Biocontrol Agents *Pseudomonas brassicacearum* DF41 & *Pseudomonas chlororaphis* PA23: Investigation of Fungal Suppression and Defense Against *Caenorhabditis elegans*

By

Munmun Santi Nandi

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology

University of Manitoba

Winnipeg

Abstract

The success of biocontrol bacteria is often restrained due to their low persistence in the rhizosphere and fluctuations in expression of antagonistic compounds. In the first part of this thesis the ability of the biocontrol agents (BCAs) *Pseudomonas brassicacearum* DF41 and *Pseudomonas chlororaphis* PA23 to resist grazing by the bacterivorous nematode *Caenorhabditis elegans* was investigated. We found that both BCAs are capable of killing the nematodes through exposure to toxic metabolites. We discovered that in addition to HCN, pyrrolnitrin (PRN) is a potent nematicide produced by PA23. Unique for a pseudomonad, DF41 was also found to kill the nematodes by forming biofilms on the nematode anterior, causing starvation. Biofilm formation was dependent upon the Gac two-component system and NaCl concentration of the media. Co-culturing these BCAs in the presence of nematodes increased expression of a number of genes associated with biocontrol. We observed elevated exoproduct formation, consistent with our gene expression analysis. The nematicidal activity exhibited by DF41 and PA23 towards *C. elegans* bodes well for their persistence in the environment.

In the second part of this thesis the role of hydrogen cyanide (HCN) and the anaerobic regulator ANR in PA23 biocontrol was explored. An *hcn* mutant was created and *in vitro* antifungal (AF) assays revealed the involvement of HCN in *Sclerotinia sclerotiorum* suppression. Addition of glycine promoted both AF activity and HCN production. In addition, HCN was found to be positively regulated by quorum sensing (QS). Besides a *phz* box, an *anr* box was identified in the *hcnA* promoter, suggesting a role for ANR in regulating *hcnA*. An *anr* mutant was generated and phenotypic characterization revealed that ANR is a key regulator governing PA23 secondary metabolite production. Through gene expression analysis, ANR was shown to positively regulate *phzI*/*phzR* and PhzR negatively regulate *anr*. Furthermore,

expressing *anr* in *trans* partially complemented the QS-deficient phenotype with respect to several biocontrol genes and exoproducts. Overall, the global regulator ANR is vital for PA23-mediated biocontrol and a significant overlap exists between the QS and ANR regulons.

Acknowledgments

Firstly, I would like to express my sincere gratitude to my supervisor Dr. Teresa de Kievit for giving me the opportunity to work in her lab. I greatly appreciate your support, guidance and encouragement that helped me stay focused, even during tough times in the PhD pursuit. Your hard work, professionalism and enthusiasm have been motivational and will be my inspiration throughout my future career.

I would like to thank my committee members, Drs Peter Loewen, Karen Brassinga and Mark Belmonte for your time and helpful suggestions. I am thankful to Dr. Loewen, your generosity and constant support throughout this journey have been greatly appreciated. I would like to thank Dr. Brassinga for teaching me the techniques required to work with *C. elegans* and extending your lab facilities to me. As well, thanks to Dr. Belmonte for sharing your lab equipments that I needed to use for my experiments.

I am grateful to Dr. Thomas Ward for letting me use the HPLC machine in your lab and would like to thank Carrie for your assistance and time devoted while performing the experiment.

I would also like to thank my past and present labmates, Carrie, Natasha, Kelly, Nidhi, Sanjay, Akrm who made this journey enjoyable, as it would not be the same without your support and friendship. I will always cherish the moments that we have spent together.

Finally, I would like to thank my family for their love, moral support, blessings and well wishes that have always helped me to stand up in the difficult times. To my father, for raising me with a love of science and supporting me in all my accomplishments, I would like to thank you and let you know that you are always in my thoughts.

Table of contents

List of Tables

List of Figures

List of Abbreviations

AF	antifungal
AHL	acylhomoserine lactone
AI	autoinducer
Amp	ampicillin
ANR	anaerobic regulator of arginine deiminase and nitrate reductase
AWA	amphid wing “A”
AWB	amphid wing “B”
AWC	amphid wing “C”
BCA	biocontrol agent
BHI	Brain Heart Infusion
bp	base pair
Carb	carbenicillin
cDNA	complementary DNA
Chl	chloramphenicol
CRP	cAMP receptor protein
Csa	cell surface alterations
DAPG	2,4-Diacetylphloroglucinol
EHEC	Enterohemorrhagic *E. coli*
EPA	Environmental Protection Agency
$FeCl_3$	ferric chloride
FNR	fumarate and nitrate reductase regulator
Gac	global activator of antibiotic and cyanide
Gm	gentamicin
HCl	hydrochloric acid
HCN	hydrogen cyanide
HPLC	High Performance Liquid Chromatography
IMS	imaging mass spectrometry
ISR	induced systemic resistance
KB	King’s B
LB	lysogeny broth

LP	lipopeptide
LPS	lipopolysaccharide
NADH	nicotinamide adenine dinucleotide
NGM	nematode growth medium
NRPS	nonribosomal peptide synthetase
2-OH-PHZ	2-hydroxy-phenazine
PCA	phenazine-1-carboxylic acid
PCN	phenazine-1-carboxamide
PCR	polymerase chain reaction
PDA	potato dextrose agar
PG	phloroglucinol
PGPR	plant growth promoting rhizobacteria
PHZ	phenazine
PIA	Pseudomonas Isolation Agar
Pip	piperacillin
PLT	pyoluteorin
ppGpp	guanosine tetraphosphate
pppGpp	guanosine pentaphosphate
PRN	pyrrolnitrin
PsrA	*Pseudomonas* sigma regulator A
PVD	pyoverdin
PYO	pyocyanin
QS	quorum sensing
RBS	ribosome binding site
Rif	rifampicin
RNAP	RNA polymerase
Rsm	regulator of secondary metabolism
SR	stringent response
Tc	tetracycline

Chapter 1

Literature Review

1.1. Canadian agriculture and crop disease

Canadian agricultural systems provide a diverse range of grain and oilseed crops for domestic and international markets. Industrialization of agriculture has provoked a significant and essential productivity increase, which has led to a greater amount of food available to the population. So it is of vital importance to protect the productive capacity of Canadian prairie farms from incidence of crop diseases. Current cropping systems include simplified, monoculture-based conditions, a model which has resulted in the development of unintended negative consequences such as diseases caused by potential plant pathogens. This in turn can cause significant crop losses followed by dramatically decreased agricultural yield. Disease control is partly achieved through using crops that have been bred for resistance to many diseases, and by cultivation approaches such as crop rotation. Although a large sector of disease management techniques includes the application of chemical pesticides and herbicides, repeated use of such chemicals can lead to pesticide-resistance. Moreover, the use of agrochemicals has raised serious concerns not only of harmful effects on human health, but also detrimental effects on surrounding ecosystems, wildlife, and other non-target organisms. An environmentally safe alternative to suppress phytopathogens, therefore, is urgently needed.

1.2. *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic soil-borne fungal pathogen, which belongs to the phylum Ascomycota. It is a destructive pathogen that is capable of infecting over 400 plant species worldwide (Purdy, 1979), including canola (Manitoba Agriculture, 2002). This fungus occurs in all the canola growing areas of Canada and causes stem rot of canola

(Nelson, 1998). Under conditions conducive for fungal infection, sclerotinia stem rot (SSR) can result up to 100% yield loss (Kutcher *et al*., 2011).

S. sclerotiorum produces white mycelia that aggregate into hard, over-wintering bodies called sclerotia to allow the species to survive in soil for up to 8 years in the absence of a plant host (Adams and Ayers, 1979). So, management of this fungus becomes difficult due to the resilience of sclerotia within soil and its unique life cycle. Sclerotia germinate when conditions are suitable to produce mycelium that begin as microscopic filaments capable of infecting plants, especially root tissues (Purdy, 1979). Alternatively, they can germinate into mushroom-like fruiting bodies termed apothecia, which release ascospores over a period of several days. Ascospores are blown by wind up to one kilometer to infect the aerial portions of plants (Venette, 1998).

Based on recent statistics related to canola crop losses in Manitoba and Saskatchewan, SSR is one of the major causes for yield losses ranging between 5 – 100% in a growing season (Canadian Phytopathological Society, 2013). Although synthetic fungicides, such as Vertisan® 7 (Dupont), Astound® (Syngenta), Quash® (Valent) were approved to control SSR in canola (Saskatchewan Ministry of Agriculture, 2013), they are considered to have broad-spectrum modes of action.

1.3. Biological control of phytopathogens by *Pseudomonas* species

1.3.1. Biological control

Biological control, or in short biocontrol, is the purposeful utilization of living organisms to suppress the activities and populations of one or more plant pathogens. The term biological control was first coined by a phytopathologist, Carl Freiherr Von Tubeuf in 1914 and since then

various biocontrol products have been developed to control plant pests (Junaid *et al*., 2013). The era of biocontrol of insects started more than 100 years ago with the highly successful introduction of the vedalia beetle from Australia to California (1888) to control cottony cushion scale of citrus (Howard, 1930). But the idea of biocontrol of plant diseases is relatively new. For example, in 1979 the first bacterium *Agrobacterium radiobacter* strain K 84 was registered with the United States Environmental Protection agency (EPA) for the control of crown gall disease (Junaid *et al*., 2013). Interestingly, the innovation of biocontrol of soil-borne pathogens was actually recognized through the discovery of naturally suppressive soils, where the roots of crop plants do not suffer from diseases even in the presence of soil-borne pathogenic microorganisms (Schroth and Hancock, 1982; Hornby, 1983). This phenomenon was found to be due to the presence of antagonistic soil-associated microorganisms which can inhibit pathogens by producing antibiotic compounds (Haas and Défago, 2005). A number of soil microorganisms, including species from the genera *Streptomyces*, *Bacillus*, *Pseudomonas*, *Agrobacterium* and *Burkholderia* have been identified as potential biocontrol agents (BCAs) (Baker and Snyder, 1965). Research on BCAs indicates three main modes of action: i) antibiosis or the production of metabolites that have a detrimental effect on other organisms; ii) induced systemic resistance (ISR) to stimulate the plant defense response and iii) pathogen-antagonist interaction (Haas and Défago, 2005).

1.3.2. Biocontrol activity of *Pseudomonas* species

Members of the genus *Pseudomonas* belong to the class gammaproteobacteria which includes both medically and ecologically important groups of bacteria. They are ubiquitous, Gram-negative, rod shaped bacteria and have one or several polar flagella, providing motility

(Palleroni, 2008; Pertot *et al*., 2015). This group is characterized for its metabolic versatility, and is capable of aerobic as well as anaerobic respiration (Haas and Défago, 2005).

Pseudomonas species found in agricultural soils are well adapted to grow in the rhizosphere and they possess many traits which make them efficient growth promoters and BCAs (Pertot *et al*., 2015). Such traits are facilitated by their ability to rapidly utilize seed and root exudates, and colonize and multiply in the rhizosphere to stimulate plant growth, hence they are called plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Additionally, by producing a wide spectrum of bioactive metabolites, biocontrol pseudomonads can aggressively compete with other microorganisms and adapt to environmental stresses (Weller, 1988; Lugtenburg *et al.,* 2001). These features result in disease suppression of various plant pathogens including fungi, nematodes and bacteria. For example, studies have shown that *Pseudomonas* strains with the ability to produce the antifungal (AF) metabolite 2,4-diacetylphloroglucinol (DAPG) were able to suppress black root rot of tobacco and take-all disease of wheat (Keel *et al*., 1992; Raaijmakers *et al*., 1997). A well known biocontrol agent, *Pseudomonas protegens* (previously, *Pseudomonas fluorescens*) strain CHA0 was found to suppress infection by the root knot-nematode *Meloidogyne javanica* and nematode egg-hatching on tomato plants (Norabadi *et al*., 2013). Several *Pseudomonas* strains have contributed to the global biopesticide market which is expected to reach $4,556.37 million by 2019 with an annual growth rate of 15.30% from 2014 to 2019 (source: www.marketsandmarkets.com, 2014; Pertot *et al*., 2015).

1.3.3. Biocontrol compounds produced by *Pseudomonas* species

Pseudomonas spp. that are efficient BCAs produce one or more antibiotic compounds, siderophores and lytic enzymes (Pal and Gardener, 2006). This section will focus on the nature and modes of action of these secondary metabolites which act in different ways to suppress the plant pathogens.

1.3.3.1. Phenazines

Fluorescent pseudomonads are the best studied phenazine (PHZ) producing bacteria responsible for almost one-third of all known PHZs. PHZs represent a large group of nitrogen-containing heterocyclic pigments (Pierson and Pierson, 2010). The first PHZ compound, Pyocyanin (PYO), was found by Fordos in 1859; subsequently several other PHZs, such as phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), 1-hydroxyphenazine (1-OH-PHZ), 2-hydroxyphenazine (2-OH-PHZ), pyoverdin or pseudobactin (PVD) and aeruginosin A and B were reported (Hassan and Fridovich, 1980; Kerr *et al*., 1999; Mavrodi *et al*., 2006; Shanmugaiah *et al*., 2010; Ra'oof and Latif, 2010; Herbert and Holiman, 1969). Over 100 different PHZ structural derivatives have been identified in nature, that exhibit broad-spectrum activity towards fungi, bacteria, plant and animal tissue (Hernandez *et* al., 2004). Biosynthesis of PHZs occurs through a branch point in the shikimic acid pathway and a conserved seven gene operon, *phzABCDEFG* is responsible for their production (Haas and Keel, 2003; Chin-A-Woeng et al., 2003). These compounds are mainly composed of a central nitrogen ring, flanked by two benzene rings containing various side chain substitutions (Mavrodi *et al*., 2006).

PHZs contribute to biocontrol capabilities as well as environmental competitiveness of the organism (Haas and Keel, 2003). Being analogous to flavin coenzymes, PHZs inhibit

electron transport chains (Ran *et al*., 2003) and catalyze the formation of hydroxyl radicals causing damage to lipids and other macromolecules (Britigan *et al*., 1992). These molecules can modify cellular redox states and release soluble iron that might contribute to iron mobilization in soil under certain conditions (Hernandez *et al*., 2004). Moreover, PHZs have been found to be involved in biofilm formation, thereby enhancing bacterial survival (Drago, 2009; Maddula *et al*., 2008; Selin *et al*., 2010). Additionally, PHZs can act as signals capable of regulating patterns of gene expression (Dietrich *et al*., 2006).

1.3.3.2. Pyrrolnitrin

Pyrrolnitrin [3-chloro-4-(3´-chloro-2´-nitrophenyl)pyrrole] (PRN) is a halogenated bacterial metabolite and *Pseudomonas pyrrocinia* was the first species found to produce this compound in 1964 (Arima *et al*., 1964). Later a few other pseudomonads, including *P. protegens* and *P. chlororaphis* were reported to produce PRN (Sarniguet *et al.*, 1995; Haas and Keel, 2003). Biosynthesis of PRN, which starts with tryptophan, is mediated by a chloroperoxidase enzyme, encoded by the operon *prnABCD,* acting on tryptophan (Lively *et al*., 1967).

PRN is an AF antibiotic and its production has been implicated in the biocontrol of fungal phytopathogens by *Pseudomonas* species (Pfender *et al*., 1993; Hammer *et al*., 1997). PRN has broad spectrum antifungal activity against a wide range of Deuteromycete, Ascomycete, Basidiomycete fungi due to its ability to inhibit the respiratory electron transport system (Tawara *et al*., 1989). In *Saccharomyces cerevisiae,* PRN was shown to inhibit the terminal electron transport system between succinate or reduced nicotinamide adenine dinucleotide (NADH) and coenzyme Q (Tripathi and Gottlieb, 1969). Additionally, several PRN

derivatives were developed, such as fenpiclonil and fludioxonil (Ciba-Geigy, now Novartis) having increased activity as agricultural fungicides (Ligon *et al*., 2000).

1.3.3.3. 2,4-Diacetylphloroglucinol

Phloroglucinols (PG) are phenolic, secondary metabolites produced by several strains of *Pseudomonas* spp. (Sharifi-Tehrani *et al*., 1998). Over 60 PG derivatives have been shown to have antimicrobial, antihelminthic and plant growth-regulating activities (Keel *et al*., 1992; Cronin *et al*., 1997; Debabrata and Naik, 2000). One of the better known PGs, a polyketide antibiotic DAPG [1-(3-acetyl-2,4,6-trihydroxyphenyl)ethanone] has been shown to have a major role in biocontrol of plant diseases (Raaijmakers and Weller, 1998). Biosynthesis of DAPG is encoded by the *phlABCD* operon (Bangera and Thomashow, 1999).

DAPG was first detected from *P. protegens* strain PFM2 and found to have antagonistic activity against the plant pathogenic fungus *Septoria tritici* (Levy *et al*., 1992). Later, DAPG producing pseudomonads were found to suppress black root rot of tobacco (Stutz *et al*., 1986), take-all of wheat (Keel *et al*., 1992), Fusarium wilt (Duffy and Défago, 1997), damping-off of sugar beet (Fenton *et al*., 1992) and many more plant diseases. Moreover, studies have shown that DAPG can cause membrane damage to zoospores of *Pythium* spp. which include a major group of phytopathogenic fungi (de Souza *et al*., 2003).

1.3.3.4. Pyoluteorin

Pyoluteorin (4,5-dichloropyrrol-2-yl 2,6-dihydroxyphenyl) (PLT) is a chlorinated polyketide antibiotic produced by certain rhizospheric pseudomonads (Bailey *et al*., 1973). This compound was first identified from *P. aeruginosa* (Tekeda, 1958), followed by its detection from

other PGPR, such as *P. protegens* CHA0 (Maurhofer *et al*., 1992), *P. protegens* Pf-5 (Corbell & Loper, 1995) and *Pseudomonas aeruginosa* M18 (Hu *et al*., 2005). PLT is composed of a bichlorinated pyrrole linked to a resorcinol moiety (Wang *et al*., 2008) and it is synthesized from proline and acetate precursors (Nowak-Thompson *et al*., 1999). The PLT biosynthetic operon consists of eight structural genes, *pltLABCDEG* and *pltM* encoding enzymes required for its synthesis (Brodhagen *et al*., 2004).

PLT has been found to effectively inhibit several phytopathogenic fungi and oomycetes, including *Pythium ultimum* (Howell & Stipanovic, 1980; Maurhofer *et al*., 1994). Furthermore, production of PLT contributes to the ecological competence of the strain in the rhizosphere (Carmi *et al*., 1994; Babalola, 2010).

1.3.3.5. Lipopeptides

Lipopeptides (LPs) produced by *Pseudomonas* spp. have received considerable attention for their natural roles having antimicrobial, cytotoxic and surfactant properties (Nybroe & Sorensen, 2004; Raaijmakers *et al*., 2006).These bioactive molecules are composed of a lipid tail linked to a linear or cyclic oligopeptide with structural diversity due to varying length and composition of the lipid moiety, as well as number and configuration of the amino acids in the peptide chain (Raaijmakers *et al*., 2010). Biosynthesis of most LPs involves multifunctional enzymes encoded by large nonribosomal peptide synthetase (NRPS) genes, organized in modules that form the building blocks for the stepwise incorporation of each amino acid into the peptide head (Maraheil *et al.*, 1997).

The AF activity of LPs is attributed primarily to their ability to form pores in cell membranes, leading to an imbalance in transmembrane ion fluxes, causing cell death (Bender *et*

al., 1999; Baltz, 2009). For example, massetolide A was identified in biocontrol strains *P. fluorescens* SS101 and *Pseudomonas* sp. strain MF-30 and found to inhibit growth of the fungal pathogens *Fusarium oxysporum* and *Drechslera teres* (De Souza *et al*., 2003; Raaijmakers *et al*., 2006). Production of a cyclic LP named tensin by *P. fluorescens* strain 96.578 was essential for inhibition of mycelial growth and seed infection caused by *Rhizoctonia solani* (Nielsen *et al*., 2000). Other natural functions of LPs described to date include involvement in swarming motility and biofilm formation (Pauwelyn *et al*., 2013).

1.3.3.6. Hydrogen cyanide

Production of the toxic, volatile compound hydrogen cyanide (HCN) is well-documented among pseudomonads (Castric, 1975). According to Lorck (1948), glycine is the metabolic precursor of HCN in *P. aeruginosa*. The genetic basis of HCN synthesis involves three contiguous structural genes *hcnABC* encoding formate dehydrogenase (*hcnA*) and amino acid oxidases (*hcnB* and *hcnC*) (Laville *et al*., 1998). But significant polymorphism exists in *hcnBC* clusters among various biocontrol *Pseudomonas* spp. (Ramette *et al*., 2003). Together, the HCN synthase enzymes can stoichiometrically oxidize glycine to HCN and carbon dioxide (CO_2) in a reaction that conserves the substrate's C-N bond in HCN and derives CO_2 from the carboxyl group of glycine (Pessi and Haas, 2004).

Cyanide ion derived from HCN was found to be a potent inhibitor of many metalloenzymes including copper-containing cytochrome *c* oxidases (Blumer and Haas, 2000). It contributes to the broad-spectrum antimicrobial activity and suppression of different plant root diseases. HCN production by *P. protegens* CHA0 was found to suppress tobacco black root rot caused by *Thielaviopsis basicola*, and root rot of tomato caused by *Fusarium oxysporum* F. sp.

radicislycopersici (Flaishman *et al*., 1996; Voisard *et al*., 1989). Additionally, HCN has been suggested to have detrimental effects on some plant-pathogenic nematodes (Gallagher and Manoil, 2001; Insunza *et al*., 2002).

1.3.3.7. Siderophores

Iron is considered one of the essential nutrients of microbial life, although it is not readily available in the preferred form. Bacteria can produce small high-affinity chelating molecules known as siderophores for iron (III) acquisition and transport into cells (Neilands, 1981). Fluorescent pseudomonads are able to produce two unique siderophores, pyoverdin (PVD) or pseudobactin, and pyochelin (Meyer and Abdallah, 1978; Cox *et al*., 1981). Under iron-deficient conditions, PVD imparts a yellow-green fluorescence due to a chromophore group (Meyer *et al*., 1998). PVD has high affinity for Fe^{3+} ions and when bound to iron, is recognized and taken up by membrane-receptor proteins (Magazin *et al*., 1986). Synthesis of these aryl-capped siderophores, including PVD and pyochelin, is primarily dependent on NRPSs (Stintzi *et al*., 1996; Quadri *et al*., 1999).

The PVD-producing pseudomonads have been found to inhibit the growth of phytopathogenic fungi (Raaijmakers *et al*., 1995). In fact, the importance of siderophore production by *Pseudomonas* strain B10 was first demonstrated to convert disease-conducive soils to disease-suppressive soils, where siderophores had a major role in suppressing take-all and *Fusarium*-wilt diseases (Kloepper *et al*., 1980). Later, these compounds were shown to inhibit pathogens like *P. ultimum*, *Gaeumannomyces graminis* var. *tritici* and others (Becker and Cook, 1988; Cook and Weller, 1987). Overall, a siderophore producer might compete with other

microorganisms by sequestering the limited supply of iron in the rhizosphere, thereby suppressing the viability of microbes having less avid iron-uptake systems.

