Characterizing the CsaI-CsaR quorum sensing system in *Pseudomonas chlororaphis* strain PA23 and investigating its role in regulating the production of secondary metabolites

By: Grace Saliga

A thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Microbiology

Faculty of Science

University of Manitoba

ABSTRACT

Pseudomonas chlororaphis strain PA23 is able to inhibit fungal phytopathogens, primarily through production of excreted secondary metabolites, deeming this organism a promising biocontrol candidate. Antibiotics, including phenazine (PHZ) and pyrrolnitrin (PRN), in addition to degradative enzymes and volatile organic compounds, make up the arsenal that combats pathogenic fungi like *Sclerotinia sclerotiorum.* Production of these exometabolites is tightly controlled via a hierarchy, which includes the Phz quorum sensing (QS) system. Previous RNA-seq analysis focusing on two QS-deficient strains, namely PA23*phzR* and PA2--AHL, revealed that 13% of the PA23 genome is QS-regulated. A number of unique differentially expressed genes in the QS-deficient strain compared to the *phzR* mutant suggested that additional regulators are involved in PA23 QS. A second system, called Csa (cell surface alterations) has been characterized in the closely related *P. chlororaphis* 30-84. While *csaI* and *csaR* are present in the PA23 genome, the role of this system in PA23 biocontrol has not yet been investigated. The purpose of this research was to characterize the PA23 Csa QS system and elucidate its role in regulating secondary metabolite production. Single mutants of the Csa and Phz QS systems, PA23*csaR,* PA23*csaI* and PA23*phzI,* in addition to double mutants, PA23*phzRcsaR* and PA23*phzIcsaI*, were generated and subjected to phenotypic analysis. Results indicated that the Csa system negatively regulates PA23 AF activity. PA23*csaR* and PA23*csaI,* as well as the *phzIcsaI* double mutant, all exhibited increased *S. sclerotiorum* inhibition in plate assays. While phenotypic analysis revealed very minor differences in chitinase, protease, and HCN production as well as biofilm formation in one or both *csa* mutants, these differences

alone are insufficient to account for the increased antifungal (AF) activity. Expression of *prnA*, which encodes the primary antibiotic involved in PA23 biocontrol, was unchanged in the *csa* mutant background suggesting that PRN is likely not affected. Therefore, the traits responsible for the enhanced AF activity have yet to be identified. Additionally, the relationship between the Csa QS system and other components of the regulatory cascade require further investigation to elucidate to what degree, directly or indirectly, the Csa QS regulates PA23 biocontrol.

ACKNOWLEDGMENTS

First and foremost, I would like to express my most sincere appreciation and gratitude to my advisor, Dr. Teresa de Kievit, for allowing me the opportunity to work in her lab. The experience and knowledge that I have gained throughout my time under her guidance and with her support, is truly invaluable.

I also extend this sentiment of gratitude to my committee members, Dr. Deb Court and Dr. John Sorensen. I consider myself fortunate to have been able to learn from the both of you as an undergraduate student, as a staff member of the Faculty of Science and most recently as a graduate student. I appreciate the guidance and advice that I received from you throughout my graduate studies career.

To the members of the de Kievit lab, past and present, I thank you for your camaraderie within and beyond the walls of 410 Buller. To April especially, who made my days brighter and taught me so much, not only about Microbiology, but also just life. I will forever be thankful to have been so lucky to share this time with you. Thank you as well, to the undergraduates whom I mentored through the years and helped to build on my thesis project.

Thank you to literally every person in the department of Microbiology. Thank you for letting me share my research woes with you on the elevator ride, introducing yourself to me while enjoying cake in the coffee room, passing the soccer ball to a fellow MSDS team player, streaking out a plate of your coveted strain and celebrating victories along the way. I can confidently say that every student, staff and faculty member has played some part in making my experience in graduate studies exciting and rewarding. To the amazing friends that I have made along the way, you have impacted my life immensely, and I hope that our friendships continue to grow in the many years ahead.

Lastly, but most importantly, thank you to my family, who have always encouraged me to believe in myself and to be the best version of myself in everything that I do. My main goal in life has always been to make you proud, which drives me to always be and do better every day.

DEDICATION

To my husband Jamie,

I don't think that I could have dreamt up a more patient, supportive, intelligent, talented and adventurous person. I consider myself lucky every single day, that I share this crazy life with you. Thank you for your encouragement and support throughout this chapter of our lives and for seeing the value in investing in my future.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AF - antifungal

AHL - acyl-homoserine lactone

Amp - ampicillin

bp - base pair

cDNA - complementary DNA

Chl - chloramphenicol

CV - crystal violet

DAHP - deoxy-arabino-heptulosonate-7-phosphate

DNA - deoxyribonucleic acid

Fe^{3+} - ferric iron

Gm - gentamicin

HCN - hydrogen cyanide

HPR - 2-hexyl, 5-propyl resorcinol

ISR - induced systemic resistance

Kan - kanamycin

mRNA - messenger RNA

NRPS - Nonribosomal peptide synthetase

PCA - phenazine-1-carboxylic acid

PCR - polymerase chain reaction

PDA - potato dextrose agar

PHZ - phenazine

Pip - piperacillin

(p)ppGpp - guanosine pentaphosphate

PRN - pyrrolnitrin

qPCR - Quantitative PCR

QS - quorum sensing

Rif - rifampicin

RNA - ribonucleic acid

RNAP - RNA polymerase

RNAseq - RNA-sequencing

rRNA - ribosomal RNA

RT - reverse transcription

SR - stringent response

Tc - tetracycline

TCST - Two-component signal transduction

1. INTRODUCTION

1.1. Canola in Canada and the threat to crops

Canola (*Brassica napus*) represents Canada's most revered and valuable crop, contributing billions annually to the economy. Aside from its well-known status as the source of one of the healthiest vegetable oils, canola holds value as a nutritional, high-protein meal for livestock as well as a more recent contender as a biofuel agent (Canola Council of Canada, 2020a). Tasked with the challenge to develop an oilseed crop that was suitable for human consumption in the 1950s, researchers Richard Downey and Baldur Stefansson from the University of Manitoba dedicated two decades to developing the rapeseed double-low variety, known today as canola (Canola Council of Canada, 2020b). What makes this variety unique is its low erucic acid content (<2%) and reduction of approximately 10-11 times the proportion of fatty acids of the traditional Argentinian and Polish rapeseed crops that were first cultivated in Canada in the 1940s (Raymer, 2002). As victorious as was the creation of this revolutionary agricultural commodity, the task of protecting it from the natural predators with which it co-habits, followed quickly in tow.

Commercial crops in Canada and worldwide constantly face the threat of destruction at the hand of fungal pathogens. This presents a unique challenge requiring intervention to prevent detrimental loss to farmers and the economy. To address this issue, management strategies like crop rotation are implemented to improve the odds of healthy, disease-free crops (Canola Council of Canada, 2020c). The effectiveness of crop rotation, however, can be hindered by factors like the persistence of dormant fungal spores within the soils for several

years, the dispersal of air-borne spores between crops and the ability of broad host-range pathogens to infect alternate crops (Canola Council of Canada, 2020c). Though in some instances crop rotation can be an effective management strategy to fight against specific fungi, it is usually executed in combination with one or more additional control measures such as tilling or fungicide application (Peng *et al*., 2014). Breeding for resistance is another strategy to combat crop pathogens, one that has been helping farmers fight blackleg caused by *Leptosphaeria maculans* in canola crops since the 1980s. Through screening and testing, resistant cultivars are recognized as growing in the presence of a fungal pathogen and, depending on the percentage of the crop infected by the pathogen, classified as resistant, intermediate or susceptible (Canola Council of Canada, 2020d). To date, there are no *S. sclerotiorum*–resistant varieties of canola*;* however, there are tolerant cultivars that in combination with other control measures, enable increased crop yields (Minogue, 2018).

1.2. *Sclerotinia sclerotiorum* infection and crop control

S. sclerotiorum is a phytopathogenic fungi with the ability to infect over 400 crops including peas, soybeans, beans, sunflower, rapeseed and canola leading to devastating loss due to its sporadic infection patterns (Bom & Boland, 2000). There are several factors that can influence the incidence of infection which include soil moisture, rainfall, field disease history, canopy density, temperature, and light penetration (Canola Council of Canada, 2020c). Though there is no way to control these factors in order to reduce the likelihood of disease, they do play an important role in prediction models (Canola Council of Canada, 2020c).

In canola, *S. sclerotiorum* causes stem rot, which presents as soft, grey-white lesions, and the presence of white mouldy growth on the stems. Black resting bodies known as sclerotia are also formed, which are resilient, densely packed clusters of fungal hyphae (Ordóñez-Valencia *et al*., 2015). The disease cycle of this pathogen begins with the germination of the sclerotia to either produce spore-forming fruiting bodies called apothecia or actively growing mycelia. The latter can colonize surrounding dead plant debris and subsequently infect adjacent plants (Hegedus & Rimmer, 2005). When apothecia mature, they burst and release ascospores into the air. If spores land on decaying canola petals, they begin to germinate and produce hyphae. Once the petals fall from the plant they can land on the stem and leaves below, at which point the fungal hyphae can penetrate and infect the plant body (Canola Council of Canada, 2020c). Because germination is dependent upon nutrients provided by the petals, fungal infection is limited to the flowering stage. This narrow timeframe of susceptibility paired with the ability of sclerotia to over-winter and survive in the soil for several years, renders crop rotation inefficient and leaves a very narrow timeframe during which foliar fungicidal treatment would be beneficial. Since stem rot can lead to yield losses of 5-100% if not properly managed (Manitoba Agriculture, 2020), several methods including crop rotation and fungicide application are used in tandem with crop monitoring and prediction models to reduce the incidence of stem rot disease (Canola Council of Canada, 2020c).

1.3. Fungicide use, the negative effects and resistance

Commercial fungicides have delivered fast and effective protection against many pathogens over decades of agricultural practices. However, the race to keep ahead of emerging

resistance and concern over the dangers posed to human health and ecosystems due to the expansive use of these compounds has proven exhaustive (Hahn, 2014). The increasing need for a safer and more natural means of managing crop disease has led to efforts being directed toward research and development of biological pesticides (biopesticides). These are classified as compounds derived from natural sources such as bacteria, minerals, fungi, plants and animals (Agriculture and Agri-Food Canada 2020). Microbial biopesticides contain living organisms like bacteria or fungi, along with their secreted metabolites and by-products (Agriculture and Agri-Food Canada 2020). The use of one organism to control or inhibit the growth of another is termed biocontrol, which will be discussed in more detail in the following sections. Such products have been tested, registered and put into practice in canola fields to curb infection by fungal phytopathogens like *S. sclerotiorum* (Manitoba Canola Growers, 2020).

1.4. Historical significance of pseudomonads as biocontrol agents

Harnessing the natural capabilities of microbial species has been in practice for centuries, if not longer, providing valuable tools for the food, pharmaceutical and agricultural industries. *Pseudomonas* spp. are Gram negative, aerobic, motile rods belonging to the class gammaproteobacteria. These bacteria have garnered significant attention as they are found to be ubiquitous in a variety of niches and are extremely versatile in their metabolism and ability to adapt to environmental fluctuations (Peix *et al.*, 2009; Silby *et al*., 2011). *Pseudomonas* spp. possess many favourable traits that render them model candidates as biocontrol agents. These include their ability to use root and seed exudates, colonize and compete for nutrients within

the rhizosphere, adapt to environmental stresses and especially, the ability to produce valuable exometabolites (antibiotics, growth-promoting compounds, volatiles) (Weller, 2007).

Historically, candidates have been isolated from suppressive soils, where a fungal phytopathogen is present but is unable to persist or cause severe disease in the crops, indicating the presence of natural fungal antagonists (Walsh *et al*., 2001). This natural antifungal (AF) activity has been frequently attributed to the compound 2,4-diacetylphloroglucinol (DAPG) that is produced by a significant number of *Pseudomonas* spp. isolated from the suppressed soils of wheat and tobacco affected by take-all disease and black root rot, respectively (Keel, 1992; Raaijmakers *et al*., 1997). Aside from DAPG, other exometabolites like phenazine (PHZ), pyrrolnitrin (PRN) and compounds with biosurfactant properties have been shown to contribute to the biocontrol activity of *Pseudomonas* spp. (Silby *et al*., 2011). Currently there are 4 microbial biopesticides registered for use against Sclerotinia stem rot in canola crops; Actinovate (*Streptomyces lydicus*), Ballad Plus (*Bacillus pumilus*), Contans WG (*Coniothyrium minitans*) and Polyversum (*Pythium oligandrum*). In addition, seven *Pseudomonas* strains are registered as biopesticides for fruit, vegetable and grain crops (Agriculture and Agri-Food Canada, 2013).

1.5. *Pseudomonas chlororaphis* PA23 as a biocontrol agent

Pseudomonas chlororaphis strain PA23 was first isolated from the root tips of soybean plants in 1995 and has been investigated extensively due to its ability to inhibit the fungal pathogen *S. sclerotiorum* (Savchuk & Dilantha Fernando, 2004). The AF capabilities of PA23 stem from the additive effects of the production of several secondary metabolites, which are

secreted by the bacterium into its surrounding environment. These exometabolites directly inhibit fungal growth and include antibiotics, volatile compounds, siderophores and degradative enzymes. Expression of the genes encoding these secondary metabolites is most prevalent in the late log phase-early stationary phase of bacterial growth and is tightly regulated at both the transcriptional and post-transcriptional level by a multi-tiered regulatory circuit (Klaponski *et al*., 2014; Manuel *et al*., 2012; Poritsanos *et al*., 2006; Selin *et al*., 2010, 2014; Shah *et al*., 2016). PA23 was also found to indirectly protect canola from Sclerotinia infection through induction of the plant immune response (Duke *et al*., 2017). A detailed description of metabolites produced by PA23 and the regulatory cascade overseeing their expression is provided below.

1.5.1. Phenazine

PHZs are a group of heterocyclic, nitrogen-containing, pigmented compounds with broad-spectrum antibiotic activity, proving toxic to many bacteria, fungi and algae (Chin-A-Woeng *et al*., 2003; Toohey *et al*., 1965). The antagonistic effects of PHZ derivatives are brought about by the production of reactive oxygen species (ROS) (Guttenberger *et al*., 2017). These molecules can either accept or donate electrons due to the characteristic aromatic ring structure, which is the building block of all PHZ derivatives (Pierson, 2010). Such redox transformations mediate the formation of ROS and hydrogen peroxide (H_2O_2) leading to oxidative stress in the surrounding communities (Mavrodi *et al*., 2006).

PHZ is almost exclusively produced by bacteria, including species belonging to the genera *Pseudomonas, Burkholderia* and *Streptomyces* (Chin-A-Woeng *et al*., 2003). The most common PHZ derivatives produced by *Pseudomonas* spp. include pyocyanin (PYO), phenazine-1-carboxamide (PCN) and phenazine-1-carboxylic acid (PCA), along with several hydroxy-

phenazines. *P. chlororaphis* PA23 produces two PHZ derivatives, namely PCA and 2-hydroxyphenazine (Turner & Messenger, 1986). Aside from the antibiotic properties of PHZs, these compounds also contribute to competency of rhizosphere colonization, biofilm formation and the activation of induced systemic resistance (ISR) in plants (Mavrodi *et al*., 2006; Pierson III & Pierson, 2010).

PHZ biosynthesis occurs through the shikimic acid pathway, which is highly conserved across most organisms and feeds into the synthesis of siderophores and aromatic amino acids (tyrosine, phenylalanine). In *Pseudomonas* spp., the seven-gene PHZ operon (*phzABCDEFG*) drives the production of PCA and other PHZ derivatives. The expression of *phzC*, which encodes a deoxy-arabino-heptulosonate-7-phosphate (DAHP) synthase, diverts the flow of C_3 and C_4 phosphates into the shikimic acid pathway. The remaining genes in the operon are responsible for carrying out the final synthesis of PCA from chorismic acid, the branching point of PHZ synthesis. It has been shown that the loss of function of PhzE, an anthranilate synthase homologue, leads to a diversion of the pathway from the production of PHZ, to the production of PRN (Mavrodi *et al*., 2006; Selin *et al*., 2010).

1.5.2. Pyrrolnitrin

As a highly active metabolite, PRN (3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrole) has been investigated as a biocontrol compound due to the broad-spectrum AF activity it exerts on many fungal phytopathogens (Hammer *et al*., 1997). PRN was first isolated and identified as an exometabolite produced by *P. pyrrocinia* in 1964 (Arima *et al*., 1964), and has since been isolated from many other *Pseudomonas* spp. including *P. chlororaphis* PA23 (Hill *et al*., 1994).

For PA23, PRN has been shown to be the focal metabolite effectively staving off fungal disease (Selin *et al*., 2010), although this isn't the case for all *Pseudomonas* biocontrol strains.

Synthesis of PRN is dependent upon the highly conserved *prnABCD* operon utilizing tryptophan as a substrate (Hammer *et al*., 1997). These four genes encode enzymes, which catalyze the modification of tryptophan by the addition of nitro and chlorine units to the benzene and pyrrole rings of the substrate molecule (Pawar *et al*., 2019). The mechanism of action of PRN in fungi such as *Saccharomyces cerevisiae* and *Penicillium oxalicum,* has been shown to be through uncoupling the normal electron flow in the respiratory electron transport system (R. K. Tripathi & Gottlieb, 1969). In *Candida utilis*, PRN inhibits growth, the intake of metabolites and RNA, DNA and protein synthesis (Nose & Arima, 1969).

1.5.3. Degradative enzymes

Several biocontrol strains are capable of producing degradative enzymes such as lipases, chitinases, cellulases and proteases (Chin-A-Woeng *et al*., 2003). Rhizobacteria benefit from the production of chitinase and cellulase, which target chitin and cellulose respectively, essential building blocks of fungi, insects and eggs of predatory nematodes (A. Veliz *et al*., 2017). These enzymes aid in not only fighting off phytopathogenic disease, but also rendering these compounds available as a source of carbon and nitrogen (A. Veliz *et al*., 2017). Proteases have been shown to work synergistically with other metabolites to successfully stave off fungal disease as well. A higher degree of protection against *Pythium*-mediated damping-off disease was seen when *Pseudomonas fluorescens* F113 and *Stenotrophomonas maltophilia* W81, producing DAPG and protease respectively, were co-inoculated onto seeds. The same degree of AF activity was not observed when the strains were inoculated individually (Dunne *et al*., 1998).

PA23 has been shown to produce and secrete proteases, lipases and chitinases; however, the precise mechanism of action of these metabolites and their contribution to biocontrol remain to be elucidated.

1.5.4. Hydrogen Cyanide

Hydrogen cyanide (HCN) is produced by several biocontrol genera including *Pseudomonas, Rhizobium, Bacillus, Alcaligenes* and *Chromobacterium* spp. and its contribution to pathogen suppression has been investigated extensively (Alemu, 2016; Bunch & Knowles, 1980; Rudrappa *et al*., 2008). HCN synthesis is governed by the *hcnABC* synthase cluster, which encodes enzymes with glycine dehydrogenase/oxidase activity, utilizing glycine as a precursor (Pessi & Haas, 2000). HCN acts to destabilize the aerobic respiratory chain by inhibiting cytochrome *c* oxidase activity and has also been shown to disrupt the activity of metalloenzymes within the rhizosphere (Knowles, 1976).

There have been only a few cases demonstrating that HCN acts directly to repress fungal pathogenicity in crop plants; it does however function in other ways to improve plant growth. A synergistic model was brought to light showing that DAPG and HCN work together to reduce bacterial canker disease in tomato plants caused by *Clavibacter michiganesis*. *Pseudomonas brassicacearum* LBUM300 secretes both HCN and DAPG that act to impede canker disease, a trait that is not seen in either *phlD*- or *hcnC*- deficient strains (Paulin *et al*., 2017). *P. fluorescens* CHA0 is also capable of HCN synthesis, which was linked to the modification of root morphology in tobacco resulting in the induction of plant resistance (Schippers *et al*., 1990). Using lettuce as a model host, Nandi and colleagues (2017) demonstrated that an *hcn* mutant left the crown and stem susceptible to *S. sclerotiorum* infection, indicating that HCN plays a significant role in

PA23 biocontrol (Nandi *et al*., 2017). Additionally, it was revealed that HCN is the metabolite responsible for PA23-mediated fast killing of *Caenorhabditis elegans*, which is hypothesized to increase persistence of this bacterium in the environment (Nandi *et al*., 2015).

1.5.5. Siderophores

Siderophores are iron-chelating metabolites with a high affinity for ferric iron (Fe^{3+}) that have the ability to inhibit pathogenic bacteria and fungi (Arya *et al*., 2018). Biocontrol strains that produce siderophores can increase the solubility of Fe^{3+}, by forming Fe-siderophore complexes ultimately facilitating uptake into the cell. Since iron is a major limiting factor for all living organisms, the ability to efficiently form these complexes and sequester ferric iron greatly impacts environmental fitness and contributes to the inhibition of pathogenic species (Shanmugaiah *et al*., 2015).

In conditions of low iron bioavailability, *Pseudomonas* spp. produce catecholate and hydroxymate siderophores, namely pyochelin and pyoverdine (Boukhalfa & Crumbliss, 2002). Pyoverdin has a remarkably high affinity for ferric iron in comparison to pyrochelin and shows a significant amount of genetic divergence across all pseudomonads (Boukhalfa & Crumbliss, 2002; Smith *et al*., 2005). Both classes of siderophores are synthesized via non-ribosomal peptide synthetase (NRPS) dependent pathways and are secreted into the surrounding environment by an RND efflux pump system (Mossialos *et al*., 2002).

1.5.6. 2-hexyl, 5-propyl resorcinol (HPR)

The compound 2-hexyl, 5-propyl resorcinol (HPR) was first isolated in 1975 from an unknown *Pseudomonas* sp. strain B-9004 showing moderate AF and antibacterial activity (Kitahara & Kanda, 1975). HPR is a phenolic lipid belonging to a group of alkylresorcinols whose

AF activity is a consequence of DNA cleavage in the presence of Cu^{2+} and O_2 (Pohanka *et al*., 2006). Five genes (*darABCSR)* direct the biosynthesis of HPR; *darABC* encodes three biosynthetic enzymes and two AraC/XylS family transcriptional regulators are products of *darSR* (Calderón *et al*., 2019).

The ability of *P. chlororaphis* PCL1606 to protect avocado roots from white root rot disease caused by *Rosselinia necatrix*, was found to be significantly dependent upon HPR production (Arrebola *et al*., 2019). While strain PCL1606 produces PRN, HCN and HPR, an HPR-deficient strain showed a much greater deficit in fungal suppression compared to HCN- and PRN-deficient strains (Calderón *et al*., 2013). Evidence shows that HPR also plays a role in biofilm formation, since HPR-deficient strains have increased flagellar motility, reduced cell adhesion and incomplete formation of a pellicle (Calderón *et al*., 2019). Finally, confocal laser scanning microscopy images confirmed that the HPR-deficient strain produced a disorganized biofilm, indicating a direct correlation between this metabolite and PCL1606 biofilm formation (Calderón *et al*., 2019). HPR production has yet to be investigated in *P. chlororaphis* PA23, although the *darABCSR* genes were identified in the genome of this bacterium.

