01$). Error bars indicate SD.

The PHZ-deficient strain PA23-63 was equivalent to the wild type in its ability to control fungal infection of stems and leaves and reduce disease severity (Fig.3.4). For strain PA23-8, which produces the diffusible antibiotic PHZ but no PRN, some biocontrol was observed but it was significantly decreased from that of the wild type (Fig. 3.4). Compared to the disease control, no difference in the incidence of leaf infection (Fig. 3.4A) and only a modest decline in disease severity were observed for the PRN-/PHZ- double mutant PA23-63-1 (Fig 3.4B). Collectively, these findings indicate that PRN is the primary antibiotic underlying PA23 biocontrol of *S. sclerotiorum* infection in canola. Although PHZ can inhibit the fungus to some degree, it plays a more minor role in disease suppression.

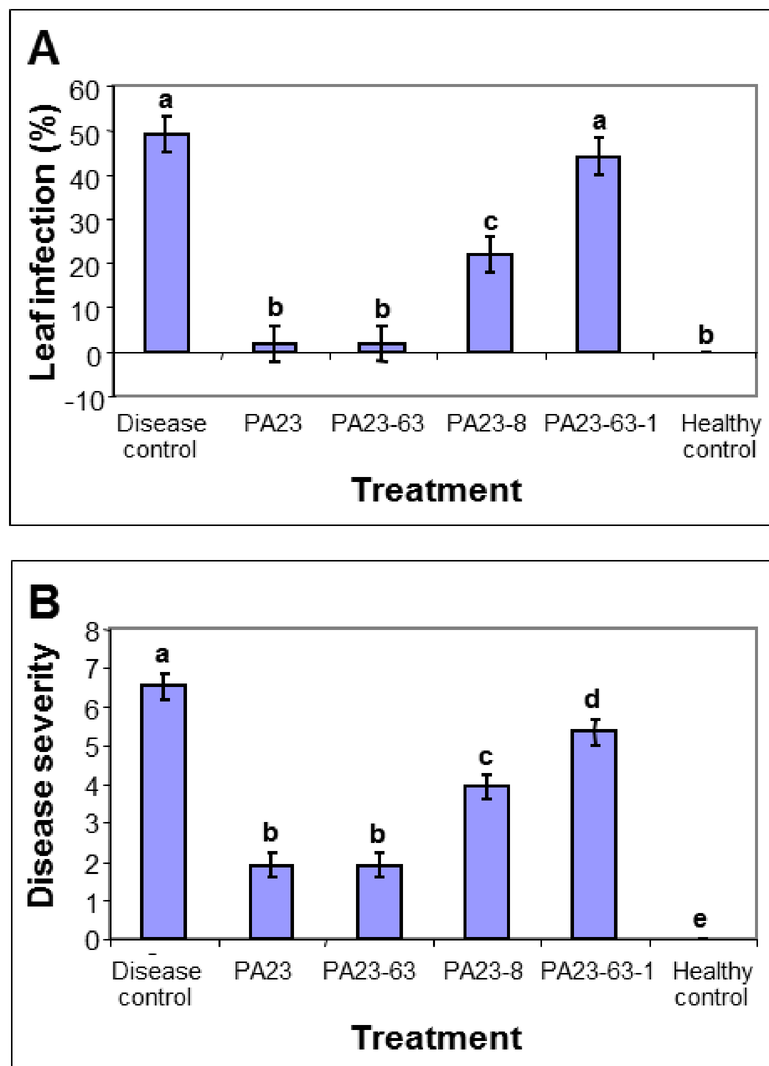

Figure 3.4. Efficiency of *Pseudomonas chlororaphis* PA23 (wild type), PA23-63 (PHZ^-) PA23-8 (PRN^-) and PA23-63-1 (PRN^- PHZ^-) in managing *Sclerotinia sclerotiorum* ascospore infection on canola plants. Panel A: percent incidence of leaf infection. Panel B: disease severity on stem. In all treatments, except the healthy control, plants were sprayed with *S. sclerotiorum* ascospores. The healthy control plants were sprayed with phosphate buffer. Column means labeled with the same letter do not differ significantly by Duncan's Multiple Range Test (DMRT; P>0.05). Error bars indicate SD.

3.3 Discussion

Sclerotinia sclerotiorum is the causative agent of *Sclerotinia* stem rot, one of the most important diseases of canola in Western Canada (Martens et al. 1994). Much of the research on *S. sclerotiorum* biocontrol is focused on hyperparasitism by fungal antagonists that degrade sclerotia in the soil (Gerlagh et al. 1999; McLaren et al. 1996). This approach is limited, however, by the fact that reducing the sclerotial soil load does not eliminate the risk of crop infection. Therefore, our group has focused on biocontrol of *S. sclerotiorum* through application of the antagonist directly at the site of pathogen entry, the canola petal. In greenhouse and field trials, *P. chlororaphis* strain PA23 has demonstrated excellent biocontrol of *S. sclerotiorum* (Fernando et al. 2007; Savchuk and Fernando, 2004; Zhang, 2004). This bacterium produces metabolites that are believed to inhibit mycelial growth (Poritsanos et al. 2006) and it also stimulates production of plant defense enzymes (Fernando et al. 2007); therefore fungal antagonism is multifactorial.

Pseudomonads that function as biocontrol agents frequently produce more than one antibiotic (Haas and Keel, 2003). *P. chlororaphis* PA23 produces the PHZ derivatives PCA and 2-OH-PHZ, together with PRN (Zhang et al. 2006). PHZ production plays an essential role in the disease suppressive ability of many biocontrol agents. For example *phzB* and *phzH* mutants of *P. chlororaphis* strain 1391, deficient in PCN, were unable to control *Fusarium oxysporum f. sp. radicis-lycopersici* tomato foot and root rot (Chin-A-Woeng et al. 1998). *P. fluorescens* 2-79 mutants no longer producing PCA exhibited dramatically decreased suppression of take-all disease of wheat (Thomashow and Weller, 1988). Similarly a PHZ-deficient mutant of *P. aeruginosa,* PNA1, exhibited reduced ability to suppress *Fusarum* wilt of chickpea or *Pythium*

damping off of bean compared to the wild type (Anjaiah et al. 1998). In light of these findings, we were surprised to discover an increased zone of inhibition surrounding the PHZ-deficient mutant PA23-63 (Fig. 3.1). The AF activity observed *in vitro* was supported by our greenhouse analysis, where PA23-63 was able to suppress stem rot of canola as well as the wild type (Fig. 3.4). We postulated that a second metabolite, most likely PRN, was upregulated in the *phz*-minus background accounting for the increased AF activity *in vitro*. Balanced metabolite production has been observed for *P. fluorescens* CHA0, which produces the antibiotics 2,4-DAPG and PLT (Baehler et al. 2005; Schnider-Keel et al. 2000). Regulation of these two antibiotics occurs at the transcriptional level; 2,4-DAPG and PLT activate their own biosynthesis while at the same time repressing synthesis of the other (Baehler et al. 2005; Schnider-Keel et al. 2000). Strain CHA0 also produces PRN but expression of this antibiotic had no impact on the 2,4-DAPG:PLT balance (Baehler et al. 2005). When we analyzed culture supernatants of the PHZ-deficient mutant PA23-63, we observed a two-fold increase in PRN production (Table 3.3). This finding led us to explore whether PHZ is acting as a transcriptional repressor of the *prn* biosynthetic operon. Analysis of a *prnA-lacZ* TS fusion revealed no difference in expression kinetics between the *phzE* mutant and the wild type (Fig. 2A). Therefore, a mechanism other than TS repression must account for the increased PRN production. As depicted in Figure 3.5, PHZ and PRN are both produced via the Shikimic acid pathway. Because PhzE is responsible for

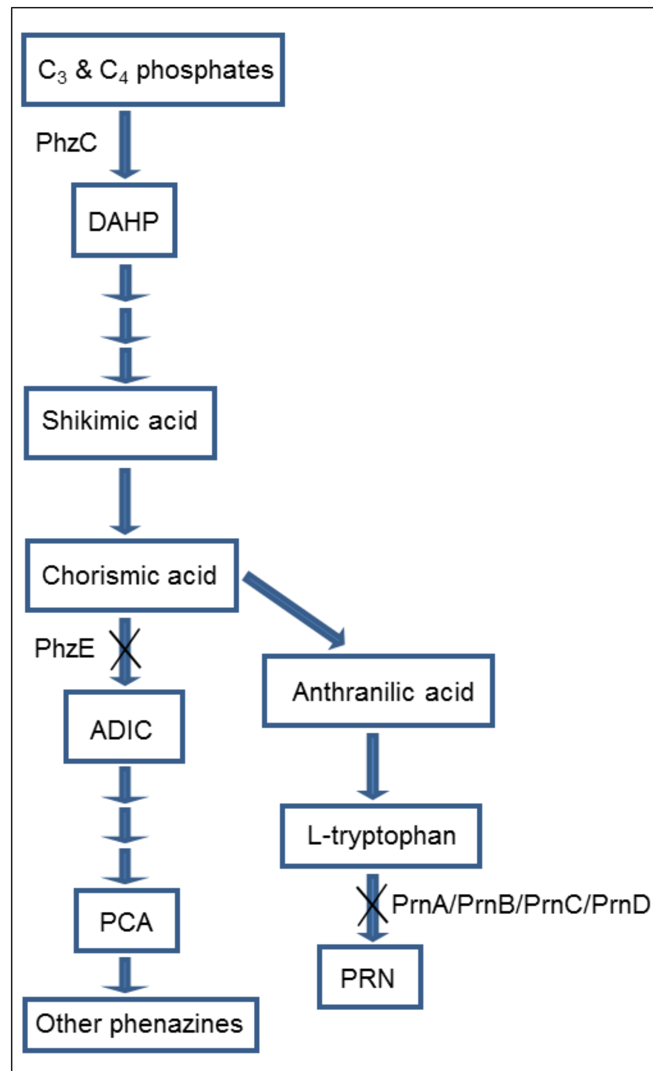

Figure 3.5. Biosynthesis of PRN and PHZ from shikimic acid. ADIC, 2-amino-4 deoxychorismic acid; PCA, phenazine-1-carboxylic acid; PRN, pyrrolnitrin. Strain PA23-63 has a Tn insertion in *phzE*. Strains PA23-8 and PA23-63-1 have a portion of *prnBC* replaced with a Gm^R cassette.

converting chorismic acid into 2-amino-2-deoxy-isochorismic acid (ADIC) (Mavrodi et al. 1998; McDonald et al. 2001; Pierson and Thomashow, 1992), a mutation in *phzE* should increase the level of chorismic acid available for PRN biosynthesis.

As a result, enhanced PRN production by PA23-63 is most likely due to increased substrate availability. Production of PHZ, however, was not increased in the PRN-minus background. This is not surprising in light of the fact that PrnB functions at a later point in the pathway and so build-up of the substrate chorismate would not be expected (Fig. 3.5).

Through our expression analysis we discovered that PRN is not subject to autoregulation as there was no change in *prn* transcription in the PRN-deficient mutants (Fig. 3.2A). Examination of the PA23 *prn* upstream region revealed the presence of genes encoding a putative efflux pump; however no regulatory genes were detected. These findings are consistent with others who have reported an absence of pathway-specific regulators for the *prn* operons of *P. fluorescens* strains BL915 and CHA0 (Baehler et al. 2005; Hammer et al. 1997; Kirner et al. 1998; van Pée and Ligon, 2000). In terms of the *phz* operon, a deficiency in PRN and/or PHZ production had no impact on TS, suggesting these genes are neither autoregulated nor controlled by PRN. In *P. chlororaphis* strains 30-84 and PCL1391, PHZ gene expression is under QS control (Chin-A-Woeng, et al. 2001; Pierson et al. 1994). We expect the same will hold true for PA23 because this bacterium produces AI signal molecules (Table 3.2), and two genes with high homology to the *phzI* and *phzR* QS genes are located immediately upstream of the PA23 PHZ operon (Selin et al. 2012).

It was previously demonstrated that strain PA23 is capable of forming a biofilm and cells within this adherent community are significantly more resistant to antibiotics (Poritsanos et al. 2006). When growing as a biofilm, the increased antimicrobial resistance as well as protection from environmental stresses, such as desiccation and UV radiation likely facilitates PA23 survival in the environment. In the current study, the role of PHZ and PRN in PA23 biofilm formation was investigated. A static, microtitre biofilm assay was employed to mimic the phyllosphere / rhizosphere, which can be best characterized as a no-flow or low-flow environment, depending on the prevailing conditions. We discovered that PHZ production enhances biofilm formation in PA23 (Fig. 3.3). A role for PHZ in biofilm development has been reported previously by Maddula and coworkers (2008). Not only did PHZ production enhance *P*. (*aureofaciens*) *chlororaphis* 30-84 biofilm production by similar amounts to what is reported here, but altering the PCA:2-OH-PCA ratio affected initial attachment, dispersal and the mature biofilm structure (Maddula et al. 2008). Our discovery that PRN does not affect biofilm development (Fig. 3.3) suggests that this trait is not linked to antibiotic production in general; rather it may be unique to PHZ. Recently, it has been proposed that antibiotics have multiple, concentration-dependent roles (see Davies et al. 2006; Fajardo and Martínez, 2008 for reviews). At lower concentrations, antibiotics act as signaling molecules capable of modulating gene expression, whereas at higher concentrations they function as inhibitors. Consistent with this, Dietrich and coworkers (2006) demonstrated that the PHZ derivative pyocyanin can induce expression of a set of *P. aeruginosa* genes called the “PYO stimulon”, indicating pyocyanin can function as both an antibiotic and a QS signal. In a number of bacterial species, a connection between QS and biofilms has

been made (Irie and Parsek, 2008). It is possible that PHZ is functioning as a signaling molecule and in this manner affects *P. chlororaphis* biofilm development.

In summary, our findings indicate that PRN is the primary antibiotic responsible for PA23 biocontrol of *S. sclerotiorum*. Our next step is to elucidate factors influencing PRN production with the end goal of maximizing disease suppression in the field. Others have shown that PHZ-producing biocontrol strains are better able to colonize roots and persist in the wheat rhizosphere compared to PHZ-minus mutants (Mazzola et al. 1992). As such, PHZ appears to play a role in the ecological competence of these bacteria. Consistent with this notion, PA23-63 (PHZ-minus) which produces 2-fold more PRN than PA23 exhibited increased AF activity on plates but only wild-type levels of disease suppression in the greenhouse. One potential explanation for these findings is that PHZ production is expendable for PA23 biocontrol but it contributes to the overall fitness of this bacterium. The PHZ-mediated increase in biofilm development may be one means by which these antibiotics facilitate PA23 establishment in the environment.