1.3.3.8. Lytic enzymes

Pseudomonads are well known for the production of lytic enzymes, such as proteases, lipases, cellulases, hemicellulases, etc. (Pal and Gardener, 2006). These degradative enzymes can hydrolyze a wide variety of polymeric compounds including fungal cell walls by degrading glucans and chitins (Chin-A-Woeng *et al*., 2003). Consequently, secretion of these enzymes may result in the suppression of various plant pathogens. For example, extracellular chitinases and glucanase synthesized by *Pseudomonas stutzeri* were shown to digest and lyse mycelia of *F. solani* (Lim *et al*., 1991). Moreover, extracellular protease of *P. protegens* CHA0 was found to play a major role in the inhibition of egg-hatch and induced mortality of the plant parasitic root-knot nematode *M. incognita* during tomato and soybean infection (Siddiqui *et al*., 2005).

1.3.4. Regulatory elements underlying biocontrol activity

The production of AF compounds by fluorescent pseudomonads is a key biocontrol determinant and so is the underlying metabolic regulation of exoproducts that occurs at both the transcriptional and post-transcriptional levels. Major regulatory pathways include the GacS/GacA two-component signal transduction, quorum sensing (QS), sigma factors, and the anaerobic regulator ANR which together form a complex regulatory hierarchy, to be discussed in more detail in the following sections.

1.3.4.1. Gac-Rsm regulatory control

In order to respond to changing environments, bacteria can adjust their cellular levels of secondary metabolite production through various regulatory pathways; the Gac-Rsm system (global activator of antibiotic and cyanide; regulator of secondary metabolism) is one of the most ubiquitous signal transduction systems employed for such purposes (Laville *et al*., 1992; Lapouge *et al*., 2008). GacS/GacA is a two-component system that forms a part of this regulatory circuit. GacS, a membrane-bound sensor kinase, was first discovered in the plant pathogen *P. syringae* pv. *syringae* and identified as a major regulator of pathogenicity (Hrabak and Willis, 1992). GacA, a cognate response regulator, was discovered in the biocontrol bacterium *P. protegens* CHA0 and found to control the production of AF metabolites (Laville *et al*., 1992). GacS and GacA are essential regulators of secreted products; as such, mutation in either *gacA* or *gacS* completely abolishes the biocontrol activity of several pseudomonads (Chancey *et al*., 1999; Chin-A-Woeng *et al*., 2001; Heeb and Haas, 2001; Koch *et al*., 2002; Van den Broek *et al*., 2003). Details regarding the Gac-Rsm system of regulation have been well described for the biocontrol bacteria *P. protegens* CHA0. In this bacterium, GacS responds to an unidentified environmental signal and autophosphorylates, followed by the transfer of the phosphate group to GacA through a phospho-relay mechanism. Upon activation, GacA can initiate the transcription of small non-coding RNA (sRNA) molecules known as *rsmX*, *rsmY*, *rsmZ* by binding to a conserved palindromic upstream sequence present in the promoter regions of *rsmX/Y/Z* (Heeb and Haas, 2001). Each of these small RNAs can form specific stem loop secondary structures which enable them to bind to small RNA-binding proteins, RsmA and RsmE. Studies have shown that in *P. protegens*, RsmA and RsmE function as post-transcriptional repressors of the genes encoding PLT, HCN, and DAPG biosynthetic enzymes (Heeb *et al*., 2002; Reimmann *et*

al., 2005). These repressors can specifically bind to the ANGGAN (N, any nucleotide) sequence motifs of target messenger RNA (mRNA), causing interruption in the ribosome-binding site (RBS) and consequently blocking translation (Kay *et al*., 2006; Lapouge *et al*., 2008). Thus, sRNAs can relieve translational repression by titrating out the repressors and allowing access to the RBS of target mRNAs.

In the absence of *rsmY/Z* in *P. aeruginosa* and *rsmX/Y/Z* in *P. protegens*, bacteria display phenotypes similar to that of a *gacA* mutant (Kay *et* al., 2005; Kay *et* al., 2006). On the other hand, single mutations in *rsmA* or *rsmE* had little effect compared to an *rsmArsmE* double mutation, which is required for derepressed production of biocontrol factors (Reimmann *et al*., 2005). Although, levels of both translational repressors vary during the growth of the organism, RsmA was found to be present in higher amounts consistently throughout all growth phases. RsmE has been predicted to play a role in termination of Gac-controlled gene expression as it was found present in low amounts at a low cell density and in highest amounts at the end of growth (Reimmann *et al*., 2005).

1.3.4.2. Quorum sensing system

Quorum sensing (QS) is a phenomenon whereby bacteria communicate with each other via chemical signals allowing regulation of target genes in a population density-dependent fashion (Bassler, 2002). Beside the GacS/GacA two component system, pseudomonads utilize QS systems to regulate the synthesis of secondary metabolites. Typically, the QS circuitry of most Gram-negative bacteria is comprised of a LuxR-type transcriptional activator and a LuxI-type autoinducer (AI) synthase (Bassler, 2002). The AI synthase generates *N*-acyl homoserine lactones (AHLs) which are the QS signalling molecules (Venturi, 2006). As the bacterial

population increases, so does the level of AHLs. Once a critical threshold concentration has been reached, AHLs bind to a LuxR-type protein, allowing it to dimerize and bind to a promoter, thereby controlling expression of target genes (Fuqua *et al*., 1994). The acyl-chain length of AHLs generally varies between C_4 to C_{18} (Marketon *et al*., 2002; Fuqua and Eberhard, 1999). Although AHLs are believed to passively diffuse through the cell envelope, in *P. aeruginosa*, the longer chain 3O-C_{12}-AHL is actively effluxed by the MexAB-OprM encoded efflux pump (Pearson *et al*., 1999). The length of the N-acyl side chain therefore appears to determine whether an AI is freely diffusible or not.

Among the biocontrol pseudomonads, PHZ-producers, such as *P. chlororaphis* strains O6, 30-84, GP72 have a PhzI/PhzR QS system which controls expression of this antibiotic (Shen *et al*., 2012; Delaney *et al*., 2001; Spencer *et al*., 2003). Additionally, a second QS system called Csa (cell surface alterations) has been identified in *P. chlororaphis* 30-84. Although Csa is not required for PHZ biosynthesis, it affects protease production and cell-surface properties of this bacterium (Zhang and Pierson, 2001). Studies have demonstrated that in the rhizosphere, *P. chlororaphis* AHL-signalling operates over a distance of up to 60 µm and it elicits tomato plant resistance to the leaf pathogen *Alternaria alternata* (Hartmann *et al*., 2004). Overall, QS controls a broad range of physiological properties, including bioluminescence, spore formation, biofilm formation and motility (Bassler, 2002; Davies *et al*., 1998; de Kievit *et al*., 2001; Shrout *et al*., 2006). Regulatory elements like Gac-Rsm, RpoS and PsrA may stimulate QS positively or negatively (Venturi, 2006) and environmental factors, such as oxygen tension, nitrogen source, pH, and temperature can significantly affect the QS machinery (van Rij *et al*., 2004).

1.3.4.3. RpoS and PsrA regulatory control

Regulation of genes that are essential upon entry into stationary phase and in response to stress requires the sigma factor (σ^{38}/ σ^{S}). This sigma factor, which is encoded by *rpoS*, is found in a variety of Gram-negative bacteria including *E. coli* and *Pseudomonas* spp. (Loewen *et al*., 1998; Suh *et al*., 1999). First discovered in *E. coli*, RpoS was found to induce more than 40 genes during the transition from exponential to the stationary phase, producing proteins generally related to stress resistance (Loewen *et al*., 1998; Hengge-Aronis, 2002). In pseudomonads, deleting *rpoS* results in altered production of secondary metabolites, including PHZs, PRN, PLT, HCN, and AHLs (Heeb *et* al., 2005; Oh *et al*., 2013; Venturi, 2003) together with increased sensitivity to oxidative stress (Sarniguet *et al*. 1995; Heeb *et al.* 2005). However, these regulatory effects may differ depending on the strain and the secondary metabolite under RpoS control. At early stationary phase, RpoS starts to accumulate within the cell and compete with other sigma factors for binding to the core RNA polymerase, thereby, altering its binding specificity for target promoters (Venturi, 2003).

In *Pseudomonas* spp., RpoS has been shown to be positively regulated at the transcriptional level by PsrA (Pseudomonas sigma regulator A) (Kojic and Venturi, 2001). Based on amino acid sequence, PsrA belongs to the TetR family of transcriptional regulators, the majority of which function as repressors in regulating the level of susceptibility to hydrophobic antibiotics and detergents (Chin-A-Woeng *et al*., 2005). In *P. aeruginosa* and *P. putida*, *rpoS* transcription was found to be 50% reduced in a *psrA* mutant compared to wild type (Venturi, 2003). Based on DNA-binding analysis, PsrA binds directly to the *rpoS* promoter region, specifically at the nucleotide sequence -35 to -59 upstream of the transcriptional start site (Kojic *et al*., 2002). In *P. aeruginosa*, *P. putida*, and *P. chlororaphis*, PsrA also controls AHL

production (Kojic *et al*., 2002; Chin-A-Woeng *et al*., 2005). Additionally, PsrA is able to repress its own expression in *P. chlororaphis* and *P. syringae* through binding a PsrA recognition sequence in the promoter region (Chin-A-Woeng *et al*., 2005; Chatterjee *et al*., 2007).

1.3.4.4. Anaerobic regulator

The anaerobic metabolism of root-colonizing biocontrol bacteria is important in terms of their ability to inhabit different environments as well as protect plants from root diseases caused by soil-borne fungi. Research related to the biocontrol strain *P. protegens* CHA0 has indicated that HCN production plays a major role in suppression of black root rot of tobacco (Voisard *et al*., 1989). In *P. protegens* and *P. aeruginosa*, optimal expression of the HCN synthase proceeds at low-oxygen concentrations and depends on ANR (anaerobic regulator of arginine deiminase and nitrate reductase) (Castric, 1983; Castric, 1994). ANR is a FNR-like (fumarate and nitrate reductase regulator) transcriptional regulator which under low oxygen supply can convert to its active form (Spiro, 1994). FNR was first discovered in the enterobacterium *E. coli* (Lambden and Guest, 1976) and it belongs to the FNR-CRP (catabolite repressor protein) superfamily of transcriptional regulators (Körner *et al*., 2003). These regulators can respond to various stimuli, such as oxygen, nitric oxide, carbon monoxide and cyclic nucleotides, and in turn regulate expression of target genes (Körner *et al*., 2003).

In *P. aeruginosa*, ANR can sense oxygen tension and dimerize via a dimerization helix (Sawers, 1991; Yoon *et al*., 2007). This dimeric form carries a $4[4Fe\text{-}4S]^{2+}$ cluster which is a cofactor for ANR (Spiro, 1994). Three N-terminal cysteine residues and one internal cysteine residues (Cys-20, Cys-23, Cys-29 and Cys-122) in ANR have been predicted to bind a $4[4Fe\text{-}4S]^{2+}$ cluster at less than 5 µM oxygen (O_2) concentration. At high O_2 concentration, the 4[4Fe-

4S]$^{2+}$ cluster is converted to [2Fe-2S]$^{2+}$ followed by inactivation of ANR (Jordan *et* al., 1997; Zimmermann *et al*., 1991). The amino acid sequence of *P. protegens* CHA0 ANR (ANR_{Ppr}) shows 88% identity with *P. aeruginosa* ANR (ANR_{Pae}) and the overall regulatory mechanism for both is similar (Laville *et al*., 1998). The active form of ANR can bind to a conserved DNA binding site (5´-TTGATNNNNATCAA-3´) referred to as *anr* box in the promoter region to regulate transcription (Winteler and Haas, 1996; Yoon *et al*., 2007). Through analysis of the *hcnA* promoter region in CHA0, an *anr* box (5´-TTGGC….ATCAA-3´) was identified which is centered at -41.5 and differs by two nucleotides from the consensus FNR/ANR recognition sequence (Laville *et al*., 1998). An FNR/ANR box located at -41.5 is the signature for anaerobically inducible promoters controlled by FNR/ANR-like regulators (Spiro, 1994). In *P. aeruginosa*, 40 experimentally confirmed *anr* box sequences were analysed through a position weight matrix-based bioinformatics approach. According to the sequence logo as shown in Figure 1.1, the level of sequence conservation peaked at four positions, namely 2, 3, 12 and 13 (Trunk *et al*., 2010).

In the Antarctic bacterium, *P. extremaustralis*, under low O_2 conditions, ANR has been shown to differentially regulate motility, aggregation and consequently affects the early stages of biofilm development (Tribelli *et al*., 2013). A recent study on ANR of *P. aeruginosa* has demonstrated that this regulator is necessary for activation of pathways providing energy under low O_2 conditions. Such pathways involve the machinery required for denitrification, genes encoding high-affinity cytochrome oxidases and arginine fermentation enzymes (Hammond *et al*., 2015). Interestingly, this research was able to link QS and ANR and has shown that the regulons attributed to ANR are expressed at higher levels in a QS-minus background suggesting that ANR might be important for the fitness of the bacteria in the absence of QS. These

researchers also found that ANR has a role in controlling expression of multiple QS-associated genes, including *hcnABC*. This correlates with the previous finding that the *hcn* operon is under the regulation of both QS regulators (*lasR*, *rhlR*) and ANR as shown in Figure 1.2 (Pessi and Haas, 2000).

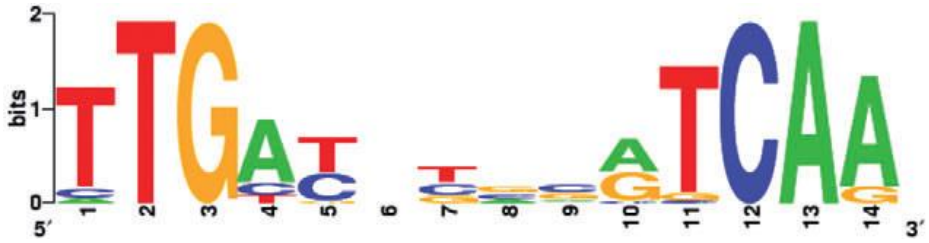


Figure 1.1. The *anr* position weight matrix sequence based on the sequence logo of the 40 functional *P. aeruginosa anr* boxes. Reprinted with permission from Society for Applied Microbiology and Blackwell Publishing Ltd; Environmental Microbiology (2010) **12**(6), 1719-1733. License no. 3814860530172

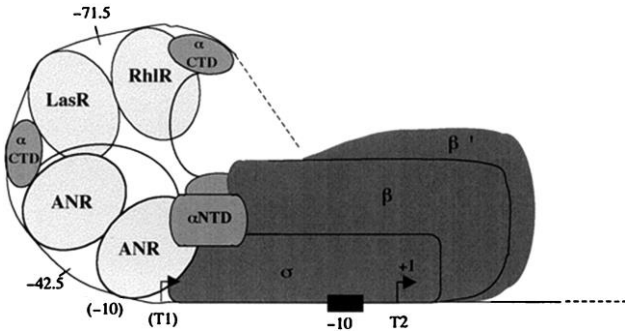


Figure 1.2. Model for recognition of the *P. aeruginosa hcn* promoter by the transcriptional regulators ANR, LasR, RhlR. Interaction of these regulators with RNA polymerase αCTD (C-terminal domain of α) and αNTD (N-terminal domain of α), joined by a linker. Model adapted from that proposed by Belyaeva *et al*., 1998 for CRP-dependent promoter activation. Reprinted with permission from Society for American Society for Microbiology; Journal of Bacteriology (2000) **182**(24), 6940-6949.

1.3.5. *Pseudomonas chlororaphis* PA23

The biocontrol bacterium *P. chlororaphis* PA23 was first isolated from soybean root tips and was found to protect canola from stem rot caused by the fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary (Savchuk and Fernando, 2004). PA23 is able to produce antibiotics, such as PRN, PHZ and HCN together with protease and lipase (Poritsanos *et al*., 2006; Zhang *et al*., 2006). It was discovered that PRN is more important than PHZ for controlling the fungal pathogen, whereas PHZ is involved in biofilm development (Selin *et al*., 2010). Whether HCN production contributes to PA23 fungal antagonism is currently unknown.

The production of secondary metabolites in PA23 is under the control of a complex regulatory hierarchy. Sitting at the top of this hierarchy is the Gac-Rsm system that works in concert with other regulatory elements including QS, RpoS, PsrA and the stringent response. In PA23, a mutation in *gacS* or *gacA* leads to loss of AF activity and dramatically decreased production of PHZ, PRN, HCN, protease, lipase and AI molecules (Poritsanos *et al*., 2006; Selin *et al*., 2014). Moreover, components of the Rsm system in PA23 have been identified which include a regulatory RNA, RsmZ, and the repressor proteins, RsmA and RsmE (Selin *et al*., 2014). Gene expression analysis revealed that in a *gacA* mutant, expression of *rsmA/E/Z* is significantly reduced. Additionally, ample cross-talk has been reported between Gac-Rsm and the other regulatory elements, such as RpoS, PsrA, QS and the stringent response (Selin *et al*., 2014).

In PA23, the PHZ QS system is crucial for regulating the production of secondary metabolites, biofilm formation as well as exhibiting cross-regulation with RpoS. The QS locus *phzI*/*phzR* was not only found to regulate the *phz* operon, but also controls the *prn* biosynthetic operon (Selin *et al*., 2012). Analysis of both *phzR*-minus and AI-deficient strains revealed a

significant decrease in PHZ, PRN, protease production, as well as loss of AF activity and greatly reduced biofilm formation. The PHZ QS system was also found to positively regulate *rpoS* expression (Selin *et al*., 2012).

In PA23, the stationary phase sigma factor RpoS positively regulates PHZ production while PRN, protease and lipase are repressed by this element (Manuel *et al*., 2012). A positive regulator of RpoS, called PsrA was found to negatively regulate the AF activity of PA23. Like the *rpoS* mutant, a *psrA*-deficient strain showed enhanced AF activity with increased PRN and protease production (Selin *et al*., 2014). Another regulatory element linked to RpoS, is a global stress response system known as the stringent response (SR). The SR enables bacteria to adapt to nutrient deprivation by undergoing a global change in gene expression triggered by the production of the alarmones, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (Potrykus and Cashel, 2008). In SR mutants of PA23, AF activity is significantly increased together with increased production of PRN, protease and lipase. The SR seems to affect AF activity through *rpoS*, as *rpoS* transcription is decreased in the SR mutant background (Manuel *et al*., 2012).

As described above, it is evident that multiple regulatory elements control the production of secondary metabolites in PA23. Recently a PA23 *anr* mutant has been generated and this mutant exhibits a complete loss of AF activity. These findings indicate ANR is another essential regulator of PA23 fungal suppression. How ANR controls expression of genes involved in biocontrol has yet to be determined.

1.3.6. *Pseudomonas brassicacearum* DF41

Pseudomonas brassicacearum DF41 is a BCA originally isolated from canola root tips (Savchuk and Fernando, 2004). In both green house and field assays, DF41 has demonstrated efficient antagonism against *Sclerotinia sclerotiorum* (Lib.) de Bary (Berry *et al*, 2010; Savchuk and Fernando, 2004). DF41 produces a LP called sclerosin, responsible for fungal antagonism (Berry *et al*., 2010). Germination of both *S. sclerotinia* ascospores and sclerotia was found to be inhibited by incubation in the presence of sclerosin (Berry *et al*., 2012). Mass spectrometry analysis revealed that sclerosin is a linear LP containing a 22-amino acid head attached to a short (C8) unsaturated lipid moiety which closely resembles the tolassin group of LPs (Berry *et al*., 2012). DF41 produces several other compounds, such as HCN, protease and alginate (Berry *et al*., 2010).

Key regulators overseeing production of AF metabolites in DF41 include GacS/GacA, the SR, and the PdfRI QS system. The Gac two-component system is essential for DF41 biocontrol, as a *gacS* mutant exhibits a loss of AF activity both *in vitro* and in the greenhouse (Berry *et al*., 2010). The SR has a repressive effect on DF41 inhibition of *S. sclerotiorum* as SR mutants show significantly increased AF activity, likely mediated through elevated LP production and protease activity. Similar to PA23, *rpoS* expression decreases in a DF41 SR-deficient background (Manuel *et al*., 2011). As a part of the regulatory network, DF41 also employs QS which is comprised of *pdfR* and *pdfI*, encoding a transcriptional activator and an autoinducer (AI) synthase, respectively (Berry *et al*., 2013). Interestingly, a third gene, called *rfiA* is located immediately downstream of *pdfI*, followed by genes encoding an efflux pump (*pdfABC*), as shown in Figure 3 (Berry *et al*., 2013). A similar genetic arrangement has been observed in the closely related pseudomonad, *P. corrugata* CFBP 5454 where RfiA regulates

secretion of LP molecules via activation of downstream efflux pump genes (Licciardello *et al*., 2009). A DF41 QS-deficient strain, no longer synthesizing autoinducer (AI) molecules, exhibited no change in AF activity and sclerosin production. Conversely, an *rfiA* mutant showed a lack of sclerosin production and, not surprisingly, was devoid of AF activity (Berry *et al*., 2013).