1.5.7. Biofilm formation and motility

Motility and biofilm formation are important attributes for biocontrol pseudomonads, as they play a crucial role in colonization and persistence in the rhizosphere. It has been shown that strains with deficiencies in motility or biofilm formation exhibit poor outcomes with regard to rhizosphere competence (Capdevila *et al*., 2004; Simons *et al*., 1996; Watnick & Kolter, 2000) In *P. fluorescens* F113*,* wild-type flagellar motility is repressed by the GacS/GacA two-component system. Isolates showing successful rhizosphere competence contain spontaneous

mutations in either *gacS* or *gacA*, causing a hyper-motile phenotype (Navazo *et al*., 2009). Similar findings were seen in *P. chlororaphis* 06 and *P. brassicacearum* DF41, where disruption of *gacS* resulted in hyper-motility *in vitro* (Kim *et al*., 2013 & Berry *et al*., 2010). Evidence also suggests that PA23 motility is QS-mediated, as a *phzR*-deficient and an AHL-deficient strain both show increased swimming motility compared to wild type (Shah *et al*., 2020).

Biofilms form on surfaces and are typically composed of a multispecies microbial community that exhibits increased resiliency to external stresses (Maddula *et al*., 2006). In several instances, biofilm formation has been found to be dependent on antibiotic production. In *P. chlororaphis* (*aureofaciens*) 30-84, biofilm formation was impaired in a PHZ-deficient strain and restored to wild type either in the presence of exogenous PHZ, or the introduction of the PHZ biosynthetic genes *in trans* (Maddula *et al*., 2006). These results have led to the hypothesis that PHZs may serve as adhesins or signalling molecules to mediate the production of biofilms (Maddula *et al*., 2006). These results were also reflected in PA23, where a mutation in *phzE* of the PHZ biosynthetic operon resulted in a significant decrease in biofilm formation (Selin *et al*., 2010).

1.6. The anti-fungal regulatory hierarchy of PA23

The AF activity of PA23 is a consequence of the additive effects of the many secondary metabolites secreted by the bacterium into its surrounding environment. They are considered secondary metabolites because their encoding genes are expressed in the late log phase-early stationary phase of bacterial growth. Moreover, a multi-tiered regulatory circuit that functions at the level of transcription and translation tightly controls gene expression. This hierarchy consists of the GacS/GacA two-component system, which works in concert with the Rsm

network, RpoS, PsrA, the stringent response (SR) and the Phz QS system (Figure 1). Each of these regulators is discussed in more detail in the following sections.

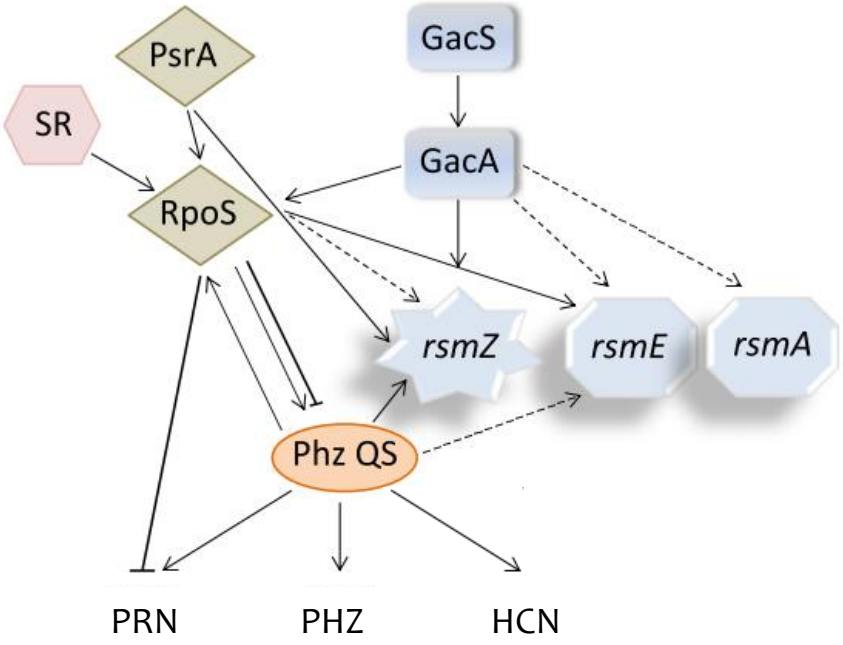


Figure 1. The production of secondary metabolites in PA23 is regulated by a multi-tiered regulatory network**.** A complex hierarchical network governs expression of target genes involved in the biocontrol activity of *P. chlororaphis* PA23. These include biosynthetic and regulatory genes, the latter activating or repressing both biosynthetic and other regulatory genes. Biosynthetic genes are responsible for the production of secreted secondary metabolites, which play a role in staving off fungal pathogens include antibiotics like phenazine (PHZ), pyrrolnitrin (PRN), exoproteases and hydrogen cyanide (HCN). This regulatory network has also been shown to influence the expression of genes that indirectly affect biocontrol such as those involved in motility and biofilm formation. The Phz QS system has been extensively characterized and shown to regulate the production of secondary metabolites both directly and indirectly in *P. chlororaphis* strain PA23. Symbols: →, positive effect; ⊣ , negative effect; solid lines, direct effect; dashed lines, indirect effect. Modified from Selin *et al.*, (2014).

1.7. Gac-Rsm system

The GacS/GacA (Global activator of Antibiotic and Cyanide) two-component regulatory system governs expression of genes responsible for PA23 AF activity and is considered a global regulator. It consists of GacS, a membrane-bound sensor kinase and GacA, a cytoplasmic response regulator (Laville *et al*., 1992). GacS undergoes autophosphorylation in response to an unknown environmental signal, after which the phosphate group transfers to and activates GacA via a phospho-relay mechanism ultimately triggering the expression of Rsm (Regulator of Secondary Metabolism) genes (Heeb & Haas, 2001). Targets of the Gac system include the small non-coding RNAs (ncRNAs) *rsmX, rsmY* and *rsmZ* whose transcription is regulated by a GacA-binding site within the promoter region of these genes (Wang *et al*., 2013). Once transcribed, RsmX/Y/Z form multiple secondary stem loop structures capable of interacting with and sequestering the post-transcriptional repressors RsmA and RsmE. These proteins block translation by binding specific motifs and hindering accessibility of the ribosome-binding sites of target mRNA (Babitzke & Romeo, 2007).

Another level of regulation intertwined within the Gac-Rsm system involves two sensor kinases, RetS and LadS, which have been identified in several pseudomonads including *P. aeruginosa, P. fluorescens* and *P. chlororaphis* (Lapouge *et al*., 2007; Sonnleitner & Haas, 2011). In *P. aeruginosa*, RetS interacts with GacS leading to the formation of a heterodimer. In this conformation, GacS is unable to autophosphorylate, resulting in the prevention of phosphotransfer to GacA. This leads to interruption in gene expression downstream of the Gac system, namely the ncRNAs *rsmY/Z* (Chambonnier *et al*., 2016). It was also discovered that through a multicomponent signal transduction system, LadS is phosphorylated followed by

phosphotransfer to GacS, ultimately activating *rsmY* and *rsmZ* expression (Chambonnier *et al*., 2016).

In PA23, disruption of either *gacS* or *gacA* leads to an inability to inhibit *S. sclerotiorum* infection in plate assays. Since fungal inhibition is driven by the production of secondary metabolites like PRN, PHZ, protease and HCN, the abolition of AF activity is evidence that the Gac system globally regulates the production of these compounds (Poritsanos *et al*., 2006; Selin *et al*., 2014). RetS and LadS have been identified in PA23; however, their relationship with the Gac two-component system and role in biocontrol activity have yet to be established (Shah *et al*., 2016).

1.8. Sigma (σ) factor RpoS and PsrA

Sigma factors are at the forefront of the hierarchal scheme that regulates gene expression in bacteria. These proteins function by binding RNA polymerase (RNAP), changing its affinity for promoter regions of a subset of target genes (Tripathi *et al*., 2014). Bacteria have at least one housekeeping sigma factor, which initiates gene transcription in the exponential growth phase, and several alternate sigma factors, which come into play in response to changes in pH, osmolarity, temperature or a lack of nutrients (Marles-Wright & Lewis, 2007). The types and the number of sigma factors that a bacterium possesses is often species-specific. It has been documented that some pseudomonads, like *Pseudomonas putida,* encode 24 different sigma factors in comparison to *E. coli*, with only 7 (Gruber & Gross, 2003). The number of distinct sigma factors that an organism harbours has been correlated with its ecological lifestyle. Because more diverse organisms are forced to adapt to environmental stress, they require a larger number of sigma factors (Gruber & Gross, 2003). One of the alternate sigma

factors, RpoS (σ^{38} / σ^{S}), regulates genes associated with stationary phase growth and stress response (Battesti *et al*., 2011). Once a threshold concentration of RpoS is reached, it begins to compete with other sigma factors for RNAP core binding, resulting in transcription of alternative sets of genes (Venturi, 2003).

The effect of RpoS on target gene expression in not universal and it regulates genes in different ways depending on the species (Chiang & Schellhorn, 2010). For example, an *rpoS*-null mutant of *P. fluorescens* Pf-5 CHAO showed abolished PRN production (Sarniguet *et al*., 1995), whereas in PA23, PRN production was found to increase in an *rpoS* mutant background (Manuel *et al*., 2012). In addition, this mutant produced elevated lipase and protease activity, enhanced biofilm and elevated fungal suppression compared to PA23 wild type (Manuel *et al*., 2012).

PsrA (Pseudomonas Sigma Regulator A) is a TetR transcription factor that has been identified in biocontrol pseudomonads like *Pseudomonas putida, P. syringae, P. aeruginosa,* and *P. chlororaphis* PCL1391 (Girard *et al*., 2006; Chatterjee *et al*., 2007). In *P. aeruginosa,* PsrA directly regulates the expression of *rsmZ* and *rpoS* and has been shown to auto-regulate, evidenced by a PsrA-binding box located within the promoter region of these target genes (Kojic & Venturi, 2001). In PA23, disruption of *psrA* results in increased AF activity marked by an increase in the production of PRN and protease as well as decreased production of PHZ (Selin *et al*., 2014). A PsrA box was also identified within the promoter region of *rsmZ* in PA23, suggesting the direct influence on gene expression by PsrA (Selin *et al*., 2014).

1.9. Stringent Response

When microorganisms experience nutrient deprivation in the environment, it becomes a necessity to adapt in order to survive. The physiological response to limited nutrient availability is governed by the SR, which results in the arrest rRNA synthesis (Boes *et al*., 2011). Studies examining *E. coli* and *P. aeruginosa* show that activation of the SR involves the induction of RelA [(p)ppGpp synthase] and SpoT [(p)ppGpp hydrolase/(p)ppGpp synthase], ultimately resulting in the production of the alarmone guanosine tetraphosphate (p)ppGpp (Cashel *et* al., 1996). Intracellular accumulation of (p)ppGpp leads to its binding to RNAP and shifting global gene expression by activating or repressing target genes (Potrykus & Cashel, 2008).

In PA23, the SR mutants *relA* and *relAspoT* show enhanced AF activity toward *S. sclerotiorium,* similar to the *rpoS* mutant, due to increased production of PRN, HCN, lipase and protease (Manuel *et al*., 2012). Introducing either *relA* or *rpoS in trans* was able to rescue the wild-type phenotype, indicating that the negative regulation of AF activity governed by the SR is mediated through RpoS (Manuel *et al*., 2012).

1.10. Quorum sensing

Bacteria have the extraordinary capability of orchestrating intercellular communication, a trait once thought to be unique amongst multicellular organisms. This phenomenon transpires via diffusible chemical signalling molecules, known as autoinducers that are synthesized and released into the environment. The most common signalling molecules utilized by Gram-negative bacteria are acyl-homoserine lactones (AHLs) (Whitehead *et al*., 2001). The production of these AHLs, coupled with the cellular response mounted by their reception, lays the

foundation for what is known as QS. The phenomenon of QS is based on the fact that single bacterial cells produce AHLs at a basal level too low to be detected. Once the cell population increases and the concentration of AHLs reach a specific threshold, they can then bind to their cognate transcriptional regulator (W. C. Fuqua *et al*., 1994). The regulator subsequently undergoes dimerization and conformational changes, allowing it to bind recognition sequences within the promoter regions of target genes called *lux* boxes (Figure 2) (C. Fuqua & Greenberg, 1998). As such, QS controls gene expression in a population-density dependent manner and works in tandem with other systems to regulate expression of target genes.

Gram-negative QS systems encompass a LuxI-type AHL-synthase responsible for the production of the AHL signalling molecule, and a LuxR-type transcriptional regulator (Hao *et al*., 2010). *Vibrio fischeri* was the first organism shown to display the production of functional and detectable chemical signalling molecules. AHLs were synthesized by the product of *luxI* and detected by the transcriptional activator LuxR, which together regulate expression of the luciferase operon (Whiteley *et al*., 2017). The regulatory effects seen through the LuxR-AHL interaction paved the road for the classification of the LuxR/LuxI QS family, and thus began investigation into which other bacterial species regulate gene expression through this unique communication system.

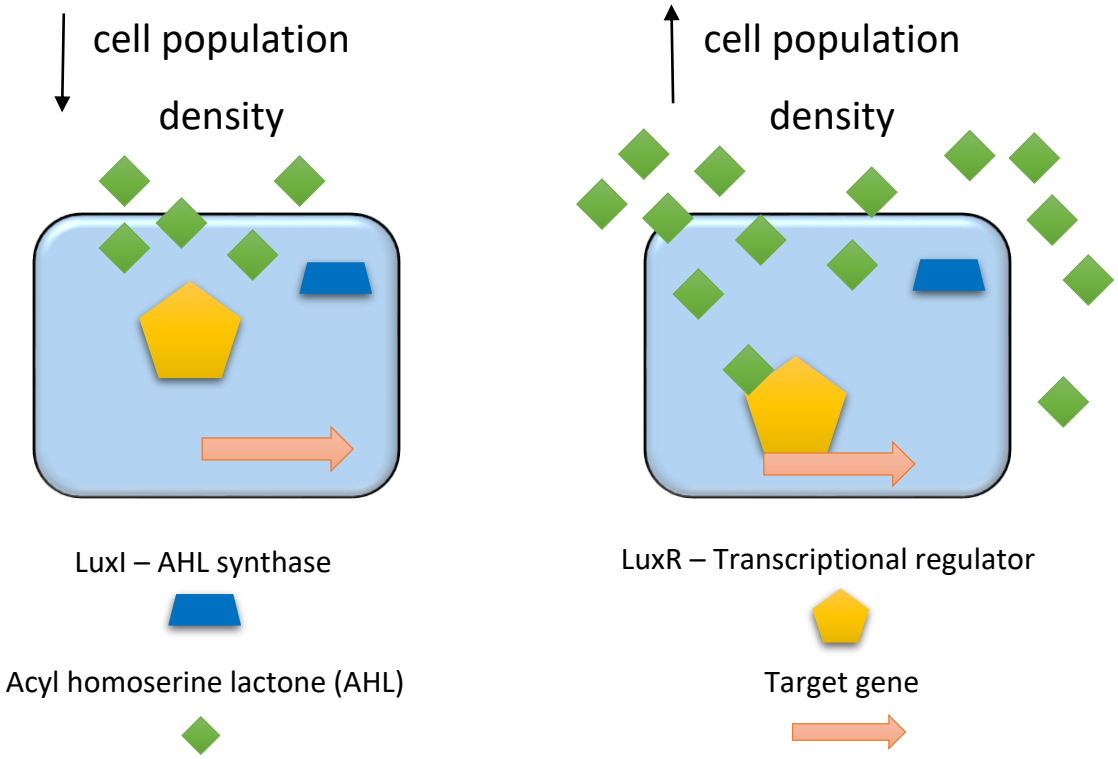


Figure 2. Mechanism of the Lux-type QS system. QS systems are classified as having a transcriptional regulator (LuxR) and an AHL synthase (LuxI). These systems regulate in accordance with population density. If the population size is low, resulting in a low concentration of AHLs, target genes are not expressed. As the population increases and the concentration of AHLs reaches a threshold concentration, they bind to the regulator protein, resulting in dimerization. The LuxR-AHL complex is now able to recognize regulatory sequences, called "*lux*-boxes", in the promoter region of target genes, controlling gene expression. See text for references.

1.10.1. AHL signalling molecules

AHLs are synthesized in various forms, all consisting of a homoserine lactone core and an acyl tail (Ng & Bassler, 2009). The variation resides in the length of the acyl tail, ranging from 4 to 20 carbons, and the functional group situated at carbon 3, which can be a hydroxy group, an oxo group or consist of no substitution at all (Rajput *et al*., 2016). The Bauer group reported that *P. chlororaphis* subsp. *aurantiaca* PB-St2 harbours four distinct QS systems, namely Phz, Csa, Aur and Hdts (Bauer *et al*., 2016). In AHL profiling experiments AurI was discovered to be the only synthase generating AHLs with an "oxo" functional group. Additionally, while Hdts did not produce any AHLs, there was significant overlap seen in signals produced by the Phz, Csa and Aur QS systems. Two AHLs, C6-HSL and C8-HSL were produced by all three systems, suggesting the possibility of cross-communication but this was not explored.

1.10.2. QS intercellular signalling

There have been approximately 20 different AHLs identified to date produced by numerous bacterial strains (Rajput *et al*., 2016). Though the specificity of the LuxR protein for its cognate AHLs is quite high, there is evidence that some LuxR proteins may be promiscuous (Wellington & Greenberg, 2019). Considering that most bacteria exist within polymicrobial communities, it can be speculated that there is some degree of crosstalk from one QS system to another (Wellington & Greenberg, 2019). A well-known example of the ability of one bacterium to detect and respond to AHLs secreted from another is the autoinducer reporter *Chromobacterium violaceum* strain CV026. The parent strain has a functional QS system (Cvi), which activates production of a purple-pigmented compound called violacein in the presence of AHLs with 4- to 8-carbon acyl side chains. *C. violaceum* CV026 has a non-functional AHL-

synthase gene (*cviI*) and the production of violacein is induced in the presence of exogenous AHLs of the same form (McClean *et al*., 1997). This clearly demonstrates QS crosstalk and the simple outcome that is seen with CV026 has made this strain an extremely valuable tool for detecting AHL production. A study carried out by Wellington and Greenberg (2019) tested LuxR protein selectivity to 19 naturally synthesized AHLs. The LuxR proteins examined belong to well-characterized QS systems from *P. aeruginosa, Burkholderia thailandensis, V. fischerii* and *C. violaceum* and were screened for both activation and repression by exogenous AHLs. It was concluded that signalling molecule selectivity varied between species and aspects dictating affinity for the LuxR proteins ranged from the length of the acyl chains to the functional groups located at the 3rd carbon (Wellington & Greenberg, 2019).

1.10.3. QS in *Pseudomonas* biocontrol

The Phz QS system has been investigated in a number of biocontrol pseudomonads, where it plays an important role in the ability of these organisms to combat fungal disease through the production and secretion of secondary metabolites (Chin-A-Woeng et al. 2001; Khan et al. 2005; Mavrodi et al. 1998; Wood and Pierson III, 1996). This network consists of the AHL synthase gene *phzI,* and its cognate transcriptional regulator gene *phzR,* which lie immediately upstream of the PHZ operon. The PhzR-AHL complex putatively binds to *phz* boxes in the promoter regions of target genes. In *P. chlororaphis* PA23 both regulatory and biosynthetic genes for compounds including PRN, PHZ, HCN, exoprotease and siderophores are under Phz QS control (Shah *et al*., 2020). To better understand the scope of the PA23 QS regulon, RNA sequencing was conducted to compare gene expression patterns of a *phzR* mutant, an AHL-deficient strain and wild type. The AHL-deficient strain harbours a plasmid-

borne copy of the *aiiA* gene originating from *Bacillus* sp. A24, which encodes a lactonase enzyme that inactivates all AHL molecules. Transcriptomic profiling revealed that approximately 13% of the PA23 genome is under QS control (Shah *et al*., 2020). Moreover, only a small proportion of these genes appear to be directly regulated as most lack an upstream *phz* box sequence. Interestingly, although there were a higher number of differentially expressed genes in PA23*phzR* (8.8%) compared to the AHL-deficient strain (8.6%), a higher degree of uniquely differentially expressed genes was discovered in the latter. Inspection of the PA23 genome revealed the presence of two additional QS systems, namely Csa (*csaR, csaI)* and Aur (*aurR*, *aurI),* similar to *P. chlororaphis* subsp. *aurantiaca* PB-St2 (Bauer *et al*., 2016). Because the lactonase cleaves all AHL molecules, the AHL-deficient strain is equivalent to a *phzI*, *csaI, aurI* triple mutant. Accordingly, it is not surprising that a larger number of unique genes was shown to be differentially expressed in this background versus the *phzR* single mutant (Shah *et al*., 2020).

While the role of the Aur QS system in *P. chlororaphis* physiology has not been investigated, the CsaRI system has been characterized in *P. chlororaphis* 30-84 (Zhang & Pierson III, 2011). Despite the fact that protease, HCN and siderophore production are all under QS control in 30-84, when genes from the Phz QS system were disrupted, wild type levels of metabolites were produced. These findings suggested that there might be a second QS system influencing gene expression (Zhang & Pierson III, 2001). Single mutants (*csaR, csaI*, *phzR* and *phzI*) and a series of double mutants (*phzRcsaR*, *phzRcsaI, phzIcsaR* and *phzIcsaI*) were generated and phenotypic assays conducted to investigate how this network affects 30-84 physiology. It was discovered that this QS system primarily regulates genes involved in cell

surface properties, and so it was named Csa (Cell Surface Alterations). The Csa system also works in concert with the Phz QS system to regulate protease production and influence rhizosphere competence (Zhang & Pierson III, 2001).

1.10.4. Regulation of QS systems

As mentioned earlier, QS forms part of a regulatory cascade that governs expression of compounds involved in PA23 biocontrol. Besides controlling production of these exometabolites, substantial interaction exists between the regulators themselves. For example, RpoS works in concert with several other regulators, like the Gac-Rsm two-component system, the SR, the Phz QS system and other transcriptional regulators like PsrA, to control genes governing secondary metabolite production (Poritsanos *et al*., 2006). PRN production is positively controlled by the Phz QS system and is conversely negatively regulated by RpoS. It has also been demonstrated that RpoS activates expression of *phzI* and represses expression of *phzR* and that lack of a functional Phz QS system leads to a decrease in *rpoS* expression (Selin *et al*., 2012). The significant overlap of target gene expression that seems to be controlled by both systems clearly indicates cross-regulation is occurring at both high cell density and during the stationary phase of growth (Bertani & Venturi, 2004). RpoS has been shown to directly control gene expression of *rsmE* and indirectly control gene expression of *rsmZ*, likely through PsrA (Selin *et al*., 2014), indicating the cross-regulation that exists between PsrA, RpoS and the Gac-Rsm system.

In many cases, QS systems self regulate via a positive autoinduction feedback loop. This was observed in PA23 where *phzR* and *phzI* were found to be positively autoregulated and *phz* boxes were identified upstream of both genes (Selin *et al*., 2012). Additionally, *phz* boxes were

identified in the promoter region of *csaI* and *csaR,* but not *aurI* and *aurR*. Quantitative PCR (qPCR) analysis of PA23*phzR* and the AHL-deficient strain revealed downregulated gene expression of both *csaI* and *csaR*, indicating that the Phz QS system plays a role in regulating the Csa QS system. qPCR analysis also revealed that *aurI* expression was upregulated and *aurR* expression was downregulated in the AHL-deficient strain but in PA23*phzR*, there was no significant difference in expression of either gene compared to wild type (Shah *et al*., 2020). The fact that there are a significant number of unique genes showing altered expression in the AHL-deficient strain compared to PA23*phzR* suggests there is notable complexity within the regulatory hierarchy and the Csa and Aur QS systems likely play an integral role in PA23 biocontrol.