3.4 Aknowledgments

I would like to thank Rhahim Habibian for his assistance in generating PA23-8 and PA23-63-1. I would also like to thank Dr. Dieter Haas for providing the plasmid pME3219.

Chapter 4

The PhzI/PhzR Quorum-Sensing System is Required for Pyrrolnitrin and Phenazine Production and Exhibits Cross Regulation with RpoS in *Pseudomonas chlororaphis* PA23.

4.1 Introduction

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

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

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

Closer inspection of the network overseeing secondary metabolite production has revealed that the regulators themselves may be subject to cross regulation. In *P. aeruginosa*, for example, RpoS and QS exert modest effects on each other. Quorum sensing induces *rpoS* transcription two-fold; whereas, RpoS has both a positive (*lasR* and *rhlR*) and negative effect (*rhlI*) on QS genes (Schuster et al. 2004). In *P. chlororaphis* PCL1391, RpoS levels remained unchanged in a QS mutant; however AHL production was found to be positively regulated by this sigma factor (Girard et al. 2006). As a final

example, two of the three *P. putida* QS genes, namely *ppuI* and *rsaL*, are repressed by RpoS. Thus, the interconnectivity between QS and RpoS is readily apparent; in terms of the metabolites they control and their cross regulation. However the nature of this regulation is variable, observed as positive, negative or non-existent. So for each bacterial strain in question, details regarding how these systems function must be uncovered anew.

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

4.2 Results

4.2.1 Generation and phenotypic characterization of PA23 QS-deficient strains.

For bacteria that harbor a copy of the *phz* biosynthetic operon, the *phzI/phzR* QS locus is typically located just upstream. Sequencing of a plasmid containing part of the PA23 *phz* operon (*phzABCDE*) revealed the presence of *phzI* and *phzR* homologs upstream of *phzA*. This QS locus showed the highest degree of identity with *phzI* and *phzR* of *P. chlororaphis* strains O6 (99%; accession #AY927995.1), 30-84 (94%; accession #L33724 & EF62944.1), and PCL1391 (94%; accession #AF19615). The genes are organized with *phzR* immediately upstream of and divergently transcribed from *phzA*. Similarly, *phzI* is upstream of *phzR* but oriented in the opposite direction (Fig. 4.1A).

To investigate the impact of QS on PA23 secondary metabolite production, a *phzR* mutant was created through allelic exchange. Replacement of the wild-type copy of *phzR* with the mutated allele was confirmed through PCR (Fig. 4.2). A quorum-quenching approach was taken to generate an AHL-deficient derivative of PA23. The *aiiA* AHL lactonase gene on plasmid pME6863 was mobilized into PA23, creating PA23-6863.

When PA23*phzR* and PA23-6863 were tested for their ability to inhibit *S. sclerotiorum in vitro*, both strains exhibited a complete loss of fungal antagonism (Table 4.1). Mobilization of pUCP23-*phzR* into the *phzR* mutant restored AF activity to near wild-type levels (Table 4.1). We have previously shown that PHZ production imparts an orange phenotype to PA23; whereas PRN is the primary antibiotic responsible for *S. sclerotiorum* antagonism (Selin et al. 2010). The reduced pigmentation and lack of

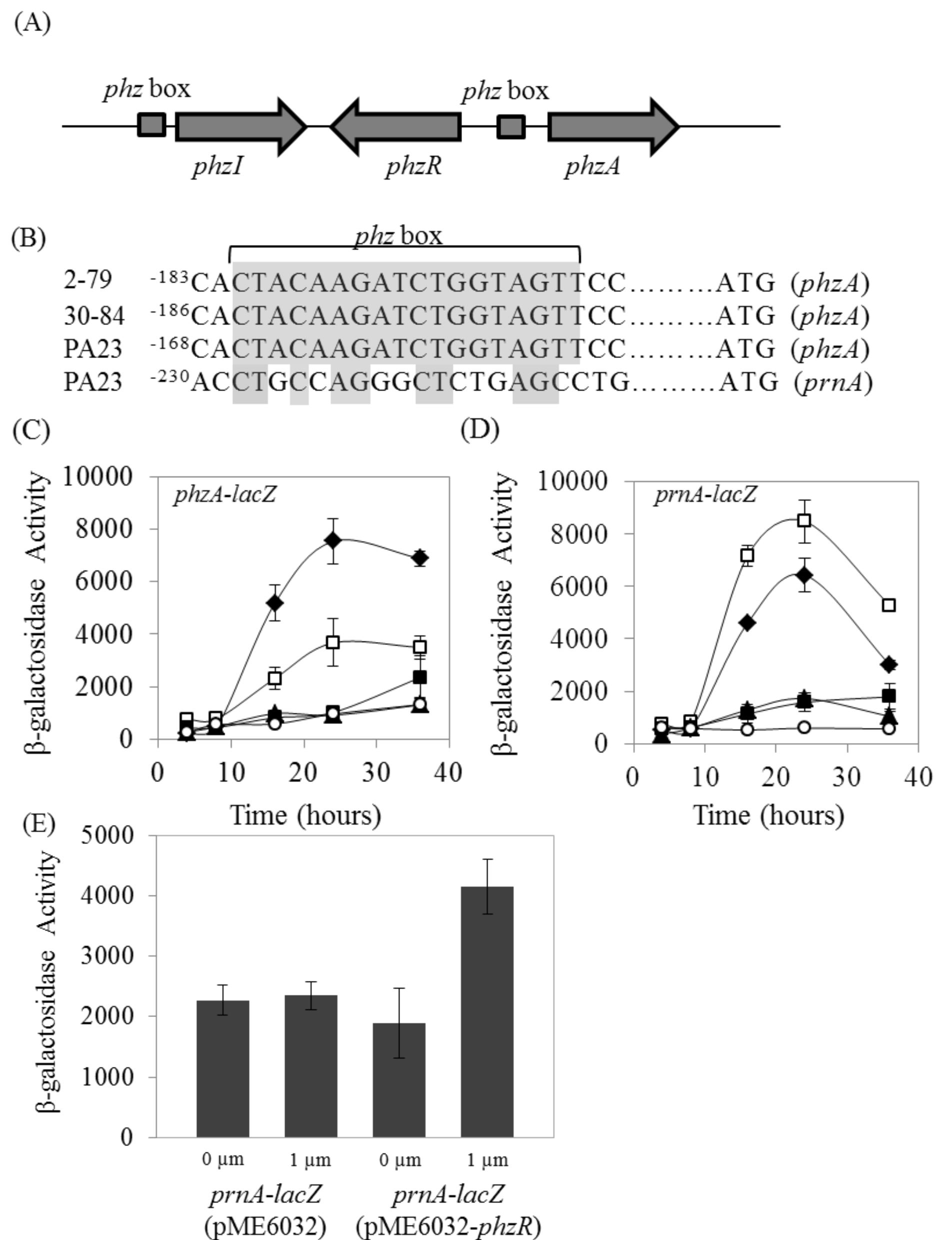

Figure 4.1 Chromosomal localization of the *phzI* and *phzR* QS genes and analysis of their impact on *phzA* and *prnA* expression in PA23. (A) The *phzI* and *phzR* genes are located upstream of the PHZ operon. The first gene of the operon, *phzA*, is illustrated along with the location of the *phz* boxes. (B) Identification of the *phz* box sequences in the *phzA* and *prnA* promoter regions of *P. chlororaphis* PA23. Alignments of the *phz* box sequences found in *P. fluorescens* 2-79, *P. chlororaphis,* 30-84, and *P. chlororaphis* PA23 are illustrated. The sequences and relative positions of the *phz* boxes and the ATG start codons of *phzA* and *prnA* are indicated. The nucleotides within the *phz* box shared among the sequences are shaded in grey. Nucleotide sequences of the *phzA* promoter regions of *P. fluorescens* 2-79 and *P. chlororaphis* 30-84 were obtained from Genbank as accession numbers L48616 and AF007801, respectively. The impact of QS on *phzA* (C) and *prnA* (D) expression in PA23. Bacterial strains are as follows: *P. chlororaphis* PA23 (closed diamond), PA23*phzR* (closed triangle), PA23*phzRrpoS* (closed square), PA23-6863 (open circle) and PA23*rpoS* (open square). Bacteria were grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose. β-galactosidase activity is expressed as Miller units. (E) The impact of QS on the expression of *prnA*-lacZ in *E.coli*. The *prnA*-lacZ fusion was co-expressed in *E .coli* with *phzR*-pME6032 and pME6032 (empty vector) with and without C6-HSL (1µm). Bacteria were grown in M9 minimal media supplemented with 1mM $MgSO_4$, 0.2% glucose and 0.4% casamino acids for 24 hours. β-galactosidase activity is expressed as Miller units.

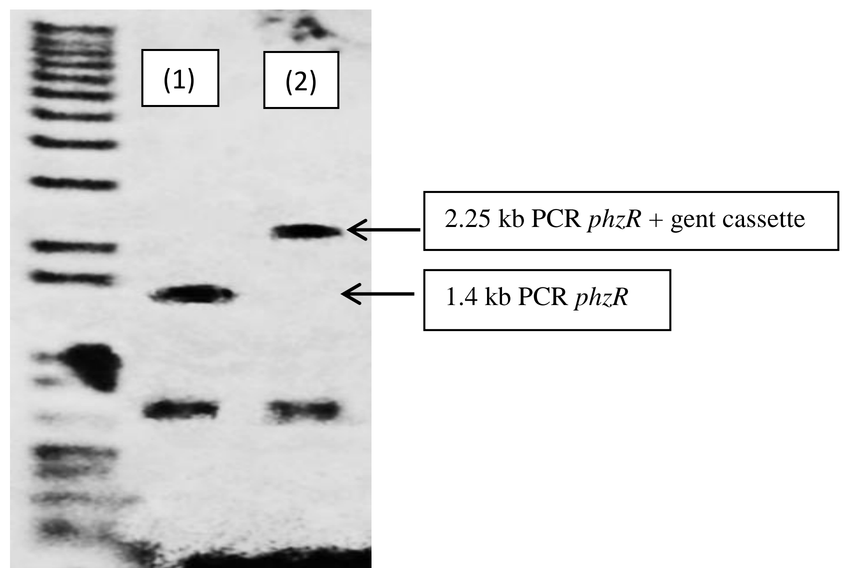

Figure 4.2. PCR verification of the PA23 *phzR* mutant. An 850-bp gentamicin cassette was used to disrupt the *phzR* gene. PCR was used to detect an increase in band size from 1.4-kb (1-wild type) to 2.25 kb (2-PA23*phzR*) which indicates that allelic exchange had taken place.

Table 4.1 Phenotypic characteristics of PA23 and its derivatives.

Strain	Extracellular Metabolite Activity			
	Antifungal (mm)*	Protease (Units of enzyme/ml)¶	Total PHZ ¶ μg/ml	PRN§¶ μg
PA23 (pUCP22)	8.6 (0.7)	0.56 (0.04)	79.4 (0.5)	16.9 (1.5)
PA23-6863	0 ø	0.09 (0.01) ø	11.5 (0.9) ø	0 ø
PA23*phzR* (pUCP22)	0 ø	0.30 (0.03) ø	14.1 (0.3) ø	0 ø
PA23*phzR* (pUCP23-*phzR*)	7.0 (0.9) ‡	0.40 (0.01) †	68.5 (1.8) †	14.8 (1.4) °
PA23*phzRrpoS* (pUCP22)	0 ø	1.15 (0.04) ø	17.7 (0.4) ø	0 ø
PA23*phzRrpoS* (pUCP22-*rpoS*)	0 ø	0.36 (0.01) †	16.0 (1.2) ø	0 ø
PA23*phzRrpoS* (pUCP23-*phzR*)	6.7 (1.1) †	0.87 (0.02) ø	63.9 (0.5) ø	14.5 (2.3) °

* Mean (standard deviation) obtained from six replicates.
¶ Mean (standard deviation) obtained from a triplicate set.
§ Mean (standard deviation) of PRN extracted from 100-ml culture volume.
† Significantly different from wild type (p <0.01).
‡ Significantly different from wild type (p < 0.05).
ø Significantly different from wild type (p < 0.001).
° Not significantly different from wild type.

AF activity exhibited by the QS mutants suggested that decreased levels of PHZ and PRN were being produced. Quantitative analysis revealed that PHZ levels were down 5- and 7-fold in PA23*phzR* and PA23-6863, respectively. This was not unexpected as the *phz* biosynthetic operon is immediately downstream of *phzI* and *phzR* and PHZs are QS regulated in other bacterial strains (Mavrodi et al. 2006). QS control of PRN (Table 4.1), on the other hand, has not been reported for other *Pseudomonas* spp. Because PA23 produces a number of other secondary metabolites that may contribute to fungal antagonism, including HCN, protease and lipase we investigated whether these compounds were QS regulated. HCN and lipase production were not affected by the lack of PhzR or AHL (data not shown). However, protease activity was reduced 2-fold and 6-fold in PA23*phzR* and PA23-6863 respectively (Table 4.1). Mobilization of the *phzR* gene into PA23*phzR* increased protease production but, for reasons unknown, only partial complementation was achieved (Table 4.1).