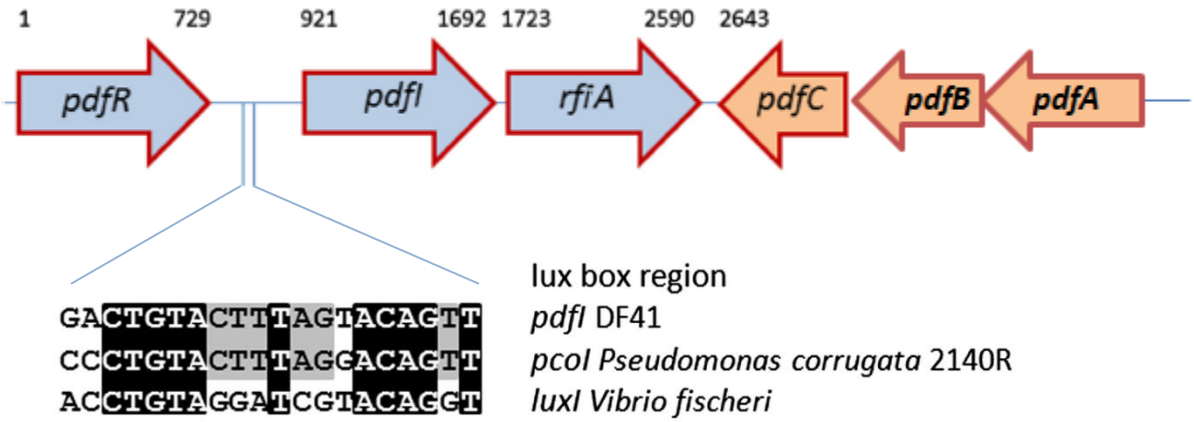


Figure 1.3. Genetic map of *Pseudomonas brassicacearum* DF41 QS locus showing genes including *pdfR*, *pdfI* and *rfiA* with the position of each gene located above. Downstream of and divergently transcribed from *rfiA* lay *pdfC*, *pdfB*, *pdfA* which are predicted to encode an efflux pump. A putative 20-bp lux-box sequence is located in the *pdfI* promoter. Light and dark shading highlights nucleotides identical to those found in the lux boxes of *Pseudomonas corrugata* 2140R and *Vibrio fischeri*. Reprinted with permission from Elsevier Inc.; Biological Control (2014) **69**, 82-89. License no. 3818390328136

1.4. Criteria of a successful biocontrol agent

In order to be a successful BCA, microbes must exhibit not only efficient pathogen suppression but the ability to establish in the environment at levels significant for pathogen control. One of the factors challenging soil bacterial communities is constant predation by microfaunal predators, such as nematodes and protozoa (Griffiths *et al*., 1999). To counteract predation, biocontrol *Pseudomonas* spp. have developed defense mechanisms such as the production of toxins, which serve to repel or kill predators (Kumar *et al*., 2005; Jousset *et al*., 2006; Neidig *et al*., 2011). Toxin production is energetically costly and according to the prediction of defense theory, bacteria should optimize the investment in defense according to the risk of predation and resources available (Steiner, 2007; Friman *et al*., 2008). Toxins enhance the competitiveness of bacteria in the rhizosphere by reducing predation pressure as well as acting antagonistically against plant pathogens, thereby promoting plant growth (Haas and Défago, 2005). Understanding predator-prey interactions is essential for the development of a promising bioproduct capable of persisting in the environment under prevailing predation risk.

1.5. Risk of predation

In the rhizosphere, high nutrient availability enables microbial communities to increase their population and subsequently become accessible to their predators (Taylor, 1978). Predators, including bacterivorous protozoa and nematodes are present in the rhizosphere at levels about 30 fold higher than those in the bulk soil (Griffiths, 1990). Thus, bacteria present in the rhizosphere are exposed to increased predation pressure. Predation can have a dramatic effect on the structure and function of root-associated bacterial populations and can be a significant cause of bacterial mortality (de Mesel *et al*., 2004; Rosenberg *et al*., 2009). In a study conducted to analyze the

effects of grazing by the free-living soil amoeba *Acanthamoeba castellanii* in the rhizosphere of *Arabidopsis thaliana*, a prominent feeding preference was observed. A significant decrease in the number of Betaproteobacteria and Firmicutes was discovered, whereas the population size of Actinobacteria, Nitrospira, Verrucomicrobia and Planctomycetes was found to increase (Rosenberg *et al*., 2009). Pedersen and coworkers (2009) investigated the feeding habitats of two different bacterial feeders in a microcosm consisting of the biocontrol strains *P. protegens* CHA0 and DSS73. In the presence of the flagellate, *Cercomonas longicauda*, DSS73 was consumed at a higher rate than CHA0, but the nematode *Caenorhabditis elegans* was found to consume CHA0 preferentially over DSS73 (Pedersen *et al*., 2009). Overall, the way microfaunal predators affect colonization and survival of soil bacteria needs further exploration to facilitate the application of suitable biocontrol inoculum in agriculture.

A number of different host models have been exploited to investigate the response of specific biocontrol pseudomonads to predation pressure. Among protists, the amoeba *A. castellanii*; flagellates including *Neobodo designis* and *C. longicauda*; ciliates like *Colpoda steinii* and the amoebo-flagellate *Naegleria americana* have been widely used (Jousset *et al*., 2006; Pedersen *et al*., 2009; Mazzola *et al*., 2009; Müller *et al*., 2013). Among bacterivorous nematodes, *C. elegans*, a free-living soil nematode is an intensively utilized model organism (Brenner, 1974).

C. elegans has proven to be an excellent model for studying bacteria with respect to their biocontrol and pathogenicity traits (Zhang and Hou, 2013). Some of the major attributes associated with *C. elegans* include, i) a short life cycle of three to five days depending on the temperature employed (15^{o} C or 20^{o} C); ii) the relative ease of handling and maintenance in the laboratory; iii) the transparency of the cuticle enabling microscopic monitoring of the infection

process and iv) the availability of the whole genome sequence as well as a plethora of genetic mutants (Brenner, 1973; Wood, 1988).

1.6. Predator-prey interaction

Predator-prey interaction is a significant parameter shaping soil bacterial communities. In the environment, bacteria and their consumers receive and respond to chemical signals and this molecular interplay, combining responses from both sides, modulates the interaction. Previous studies have revealed prey and predator species responses that are described in detail in the following sections.

1.6.1. Bacterial defense against predators – secondary metabolites

While bacteria can serve as food for protozoa and nematodes, they are also able to respond to predators or predator-specific cues and escape predation through various mechanisms. One of the most effective defense strategies involves production of unpalatable compounds or toxic metabolites, which act to repel or kill predators. Earlier it was found that *P. aeruginosa* PAO1 can cause lethal paralysis and death of *C. elegans* through diffusible factors, the production of which is dependent upon the Las and Rhl QS systems. Mutation in either *lasR* or *rhlR* reduces paralysis and nematode mortality by almost 100% (Darby *et al*., 1999). This QS-mediated response of *P. aeruginosa* is also highlighted in another recent finding. In the presence of QS inhibitors, like meta-bromo-thiolactone that partially inhibit QS *in vitro*, nematode death was reduced by 60% at 24 h (O'Loughlin *et al*., 2013).

P. protegens CHA0 is able to produce exoproducts, such as DAPG, PLT, PRN, HCN and protease (Heeb *et al*., 2002). Among these, DAPG exhibited acute toxicity towards the amoeba

Vahlkampfia and the ciliate *Colpoda steinii*, resulting in rapid lysis of both protists. In addition, PLT, PRN and protease were found to induce encystation of the protists (Jousset *et al*., 2006). Therefore, it can be assumed that DAPG, PLT, PRN and protease interfere with key cellular processes, including membrane permeability and electron transport. Additionally, the presence of cell free supernatants of *A. castellanii* lead to elevated *phlA* (DAPG), *prnA* and *hcnA* gene expression in *P. protegens* CHA0 indicating an upregulated defense mechanism in response to soluble predator cues. In contrast, these researchers observed that coculturing CHA0 with amoeba decreased gene expression, suggesting that direct contact with bacteria enables the amoeba to repress bacterial toxicity (Jousset *et al*., 2010).

According to Neidig and coworkers (2011), CHA0 metabolites were found to be nematicidal to *C. elegans* and notably DAPG, PLT and HCN were reported to induce the nematode stress response. It is well established that HCN, being a potent inhibitor of respiratory pathways, can cause hypoxia and paralytic death of nematodes (Gallagher and Manoil, 2001). Moreover, *P. chlororaphis* O6 exometabolites displayed strong nematicidal activity; in particular HCN has been predicted to be the major factor in the biological control of the root-knot nematode *Meloidogyne hapla* (Lee *et al*., 2011).

The BCA *P. protegens* DR54 can hinder the growth of the soil amoebae *Hartmanella vermiformis* as well as *Acanthamoeba* species. These detrimental effects have been attributed to the production of a cyclic LP called viscosinamide, a known antimicrobial compound with surfactant properties (Andersen and Winding, 2004). Moreover, *P. protegens* strains SS101 and SBW25 were found to protect themselves from *N. americana* grazing by producing the cyclic LPs massetolide (*massABC*) and viscosin (*viscABC*), respectively. Placing strains SS101 and SBW25 in close proximity or in direct contact with the protozoa, lead to upregulation of both

massABC and *viscABC* (Mazzola *et al*., 2009). Recently, transcriptomic analyses revealed that in addition to *massABC* and *viscABC*, a total of 55 genes in strain SS101 and 65 genes in strain SBW25 were upregulated upon interaction with *N. americana* (Song *et al*., 2015). Based on transcriptomic and live imaging mass spectrometry (IMS), SS101 was found to upregulate putrescine biosynthesis during challenge with the protozoa. Interestingly, this compound was discovered to induce trophozoite encystment and affect cyst viability (Song *et al*., 2015).

The number of pathogenic bacteria affecting predators through secondary metabolite production surpasses the list of biocontrol bacteria doing the same. *P. aeruginosa* strain PA14 is able to kill nematodes within a matter of hours by producing the PHZ molecules PCA, 1-OH-PHZ and PYO (Cezairliyan *et al*., 2013). Gluconic acid production by *Enterobacter intermedium* was reported to be the active compound that helps bacteria to escape or defend themselves against protozoan grazing in the rhizosphere (Gomez *et al*., 2010). Specific exoproducts of other pathogenic bacteria including *Vibrio cholerae*, *E. coli*, *Serratia marcescens* have also been reported to affect predation (Vaitkevicius *et al*, 2006; Lainhart *et al*., 2009; Pradel *et al*., 2007).

1.6.2. Morphological adaptations

In an effort to avoid consumption, bacteria have adopted morphological defenses to make themselves inaccessible to their predators. One of the basic defense strategies employed by bacteria involves growing in aggregates or biofilms. Bacterial biofilms are defined as groups of microcolonies embedded within a hydrated extra polymeric substance matrix adhering to a biotic or abiotic surface (Costerton and Lewandowski, 1995). Human pathogens *Yersinia pestis*, *Y. pseudotuberculosis* and the insect pathogen, *Xenorhabdus nematophila* can block the feeding of *C. elegans* by forming biofilms on the nematode's anterior cuticle, especially the head (Darby *et*

al., 2002; Tan and Darby, 2004; Drace and Darby, 2008). *S. marcescens* can form different kinds of biofilms mediating resistance to various modes of protozoan grazing. For example, microcolony-type biofilms provide protection from the suspension feeder flagellate *Bodo saltans*; whereas, a filamentous biofilm protects against the surface feeder, *Acanthamoeba polyphaga* (Queck *et al*., 2006). In addition, *P. aeruginosa* PAO1 forms grazing-resistant microcolonies in the presence of the flagellate *Rhynchomonas nasuta* (Matz *et al*., 2004). Another interesting way bacteria can avoid predation is by filament formation, which results when rod-shaped cells cease to divide but continue to grow. Such morphological plasticity is prominent in freshwater *Flectobacillus* spp. that can form filamentous cells resistant to grazing by flagellate *Ochromonas* spp. (Corno and Jürgens, 2006).

1.6.3. Surface properties

Besides toxins and morphological alterations, bacterial surface properties can affect grazing predators. Studies have shown that the bacterial cell wall can afford protection against predation and as such, Gram-positive enteric bacteria are consumed by protists at significantly lower rates than Gram-negative organisms (Iriberri *et al*., 1994). For example, heterotrophic nanoflagellates actively avoid feeding on Gram-positive Actinobacteria (Pernthaler, 2005). As parameters, bacterial cell surface charge and hydrophobicity have been implicated in affecting predator feeding behavior (Matz and Jürgens, 2001). Lipopolysaccharide (LPS) in the outer membrane (OM) of Gram-negative bacteria has been found to be critical for persistence of *Salmonella enterica* and *Shigella flexneri* in the *C. elegans* intestine (Aballay *et al*., 2003; Paciello *et al*., 2013). Additionally, LPS O-side chains are crucial for colonization of enterohemorrhagic *E. coli* (EHEC) O157:H7 in the nematode intestinal tract (Youn *et al*., 2013).

An LPS O-side chain-defective *perA* mutant was unable to colonize at 24 h post-feeding, whereas the wild type started colonizing at 3 h post-feeding (Youn *et al*., 2013). Another example of bacterial adaptation to evade predation involves the formation of an S-layer (surface layer), a monomolecular layer of identical protein or glycoprotein subunits (Sára and Sleytr, 2000). Indeed, the S-layer of Actinobacteria has been reported to affect both ingestion and digestion by the nanoflagellate *Poterioochromonas* species (Tarao *et al*., 2009). A discussion of surface properties and bacterial defense would be incomplete without mentioning bacterial motility, which can both negatively and positively affect predation. On the one hand, swimming motility enhances the probability of predator-prey contact, thereby increasing the chances of bacterial consumption by reducing predator search time for prey (González *et al*., 1993). On the other hand, fast swimming bacteria (>25 mm s^{-1}) exhibit decreased predator capture efficiency, which ultimately protects them from grazing (Matz and Jürgens, 2005).

1.6.4. Response of predator species

C. elegans has a highly sophisticated chemosensory system that enables it to sense and respond to a wide range of volatile (olfactory) and water-soluble (gustatory) cues associated with food, danger or other organisms (Bargmann, 2006). The phenomenon of chemotaxis elicited by chemosensory cues enabling a response to amino acids, cyclic nucleotides, cations and anions was first elucidated by Ward (Ward, 1973). Later, chemosensory cues were found to elicit other responses, including changes in overall motility, rapid avoidance of certain compounds and entry into or exit from the alternative dauer developmental stage due to adverse environmental conditions (Pierce-Shimomura *et al*., 1999; Golden and Riddle, 1984; Pradel *et al*., 2007). Previous studies have shown that *C. elegans* is attracted to AHLs (3O-C_{12}-AHL and C_4-AHL)

synthesized by *P. aeruginosa* indicating that these compounds can elicit aversive or attractive learning in the nematode (Beale *et al*., 2006). A similar effect was observed for another Gram negative bacterium, *V. cholera* producing specific AI molecule, termed CAI-1 [(S)-3-hydroxytridecan-4-one], that can influence *C. elegans* chemotaxis (Werner *et al*., 2014). It was discovered that a ketone moiety present in CAI-1 is responsible for the AI-driven nematode behavior with the chemical being detected by the amphid sensory neuron AWC (amphid wing "C") (Werner *et al*., 2014). Basically, any particular behavioral response is preferentially linked to a sensory neuron in *C. elegans*. For example, volatile attraction occurs through two pairs of amphid sensory neurons, AWA (amphid wing "A") and AWC (Bargmann *et al*., 1993), whereas volatile avoidance is mediated by another pair of amphid sensory neurons AWB (amphid wing "B") (Troemel *et al*., 1997). Unlike AHLs, *C. elegans* can detect and avoid a cyclic lipodepsipentapeptide, called serrawettin produced by the pathogenic bacterium *S. marcescens* and the response is primarily mediated by the two AWB sensory neurons (Pradel *et al*., 2007). Overall, these findings indicate that *C. elegans* analyzes the condition of its territory and responds accordingly through a versatile chemosensory system.

Nematodes living in habitats with diverse microbial environments are exposed to numerous elements that challenge their health and survival. Thus, it is not surprising that they possess an arsenal of detoxification and defense genes for decomposing various organic materials (Lindblom and Dodd, 2006). In a recent study, *C. elegans* was shown to be capable of modifying small molecule toxins via *O*- and *N*-glucosylation along with the unusual 3´-*O*-phosphorylation of the resulting glucosides. Nematodes were found to glycosylate the bacterial toxins 1-OH-PHZ and indole, released by *P. aeruginosa* and *E. coli* respectively, in an effort to minimize their toxicity (Stupp *et al*., 2013). In conjunction with biotic factors, abiotic

environmental stressors can significantly impact the response and survival of *C. elegans*. Nematodes are subjected to daily changes in their environment with varying oxidative, osmotic and other stresses; an underlying circadian rhythm allows the organism to predict these changes and respond accordingly (Saigusa *et al*., 2002; Kippert *et al*., 2002). In HCN-mediated lethal paralysis of *C. elegans* by *P. protegens* CHA0, HCN tolerance was found to be higher during the day, as was nematode survival (Romanowski *et al*., 2011). Although *C. elegans* has been reported to have photoreceptors, how they are coupled to the circadian system remains to be explored (Ward *et al*., 2008).

In comparison to prey recognition by nematodes, in particular *C. elegans*, protozoan-prey encounters are quite straightforward. According to the model described by Jeschke and colleagues (2002), protozoan predation involves four basic steps, namely prey finding, recognition, consumption and digestion. Like nematodes, chemotaxis plays an important role in prey search and recognition by protists. For example, the marine heterotrophic nanoflagellate, *Jakoba libera* is predicted to have a chemosensory response towards *Pseudomonas*, *Flavobacterium* and *Aeromonas* spp. in order to detect prey in marine environments (Mohapatra and Fukami, 2007). The social amoeba *Dictyostelium discoideum* relies on chemotaxis in order to find food and survive starvation (Willard and Devreotes, 2006). Moreover, certain protozoa possess distinct secretory organelles called extrusomes that discharge specific substances for both defensive and offensive purposes (Hausmann, 1978). Based on a study reported in 1964, an opsonin secreted by *Amoeba proteus* is responsible for detection and phagocytosis of *Salmonella* species (Savanat and Pavillard, 1964). The heliozoon *Actinophrys sol* secretes an adhesive substance identified as a glycoprotein that helps in immobilization and ingestion of prey (Sakaguchi *et al*., 2001).

1.7. Thesis objectives

In order for an introduced BCA to be successful, it must be able to establish itself in the environment. Colonization depends upon competition with indigenous microbes for available resources and the ability to resist grazing predators. At present it is not known whether *P. brassicacearum* DF41 and *P. chlororaphis* PA23 can avoid consumption by predators such as the bacterivorous nematode *C. elegans*. In the first part of the thesis, PA23- and DF41 - *C. elegans* interaction studies were undertaken to address this question.

PA23 produces an arsenal of secondary metabolites, some of which are essential for biocontrol activity. In addition to diffusible antibiotics and degradative enzymes, PA23 releases HCN; however, the contribution of this volatile to fungal antagonism is currently unknown. Additionally, the role of the anaerobic regulator ANR in PA23 biocontrol has yet to be explored. In the second half of this thesis, the role of HCN in PA23 antifungal activity together with factors contributing to PA23 biocontrol were investigated.

The specific objectives of this thesis are as follows:

1. To investigate the role of DF41 exoproducts in defense against the predator *C. elegans* and to determine whether the presence of nematodes elicits changes in DF41 gene expression.

2. To analyze the effect of PA23 secondary metabolites on the survival and chemotactic behavior of *C. elegans* and nematode co-culturing on PA23 gene expression.

3. To identify the role of HCN in the biocontrol capabilities of PA23 and the effect of ferric chloride and glycine on HCN production.

4. To characterize the role of the anaerobic regulator ANR in PA23 fungal suppression.

Chapter 2

Materials and Methods

2.1. Bacterial strains and growth conditions

For a list of bacterial strains and plasmids used in this study see, Table 2.1. *E. coli* was cultured at 37ºC on Lysogeny Broth (LB) agar (Difco Laboratories, Detroit, MI, USA). *Pseudomonas* strains were cultured on LB or King's B (KB) (King *et al.*, 1954) medium at 28ºC or in M9 minimal salts medium amended with 0.2% glucose and 1mM magnesium sulfate ($MgSO_4$). Antibiotics were used at the following concentrations: ampicillin (Amp; 100 µg/mL), chloramphenicol (Chl; 12.5 µg/mL), gentamicin (Gm; 15 µg/mL), tetracycline (Tc; 15 µg/mL) for *E. coli*, and piperacillin (Pip; 40 µg/mL), Gm (20 µg /mL), Tc (15 µg /mL) and rifampicin (Rif; 50 µg/mL) for *Pseudomonas* spp. All antibiotics were obtained from Research Products International Corp. (Mt. Prospect, IL, USA).

2.2. Nematode strains and culture conditions

The *C. elegans* strains used in this work include wild type Bristol N2, AT6 [*srf-2*(*yj262*)], DC9 [*bah-3*(*br9*)], wild type Hawaiian isolate CB4856 (Table 2.1). The strains were maintained at 15ºC on nematode growth medium (NGM) (Brenner, 1974) supplemented with *E. coli* OP50. Synchronous cultures were produced according to the protocols available in NematodeBook (Girard *et al*., 2007). L4-stage hermaphrodites were used in the studies described herein.

2.3. Nucleic acid manipulation

Standard techniques were employed for purification, cloning and other manipulations of DNA (Sambrook *et al*., 1989). Polymerase chain reaction (PCR) was performed following standard conditions suggested by Thermo Fisher Scientific data sheets supplied with their *Taq* polymerase.

Table 2.1. Strains, plasmids, and primers used in the study.