1.11. Thesis objectives

In *P. chlororaphis* strain PA23, a highly complex network of regulators tightly controls production of AF metabolites. The Phz QS system enables PA23 to coordinate expression of biocontrol compounds in accordance with population density. This bacterium has two additional QS circuits, CsaIR and AurIR. In the related bacterium, *P. chlororaphis* 30-84, the Csa system is important for the regulation of cell surface properties, exoprotease production and rhizosphere competence (Zhang & Pierson III, 2001). Thus far, the role of the Csa system in PA23 physiology has not yet been explored, which forms the basis of this research. The specific thesis objectives are as follows:

1. To investigate the role played by the Csa QS system in the regulation of secondary metabolite production in *P. chlororaphis* PA23.

2. To inform on how the Csa QS system affects expression of regulatory and biosynthetic genes involved in PA23 biocontrol.

2. MATERIALS AND METHODS

2.1. Bacterial growth conditions

All bacterial strains used in this research are listed in Table 1. *P. chlororaphis* PA23 and derivative strains as well as *C. violaceum* strain CV026 were cultured at 28°C; *Escherichia coli* strains were cultured at 37°C. All bacteria were grown and maintained on LB agar or in LB broth (BD Difco, Ottawa, Canada). *S. sclerotiorum* was grown and maintained on Potato-Dextrose Agar (PDA) plates (BD Difco). For phenotypic analysis and cDNA library synthesis, strains were grown in M9 minimal media (BD Difco) supplemented with 0.2% glucose and 1mM $MgSO_4$ (M9-glc). When required, antibiotics were added at the following concentrations: ampicillin (Amp, 100 mg/mL), chloramphenicol (Cm 25 mg/mL), gentamicin (Gm, 20 mg/mL), kanamycin (Kan 30 mg/mL), piperacillin (Pip, 40 mg/mL), rifampicin (Rif, 100 mg/mL) and tetracycline (Tc, 15 mg/mL) and, which were purchased from Research Products International Corp (Mt. Prospect, IL, USA).

2.2. Nucleic acid manipulations

2.2.1. Genomic and plasmid DNA extractions

Genomic and plasmid isolation and purification involving PA23 strains was performed according to the protocols described in Sambrook *et al.* (1989). Plasmid isolation and purification pertaining to *E. coli* was conducted using the Froggabio DNA Purification Kit (Froggabio, Concord, ON, Canada).

Table 1. Bacterial strains and plasmids

Strains	**Relevant Genotypes**	**Source/Reference**
Pseudomonas chlororaphis		
PA23	PHZ+PRN+RifR wild type (soybean root tip isolate)	Savchuk & Fernando (2004)
PA23*phzR*	PA23 with GmR cassette inserted into the *phzR* gene	Selin *et al.* (2012)
PA23*phzI*	PA23 Δ*phzI* deletion mutant	This study
PA23*csaR*	PA23 with pKNOCK-Tc vector inserted into *csaR* gene, Tet^R	This study
PA23*csaI*	PA23 with pKNOCK-Tc vector inserted into *csaI* gene, Tet^R	This study
PA23*phzRcsaR*	PA23*phzR* with pKnock-Tc-*csaR* vector inserted into *csaR* gene, Tet^R	This study
PA23*phzIcsaI*	PA23*phzI* with pKnock-Tc-*csaI* vector inserted into *csaI* gene, Tet^R	This study
PA23--AHL	PA23 carrying pME6863; rendering it AHL-deficient	Selin et al. (2012)
Escherichia coli		
DH5α	*supE44 ΔU169 (φ80lacZΔM15) hadR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco
DH5α λpir	DH5 λpir lysogen of DH5α	House *et al.* (2004)
Chromobacterium violaceum		
CV026	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31532; autoinducer biosensor	Latifi *et al*. (1995)
Plasmids		
pME6863	pME6000 carrying *aiiA* gene from Bacillus sp. A24 under P_{lac} promoter	Reimmann *et al*. (2002)
pCR2.1	TA-cloning vector, Amp^R	Invitrogen
pCR-*csaR*	644-bp internal PA23 *csaR* fragment in pCR2.1	This study
PCR-*csaI*	570-bp internal PA23 csaI fragment in pCR2.1	This study
pKNOCK-Tc	Suicide vector for insertional mutagenesis; R6K ori Rp4 oriT Tet^R	Alexeyev (1999)
pKNOCK-Tc-*csaR*	644-bp internal fragment of csaR cloned into pKNOCK-Tc	This study
pKNOCK-Tc-*csaI*	570-bp internal fragment of csaI cloned into pKNOCK-Tc	This study
pTNS2	Plasmid expressing transposon insertion machinery	Choi et al. (2005)
pRK600	Mobilization helper plasmid, Chl^R	Finan *et al*. (1986)
pRK2013	Mobilization helper plasmid, Kan^R	Figurski & Helinski (1979)

pEX18-Tc	Suicide plasmid, Tet^R	Hoang *et al,* (1998)
pEX18Tc-*phzI*	Gene fragment containing *phzI* gene cloned into pEX18-Tc, Tet^R	This study
pUCP22	Broad-host-range vector, Amp^R, Gm^R	West *et al.* (1994)
pUCP23	Broad-host-range vector, Amp^R, Gm^R	West *et al.* (1994)
pUCP23-*phzR*	1.68 kb fragment containing *phzR* in pUCP23	Selin *et al*. (2012)
pUCP22*csaR*	804-bp fragment containing *csaR* in pUCP22	This study
pUCP22*csaI*	746-bp fragment containing *csaI* in pUCP22	This study
pUCP22*phzI*	639-bp fragment containing *phzI* in pUCP22	This study
pUC18T-mini-Tn7T-LAC-Gm	Single-copy insertion vector, Gm^R, Amp^R	Choi *et al.* (2005)
pUC18T-mini-TN7T-LAC-Gm-*phzRA*	1.68-kb fragment containing *phzR* in pUC18T-mini-Tn7T-LAC-Gm	This study
pUC18T-mini-Tn7T-LAC-Gm-*csaR*	1.6-kb fragment containing *csaR* in pUC18T-mini-Tn7T-LAC-Gm	This study

2.2.2. Gel extractions

Gel extractions were carried out using the Invitrogen Purelink Purification kit (Invitrogen, Waltham, Massachusetts, USA) following manufacturer's instructions.

2.2.3. Polymerase Chain Reaction

Polymerase chain reactions (PCR) were run to both amplify fragments for molecular cloning and to confirm the ligation, transformation and chromosomal integration of DNA into PA23 strains. PCR primers used in the current study are listed in Table 2. These reactions were performed using the BIO-RAD T100 Thermal Cycler (BIO-RAD, Mississauga, ON, Canada) and the Q5 High-Fidelity DNA Polymerase reaction kit (NEB, Ipswich, USA), following the protocols and instructions provided with the DNA polymerase. In the case that PCR amplification was accomplished using a bacterial colony suspension instead of purified genomic or plasmid DNA, 100 µl colony suspensions were loaded onto the thermal cycler and heated to 98°C for 10 minutes prior to including it as the template for the subsequent PCR reaction.

2.2.4. Restriction digest and ligations

Restriction digests were conducted using ThermoFisher Anza Restriction Enzymes (ThermoFisher, Waltham, Massachusetts, USA) according to manufacturer's instructions. Enzymatic reactions were subjected to a clean-up step using the Purelink Purification Kit (Invitrogen) or through gel electrophoresis and purification.

Table 2. Oligonucleotide sequences

Primer	**Sequence**	**Source/Reference**
Cloning		
csaR-KO1-F	5'-CATGCACTGGTGGAACGACC-3'	This study
csaR-KO1-R	5'-GCCAGAGTCTTGTTGGCTGC-3'	This study
csaR-pKnock-R2	5'-CTGACGCTTCATCCGACGAT-3'	This study
csaI-KO1-F	5'-ATGCCTGATTCAGCGAAGCC-3'	This study
csaI-KO1-R	5'-AAAGCCAGCTTTCACCGACC-3'	This study
csaI-pKnock-R2	5'-CTGACGCTTCATCCGACGAT-3'	This study
pKnock-R2	5'-TGAAGAAGGAACACCCGCTC-3'	
phzI UP-F	5'-GACGCGGTACCGTTCTACGCGAATAATGCGCG-3'	This study
phzI UP-R	5'CAAAAAGGACGACTCGGCCCGTATCACATTGCTGGG GTTCCTTAGGGA-3'	This study
phzI DOWN-F	5'-TGATACGGGCCGAGTCGTC-3'	This study
phzI DOWN-R	5'-CCGGGGATCCGCGGACCTGTGGTCTGAAG-3'	This study
phzI-ext-insert F1	5'-GATTCCACCGAAGCCCCTACCAG-3'	This study
phzI-ext-insert R1	5'-CGCGTGCAGGCAGTGTCTTAC-3'	This study
pUCP22-csaI-F	5'-GGCCAAGCTTCCAACGTGTTCGTCACCCT-3'	This study
pUCP22-csaI-R	5'-GGGCTGGATCCGACGCTTCATCCGACGAT-3'	This study
csaR-*Hin*dIII-F2	5'-GTGAAAGCTTGCTTTCATGCCGTGAAACCAC-3'	This study
csaR-*Bam*HI -R2	5'-GCAAGGATCCAAGGCAAACGCCATGGAGAAACG-3'	This study
phzIpUCPF2-with *Kpn*I	5'-CTAAGGTACCCCAGCAATGCACATGG-3'	This study
phzIpUCPR2-with *Bam*HI	5'-AGGGGGATCCAAAAAGGACGACTCG-3'	This study
M13-F	5'-GTAAAACGACGGCCAG-3'	Invitrogen
M13-R	5'-CAGGAAACAGTATGAC-3'	Invitrogen
csaR-Tn7T-F	5'-ATAGAAAAAAGCTTAACACGTCCGCCAGCAG-3'	This study
csaR-Tn7T-R	5'-TAGCATTAGGATCCTCGAACAGGCGCTCAGG-3'	This study
FORmini-Tn7T-csaR	5'-CGTTCCACCAGTGCATGAAATCCTG-3'	This study
pUC18-mini-tn7T-backbone	5'-GCATAATTCGTGTCGGCTCAAGGCG-3'	This study
FORmini-tn7T-phzR	5'-CAGCAGTTGGGATGGGATGCGTA-3'	This study
qRT-PCR		
rpoB RT-PCR FWD	5'- CGTGTTCCTGCCGCTATC-3'	Shah et al, 2016
rpoB RT-PCR REV	5'-GCCGCAACCGAAACTACC- 3'	Shah et al, 2016
prnA RT-PCR FWD	5'- CTGTCGTCGTGCTTTCTG-3'	Shah et al, 2016
prnA RT-PCR REV	5'-GATCTCGGCGTTGAATGC-3'	Shah et al, 2016

2.3. Preparation of competent cells

2.3.1. Competent *E. coli* DH5α cells

An overnight culture of *E. coli* DH5α in LB broth was subcultured (1:100 v/v) in a final volume of 30mL of fresh LB broth and incubated at 37°C with shaking until an OD_{600} of between 0.4 and 0.5 was reached. The culture was pelleted by centrifugation at 4,000 rpm for 10 minutes at 4°C and the supernatant decanted. The remaining pellet was resuspended in $1/10^{th}$ the volume of the starting culture volume (3mL for a 30mL culture) in ice-cold, sterile TSS buffer (4.5mL LB broth, 0.5g PEG 6000/8000, 250µL dimethyl sulfoxide, 500µL 1M $MgSO_4$). Cells were transferred to sterile, pre-chilled 1.5mL Eppendorf tubes in 50µl aliquots and immediately frozen to -70°C. Cells were considered viable for transformation up to 90 days following preparation.

2.3.2. Electro-competent PA23 cells

Recipient strains were cultured overnight in LB broth and subsequently subcultured (1:100 v/v) in a total final volume of 30mL fresh LB broth. They were then incubated at 28°C with shaking until an OD_{600} of 0.5-0.8 was reached. Next, cultures were centrifuged at 4,000 rpm for 10 minutes at room temperature, the supernatant was removed, and the remaining pellet was resuspended in 30mL of sterile 300mM sucrose. The suspension was centrifuged again at 4,000 rpm for 10 minutes, and the pellet was resuspended in 6mL of 300mM sucrose. After a final centrifugation at 4,000 rpm for 10 minutes, the pellet was resuspended in 150 µl of 300mM sucrose. Electro-competent cells were utilized immediately for electroporation.

2.4. Mobilization of plasmid DNA

2.4.1. Conjugation

Tri-parental and four-parental matings were conducted in order to mobilize plasmid DNA into PA23 strains. For tri-parental matings, the recipient strain [PA23], the helper strain [*E. coli* DH5α(pRK600)] and the donor strain [*E. coli* DH5α (harbouring the vector containing gene of interest)] were cultured individually in 3mL of LB broth supplemented with selective antibiotics where appropriate, overnight at 28°C with shaking. All strains were subcultured at a 1:10 ratio in fresh LB broth and incubated at 28°C with shaking until an OD_{600} of between 0.3-0.8nm was reached. Cultures were pelleted by centrifugation at 4,000 rpm for 10 minutes, the supernatant was removed, and the pellet was resuspended in 5mL fresh LB broth and the process was repeated again. The bacterial strains were mixed in sterile 1.5-mL Eppendorf tubes in 3 different ratios (1 = 300µL; donor:recipient:helper): 1:1:1, 1:2:1, 2:1:1. The mating mixtures were centrifuged for 5 minutes at 6,000 rpm, supernatant was removed and cells were resuspended in 100µL of fresh LB broth. The entirety of each mating reaction was then spotted onto LB agar supplemented with 10mM $MgSO_4$ and incubated upright at 28°C overnight. Cells were removed from the plate and resuspended in 1mL 1X PBS buffer (pH 7.4). After centrifuging at 6,000 rpm for 5 minutes, the supernatant was removed, and the pellet was resuspended in 1mL of fresh 1X PBS buffer (pH 7.4). Serial dilutions (undiluted, 10^{-1}, 10^{-2}, 10^{-3}) of the final mating mixture were plated on selective media and grown at 28°C until sufficient colony growth was present. The four-parental mating was carried out as described by Choi & Schweizer (2006), and involved a recipient [PA23], 2 helper strains [*E. coli* DH5α(pRK2013/pTNS2)] and the donor strain [*E. coli* DH5α (harbouring the vector containing

gene of interest)]. All strains were grown overnight in LB broth with corresponding antibiotics at 28°C with shaking. A 100µL volume of each of the 4 strains was combined in a sterile 1.5mL eppendorf tube along with 600µL of LB broth. Cell mixtures were spun down by centrifugation at 6,000 rpm for 5 minutes, the supernatant was removed, and cells were resuspended in fresh LB broth. The process was repeated, and the pellet was resuspended in final volume of 100µL fresh LB broth. The 100µl aliquot was then plated on LB agar supplemented with 10mM $MgSO_4$ and incubated overnight at 28°C. The remaining steps were identical to those outlined in the triparental mating protocol.

2.4.2. Transformation of chemically competent cells

Approximately 1-5µg of purified plasmid DNA was added to 50µL of chemically competent *E. coli* cells in a sterile 1.5mL eppendorf tube and placed on ice for 30 minutes. Cells were then heat-shocked at 42°C for 1 minute and placed on ice for an additional 5 minutes, after which 950µL of fresh LB broth was added and cells were incubated at 37°C with shaking. Serial dilutions (undiluted, 10^{-1}, 10^{-2}, 10^{-3}) were plated on selective media and incubated overnight at 37°C.

2.4.3. Transformation of electro-competent cells

A 40µL volume of electro-competent cells (PA23 strains) and 1-5µL of purified plasmid DNA was transferred to the inner well of an electroporation cuvette (BIO-RAD, Mississauga, ON, Canada). The cuvette was placed in the electroporation chamber of a BTX ECM600 Electro Cell Manipulator (BTX, Holliston, MA). The following parameters were set on the device: 1.6 kV/cm voltage; 25 µFD capacitance and 200 ohms resistance. The “pulse” button was pressed to discharge the electrical current, the cuvette was removed from the chamber and 1mL of fresh

LB broth was added directly into the cuvette. The reaction mixture was mixed gently with a micropipette before transferring the entire reaction volume to 2mL of LB broth in a test tube. Cells were incubated with shaking at 28°C for 60 minutes. A 100µL volume of both undiluted and a 10^{-1} dilution were plated on selective media. Additionally, the remaining suspension was centrifuged at 6,000 rpm, the supernatant was removed, and the pellet resuspended in 100µL of fresh LB broth and plated on selective media.

2.5. Generation of mutants

2.5.1. Generation of PA23*csaR* and PA23*csaI*

Individually, the AHL-synthase gene (*csaI*) and the transcriptional regulator (*csaR*) of the Csa QS system were targeted and interrupted using the pKnock insertional mutagenesis system. To create PA23*csaR*, primers (csaR-KO1-F/csaR-KO1-R) were designed to amplify a 652-bp fragment, which was then cloned into pCR2.1-TOPO, generating pCR-*csaR*. Successful cloning was confirmed with the same set of primers. pCR-*csaR* was digested with *Hin*dIII and *Bam*HI and the resulting 644-bp fragment containing *csaR* was subcloned into the same restriction sites of the suicide vector pKnock-Tc, generating pKnock-Tc-*csaR*. This vector was then mobilized into *E. coli* DH5α λpir and plated on LB agar supplemented with Tc to select for transformants. To mobilize this suicide plasmid into PA23 wild type, a triparental mating was performed, including *E. coli* DH5α-pKnock-Tc-*csaR* as the donor and *E. coli* DH5α-pRK600 as the helper strain. Transconjugants were selected for by plating on LB agar supplemented with Rif and Tc. Putative mutants were screened via PCR using an internal primer, designed to anneal to the pKnock backbone (pKnock-R2) and an external primer, designed to anneal to the upstream flanking

region of the chromosomal *csaR* gene (csaR-pknock-R1). The same procedure was followed to create PA23*csaI,* except primers specific to *csaI* (csaI-KO1-F/csaI-KO1-R) were used to generate a 570-bp amplicon, which was cloned into pCR2.1-TOPO generating pCR-*csaI*. An external primer designed to anneal to the upstream flanking region of the chromosomal *csaI* gene (csaI-pKnock-R2) was used in conjunction with the internal primer (pKnock-R2) to screen for putative mutants.

2.5.3. Generation of PA23*phzI*

An in-frame deletion of *phzI* in PA23 was created using allelic exchange as outlined by Hmelo and colleagues (2015). One set of primers (phzI UP-F/phzI UP-R) was designed to amplify the 450-bp region directly upstream of *phzI* and another (phzI DOWN-F/phzI DOWN-R), to amplify the 450-bp region directly downstream of *phzI*. Recognition sequences for 2 restriction enzymes, *Kpn*I and *Bam*HI, were built in to the 5' ends of phzI UP-F and phzI DOWN-R respectively, to allow for directional cloning into the suicide vector. The 3' end of phzI UP-R contains a 27-bp sequence that has reverse-complementarity to the phzI DOWN-F primer. Both flanking fragments were amplified using the Q5 DNA polymerase system (NEB) with a 30-second denaturation at 98°C, followed by 35 cycles consisting of a 10-second denaturation at 98°C, 15-second annealing at 62.2°C, a 30-second extension at 72°C and a final extension at 72°C for 5 minutes (Hmelo *et al*., 2015). The reaction products were purified using the Invitrogen Clean-up kit (Invitrogen). Both fragments were used as template DNA in a splicing-overlap-extension (SOE) PCR reaction using High-fidelity Q5 DNA polymerase (NEB) following the same PCR program mentioned previously, except with an annealing temperature of 71.0°C. The newly assembled *phzI*-SOE fragment was then analyzed by gel electrophoresis to confirm

the desired fragment size of 900 bp. The *phzI*-SOE amplicon and the pEX18Tc suicide vector were subjected to restriction digest by *Bam*HI and *Kpn*I, followed by an overnight ligation reaction using the T4 DNA ligase system (NEB). The ligation mixture was transformed into chemically competent *E. coli* DH5α cells, which were plated on LB agar supplemented with Tc to select for transformants containing the desired deletion construct (pEX18Tc-*phzI*). Plasmids were extracted from colonies displaying resistance to Tc and the presence of the *phzI*-SOE fragment was confirmed through PCR amplification using the phzI UP-F and phzI DOWN-R primers. PA23 wild type was combined with *E. coli* DH5α(pEX18Tc-*phzI*) as the donor strain and *E. coli* DH5α(pRK600) as the helper strain in a triparental mating reaction. Transconjugants were selected by plating the mating reaction on LB agar supplemented with Rif and Tc. Putative *phzI* merodiploids, generated via a single crossover event, were screened by PCR amplification of the chromosomal *phzI* gene using primers phzI-ext-insert F1 and phzI-ext-insert R1. To counter select against merodiploids, bacteria were streaked onto no-salt LB agar containing 15% sucrose (w/v). Colonies showing sensitivity to Tc and resistance to sucrose were considered to harbour the chromosomal *phzI* deletion allele. Allelic exchange of the wild-type gene with the deletion allele was confirmed by PCR using primers that anneal to the chromosomal *phzI* flanking regions (phzI ext insert F1 and phzI ext insert R1). An amplicon corresponding to the size of the intact *phzI* gene (~784 bp) indicated unsuccessful allelic exchange and an amplicon corresponding to the absence of *phzI* (~154 bp) indicated successful allelic exchange and deletion of *phzI*.

2.5.2. Generation of PA23*phzRcsaR* and PA23*phzIcsaI*

To create the PA23*phzRcsaR* double mutant, pKnock-Tc-*csaR* was mobilized into the PA23*phzR* strain through a triparental mating, using *E. coli* DH5α(pRK600) as the helper strain. The mating was plated on LB supplemented with Rif and Tc and putative mutants were screened via PCR using pKnock-R2 and csaR-pKnock-R1. The same triparental mating procedure was used to generate the PA23*phzIcsaI* double mutant, where pKnock-Tc-*csaI* was mobilized into the PA23*phzI* strain. Transconjugants were selected by plating on LB agar supplemented with Rif and Tc. Putative double mutants were screened via PCR using primers pKnock-R2 and csaI-pKnock-R2.

2.6. Complementation of mutant strains

2.6.1. Complementation of PA23*csaI,* PA23*phzI,* PA23*csaR* and PA23*phzR*

To complement mutations in the four QS genes, a wild-type copy of the interrupted gene was expressed from a multi-copy cloning vector (pUCP22). The same system was employed to cross-complement mutations, meaning that *csaI* was expressed *in trans* in the PA23*phzI* background, and *vice versa*. Similarly, *csaR* and *phzR* were cloned for complementation and cross-complementation of their respective mutants. To generate pUCP22-*csaI*, a 746-bp amplicon containing the entire coding sequence of *csaI* was generated using pUCP22-csaI-F2 and pUCP22-csaI-R2. To facilitate directional cloning, the 5’ ends of both forward and reverse primers contained the recognition sequence for *Hin*dIII and *Bam*HI, respectively. The fragment was then digested with *Bam*HI and *Hin*dIII and cloned into the same

sites of pUCP22 under control of the lac-promoter, generating pUCP22-*csaI.* This vector was then mobilized into chemically competent *E. coli* DH5α and transformants were selected using LB agar supplemented with Gm. Plasmids were purified from colonies showing resistance to Gm and subsequently screened by PCR utilizing the same primers used to generate the original 746-bp amplicon. Once confirmed, pUCP22*csaI* was mobilized into electro-competent PA23*csaI* and PA23*phzI* cells via electroporation. Reactions were plated on LB agar supplemented with Gm to select for transformants. Colonies showing resistance to Gm were screened for the insert by PCR using commercial M13-F and M13-R primers. Generation of pUCP*csaR* and pUCP*phzI* and mutant complementation was carried out as described above, with a few modifications. Specifically, [*phzI(*phzIpUCPF2-with *Kpn*I/phzIpUCPR-with *Bam*HI) and *csaR*(csaR-*Hin*dIII-F1/csaR-*Bam*HI-R1)] were used to amplify the original gene fragments [*phzI*(639 bp) and *csaR*(818 bp)] and to confirm the mobilization of pUCP*phzI* and pUCP*csaR* into various backgrounds. pUCP23-*phzR,* which was previously generated by Selin *et al.* (2012), was mobilized into PA23*csaR* and PA23*phzR* as described above.