We also examined whether biofilm development and motility were altered in the QS-deficient strains. As shown in Figure 4.3, biofilm formation was reduced over 5 fold in PA23*phzR* and PA23-6863. The presence pUCP23-*phzR* in PA23*phzR* increased the adherent biomass close to that of PA23. Taken together, these findings indicate that a functional PHZ QS system facilitates establishment of PA23 biofilms. Flagellar motility is important for not only biocontrol (Haas and Défago, 2005), but also the early stages of biofilm formation (Davey and O'Toole, 2000). Therefore, we examined whether there were any differences in swimming motility between PA23 and the PhzR- and AHL-deficient strains. We discovered that PA23*phzR* was as motile as PA23; PA23-6863 on the other hand exhibited decreased motility at both 24 and 48 h (Table 4.2). As both QS-

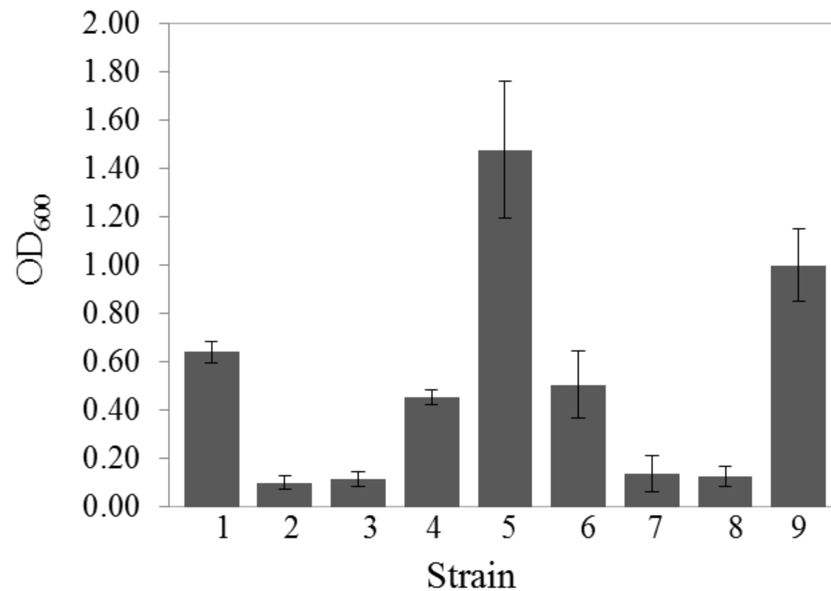

Figure 4.3 Biofilm formation in PA23 and its derivatives. Bacterial strains are as follows: 1, PA23 (pUCP22); 2, PA23-6863 (pUCP22); 3, PA23*phzR* (pUCP22); 4, PA23*phzR* (pUCP23-*phzR*); 5, PA23*rpoS* (pUCP22); 6, PA23*rpoS* (pUCP22-*rpoS*); 7, PA23*rpoSphzR* (pUCP22); 8, PA23*phzRrpoS* (pUCP22-*rpoS*); 9, PA23*rpoSphzR* (pUCP23-*phzR*). Cultures were grown in 96-well microtiter plates containing M9 media (1 mM $MgSO_4$ and 0.2% glucose) for 24 hours at 28ºC. Biofilm formation, indicated by crystal violet staining, was measured at an absorbance of 600 nm.

TABLE 4.2. Flagellar motility of *Pseudomonas chlororaphis* PA23 and derivative strains.

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

* Mean (standard deviation) from triplicates.
† Significantly different from wild type (p <0.01).
‡ Significantly different from wild type (p < 0.05).
ø Significantly different from wild type (p < 0.001).
° Not significantly different from wild type.

deficient strains exhibited diminished biofilm formation, but only PA23-6863 showed altered motility, other factors must be responsible for the impaired biofilm development.

4.2.2 QS regulates *phzA* and *prnA* expression.

Expression of *phzA*- and *prnA*-*lacZ* transcriptional fusions were analyzed in PA23 and the QS-deficient strains. The transcription of both genes was markedly reduced in PA23*phzR* and PA23-6863 (Fig 4.1 C & D), indicating that QS positively regulates *phzA* and *prnA* expression. Next, we searched for the presence of a phz-box element upstream of the aforementioned genes. A sequence was identified upstream of *phzA* (168 bp from the ATG start) that was a 100% match (18/18 nt) with the phz-box found in other *P. chlororaphis* strains (Fig. 4.1B). Similarly, we found an 18-bp region (54 bp upstream of the *phzI* ATG start) that is 100% identical to the phz-box consensus upstream of *phzI* (Chin-A-Woeng et al. 2001; data not shown). Inspection of the *prnA* promoter region revealed a sequence that has 9/18 nt in common with the phz box consensus of *phzA* (Fig. 4.1B). To confirm that QS has a direct effect on *prn* expression, the activity of a *prnA-lacZ* transcriptional fusion was monitored in *E. coli* in the presence and absence of pME6032-*phzR*. This plasmid carries *phzR* under control of the *tac* promoter. As shown in figure 4.1E, no difference in *prnA-lacZ* activity was observed between cells carrying pME6032-*phzR* versus those carrying the empty vector. However in the presence of PhzR and 1 μM C_6-HSL, *prnA*-*lacZ* activity increased two fold (Fig. 4.1E), indicating that QS directly controls *prnA* transcription.

4.2.3 The Phz QS system is subject to positive autoregulation.

Quorum-sensing systems are typically arranged as an autoinduction circuit with both the I- and R-genes subject to positive autoregulation. To determine if the same holds true for PA23, the activity of *phzI-lacZ* and *phzR-lacZ* transcriptional fusions was analyzed in PA23, PA23*phzR* and PA23-6863. Expression of *phzI* in both PA23*phzR* and PA23-6863 remained at low levels throughout growth, unlike PA23 in which *phzI* expression peaked maximally at 24 hours (Fig. 4.4A). The same trend was observed for *phzR* transcription (Fig. 4.4B). Thus, it appears that the expression of the Phz QS genes requires both the AHL signaling molecule and PhzR. Next, we analyzed the amount of AHL present in culture extracts using *P. aeruginosa* QSC105 (pEAL01), a strain capable of detecting a broad range of AHLs (Ling et al. 2009). Levels of AHL were reduced 2 fold and 8 fold in the *phzR* mutant and PA23-6863, respectively. When strain PA23*phzR* was complemented with *phzR* in trans, AHL levels exceeded those of PA23 (Fig. 4.5).

4.2.4 QS positively regulates *rpoS* expression.

To determine if QS has an impact on *rpoS* expression, an *rpoS-lacZ* fusion was monitored in PA23, PA23*phzR*, and PA23-6863. At 24 h, a 1.5-fold and a 3.5-fold decrease in *rpoS* transcription was demonstrated by PA23*phzR* and PA23-6863, respectively (Fig. 4.6A). We also examined the total amount of RpoS protein present through Western blot analysis. Protein levels were significantly reduced in PA23*phzR* compared to the wild type, while PA23-6863 did not produce any detectable RpoS (Fig. 4.6B). Taken together, both the transcriptional fusion data and Western blot analysis indicate that RpoS is positively regulated by QS.

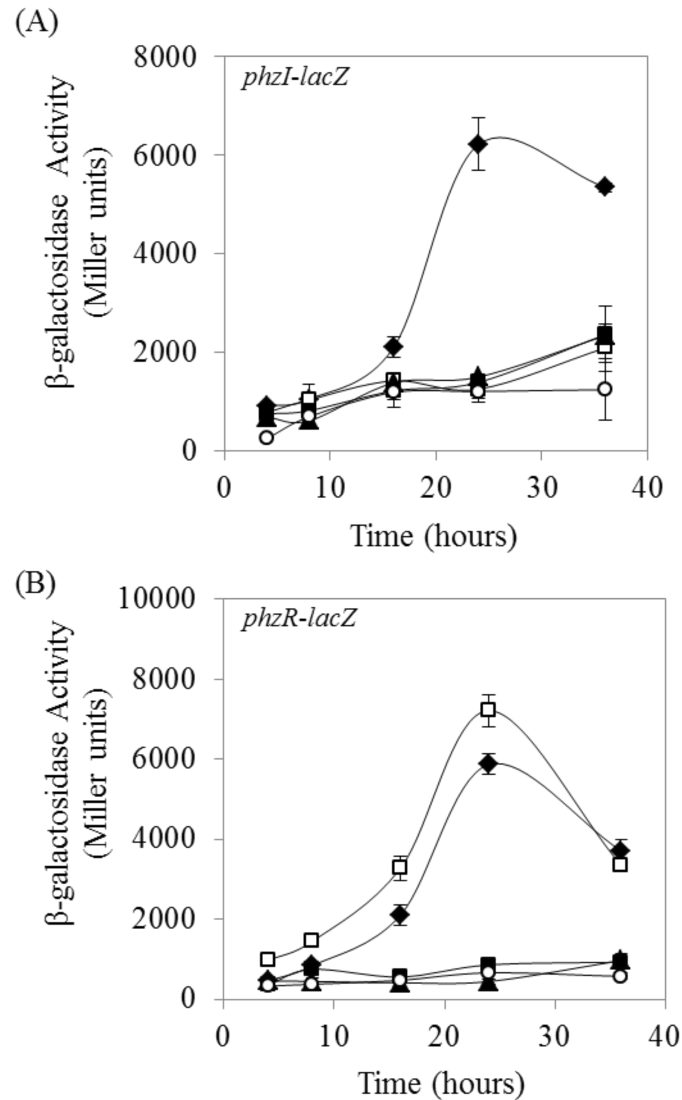

Figure 4.4. The effect of QS and RpoS on *phzI* (A) and *phzR* (B) transcription. Bacterial strains are as follows: *P. chlororaphis* PA23 (closed diamond), PA23*phzR* (closed triangle), PA23-6863 (open circle), PA23*phzRrpoS* (closed square) and PA23*rpoS* (open square). Bacteria were grown in M9 minimal media supplemented with 1mM $MgSO_4$ and 0.2% glucose.

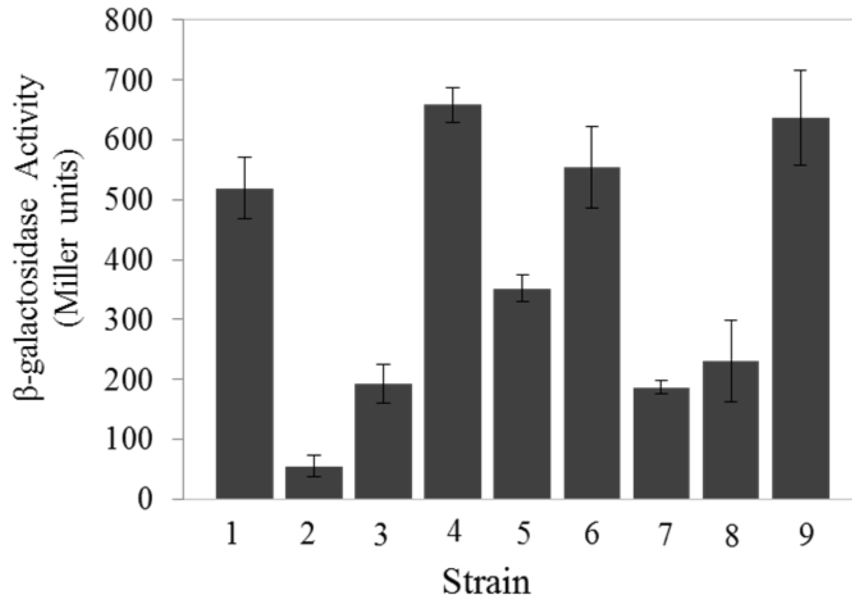

Figure 4.5 β-galactosidase activity of *lasB-lacZ* in *Pseudomonas aeruginosa* spiked with autoinducer extracts from PA23 and derivative strains. Extracts were analyzed from the following bacteria: 1, PA23 (pUCP22); 2, PA23-6863 (pUCP22); 3, PA23*phzR* (pUCP22); 4, PA23*phzR* (pUCP23-*phzR*); 5, PA23*rpoS* (pUCP22); 6, PA23*rpoS* (pUCP22-*rpoS*); 7, PA23*rpoSphzR* (pUCP22); 8, PA23*phzRrpoS* (pUCP22-*rpoS*) and 9, PA23*rpoSphzR* (pUCP23-*phzR*). Cell-free supernatants of bacterial cultures grown in M9 minimal media (1mM $MgSO_4$, 0.2% glucose) were extracted using ethyl acetate. The concentrated AHL preparations were added to cultures of *P. aeruginosa* QSC105 (pEAL01) which were grown for 18 hours prior to β-galactosidase analysis. Assays were carried out three times in triplicate and a representative data set is shown.

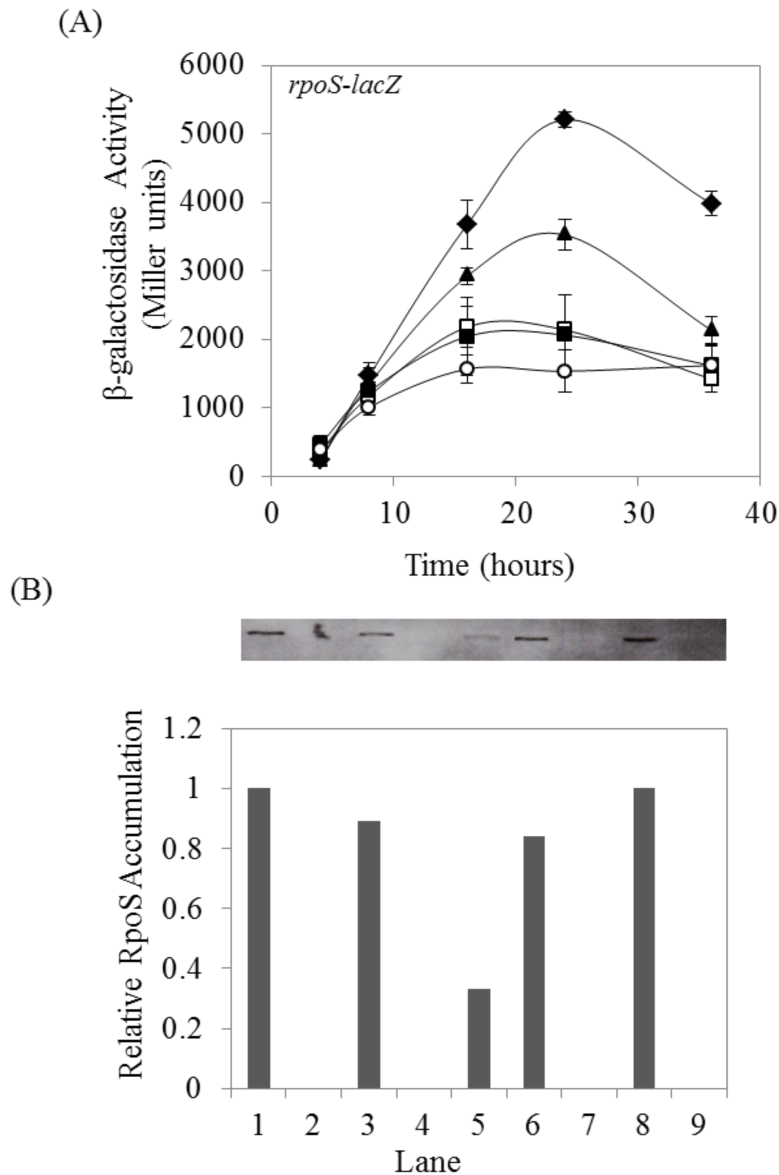

Figure 4.6. Analysis of *rpoS-lacZ* activity and relative accumulation of RpoS protein in *Pseudomonas chlororaphis* PA23 and its derivatives. (a) Strains used are as follows: PA23 (closed diamond), PA23*phzR* (closed triangle), PA23-6863 (open circle), PA23*phzRrpoS* (closed square), and PA23*rpoS* (open square). All strains harbored the *rpoS-lacZ* reporter plasmid pRPOS-*lacZ*. (b) RpoS-specific antiserum was used to visualize RpoS from Western blots of protein extracted from cultures grown to stationary phase. Each lane contains 100 μg of protein. Lanes are as follows: 1, PA23 (pUCP22); 2, PA23*rpoS* (pUCP22); 3, PA23*rpoS* (pUCP22-*rpoS*); 4, PA23-6863; 5, PA23*phzR* (pUCP22); 6, PA23*phzR* (pUCP23-*phzR*); 7, PA23*phzRrpoS* (pUCP22); 8, PA23*phzRrpoS* (pUCP22-*rpoS*); 9 PA23*phzRrpoS* (pUCP23-*phzR*). (c) 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.