Strain, plasmid, or primer	Relevant genotype, phenotype or sequence	Reference or source
Strains		
C. elegans		
N2	Wild type isolate	CGC, University of Minnesota, MN
AT6	*srf-2*(*yj262*)	CGC, University of Minnesota, MN
DC9	*bah-3*(*br9*)	CGC, University of Minnesota, MN
CB4856	Wild type Hawaiian isolate	CGC, University of Minnesota, MN
P. brassicacearum		
DF41	Rif^R wild-type (canola root tip isolate)	Savchuk & Fernando (2004)
DF41-1278	Rif^R lp::Tn5-1063 genomic insertion	Berry *et al*. (2010)
DF41*hcn*	DF41 with the pKNOCK-Tc vector inserted into the *hcn* gene	This study
AI-deficient	DF41 carrying pME6863	Berry *et al.* (2013)
DF41*rfiA*	DF41 with Gm^R cassette inserted into the *rfiA* gene	Berry *et al.* (2013)
DF41*gacS*	Rif^R *gacS*::Tn5-1063 genomic insertion	Berry *et al.* (2010)
DF41-*rfp*	DF41 containing mCherry expressed from pMCh-23	Berry *et al*. (2012)
DF41-1278-*rfp*	DF41-1278 containing mCherry expressed from pMCh-23	Berry *et al*. (2012)
DF41*hcn-rfp*	DF41*hcn* containing mCherry expressed from pMCh-23	This study
AI-deficient-*rfp*	DF41-6863 containing mCherry expressed from pMCh-23	This study
DF41*rfiA-rfp*	DF41*rfiA* containing mCherry expressed from pMCh-23	This study
DF41*gacS-rfp*	PA23gacS containing mCherry expressed from pMCh-23	This study
P. chlororaphis		
PA23	PRN^+ PHZ^+ Rif^R; wild-type (soybean root tip isolate)	Savchuk & Fernando (2004)

PA23-8	PRN$^-$ Rif^R *prnBC* deletion mutant	Selin *et al*. (2010)
PA23-63	PHZ$^-$ Rif^R *phzE*::Tn5-OT182 genomic fusion	Selin *et al*. (2010)
PA23-63-1	PRN$^-$ PHZ$^-$ Rif^R *phzE*::Tn5-OT182 genomic fusion; *prnBC* deletion mutant	Selin *et al*. (2010)
PA23*hcn*	PA23 with the pKNOCK-Tc vector inserted into the *hcn* gene	This study
PA23*anr*	PA23 with a tet^R marker inserted into *anr*	This study
PA23-6863	PA23 carrying pME6863; AHL deficient	Selin *et al*. (2012)
PA23*phzR*	PA23 with Gm^R marker inserted into *phzR* gene	Selin *et al*. (2012)
PA23*phzRanr*	PA23*phzR* with a tet^R marker inserted Into *anr*	This study
PA23*rpoS*	PA23 with pKNOCK-Tc vector inserted into *rpoS* gene	Selin *et al*. (2012)
PA23*psrA*	PA23 with pKNOCK-Tc vector inserted into *psrA* gene	Selin *et al*. (2014)
PA23*gacA*	Gm^R marker inserted into the *gacA* gene	Selin *et al*. (2014)
PA23-314	Rif^R *gacS*::Tn-OT182 genomic fusion	Poritsanos *et al*. (2006)
PA23-*rfp*	PA23 containing mCherry expressed from pMCh-23	This study
PA23-8-*rfp*	PA23-8 containing mCherry expressed from pMCh-23	This study
PA23-63-*rfp*	PA23-63 containing mCherry expressed from pMCh-23	This study
PA23-63-1-*rfp*	PA23-63-1 containing mCherry expressed from pMCh-23	This study
PA23*hcn*-*rfp*	PA23*hcn* containing mCherry expressed from pMCh-23	This study
PA23-6863-*rfp*	PA23-6863 containing mCherry expressed from pMCh-23	This study
PA23*phzR*-*rfp*	PA23*phzR* containing mCherry expressed from pMCh-23	This study
PA23*rpoS*-*rfp*	PA23*rpoS* containing mCherry expressed from pMCh-23	This study

PA23*psrA-rfp*	PA23*psrA* containing mCherry expressed from pMCh-23	This study
PA23*gacS-rfp*	PA23gacS containing mCherry expressed from pMCh-23	This study
P. aeruginosa		
QSC105	Strain carrying pEAL01 (*lasB-lacZ* transcriptional fusion), $Carb^R$	Ling *et al.* (2009)
E. coli		
DH5α	*supE44* Δ*U169* (*Φ*80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco
DH5α λpir	λpir lysogen of DH5α	House *et al.* (2004)
OP50	Laboratory strain for maintenance of	Brenner (1974)
Chromobacterium violaceum CVO26	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31532 autoinducer biosensor	Latifi *et al.* (1996)
Plasmids		
pME6863	pME6000 carrying the *aiiA* gene from Bacillus sp.A24 under the constitutive P_{lac} promoter	Reimmann et al. (2002)
pCR2.1	TA cloning vector, Amp^R	Invitrogen
pKNOCK-Tc	Suicide vector for insertional mutagenesis; R6K ori Rp4 oriT Tc^R	Alexeyev (1999)
pRK600	Contains *tra* genes for mobilization, Chl^R	Finan *et al.* (1986)
pCR*hcnABC*-41	1.6 kb DF41 *hcnABC* fragment in pCR2.1	This study
pKNOCK*hcnABC*-41	1.6 kb fragment from *hcnABC* (DF41) in pKNOCK-Tc	This study
pCR*hcnABC*-23	1.9 kb PA23 *hcnABC* fragment in pCR2.1	This study
pKNOCK*hcnABC*-23	1.9 kb fragment from *hcnABC* (PA23) in pKNOCK-Tc	This study
pCR*anr*-23	695 bp PA23 *anr* fragment in PCR2.1	This study
pKNOCK*anr*-23	695 bp fragment from *anr* (PA23) in pKNOCK-Tc	This study
pUCP23	Broad-host-range vector, Amp^R Gm^R	West *et al.* (1994)
pUCP23-*anr*	*anr* in pUCP23	This study
pUCP23-*phzR*	*phzR* in pUCP23	Selin *et al.* (2012)

pRSET-B mCherry	mCherry expression vector, f1 ori, Amp^R	Shaner *et al.* (2004)
pMCh-23	pUCP23 carrying the mCherry red fluorescent protein gene	Berry *et al.* (2012)
pLP170	*lacZ* transcriptional fusion vector	Preston *et al.* (1997)
pPRNA-*lacZ*	*prnA* promoter in pLP170	Selin *et al.* (2010)
pPHZA-*lacZ*	*phzA* promoter in pLP170	Selin *et al.* (2010)
pHCNA-*lacZ*	963 bp fragment containing the *hcnA* promoter (PA23) in pLP170	This study
pPHZI-*lacZ*	674 bp fragment containing the *phzI* promoter in pLP170	Selin *et al.* (2012)
pPHZR-*lacZ*	1.1 kb fragment containing the *phzR* promoter in pLP170	Selin *et al.* (2012)
pRPOS-*lacZ*	*rpoS* promoter in pLP170	Poritsanos et al. (2006)
pPSRA-*lacZ*	*psrA* promoter in pLP170	Selin (unpublished)
pGACS-*lacZ*	*gacS* promoter in pLP170	Manuel (unpublished)
pGACA-*lacZ*	*gacA* promoter in pLP170	Manuel (unpublished)
Primers		
41*hcnA*-FOR	5´-atgggcgtatgccactgc-3´	This study
41*hcnC*-REV	5´-taagcacacgacgcgccg-3´	This study
23*hcnA*-FOR	5′-atgcggacatgaccatcagc-3′	This study
23*hcnC*-REV	5′-aatccaccaccaccgccgaacc-3′	This study
23*hcnA*-FOR1	5´-atgctgttgcaggcggtg-3´	This study
23*hcnA*-REV1	5´-atgcggttgacctcggtctg-3´	This study
anr-FOR	5´-attcggtctggtaaccctc-3´	This study
anr-REV	5´-cctgccactttctctgaatc-3´	This study
anr(comp)-FOR	5´-agtgtctagacgggcatgaagtggag-3´	This study
anr(comp)-REV	5´-tgcacgagctcattttcccggagtg-3´	This study
qRT-PCR primers		
DF41		
sclerosin-FOR	5´-ccacaaacggcatttgctgg-3´	This study
sclerosin-REV	5´-agtttgctaaggaccgctgc-3´	This study
hcnC-FOR	5´-tacgtggcgcagaaagacaacg-3´	This study
hcnC-REV	5´-ttgcaccaacccttcgatctcg-3´	This study
pdfI-FOR	5´-accgttgacagacgcaatatcg-3´	This study
pdfI-REV	5´-agcgttcttgctaaggacctcc-3´	This study
pdfR-FOR	5´-agcatcatcgccaaccaacacc-3´	This study
pdfR-REV	5´-gttttttcccagtgcgagccag-3´	This study
rfiA-FOR	5´-gcacctgaacttgccgaacaac-3´	This study

rfiA-REV	5´-gcatccatcggataagcgaacg-3´	This study
gacS-FOR	5´-tggtgcaaaccctgctcgaag-3´	This study
gacS-REV	5´-tctgcacgtccatcaacaccag-3´	This study
PA23		
phzA-FOR	5´-gactggcaatggcacaac-3´	Klaponski (unpublished)
phzA-REV	5´-gcaataaccttcgggataacc-3´	Klaponski (unpublished)
prnA-FOR	5´-ctgtcgtcgtgctttctg-3´	Klaponski (unpublished)
prnA-REV	5´-gatctcggcgttgaatgc-3´	Klaponski (unpublished)
hcnC-FOR	5´-aactgctcaacggttgcctg-3´	This study
hcnC-REV	5´-tgctgatgtcgaagcccttg-3´	This study
phzI-FOR	5´-gcgatgccgttgttctgg-3´	Klaponski (unpublished)
phzI-REV	5´-agccgttcgtagtggactc-3´	Klaponski (unpublished)
phzR-FOR	5´-gaatccttggcttcagacc-3´	Klaponski (unpublished)
phzR-REV	5´-atcaggcggctaactacg-3´	Klaponski (unpublished)
anr-FOR	5´-atagacggagtcgaatgcg-3´	This study
anr-REV	5´-tctggaagacatggatgcg-3´	This study
rpoS-FOR	5´-tggctttccgaattgacc-3´	Klaponski (unpublished)
rpoS-REV	5´-cagacgcttgagaccttc-3´	Klaponski (unpublished)
rpoB-FOR	5´-cagctgacctttgtcgttca-3´	Reimer (unpublished)
rpoB-REV	5´-tatgctcgcaccaaccagta-3´	Reimer (unpublished)

Rif, rifampicin; Tc, tetracycline; Gm, gentamicin; Carb, carbenicillin; Chl, chloramphenicol; Amp, ampicillin.

2.4. Generation of PA23*hcn*, PA23*anr*, PA23*phzRanr* and DF41*hcn* mutants

Primers used for the construction of the mutants are listed in Table 2.1. The PA23 *hcn* mutant strain was generated as follows. A portion of the PA23 *hcn* gene cluster was PCR amplified using primers 23*hcnA*-FOR and 23*hcnC*-REV. Primers were designed from the *Pseudomonas fluorescens* CHA0 *hcnABC* gene sequence (accession no. AF053760). A TOPO® kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to clone the 1.9-kb PCR product into the pCR®2.1-TOPO® vector generating pCR*hcnABC*-23. Plasmid pCR*hcnABC*′-23 was digested with *Hin*dIII and *Xho*I, and the 1.9-kb insert was subcloned into the same sites of the suicide vector pKNOCK-Tc. Triparental mating between the donor [*E. coli* DH5α λpir (pKNOCK*hcnABC*-23)], helper [*E. coli* DH5α (pRK600)] and recipient (PA23) was performed to insertionally interrupt the wild type *hcnABC* gene cluster. The *hcn* mutation in PA23*hcn* was confirmed by PCR and testing for a lack of HCN production using Cyantesmo paper (Machery-Nagel GmbH & Co., Germany).

To generate PA23*anr*, an internal region of the *anr* gene cluster was PCR amplified using primers *anr*-FOR and *anr*-REV. Primers were designed based on the sequence of the *anr* gene obtained from *P. chlororaphis* PA23 (GenBank accession no. NZ_CP008696). A TOPO® kit was used to clone the 726-bp PCR product into the pCR2.1-TOPO vector following the manufacturer's instructions to generate pCR*anr*-23. To liberate the insert, pCR*anr*-23 was digested with *Hin*dIII and *Xba*I, and subcloned into the same sites of the suicide vector pKNOCK-Tc. Triparental mating between the donor [*E. coli* DH5α λpir (pKNOCK*anr*-23)], helper [*E. coli* DH5α (pRK600)] and recipient (PA23) was performed to insertionally interrupt the wild type *anr* gene. Pseudomonas Isolation Agar (PIA; Difco) supplemented with Tc (50

μg/mL) was used to screen for transconjugants. To verify that pKNOCK*anr*-23 had correctly inserted into the *anr* gene, PCR analysis was performed using primers *anr*-FOR and tet-REV.

To create a PA23*phzRanr* double mutant, triparental mating was performed between the donor [*E. coli* DH5α λpir (pKNOCK*anr*-23)], helper [*E. coli* DH5α (pRK600)] and recipient (PA23*phzR*) strains. The *anr* mutation in PA23*phzRanr* was confirmed by PCR analysis using *anr*-FOR/tet-REV and *anr*-REV/tet-FOR primers.

The DF41 *hcn* mutant strain was generated as follows. An internal region of the DF41 *hcn* gene cluster was PCR amplified using primers 41*hcnA*-FOR and 41*hcnC*-REV. Primers were designed based on the sequence of the *hcnABC* gene obtained from *P. brassicacearum* DF41 (GenBank accession no. NZ_CP007410). A TOPO® kit was used to clone the 1.6-kb PCR product into the pCR2.1-TOPO vector generating pCR*hcnABC*-41. To liberate the insert, pCR*hcnABC*-41 was digested with *Hin*dIII and *Xho*I, and subcloned into the same sites of the suicide vector pKNOCK-Tc. Triparental mating between the donor [*E. coli* DH5α λpir (pKNOCK*hcnABC*-41)], helper [*E. coli* DH5α (pRK600)] and recipient (DF41) was performed to insertionally interrupt the wild type *hcnABC* gene cluster. The *hcn* mutation in DF41*hcn* was confirmed by PCR and testing for a lack of HCN production using Cyantesmo paper.

2.5. Construction of plasmids

2.5.1. Generation of pUCP23-*anr*

To complement PA23*anr*, pUCP23-*anr* was generated as follows. A 959-bp PCR fragment containing the entire *anr* gene was obtained using *anr*(comp)-FOR and *anr*(comp)-REV and cloned into pCR2.1-TOPO to yield pCR*anr*(comp)-23. For *anr* complementation, the

959-bp *Sac*I-*Xba*I fragment was excised from pCR*anr*(comp)-23 and subcloned into the same sites of pUCP23, creating pUCP23-*anr*.

2.5.2. Generation of *hcnA-lacZ* transcriptional fusion

To monitor PA23 *hcn* gene expression, an *hcnA-lacZ* transcriptional fusion was created using PCR primers *hcnA*-FOR2 and *hcnA*-REV2 (Table 2.1). Primers were designed based on the promoter sequence of *hcnA* obtained from *P. chlororaphis* PA23 (GenBank accession no. NZ_CP008696). The *hcnA* promoter region was PCR amplified and a 963-bp amplicon was cloned into pCR2.1 to generate pCR2.1-*hcnA*. The construct pCR2.1-*hcnA* was digested with *Hin*dIII and *Xba*I and the insert was subcloned into the same sites of pLP170, creating *hcnA-lacZ*.

2.6. Growth rate analysis

The PA23 wild type was inoculated at a starting OD_{600} of 0.01 in M9 minimal medium (0.2% glucose, 1 mM $MgSO_4$) alone or supplemented with 100 µM ferric chloride ($FeCl_3$) and 20 mM glycine separately as well as in combination. OD_{600} readings were taken at 10 h, 12 h and 15 h, followed by readings every 2 h thereafter for a total of 27 h. Triplicate samples were analyzed.

2.7. Nematode Assays

2.7.1. *C. elegans* slow- and fast-killing assays

C. elegans slow-killing assays were performed by spotting 10 µl of a 1/10 dilution of an overnight bacterial culture grown in NGM or KB broth onto a 35x10 mm NGM or KB agar

plate. After 24 h incubation at 28^{o}C, the plates were cooled to room temperature (RT) and seeded with 25 to 30 L4-stage nematodes. The plates were then incubated at 25^{o}C (for PA23) or 20^{o}C (for DF41) and the nematodes were scored for viability by examining them with a stereomicroscope over a ten-day period. Nematodes without detectable movement were considered dead after confirmatory prodding with a nematode pick. Three replicates were included for each trial and the assays were repeated three times independently. Fast-killing assays were executed in a similar manner except that brain heart infusion (BHI) agar was used instead of NGM agar and the nematodes were monitored every hour for 9 h (PA23), or at 12 h and 24 h (DF41) timepoints.

2.7.2. Effect of purified PRN on *C. elegans* viability and egg hatching

All assays were conducted in 96-well culture plates. *C. elegans* L4-stage nematodes and eggs were collected separately in sterile water; approximately 20 nematodes and 15 eggs were used per well. Nematodes and eggs were incubated at 25^{o}C in purified PRN (Sigma-Aldrich Co., Missouri, USA) at the following concentrations: 0 µg/mL (water control), 0.1, 0.5, 1.0, 5.0 and 10 µg/mL. Nematode viability was assessed at 1, 3, 6, 12, 18, 24, 48 and 72 h and percent egg hatch was determined at 1, 3, 6, 24 and 48 h. Five replicate wells were used per trial and the assays were repeated three times. One representative data set is shown.

2.7.3. Chemotaxis Assays

Overnight cultures grown in NGM broth were diluted 10-fold and 10 µl volumes of the two bacterial strains being tested were spotted equidistant from a central point on a 60x15 mm NGM plate. Plates were incubated for 24 h at 28^{o}C to allow for bacterial growth. To obtain

synchronous L4-stage nematodes, 5 adult hermaphrodites were transferred to NGM agar plates spotted with *E. coli* OP50 and allowed to lay eggs. After flame-killing the adults, plates were incubated at 15^{o}C for 4 days to allow the nematodes to reach the L4-stage. Nematodes were collected in M9 buffer, and a 20 µl aliquot (containing 50 – 100 nematodes) was spotted onto the centre of each plate. The number of nematodes on the two bacterial colonies was counted 24 h after transfer. The chemotaxis index was calculated based on the formula = {(number on spot 2 – number on spot 1)/total number of nematodes on spot 1 + spot 2}.

2.8. Generation of bacteria expressing the mCherry red fluorescent protein

As plasmid pMCh-23 contains the mCherry red fluorescent protein gene, bacteria harboring this plasmid are easily visualized under the fluorescence microscope (Berry *et al.*, 2012). pMCh-23 was electroporated into the wild type PA23, and DF41, as well as their derivatives. Derivative strains of PA23 include Δ*prn*, Δ*phz*, Δ*prn/phz*, Δ*hcn*, Δ*phzR*, Δ*rpoS*, Δ*psrA*, Δ*gacS* mutants and the AI-deficient PA23 (pME6863). Derivative strains of DF41 include DF41-1278, Δ*hcn*, Δ*rfiA*, Δ*gacS* and AI-deficient DF41 (pME6863). The mCherry RFP was visualized using 587 nm excitation and 610 nm emission wavelengths.

2.9. Microscopic imaging of *C. elegans*

NGM or KB plates were spotted with the aforementioned bacterial strains harboring pMCh-23 and incubated overnight at 28^{o}C. Plates were cooled to RT prior to seeding with nematodes, followed by incubation at 25^{o}C (for PA23) or 20^{o}C (for DF41). For microscopic examination, nematodes were mounted on 2% agarose pads on glass microscope slides and anesthetized with 10 mMol/L Levamisole (Sigma) in M9 buffer. Nematodes were examined with

a Zeiss LSM 700 scanning confocal laser microscope and a Zeiss Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany).

2.10. Analysis of PA23 transcriptional fusions

The activity of *prnA*-, *phzA*-, *phzI*-, *phzR*-, *gacA*-, *gacS*-, *rpoS*- and *psrA*-*lacZ* transcriptional fusions was determined in PA23 cultured in the presence and absence of *C. elegans*. Nematodes were collected in M9 buffer and ~200 animals were added to 3 mL of PA23 cultures (OD_{600}=0.1) carrying the *lacZ* fusion plasmids. Cells were grown at RT (22 – 23^{o}C) for 24, 48 and 72 h in M9 minimal medium supplemented with 1 mM $MgSO_4$ and 0.2 % glucose prior to analysis of *β*-galactosidase activity (Miller, 1972). The activity of an *hcnA*-*lacZ* transcriptional fusion was determined in PA23 and the QS-deficient strains, Δ*phzR* and PA23 (pME6863). Strains carrying the *lacZ*-fusion plasmids were grown for 24 h in M9 (0.2% glucose, 1 mM $MgSO_4$) alone or supplemented with $FeCl_3$ (100 μM), glycine (20mM), or both prior to β-galactosidase analysis (Miller, 1972). The activity of *phzI*-, *phzR*-, *phzA*- and *prnA*-*lacZ* transcriptional fusions was also measured in PA23 under the same conditions. Samples were analyzed in triplicate and the experiment was repeated three times.

2.11. Gene expression analysis using qRT-PCR

To monitor expression of metabolite and regulatory genes involved in biocontrol, quantitative real-time (qRT) PCR was used. The expression of *prn*, *phz*, *hcn*, *phzI*, *phzR*, *anr* and *rpoS* was determined in PA23 and its derivative strains. Primers were designed based on the sequences of the respective genes obtained from *P. chlororaphis* PA23 (GenBank accession no. NZ_CP008696). Cultures were grown upto early stationary phase in 3 mL M9 minimal medium

(0.2% glucose, 1 mM $MgSO_4$) at 28ºC. Total RNA was extracted from a 0.5-mL volume of culture using an RNeasy Mini Kit (QIAGEN, Valencia, USA) and residual genomic DNA was removed by treatment with TURBO RNAse-free DNAse I (Ambion, Carlsbad, USA). The cDNA was generated by reverse transcription using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, USA) and the following conditions were employed: initial heating at 25ºC for 10 min, 50ºC for 15 min for reverse transcription and 85ºC for 5 min for enzyme denaturation. The qRT-PCR was performed using a CFX Connect™ Real-Time System (BIO-RAD) and SsoFast™ EvaGreen® Supermix (BIO-RAD). PCR reaction conditions included an initial denaturation at 98ºC for 2 min, followed by 40 cycles of 98ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 30 sec. The reactions were performed in triplicate and experiments were repeated three times with four biological replicates. Relative gene expression was determined using the ΔΔCt method as described by Livak and Schmittgen (2001).

The expression of *hcn*, sclerosin, *pdfR*, *pdfI*, *rfiA* and *gacS* was determined in DF41 cultured in the presence and absence of *C. elegans*. Primers were designed based on the sequences of the respective genes obtained from *P. brassicacearum* DF41 (GenBank accession no. NZ_CP007410). Nematodes were collected in M9 buffer and ~200 animals were added to 3 mL of DF41 cultures (OD_{600}=0.1). Cells were grown at RT (22–23°C) for 72 h in M9 minimal medium (0.2% glucose, 1 mM $MgSO_4$). Bacteria-nematode suspensions were centrifuged at 500 rpm for 1 min to separate the nematodes from the supernatants containing bacteria. Total RNA was extracted from 0.5 mL of the supernatants, which was processed as described above. The primer sequences for the genes of interest for both PA23 and DF41 are listed in Table 2.1.

2.12. Phenotypic assays

2.12.1. Antifungal activity

To study the AF activity of bacterial strains against *S. sclerotiorum*, radial diffusion assays were performed. Overnight cultures (5 µL) were spotted onto 1/5th PDA plates and incubated at 28°C for 24 h. An agar plug containing fungal mycelia was then placed in the centre of the plate and plates were incubated for 3 days at 22^{o}C to allow for growth of *S. sclerotiorum*. Fungal inhibition was assessed by measuring the zone of clearing between the edges of the bacterial colony and the fungal growth front as described by Poritsanos *et al*. (2006). To assess the impact of *C. elegans* co-culturing on PA23 AF activity, cultures of PA23 were grown in the presence and absence of the nematodes at RT (22–23°C) for 72 h before spotting onto PDA plates. To determine the role of HCN in PA23 fungal suppression, radial diffusion assays were performed using the wild type PA23 and *hcn* mutant on 1/5th PDA alone or supplemented with 100 µM $FeCl_3$ and 20 mM glycine separately as well as in combination. Plates were parafilmed to avoid the escape of the volatile HCN. Five replicates were analyzed for each strain or condition and experiments were repeated three times.

2.12.2. Autoinducer assay

The production of AHL molecules was analyzed qualitatively by spotting 5 µL of an overnight culture onto LB agar plates seeded with *C. violaceum* CV026. This AI-deficient strain is able to detect exogenous AHLs with carbon chain length ranging from C_4 to C_8, resulting in a purple halo (violacein) surrounding the colonies (Latifi *et al*., 1995). The diameter of the purple zones was measured at 24 h. Five replicates were analyzed for each strain or condition and experiments were repeated three times.

2.12.3. Qualitative HCN analysis

Qualitative assay of HCN production was performed using Cyantesmo paper, which turns blue in the presence of HCN. Three replicates were analyzed for each strain or condition and experiments were repeated twice.