2.6.2. pUC18-mini-Tn7 for complementation

Due to toxicity issues associated with multiple copies of *phzR* and *csaR in trans*, a mini-Tn7 system was used to create single copy chromosomal insertions of these genes for complementation (Choi *et al*., 2005). Primers were designed to amplify the entire coding sequence of *csaR,* including ~650 bp upstream in order to account for the putative promoter-binding site. The 5' end of primers csaR-Tn7-F and csaR-Tn7-R contained the recognition sequence of *Hin*dIII and *Bam*HI, respectively. Following PCR amplification, the resulting 1.68-kb fragment was digested and directionally cloned into the corresponding sites of a suicide cloning

vector, generating pUC18-mini-Tn7T-LAC-Gm-*csaR*, which was thereafter transformed into *E. coli* DH5α. Plating cells on LB agar with Amp was used to select for transformants. Plasmids were extracted and purified from colonies showing resistance to Amp and the construct was confirmed by PCR amplification using the primers used to generate the original fragment. In order to mobilize pUC18-mini-Tn7T-LAC-Gm-*csaR* into PA23*csaR* and PA23*phzR*, these strains were mixed with two helper strains, *E. coli* DH5α(pRK2013/pTNS2) in a mating reaction. After 24 hours' incubation at 28°C on LB agar supplemented with 10mM $MgSO_4$, cells were plated on LB with Rif and Gm to select for transconjugants. Successful integration of pUC18T-mini-Tn7T-LAC-Gm-*csaR* into the chromosome was confirmed via PCR amplification using the FORmini-Tn7T-csaR primer and pUC18-mini-Tn7T-backbone primer, the latter having been designed to anneal to pUC18-mini-Tn7T-LAC-Gm. The same procedure was used to mobilize pUC18T-mini-Tn7T-LAC-Gm-*phzR* into PA23*phzR* and PA23*csaR.* In this case, the primer (FORmini-Tn7T-phzR) was used in combination with the pUC18-mini-Tn7T-backbone primer to confirm the integration of the vector. The *phzR* gene, including the promoter region, was excised as a 1.6kb *Bam*HI-*Hin*dIII fragment from pUCP23*phzR* (Selin *et al*., 2012) and cloned into the same sites of pUC18-mini-Tn7T-LAC-Gm.

2.7. Growth curves

PA23 wild type, the AHL-deficient strain and all mutants were subjected to growth rate analysis in order to determine at which time point the stationary phase of growth is reached. All strains were cultured in M9-glc and incubated overnight at 28°C with shaking. Bacteria were then subcultured into fresh M9-glc at a starting OD_{600} of 0.1 and incubated at 28°C with

shaking. OD_{600} readings were taken at 8-, 12-, 15-, 18-, 21-, 23- and 25-hours postinoculation. All strains were analyzed as three biological replicates in

2.8. Phenotypic assays

2.8.1. Antifungal activity

S. sclerotiorum was propagated approximately four days prior to the anticipated execution of the fungal radial diffusion assay. To obtain newly growing mycelia, the outer surface of a dormant sclerotial body was surface sterilized in a 10% bleach solution and then rinsed in sterile distilled water. After "sterilization", sclerotia were cut in half and placed cut-side down onto the centre of a full-strength PDA plate. Plates were wrapped with parafilm and incubated for 96 hours at room temperature (~21°C). At the 48-hour time point of *S. sclerotiorum* propagation, the PA23 strains were inoculated into M9-glc and incubated overnight at 28°C with shaking. Cultures were standardized to an OD_{600} of 1.0, and a 5µl aliquot was spotted onto $1/5^{th}$ strength PDA and incubated at 28°C for 24-hours. A plug containing fungal mycelia was placed in the centre of the plate inoculated with the bacterial strains, mycelia-face down. Plates were then incubated at 21°C. AF activity was assessed by measuring the zone of inhibition, or the distance between the edge of the bacterial colony and the fungal growth front (Poritsanos *et al*., 2006). Three technical replicates were measured for each strain and the experiment was repeated three times.

2.8.2. Autoinducer reporter assay

The bacterial reporter strain, *C. violaceum* CV026, was used to semi-quantitatively assess AHL production in the PA23 strains. In the presence of exogenous AHLs with side-chain

lengths ranging from 4 to 8 carbons, CV026 produces a violet-pigmented compound (violacein), which diffuses into the surrounding media in the form of a measurable halo (Latifi *et al*., 1995). Overnight cultures, grown in M9-glc broth, were standardized to an OD_{600} of 1.0 and 5µl was spotted onto LB agar that had been seeded with an overnight culture of *C. violaceum* CV026. Plates were incubated for 24 hours at 28°C, at which point the diameter of the violet halo was measured. Three technical and 3 biological replicates were analyzed for each strain. 2.8.3.

Protease production assay

Bacterial strains were grown overnight in M9-glc broth at 28°C with shaking. Cultures were standardized to an OD_{600} of 1.0 and a 5µl aliquot was spotted onto 2% skim milk agar and incubated at 28°C for 48-72 hours. Proteolytic activity was semi-quantitatively determined by measuring the zone of clearing surrounding the edge of the bacterial colony (Poritsanos *et al*., 2006). Each strain was spotted in triplicate and the experiment was repeated with 3 biological replicates.

2.8.4. Chitinase assay

Chitinase activity was monitored following a protocol described by Wirth & Wolf (1990). Strains were grown overnight in M9-glc at 28°C with shaking. Bacteria were then subcultured into fresh M9-glc at a starting OD_{600} of 0.1 and incubated at 28°C until the stationary-phase of growth was reached. A 250µl volume of cell-free supernatant from each culture was combined with equal volumes of 0.1M sodium acetate (pH 5.2) and carboxymethyl-chitin-Remazol brilliant violet aqueous solution (Blue Substrates, Göttingen, Germany) in a sterile 1.5mL eppendorf tube and incubated statically at 28°C for 1 hour. Adding 250µl of a 1M HCl solution to each tube stopped the reactions. Tubes were placed on ice for 10 minutes and then

centrifuged at 12,000 xg for 10 minutes. A 250µl aliquot from each reaction was transferred to a 96-well plate from which absorbance readings were taken at a wavelength of 550nm. Three technical and 5 biological replicates were analyzed for each strain. 2.8.5. Swimming assay

Strains were grown overnight in M9-glc broth at 28°C with shaking, after which cultures were standardized to an OD_{600} of 1.0. A sterile, pointed-end toothpick dipped into the culture was used to stab-inoculate plates containing M9-glc media, solidified with 0.3% agar. Flagellar-mediated ((swimming) motility was assessed by measuring the swim zone diameter (Poritsanos *et al*., 2006). Three technical and 3 biological replicates were analyzed for each strain.

2.8.6. HCN assay

The ability of PA23 and the QS derivatives to produce HCN was determined using Cyantesmo paper (Machery-Nagel GmbH & Co). Strains grown overnight in M9-glc broth at 28°C with shaking were standardized to an OD_{600} of 1.0 and subsequently inoculated onto M9-glc agar to obtain a lawn of bacterial growth. A strip of Cyantesmo paper was affixed to the inside of the lid of the petri dish after which plates were sealed with parafilm and incubated at 28°C for 24 hours. A change in color observed on the Cyantesmo paper from colorless to blue was indicative of HCN production. Three technical and 3 biological replicates were analyzed for each strain.

2.8.9. Biofilm formation assay

The ability of PA23 derivative strains to successfully form biofilms was assessed by employing the protocol previously outlined by Berry *et al.* (2010) with slight modifications. Strains were grown overnight in M9-glc at 28°C with shaking and then diluted 1:100 in fresh

media. A 200µL aliquot of each strain was transferred to 6 separate wells in a 96 well polystyrene, round-bottom microtiter plate (Sarstedt, Montreal, QC, Canada). Following stationary incubation for 48 hours at 28°C, a 25µL aliquot of a 1% Crystal Violet (CV) solution was added to each well. After 15 minutes of incubation at room-temperature, wells were washed 3 times with sterile water to remove any unbound cells. The remaining CV fixed within the adherent biomass was solubilized by adding a 200µL aliquot of 95% ethanol to each well. Optical density was then measured at a wavelength of 550nm. Six technical replicates were tested for each strain and the experiment was repeated with 5 biological replicates.

2.9. RNA extraction, DNase treatment and cDNA synthesis

Bacterial strains were inoculated in M9-glc and incubated overnight at 28°C with shaking. The OD_{600} was adjusted to 0.1 in fresh M9-glc and resulting cultures were incubated at 28°C with shaking until the stationary phase of growth was reached. Qiagen RNAprotect (Qiagen, Valencia, USA) was combined in a 2:1 ratio with each culture in an RNase-free, 1.5mL eppendorf tube. Each tube was mixed using a vortex and then statically incubated at room temperature for 5 minutes. Immediately following incubation, RNA was extracted with the Qiagen RNeasy Mini Kit (Qiagen, Valencia, USA) following the manufacturer's protocol. RNA quality was assessed by analysis using a Nanodrop 2000 spectrophotometer and only samples with a 260/280-ratio value between 2.0-2.2 were used. A total of 4µg from each RNA extraction reaction was then treated with Ambion TURBO DNA-free DNase (ThermoFisher), according to manufacturer's instructions, to remove any residual genomic DNA. Complementary DNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis Kit (ThermoFisher) through reverse transcription (RT) using random hexamer primers in total reaction volumes of

20µl. Alongside cDNA synthesis, a no-RT control was also synthesized for each strain to detect genomic DNA contamination. The thermal cycler programme was set according to manufacturer's instructions as follows: initial heating at 25°C for 10 minutes, reverse-transcription at 50°C for 15 minutes followed by an enzyme denaturation step for 5 minutes at 85°C.

2.10. Quantitative reverse transcriptase PCR (qRT-PCR)

qRT-PCR reactions were run on a CFX96 Connect™ Real-Time PCR Detection System (BIO-RAD, Hercules, USA) using the SsoAdvanced Universal SYBR Green Supermix. Each reaction had a final volume of 10µL, consisting of 0.4µL of both forward and reverse primers (12µM), 1µL of 1:5 diluted cDNA template, 5µL of SsoAdvanced Universal SYBR Green Supermix (BIO-RAD) and 3.2µL of nuclease-free water. PCR primers for genes of interest were previously designed by referencing GenBank (gi: NZ_CP008696); oligonucleotide sequences corresponding to these primers can be found in Table 2. The PCR reaction conditions were programmed as follows: initial denaturation at 98°C for 2 minutes, followed by 39 cycles of 98°C for 5 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Melt curves were performed on each primer pair according to program specifications outlined by BIO-RAD, to assess the formation of primer dimers or other artefacts. Three biological replicates per strain were analyzed in triplicate using the housekeeping gene *rpoB* as the reference and relative gene expression was calculated using the ΔΔCt method as described by Livak & Schmittgen (2001).

3. SUMMARY OF RESULTS

3.1. Generation of single QS mutants PA23*csaR*, PA23*csaI* and PA23*phzI* and double QS mutants PA23*phzRcsaR* and PA23*phzIcsaI*

In order to elucidate the role of the Csa QS system in PA23 biocontrol, mutations were generated in *csaR* and *csaI.* Both genes were separately disrupted using insertional mutagenesis to render the gene products non-functional and allow for the investigation of changes in phenotypic characteristics. Subsequent to the cloning of the target gene fragment into the pKnock-Tc suicide vector and mobilization into PA23 via triparental mating, integration of the plasmid into the chromosome was confirmed for both *csaI* and *csaR* by PCR amplification (Figure 3). The primers csaR-pKnock-R2 and csaI-pKnock-R2, which annealed upstream of *csaR* and *csaI* respectively, were paired with pKnock-R2, which annealed to the pKnock-Tc backbone, in the corresponding PCR reactions. These primer sets were designed with the intention that amplification would occur exclusively if the plasmid had integrated via homologous recombination into the PA23 chromosome. Depending on the point of cross-over, the amplicon was expected to be 1,000 - 1,300 bp for the *csaI* transconjugants and 1,200 - 1,500 bp for the *csaR* transconjugants. Nine isolates from the pKnock-Tc-*csaI* mating showed bands of approximately 1,200 – 1,300 bp and 10 isolates from the pKnock-*csaR* mating showed bands just below 1,500 bp. PA23 was included as a negative control to ensure that no amplification was occurring in the wild type background.

While a *phzR* mutant of PA23 had been previously created, multiple attempts to generate a *phzI* mutant were unsuccessful until now. Employing the two-step allelic exchange

method with SOE-PCR, a *phzI* clean deletion mutant was generated. Integration of the suicide vector pEX18Tc-*phzI* into the PA23 wild type chromosome initially resulted in a merodiploid. This first crossover event was confirmed by PCR using primers phzI-ext-insert F1 and phzI-ext-insert R1, generating two amplicons, one corresponding to the wild type (591 bp) and the other to the mutant allele (173 bp) (Figure 4A). Following counterselection, the second crossover event was confirmed by PCR using the same primer set. The absence of the wild type allele and the presence of the mutant allele indicated successful deletion by allelic exchange as demonstrated in Figure 4B. Generating the double mutants involved mobilizing pKnock-*csaR* into the PA23*phzR* background and pKnock-*csaI* into the PA23*phzI* background. Disruption of these genes was confirmed as previously described and results from the PCR amplification can be seen in Figure 5.

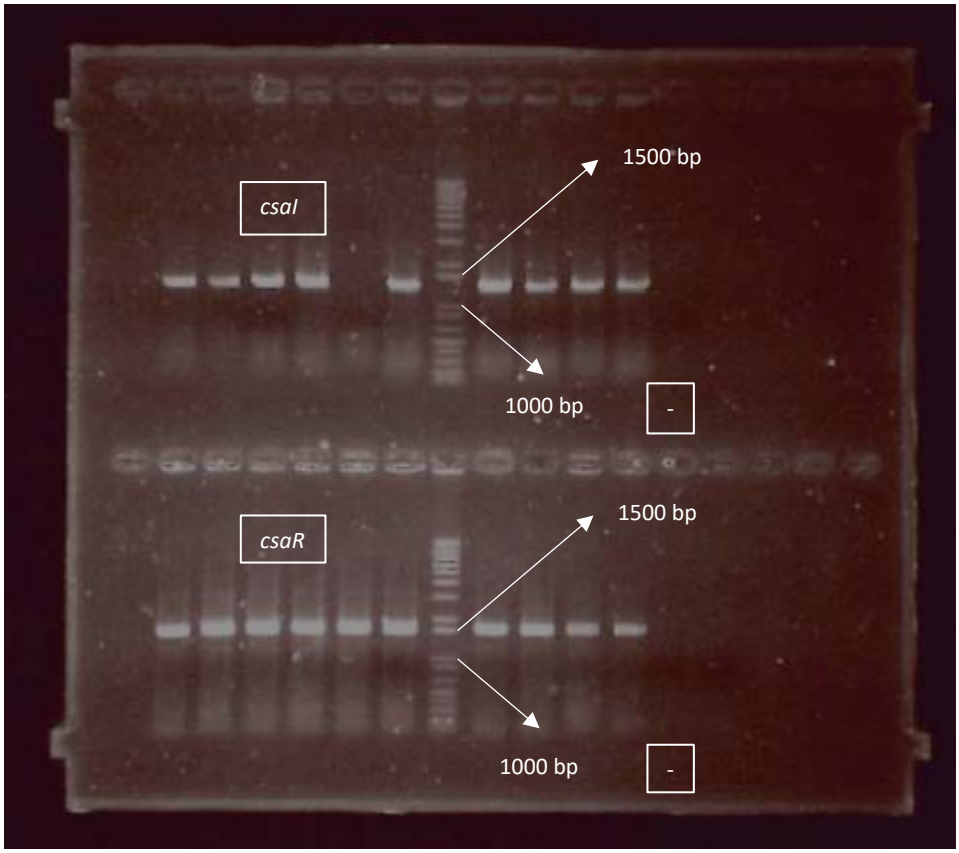
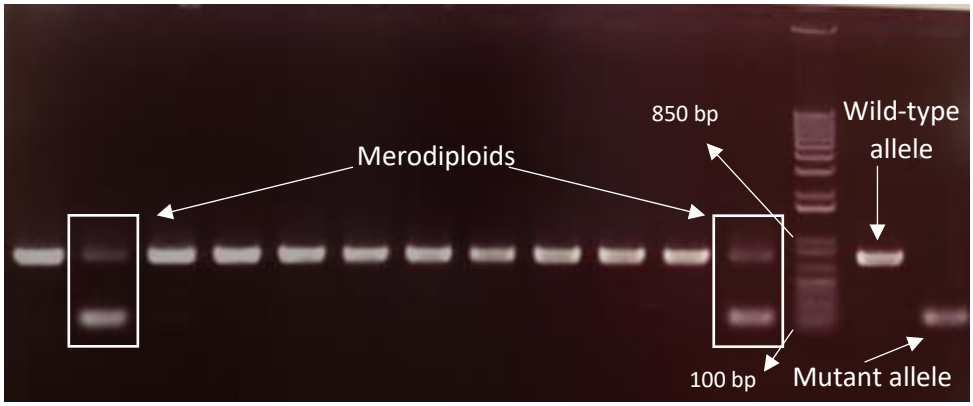


Figure 3. Screening transconjugants by PCR to confirm integration of the suicide vector pKnock-*csaI* and pKnock-*csaR* into the PA23 chromosome. (-) Represents PA23 wild type as a negative control. Twenty candidates were screened resulting in 10 successful transconjugants for *csaR* and 9 for *csaI.*

A



B

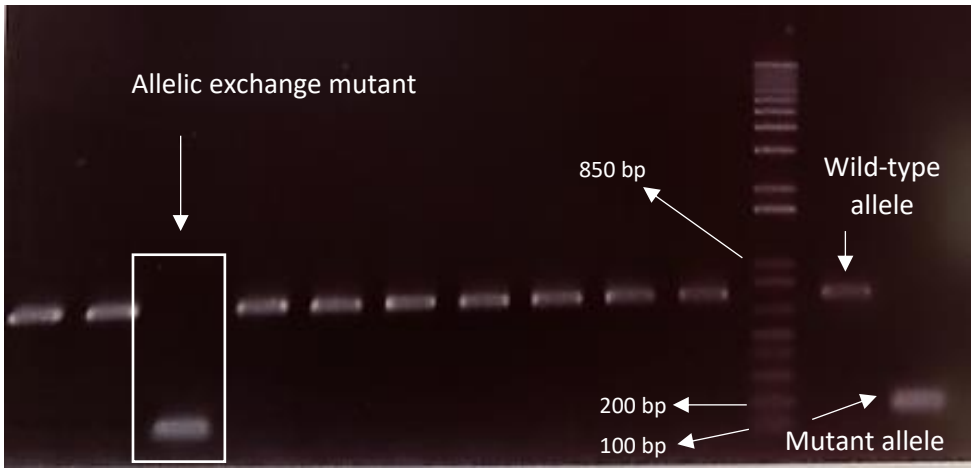
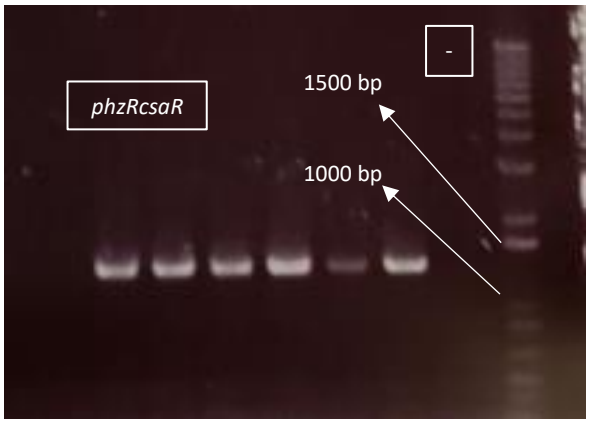


Figure 4. Generation of a PA23 *phzI* mutant by allelic exchange. (A) Confirmation of the first crossover event. The presence of both wild-type allele (590bp) and mutant allele (173bp) indicates successful generation of a merodiploid. (B) Confirmation of the second crossover event. Absence of the wild-type allele and the presence of only the mutant allele indicate successful allelic exchange.

A



B

Figure 5. Generation of QS double mutants. (A) Transconjugants were screened for the presence of pKnock-*csaR* in the PA23*phzR* background via PCR using the same primers designed to screen for the PA23*csaR* single mutant. (-) Represents PA23 wild type as a negative control. Amplification is indicative of successful integration of the suicide vector and disruption of *csaR.* (B) Transconjugants were screened for the integration of pKnock-*csaI* into the chromosome using the same primers designed to screen for the PA23*csaI* single mutant. Amplification is indicative of successful integration of the suicide vector and disruption of *csaI.*

3.2. Phenotypic characterization of the Csa QS and *phzI* mutants: Production of AF secondary metabolites

Phenotypic assays were conducted with the newly generated *phzI*, *csaI*, *csaR*, *phzIcsaI* and *phzRcsaR* mutants to determine whether these genes play a role in protease, PHZ, AHL, and chitinase production. Additionally, how these QS elements impact PA23 motility and biofilm formation was assessed.

3.2.1. Disruption of the Csa QS system in PA23 does not affect growth

Growth rate analysis was conducted to reveal at which point in time PA23 wild type and the derivative strains reached the stationary phase of growth. Strains were grown in M9-glc media, as this media was used for all of the following phenotypic assays. Compared to PA23 wild type, no changes in growth were brought on by the absence of a functional *csaR* or *csaI* gene in PA23 (Figure 6). All 3 strains reached stationary phase at the same time (~25 hours) and followed a similar pattern of growth. The *phzI* and *phzIcsaI* mutants grew similarly to wild type and the Csa QS mutants. The *phzR, phzRcsaR* mutants and the AHL-deficient strain, however, were found to enter the stationary phase of growth earlier (12 hours). This was marked by a short lag phase, followed by another peak in growth before entering the stationary phase (18 hours). Additionally, these strains attained a much lower total cell population density compared to the other strains (Figure 6).