4.2.5 RpoS regulates *phzI* and *phzR*.

Since QS positively regulates *rpoS* expression, we were interested to see if there was reciprocal cross-regulation of QS by RpoS. Therefore, expression of *phzI-lacZ* and *phzR-lacZ* were measured in PA23 and PA23*rpoS*. As shown in Figure 4.4, *phzI* transcription was reduced over 3 fold at 24 hours of growth in PA23*rpoS* (Fig. 4.4A). The opposite was observed for *phzR*, where transcription levels were elevated in the *rpoS* single mutant (Fig. 4.4B). Collectively these results suggest that under minimal conditions, transcription of *phzI* but not *phzR* is dependent on RpoS. Quantitative analysis revealed reduced levels of AHL in PA23*rpoS* culture extracts (Fig. 4.5), which is consistent with the *phzI* transcriptional fusion data.

4.2.6 RpoS controls PA23 antifungal activity indirectly through the Phz QS system.

Next we addressed whether constitutively expressed *phzR* and/or *rpoS* would be able to complement PA23*phzRrpoS*. When pUCP23-*phzR* was mobilized into the *phzRrpoS* double mutant, AF activity and antibiotic production were restored to wild-type levels (Table 4.1). Conversely, PA23*phzRrpoS* harboring pUCP22-*rpoS* was virtually indistinguishable from PA23*phzR* (Table 4.1). Regulation of protease production was found to be somewhat unique. In an earlier study, we discovered that RpoS has a repressive effect on protease production (Manuel et al. 2012). Thus, it was not surprising that the double mutant harboring pUCP22-*rpoS* produced less protease than the mutant carrying empty vector (Table 4.1). As protease production is positively regulated by QS, we expected to see an increase in protease levels in the *rpoSphzR* double mutant harboring pUCP23-*phzR*. As illustrated in Table 4.1, this was the case.

Next we examined the ability of PA23*phzRrpoS* and PA23*rpoS* to form biofilms and translocate via flagellar motility. Biofilm analysis revealed that PA23*phzRrpoS* closely resembles the QS-deficient strains, producing significantly less adherent biomass (Fig.4.3). Constitutively expressed *phzR* restored PA23*phzRrpoS* biofilm formation to wild-type levels. Compared to PA23 and the QS-deficient strains, the *rpoS* single mutant showed increased biofilm formation. Addition of pUCP22-*rpoS* had no effect on PA23*phzRrpoS*; however for PA23*rpoS,* it reduced biofilm formation (Fig. 4.3). When the aforementioned strains were assessed for flagellar motility, the double mutant showed increased swim zones, much like the *rpoS* single mutant (Table 4.3).

Lastly, we examined the activity of the *phzI*-, *phzR*-, *phzA*-, and *prnA*-*lacZ* transcriptional fusions in PA23*phzRrpoS*. No differences in expression were observed between PA23*phzRrpoS* and PA23*phzR* (Fig. 4.4A & 4.4B; Fig. 4.2C &4.2D).

4.3 Discussion

The aim of the current study was to discover how QS impacts production of AF compounds, biofilm formation and motility in *P. chlororaphis* strain PA23. As the majority of secondary metabolites are produced at the onset of stationary phase, we were also interested to learn whether cross-regulation occurs between QS and the stationary-phase sigma factor RpoS. A *phzR* mutant (PA23*phzR*) and an AHL-deficient strain (PA23-6863) were created that no longer inhibited *S. sclerotiorum* (Table 4.2), indicating that QS is required for fungal antagonism. A number of metabolites contribute to PA23 biocontrol, including diffusible antibiotics (PHZ, PRN), HCN, and extracellular enzymes (Poritsanos et al. 2006). We hypothesized that the lack of AF activity exhibited by the QS-deficient derivatives was due to diminished production of one or more of these compounds. The observed reduction in PHZ levels was expected because PHZs are positively regulated by QS in other *P. chlororaphis* strains (Chin-A-Woeng et al. 2001; Pierson et al. 1994; Wood and Pierson, 1996). Conversely, the markedly lower amounts of PRN indicated that this antibiotic was also under QS control, which has not been reported in *P. chlororaphis* or other *Pseudomonas* sp. Studies of PRN regulation have lagged behind those of other antibiotics in biocontrol pseudomonads. In *P. chlororaphis* for example, PHZs are essential for biocontrol; consequently, this antibiotic has been the focus of most investigations (Chin-A-Woeng et al. 1998; Pierson and Thomashow, 1992; Spencer et al. 2003). Furthermore, while *P. fluorescens* strains Pf-5 and CHA0 both produce PRN, neither strain has a QS system (Haas & Keel, 2003).

LuxR-type transcriptional regulators bind to distinct elements upstream of target genes called “lux boxes” or in the case of the *phz* biosynthetic operon, “phz boxes”.

Upstream of *phzI* and *phzA*, we found sequences that were 100% identical to phz boxes present in the promoter regions of homologous genes from other pseudomonads. In addition, a less conserved (9/18 nt) phz box lies upstream of *prnA*. To further establish that QS has a direct effect on *prnA,* we monitored *prnA-lacZ* activity in *E. coli* in the presence and absence of PhzR-C_6-HSL. A similar approach has been used to demonstrate direct gene activation by the *P. aeruginosa* Las and Rhl QS systems (de Kievit et al. 1999; Pesci et al. 1997). In the *E. coli* background, *prnA-lacZ* activity increased two fold in the presence of pME6032-*phzR* and exogenous C6-HSL (Fig. 4.1E). These findings suggest that QS activates the *prn* operon directly; however, DNA binding assays are required to confirm a direct interaction between PhzR-C6-HSL and the *prn* promoter region. Although this is the first report of PRN being under QS control in a pseudomonad, it has been observed in *Serratia plymuthica* and certain *Burkholderia* spp. (Lui et al. 2007; Schmidt et al. 2009). Quorum sensing-deficient strains of *S. plymuthica* and *B. lata* failed to produce PRN and no longer exhibited antagonistic activity against the fungal pathogen in question (Lui et al. 2007; Schmidt et al. 2009). While Lui and coworkers did not search for lux-box sequences in *S. plymuthica*, a survey of the *prnABCD* promoter regions in members of the *B. cepacia* complex (Bcc) revealed the presence of a "cep-box" in some but not all cases (Schmidt et al. 2009). These findings led the authors to conclude that QS may not be required for PRN expression in all members of the Bcc (Schmidt et al. 2009).

In many *Pseudomonas* sp., there is evidence that RpoS and QS are subject to cross regulation (Bertani & Venturi, 2004; Girard et al. 2006; Schuster et al. 2004; Whiteley et al. 2000). Therefore, we sought to determine if a similar link exists between

QS and RpoS. In PA23*rpoS*, *phzI* transcription was reduced, while that of *phzR* was elevated (Fig 4.3), indicating that RpoS both activates *phzI* and represses *phzR*. Although differential regulation of "partners" within a QS network seems counterintuitive, similar findings were observed in *P. aeruginosa* strain PAO1 where *rhlR* was positively regulated by RpoS, while *rhlI* was repressed (Schuster et al. 2004). Moreover, an extensive study looking at *lasR/rhlR* and *lasI/rhlI* gene transcription revealed little correlation between the expression profiles of the cognate I- and R-genes under most growth conditions (Duan and Surette, 2007). Approaching cross-regulation from the other side, we found RpoS to be positively controlled by QS in PA23. In other pseudomonads, the involvement of QS in RpoS regulation varies. For instance in *P. chlororaphis* PCL1391, a *phzI* mutant showed no difference in RpoS levels (Girard et al, 2006). Whereas, in *P. putida* WCS358 and *P. aeruginosa* PAO1, QS system positively regulates *rpoS* expression (Bertani and Venturi, 2004; Schuster et al. 2004), similar to what was found here.

To better understand the regulatory roles of QS and RpoS in PA23, a *phzRrpoS* double mutant was created. Characterization of this strain revealed that it resembled PA23*phzR* in terms of AF activity, production of PHZ and PRN and expression of *phzA*-, *prnA*-, *phzI*- and *phzR-lacZ* fusions. What's more constitutive expression of *phzR* but not *rpoS* was able to complement PA23*phzRrpoS* for both antibiotic expression and AF activity (Table 4.2). These results, together with the fact that RpoS positively regulates *phzI* transcription, suggest that RpoS regulation is mediated at least in part through QS. Girard and coworkers (2006) reported similar findings, wherein constitutively expressed *phzR* was found to complement *rpoS*-, *psrA*-, and *gacS*-mutants of *P. chlororaphis* strain

PCL1391. The authors concluded that PhzR is a master regulator controlling expression of AF metabolites in strain PCL1391 (Girard et al. 2006). Herein, the only difference in secondary metabolite production between the *phzR* mutant and the *phzRrpoS* double mutant was protease levels, which were greatly enhanced in the latter (Table 4.2). In a previous study, we discovered that RpoS represses protease production (Manuel et al. 2012). Thus, it appears that RpoS-mediated repression of this exoproduct does not involve QS. In *P. chlororaphis* 30-84, protease expression was found to be regulated by both the PhzI/PhzR and CsaI/CsaR QS systems as only a double *phzR/csaR* or *phzI/csaI* knockout abolished protease production (Zhang & Pierson, 2001). Although, we have yet to identify a second QS system in PA23, it should be noted that greatly diminished protease activity was only observed in PA23-6863 (Table 4.2). The *phzR* mutant conversely demonstrated a modest (1.5-fold) decrease in protease levels (Table 4.2). The possible involvement of a second QS system and repression by RpoS may help to explain why protease levels were not completely restored in PA23*phzR* (pUCP23-*phzR*). Taken together, the results of this and other studies suggest that the regulatory network overseeing protease expression in *P. chlororaphis* is quite complex.

Bacteria that are able to establish themselves as an adherent biofilm community are afforded protection from assaults that would otherwise threaten their planktonic counterparts, including dessication, UV-radiation, grazing predators, etc. As such, biofilm formation may facilitate bacterial persistence in the environment. To date, an in-depth analysis of genes and gene products essential for *P. chlororaphis* biofilm development has not been undertaken. In the current study, the QS-deficient strains were found to produce less biofilm than the wild type (Fig. 4.3). In *P. chlororaphis* 30-84, QS

is involved in biofilm formation; specifically the production of PHZ was found to be critical (Maddula et al, 2008). Similarly, we have shown that a *phzA* mutant produces slightly less biofilm than the wild type (Selin et al. 2010). In the present study, PA23*rpoS* exhibited increased biofilm formation despite the reduction in PHZ levels (Fig. 4.3), suggesting that PHZs play only a minor role during the initial attachment and colonization process. For many bacteria, flagella are involved in interaction with surfaces during the early stages of biofilm formation (Davey and O'Toole, 2000). The enhanced flagellar motility exhibited by the *rpoS* mutant may correlate with the increased biomass on the plates. Consistent with our findings, an *rpoS* mutant of *P. aeruginosa* produced more biofilm than the PAO1 parent, and the biofilms showed elevated resistance to the antibiotic tobramycin (Whiteley et al. 2001). Thus it appears that similar to what has been observed in other bacteria; multiple factors come in to play during the process of PA23 biofilm establishment.

In conclusion, our results provide insight into the regulatory mechanisms governing secondary metabolite production in *P. chlororaphis* strain PA23. We demonstrate that the Phz QS system is required for PA23 AF activity, with PRN, PHZ and protease expression all under QS control. In addition, RpoS and QS are subject to cross regulation. Quorum sensing induces *rpoS* expression; whereas RpoS exerts positive and negative control over *phzI* and *phzR*, respectively. We also discovered that both global regulators affect PA23 biofilm formation. The Phz QS system positively regulates biofilm development; RpoS, on the other hand, reduces the adherent biomass. Studies in other pseudomonads show that such complexity is not exclusive to PA23 (Haas and Keel, 2003; Haas and Défago, 2005). Integration of the QS network with other global

regulators presumably facilitates sensing and processing of multiple signals, allowing tight control of secondary metabolite production under fluctuating environmental conditions. Future studies will focus on the biocontrol capabilities of these strains and their persistence in the environment.

4.4. Acknowledgments

I would like to thank Dr. Everett Pesci for providing *P. aeruginosa* QSC105 (pEAL01) and Dr. Kan Tanaka for providing anti-RpoS antisera.

Chapter 5

Regulation of the PA23 Rsm System by GacA, PsrA, RpoS and QS

5.1 Introduction

The GacS/GacA two-component regulatory system controls the expression of secondary metabolites in a number of different bacterial species (Heeb and Haas, 2001). In *P. fluorescens* strains CHA0 and Pf-5, and *P. chlororaphis* strains PCL1391 and 30-84, mutations within *gacA* or *gacS* significantly impairs the production of AF compounds (Duffy and Défago, 2000; Corbell and Loper, 1995; Chin-A-Woeng, 2000; Chancey et al. 1999). Studies have shown that the GacS/GacA system primarily regulates secondary metabolism at the posttranscriptional level involving another regulatory system termed Rsm (Regulator of secondary metabolism). The Gac/Rsm system has been characterized in a number of bacteria including *E. coli*, *P. fluorescens*, *P. aeruginosa* and *E. carotovora* (Mulcahy et al. 2008; Dubey et al. 2003; Hyytiäninen et al. 2001). In all cases, the Gac/Rsm circuitry serves to modulate the expression of secondary metabolites and extracellular enzymes during the transition from low- to high-cell density states (Berjerano-Sagie and Xavier, 2007). In *P. fluorescens* CHA0, the Rsm system is comprised of two RNA binding proteins RsmA/E and three small regulatory RNAs, RsmZ, RsmX, and RsmY (Lapouge et al. 2008). The RsmA/E proteins function as posttranscriptional repressors by binding to a specific ribosome-binding sequence in the target mRNA and blocking translation. Translational repression can be alleviated by the action of RsmZ, RsmY and RsmX, which bind multiple copies of RsmA/E, rendering the ribosome binding site (RBS) of target genes accessible to the translational machinery (Haas and Keel, 2003).