2.12.4. Protease assay

Extracellular protease activity was determined by inoculating 5 µL of an overnight culture onto 1.5% agar plates containing 2 % skim milk. Zones of proteolysis were observed around the colony after 36 – 48 h growth at 28ºC (Poritsanos *et al*., 2006). Zones were measured from the edge of the bacterial colony to the edge of the zone of clearing. Data represent the average of five replicates and the assay was repeated three times.

2.12.5. Motility analysis

Flagellar (swimming) motility was monitored for the PA23 cultures grown in the presence and absence of *C. elegans*. Motility was assessed by inoculating 5 µL of a 72 h culture onto the center of a 0.3% LB agar plate and the swim zone diameters were measured at 24 and 48 h according to Poritsanos *et al*. (2006). Five replicates were analysed for this assay and the experiment was repeated three times.

2.13. AHL signal analysis

Total autoinducer production was monitored according to Ling *et al*. (2009), with the following modifications. PA23 was grown in the presence or absence of *C. elegans* for 72 h at RT in 30 mL M9 minimal medium (0.2% glucose, 1 mM $MgSO_4$). Cells were pelleted and cell-

free supernatants were extracted twice with an equal volume (30 mL) of acidified ethyl acetate. The ethyl acetate fractions were pooled and concentrated to a final volume of 1 mL. For AHL quantification, 100 µl aliquots of each extract were tested according to Selin *et al*. (2012). Extracts were added to test tubes and dried under a stream of air. An overnight culture of *P. aeruginosa* QSC105 (pEAL01) grown in PTSB with carbenicillin (200 µg/mL) was diluted to a final OD_{600} of 0.1 and 1 mL aliquots were added to tubes containing dried extracts. The cultures were grown for 18 hours at 37°C with vigorous shaking and then analyzed for β-galactosidase activity (Miller, 1972). Samples were analyzed in triplicate and the experiments were repeated three times.

2.14. Quantitative analysis of PHZ

Production of PCA and 2-OH-PHZ was quantified according to the methods outlined by Selin *et al*. (2010). Cultures (5 mL) were grown overnight in M9 minimal medium (0.2% glucose, 1mM $MgSO_4$) at 28ºC. Cell supernatants were collected and combined with 5 mL of benzene and one drop of concentrated HCl. Following one hour of shaking at RT, the top organic layer was removed and dried under air. The dried extracts were resuspended in 1 mL 0.1 M NaOH and filtered to remove particulate matter. Spectrophotometric quantification was performed at 367 nm and 490 nm for PCA and 2-OH-PHZ, respectively, according to Maddula *et al.* (2008). To determine the relative amounts of PHZ present, absorption maxima were divided by their standard extinction coefficients (PCA: 3019 $M^{-1}cm^{-1}$; 2-OH-PHZ: 7943 $M^{-1}cm^{-1}$). Each strain or condition was tested in triplicate and the experiments were repeated twice.

2.15. Quantitative analysis of PRN

Production of PRN was quantified by HPLC according to the methods outlined by Selin *et al.* (2010). Briefly, 30 mL cultures of PA23 and its derivatives were grown for 5 days in M9 minimal medium (0.2% glucose, 1 mM $MgSO_4$) at 28^oC. Cells were combined with an equal volume of ethyl acetate (20 mL) and 5 mL toluene (internal control). Following 30 mins of shaking at RT, the top organic layer was removed and dried under air. Peaks corresponding to toluene and PRN were analysed by UV absorption at 225 nm using a Varian 335 diode array detector. PRN peaks were detected at 4.8 mins. Samples were analyzed in triplicate.

2.16. Biofilm formation

To analyze the ability of DF41 and derivative strains to form biofilms, a 96-well plate assay was employed as detailed in Berry *et al*., 2010. The media used for this assay include KB, NGM, and LB broth. Overnight cultures were diluted 1:100 in fresh medium and a 200 µL aliquot was added to each well of the microtitre plate (Becton-Dickenson, Oakville, ON). Following 24 h incubation at 28^oC, a 25-µL volume of 1% Crystal Violet (CV) solution was added to each well and incubated for 15 mins. Unbound cells were removed by washing the plate with distilled water at least three times. A 200 µL aliquot of 95% ethanol was added to each well to solubilize the CV contained in the adherent cell population. The optical density was then measured at a wavelength of 550 nm to quantify biofilm formation. Each strain was tested in five replicates and the experiments were repeated twice.

2.17. Statistical analysis

An unpaired Student's *t* test was used for statistical analysis of PHZ, PRN, and AHL production, biofilm formation, swimming motility, antifungal activity, protease production and gene expression using qRT-PCR. The Bonferroni test was applied to determine the chemotactic preference of nematodes for PA23 and derivative strains. The log-rank (Mantel-Cox) test was applied for statistical analysis of pairwise comparisons in the fast and slow killing assays of nematodes by PA23, DF41 and derivative strains. Nematodes that died from crawling off the plate were censored from statistical analysis.

Chapter 3

Pseudomonas brassicacearum strain DF41 kills *Caenorhabditis elegans* through biofilm-dependent and biofilm-independent mechanisms

The work presented in this chapter has been accepted by the *Applied and Environmental Microbiology* journal with minor revisions (article # AEM02199-16).

3.1. Introduction

Production of extracellular metabolites by biocontrol bacteria is energetically costly; as such, these compounds are expected to impart a fitness advantage to the producer. It can be argued that reduced competition for resources is not sufficient to warrant synthesis of inhibitory compounds. Rather these products must provide additional advantages, such as reducing the threat of grazing predators through their repellent and/or cidal activities (Jousset *et al.*, 2009). Nematodes are among the most abundant animals on the planet and through their grazing pressure they are believed to influence microbial community structure. Bacteria, in turn, have evolved defensive mechanisms to resist nematode grazing, including the production of exometabolites that act to deter and/or reduce predator populations. The model organism *Caenorhabditis elegans* has been widely employed in bacterial-nematode interaction studies because of its genetic tractability and the comprehensive array of mutants available (Hope, 1999). For pseudomonads that exhibit pathogenicity towards *C. elegans*, killing ensues through two distinct mechanisms. On rich media, which supports production of high levels of toxic metabolites, death occurs quickly through intoxication, a mechanism known as "fast-killing" (Gallagher and Manoil, 2001; Tan *et al*., 1999; Darby *et al*., 1999; Nandi *et al*., 2015). Conversely, low-nutrient media results in production of sublethal levels of exometabolites and "slow killing" occurs over the course of days. In this instance, bacterial colonization of the intestinal tract ultimately leads to nematode death (Tan *et al*., 1999).

In nature, the bulk of bacterial biomass exists as an adherent community of cells encased in an extrapolymeric matrix, collectively known as a biofilm. The ability to form biofilms has many advantages, including protection from environmental assaults that would threaten planktonic cells (Costerton *et al*., 1995). By way of example, biofilms exhibit elevated resistance

to biocidal agents, immune components, desiccation, and UV-radiation (Costerton *et al*., 1995; Davey and O'Toole, 2000). For a select group of bacteria, the ability to form biofilms allows them to escape predation by grazing nematodes. In 2002, Darby and coworkers reported that *Y. pestis* is able to form biofilms on the head and body of *C. elegans*, which blocks feeding and ultimately causes starvation. Similarly, *Xenorhabdus nematophila* (Couillalt and Eubank, 2002) and some but not all strains of *Y. pseudotuberculosis* (Joshua *et al*., 2003) are capable of establishing nematode-associated biofilms. Screening an array of 26 pathogenic bacteria revealed that *C. elegans* surface colonization is not a common trait (Alegadro *et al*., 2003; Tan and Darby, 2004). Even the notorious infection-related biofilm formers *P. aeruginosa* and uropathogenic *E. coli* were found to lack this ability, leading to the conclusion that this defensive strategy is employed by only few bacterial species (Tan and Darby, 2004).

Pseudomonas brassicacearum strain DF41 is a biocontrol agent capable of suppressing disease caused by the pathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Savchuk and Fernando, 2004). This bacterium produces an arsenal of extracellular metabolites that are believed to contribute to fungal antagonism including degradative enzymes, hydrogen cyanide (HCN) and a novel lipopeptide called sclerosin (Berry *et al*., 2010, 2012). The latter has been confirmed to be essential for biocontrol as a sclerosin-deficient mutant, DF41-1278, exhibits dramatically reduced fungal suppression (Berry *et al*., 2010). A complex regulatory cascade oversees production of DF41 exometabolites, including the GacS/GacA two-component system, the stationary phase sigma factor RpoS and a global stress response known as the stringent response (Berry *et al*., 2010; Manuel *et al*., 2011). In addition, DF41 has a quorum-sensing (QS) system comprised of a LuxR-type transcriptional activator (PdfR) and an acyl-homoserine lactone (AHL) synthase (PdfI), encoded by *pdfR* and *pdfI*, respectively (Berry *et al*., 2014).

Immediately downstream of and co-transcribed with *pdfI* lays a third gene in the QS locus, called *rfiA.* RfiA belongs to a distinct group of LuxR regulators characterized as having a C-terminal helix-turn-helix DNA binding motif but lacking an N-terminal AHL binding domain (Berry *et al*., 2014; de Bruijn and Raaijmakers, 2009). We have previously shown that AHL signaling molecules are not involved in DF41 biocontrol because an AHL-deficient strain exhibits no discernable phenotype (Berry *et al*., 2014). RfiA, on the other hand was found to be essential, as an *rfiA* mutation results in a complete loss of antifungal activity (Berry *et al*., 2014). RfiA is believed to control expression of the PdfABC efflux pump involved in exometabolite export. In the Δ*rfiA* background, metabolites that are normally excreted accumulate to elevated levels within the mutant cells (Berry *et al*., 2014).

The aim of the current study was to elucidate the interaction between DF41 and the bacterivorous predator *C. elegans*. Specifically, we were interested to learn whether DF41 is able to resist nematode grazing and what role, if any, DF41 exoproducts play in the bacterial-nematode interaction. We discovered that DF41 is capable of killing *C. elegans* through two distinct modes; the first involves exposure to toxic metabolites while the second entails biofilm formation on the nematode surface. It has been reported that bacteria are capable of responding to predator cues through altered expression of toxin-encoding genes (Jousset *et al*., 2010; Mazzola *et al*., 2009; Nandi *et al*., 2015). To see if the same holds true for DF41, we co-cultured bacteria with *C. elegans* and monitored expression of genes associated with exometabolite production. In the presence of *C. elegans*, several genes were upregulated including *hcnA* (hydrogen cyanide) and the QS genes *pdfRI* and *rfiA*. Our findings indicate that through soluble chemical cues and/or direct contact, DF41 is able to perceive the presence of *C. elegans* and adjust its physiology accordingly.

3.2. Results

3.2.1. DF41 kills *C. elegans* through production of toxic metabolites

For pseudomonads that exhibit toxicity towards *C. elegans*, these effects are mediated through two distinct mechanisms known as fast and slow killing. One of the primary factors dictating which killing mechanism ensues is the nutrient content of the media. With that in mind, several different media were employed for nematode feeding assays to fully explore the interaction between DF41 and *C. elegans*. Bacterial strains expressing the fluorescent reporter mCherry were grown on BHI, a media previously shown to support fast killing by pseudomonads (Nandi *et al*., 2015; Tan *et al*., 1999). As outlined in Figure 3.1.a, differences in lethality were observed; however, none of the strains elicited 100% nematode killing at 24 hours. The *gacS* and *hcn* mutants showed the lowest level of toxicity. Because HCN is known to cause lethal paralysis in *C. elegans* (Gallagher and Manoil, 2001; Tan *et al*., 1999) and the *gacS* mutant is devoid of HCN production (Berry *et al*., 2010), these findings are not unexpected. The sclerosin-deficient strain, DF41-1278, was similar to wild type, indicating that this LP does not contribute to fast killing. Similarly, the AI-deficient DF41 (pME6863) exhibited no change in lethality. We have previously shown that a loss of AI production leads to no discernable phenotypic changes in DF41 (Berry *et al*., 2014). The most toxic strain was the Δ*rfiA* mutant. RfiA is a transcriptional regulator that is predicted to activate expression of the downstream *pdfABC* efflux pump-encoding genes (Berry *et al*., 2014). Previous characterization revealed intracellular accumulation of secondary metabolites and elevated *hcn* gene expression in this strain (Berry *et al*., 2014). The fact that toxic compounds are being trapped within the Δ*rfiA* cells which are also producing increased levels of HCN likely accounts for the high nematicidal activity.

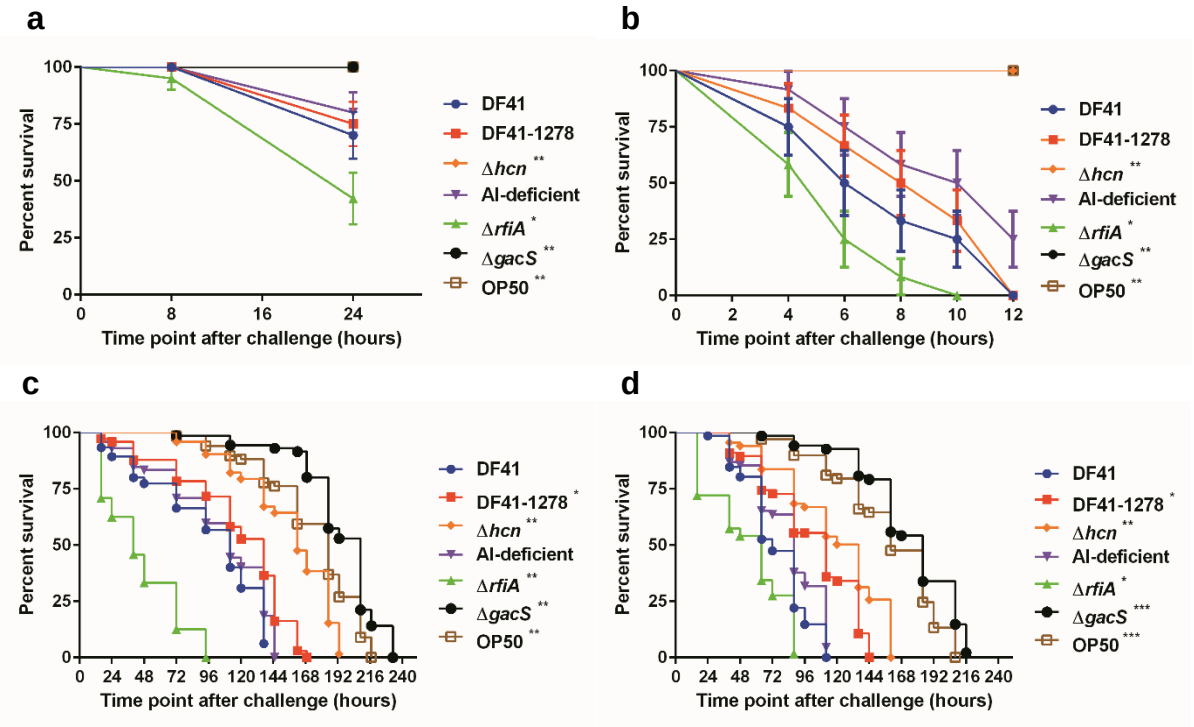


Figure 3.1. Fast-killing (a, b) and slow-killing (c, d) of *C. elegans* by *Pseudomonas brassicacearum* DF41. Kaplan-Meier survival plots of *C. elegans* N2 (n = 25) fed *E. coli* OP50, DF41 or derivative strains propagated on a. BHI agar, b. BHI agar supplemented with 100 µM $FeCl_3$, c. NGM agar and d. KB agar. Nematode viability was monitored for 24 h and 12 h for a and b respectively. Nematodes were assessed for viability over a ten and nine-day period for c and d respectively. Each data point represents the average of three biological replicates. Experiments were performed three times; one representative data set is shown. Asterisks indicate significant difference from the wild type as determined by the log-rank test (*, P<0.01; **, P<0.001; ***, P<0.0001).

Although DF41 produces HCN, it does so at levels lower than what we have observed for other biocontrol strains, including *Pseudomonas chlororaphis* PA23 and *P. protegens* Pf5 (data not shown). Supplementing media with iron has been reported to elevate HCN production (Michelsen and Stougaard, 2012); therefore, BHI containing 100 µm $FeCl_3$ was employed in the aforementioned assays to see if more rapid killing would ensue. While the same relative toxicity pattern was observed, the presence of iron did enhance overall lethality (Figure 3.1.b). When nematodes were placed on BHI (100 µM $FeCl_3$), the DF41 wild type and sclerosin mutant caused 100% killing after 12 hours. As before, the Δ*rfiA* strain was the most toxic killing 100% of the nematodes by 10 hours, while the HCN-deficient *hcn*- and *gacS*-mutants were completely benign (Figure 3.1.b). Collectively, these findings indicate that on BHI, HCN is the primary compound responsible for fast killing of *C. elegans*. Due to low-level production of this volatile compound, however, rapid intoxication does not result.

3.2.2. Slow killing of *C. elegans* by biofilm-dependent and biofilm-independent mechanisms

For the slow-killing assays, DF41 and mutant strains were grown on NGM and KB agar prior to seeding with nematodes to assess the survival rate. On NGM agar, the survival of the nematodes decreased over the course of days (Figure 3.1.c), the flux in survival rates strain-dependent but nevertheless consistent with lethality mediated by bacterial colonization rather than intoxication. Under these conditions, the *gacS* mutant was found to be less toxic than the *hcn* mutant. In addition to HCN, the *gacS* mutant fails to produce degradative enzymes and antibiotic compounds including sclerosin (Berry *et al*., 2010), some of which may be contributing to toxicity. The sclerosin- minus mutant (DF41-1278) showed reduced nematicidal effects compared to the wild type, indicating that this LP is involved in slow killing, albeit

modestly. As before, the greatest toxicity was observed for the Δ*rfiA* mutant, which killed 100% of the nematodes within 96 hours (Figure 3.1.c).

Slow killing of *C. elegans* was also observed when nematodes were fed bacteria growing on KB media (Figure 3.1.d). However, there was one dramatic difference compared to nematodes fed DF41 and derivative strains grown on NGM. After 24 hours, we noticed that biofilms began to form on the *C. elegans* head and body, which dramatically impacted nematode physiology and behavior (Figure 3.2). Microscopic visualization at low magnification revealed that when nematodes were placed on NGM supplemented with the standard lab strain *E. coli* OP50, the nematodes moved freely through the bacterial lawns generating sinusoidal track marks known as "skd" marks (Figure 3.2.a). Conversely on DF41 lawns grown on KB media, aberrant skd marks were formed (Figure 3.2.b, c) and the nematodes fishtailed through the viscous bacterial lawn like a car moving through deep snow. Moreover, the animals were frequently observed to migrate away from the bacteria in an attempt to remove their matrix entrapment (Figure 3.2.c). Over time, the biofilm-coated *C. elegans* became progressively thinner, with some disintegrating completely, presumably due to starvation (data not shown).

3.2.3. DF41 Biofilm formation on *C. elegans* is dependent on GacS

Next, we examined whether all of the DF41 derivative strains were capable of forming biofilms on the *C. elegans* head and body. On KB media, the Δ*hcn*, Δ*rfiA*, sclerosin-minus (DF41-1278), and AI-deficient strains all coated the surface of *C. elegans* and resulted in aberrant skd marks (data not shown); however, *C. elegans* growth on and movement through the *gacS* bacterial lawn resembled that observed for *E. coli* OP50 (Figure 3.2.d). Confocal microscopic analysis of RFP-expressing bacteria revealed that, with the exception of the *gacS*

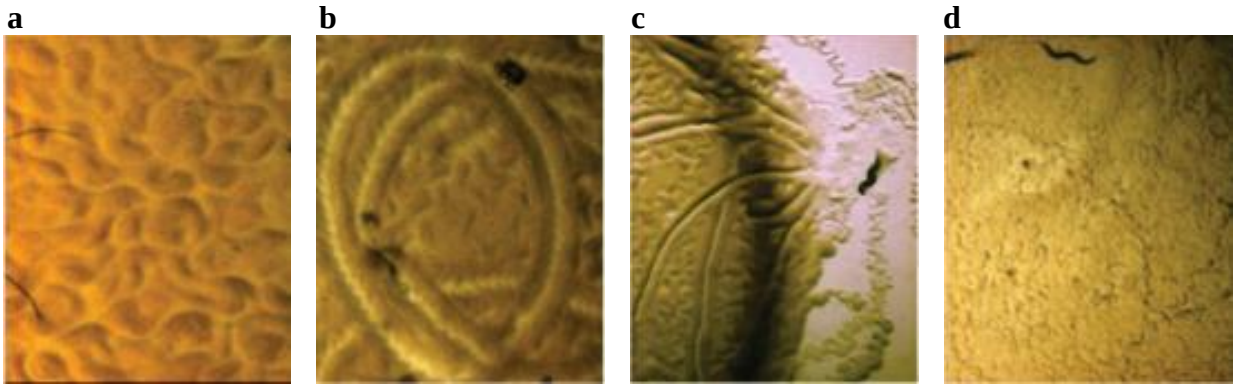
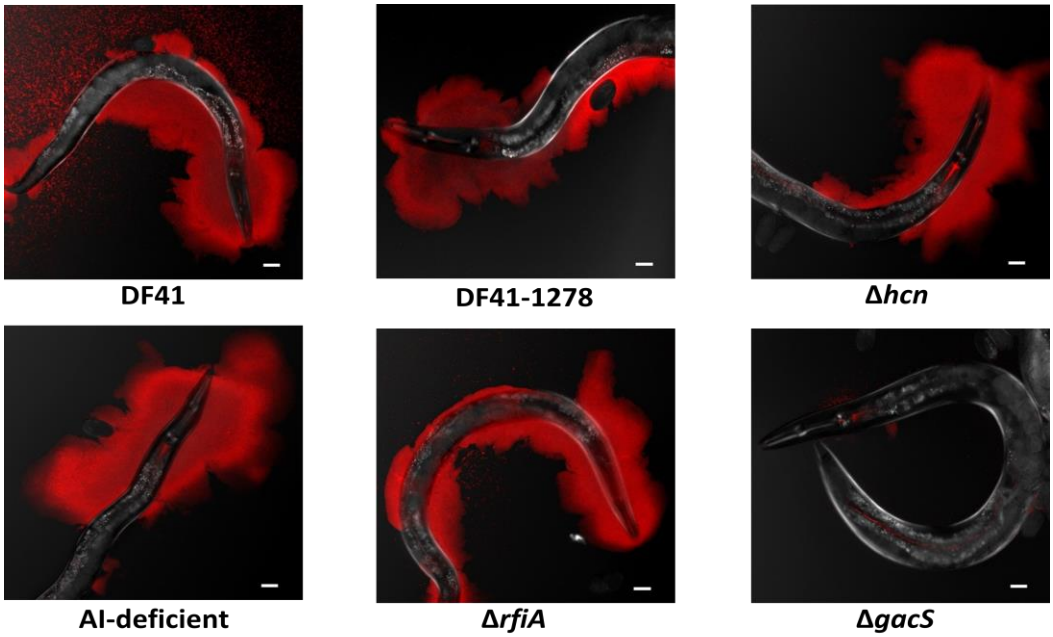


Figure 3.2. Track marks of nematodes fed on *E. coli* OP50 (panel a), DF41 (panels b and c) and a DF41 *gacS* mutant (panel d). Image courtesy for Chrystal Berry.

a



b

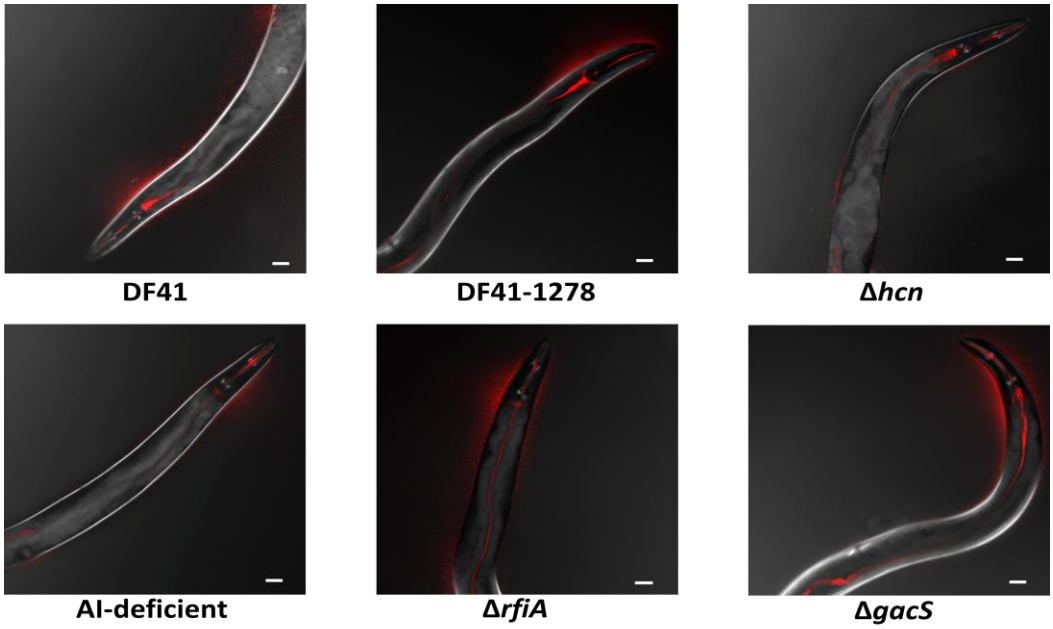


Figure 3.3. Microscopic analysis of (a) N2 and (b) Δ*srf*2 (strain AT6) worms fed *rfp*-tagged DF41 and derivative strains on KB. Confocal images were taken with Zeiss LSM 700 scanning confocal laser microscope under 10x magnification. Scale bar represents 20 µm.

mutant, all of the strains formed a thick biofilm on the *C. elegans* surface (Figure 3.3.a). In all cases, bacteria are visible in the mouth, grinder and intestinal track of the nematodes indicating that bacterial ingestion had occurred prior to biofilm formation blocking the buccal cavity.