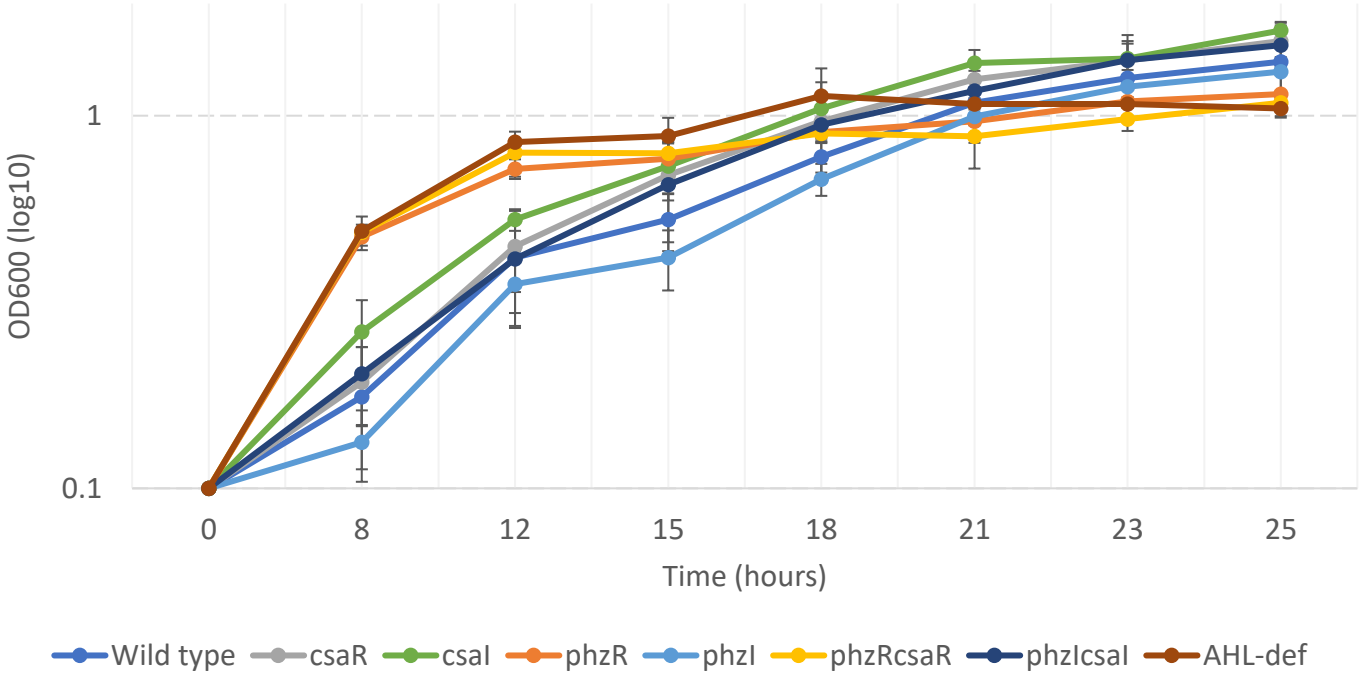


Figure 6. Growth analysis of PA23 wild type and QS mutants grown in M9-glc at 28°C. Spectrophotometric readings were taken at 600nm and each time point reading is an average of 3 biological replicates ± standard error.

3.2.2. The Csa QS system does not appear to regulate PHZ production in PA23

PHZ production is directly controlled by the Phz QS in PA23; however, whether or not the Csa QS system also plays a role in regulating production has yet to be elucidated. PA23 strains were grown in either M9-glc or LB overnight in 96-well plates as a means to qualitatively determine whether the absence of either *csaR* or *csaI* lead to any changes in the production of PHZ. Figure 7 shows that in M9-glc, PA23*csaR* and PA23*csaI* both produce PHZ, noted by the orange colour of the cultures, although the *csaI* mutant appears to produce notably less. Conversely, the AHL-deficient strain, the single *phzI* and *phzR* mutants and both double mutants exhibited no PHZ production. In LB broth, both PA23*csaR* and PA23*csaI* show wild-type levels of PHZ production while the AHL-deficient strain as well as PA23*phzR* and PA23*phzRcsaR* show no signs of PHZ production. The *phzI* single and *phzIcsaI* double mutant, however, appear to produce low levels of PHZ with the colour of the single mutant less intense than wild type and that of the double mutant less intense than the single mutant.

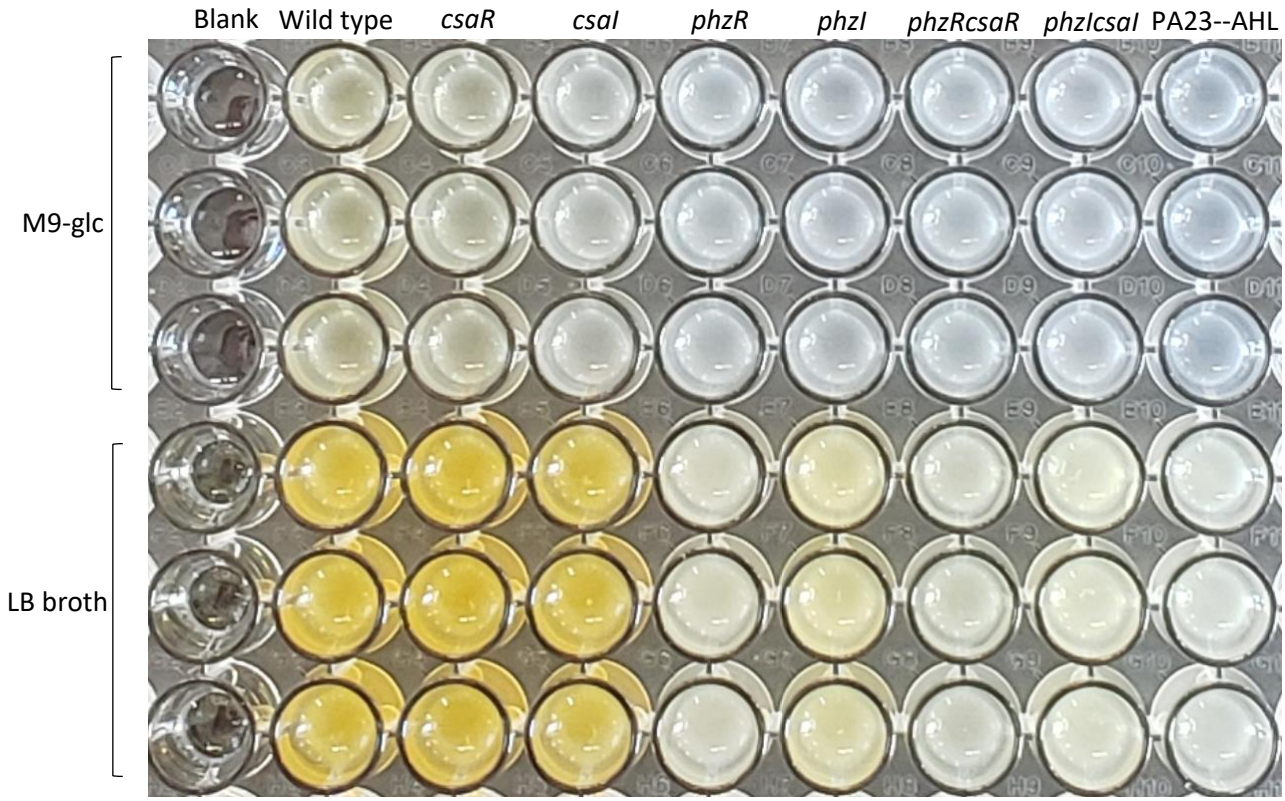


Figure 7. Qualitative analysis of PHZ production by PA23 wild type and derivative strains in M9-glc and LB broth. PA23 wild type and QS mutants were standardized to an OD_{600} of 1.0 before growing overnight at 28°C with shaking in 2 media types, M9-glc and LB broth.

3.2.3. The Csa QS system does not control protease production in PA23

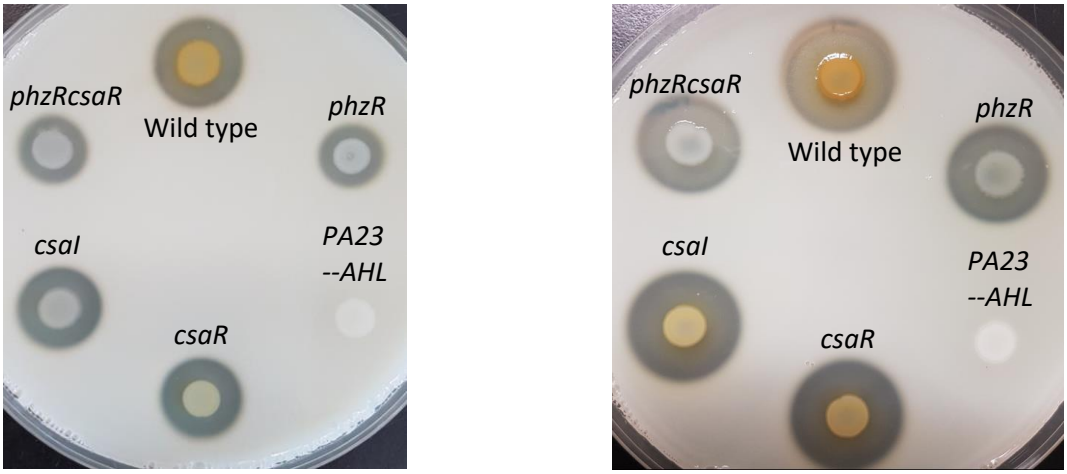
In a previous study, protease activity was reported to be completely absent in the PA23 AHL-deficient strain and decreased 2-fold in PA23*phzR*, with only partial complementation with the introduction of *phzR in trans* (Selin *et al*., 2012). These results suggest that the Phz QS system may work in tandem with another regulatory element to control protease production. Qualitative analysis of protease production was conducted with the PA23 Csa mutants to determine if this system regulates protease production. Strains were individually grown overnight in M9-glc at 28°C and then plated on 2% skim milk agar plates to measure protease activity.

Zones of clearing did not begin to appear until after 24 hours (data not shown) and a delay was seen in the *phzR* and *phzRcsaR* mutants at the 48-hour point (Figure 8). At the 72-hour time point, there was no significant difference in protease production in any of the QS mutants, single or double, in comparison to wild type, other than the AHL-deficient strain, which showed no protease activity (Table 3). By 72 hours all strains measured zones of clearing that were similar to wild type. Additionally, compared to wild type, a delay in PHZ production in the *csaR* and *csaI* mutants at 48- and 72-hours postinoculation onto 2% milk agar was apparent (Figure 8). This phenotype is only apparent on this media type and did not present in any other phenotypic assay that was performed.

Table 3. Protease production in PA23 and derivative strains

Strains	Zone at 72 hours (mm) [§]
PA23 wild type	6.00 (1.1)
PA23*csaR*	6.11 (0.7)
PA23csaI	6.22 (0.9)
PA23*phzR*	6.56 (1.5)
PA23*phzI*	6.11 (1.1)
PA23*phzRcsaR*	6.33 (1.0)
PA23*phzIcsaI*	6.33 (0.8)
PA23--AHL	0 [¥]

[§] Mean (standard deviation) obtained from three biological replicates analyzed in triplicate.
[¥] Significantly different from wild type ($p < 0.005$).



48 hours postinoculation

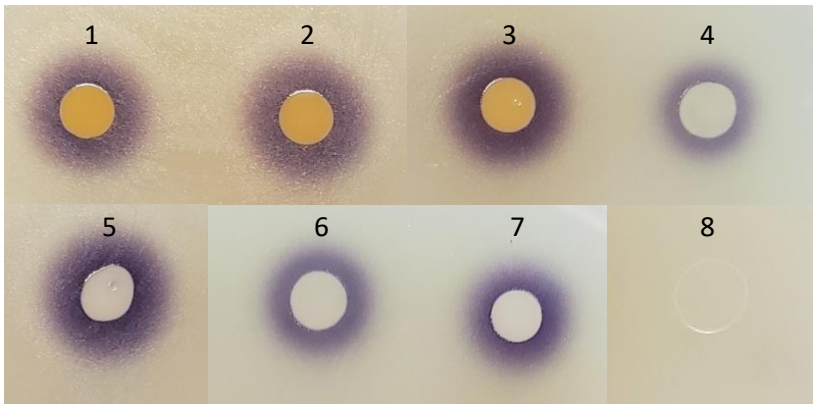
72 hours postinoculation

Figure 8. PHZ and protease production by QS mutants on 2% skim milk agar plates. A delay in protease production at 48 hours postinoculation versus 72-hours postinoculation is evident in the *phzR* and *phzRcsaR* mutants. Additionally, PHZ production in the *csaR* and *csaI* mutants fails to reach wild-type levels, as evidenced by the reduction in the orange pigmentation of the bacteria.

3.2.4. AHL signalling molecules that activate the CV026 reporter strain are predominately produced by the Phz QS system

For bacteria that utilize AHL-based QS systems, a range of different AHL molecules are produced by a given LuxI-type AHL synthase. While the AHL profile of PA23 has yet to be elucidated, we do know that it produces signals that can be detected by the *C. violaceum* CV026 biosensor strain. In the presence of exogenous AHLS with 4-8 carbon acyl chain lengths, CV026 bacteria change from colorless to purple (Latifi *et al*., 1995). No significant change in the production of violacein was observed for the Csa QS single mutants compared to wild type (Figure 9A and 9B). These results indicate that the predominant AHLs produced by *csaI* are variants other than those that can induce violacein production in CV026. Conversely, the *phzR* and *phzRcsaR* mutants show a significant decrease in violacein production in comparison to wild type. The lack of a compounding effect seen in the double mutant supports the notion that the Csa QS system produces AHLs other than those that activate violacein production. Interestingly, though there is a decrease in violacein production observed in both *phzR* mutants, no detectable change was noted in the *phzI* single or *phzIcsaI* double mutant.

A



B

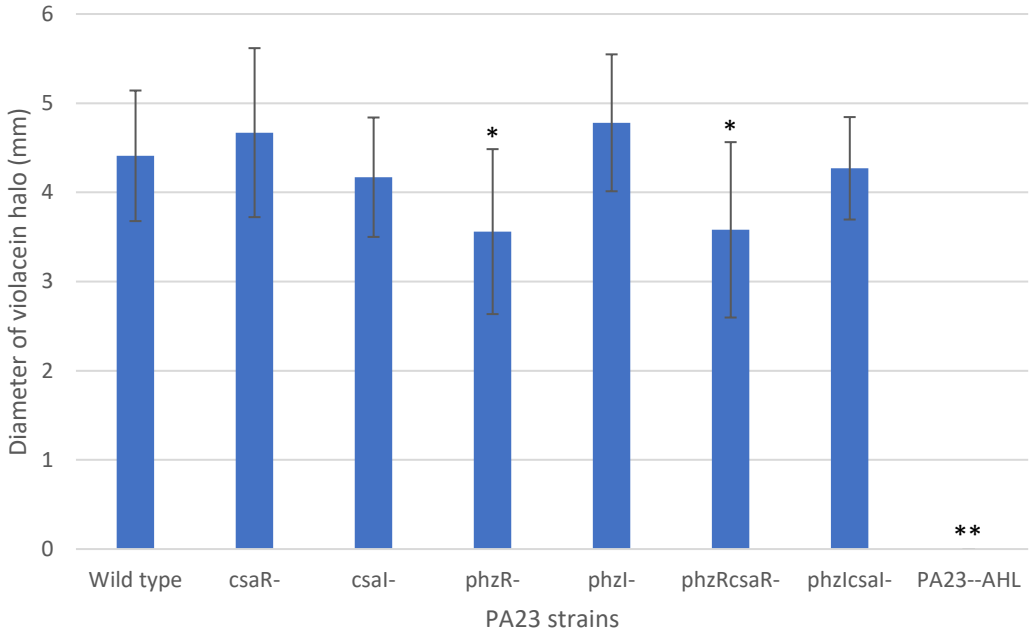


Figure 9. Autoinducer production by PA23 and derivative strains using *C. violaceum* CV026 as a reporter strain. (A) The production of violacein (violet-pigmented compound) is used to qualitatively detect synthesis of AHL signalling molecules. Strains are as follows: 1, PA23 wild type; 2, PA23*csaR*; 3, PA23*csaI*; 4, PA23*phzR*; 5, PA23*phzI*; 6, PA23*phzRcsaR*; 7, PA23*phzIcsaI*; 8, PA23--AHL (B) The diameter of the halo surrounding the colony can be measured to give a semi-quantitative measurement of violacein production corresponding to production by the reporter strain. (*, $p<0.05$; **, $p<0.01$)

3.2.5. The Phz QS system plays a more significant role in regulating chitinase production than the Csa QS system

To better understand how QS affects chitinase activity in PA23, the wild type and QS derivatives were subjected to quantitative chitinase analysis. Chitinase activity was found to be elevated in PA23*csaI* compared to wild type, suggesting that the AHLs synthesized by CsaI may somehow negatively regulate chitinase production. Interestingly, PA23*csaR* showed wild-type levels of chitinase activity, a phenotype also shared with PA23*phzI* and PA23*phzIcsaI* (Table 4). In the case of PA23*phzR*, PA23*phzRcsaR* and the AHL-deficient strain, no chitinase activity was observed.

Table 4. Chitinase activity in PA23 and derivative strains

Strains	**Absorbance at 550nm** [§]
PA23 wild type	0.131 (0.01)
PA23*csaR*	0.135 (0.06)
PA23*csaI*	0.168 (0.01) [¥]
PA23*phzR*	0 (0) [ǂ]
PA23*phzI*	0.123 (0.03)
PA23*phRcsaR*	0 (0) [ǂ]
PA23*phzIcsaI*	0.131 (0.02)
PA23--AHL	0 (0) [ǂ]

[§] Mean (standard deviation) obtained from a set of 5 replicates.

[¥] Significantly different from wild type ($p < 0.005$).

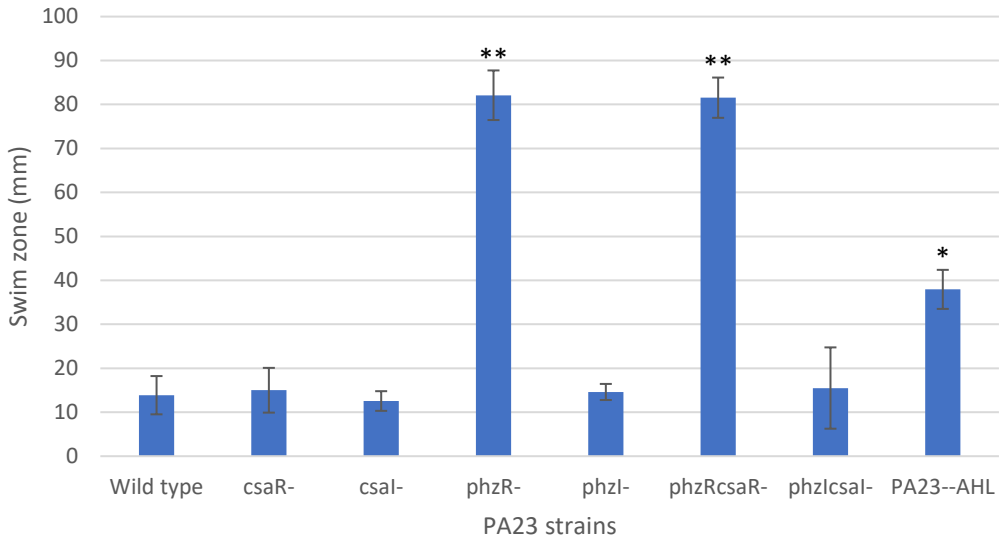
[ǂ] Significantly different from wild type ($p < 0.001$).

3.2.6. The Csa QS system does not regulate flagellar motility in PA23 but may play a role in biofilm formation

Bacterial motility and biofilm formation are important aspects of biocontrol and have been found to be vital in controlling aspects of root colonization and rhizosphere competence (Capdevila *et al*., 2004; Simons *et al*., 1996; Watnick & Kolter, 2000). The Csa QS and *phzI* mutants were assessed for flagellar motility by measuring their ability to move through semi-solid media. Compared to PA23 wild type, there was no significant change in motility observed by PA23*csaI,* PA23*csaR,* PA23*phzI* or PA23*phzIcsaI* (Figure 10A). The AHL-deficient strain however, exhibited a 2.5-fold increase in its swim zone while PA23*phzR* as well as PA23*phzRcsaR* exhibited a 5-fold increase in the size of the swim zone. Collectively, these findings suggest that the Csa system plays little to no role in flagellar motility, whereas the Phz QS system exerts a repressive effect (Figure 10A). The fact that the *phzRcsaR* double mutant was similar to PA23*phzR* substantiates that the Csa QS system does not influence flagella-mediated motility in PA23.

Results from the biofilm assay indicate that in comparison to wild type, PA23*csaR* established a more robust biofilm, whereas no significant difference was seen for PA23*csaI,* PA23*phzI* or the double mutant PA23*phzIcsaI* (Figure 10B). Conversely, the AHL-deficient strain, PA23*phzR* and PA23*phzRcsaR* are significantly deficient in their ability to establish a biofilm. Thus, it appears that while the Phz QS positively regulates PA23 biofilm formation, CsaR has a modest repressive effect.

A



B

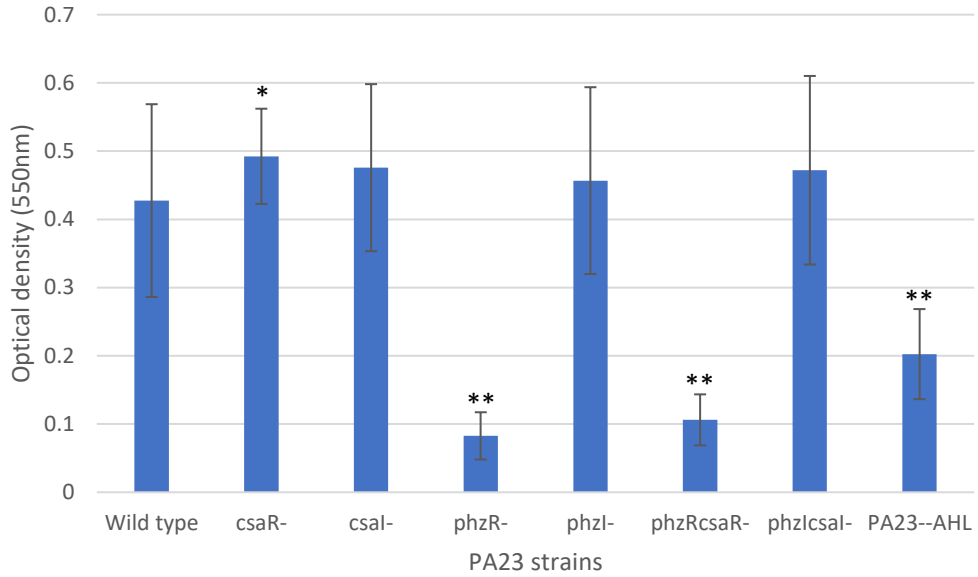


Figure 10. Assessment of (A) flagella-dependent motility and (B) biofilm formation of PA23 wild type and derivative strains. Swimming motility was determined using M9-glc media solidified with 0.3% agar, allowing cells to migrate through the semi-fluid contents of the plate. To assess biofilm formation, strains were grown to stationary phase in M9-glc, stained with crystal violet and measured at an absorbance of 550nm. (*, $p<0.05$; **, $p<0.005$).

3.2.7. The Phz and Csa QS systems interact to regulate HCN production

In order to understand how the Phz and Csa QS systems affect HCN production qualitative assays were undertaken using Cyantesmo paper, which turns blue in the presence of this compound. All of the strains were capable of HCN production, except for the PA23 *hcn* mutant, which was included as a control (Figure 11). Though this is solely a qualitative means of measuring HCN production, it is worth noting that the intensity of the blue color on the Cyantesmo paper differs between the strains. For example, PA23*csaR*, PA23*csaI,* PA23*phzI* and PA23*phzIcsaI* appear to produce increased levels of HCN compared to wild type and PA23*phzR.* Interestingly, PA23*phzRcsaR* shows a noticeable lack in color intensity on the Cyantesmo paper compared to PA23*phzR*, suggesting that there is some level of interaction between the two QS systems.