P. chlororaphis strain PA23 produces a number of secondary metabolites, including PHZ, PRN, HCN, proteases, lipases and siderophores. Our studies have

identified multiple regulatory elements that influence production of these metabolites including the GacS/GacA two-component system, the activator of *rpoS* transcription, PsrA, the stationary phase sigma factor, RpoS, and the PhzI/PhzR QS system (Poritsanos et al. 2006; Manuel et al. 2012; Selin et al. 2012; Selin and de Kievit unpublished data). Although each of these members appears to regulate secondary metabolite production, it has become apparent that there is substantial interaction between the elements within the cascade. For example, we have recently demonstrated that in PA23, RpoS positively and negatively controls the expression of *phzI* and *phzR*, respectively, while *rpoS* expression was positively regulated by QS (Selin et al. 2012). Similar interactions between regulatory elements and the Rsm system have been reported in *P. fluorescens* CHA0 and *P. aeruginosa*, where the expression of the sRNAs appears to be positively regulated by GacA in both strains (Kay et al. 2005; Kay et al. 2006). The activation of the regulatory RNAs by GacA is believed to occur through binding to an upstream promoter element termed the GacA-box (TGTAAGN$_6$CTTACA) (Valverde et al. 2003; Kay et al. 2005; Lenz et al. 2005; Kulkarni et al. 2006). Moreover, a recent study in *P. fluorescens* CHA0 revealed that in addition to GacA, PsrA positively stimulates *rsmZ* transcription (Humair et al. 2010). By uncovering how these elements interact, a better understanding of the cascade governing secondary metabolite production in *Pseudomonas* is gained.

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

5.2 Results

5.2.1 Identification of *rsmZ*, *rsmA*, and *rsmE* in PA23.

To identify the presence of the *rsmZ*, *rsmA* and *rsmE* genes in strain PA23, PCR primers were designed using the *rsmZ*, and *rsmA* sequences from *P. fluorescens* CHA0 (accession Nos. AF245440 and AF136151), *P. aeruginosa* PA01 (accession No. AE004091) and *Pseudomonas entomophila* (accession No. NC_008027). The *rsmE* sequence of *P. fluorescens* CHA0 (accession No. AY547575) was used to design primers to amplify the *rsmE* gene in PA23. The resulting PCR products were cloned into pCR2.1 topo, generating pCR-*rsmZ*, pCR-*rsmA* and pCR-*rsmE*. Sequence analysis of these plasmids revealed that the PA23 *rsmZ* gene demonstrated the highest sequence identity with *rsmZ* of *P. fluorescens* Pf-5 (96%; accession No. CP000076), *P. fluorescens* CHA0 (96%; accession No. AF245440) and *P. fluorescens* Pf0-1 (91%; accession No. CP000094). The PA23 *rsmA* sequence exhibited the highest degree of identity with the *rsmA* genes of *P. fluorescens* Pf-5 (96%; accession No. NC004129), *P. fluorescens* Pf0-1 (93%; accession No. CP000094), and *P. fluorescens* CHA0 (92%; accession No. AF136151). The *rsmE* sequence demonstrated the highest degree of identity with the *rsmE* genes of *P. fluorescens* CHA0 (88%; accession No AY547575), and *P. fluorescens* Pf-5 (88%; accession No. CP000076). Looking at the arrangement of these genes in the PA23 chromosome, *rsmZ* is localized downstream of the *rpoS* gene, similar to *rsmZ* in *P. fluorescens* CHA0 and *P. aeruginosa* (Heeb et al. 2002; Kay et al. 2006). Blast analysis revealed that an open reading frame with 87% identity to a gene found in *P. chlororaphis* PCL1391 is located downstream in the same orientation as the *rsmZ* gene in PA23 (Fig. 5.1A).

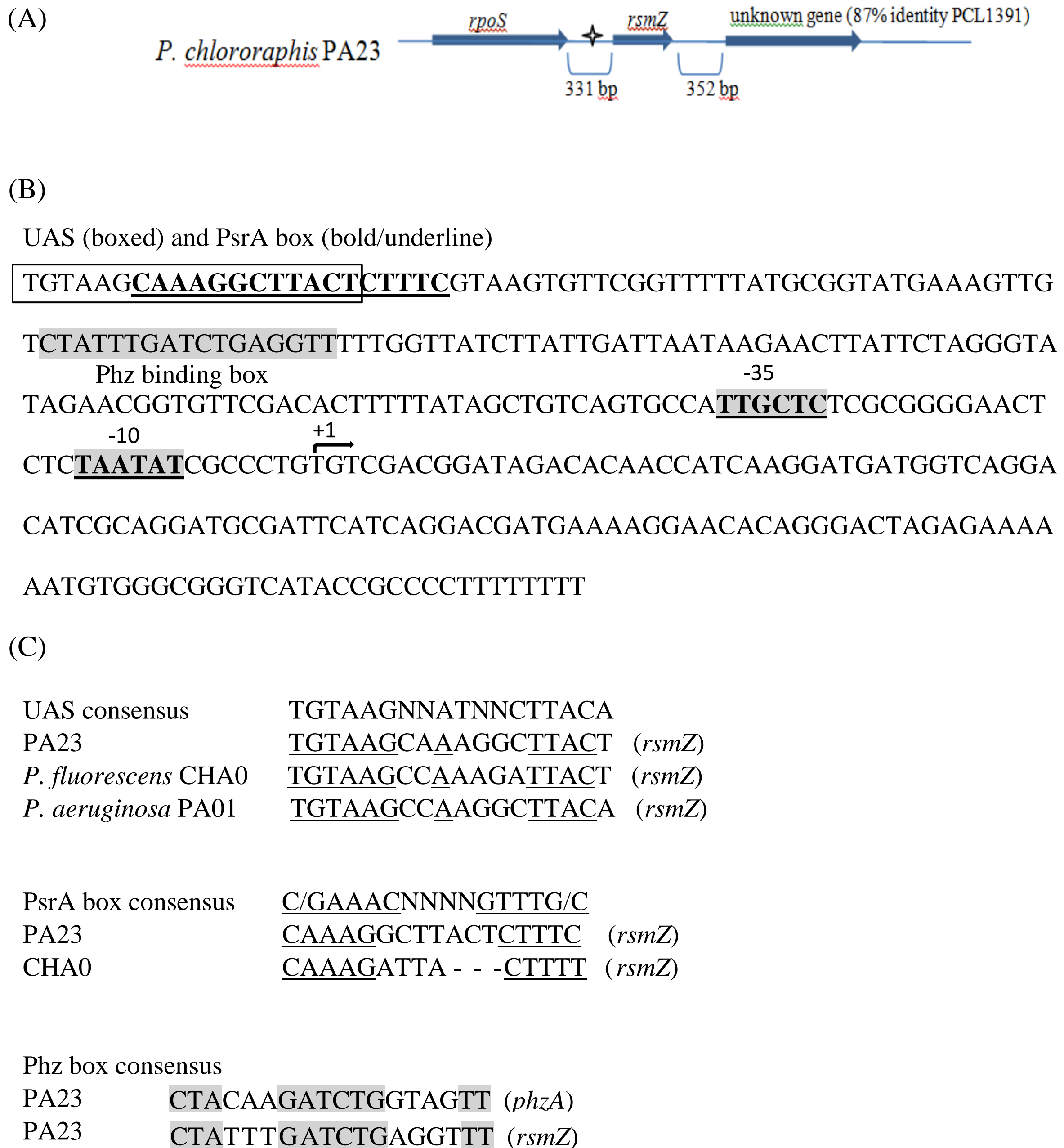

Figure 5.1 Analysis of the PA23 *rsmZ* gene. A) Localization of *rsmZ* within the PA23 chromosome, B) The PA23 *rsmZ* sequence determined through sequence analysis; the upstream activating sequence (UAS), the PsrA binding box, the Phz binding box are all clearly defined. For orientation, the transcriptional +1 site as well as the putative -10 and -35 sites are highlighted as reported for *P. fluorescens* CHA0 (Heeb and Haas, 2002), (C) Alignment of the UAS, PsrA and Phz box consensus sequences of PA23 and other pseudomonads.

This is in contrast to the genetic arrangement observed in *P. fluorescens* CHA0 and *P. aeruginosa*, where *fdxA* is located downstream of *rsmZ* but in the opposite orientation (Heeb et al. 2002; Kay et al. 2006). The PA23 *rsmA* gene is located downstream from the *lysC* gene (data not shown), analogous to the gene arrangement found in *P. fluorescens* CHA0 (Blumer et al. 1999). The *rsmE* gene of PA23 is localized upstream of a gene sequence encoding a putative protein (data not shown).

5.2.2 Predicted secondary structure of *rsmZ*

Regulatory RNAs, such as *rsmZ,* form unique stem-loop secondary structures which are important for their function (Haas and Défago, 2005). In order to determine if RsmZ from PA23 adopts a similar secondary structure, the RNA sequence was analyzed by the computer software program M-fold (Zuker, 2003). As shown in Figure 5.2, the M-fold program predicted RsmZ to have a flower-like secondary structure, with five stem-loop structures, all of which contain GGA-motifs.

5.2.3 Transcription of *rsmZ*, *rsmA*, and *rsmE* depends upon GacS/GacA

To investigate the regulatory effects of the Gac system on *rsmZ*, *rsmA* and *rsmE* expression, *rsmZ*-, *rsmA*-, *rsmE*-*lacZ* transcriptional fusions were mobilized into PA23 and PA23*gacA* and analyzed for β-galactosidase activity. As shown in figure 5.3A, the expression of *rsmZ* was significantly reduced compared to the wild type, indicating that GacA is required for optimal expression of *rsmZ.* In *P. fluorescens* CHA0, GacA-mediated control of *rsmZ* expression is reportedly through a conserved palindromic upstream activating sequence (UAS) within the *rsmZ* promoter region, predicted to be bound by the phosphorylated GacA protein (Heeb et al. 2002). Therefore, we analyzed

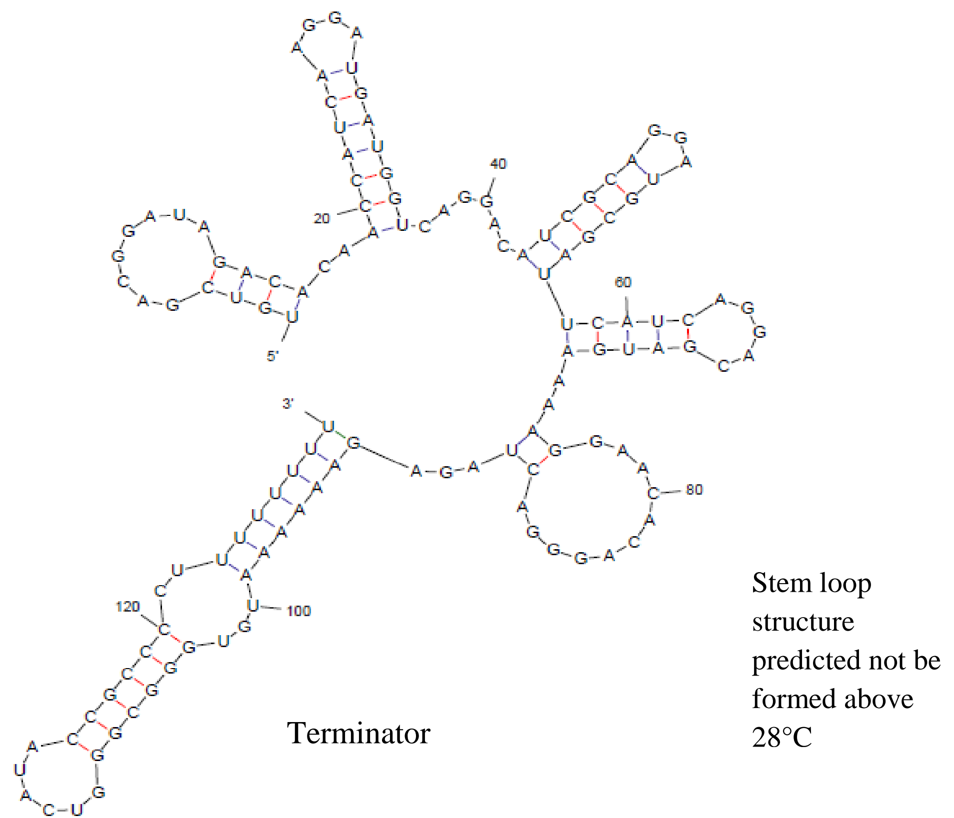

Figure 5.2 The predicted secondary structure of RsmZ from *P. chlororaphis* PA23 determined using the Mfold program (Zuker 2003).

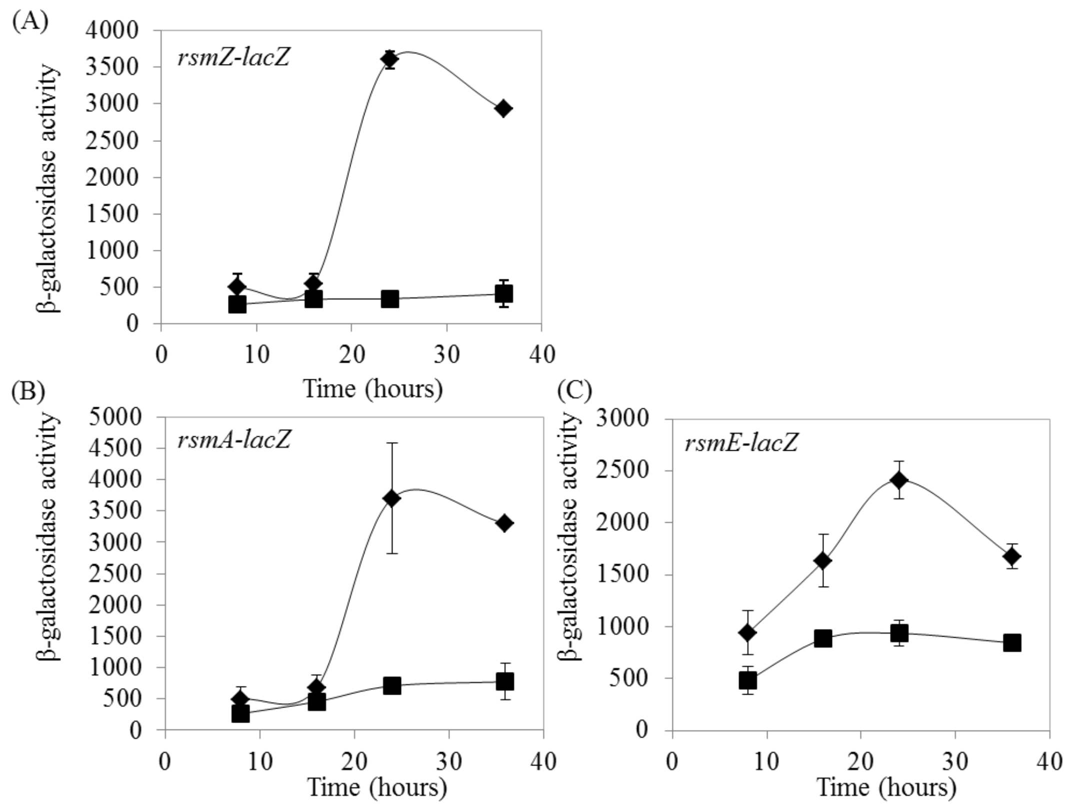

Figure 5.3 Expression analysis of (A) *rsmZ-lacZ* (B) *rsmA-lacZ* and (C) *rsmE-lacZ* in PA23 (closed diamonds) and PA23*gacA* (closed squares).

the sequence of the PA23 *rsmZ* promoter region to locate a possible match to the UAS identified in *P. fluorescens* CHA0 (Heeb et al. 2002). As expected, a conserved UAS consisting of the sequence TGTAAGCAAAGGCTTACT was identified upstream of the *rsmZ* promoter (Fig. 5.1B and C). This sequence matched 15 of the 18 nucleotides within the UAS of *P. fluorescens* CHA0 (Humair et al. 2010) and 16 of 18 nucleotides within the UAS found in *P. aeruginosa* (Brencic et al. 2009) (Fig. 5.1C). These results suggest that GacA positively influences the expression of *rsmZ*, likely via recognition of the UAS upstream of the *rsmZ* promoter region. As illustrated in figure 5.3B and C, the expression of both *rsmA-lacZ* and *rsmE-lacZ* were reduced in the *gacA* mutants compared the wild type, suggesting that a functional Gac system is required to obtain maximum expression of both *rsmA* and *rsmE*. A UAS consensus was not located upstream in the *rsmA* and *rsmE* promoter regions, indicating that GacA-mediated control of these genes may be indirect.