Biofilm formation on the surface of *C. elegans* has only been reported for *Y. pestis, Y. pseudotuberculosis*, and *X. nematophila* (Couillalt and Eubank, 2002; Darby *et al*., 2002; Tan and Darby, 2004). Moreover *C. elegans* mutants resistant to biofilm formation by all three bacteria have been identified (Darby *et al*., 2007; Drace and Darby, 2008; Joshua *et al*., 2003). To determine whether similar mechanisms of DF41 nematode attachment were at play, we examined several biofilm-resistant *C. elegans* strains, namely *srf-2*(*yj262*), *bah-3*(*br9*) and a biofilm-minus Hawaiian isolate (CB4856), growing on DF41. In all cases, biofilm formation was markedly reduced on the nematodes (Figure 3.3.b). Figure 3.4 highlights the difference in biofilm formation on the anterior surface of N2 compared to the *srf-2*(*yj262)* mutant nematodes.

3.2.4. NaCl impacts DF41 biofilm development on the surface of *C. elegans*

The fact that biofilms developed on the nematode surface when bacteria were grown on KB, but not BHI, NGM or LB suggested that media composition is having an impact on this trait. One obvious difference in the composition of these media is salt content. The three media that did not promote biofilm formation contained NaCl [NGM (0.3%), BHI (0.5%) and LB (1%)]; whereas KB medium does not. This finding prompted us to investigate whether NaCl affects the ability of DF41 to form biofilms on the surface of *C. elegans*. Nematodes fed on DF41 grown on KB media containing 0.5% NaCl resulted in a noticeable decrease in biofilm formation on the head and body (Figure 3.5.a). The effects were even more dramatic when the concentration of NaCl increased to 1%, in which case virtually no biofilms were observed on the

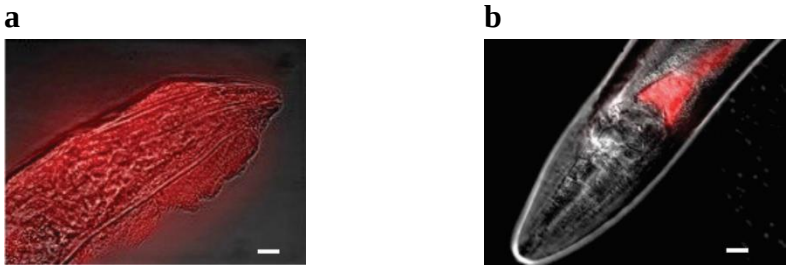


Figure 3.4. (a) Biofilm formation on wild type N2 worms feeding on *rfp*-tagged DF41. (b) Absence of biofilm on Δ*srf*2 (strain AT6) worms feeding on *rfp*-tagged DF41. Confocal images were taken with Zeiss LSM 700 scanning confocal laser microscope. Under 10x magnification. Scale bar represents 40 µm. Image courtesy for Chrystal Berry.

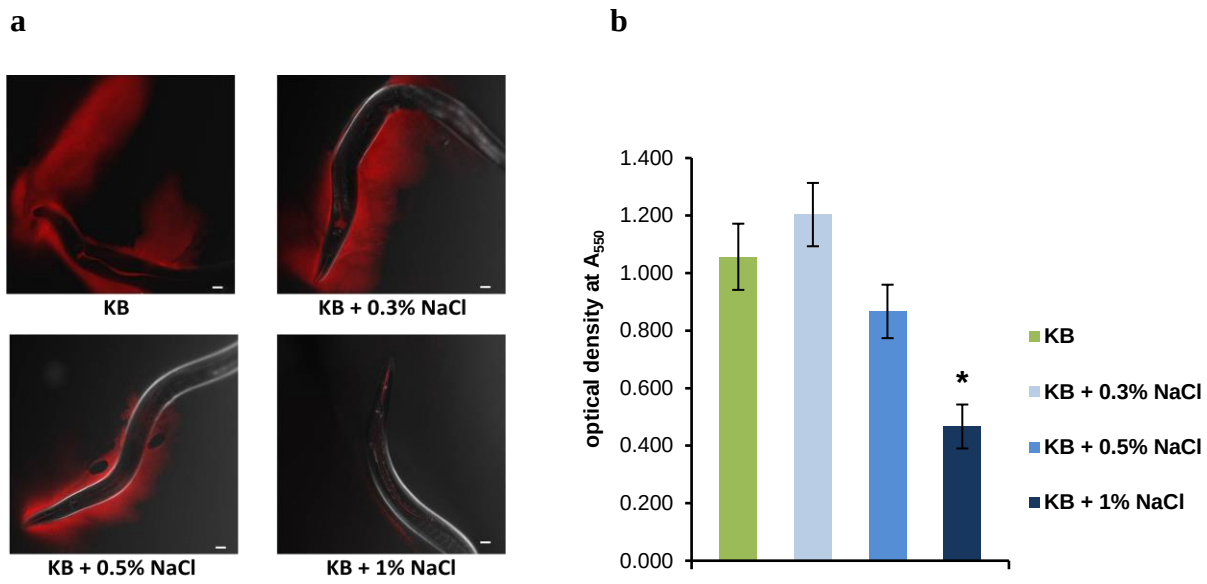


Figure 3.5. (a) Microscopic analysis of N2 worms fed *rfp*-tagged DF41 propagated on KB supplemented with NaCl (0 - 1%). Confocal images were taken with Zeiss LSM 700 scanning confocal laser microscope under 10x magnification. Scale bar represents 20µm. (b) *In vitro* biofilm formation by DF41 in KB broth supplemented with NaCl (0 - 1%). Each value represents the mean from three biological replicates ± standard error. The data point marked with an asterisk indicates statistical significance (*, P<0.01).

head and body of the nematodes (Figure 3.5.a). Collectively these findings indicate that NaCl is able to modulate biofilm formation on the *C. elegans* surface.

3.2.5. Biofilm formation on abiotic surfaces is affected by NaCl but not GacS

Factors affecting attachment to abiotic surfaces are expected to be dramatically different from those involved in biofilm formation on biotic surfaces. As such, we employed a 96-well microtitre plate assay to assess the ability DF41 and derivative strains to form adherent biomass under different media conditions. While there was no significant difference in the ability of the strains to form biofilms on plastic, we did notice that in KB, biofilm formation was nearly double that observed for bacteria grown in NGM and LB (Figure 3.6). These findings suggest that NaCl may be impacting biofilm formation not only on biotic surfaces but abiotic substrates as well. To test this hypothesis, we examined the adherent DF41 biomass after 24 h static growth in KB supplemented with NaCl (0% - 1%). As illustrated in Figure 3.5b, a significant decrease in biofilm formation was observed in KB containing 1% NaCl.

3.2.6. Identification of an *hmsHFRS* operon in the DF41 genome

In *Y. pestis*, *Y. pseudotuberculosis* and *X. nematophila*, biofilm development on *C. elegans* is dependent upon the *hmsHFRS* operon. Through BlastX analysis we identified an *hmsHFRS* homolog in the DF41 genome. This operon showed the highest degree of similarity at the amino acid level with PgaABCD of *Pseudomonas fluorescens*. The DF41 HmsH protein is 91% identical to PgaA, a predicted poly-beta-1,6-*N*-acetyl-D-glucosamine (PGA) export porin (gb ALI07281.1). HmsF exhibits 94% sequence identity with PgaB, a PGA N-deacetylase (WP

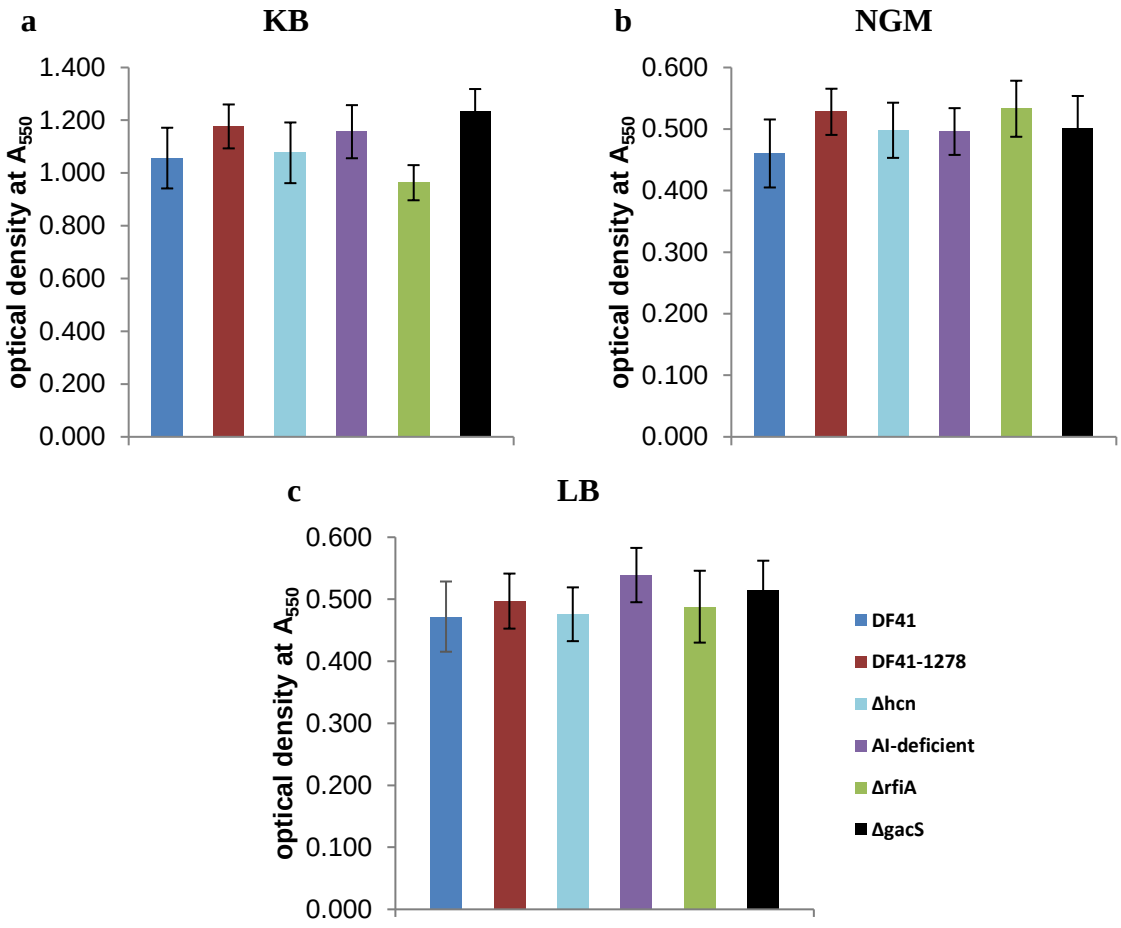


Figure 3.6. *In vitro* biofilm formation by DF41 and derivative strains in (a) KB, (b) NGM and (c) LB media.

024618876.1). HmsR is 99% identical to the PGA synthase PgaC (gb ALI07279.1) and HmsS exhibits 93% identity with the PGA biosynthesis protein PgaD (WP 003186997.1).

3.2.7. DF41 gene expression is affected by growth in the presence of *C. elegans*

To investigate whether DF41 is able to sense the presence of *C. elegans*, either through direct contact or soluble chemical cues, we monitored bacterial gene expression upon growth in the presence and absence of these predators. Both regulatory genes (*gacS, pdfR, pdfI, rfiA*) and the biosynthetic loci encoding HCN and sclerosin were analyzed. The most dramatic increase in gene expression was observed for *hcnA*, which was elevated over five-fold in the presence of the nematodes (Figure 3.7). Sclerosin biosynthetic gene activity was not affected by the presence of *C. elegans* (Figure 3.7). Expression of the QS-regulatory genes *pdfR*, *pdfI* and *rfiA* were all significantly elevated upon *C. elegans* co culture (Figure 3.7). Transcription of *gacS*, on the other hand, remained unaffected. We observed increased HCN and AHL signal production in DF41 cells co-cultured with *C. elegans* (Figure 3.8) consistent with the increase in *hcnA* and *pdfI* transcription, respectively.

3.3. Discussion

In order for a biocontrol agent to be effective, it must colonize and persist in a particular environment at levels sufficient for pathogen control. There are a number of barriers that challenge successful establishment including competition for resources and predation by bacterivorous organisms, such as nematodes. The focus of this study was to determine whether DF41 is able to resist nematode grazing and what role if any DF41 exoproducts play in the bacterial-nematode interaction. We discovered that DF41 is capable of killing *C. elegans* through

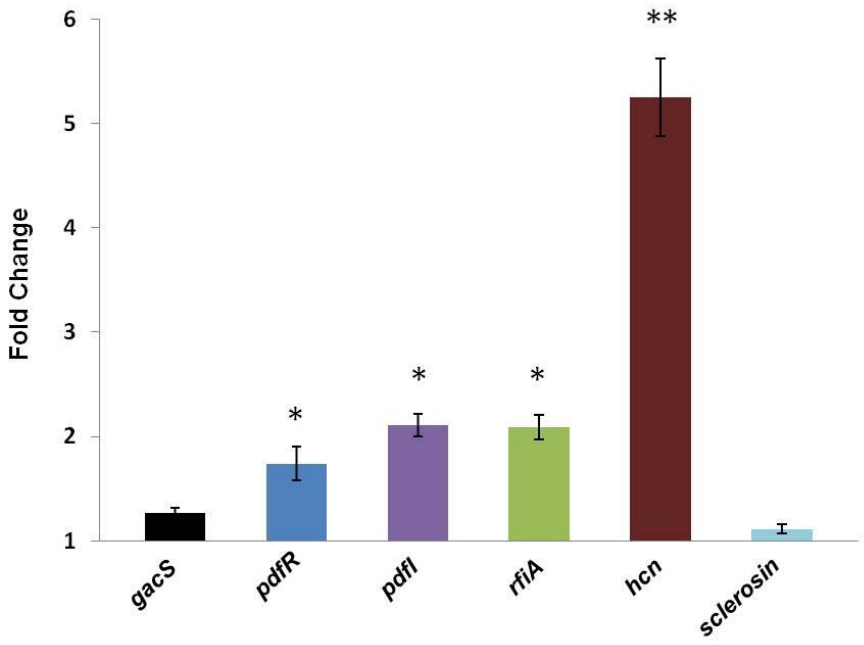
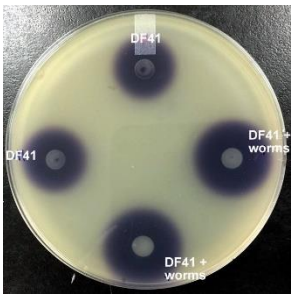


Figure 3.7. The impact of *C. elegans* co-culture on DF41 biocontrol gene expression. qRT-PCR analysis was used to quantitatively assess gene expression in the presence and absence of nematodes. Expression levels in the absence of *C. elegans* were normalized to 1; differentially expressed genes are indicated with an asterisk (*, $P<0.05$; **, $P<0.001$).

a



Organism	Qualitative AI assay (mm)
DF41	6.68 (0.93)
DF41 + *C. elegans*	8.57 (0.87)*

b

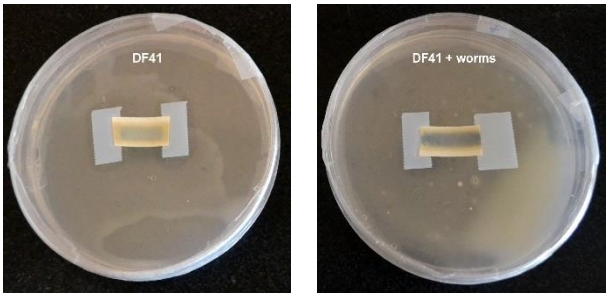


Figure 3.8. (a) Autoinducer assay of DF41 in the presence and absence of worms on CVO26-seeded agar. Mean (SD) of zones of activity (mm) obtained from ten replicates. Asterisk indicates significant difference from the wild type (P<0.001). (b) HCN detection by cyantesmo paper in the presence of DF41 and DF41 with worms.

two different mechanisms: the first involves biofilm-formation on the nematode surface; and the second relies on exposure to toxic DF41 metabolites.

It is well established that media composition has a profound impact not only on secondary metabolites produced by pseudomonads (Haas and Keel, 2003) but also the nematicidal activity associated with these organisms (Cezairliyan *et al*., 2013; Darby *et al*., 1999; Gallagher and Manoil, 2001; Tan *et al*., 1999; Nandi *et al*., 2015). With this in mind, several different media were employed as part of our DF41-*C. elegans* interaction studies. On nutrient-rich BHI, which has been previously reported to support high exometabolite production and fast-killing of *C. elegans* (Gallagher and Manoil, 2001; Nandi *et al*., 2015), none of the strains tested lead to 100% killing at 24h (Figure 3.1.a). Because HCN is a nematicidal agent causing rapid paralysis of *C. elegans* (Gallagher and Manoil, Tan *et al*., 1999), we hypothesized that HCN levels were below that which was required for fast killing. When $FeCl_3$ was added to the media to boost HCN production, the rate of killing by DF41 was enhanced such that all of the nematodes were dead by 12h (Figure 3.1.b). With respect to relative toxicity, both in the presence and absence of $FeCl_3$, the strain exhibiting the highest level of nematicidal activity was the *rfiA* mutant (Figure 3.1.a, b). We have previously demonstrated increased expression of *hcnA-lacZ* and *pdfI-lacZ* fusions coupled with intracellular accumulation of AI molecules in the *rfiA* mutant (Berry *et al*., 2014). As RfiA is a putative activator of the downstream PdfABC efflux pump, we believe that metabolites trapped within the RfiA-deficient cells lead to increased nematicidal activity. HCN is clearly an important toxin since the Δ*hcn* cells show significantly reduced toxicity (Figure 3.1.a, b). Sclerosin on the other hand did not contribute to killing under these conditions.

In slow killing assays, which depend upon infection of the *C. elegans* intestine, killing occurred over the course of days (Figure 3.1.c, d). On both NGM and KB media, HCN and to a lesser degree sclerosin, contributed to the nematicidal effects; however, one dramatic difference was observed between the two media. After 24h on KB, biofilms began to accumulate on the surface of the nematodes as they translocated through the bacterial lawns. This phenomenon is unique to a small number of bacteria including the human pathogens *Y. pestis* and *Y. pseudotuberculosis*, and the insect pathogen *X. nematophila*. To the best of our knowledge, biofilm formation on *C. elegans* has never been reported for a pseudomonad, pathogenic or otherwise. For both *Yersinia* spp. and *Xenorhabdus*, *C. elegans* biofilm formation is dependent upon the *hmsHFRS* gene cluster (Darby *et al*.., 2002; Drace and Darby, 2008). Analysis of the DF41 genome revealed the presence of a homologous *hmsHFRS* operon. The translated protein products share the greatest identity (91-99%) with *P. fluorescens* PgaABCD (HmsHFRS). In *E. coli* PGA biosynthesis, mediated by *pgaABCD*, is essential for biofilm formation (Itoh *et al*., 2008; Wang *et al*., 2004). Whether these genes are involved in the development of DF41 biofilms remains to be established. The fact that *C. elegans* mutants that are resistant to *Yersina* and *Xenorhabdus* biofilms are resistant to those of DF41 suggests a common ligand-receptor interaction is likely involved (Drace and Darby, 2008; Tan and Darby, 2004).