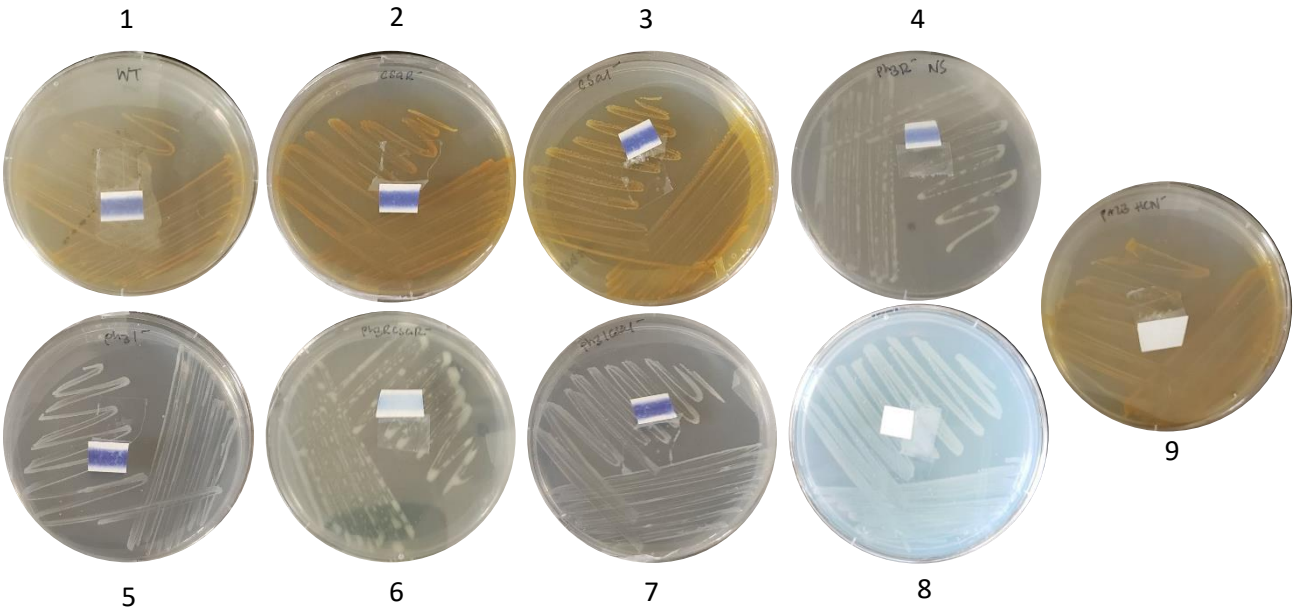


Figure 11. Monitoring HCN production in PA23 and derivative strains using Cyantesmo paper as an indicator. Strains were streaked onto M9-glc agar, sealed with parafilm and grown in the presence of Cyantesmo paper at 28°C. Pictures were taken at 48-hours postinoculation. Strains are as follows: 1, PA23 wild type; 2, PA23*csaR*; 3, PA23*csaI*; 4, PA23*phzR*; 5, PA23*phzI*; 6, PA23*phzRcsaR*; 7, PA23*phzIcsaI*; 8, AHL-deficient; 9, PA23 HCN-deficient.

3.2.8. The ability of PA23 to antagonise the growth of *S. sclerotiorum* increases when the Csa QS system is disrupted

The ability of PA23 and QS mutants to antagonize *S. sclerotiorum* was analyzed using fungal radial diffusion assays. Strains were grown in M9-glc media, spotted onto 1/5 strength potato dextrose agar (PDA) and incubated at 28°C for 24 hours before introducing infectious *S. sclerotiorum* fungal hyphae. As demonstrated in Figure 12, all bacteria except the AHL-deficient strain exhibited some level of fungal inhibition by 72-hours postinoculation. Compared to wild type, PA23*csaR*, PA23*csaI* and PA23*phzIcsaI* demonstrated significantly increased fungal inhibition, with PA23*csaR* showing the greatest degree of AF activity (Figure 12). PA23*phzR* and PA23*phzRcsaR* exhibited wild-type levels of fungal inhibition while PA23*phzI* was impaired in its ability to suppress *S. sclerotiorum*.

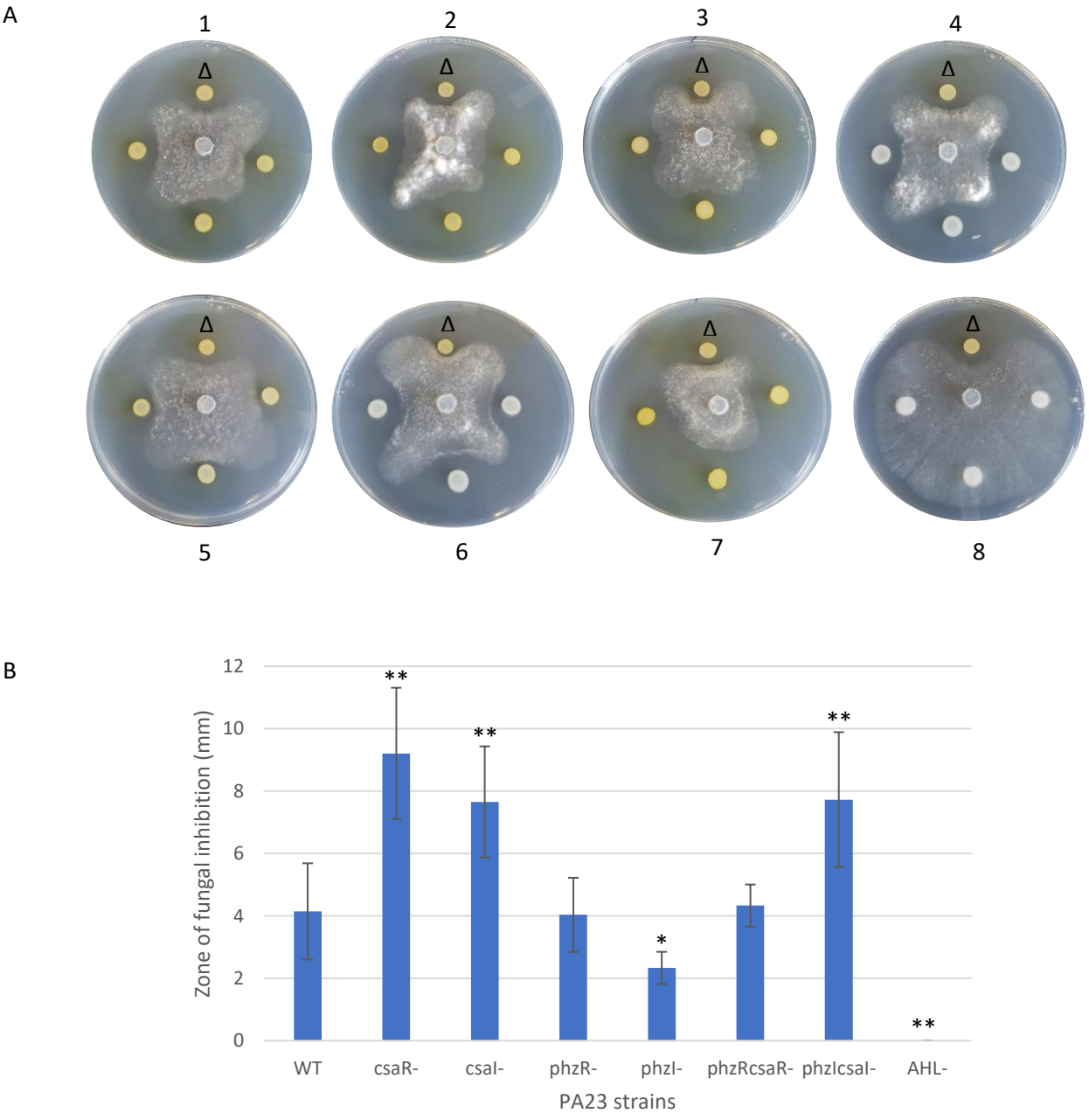


Figure 12. Inhibition of *S. sclerotiorum* by PA23 and derivative strains. Test strains grown in M9-glc were spotted onto 1/5 PDA before introducing a fungal plug containing *S. sclerotiorum* hyphae. (A) Radial diffusion assays showing AF activity exhibited by PA23 wild type (Δ) and three replicates of the test strain. Plates contain the following test strains: 1, PA23 wild type; 2, PA23*csaR*; 3, PA23*csaI*; 4, PA23*phzR*; 5, PA23*phzI*; 6, PA23*phzRcsaR*; 7, PA23*phzIcsaI*; 8, PA23--AHL. (B) Mean of zones of inhibition of three replicates from radial diffusion assays, obtained from measuring the zone of clearing between the edge of the bacterial colony and fungal growth front (*, $p<0.01$; **, $p<0.001$).

3.3. Gene expression analysis of *prnA* in PA23 derivative strains

The production of PRN has been deemed a vital aspect of PA23 biocontrol and could justify the increased AF activity seen in the Csa QS mutants. For this reason, expression of *prnA,* the first gene of the *prnABCD* operon driving PRN biosynthesis, was analyzed by qPCR. Significant downregulation in gene expression is noted in the *phzR* single mutant, the *phzRcsaR* double mutant and the AHL-deficient strain, exhibiting a log2 fold change of -6.79, -6.74 and -6.49 respectively (Figure 13). Wild-type expression is seen in PA23*csaR,* PA23*csaI,* PA23*phzI* and PA23*phzIcsaI*.

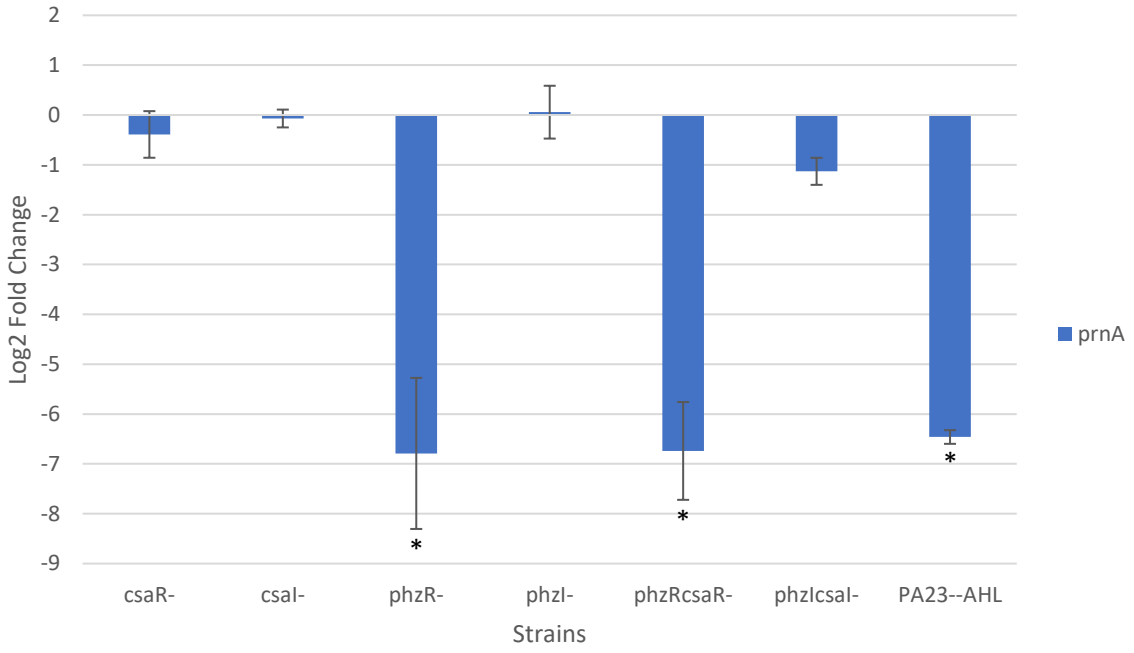


Figure 13. Log2 fold change in gene expression of *prnA* in PA23 QS mutant strains in comparison to PA23 wild type as measured by qPCR. The gene of interest was analyzed against *rpoB* as the housekeeping gene and wild type gene expression was normalized to 0.0. (*, p< 0.001).

4. DISCUSSION

The AF properties of *P. chlororaphis* PA23 have been proven to curb infection of canola by *S. sclerotiorum* in laboratory and greenhouse settings. In order to be considered a viable contender as a biocontrol agent in agricultural practice, however, the mechanisms that drive this antagonistic behaviour require thorough investigation. The hierarchical regulatory system that governs PA23 secondary metabolite production has been previously studied. One unexplored aspect of this regulatory cascade, the Csa QS system, was the focal point of this research. The genes of the Csa QS system were disrupted individually generating single mutants (PA23*csaR,* PA23*csaI*) and disrupted along with their counterparts of the Phz QS system, generating double mutants (PA23*phzRcsaR*, PA23*phzIcsaI*). These strains together with PA23*phzR*, PA23-AHL and the newly-created *phzI* mutant were phenotypically characterized to better understand the role of QS in PA23 physiology.

Growth rate analysis showed a growth deficiency in the *phzR* single mutant, the *phzRcsaR* double mutant and the AHL-deficient strain when propagated in the minimal medium, M9-glc (Figure 6). In these conditions, aspects of cell biology such as cell division, motility, growth and secondary metabolite production may be affected in response to elevated (p)ppGpp (Potrykus & Cashel, 2008), leading to SR activation. The SR, along with RpoS, has been shown to negatively regulate *phzR* expression (Manuel *et al*., 2012; Selin *et al*., 2012). It is possible that the altered growth rate exhibited by the *phzR* single and double mutant might be due to the effects of the SR and RpoS, which ultimately result in early entry into stationary phase and a reduced total cell population.

The Csa QS mutants were grown in the presence of the reporter strain CV026, to determine if disruption of this regulatory system resulted in a change in AHL synthesis. In terms of the size of the zone of purple pigmentation, there was no significant change in AHL production by PA23*csaR*, PA23*csaI,* PA23*phzI* and PA23*phzIcsaI* (Fig. 9). The *phzR* single and the *phzRcsaR* double mutants, on the other hand, showed a significant deficit in violacein production. We did note a difference in the intensity of violacein production from one strain to the other. For example, the purple pigmentation is significantly more intense around the PA23*phzI* colony compared to PA23 (Figure 9A). This is likely due to the biosensor being activated to different degrees depending on the structure of the AHL molecules being synthesized. McClean and colleagues (1997), the developers of the CV026 bioassay, discovered that different homoserine lactone (HSL) compounds showed variation in their ability to induce violacein production. *N*-Hexanoyl-L-HSL (HHL; C6-HSL), which is the AHL produced by the parent strain *C. violaceum*, is the most active AHL, followed by *N*-Butanoyl-L-HSL (BHL; C4-HSL) with a 30-fold decrease in activity (McClean *et al*., 1997). Additionally, *N*-Octanoyl-L-HSL (OHL; C8-HSL) was able to activate violacein production at significantly lower quantities than C4-HSL. It was noted that HSL compounds with acyl chains longer than C8 were not only incapable of inducing violacein production, but when in proximity to compounds with acyl chains shorter than C8, they antagonized violacein production by these compounds (McClean *et al*., 1997).

Bauer *et al.* (2016) demonstrated that PhzI, CsaI and AurI in the related strain *P. chlororaphis* subsp. *aurantiaca* PB-St2 produce a variety of AHL variants. While there are some AHLs that are produced by all three synthases, there are signals that are unique to each QS system (Bauer *et al*., 2016). All three AHL synthases in PB-St2 produce C6- and C8-HSLs however

only CsaI and AurI produce C4-HSL. Additionally, PhzI synthesizes three long-chain AHL signalling molecules, C8-HSL/3-OH-C8-HSL/3-OH-C10-HSL where CsaI and AurI synthesize C8-HSL/3-OH-C10-HSL and C8-HSL/3-oxo-C8-HSL, respectively. If we assume that the QS systems in PA23 produce AHLs on the same spectrum as PB-St2, the more intense pigmentation seen in the *phzI* single and *phzIcsaI* double mutants may be the result of a decreased concentration of AHLS that act as inhibitors and increased levels of those that activate CVO26. This is a rudimentary means of justifying the results that were exhibited in the AHL reporter assay therefore further investigation into which AHL signalling molecules are produced by the three QS systems in PA23 is warranted. This would not only confirm the aforementioned hypothesis but also allow for exploration into the crosstalk that exists between the QS systems in PA23.

PA23 produces PHZ, which imparts an orange colour to the bacteria; as such, it is easy to monitor production of this antibiotic by assessing the colour of the strains. We noticed differences in the pigmentation of the QS mutants in a medium-dependent fashion. When grown in M9-glc broth, PHZ levels appear to be altered in all of the mutants, with very little being produced by the single Csa mutants, and virtually none for the Phz single mutants, the double mutants and PA23--AHL (Figure 7). Interestingly on M9-glc agar, PA23*csaR,* PA23*csaI* and PA23*phzI* appear to produce near wild-type levels of PHZ, while PA23*phzIcsaI* shows only the slightest bit of pigmentation. At present it is unclear why we see differences between the M9 liquid and solid media, but it may be related to oxygen levels which is known to affect PHZ production (Mavrodi *et al*, 2006). When bacteria were grown in LB broth, PHZ production was similar to that seen on M9-glc solid media. These observations differ from that reported for *P. chlororaphis* (*aureofaciens*) 30-84, where *phzR* and *phzI* mutants showed a 90% reduction, and

phzRcsaR/phzIcsaI double mutants were totally devoid of PHZ production when grown in Pigment-Producing Medium (PPM: Tryptone, glucose, KNO_3) (Zhang & Pierson III, 2001). Interestingly, we observed delayed PHZ production by the *csaR* and *csaI* mutants when grown on the skim milk agar used for the protease assay (Figure 8). It is not uncommon for variations in media components to alter phenotypes, specifically those concerning the production of secondary metabolites. Nandi *et al.* (2017) demonstrated that supplementing minimal M9-glc media with glycine increased the production of PHZ, PRN and HCN indirectly through upregulation of the Phz QS system. Since this phenotype was observed exclusively with skim milk agar, it is possible that a compound in this medium indirectly influences PHZ production through the Csa QS system. These results suggest that the Csa QS does in fact play a role in PHZ production, albeit a smaller role than that of the Phz system.

Many biocontrol pseudomonads produce HCN, which in some cases, has proven to be a vital component to overall AF activity. When applied as a root drench on lettuce plants, mutants devoid of HCN production left the plants susceptible to fungal infection, deeming HCN a necessary component of PA23 biocontrol activity (Nandi *et al*., 2017). A putative *phz*-box sequence has been identified in the promoter region of the HCN biosynthetic gene cluster, suggesting that the Phz QS system governs expression of this compound. To assess whether the Csa QS system or PhzI plays a significant role in regulating this metabolite, mutants were grown in the presence of an HCN indicator, Cyantesmo paper. In figure 11, results show that with the exception of the HCN-null mutant and the AHL-deficient strain, all strains were capable of producing HCN, although the blue coloring differs in intensity from pale to dark blue. Color intensity is directly proportional to the concentration of HCN being released by the bacteria

(Rella *et al*., 2004). When compared to the wild type, the *csaR, csaI, phzI* and *phzIcsaI* mutants all presented dark blue on the Cyantesmo paper, while PA23*phzR* presented a lighter blue and PA23*phzRcsaR* exhibited a much paler blue. Previous qPCR and RNAseq analysis demonstrated that expression of *hcnC* was decreased in PA23--AHL and PA23*phzR* (Shah *et al*., 2020). Additionally, the paler blue seen with the *phzRcsaR* double mutant is not reflected in either the *csaR* or *phzR* single mutants. These findings are in keeping with the Cyantesmo qualitative analysis and suggest that PhzR acts to positively regulate HCN. The darker blue seen in the other strains implies Csa exerts a repressive effect on HCN production and the paler blue seen in PA23*phzRcsaR* suggests that both QS systems may work in tandem to regulate HCN production. Clearly, more work is required to reveal the molecular mechanisms involved.

Results from the protease assays established that with the exception of the AHL-deficient strain, all of the QS mutants exhibited wild-type levels of protease activity by 72 hours. A slight delay in protease production was noted at the 48-hour time point in PA23*phzR* and PA23*phzRcsaR*; however, protease production reached wild-type levels by 72 hours (Figure 8). These results differ from those noted by the Zhang and Pierson (2001), where the double transcriptional regulator mutant as well as the double AHL-synthase mutant in 30-84 were incapable of producing protease, while the single QS mutants exhibited wild-type protease activity. In our case, PA23*phzRcsaR* and PA23*phzIcsaI* produced wild-type levels of protease, further emphasizing that production of this enzyme occurs even when both QS systems are disabled.

Previous data demonstrated a link between QS and chitinase production in PA23 (Shah *et al*., 2020). A putative *phz*-box was identified in the promoter region of a chitinase

biosynthetic gene and through RNAseq data, it was discovered that expression of this gene was significantly decreased in the *phzR* mutant and AHL-deficient strain (Shah *et al*., 2020). In the current study, we observed a complete eradication of chitinase activity in PA23*phzR*, PA23*phzRcsaR* and PA23--AHL, consistent with the notion that the Phz QS positively regulates chitinase expression. Conversely, PA23*csaR*, PA23*phzI* and PA23*phzIcsaI* all exhibited wild-type levels of chitinase activity. The *csaI* single mutant is the only strain exhibiting increased chitinase activity. It is possible that the increase in chitinase production in the *csaI* mutant is related to the AHLs synthesized by CsaI. In this case, AHLs would act to repress expression of chitinase genes directly or indirectly through some other component of the multi-tiered regulatory cascade overseeing expression. Further investigation to identify which AHLs might be uniquely synthesized by CsaI paired with gene expression analysis could reveal how the Csa QS regulates chitinase production in PA23.

When we examined swimming motility, we discovered that the Csa QS system has little impact on this trait (Figure 10A). The *csaR, csaI* and *phzI* single mutants as well as the *phzIcsaI* double mutant exhibit wild-type levels of swimming motility. Conversely, the *phzR* and *phzRcsaR* mutants as well as the AHL-deficient strain exhibit increased motility. Previous RNAseq work revealed that genes involved in flagellar-mediated motility, namely those encoding the alternative stators MotD/MotY, flagellin and a diguanylate phosphodiesterase, showed increased expression in the *phzR* mutant (Shah *et al.,* 2020). Chemotaxis signal transduction- and cellular cyclic di-GMP-associated genes were also upregulated in one or both of the *phzR* and AHL-deficient strains (Shah *et al*., 2020). Collectively these findings indicate that

the Phz QS system negatively regulates PA23 flagellar-mediated motility, whereas the Csa system and PhzI have little impact on this trait.

Investigation of *P. chlororaphis* (*aureofaciens*) strain 30-84 revealed that the Csa QS system played a significant role in the formation of biofilms in the rhizosphere, with single and double *csa* mutants showing deficits in biofilm formation on wheat seeds (Maddula *et al*., 2006). Common to both 30-84 and PA23, the production of the antibiotic PHZ is necessary for biofilm formation (Maddula *et al*., 2006; Selin *et al*., 2010). Figure 10B shows that when the QS mutants were grown statically in a 96-well plate, a slight increase in biofilm formation is seen in the *csaR* mutant compared to wild type, where no change is seen in the *csaI* mutant. The PA23*phzR,* PA23*phzRcaR* and AHL-deficient strains, on the other hand, show significant deficiencies in biofilm formation. These results, paired with the lack of PHZ production in the three strains, coincide with previous claims that PHZ production is important for biofilm formation. The slight increase in biofilm production exhibited by PA23*csaR* may be the result of alterations in cellular attributes, as the name Csa (Cellular Surface Alterations) implies. Factors may include extracellular components such as lipoproteins, polysaccharides, proteins and lipids, which are just a portion of the biomolecules associated with biofilm assembly (Karygianni *et al*., 2020).