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

GacA appears to regulate PsrA, and RpoS (Selin and de Kievit unpublished); therefore it is possible that the regulatory effects of GacA on the Rsm system are mediated through these transcription factors. To determine if PsrA or RpoS regulate the expression of *rsmZ*, *rsmE* and *rsmA*, the transcriptional activity of the aforementioned was measured in PA23, PA23*psrA*, and PA23*rpoS*. As shown in figure 5.4A, the expression levels of *rsmZ* at 24 hours in PA23*psrA* and

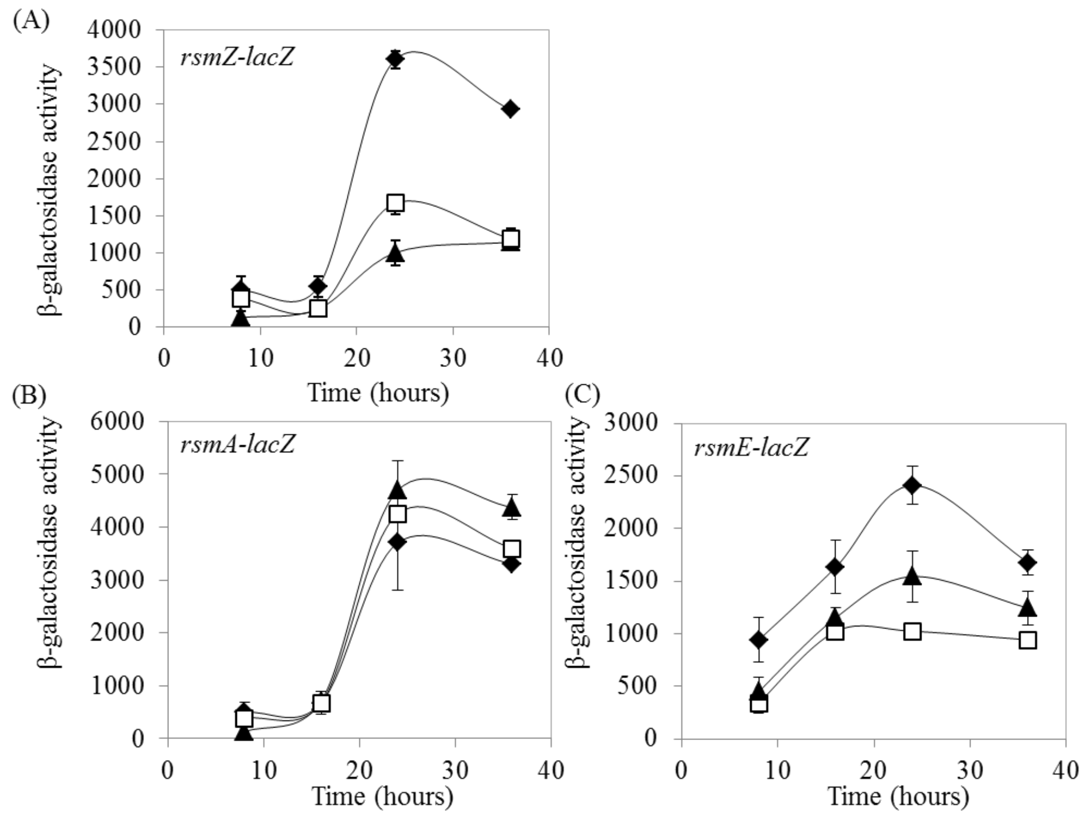

Figure 5.4 Expression analysis of (A) *rsmZ-lacZ* (B) *rsmA-lacZ* and (C) *rsmE-lacZ* in PA23 (closed diamonds), PA23*psrA* (closed triangles), and PA23*rpoS* (open squares).

PA23*rpoS* were reduced approximately 3.6- and 2.1-fold, respectively, compared to the wild type. A similar result was observed where the expression of *rsmE* was reduced 1.5- and 2.3-fold in both the *psrA* and the *rpoS* mutant, respectively (Fig. 5.4C). The transcription of *rsmA*, however, remained relatively close to wild-type levels (Fig. 5.4B). These results suggest that *rsmZ* and *rsmE* transcription is positively influenced by PsrA and RpoS, and that *rsmA* expression occurs independently of these regulators. In addition, GacA regulation of *rsmZ* transcription may be indirect through these transcriptional regulators.

Next, we sought to determine if the positive effects of PsrA and RpoS on *rsmZ* and *rsmE* transcription were direct. Towards this end, *rsmZ* and *rsmE-lacZ* transcriptional fusions were co-expressed with either pBAD-*psrA or* pBAD-*rpoS* in an *E. coli* background. The expression of the *rsmZ-lacZ* fusion, when co-expressed with pBAD-*psrA*, demonstrated a 2.8-fold increase compared to the *E. coli* strain carrying the *rsmZ-lacZ* plasmid and empty vector (Table 5.1); however no direct effects on *rsmZ* transcription was observed in the presence of RpoS (pBAD-*rpoS*) (Table 5.1). The opposite was observed when *rpoS* (pBAD-*rpoS*) and *psrA* (pBAD-*psrA*) were co-expressed with the *rsmE-lacZ* fusion. In this case, a 2-fold increase in the transcription of *rsmE* was observed in the presence of pBAD-*rpoS*, while pBAD-*psrA* had no effect on expression (Table 5.1). As predicted, no change in the expression of *rsmA* was observed upon the addition of pBAD-*rpoS* or pBAD-*psrA* (Table 5.1). These results suggest that PsrA directly regulates *rsmZ* expression, while RpoS directly effects *rsmE* transcription.

Table 5.1 The effect of RpoS and PsrA on *rsmZ*, *rsmA* and *rsmE* expression in an *E. coli* background.

	pBAD-ACYC [*]	pBAD-*psrA* [*]	pBAD-*rpoS* [*]
Construct	β-galactosidase activity (Miller units) [§]		
pLP170-*rsmZ*	1526 (52)	4352 (503)[†]	1635 (352)[°]
pLP170-*rsmA*	3369 (695)	3068 (40)[°]	3132 (177)[°]
pLP170-*rsmE*	1675 (76)	1542 (104)[°]	3354 (289)[‡]

[*] Expression from the pBAD promoter was induced using 0.2% arabinose.
[§] Mean (standard deviation) obtained from a triplicate set.
[†] Significantly different from empty vector ($p < 0.0001$).
[‡] Significantly different from empty vector ($p < 0.001$).
[°] Not significantly different from empty vector.

Direct control by a transcriptional regulator usually involves recognition of a sequence within the promoter region of a given gene. In *P. putida*, it has been shown that PsrA binds directly to a palindromic sequence, termed the PsrA box (Kojic et al. 2002), located within both the *rpoS* and *psrA* promoter regions. In *E. coli* and *P. aeruginosa,* a -10 promoter consensus has been identified within genes that are predicted to be positively regulated by the stationary phase sigma factor RpoS (Espinosa-Urgel et al. 1996; Schuster et al. 2004). Therefore, the promoter regions of both *rsmZ* and *rsmE* were analyzed to identify any potential PsrA or RpoS binding consensus sequences. A conserved sequence matching the PsrA box previously identified in the *rsmZ* promoter of *P. fluorescens* CHA0 (Humair et al. 2010) is located upstream from the *rsmZ* promoter region (Fig. 5.1B and C); as expected, no sequence matching the PsrA box consensus sequence was found in the *rsmE* promoter. A potential RpoS binding sequence (CTGTATA) was located in the predicted -10 region of the *rsmE* promoter, matching 4 out of the 7 nucleotides with the -10 RpoS consensus (CTATACT) suggested for *E. coli* (Espinosa-Urgel et al. 1996) and *P. aeruginosa* (Schuster et al. 2004; Irie et al. 2010). Interestingly, a similar sequence (CTAATAT) was also identified in the -10 promoter region of *rsmZ.*

5.2.5 RpoS indirectly regulates *rsmZ* transcription through the Phz QS system.

Although RpoS was shown to positively stimulate *rsmZ* transcription, the regulatory effect on *rsmZ* expression did not appear to be direct. We have previously shown that RpoS positively regulates *phzI* and negatively regulates *phzR* expression (Selin et al. 2012). Therefore, it is possible that the regulatory effect exhibited by RpoS on *rsmZ* expression is through the PhzI/PhzR QS system. To determine if QS had an

impact on the transcription of *rsmZ* in PA23, the *rsmZ-lacZ* transcriptional fusion was monitored in PA23, PA23*phzR*, PA23*rpoSphzR*, and PA23-6863. As shown in Figure 5.5A, the expression of *rsmZ* was reduced in all three mutants compared to the wild type. The highest reduction occurred in PA23-6863 with a 6.5-fold decrease in *rsmZ* transcription, while PA23*phzR* and PA23*rpoSphzR* exhibited a 5-fold reduction in *rsmZ* expression (Fig. 5.5A). These findings suggest that QS governs expression of *rsmZ.*

To determine if there is a direct relationship between *rsmZ* and QS, β-galactosidase analysis was carried out in *E. coli* co-expressing the *rsmZ-lacZ* transcriptional fusion and pME6032-*phzR* in the presence and absence of C6-HSL. In the presence of PhzR and autoinducer, *rsmZ* expression was enhanced 3-fold (Fig. 5.6). As expected, there was no induction of *rsmZ* transcription in *E. coli* carrying the empty vector (+/- C6-HSL) or in *E. coli* with pME6032-*phzR* in the absence of autoinducer (Fig 5.6). These results suggest that the expression of *rsmZ* is positively regulated by PhzR and C6-HSL. The transcriptional activator, PhzR, when bound to C6-HSL, is believed to activate the transcription of the *phz* operon by binding to the Phz box located within the *phzA* promoter (Chin-A-Woeng et al. 2001). Although direct binding has not been elucidated in PA23, the Phz box sequence has been identified in both the *phzA* and *prnA* promoter regions, both of which have been shown to be directly regulated by PhzR (Selin et al. 2012).

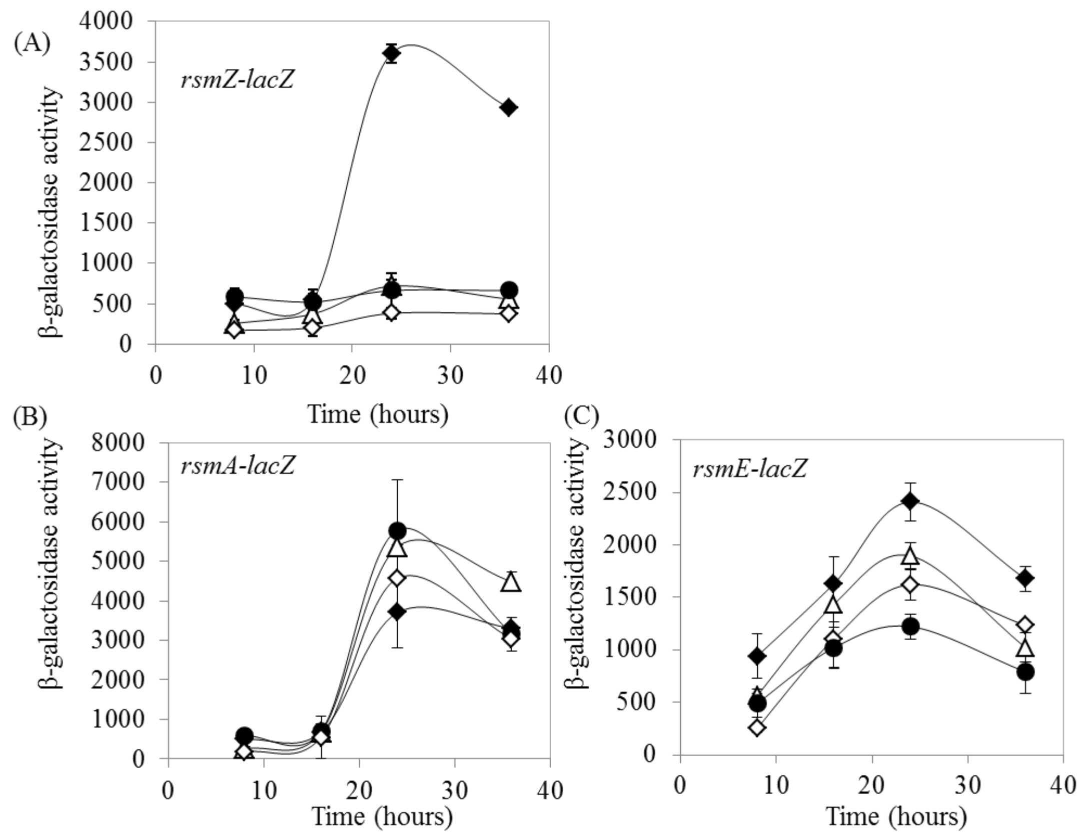

Figure 5.5 Expression analysis of (A) *rsmZ-lacZ* (B) *rsmA-lacZ* and (C) *rsmE-lacZ* in PA23 (closed diamonds), PA23*phzR* (open triangles), PA23*phzRrpoS* (closed circles) and PA23-6863 (open diamonds).