In *E. coli*, a *csrA* (*rsmA*) mutant was found to overproduce PGA and showed increased biofilm development (Jackson *et al*., 2002; Wang *et al*., 2005). CsrA belongs to the RsmA family of repressor proteins that bind mRNA at the ribosome-binding site, thereby blocking translation (Lapouge *et al*., 2008). The Gac two-component system is a positive activator of small RNAs that titrate out the RsmA proteins allowing target gene expression. Consequently in a *gac* mutant, targets remain permanently repressed. The findings presented herein suggest that genes

underlying DF41 biofilm formation on the surface of *C. elegans* are subject to Gac regulation, consistent with the CsrA control observed in *E. coli*. In another study, the LysR-type regulator NhaR was found to activate *pgaABCD* expression in response to 100 mM NaCl and high pH in *E. coli* (Goller *et al*., 2006). We on the other hand observed that addition of sodium ions [0.5% (86 mM) and 1% (171 mM)] diminished establishment of DF41 biofilms on both biotic and abiotic surfaces (Figure 3.5). A negative correlation between NaCl and biofilm formation has been previously reported for *Clostridium difficile* and *Streptococcus suis* (Dapa *et al*., 2013; Dawei *et al*., 2012). Collectively these findings suggest that the impact of sodium on biofilm development is species dependent.

Prokaryotes and eukaryotes have cohabited the earth for millions of years; it is not surprising, therefore, that chemical communication between the two facilitates mutual perception (Hughes and Sperandio, 2008). The production of toxic secondary metabolites is energetically costly and so limiting production to situations where these compounds provide a fitness advantage, when confronted by predators for example, would be beneficial to the producer. We co-cultured DF41 in the presence of *C. elegans* to see whether chemical cues and/or direct contact would lead to changes in bacterial gene expression. Upon co-culturing the most highly upregulated DF41 gene was *hcnA* (Figure 3.7). Intriguingly, HCN also exhibited the greatest nematicidal activity (Figure 3.1). Sclerosin, which exhibited almost no toxicity towards *C. elegans*, was unchanged with respect to gene expression in DF41 grown with the nematodes. These findings are similar to those reported by Jousset and coworkers (2010) wherein cell-free supernatants of the amoeba *Acanthamoeba castellani* were found to increase expression of *phlA* (DAPG), *prnA* (pyrrolnitrin) and *hcnA* in *P. fluorescens* CHA0. Notably, there was a correlation between the level of expression and the toxicity of the encoded product, with the greatest

increase in gene expression observed for DAPG, the most toxic metabolite tested (Jousset *et al*., 2010). We also examined the impact of co-culturing on regulatory gene expression. In this case, *pdfR*, *pdfI* and the co-transcribed *rfiA* were upregulated in the presence of *C. elegans*. We have previously shown that *hcn* expression is under control of the PdfRI QS system; whereas the sclerosin biosynthetic genes are not (Berry *et al*., 2014). At present, it is unclear whether upregulation of the *hcn* genes is mediated directly or indirectly through increased QS gene expression. When we analyzed HCN and AHL signal production by DF41, we observed that both endproducts were upregulated by the presence of the nematodes, consistent with our gene expression analysis.

In summary, DF41 is able to kill *C. elegans* through two independent mechanisms; exposure to secondary metabolites and biofilm formation. This is the first report of a pseudomonad establishing biofilms on the *C. elegans* surface, blocking feeding and ultimately killing the nematodes through starvation. Future studies will focus on creation of an *hmsHFRS* mutant to determine if these genes play a role in DF41 biofilm formation on *C. elegans* as well as plant surfaces. Factors that impacted nematode biofilm formation, namely the Gac system and salt, together with root exudates, pH, water availability and other relevant parameters will be explored for their influence on *hmsHFRS* expression. Finally, we will determine whether DF41 exhibits nematicidal activity against plant pathogenic organisms both in the lab and in the field.

3.4. Acknowledgments

I would like to thank Dr. Chrystal Berry for the initial investigation regarding the DF41-*C. elegans* interaction.

Chapter 4

Pyrrolnitrin and hydrogen cyanide production by *Pseudomonas chlororaphis* strain PA23 exhibits nematicidal and repellent activity against *Caenorhabditis elegans*

The work presented in this chapter has been published in PLoS One/doi:10.1371/Journal.poine. 0123184.

4.1. Introduction

Successful establishment of a biocontrol agent in a particular environment depends upon a number of factors including competition with indigenous microflora for available resources and resisting the deleterious effects of grazing predators. Bacteria fall prey to a number of organisms; among these, bacterivorous nematodes are thought to play a major role in shaping the microbial community structure. In response, bacteria have developed sophisticated defense strategies to avoid nematode grazing. A small number of bacterial species are capable of forming biofilms on the surface of the nematode, ultimately causing starvation (Darby *et al*., 2002; Tan and Darby 2004). However, a more common mechanism involves the production of exometabolites that have repellent and/or inhibitory effects on nematodes (Gallagher and Manoil, 2001; Lee *et al*., 2010; Neidig *et al*., 2011; Burlinson *et al*., 2013; Cevairliyan *et al*., 2013). The model organism *Caenorhabditis elegans* is frequently employed for studies of bacterial-nematode interactions. For bacteria that exhibit pathogenicity towards *C. elegans*, nutrient availability plays a significant role in determining how these harmful effects are mediated. Under nutrient-limiting conditions, sublethal levels of toxic bacterial metabolites are produced and nematode death proceeds over the course of days. In this case, the so-called "slow-killing" effect is reliant upon bacterial colonization of the nematode intestine (Tan *et al*., 1999). On nutrient-rich media, "fast killing" of the nematodes occurs through intoxication (Gallagher and Manoil, 2001; Tan *et al*., 1999; Darby *et al*., 1999). For the well-studied pathogen *Pseudomonas aeruginosa*, different mechanisms of fast-killing have been reported depending on the bacterial strain in question and the growth medium employed. Strain PAO1 propagated on brain-heart infusion (BHI) agar causes rapid paralysis and nematode death through HCN poisoning (Gallagher and Manoil,

2001), whereas on peptone, glucose and sorbitol (PGS) medium, phenazines (PHZ) are responsible for PA14-mediated intoxication (Cezairliyan *et al*., 2013; Tan *et al*., 1999).

Organisms are constantly receiving and responding to chemical signals in their environment and as such, it is not surprising that molecular signalling modulates predator-prey interactions. Studies have shown that bacteria are able to respond to protozoan predator cues by upregulating expression of toxin genes (Mazzola *et al*., 2009; Jousset *et al*., 2010). Similarly, bacterial metabolites such as N-acylhomoserine lactone molecules and biosurfactants can act as either chemoattractants or repellents for nematodes (Beale *et al*., 2006; Pradel *et al*., 2007). This mutual perception and response to chemical signals drives the predator-prey warfare.

Pseudomonas chlororaphis strain PA23 is able to protect canola against sclerotinia stem rot caused by the pathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary through a process known as biocontrol (Savchuk and Fernando, 2004). PA23 produces a number of metabolites, many of which are thought to contribute to fungal antagonism including the antibiotics phenazine-1-carboxylic acid (PCA), 2-hydroxy-phenazine (2OH-PHZ) and pyrrolnitrin (PRN), together with hydrogen cyanide (HCN), protease, lipase, and chitinase (Zhang *et al*., 2006; Poritsanos *et al*., 2006; Selin *et al*., 2010). We have determined that PRN is essential for suppression of *S. sclerotiorum* (Selin *et al*., 2010). While PHZ play a more minor role in fungal antagonism, they have been found to facilitate PA23 biofilm formation (Selin *et al*., 2010). Production of these metabolites is heavily regulated at both the transcriptional and posttranscriptional levels (Figure 4.1). Regulatory elements include the GacS-GacA two component system which functions together with Rsm to positively control antifungal metabolite production (Poritsanos *et al*., 2006; Selin *et al*., 2014). Additional regulators include the stationary phase sigma factor RpoS, a sigma regulator called PsrA and the PhzRI quorum-

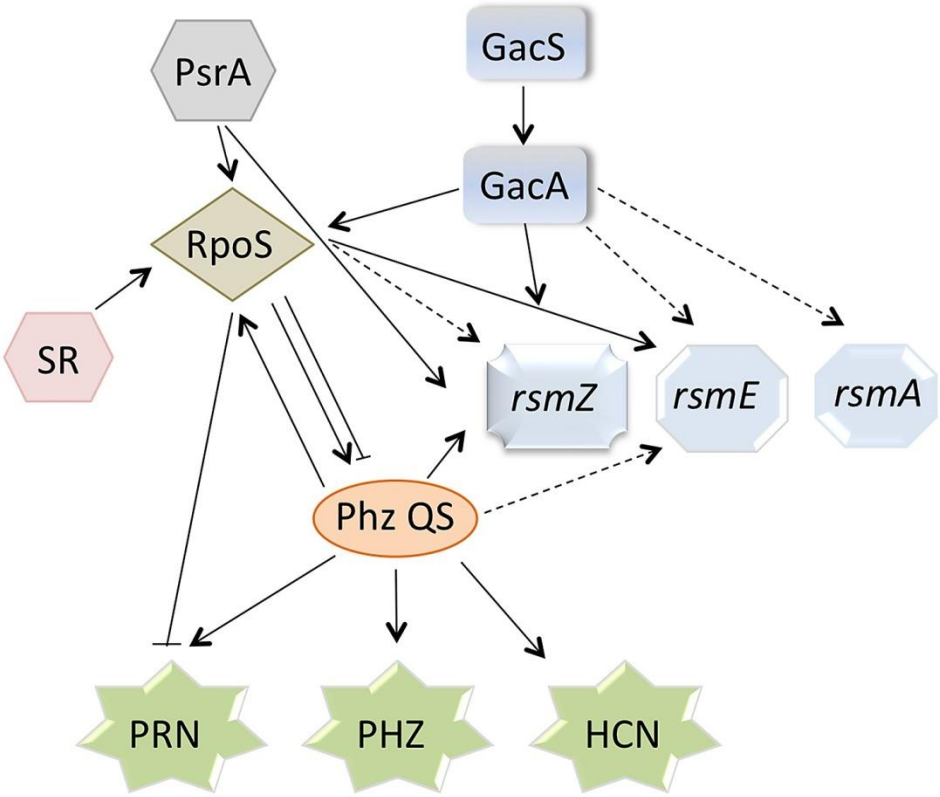


Figure 4.1. Model for the regulatory network overseeing production of *Pseudomonas chlororaphis* PA23 antifungal factors. Evidence for the proposed pathway comes from previous studies (Poritsanos *et al*., 2006; Selin *et al*., 2012; Selin *et al*., 2014; Manuel *et al*., 2012). In response to an unknown signal, the sensor kinase GacS undergoes autophosphorylation and phosphotransfer to the response regulator GacA. Activated GacA induces expression of the non-coding RNA RsmZ, the post transcriptional repressors RsmA and RsmE, and the sigma factor RpoS. RpoS is under positive control of PsrA and the SR. RpoS activates expression of *phzI*, but represses *phzR* and the pyrrolnitrin biosynthetic genes. The Phz QS system positively regulates *rpoS* as well as the phenazine, pyrrolnitrin and HCN biosynthetic loci. Symbols: ↓, positive effect; ⊥, negative effect; solid lines, direct effect; broken lines, indirect effect.

sensing (QS) system (Selin *et al*., 2014; Selin *et al*., 2012). Finally, a global stress response known as the stringent response negatively regulates PRN and therefore antifungal activity, primarily through RpoS (Manuel *et al*., 2012).

At present, the impact of PA23 metabolites on the survival and chemotactic behavior of *C. elegans* is unknown. The aim of the current study, therefore, was to determine whether PA23 is able to resist grazing by *C. elegans* and to define the role played by PA23 exoproducts in the bacterial-nematode interaction. We discovered that PRN and HCN have repellent and nematicidal activity against *C. elegans*. Moreover, co-culturing with *C. elegans* leads to altered expression of biocontrol genes and toxic metabolites, suggesting that PA23 is able to detect the presence of this predator and adjust its physiology accordingly.

4.2. Results

4.2.1. Cyanide is the primary metabolite responsible for rapid killing of *C. elegans* by PA23

Pseudomonads can cause *C. elegans* lethality via two non-mutually exclusive mechanisms known as fast and slow killing. We were interested to learn whether PA23 exhibited lethality to *C. elegans* through one or both of these means. In fast-kill assays, no toxicity was observed with either the *hcn* or *gacS* mutant (Figure 4.2). The *hcn* mutant does not produce HCN, while the *gacS* mutant is completely devoid of toxic metabolites including PRN, PHZ and HCN (Poritsanos *et al*., 2006). The QS-deficient strains, PA23*phzR* and PA23 (pME6863), exhibited intermediate nematicidal activity with only 50% of the nematodes viable at 9 h. We have previously reported that these strains produce markedly reduced PHZ and PRN (Selin *et al*., 2012). When these strains were analyzed for HCN production in the current study, only a low amount of this compound was produced (Figure 4.3). These findings are supported by *hcnA-lacZ*

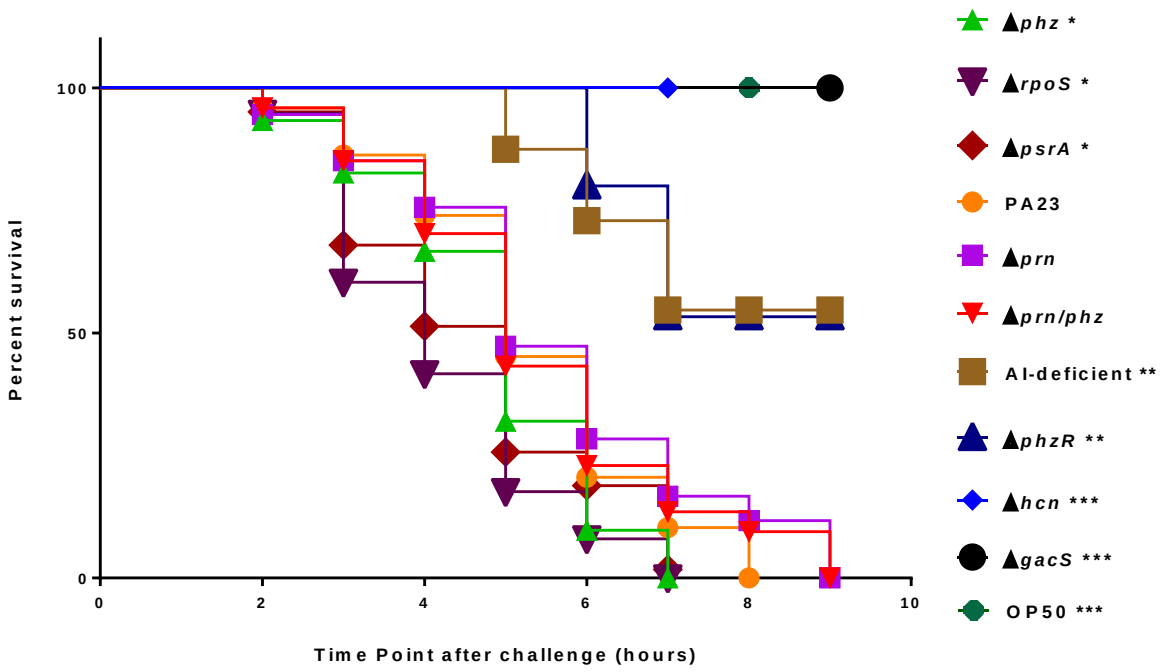


Figure 4.2. Fast-killing of *C. elegans* by *P. chlororaphis* PA23. Kaplan-Meier survival plots of *C. elegans* N2 (*n*=25) fed *E. coli* OP50, PA23 or derivative strains propagated on BHI agar. Nematode viability was monitored every hour for 9 h. Each data point represents the average of three biological replicates. Experiments were performed three times; one representative data set is shown. Asterisks indicate significant difference from the wild type as determined by the log-rank test (*, P<0.05; **, P<0.01; ***, P<0.001).

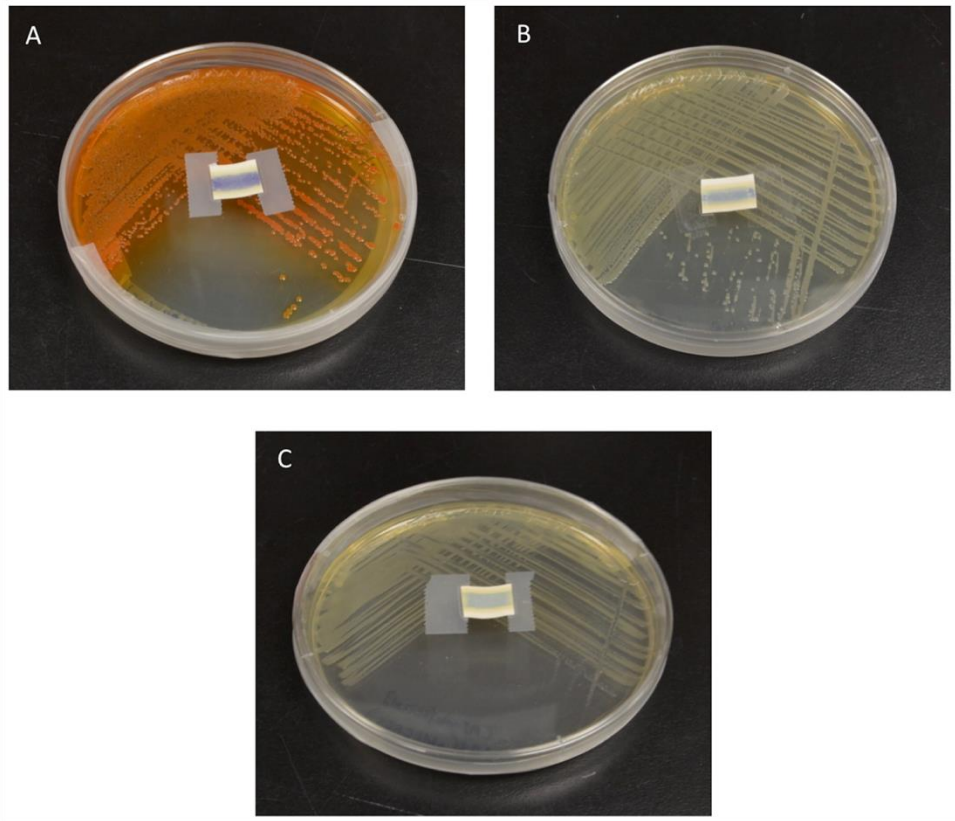


Figure 4.3. HCN is under quorum sensing control in *P. chlororaphis* PA23. HCN production by the PA23 wild type (panel A), *phzR* mutant (panel B) and AI-deficient strain (panel C) was assessed using cyantesmo paper, which turns blue in the presence of HCN. Note the reduced HCN production by the two quorum-sensing deficient strains compared to the wild type.

analysis where expression levels were five-fold lower in the *phzR* mutant (856.4 ± 57.6) compared to the wild type (4967.4 ± 437.9). The residual HCN expression likely accounts for the increased nematicidal activity associated with the QS-deficient strains compared to the *hcn* mutant. There was no difference in the rate of killing of the *prn* knock out mutants PA23-8 (Δ*prn*) and PA23-63-1 (Δ*prn/phz*) compared to the wild type (Figure 4.2). Interestingly, the highest rate of mortality was observed when nematodes were fed the *phz, rpoS* and *psrA* mutants, with 100% mortality occurring at 7 h (Figure 4.2). All three strains have been found to produce elevated PRN (Selin *et al*., 2010; Selin *et al*., 2014; Manuel *et al*., 2012). Collectively these findings indicate that HCN is the primary metabolite responsible for fast killing of *C. elegans* by PA23 grown on BHI media; however, at levels over and above that of wild type, PRN increases the rate of killing.

4.2.2. Slow killing assays

In contrast to fast killing, slow killing is brought about by culturing bacteria on low-nutrient media which doesn't support production of high levels of toxic compounds. Under these conditions, death or disease is mediated by bacterial colonization of the *C. elegans* gut. As illustrated in Figure 4.4, when we assayed PA23 and derivative strains for their slow-killing effects, the highest degree of lethality was observed when nematodes were grown on PA23-63, which produces 2.2 times as much PRN as the wild type (Selin *et al*., 2010). For the *rpoS* and *psrA* mutants which produce approximately 1.5 times as much PRN as PA23 (Selin *et al*., 2014; Manuel *et al*., 2012), there was no significant difference in killing. The *prn* mutants PA23-63-1 (Δ*prn/phz*) and PA23-1 (Δ*prn*), and the *hcn* mutant showed reduced lethality, resulting in 100% mortality at 88, 96 and 120 h, respectively (Figure 4.4). Viable nematodes were observed at 160

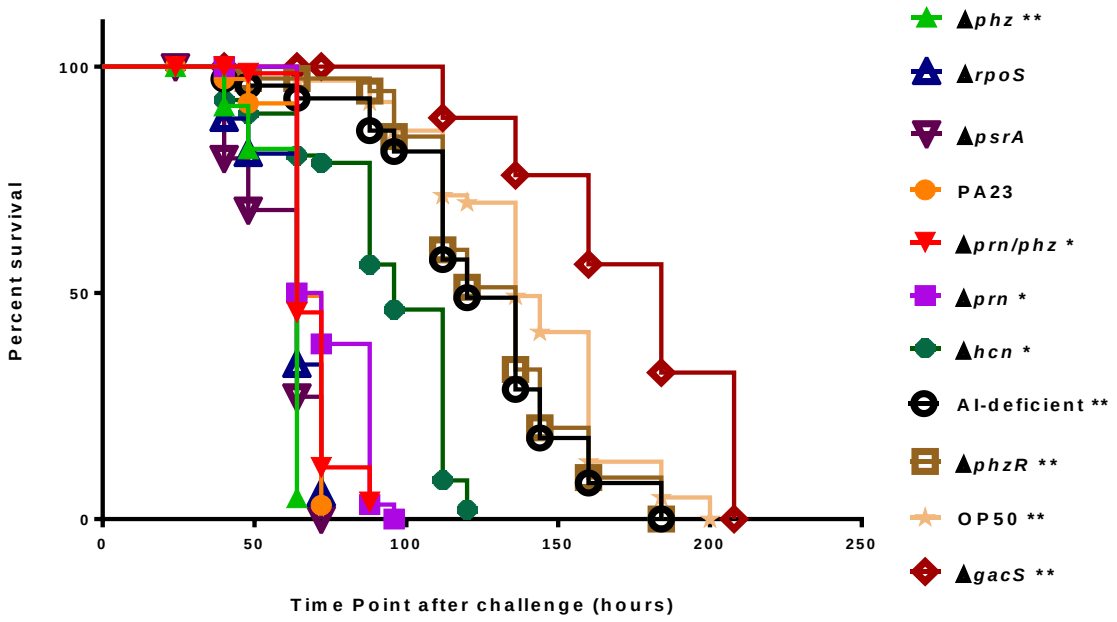


Figure 4.4. Slow-killing of *C. elegans* by *P. chlororaphis* PA23. Kaplan-Meier survival plots of *C. elegans* N2 (*n*=25) fed *E. coli* OP50, PA23 or derivative strains propagated on NGM agar. Nematodes were assessed for viability over a ten-day period. Each data point represents the average of three biological replicates. Experiments were performed three times; one representative data set is shown. Asterisks indicate significant difference from the wild type as determined by the log-rank test (*, P<0.001; **, P<0.0001).

h when growing on the QS-deficient strains and even longer (184 h) on the *gacS* mutant (Figure 4.4).