The fungal radial diffusion assay is commonly used as a means of determining overall AF capabilities, without considering individual entities such as degradative enzymes or antibiotic levels. It allows for the direct challenge of *S. sclerotiorum* by bacteria, and the degree of fungal inhibition is easily measured. In radial diffusion assays (Figure 12) PA23*csaR*, PA23*csaI* and PA23*phzIcsaI* exhibited significantly increased AF activity compared to wild type. PA23*phzR* and

PA23*phzRcsaR,* on the other hand, showed wild-type AF activity and PA23*phzI* had reduced fungal antagonism. The AHL-deficient strain exhibited a complete loss of AF activity; therefore QS is necessary for PA23 to suppress *S. sclerotiorum* infection. The residual AF activity exhibited by the Csa and Phz QS mutants supports the idea that PA23 is still capable of antagonizing *S. sclerotiorum* in the absence of one or both QS systems. One explanation for the discrepancy between the AHL-deficient strain and the single and double QS mutants is the presence of the Aur QS system. Though the regulatory mechanisms of this QS system have yet to be elucidated, it suggests a role in PA23 biocontrol.

The increased levels of AF activity noted by PA23*csaR* and PA23*csaI* hints at the likelihood that the Csa QS system either negatively regulates expression of genes involved in AF metabolite production, or it is functioning to regulate AF activity indirectly through another regulator. Our phenotypic analysis failed to reveal any major differences between the *csa* mutants and the PA23 wild type that would account for the increased fungal suppression. Previous studies have identified PRN as the primary exometabolite involved in PA23 biocontrol (Selin *et al*., 2010). Through qPCR and RNAseq analysis, expression of the PRN biosynthetic gene, *prnA* was found to be downregulated in PA23*phzR* and PA23--AHL, and both strains produce no detectable PRN (Selin *et al*., 2012 Shah *et al*., 2020). In the current study, qPCR analysis was carried out on the Csa QS mutants to determine if the enhanced AF activity was due to increased *prn* expression. Results indicate that rendering the Csa QS system non-functional does not impact transcription of the *prn* genes (Figure 13). The possibility that these genes are regulated at the post-transcriptional level cannot be ruled out. Monitoring PRN levels by HPLC should be done to verify that this antibiotic is not impacted by the *csa* mutations.

An alternative explanation for the increased fungal inhibition seen in the Csa QS single mutants and PA23*phzIcsaI* is the role that HPR may play in PA23 AF activity. This exometabolite, which is the product of the *darABSCR* operon, is considered the prominent exometabolite eliciting fungal antagonism in *P. chlororaphis* PCL1606 (Calderón *et al*., 2013). The effects of HPR in impeding fungal growth by this biocontrol strain surpass those imposed by both PRN and HCN. Homologs of these genes have been identified in PA23, but their role in fungal suppression remains unknown. Furthermore, no link has been established between HPR and QS. Future studies should be directed at elucidating the role of HPR in PA23 biocontrol as well as determining whether expression of this compound is under QS control.

Because QS functions at the level of transcription, we had intended to expand the qPCR analysis to reveal how disruption of the Csa QS system affects biocontrol gene expression. Both biosynthetic genes; *phzA, hcnC, aprA* (protease)*, darA/darB* (HPR)*,* and regulatory genes; *phzR/I, csaR/I, aurI/aurR* and *rpoS* were to be included. Unfortunately, due to circumstances surrounding the SARS-CoV-2 pandemic in 2020, this analysis was not completed.

5. CONCLUSIONS & FUTURE DIRECTIONS

In laboratory and greenhouse settings, *P. chlororaphis* strain PA23 proves to be a valuable tool in the inhibition of fungal growth, classifying it as a promising candidate for microbial biocontrol. The AF capabilities of PA23 are elicited by production and secretion of several secondary metabolites, including PHZ, PRN, HCN, protease and chitinase (Poritsanos *et al*., 2006; Selin *et al*., 2010). The multi-tiered regulatory system, which governs the expression of genes involved in production of these exometabolites has been broadly explored, revealing layers of complexity. The Gac-Rsm system, RpoS, SR, transcriptional regulator PsrA and Phz QS system are all key players in the regulation of PA23 secondary metabolites and a significant level overlap between these systems has been previously described (Klaponski *et al.*, 2014,2016; Manuel *et al.*, 2012; Nandi *et al*., 2016; Poritsanos *et al.*, 2006; Selin *et al.*, 2010, 2012, 2014; Shah *et al*., 2016). Previous RNA sequence analysis of the AHL-deficient and *phzR* mutant revealed that approximately 13% of the PA23 genome is under QS control (Shah *et al*., 2020). Highly conserved *phz*-box sequences, corresponding to putative PhzR-AHL binding sites, were identified upstream of 58 of 807 differentially expressed genes (Selin *et al*., 2012). Included in these 58 were genes responsible for the production of PRN, PHZ, HCN and chitinase, the *csaR/I, phzR/I, mvaV* and *rpoS* regulators, and those involved in motility (Shah *et al*., 2020). The PhzR-AHL complex is believed to control production of secondary metabolites directly through binding to these *phz*-boxes, as well as indirectly through regulators such as RpoS.

PA23 harbours a second QS system called the Csa system, which had not yet been characterized. The objective of this study was to generate Csa mutants and to conduct a series

of phenotypic assays to determine if the disruption of the Csa QS system resulted in alterations in the production of secondary metabolites and AF capabilities of PA23. Gene expression analysis would then follow, exploring the role that the Csa QS system plays in regulating expression of biosynthetic and regulatory genes involved in PA23 biocontrol.

Single mutants, PA23*csaR* and PA23*csaI,* as well as double mutants, PA23*phzRcsaR* and PA23*phzIcsaI,* were generated via insertional mutagenesis. Multiple earlier attempts to create a *phzI* mutant had been unsuccessful; however, a combination of SOE PCR and allelic exchange finally resulted in PA23*phzI*. The previously generated *phzR* mutant and AHL-deficient strain were also included to round out this study. Phenotypic analysis revealed that the *phzR* mutant exhibited decreased production of PHZ, AHL signalling molecules, chitinase, HCN and a delay in protease production. It also exhibited increased motility and impairment in the ability to form biofilms. Despite decreased levels of secondary metabolites, PA23*phzR* was able to inhibit *S. sclerotiorum* at the same rate as PA23 wild type. Similarly, the AHL-deficient strain showed significant deficiencies across all phenotypic assays, with no detectable levels of PHZ, AHLs, chitinase or protease and impairment in the production of HCN. This strain also exhibited increased motility and the inability to form biofilms but unlike PA23*phzR*, it was completely devoid of AF activity. These results correspond well with previous RNAseq and qPCR data presented by Shah *et al.* (2020) demonstrating that the Phz QS system regulates genes encoding secondary metabolites together with those indirectly linked to biocontrol (motility and biofilms).

Despite showing near wild-type levels of secondary metabolite production through phenotypic analysis, PA23*csaR* and PA23*csaI* exhibited increased AF activity when challenged

with *S. sclerotiorum*. PA23*csaR* demonstrated a slight but significant increase in biofilm formation and PA23*csaI* exhibited elevated chitinase activity. Furthermore, both strains produced higher levels of HCN compared to wild type, based on the increased blue color of the Cyantesmo indicator paper. While HCN contributes to PA23-mediated control of *Sclerotinia* in a root-rot model of infection (Nandi *et al*., 2017), the role of chitinase and biofilms in fungal suppression is unknown. Therefore, it is not clear whether the observed phenotypic differences account for the increased AF activity demonstrated by these strains.

Quantification of PRN and HPR in the Csa QS mutants should be conducted to establish whether increased production of either secondary metabolite accounts for the observed increased AF activity. The current study looked at *prnA* gene expression in the Csa QS mutants, which showed no change compared to wild type. In order to confidently confirm these results, PRN extraction and quantification should be executed by ethyl acetate/toluene extraction and HPLC analysis. HPR is the key secondary metabolite responsible for the ability of *P. chlororaphis* PCL1606 to combat avocado root rot and tomato foot and root rot, caused by *Rosellinia necatrix* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* ZUM2407, respectively (Calderón *et al*., 2013). Disruption in the biosynthesis of HPR resulted in a significant decrease in the ability of PCL1606 to inhibit fungal growth *in vitro*, a phenotype more drastic than what was observed by the abolition of PRN or HCN production. The genes involved in HPR production have been identified in PA23 and future exploration into the role of HPR in PA23-mediated control of *Sclerotinia* could prove enlightening.

Complementation and cross-complementation of the single QS mutants followed by phenotypic analysis has been started; however, the assays are not complete. Data has been

obtained for all QS complemented and cross-complemented strains, which can be found in Appendix Figure 1. Generation of control strains, consisting of the mutant background with empty vector, remains to be completed. These include the transcriptional regulator single mutants with the pUC18T-mini-Tn7T-LAC-Gm suicide vector mobilized and integrated into the chromosome as well as the AHL synthase single mutants harbouring the pUCP22 overexpression vector.

The degree of regulation by the Csa QS system can be further explored by carrying out gene expression analysis. Though *prnA* expression has been assessed in the Csa QS mutants, a significant number of genes still need to be examined. The remaining biosynthetic genes, which are related to the production of PHZ, HCN, protease, and HPR as well as the regulatory genes *rpoS, phzR/I, csaR/I* and *aurR/I* should be analyzed in the Csa and Phz single mutants as well as the double mutants. This could reveal whether the Csa QS system is directly or indirectly linked to the production of secondary metabolites as well as any connections that may exist with other elements within the regulatory network. Preliminary results from the current study indicate that the Csa system does not play a major role in the QS regulon of PA23. A third QS system, the Aur QS system, has been identified in PA23, which has yet to be characterized. Disrupting the Aur QS system and conducting phenotypic and gene expression analysis via qPCR, similar to what has been outlined in this study, would reveal connections that exist between QS and the other regulatory systems governing AF activity in PA23.

Future studies should also focus on defining the structure and quantity of AHL signalling molecules produced by PhzI, CsaI and AurI. The general lack of a discernible phenotype exhibited by the *csaI* and *phzI* single mutants compared to PA23*phzR* and the AHL-deficient

strain suggests that a significant level of overlap exists amongst the QS systems in PA23. The Bauer group previously explored the AHL spectrum secreted by *P. chlororaphis* PB-St2. Their study revealed that there are similarities between the AHLs produced across all three QS systems, while AHLs unique to specific AHL synthase genes were also identified (Bauer *et al*., 2016). Considering the AHL spectrum and measuring the concentration of specific classes of AHLs across bacterial growth, allowed for the estimation of when each QS system was being activated. Instances where similarities were observed, such as CsaI and PhzI producing 3-OH-C6-HSL at similar time intervals, are predicted to cause activation of CsaR and PhzR by the AHL from the non-cognate LuxI protein. Exploring the AHL spectra of the three QS systems in PA23 can be conducted by extracting AHLs from PA23 wild type and AHL- mutant cultures and employing LC-MS/MS. Keeping in mind that certain classes of AHLs have been shown to activate or inhibit gene expression, knowledge of the AHL profile would further expand our understanding of how the three QS systems positively or negatively influence PA23 gene expression.

Finally, the very characteristics, which have defined the Csa QS system in other strains in the past, have yet to be examined in PA23. These include rhizosphere competence and cell surface alterations. Zhang & Pierson (2001) concluded that the Csa QS system, specifically CsaR, plays a significant role in wheat rhizosphere competence. Taking into consideration the enhanced AF capabilities of the Csa QS mutants in PA23, it would be interesting to see if that phenotype is transferrable to *in planta* scenarios. Challenging these strains to compete in the rhizosphere and to curb fungal infection as a foliar application may in fact reveal that the Csa QS system substantially contributes to PA23 biocontrol.

6. BIBLIOGRAPHY

Agriculture and Agri-Food Canada. 2020. Biopesticides. https://www.agr.gc.ca/eng/agriculture-and-climate/agricultural-practices/agricultural-pest-management/biopesticides/?id=1531920003497#a1

Agriculture and Agri-Food Canada. 2013. Directory of Biopesticides for Agricultural Crops in OECD Countries. Retrieved from https://www5.agr.gc.ca/MPDD-CPM/searchResult-resultatRecherche.do?lang=eng

Alemu, F. (2016). Isolation of *Pseudomonas fluorescens* species from faba bean rhizospheric soil and assessment of indole acetic acid production: in vitro study, Ethiopia. *American Journal of BioScience*, 4(2), 9. https://doi.org/10.11648/j.ajbio.20160402.11

Alexeyev, M. F. (1999). The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria. *BioTechniques*, 26(5), 824–828.

Anderson, A. J., Kang, B. R. & Kim, Y. C. (2017). The Gac/Rsm signaling pathway of a biocontrol bacterium*, Pseudomonas chlororaphis* O6. *Research in Plant Disease*, 23(3), 212–227. https://doi.org/10.5423/rpd.2017.23.3.212

Arima, K., Imanaka, H., Kousaka, M., Fukuta, A. & Tamura, G. (1964). Pyrrolnitrin, a new antibiotic substance, produced by *Pseudomonas*. *Agricultural and Biological Chemistry*, 28(8), 575–576. https://doi.org/10.1080/00021369.1964.10858275

Arrebola, E., Tienda, S., Vida, C., de Vicente, A., & Cazorla, F. M. (2019). Fitness features involved in the biocontrol interaction of *Pseudomonas chlororaphis* with host plants: The case study of PcPCL1606. *Frontiers in Microbiology*, 10(719), 1–8. https://doi.org/10.3389/fmicb.2019.00719

Arya, N., Rana, A., Rajwar, A., Sahgal, M., & Sharma, A. K. (2018). Biocontrol efficacy of siderophore producing indigenous *Pseudomonas* strains against Fusarium wilt in tomato. *National Academy Science Letters*, 41(3), 133–136. https://doi.org/10.1007/s40009-018-0630-5

Babitzke, P., & Romeo, T. (2007). CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Current Opinion in Microbiology*, 10(2), 156–163. https://doi.org/10.1016/j.mib.2007.03.007

Baehler, E., de Werra, P., Wick, L. Y., Péchy-Tarr, M., Mathys, S., Maurhofer, M., & Keel, C. (2006). Two novel MvaT-like global regulators control exoproduct formation and biocontrol activity in root-associated *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interactions*, 19(3), 313–329. https://doi.org/10.1094/MPMI-19-0313

Battesti, A., Majdalani, N., & Gottesman, S. (2011). The RpoS-mediated general stress response in *Escherichia coli. Annual Review of Microbiology*, 65(1), 189–213. https://doi.org/10.1146/annurev-micro-090110-102946

Bauer, J. S., Hauck, N., Christof, L., Mehnaz, S., Gust, B., & Gross, H. (2016). The systematic investigation of the quorum sensing system of the biocontrol strain *Pseudomonas chlororaphis* subsp. *aurantiaca* PB-St2 unveils *aurI* to be a biosynthetic origin for 3-Oxo-homoserine lactones. *PLoS ONE*, 11(11), 1–21. https://doi.org/10.1371/journal.pone.0167002

Berry, C., Fernando, W.G.D., Loewen, P.C. and de Kievit, T.R. 2010. Lipopeptides are essential for *Pseudomonas* sp. DF41 biocontrol of *Sclerotinia sclerotiorum*. Biological control. **55**: 211-218.

Bertani, I., & Venturi, V. (2004). Regulation of the N-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase RpoS sigma factor and the global regulator GacA. *Applied and Environmental Microbiology*, 70(9), 5493–5502. https://doi.org/10.1128/AEM.70.9.5493-5502.2004

Bom, M., & Boland, G. J. (2000). Evaluation of disease forecasting variables for *Sclerotinia* stem rot (*Sclerotinia sclerotiorum*) of canola. *Canadian Journal of Plant Science*, 80(4), 889–898. https://doi.org/10.4141/P99-071

Boukhalfa, H., & Crumbliss, A. L. (2002). Chemical aspects of siderophore mediated iron transport. *BioMetals*, 15(4), 325–339. https://doi.org/10.1023/A:1020218608266

Bunch, A. W., & Knowles, C. J. (1980). Cyanide production and degradation during growth of the snow mould fungus. *Journal of General Microbiology*, 116(1), 9–16. https://doi.org/10.1099/00221287-116-1-9

Calderón, C. E., Pérez-García, A., de Vicente, A., & Cazorla, F. M. (2013). The *dar* genes of *Pseudomonas chlororaphis* PCL1606 are crucial for biocontrol activity via production of the antifungal compound 2-hexyl, 5-propyl resorcinol. *Molecular Plant-Microbe Interactions*, 26(5), 554–565. https://doi.org/10.1094/MPMI-01-13-0012-R

Calderón, C. E., Tienda, S., Heredia-Ponce, Z., Arrebola, E., Cárcamo-Oyarce, G., Eberl, L., & Cazorla, F. M. (2019). The compound 2-hexyl, 5-propyl resorcinol has a key role in biofilm formation by the biocontrol rhizobacterium *Pseudomonas chlororaphis* PCL1606. *Frontiers in Microbiology*, 10(396), 1–11. https://doi.org/10.3389/fmicb.2019.00396

Canola Council of Canada. (2020a). What is Canola? Retrieved from https://www.canolacouncil.org/oil-and-meal/what-is-canola/

Canola Council of Canada (2020b). History of Canola. Retrieved from https://canolahistory.ca/

Canola Council of Canada (2020c). Canola Encyclopedia: Sclerotinia Stem Rot. Retrieved from https://www.canolacouncil.org/canola-encyclopedia/diseases/sclerotinia-stem-rot/#variety-selection-and-genetic-resistancetolerance

Canola Council of Canada (2020d). Canola Encyclopedia: Control Clubroot. Retrieved from https://www.canolacouncil.org/canola-encyclopedia/diseases/clubroot/control-clubroot/#geneticresistance

Capdevila, S., Martínez-Granero, F. M., Sánchez-Contreras, M., Rivilla, R., & Martín, M. (2004). Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization. *Microbiology*, 150(11), 3889–3897. https://doi.org/10.1099/mic.0.27362-0

Cashel M, Gentry DR, Hernandez VJ, and Vinella D. 1996. The stringent response, p. 1458-1496. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.

Chambonnier, G., Roux, L., Redelberger, D., Fadel, F., Filloux, A., Sivaneson, M., de Bentzmann, S., & Bordi, C. (2016). The hybrid histidine kinase LadS forms a multicomponent signal transduction system with the GacS/GacA two-component ystem in *Pseudomonas aeruginosa*. *PLoS Genetics*, 12(5), 1–30. https://doi.org/10.1371/journal.pgen.1006032

Chatterjee, A., Cui, Y., Hasegawa, H., Chatterjee, A. K. 2007. PsrA, the Pseudomonas Sigma Regulator, Controls Regulators of Epiphytic Fitness, Quorum-Sensing Signals, and Plant Interactions in Pseudomonas syringae pv. tomato Strain DC3000. *Applied and Environmental Microbiology,* 73(11), 3684-3694.

Chiang, S. M., & Schellhorn, H. E. (2010). Evolution of the RpoS regulon: Origin of RpoS and the conservation of RpoS-dependent regulation in bacteria. *Journal of Molecular Evolution*, 70(6), 557–571. https://doi.org/10.1007/s00239-010-9352-0

Chin-A-Woeng, T. F. C., Bloemberg, G. v., & Lugtenberg, B. J. J. (2003). Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytologist*, 157(3), 503–523. https://doi.org/10.1046/j.1469-8137.2003.00686.x

Choi, Kyoung H., & Schweizer, H. P. (2006). mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. *Nature Protocols*, 1(1), 153–161. https://doi.org/10.1038/nprot.2006.24

Choi, K.H., Gaynor, J. B., White, K. G., Lopez, C., Bosio, C. M., Karkhoff-Schweizer, R. A. R., & Schweizer, H. P. (2005). A Tn7-based broad-range bacterial cloning and expression system. *Nature Methods*, 2(6), 443–448. https://doi.org/10.1038/nmeth765

de Kievit, T. R., Kakai, Y., Register, K., Pesci, E. C., Iglewski, B. H. (2002). Role of the *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in *rhlI* regulation. *FEMS Microbiology Letters*, 12(2002), 101-106.

Duke, K. A., Becker, M. G., Girard, I. J., Millar, J. L., Dilantha Fernando, W. G., Belmonte, M. F., & de Kievit, T. R. (2017). The biocontrol agent *Pseudomonas chlororaphis* PA23 primes *Brassica napus* defenses through distinct gene networks. *BMC Genomics*, 18(1), 467. https://doi.org/10.1186/s12864-017-3848-6

Dunne, C., Moënne-Loccoz, Y., McCarthy, J., Higgins, P., Powell, J., Dowling, D. N., & O'Gara, F. (1998). Combining proteolytic and phloroglucinol-producing bacteria for improved biocontrol of *Pythium*-mediated damping-off of sugar beet. *Plant Pathology*, 47(3), 299–307. https://doi.org/10.1046/j.1365-3059.1998.00233.x

Figurski, D. H., & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans (plasmid replication/replication origin/trans-complementation/broad host range/gene cloning). *Proc. Nati*. Acad. Sc, 76(4), 1648–1652.