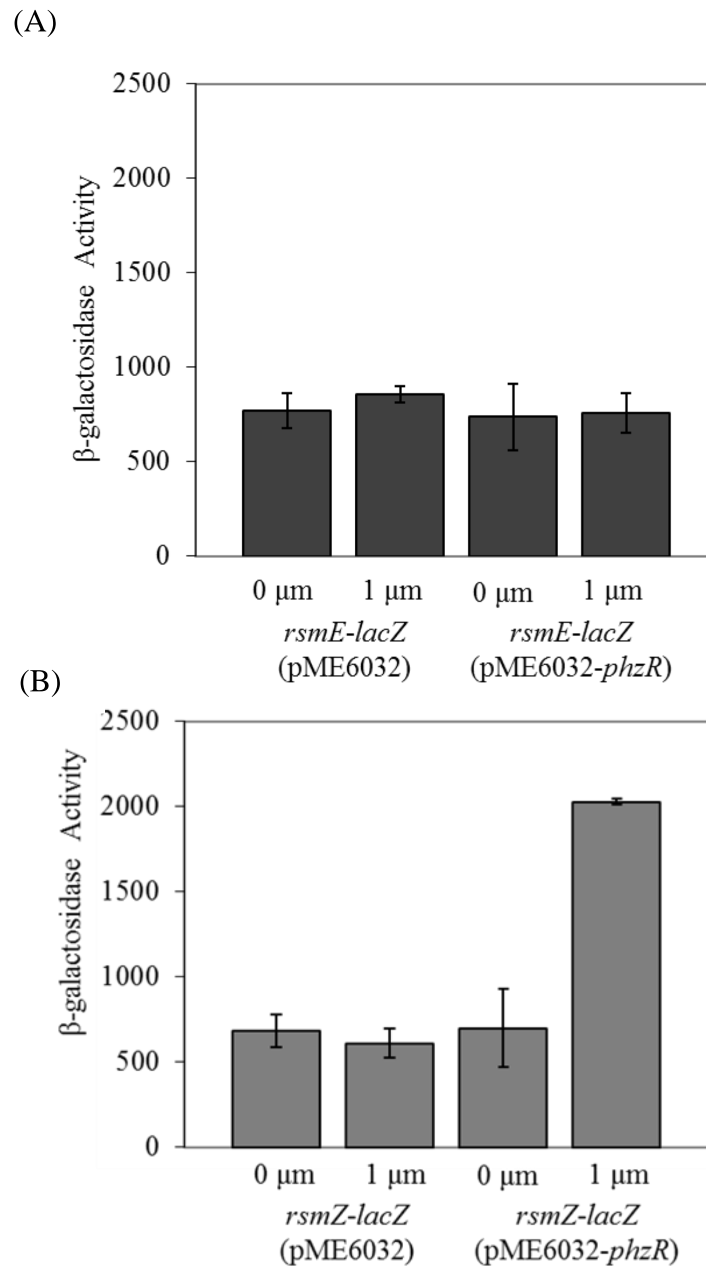

Figure 5.6 The impact of QS on the expression of (A) *rsmZ-lacZ* and (B) *rsmE-lacZ*. The *rsmZ-* and *rsmE-lacZ* fusions were co-expressed in *E. coli* with *phzR*-pME6032 and pME6032 (empty vector) in the presence and absence of C6-HSL (1µm). Bacteria were grown in M9 minimal media supplemented with 1mM $MgSO_4$, 0.2% glucose and 0.4% casamino acids. Cultures were grown for 24 hours prior to measuring β-galactosidase activity (Miller units).

Therefore, the *rsmZ* promoter was examined for the presence of a Phz box consensus sequence. A potential Phz box was identified 131-bp upstream from the putative +1 transcriptional start site (Fig. 5.1A). This sequence matched 11 of the 18 nucleotides within the Phz box sequence in the PA23 *phzA* promoter region (Fig. 5.1C), suggesting that the activation of *rsmZ* transcription by PhzR-C6-HSL may be direct through promoter sequence recognition.

5.2.6 The Phz QS system indirectly regulates *rsmE* transcription, and is not required for *rsmA* expression.

To determine if QS regulates transcription of *rsmE* and/or *rsmA*, the expression of the *rsmE*- and *rsmA-lacZ* transcriptional fusions were monitored in PA23, PA23*phzR*, PA23*phzRrpoS* and PA23-6863. The results indicate that the expression of *rsmA* is not affected by QS, as *rsmA* transcription remained at wild-type levels or slightly higher in the QS-deficient strains (Fig. 5.5B). The expression of *rsmE,* however, appeared to be positively regulated by QS, as the QS-deficient strains showed reduced *rsmE* transcription compared to the wild type (Figure 5.5C). To verify if the Phz QS system directly stimulates *rsmE* expression, transcription of the *rsmE-lacZ* fusion was analyzed in *E. coli* expressing pME6032-*phzR* in the presence and absence autoinducer. As shown in Figure 5.6, the expression of *rsmE* was not affected by the addition of PhzR and C6-HSL, suggesting an indirect relationship between QS and *rsmE* transcription. A Phz box consensus sequence was not detected in the *rsmE* promoter region, supporting the idea that the positive effect of the Phz QS on *rsmE* expression is likely mediated through other regulatory element(s).

5.3 Discussion

The GacS/GacA system primarily regulates secondary metabolism at the posttranscriptional level through the Rsm system (Haas and Keel, 2003). In *P. fluorescens* CHA0, the Rsm system is comprised of two RNA binding proteins RsmA and RsmE and three small regulatory RNAs, RsmZ, RsmX, and RsmY (Lapouge et al. 2008). In the present study, we identified genes that were homologous to *rsmZ*, *rsmA* and *rsmE* of *P. fluorescens* strains CHA0 and Pf-5 in *P. chlororaphis* PA23. These regulatory elements further expand the list or previously identified regulators including GacS/GacA, PsrA, RpoS and QS. The relationship between GacA and the expression of *rsmZ* in *P. fluorescens* and *P. aeruginosa* has been elucidated, and it is well recognized that GacA is required for the transcription of *rsmZ* (Heeb et al. 2002; Kay et al. 2006). Furthermore, additional transcriptional regulators such as PsrA have been shown to positively regulate *rsmZ* transcription in *P. fluorescens* CHA0 (Humair et al. 2010). These findings suggest that the Rsm circuitry is enmeshed in the complex cascade overseeing expression of secondary metabolites and as such may be subject to regulation by other elements within the cascade. The goal of the current study was to determine how the Rsm system is regulated by members of the PA23 regulatory hierarchy; specifically elucidating the regulatory role of GacA, PsrA, RpoS and the Phz QS system on the expression of *rsmZ*, *rsmA* and *rsmE*.

First, the link between GacA and the transcription of *rsmZ*, *rsmA* and *rsmE* in PA23 was examined. Reduced expression of all three genes was observed in the *gacA* mutant compared to the wild type (Fig. 5.3A, B and C). These results indicate that the transcription of *rsmZ*, *rsmA* and *rsmE* in PA23 is dependent upon GacA. In *P.*

fluorescens CHA0, the expression of both *rsmZ* and *rsmE* were reduced in a *gacA* mutant (Heeb et al. 2002; Reimmann et al. 2005), similar to the levels observed here (Fig. 5.3A and C); however, the expression of *rsmA* in CHA0 did not appear to require GacA, as RsmA protein levels were unaffected by a *gacA* mutation (Heeb et al. 2002). This suggests that the expression of *rsmA* may not involve GacA in all pseudomonads. Studies have indicated that GacA-mediated control may be governed by the recognition of an upstream activation sequence (UAS), which has been previously identified in the *rsmZ* promoter region of *P. aeruginosa* PAO1 and *P. fluorescens* CHA0 (Heeb et al. 2002; Kay et al. 2005). Consistent with these findings, a similar sequence was identified within the *rsmZ* promoter region in PA23 (Fig. 5.1A). A UAS consensus was not identified in the *rsmA* or *rsmE* promoter region, which suggests that the regulatory effect of GacA on these genes is likely indirect.

Next, we examined whether PsrA or RpoS control *rsmZ*, *rsmA* or *rsmE* transcription. When we analyzed the expression of the *rsmZ*, *rsmA*, and *rsmE-lacZ*-fusions in PA23*rpoS* and PA23*psrA*, transcription of *rsmZ* and *rsmE* was reduced compared to the wild type; however, *rsmA* transcription was unchanged (Fig. 5.4A, B and C). These findings suggest that in PA23, *rsmE* and *rsmZ* transcription is be positively regulated by PsrA and RpoS, whereas *rsmA* transcription does not require either of these regulators. To the best of our knowledge, this is the first report illustrating the involvement of PsrA and RpoS on *rsmE* expression in *Pseudomonas*; whether this is exclusive to PA23 has yet to be determined. In terms of *rsmZ* expression, a *psrA* mutant of *P. fluorescens* CHA0 exhibited a slight decrease in *rsmZ* transcription compared to the wild type (Humair et al. 2010), which is consistent with our findings. However, the effect

of the *psrA* mutation on *rsmZ* transcription was more pronounced in PA23 then *P. fluorescens* CHA0. A possible explanation for the discrepancy is the involvement of RpoS. Our results show, that unlike *P. fluorescens* CHA0, the stationary phase sigma factor, in addition to PsrA, positively regulates *rsmZ* transcription in PA23. Because PsrA has been shown to positively regulate *rpoS* expression, it is possible that PsrA activates *rsmZ* transcription directly as well as indirectly through RpoS. Therefore, we elucidated whether PsrA or RpoS exhibited a direct effect on *rsmZ* and *rsmE* expression. The expression of *rsmZ* was enhanced in the presence of PsrA, but not RpoS (Table 5.1), indicating that PsrA directly controls *rsmZ* transcription. Conversely, *rsmE* transcription only increased in the presence of RpoS (Table 5.1). Thus it appears that PsrA-medatied control of *rsmE* expression occurs through RpoS. In addition, a potential PsrA binding sequence similar to the sequence found in *P. fluorescens* CHA0 (Fig. 5.1C) was located within the PA23 *rsmZ* but not the *rsmE* promoter regions. In terms of an RpoS binding region, a -10 RpoS consensus (CTATACT) has been suggested in *P. aeruginosa* (Schuster et al. 2004) and *E. coli* (Espinosa-Urgel et al. 1996). Putative RpoS consensus sequences were found in the -10 promoter regions of *rsmZ* (CTAATAT) and *rsmE* (CTGTATA), suggesting that these genes are directly regulated by RpoS. However the *E. coli* expression analysis indicated that only *rsmE* is directly controlled by RpoS. We believe that *rsmZ* transcription is under control of a sigma factor other than RpoS.

Lastly, we examined whether QS plays a role in regulating the transcription of *rsmZ*, *rsmA*, or *rsmE*. Expression of both *rsmZ*- and *rsmE-lacZ* were reduced in the QS-deficient strains, while *rsmA* transcription remained at wild-type levels (Fig. 5.5A, B and C). These findings indicate that QS may play a role in the regulation of *rsmZ* and *rsmE*

transcription. To clarify whether these effects were directly linked to QS we co-expressed pME6032-*phzR* with the *rsmZ-* and *rsmE-lacZ* transcriptional fusions in an *E. coli* background with and without autoinducer. Interestingly, the expression of *rsmZ* but not *rsmE* was enhanced in the presence of PhzR and C6-HSL, indicating a direct relationship between PhzI/PhzR QS and *rsmZ* transcription. Support for a direct relationship was further demonstrated through identification of a Phz box consensus within the *rsmZ* promoter (Fig 5.1B). This sequence is believed to be required for transcriptional activation by PhzR-C6-HSL in *P. chlororaphis* strain PCL1391 (Chin-A-Woeng et al. 2001). To our knowledge this is the first report to link PhzI/PhzR QS to *rsmZ* transcription in *Pseudomonas*. Although an earlier study in *P. fluorescens* CHA0 revealed that a QS–like mechanism positively enhanced *rsmZ* transcription, the autoinducing signal was unrelated to AHL (Heeb et al. 2002). A connection between QS and RsmZ was observed in *P. aeruginosa*; but in this situation, RsmZ was found to positively regulate the Rhl QS system (Kay et al. 2006). We did not establish a direct relationship between QS and *rsmE* expression in PA23 and no Phz binding consensus was identified within the *rsmE* promoter. As RpoS has been shown to be positively regulated by QS in PA23 (Selin et al. 2012), the reduced *rsmE* expression in the PA23 QS-deficient strains is likely due to a reduction in RpoS protein levels, as opposed to a direct effect by QS.

In conclusion, our results provide insight into the regulatory roles of GacA, PsrA, RpoS and QS in the expression of *rsmZ, rsmA*, and *rsmE*. We have shown that in addition to GacA and PsrA, the PhzI/PhzR QS system positively regulates *rsmZ* expression in PA23. Furthermore, GacA, PsrA and RpoS are all required for maximal

rsmE expression, whereas *rsmA* expression only requires GacA. Identification of the Rsm components RsmZ, RsmA and RsmE add to the increasingly complex regulatory network overseeing expression of biocontrol factors in PA23. It is hypothesized that multiple signals from both the environment and the bacteria control expression of *rsmZ*, *rsmA* and *rsmE* allowing the bacteria to fine-tune expression of AF factors. Future studies will be focused on the regulatory role of the Rsm system in the production of secondary metabolites and its overall contribution to PA23 biocontrol.

5.4 Acknowledgments

I would like to thank Jer Manuel for her assistance in generating pLP170-*rsmA*, pLP170-*rsmE* and pACYC-BAD. I am extremely grateful to Dr. Dieter Haas for providing plasmid pME6032.

Chapter 6

Conclusions and Future Directions

6.1 Conclusions and Future Directions

Arguably, biological control is a safe, environmentally friendly approach to managing plant pathogens. Fluorescent pseudomonads are present in high numbers in natural soils and various strains have been found to produce secondary metabolites that are antagonistic towards fungal pathogens. One such promising isolate is *P. chlororaphis* PA23 which was isolated from soybean root tips. In both greenhouse and field studies, this bacterium is able to protect canola from the devastating effects of stem rot caused by the fungus *S. sclerotiorum* (Lib.) de Bary (Fernando et al. 2007; Savchuk and Fernando, 2004; Zhang, 2004). The AF activity of strain PA23 has been attributed to the production of secondary metabolites which include PHZ, PRN as well as HCN, proteases and siderophores (Zhang et al. 2006, Poritsanos et al. 2006).

Preliminary experiments suggested that the primary mechanism of pathogen inhibition by PA23 was due to antibiotic production (Portitsanos et al. 2006; Savchuk and Fernando, 2004). Since PA23 produces two antibiotics, one of the goals of this research was to determine the individual contribution of PHZ and PRN to PA23 inhibition of *S. sclerotinia*. Therefore, strains lacking one or both of these antibiotics were analyzed for their ability to inhibit *S. sclerotinia*. Greenhouse and AF plate assays revealed that PRN production was more essential for PA23-mediated antagonism, as only the *prn* mutants were impaired in their ability to inhibit *S. sclerotiorum*. When the PRN levels in PA23 and these derivative strains were quantified, the *phz* mutant was found to produce 2-fold higher levels of PRN than the wild type. Co-regulation of some antibiotics has been shown to occur in other *Pseudomonas* strains. In *P. fluorescens* strain CHA0 and Pf-5 for example, the antibiotic 2-4-DAPG strongly represses PLT biosynthesis and vice-

versa. (Baehler et al. 2001; Brodhagen and Loper, 2001; Schnider-Keel et al. 2000). Therefore, we examined whether the 2 antibiotics were subject to cross-regulation; however no evidence for this was observed. Instead, we believe that the enhanced production of PRN by the Phz mutant is brought about because PHZ and PRN share a common metabolic pathway. Although we discovered PHZ is not essential for PA23-mediated biocontrol of *S. sclerotiorum,* production of this antibiotic does result in enhanced biofilm establishment. A link between biofilm formation and PHZ production has been reported in *P. chlororaphis* 30-84 by Maddula et al. (2008). Since biofilms may enhance environmental fitness, it is possible that PHZs enable the bacteria to maintain a stable population within the environment.

Studies of other pseudomonads have revealed that several exoproducts, including PHZ, are QS controlled (Chin-A-Woeng et al. 2005; Schuster et al. 2004). Thus, we hypothesized that the same would hold true for PA23. To investigate the role of QS in the production of AF compounds, we phenotypically characterized PA23*phzR* and PA23-6863 (AI^{-}). Our results revealed that PHZ, autoinducer and protease production are all QS controlled, as none of these compounds were produced in the QS-deficient strains. Furthermore, PRN was positively regulated by QS. Although this is the first report to show this connection in *Pseudomonas*, QS positively regulates PRN production in *B. cepacia* (Schmidt et al. 2009). Interestingly, Schmidt and colleagues (2009) identified a "cep-box" within the *prnABCD* promoter in a majority of the *Burkholderia* strains that were studied, which suggested a direct mechanism for QS-control of PRN production. In PA23, a Phz box consensus was identified in the *phzA* promoter, as well as the *prnA* promoter. This Phz box consensus is assumed to be recognized and bound by PhzR-C6-

HSL, thus activating the transcription of QS-controlled genes. The putative Phz box together with the fact that *prn* expression was enhanced in the presence of pME6032-*phzR* and C6-HSL in an *E. coli* background, strongly suggest that the PhzI/PhzR QS system directly controls *prn* expression. Nevertheless, DNA binding assays are required to firmly establish that the PhzR-C6-HSL complex binds directly to the *prnA* promoter.

RpoS provides another layer of regulation controlling secondary metabolite production in pseudomonads (Chin-A-Woeng et al. 2005; Girard et al. 2006; Sarniguet et al. 1995) and a connection between QS and RpoS has been observed (Schuster et al. 2004; Girard et al. 2006). Here, we show that RpoS positively regulates the expression of *phzI* and *phzA*, leading to PHZ production in PA23. Furthermore, the PhzI/PhzR QS system positively regulates RpoS in terms of transcription and protein expression. This indicates that significant regulatory overlap occurs between RpoS and QS. In PA23 it appears that the production of PHZ and PRN is directly regulated by QS, as a *phzR* mutation was epistatic to a *rpoSphzR* double mutation in terms of AF activity, production of PHZ and PRN, and expression of the *phzA*–, *prnA*–, *phzI*–and *phzR*–*lacZ* fusions. Furthermore, constitutive expression of *phzR* but not *rpoS* was able to complement the double mutant for both antibiotic production and AF activity. Collectively, these findings suggest that the effects of RpoS on biocontrol traits of PA23 are indirect and primarily mediated through activation of *phzI*.

Besides GacS/GacA, PsrA, RpoS and the PhzI/PhzR QS, members of the Rsm system were identified in PA23, adding yet another layer of complexity to the regulatory cascade. The PA23 Rsm system is comprised of a single small non-coding regulatory RNA termed RsmZ, and two RNA binding proteins RsmA and RsmE. The *rsmZ*, *rsmA*,

and *rsmE* genes that encode these elements shared the highest % identity with the genes identified in *P. fluorescens* CHA0. Evidence from our studies indicate that expression of the aforementioned genes is dependent upon GacA. However, *rsmZ* may be the only gene directly regulated by GacA, as a UAS, believed to be required for GacA control, was identified within the promoter region of *rsmZ*, but not those of *rsmA* and *rsmE*. PsrA and QS were also shown to positively regulate *rsmZ* transcription, which suggests that a multi-input system is involved in maintaining appropriate RsmZ levels. We identified PsrA- and Phz-box consensus sequences within the *rsmZ* promoter. Furthermore, *rsmZ* expression was enhanced in the presence of PsrA and PhzR-C6-HSL in an *E. coli* background, suggesting that these regulators directly activate of *rsmZ* transcription. In the case of *rsmE*, we believe that GacA indirectly regulates expression through PsrA, RpoS and QS, as all three regulators control *rsmE* transcription. Given that PsrA and QS positively regulate *rpoS* expression but only RpoS directly regulated *rsmE* expression in an *E. coli* background, we believe that the positive effects of PsrA and QS on *rsmE* transcription are likely mediated through RpoS. Furthermore, a potential -10 RpoS binding consensus was located within the promoter region of *rsmE* supporting the idea of a direct relationship between RpoS and *rsmE* expression. It should be noted here, that even though we have identified potential binding sites for RpoS, PsrA and PhzR-C6-HSL within the *rsmE* and *rsmZ* promoters, specific interaction between the transcriptional regulators and these promoters need to be verified through DNA binding assays.

In summary, the findings from this thesis have shed light on the regulatory network that governs secondary metabolite production in PA23. We have shown that the regulatory cascade is complex and involves several members, including GacS/GacA,

PsrA, RpoS and the PhzI/PhzR QS system. Furthermore, we have demonstrated that the PA23 Rsm system is subject to regulation by other members of the regulatory hierarchy. A summary of the PA23 regulatory network is depicted in Fig. 6.1. There are several layers of regulation controlling the expression of biocontrol factors in this bacterium. The exact reason for so much fine-tuning remains unclear. It may be that these compounds play distinct roles under different environmental conditions and in a concentration-dependent manner. An emerging notion is that antibiotics are not solely bacterial weapons, and at subinhibitory concentrations they can act as signaling molecules (Davies, 1990; Davies et al. 2006; Seshasayee et al. 2006; Yim et al. 2006). For example, in *P. aeruginosa*, 4-hydroxy-2-heptylquinilone (HHQ) is not only a precursor for 3,4-dihydroxy-2-heptylquinilone (PQS) but it also serves as a signaling molecule that is released and taken up by the cells to induce PQS production (Déziel et al. 2004). Antibiotic-mediated signaling has been reported for biocontrol strain *P. fluorescens* Pf-5, where exogenous PLT within the plant rhizosphere positively stimulates expression of traits contributing to the biocontrol activity of *Pseudomonas* spp. (Brodhagen et al. 2004).

Part of the interest in unravelling the regulatory network governing expression of AF factors is to optimize performance, as biocontrol agents have historically suffered from inconsistent performance in the field. It is interesting, that biocontrol strains overproducing key AF metabolites do no exhibit enhanced biocontrol in greenhouse assays (Huang et al. 2004; Haas and Keel, 2003; Delaney et al. 2001). For example, in *P. fluorescens* CHA0, constitutive DAPG production by a *phlF* mutant did not lead to enhanced protection of tomato against *F. oxysporum* (Haas and Keel, 2003).

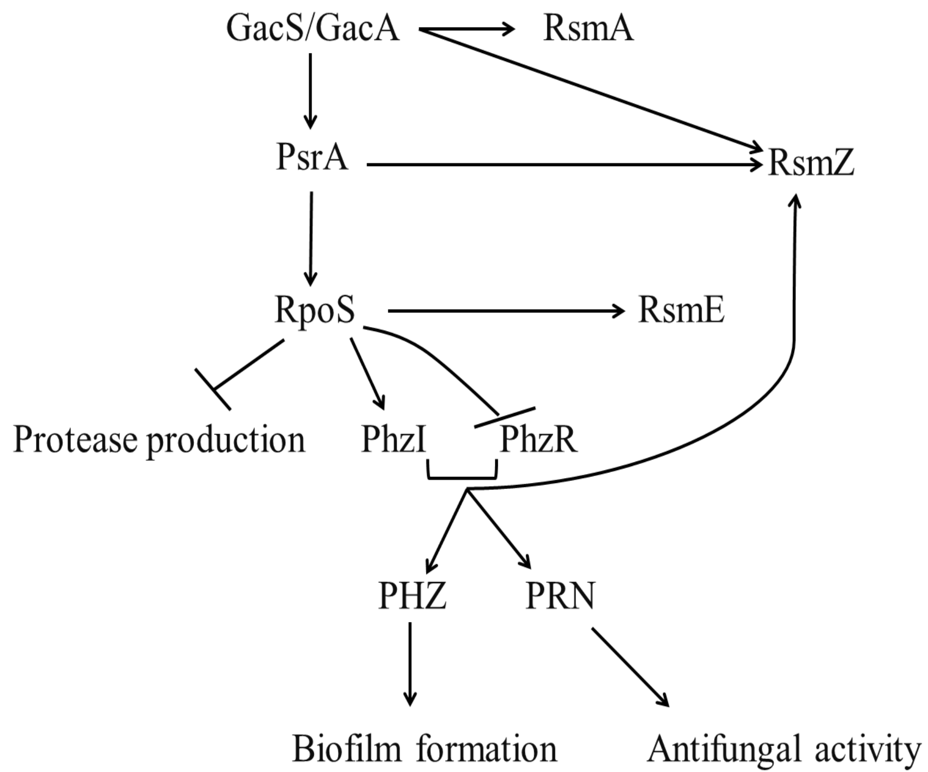

Figure 6.1 Model of the regulatory cascade governing PA23-mediated antagonism of *S. sclerotinia* in vitro and the regulatory impact on the expression of the Rsm system. Arrows indicate a positive influence while bars represent a negative impact.

Similarly, a DAPG-overproducing F113 derivative, overexpressing the *phl* operon on a multicopy plasmid, did not exhibit superior protection of sugarbeet from *P. ultimum* (Delaney et al. 2001). Although these findings suggest that balanced production of biocontrol compounds may be essential for environmental fitness, it remains unclear as to why the producing organisms are secreting these compounds at all. Obviously, production of these metabolites is energetically costly and, as such, they must be of some benefit to the producing organism. It has been proposed that these antagonistic compounds may protect bacteria from soil predators (Neidig et al. 2011; Jousset et al. 2006). A study in *P. fluorescens* CHA0 explored the ability of this bacterium to resist grazing by different soil protists by comparing resistance between the wild-type strain and metabolite-deficient mutants. It was observed that production of secondary metabolites significantly prevented protist grazing (Jousset et al. 2006). In another study, Neidig and colleagues (2011) demonstrated that HCN and 2-4-DAPG served as a repellent and also exhibited toxic effects on the nematode *Caenorhabditis elegans* (Neidig et al. 2011). Presumably these antipredator strategies are advantageous, as they would allow the bacteria to sustain higher populations within the plant rhizosphere, contributing not only to environmental fitness, but possibly to their biocontrol activity as well. In PA23, the production of PHZ is not required for biocontrol activity; however, this antibiotic enhances biofilm establishment. We have yet to determine if biofilm formation by PA23 improves the environmental fitness of this strain.

Although the signals that induce the Gac system remain to be determined in PA23 as well as other pseudomonads, it is likely that both environmental factors and bacteria-mediated signals trigger the GacS-GacA circuitry. Studies in other *Pseudomonas* strains

have begun to uncover some of the compounds that elicit a Gac-mediated response. One study by Dubuis and Haas (2007) investigated whether the Gac/Rsm cascade could be activated by signals produced by *P. fluorescens* CHA0 and other *Pseudomonas* species. It was discovered that *P. fluorescens* CHA0, *P. fluorescens* SBW25, *P. aeruginosa,* and *P. corrugata* culture extracts could all induce the Gac/Rsm network in *P. fluorescens* CHA0. In addition plant root exudates have been found to stimulate the Gac system. In *Pseudomonas* sp. strain DSS73, beet root exudates induced the expression of *asmY*, a gene that encodes for amphisin (Koch et al. 2002). Because the induction of amphisin was only achieved in the presence of a functional Gac system, it was concluded that the exudates mediated induction through GacS-GacA. In another study by Yamazaki et al. (2012), derivatives of plant phenolic compounds were tested for their ability to target virulence factors produced by *P. aeruginosa* PA01. It was discovered that the Gac/Rsm circuitry was stimulated by these plant metabolites which indirectly effected the expression of the virulence gene *exoS,* (Yamazaki et al. 2012). Collectively these findings indicate that organisms in the surrounding environment, including both prokaryotes and eukaryotes, are key contributors to the pool of signals recognized by the Gac regulatory network.

Beyond the Gac system, other types of signalling are expected to occur between bacteria, plants and other eukaryotic organisms in the environment. For example in *P. fluorescens* CHA0, fusaric acid, an antibiotic produced by the fungal pathogen *Fusarium oxysporum,* was shown to repress the transcription of the genes required for DAPG production (Notz et al. 2002). There may be thousands of other signal molecules that could affect biocontrol factors produced by a given strain. It is unknown what types of

signalling or communication is occurring between these eukaryotic organisms and the biocontrol bacteria; it is also possible that bacterial-mediated secondary metabolites play a role in signalling between the bacteria and the plant, facilitating establishment in a particular environment. Undoubtedly, there are many questions left to be answered in terms of signalling and their effects on the production of biocontrol factors. To better understand these interactions, a more wholistic approach is necessary. By looking at bacterial gene expression in the presence and absence of fungi, as well as in presence and absence of plants or root exudates, we may uncover new ways to enhance the overall abundance and activity of biocontrol agents, providing a better alternative for disease suppression.

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