Fuqua, C., & Greenberg, E. P. (1998). Self perception in bacteria: Quorum sensing with acylated homoserine lactones. *Current Opinion in Microbiology*, 1(2), 183–189. https://doi.org/10.1016/S1369-5274(98)80009-X

Fuqua, W. C., Winans, S. C., & Greenberg, E. P. (1994). Quorum sensing in bacteria: The LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology*, 176(2), 269–275. https://doi.org/10.1128/jb.176.2.269-275.1994

Girard, G., van Rij, E. T., Lugtenberg, B. J. J., & Bloemberg, G. v. (2006). Regulatory roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL1391. *Microbiology*, 152(1), 43–58. https://doi.org/10.1099/mic.0.28284-0

Gruber, T. M., & Gross, C. A. (2003). Multiple sigma subunits and the partitioning of bacterial transcription space. *Annual Review of Microbiology*, 57(1), 441–466. https://doi.org/10.1146/annurev.micro.57.030502.090913

Guo, J. H., Qi, H. Y., Guo, Y. H., Ge, H. L., Gong, L. Y., Zhang, L. X., & Sun, P. H. (2004). Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. *Biological Control*, 29(1), 66–72. https://doi.org/10.1016/S1049-9644(03)00124-5

Guttenberger, N., Blankenfeldt, W., & Breinbauer, R. (2017) Recent developments in the isolation, biological function, biosynthesis, and synthesis of phenazine natural products. *Bioorganic & Medicinal Chemistry*, 25(22), 6149–6166. https://doi.org/10.1016/j.bmc.2017.01.002

Hahn, M. (2014). The rising threat of fungicide resistance in plant pathogenic fungi: *Botrytis* as a case study. *Journal of Chemical Biology*, 7(4), 133–141. https://doi.org/10.1007/s12154-014-0113-1

Hammer, P. E., Hill, D. S., Lam, S. T., van Pée, K. H., & Ligon, J. M. (1997). Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. *Applied and Environmental Microbiology*, 63(6), 2147–2154. https://doi.org/10.1128/aem.63.6.2147-2154.1997

Hao, Y., Winans, S. C., Glick, B. R., & Charles, T. C. (2010). Identification and characterization of new LuxR/LuxI-type quorum sensing systems from metagenomic libraries. *Environmental Microbiology*, 12(1), 105–117. https://doi.org/10.1111/j.1462-2920.2009.02049.x

Heeb, S., & Haas, D. (2001). Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. *Molecular Plant-Microbe Interactions*, 14(12), 1351–1363. https://doi.org/10.1094/MPMI.2001.14.12.1351

Hegedus, D. D., & Rimmer, S. R. (2005). *Sclerotinia sclerotiorum*: When "to be or not to be" a pathogen? *FEMS Microbiology Letters*, 251(2), 177–184. https://doi.org/10.1016/j.femsle.2005.07.040

Hill, D. S., Stein, J. I., Torkewitz, N. R., Morse, A. M., Howell, C. R., Pachlatko, J. P., Becker, J. O., & Ligon, J. M. (1994). Cloning of genes involved in the synthesis of pyrrolnitrin from *Pseudomonas fluorescens* and role of pyrrolnitrin synthesis in biological control of plant disease. A*pplied and Environmental Microbiology,* 60(1), 78–85. https://doi.org/10.1128/aem.60.1.78-85.1994

Hmelo, L. R., Borlee, B. R., Almblad, H., Love, M. E., Randall, T. E., Tseng, B. S., Lin, C., Irie, Y., Storek, K. M., Yang, J. J., Siehnel, R. J., Howell, P. L., Singh, P. K., Tolker-Nielsen, T., Parsek, M. R., Schweizer, H. P., & Harrison, J. J. (2015). Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. *Nature Protocols*, 10(11), 1820–1841. https://doi.org/10.1038/nprot.2015.115

Karygianni, L., Ren, Z., Koo, H., & Thurnheer, T. (2020). Biofilm matrixome: extracellular components in structured microbial communities. *Trends in Microbiology*, article in press, 1–14. https://doi.org/10.1016/j.tim.2020.03.016

Keel, C. (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Molecular Plant-Microbe Interactions*, 5(1), 4-13. https://doi.org/10.1094/mpmi-5-004

Khan, S. R., Mavrodi, D. V., Jog, G. J., Suga, H., Thomashow, L. S. & Ferrand, S. K. (2005). Activation of the *phz* operon of *Pseudomonas fluorescens* 2-79 requires the LuxR homolog PhzR, *N*-(3-OH-Hexanoyl)-L-homoserine lactone produced by the LuxI homolog PhzI, and a *cis*-acting *phz* box. *J Bacteriol* 187, 6517-6527.

Kim, S. J., Kim, Y. H., Anderson, A. J., Kim, Y. C. 2013. The sensor kinase GacS negatively regulates flagellar formation and motility in a biocontrol bacterium, *Pseudomonas chlororaphis* 06. *Plant Pathol J.,* 30(2), 215-219. http://dx.doi.org/10.5423/PPJ.NT.11.2013.0109

Kitahara, T. & Kanda, N. (1975). DB-2073, a new alkylresorcinol antibiotic. II. The chemical structure of DB-2073. J Antibiot (Tokyo) 28, 943–946

Klaponski, N., Selin, C., Duke, K., Spicer, V., Fernando, D. W., Belmonte, M. F., & de Kievit, T. R. (2014). The requirement for the LysR-type regulator PtrA for *Pseudomonas chlororaphis* PA23 biocontrol revealed through proteomic and phenotypic analysis. *BMC Microbiology*, 14(1), 94. https://doi.org/10.1186/1471-2180-14-94

Knowles, C. J. (1976). Microorganisms and cyanide. *Bacteriological Reviews*, 40(3), 652–680. https://doi.org/10.1128/mmbr.40.3.652-680.1976

Kojic, M., & Venturi, V. (2001). Regulation of rpoS gene expression in *Pseudomonas*: Involvement of a TetR family regulator. *Journal of Bacteriology*, 183(12), 3712–3720. https://doi.org/10.1128/JB.183.12.3712-3720.2001

Lapouge, K., Schubert, M., H-T Allain, F., & Haas, D. (2007). MicroReview Gac/Rsm signal transduction pathway of g-proteobacteria: from RNA recognition to regulation of social behaviour. *Molecular Microbiology*, 67(2), 241-253. https://doi.org/10.1111/j.1365-2958.2007.06042.x

Latifi, A., Winson, M. K., Foglino, M., Bycroft, B. W., Stewart, G. S. A. B., Lazdunski, A., & Williams, P. (1995). Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Molecular Microbiology*, 17(2), 333–343. https://doi.org/10.1111/j.1365-2958.1995.mmi_17020333.x

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G., & Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proceedings of the National Academy of Sciences of the United States of America*, 89(5), 1562–1566. https://doi.org/10.1073/pnas.89.5.1562

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods,* 25, 402–408.

Maddula, V. S. R. K., Zhang, Z., Pierson, E. A., & Pierson, L. S. (2006). Quorum sensing and phenazines are involved in biofilm formation by *Pseudomonas chlororaphis* (*aureofaciens*) strain 30-84. *Microbial Ecology*, 52(2), 289–301. https://doi.org/10.1007/s00248-006-9064-6

Makhlouf, A. H., & Hamedo, H. A. (2013). Suppression of bacterial wilt disease of tomato plants using some bacterial strains. *Life Science Journal*, 10(3), 1732–1741.

Manitoba Agriculture (n.d.). Sclerotinia in Canola. Retrieved from https://www.gov.mb.ca/agriculture/crops/plant-diseases/sclerotinia-canola.html

Manitoba Canola Growers. 2020. Biopesticides as a Novel Management Strategy for Sclerotinia in Canola. Retrieved from https://canolagrowers.com/research-article/biopesticides-as-a-novel-management-strategy-for-sclerotinia-in-canola/

Manuel, J., Selin, C., Dilantha Fernando, W. G., & de Kievit, T. (2012). Stringent response mutants of *Pseudomonas chlororaphis* PA23 exhibit enhanced antifungal activity against *Sclerotinia sclerotiorum* in vitro*. Microbiology,* 158(1), 207–216. https://doi.org/10.1099/mic.0.053082-0

Marles-Wright, J., & Lewis, R. J. (2007). Stress responses of bacteria. *Current Opinion in Structural Biology*, 17(6), 755–760. https://doi.org/10.1016/j.sbi.2007.08.004

Mavrodi, D. V, Ksenzenko, V. N., Bonsall, R. F., Cook, R. J., Boronin, A. M., & Thomashow, L. S. (1998). A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79. *Journal of Bacteriology,* 180(9), 2541–2548.

Mavrodi, D. V, Blankenfeldt, W., & Thomashow, L. S. (2006). Phenazine compounds in fluorescent *Pseudomonas*. *Annual Review of Phytopathology*, 44, 417-445. https://doi.org/10.1146/annurev.phyto.44.013106.145710

Minogue, Leanne. November 6, 2018. New Canola Varieties for 2019. *Canola News*. https://www.grainews.ca/crops/canola/new-canola-varieties-for-2019/

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S. A. B., & Williams, P. (1997). Quorum sensing and *Chromobacterium violaceum*: Exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology*, 143(12), 3703–3711. https://doi.org/10.1099/00221287-143-12-3703

Mossialos, D., Ochsner, U., Baysse, C., Chablain, P., Pirnay, J. P., Koedam, N., Budzikiewicz, H., Fernández, D. U., Schäfer, M., Ravel, J., & Cornelis, P. (2002). Identification of new, conserved, non-ribosomal peptide synthetases from fluorescent pseudomonads involved in the biosynthesis of the siderophore pyoverdine. *Molecular Microbiology*, 45(6), 1673–1685. https://doi.org/10.1046/j.1365-2958.2002.03120.x

Nandi, M., Selin, C., Brassinga, A. K. C., Belmonte, M. F., Fernando, W. G. D., Loewen, P. C., & de Kievit, T. R. (2015). Pyrrolnitrin and hydrogen cyanide production by *Pseudomonas chlororaphis* strain PA23 exhibits nematicidal and repellent activity against *Caenorhabditis elegans*. *PLoS ONE*, 10(4), 1–19. https://doi.org/10.1371/journal.pone.0123184

Nandi, M., Selin, C., Brawerman, G., Fernando, W. G. D., & de Kievit, T. (2017). Hydrogen cyanide, which contributes to *Pseudomonas chlororaphis* strain PA23 biocontrol, is upregulated in the presence of glycine. *Biological Control*, 108, 47–54. https://doi.org/10.1016/j.biocontrol.2017.02.008

Navazo, A., Barahona, E., Redondo-Nieto, M., Martínez-Granero, F., Rivilla, R., & Martín, M. (2009). Three independent signalling pathways repress motility in *Pseudomonas fluorescens* F113. *Microbial Biotechnology*, 2(4), 489-498. https://doi.org/10.1111/j.1751-7915.2009.00103.x

Ng WL, Bassler BL. Bacterial quorum-sensing network architectures. 2009. *Annu Rev Genet*, 43, 197–222. doi: 10.1146/annurev-genet-102108-134304 PMID: 19686078

Ochsner, U. A., Koch, A. K., Fiechter, A., & Reiser, J. (1994). Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 176(7), 2044–2054. https://doi.org/10.1128/jb.176.7.2044-2054.1994

Ordóñez-Valencia, C., Ferrera-Cerrato, R., Quintanar-Zúñiga, R. E., Flores-Ortiz, C. M., Guzmán, G. J. M., Alarcón, A., Larsen, J., & García-Barradas, O. (2015). Morphological development of sclerotia by *Sclerotinia sclerotiorum*: a view from light and scanning electron microscopy. *Annals of Microbiology*, 65(2), 765–770. https://doi.org/10.1007/s13213-014-0916-x

Paulin, M. M., Novinscak, A., Lanteigne, C., Gadkar, V. J., & Filion, M. (2017). Interaction between 2,4-diacetylphloroglucinol- and hydrogen cyanide-producing *Pseudomonas brassicacearum* LBUM300 and *Clavibacter michiganensis* subsp. *michiganensis* in the tomato rhizosphere. *Applied and Environmental Microbiology,* 83(13), 1–13.

Pawar, S., Chaudhari, A., Prabha, R., Shukla, R., & Singh, D. P. (2019). Microbial pyrrolnitrin: Natural metabolite with immense practical utility. *Biomolecules*, 9(443), 1-26. https://doi.org/10.3390/biom9090443

Peix, A., Ramírez-Bahena, M-H., Velázquez, E. (2009). Historical evolution and current status of the taxonomy of genus *Pseudomonas*. *Infection, Genetics and Evolution*, 9, 1132–1147. https://doi.org/10.1016/j.meegid.2009.08.001

Peng, G., Lahlali, R., Hwang, S. F., Pageau, D., Hynes, R. K., McDonald, M. R., Gossen, B. D., & Strelkov, S. E. (2014). Special Issue: Crop rotation, cultivar resistance, and fungicides/ biofungicides for managing clubroot (*Plasmodiophora brassicae*) on canola. *Canadian Journal of Plant Pathology*, 36(S1), 99–112. https://doi.org/10.1080/07060661.2013.860398

Pessi, G., & Haas, D. (2000). Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 182(24), 6940–6949. https://doi.org/10.1128/JB.182.24.6940-6949.2000

Pierson III, L. S., Keppenne, V. D., & Wood, D. W. (1994). Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *Journal of Bacteriology*, 176(13), 3966–3974.

Pierson III, L. S., & Pierson, E. A. (2010). Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Applied Microbial Biotechnology*, 86, 1659–1670. https://doi.org/10.1007/s00253-010-2509-3

Pohanka, A., Levenfors, J., & Broberg, A. (2006). Antimicrobial dialkylresorcinols from *Pseudomonas* sp. Ki19. *Journal of Natural Products*, 69(4), 654–657. https://doi.org/10.1021/np0600595

Poritsanos, N., Selin, C., Fernando, W. G. D., Nakkeeran, S., & de Kievit, T. R. (2006). A GacS deficiency does not affect *Pseudomonas chlororaphis* PA23 fitness when growing on canola, in aged batch culture or as a biofilm. *Canadian Journal of Microbiology*, 52(12), 1177–1188. https://doi.org/10.1139/w06-079

Potrykus, K., & Cashel, M. (2008). (p)ppGpp: Still Magical? *Annual Review of Microbiology*, 62(1), 35–51. https://doi.org/10.1146/annurev.micro.62.081307.162903

Raaijmakers, J. M., Weller, D. M., & Thomashow, L. S. (1997). Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied and Environmental Microbiology*, 63(3), 881–887. https://doi.org/10.1128/aem.63.3.881-887.1997

Rajput, A., Kaur, K., & Kumar, M. (2016). SigMol: Repertoire of quorum sensing signaling molecules in prokaryotes. *Nucleic Acids Research*, 44(D1), D634–D639. https://doi.org/10.1093/nar/gkv1076

Raymer, P. L. (2002). Canola: An emerging oilseed crop. *Trends in New Crops and New Uses*, 122–126.

Rella, J., Marcus, S., & Wagner, B. J. (2004). Rapid cyanide detection using the cyantesmo® kit. *Journal of Toxicology - Clinical Toxicology,* 42(6), 897–900. https://doi.org/10.1081/CLT-200035349

Rudrappa, T., Splaine, R. E., Biedrzycki, M. L., & Bais, H. P. (2008). Cyanogenic pseudomonads influence multitrophic interactions in the rhizosphere. *PLoS ONE*, 3(4). https://doi.org/10.1371/journal.pone.0002073

Sarniguet, A., Kraus, J., Henkels, M. D., Muehlchen, A. M., & Loper, J. E. (1995). The sigma factor σ^S affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. *Proceedings of the National Academy of Sciences of the United States of America*, 92(26), 12255–12259. https://doi.org/10.1073/pnas.92.26.12255

Savchuk, S., & Dilantha Fernando, W. G. (2004). Effect of timing of application and population dynamics on the degree of biological control of *Sclerotinia sclerotiorum* by bacterial antagonists. *FEMS Microbiology Ecology*, 49(3), 379–388. https://doi.org/10.1016/j.femsec.2004.04.014

Schippers, B., Bakker, A. W., Bakker, P. A. H. M., & van Peer, R. (1990). Beneficial and deleterious effects of HCN-producing pseudomonads on rhizosphere interactions. *Plant and Soil*, 129(1), 75–83. https://doi.org/10.1007/BF00011693

Schuster, M., Urbanowski, M. L., Greenberg, E. P. 2004. Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci U S A*, 101(45):15833–15839. https://doi.org/10.1073/pnas.0407229101 PMID: 15505212

Selin, C., Dilantha Fernando, W. G., & de Kievit, T. R. (2012). The PhzI/PhzR quorum-sensing system is required for pyrrolnitrin and phenazine production, and exhibits cross-regulation with RpoS in *Pseudomonas chlororaphis* PA23. *Microbiology*, 158(4), 896–907. https://doi.org/10.1099/mic.0.054254-0

Selin, C., Habibian, R., Poritsanos, N., Athukorala, S. N. P., Fernando, D., & de Kievit, T. R. (2010). Phenazines are not essential for *Pseudomonas chlororaphis* PA23 biocontrol of *Sclerotinia sclerotiorum*, but do play a role in biofilm formation. *FEMS Microbiology Ecology*, 71(1), 73–83. https://doi.org/10.1111/j.1574-6941.2009.00792.x

Selin, C., Manuel, J., Fernando, W. G. D., & de Kievit, T. (2014). Expression of the *Pseudomonas chlororaphis* strain PA23 Rsm system is under control of GacA, RpoS, PsrA, quorum sensing and the stringent response. *Biological Control*, 69, 24–33. https://doi.org/10.1016/j.biocontrol.2013.10.015

Shah, N., Gislason, A. S., Becker, M., Belmonte, M. F., Fernando, W. G. D., & de Kievit, T. R. (2020). Investigation of the quorum-sensing regulon of the biocontrol bacterium *Pseudomonas chlororaphis* strain PA23. *PloS One*, 15(2), e0226232. https://doi.org/10.1371/journal.pone.0226232

Shah, N., Klaponski, N., Selin, C., Rudney, R., Fernando, W. G. D., Belmonte, M. F., & de Kievit, T. R. (2016). PtrA is functionally intertwined with GacS in regulating the biocontrol activity of *Pseudomonas chlororaphis* PA23. *Front Microbiol*, 7(1512), 1–14. https://doi.org/10.3389/fmicb.2016.01512

Shanmugaiah, V., Nithya, K., Harikrishnan, H., Jayaprakashvel, M., & Balasubramanian, N. (2015). Biocontrol mechanisms of siderophores against bacterial plant pathogens. *Sustainable Approaches to Controlling Plant Pathogenic Bacteria*, 167–190. https://doi.org/10.1201/b18892-9

Silby, M. W., Winstanley, C., Godfrey, S. A. C., Levy, S. B., & Jackson, R. W. (2011). *Pseudomonas* genomes: Diverse and adaptable. *FEMS Microbiology Reviews*, 35(4), 652–680. https://doi.org/10.1111/j.1574-6976.2011.00269.x

Simons, M., van der Bij, A. J., Brand, I., de Weger, L. A., Wijffelman, C. A., & Lugtenberg, B. J. J. (1996). Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Molecular Plant-Microbe Interactions*, 9(7), 600–607. https://doi.org/10.1094/MPMI-9-0600

Smith, E. E., Sims, E. H., Spencer, D. H., Kaul, R., & Olson, M. v. (2005). Evidence for diversifying selection at the pyoverdine locus of *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 187(6), 2138–2147. https://doi.org/10.1128/JB.187.6.2138-2147.2005

Sonnleitner, E., & Haas, D. (2011). Small RNAs as regulators of primary and secondary metabolism in *Pseudomonas* species. *Applied Microbiology and Biotechnology*, 91(1), 63–79. https://doi.org/10.1007/s00253-011-3332-1

Takeuchi, K., Yamada, K., & Haas, D. (1440). ppGpp controlled by the Gac/Rsm regulatory pathway sustains biocontrol activity in *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interactions MPMI*, 25(11), 1440–1449. https://doi.org/10.1094/MPMI-02-12-0034-R

Toohey, J. I., Nelson, C. D., & Krotkov, G. (1965). Toxicity of phenazine carboxylic acids to some bacteria, algae, higher plants, and animals. *Canadian Journal of Botany*, 43(9), 1151–1155. https://doi.org/10.1139/b65-128

Tripathi, L., Zhang, Y., & Lin, Z. (2014). Bacterial sigma factors as targets for engineered or synthetic transcriptional control. *Frontiers in Bioengineering and Biotechnology*, 2(33), 1–7. https://doi.org/10.3389/fbioe.2014.00033

Tripathi, R. K., & Gottlieb, D. (1969). Mechanism of action of the antifungal antibiotic pyrrolnitrin. *Journal of Bacteriology*, 100(1), 310–318. https://doi.org/10.1128/jb.100.1.310-318.1969

Van Delden, C., Comte, R., & Bally, M. (2011). Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. *Biosensors and Bioelectronic*s, 183(18), 5376–5384. https://doi.org/10.1128/JB.183.18.5376

Veliz, A. E., Martínez-Hidalgo, P. & M. Hirsch, A. (2017). Chitinase-producing bacteria and their role in biocontrol. *AIMS Microbiology*, 3(3), 689–705. https://doi.org/10.3934/microbiol.2017.3.689

Venturi, V. (2003). Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: Why so different? *Molecular Microbiology*, 49(1), 1–9. https://doi.org/10.1046/j.1365-2958.2003.03547.x

Venturi, V. (2006). Regulation of quorum sensing in *Pseudomonas. FEMS Microbiology Reviews*, 30(2), 274–291. https://doi.org/10.1111/j.1574-6976.2005.00012.x

Walsh, U. F., Morrissey, J. P., & Gara, F. O. (2001). *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation*. Current Opinion in Biotechnology*, 12, 289–295.

Wang, D., Lee, S. H., Seeve, C., Yu, J. M., Pierson, L. S., & Pierson, E. A. (2013). Roles of the Gac-Rsm pathway in the regulation of phenazine biosynthesis in *Pseudomonas chlororaphis* 30-84. *MicrobiologyOpen*, 2(3), 505–524. https://doi.org/10.1002/mbo3.90

Watnick, P., & Kolter, R. (2000). Biofilm, city of microbes. *Journal of Bacteriology*, 182(10), 2675–2679. https://doi.org/10.1128/JB.182.10.2675-2679.2000

Weller, D. M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. 97(2), *Phytopathology*, 250-256.

Wellington, S., Greenberg, P. E. (2019). Quorum sensing signal selectivity and the potential for interspecies cross talk. *Molecular Biology and Physiology*, 10(2), 1–14.

West, S. E. H., Schweizer, H. P., Dall, C., Sample, A. K., & Runyen-Janecky, L. J. (1996). Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the

region required for their replication in *Pseudomonas aeruginosa*. *Behavioral Interventions*, 11(3), 141–146.

Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L., & Salmond, G. P. C. (2001). Quorum-sensing in Gram-negative bacteria. *FEMS Microbiology Reviews*, 25(2001), 365-404.

Whiteley, M., Diggle, S. P., & Greenberg, E. P. (2017). Bacterial quorum sensing: the progress and promise of an emerging research. *Nature*, 551(7680), 313–320. https://doi.org/10.1038/nature24624

Wirth, S. J., & Wolf, G. A. (1990). Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. *Journal of Microbiological Methods*, 12(3–4), 197–205. https://doi.org/10.1016/0167-7012(90)90031-Z

Wood, D. W. & Pierson, L. S. III. (1996). The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* 168, 49-53.

Zhang, Z., & Pierson, L. S. 3rd. (2001). A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Applied and Environmental Microbiology*, 67(9), 4305–4315. https://doi.org/10.1128/AEM.67.9.4305

7. APPENDIX

Appendix Table 1. **Phenotypic characterization of *Pseudomonas chlororaphis* PA23 complement and cross-complement strains.**

	Exometabolite Activity				
Strains	Protease activity [§]	Fungal inhibition [§]	Autoinducer production [§]	Swimming motility [Ŧ]	HCN [Ŧ,‡]
PA23 wild type	6.70 (0.48)	4.16 (1.09)	4.50 (0.52)	14.50 (0.70)	+
PA23c*saR* C'	7.07 (0.47)	6.66 (1.87) **	4.91 (0.26)	22.71 (3.98) *	+
PA23*csaRXC'*	6.70 (0.64)	8.26 (1.62) **	4.86 (0.63)	9.83 (1.72) *	+
PA23*csaI* C'	5.04 (0.78)	8.56 (0.92) **	5.13 (1.21) **	11.37 (2.24)	+
PA23*csaI* XC'	4.50 (0.66)	8.50 (1.60) **	4.81 (0.75)	13.37 (5.73)	+
PA23*phzR* C'	6.73 (0.70)	4.80 (0.86)	5.00 (0.53) *	15.25 (3.65)	+
PA23*phzR* XC'	4.40 (0.82) **	0.73 (1.22) **	1.06 (0.70) **	77.37 (3.46) **	+
PA23*phzI* C'	5.00 (1.32) **	4.50 (1.22)	4.66 (0.44)	12.41 (5.13)	+
PA23*phzI* XC'	5.25 (0.50) **	3.85 (1.71)	4.43 (0.51)	11.62 (2.44)	+

[§] Mean (standard deviation) of the zones of activity (mm) obtained from a triplicate set.

Ŧ Mean (standard deviation) of the zones of activity (mm) obtained from 5 biological replicates.

‡ Results (+/-) determined using Cyantesmo paper.

Significantly different from wild type (*$p < 0.05$; **$p < 0.005$)