To better understand how PA23 affects nematode physiology, microscopic analysis of *C. elegans* propagated on bacteria labeled with the fluorescent reporter mCherry was undertaken. Initially, L4-stage nematodes were assessed for growth and fertility defects under low magnification. When cultured on the *phz* mutant, growth was dramatically inhibited with 63.2% of the nematodes arrested at the L4 stage compared to 45.2% for the wild type (Table 4.1). More modest growth delays were observed when propagated on the Δ*prn* (25.5%), Δ*prn/phz* (26.5%) and Δ*hcn* (22.3%) strains (Table 4.1). On the *rpoS* and *psrA* mutants, which produce elevated PRN, growth inhibition was 58% and 55%, respectively. No growth aberrations were noted when either the QS-deficient strains or the *gacS* mutant were provided as the food source (Table 4.1). *C. elegans* fertility defects were monitored by assessing the quantity of eggs produced as well as the hatching frequency. We discovered that growth on bacteria producing PRN, PHZ, or HCN, characterized by the wild type and the *phz, prn, prn/phz, hcn, rpoS* and *psrA* mutants, lead to a 4- to 5-fold reduction in the number of eggs laid compared to growth on *E. coli* OP50 (Table 4.1). On the *gacS* mutant and QS-deficient strains, which do not produce these compounds, high numbers of eggs were produced. Collectively, these findings indicate that PA23 exoproducts reduce *C. elegans* egg production. With respect to egg hatch frequency, only bacteria overproducing PRN (Δ*phz*, Δ*rpoS* and Δ*psrA*) showed less than 100% hatch (Table 4.1).

4.2.3. The effect of purified pyrrolnitrin on *C. elegans* viability and egg hatch frequency

To further establish a role for PRN in *C. elegans* lethality, we exposed L4-stage nematodes to purified PRN at concentrations ranging from 0.1 to 10 μg/mL consistent with PRN

Table 4.1. Behaviour and pathological symptoms of *C. elegans* (N2) on lawns of PA23 and derivative strains at 48 h on NGM

Strains	Growth defects[1] (%)	Number of eggs laid[2]	Egg hatch (%)[2]	Colonization[3]	Swollen tail (%)[3]	Enlarged excretory canals (%)[3]	Disintegrated gonads (%)[3]
PA23	45.2 (5.0)	260 (20)	100	extensive[a]	28.3 (1.5)	39.7 (2.5)	22.8 (1.6)
Δ*phz*	63.2 (10)	210 (30)	69.1 (7.0)	extensive[a]	47.7 (2.5)	39.8 (2.3)	29.8 (1.3)
Δ*pm*	25.4 (5.0)	275 (25)	100	mild[b]	0	0	20.7 (2.7)
Δ*pm*/*phz*	26.5 (5.0)	278 (20)	100	mild[b]	0	0	17.7 (3.3)
Δ*hcn*	22.3 (2.5)	328 (30)	100	mild[b]	12.6 (2.7)	21.3 (2.9)	0
AI-deficient	none	953 (50)	100	none	0	0	0
Δ*phzR*	none	1003 (45)	100	none	0	0	0
Δ*rpoS*	58.0 (4.5)	221 (20)	77.4 (4.5)	extensive[a]	41.1 (4.0)	30.4 (3.8)	26.8 (3.3)
Δ*psrA*	55.1 (5.0)	225 (25)	84.4 (4.4)	extensive[a]	44.7 (4.6)	29.8 (3.3)	21.6 (4.5)
Δ*gacS*	none	1160 (92)	100	none	0	0	0
E. coli OP50	none	1307 (101)	100	none	0	0	0

[1]Growth defects include growth arrest of the original L4-stage subjects or the L1/L2-stage progeny; mean (SD) obtained from a triplicate set.

[2]Mean (SD) obtained from three replicates examining 5 adult hermaphrodites for each.

[3]Thirty nematodes were examined in each trial; mean (SD) obtained from three trials.

[a]extensive—colonization throughout the entire gut.

[b]mild—colonization in only specific areas of gut, either upper or lower intestine.

doi:10.1371/journal.pone.0123184.t001

levels produced by PA23. As outlined in Figure 4.5.A, in the presence of 0.1 μg/mL PRN, all of the nematodes were dead by 48 h versus 72 h for the control. The percent survival continued to decrease in a dose-dependent fashion underscoring the nematicidal effects of PRN on *C. elegans*. In terms of egg hatching, exposure to lower concentrations of PRN (0.1 and 0.5 μg/mL) extended the time required for the eggs to hatch. Whereas exposure to higher PRN concentrations (1.0 – 10.0 μg/ml) reduced hatching to less than 50% after 24 h exposure (Figure 4.5.B); this remained unchanged at 48 h.

4.2.4. Microscopic analysis of *C. elegans* feeding on PA23

Next, we employed scanning confocal laser microscopy to reveal the extent of colonization and pathological effects in the nematodes (Figure 4.6). Pathological indicators included the presence of a swollen tail (Figures 4.6.E and 4.6.F), enlarged excretory canals (Figures 4.6.H and 4.6.I), and disintegrated gonads (Figure 4.6.J). After 72 h, the wild type and the Δ*phz*, Δ*rpoS* and Δ*psrA* mutants all showed extensive colonization of the nematode gastrointestinal tract (Table 4.1). The highest incidence of swollen tails (50%), enlarged excretory canals (40%), and disintegrated gonads (30%) was found in *C. elegans* colonized with the *phz* mutant. These traits were observed to a lesser degree in nematodes colonized by the Δ*rpoS* and Δ*psrA* strains (Table 4.1). Reduced colonization by the *prn* knock out strains was accompanied by the absence of swollen tails and enlarged canals; however, disintegrated gonads were observed in 20% (Δ*prn*) and 17% (Δ*prn/phz*) of the nematodes (Table 4.1). Collectively, these findings indicate that PRN production facilitates PA23 colonization and leads to increased pathology in *C. elegans*. The Δ*hcn* strain, which also demonstrated limited colonization, induced swollen tails (10%) and enlarged excretory canals (20%) but did not impact the gonads. At 144

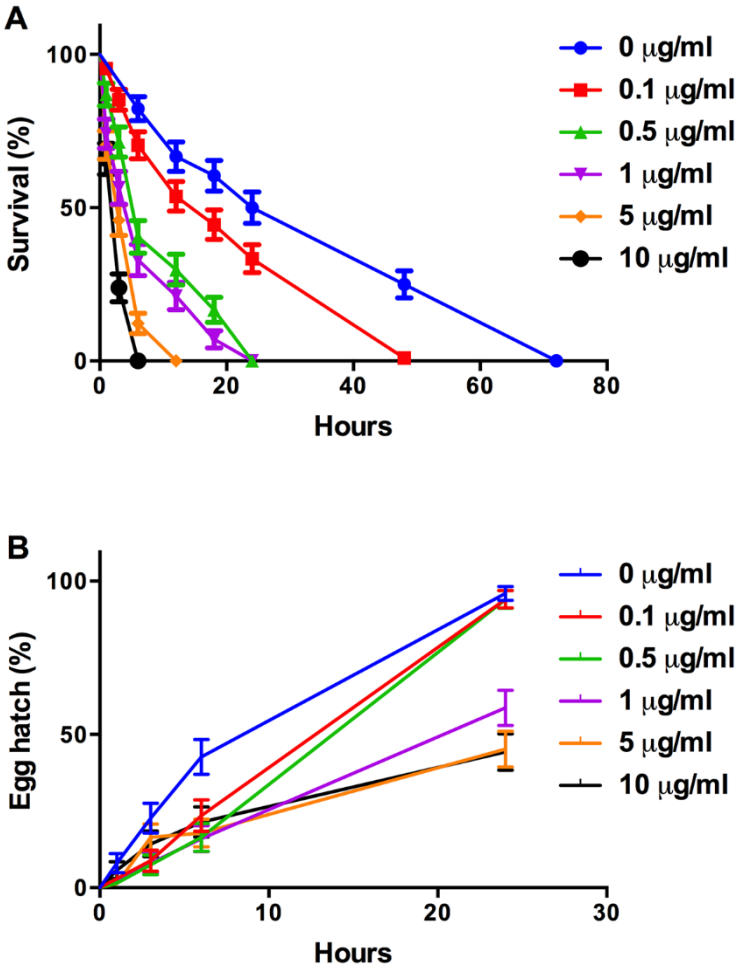


Figure 4.5. Pyrrolnitrin reduces *C. elegans* viability and egg hatching. *C. elegans* L4-stage nematodes and eggs were incubated in microtitre dishes containing purified PRN at the following concentrations: 0 µg/mL (water control), 0.1, 0.5, 1.0, 5.0 and 10 µg/mL. (A) Nematode viability was assessed at 1, 3, 6, 12, 18, 24, 48 and 72 h; (B) percent egg hatch was determined at 1, 3, 6, 24 and 48 h. Five replicate wells were used per trial and the assays were repeated three times. One representative data set is shown.

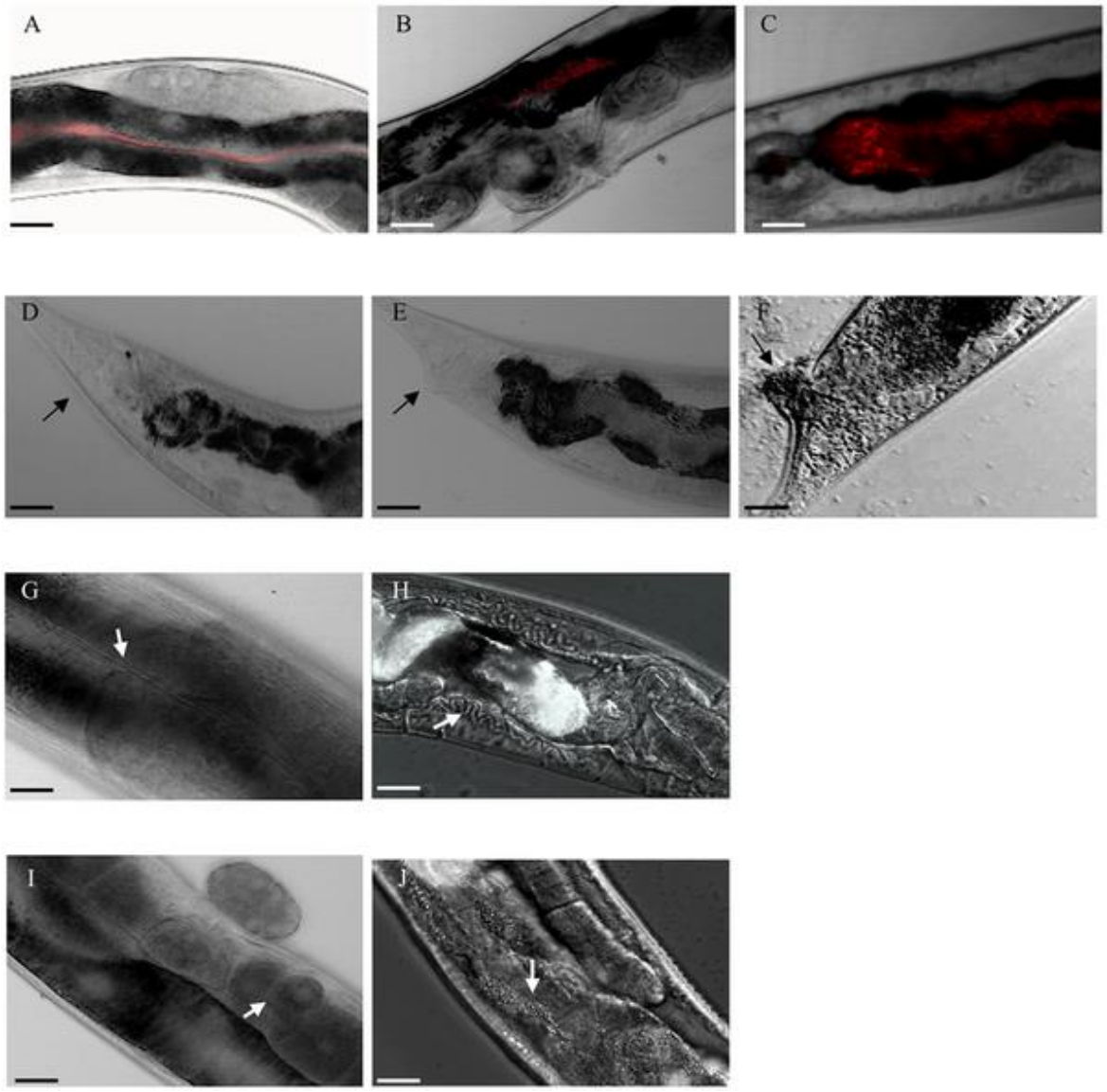


Figure 4.6. Microscopic analysis of *C. elegans* propagated on *P. chlororaphis* PA23. *C. elegans* was grown on PA23 and its derivatives harboring the mCherry red fluorescent protein (RFP) gene on pMCh-23. Nematodes were found to exhibit assimilation of bacteria but no colonization (A), mild colonization (B), or extensive colonization (C) depending on the bacterial strain tested. *C. elegans* was examined for the following pathological indicators: swollen tail; enlarged excretory canals; disintegrated gonads. Panels D, E and F reveal normal, mildly swollen and extensively swollen tails, respectively (black arrows). Panels G and H depict healthy and enlarged excretory canals, respectively (white arrows). Panels I and J show healthy and disintegrated gonads, respectively (white arrows). Images shown in Panels A – E, G and I were taken using bright-field microscopy; those in Panels F, H and J were taken using differential interference contrast microscopy. Scale bar represents 25 μM.

h, there were no surviving nematodes on lawns of the wild type or the *phz*, *rpoS* and *psrA* mutants, further establishing the impact of elevated PRN production on nematode lethality. At this point, colonization by the *prn* and *prn/phz* mutants became more extensive with some of the nematodes exhibiting swollen tails and disintegrated gonads (Table 4.2). The *hcn* mutant showed no changes in colonization or pathology compared to what was observed at 48 h, while the QS-deficient strains began to colonize but they exhibited no adverse effects on the nematode tissues (Table 4.2). It was only after 88 h that the *gacS* mutant showed limited colonization with no accompanying pathological changes.

4.2.5. Binary choice assays

Bacterial exoproducts can act as either attractants or repellants which in turn impacts nematode grazing. To understand how secondary metabolites produced by PA23 affect chemotactic traits of *C. elegans*, binary choice assays were performed. Nematodes were able to choose between colonies of the control strain, either the PA23 wild type (Figures 4.7.A and 4.7.B) or the *gacS* mutant (Figures 4.7.C and 4.7.D), and the test strain. In choice assays employing PA23 as the control, PRN was found to have a powerful repellent effect. As outlined in Figure 4.7.A, nematodes preferred the *prn* mutants (Δ*prn* and Δ*prn/phz*) over with wild type; while all the PRN overproducing strains (Δ*phz*, Δ*rpoS* and Δ*psrA*) exhibited repulsive properties. HCN was also found to repel the nematodes, with the *hcn* mutant being preferred over the wild type. The QS-deficient strains and *gacS* mutant, which all produce little to no PRN, HCN and PHZ, were highly attractive to the animals (Figure 4.7.A). As HCN is a volatile compound, we were interested to learn whether removing the lids from the petri plates would impact chemotaxis. While the overall pattern of chemotaxis remained the same, a few differences

Table 4.2. Behaviour and pathological symptoms of *C. elegans* (N2) on lawns of PA23 and derivative strains at 88 h on NGM

Strains	Time of 100% Mortality (h)	Colonization	Swollen tail (%)[1]	Enlarged excretory canals (%)[1]	Disintegrated gonads (%)[1]
PA23[a]	72	-	-	-	-
Δ*phz*[a]	64	-	-	-	-
Δ*rpoS*[a]	72	-	-	-	-
Δ*psrA*[a]	72	-	-	-	-
Δ*prn*	96	extensive[b]	36.8 (2.9)	0	30.5 (3.7)
Δ*prn*/*phz*	96	extensive[b]	29.9 (4.2)	0	17.8 (2.3)
Δ*hcn*	120	mild[c]	11.0 (4.0)	20.0 (3.3)	0
AI-deficient	184	mild[c]	0	0	0
Δ*phzR*	184	mild[c]	0	0	0
Δ*gacS*	208	none	0	0	0
E. coli OP50	200	none	0	0	0

[1]Twenty nematodes were examined for each strain; mean (SD) obtained from three trials.

[a] viable nematodes were not present at 88h; consequently, colonization and pathology was not determined.

[b]extensive—colonization throughout the entire gut.

[c]mild—colonization in only specific areas of gut, either upper or lower intestine.

doi:10.1371/journal.pone.0123184.t002

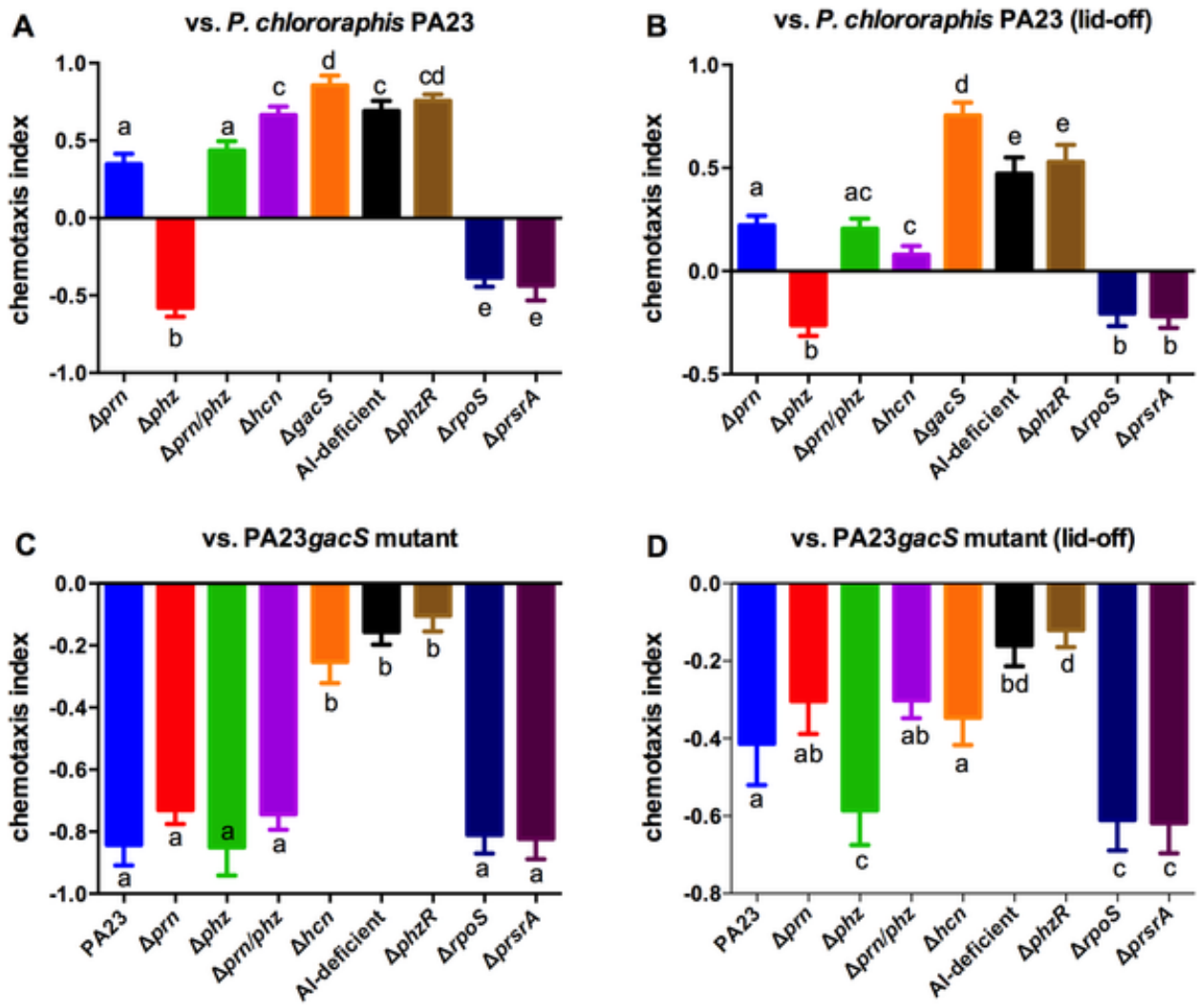


Figure 4.7. Pyrrolnitrin and hydrogen cyanide are repellents for *C. elegans*. Nematode preference was assessed against the wild-type strain PA23 (A) and the non-toxic strain Δ*gacS* (B). Chemotaxis was monitored by inoculating an NGM plate with the two bacterial strains to be tested. After 24 h, nematodes (50 -100) nematodes were spotted on the centre of each plate. The number of nematodes at each bacterial colony was counted after 24h. The chemotaxis index was calculated as (number on spot 2 – number on spot 1)/total number of nematodes at both spots. Assays were performed with the petri plate lids on (A,C) and off (B,D). Error bars indicate ± standard error; letters represent statistical groupings of means compared to the same reference strain (95% confidence, Bonferroni test).

emerged. First, the nematodes were less attracted to the Δ*hcn* strain, which is not surprising since the bulk of the volatile HCN produced by PA23 would presumably have escaped. Second, for most of the test strains, the scale of the chemotaxis index was decreased indicating that the attractive/repulsive forces were minimized through volatile release. When the same studies were performed using the *gacS* mutant as the control, in all instances this bacterium was preferred over the test strain (Figures 4.7.C and 4.7.D). No differences were observed between the Δ*prn* mutant, the wild type and the PRN overproducing strains; however, the AI-deficient, the Δ*phzR* and the Δ*hcn* mutant were found to have a less repulsive effect (Figure 4.7.C). Removal of the petri plate lids lead to some interesting changes in the pattern of chemotaxis (Figure 4.7.D). Now the PRN overproducers had the greatest repulsive effect, while the *hcn* mutant exhibited similar repulsion to the wild type. Thus it appears that both PRN and HCN act as repellents for *C. elegans* with the impact of the latter being mitigated in situations where the gases are not contained.

4.2.6. Growth in the presence of *C. elegans* affects PA23 gene expression

To investigate whether chemical cues from *C. elegans* are perceived by PA23, we monitored the influence of *C. elegans* co-culturing on bacterial gene expression. Both biosynthetic (*prnA, phzA, hcnA*) and regulatory genes (*phzI, phzR, rpoS, psrA, gacS, gacA*) were analyzed. As outlined in Figure 4.8, the presence of *C. elegans* lead to increased *phzA* gene expression and the magnitude of difference increased over time. For *prnA*, a different pattern of expression was observed. Initially at 24 h, *prnA* transcription was lower in *C. elegans* co-cultured bacteria but by 72 h, gene activity was significantly higher than in cultures containing bacteria alone (Figure 4.8). For *hcnA*, expression was consistently elevated in the presence of the

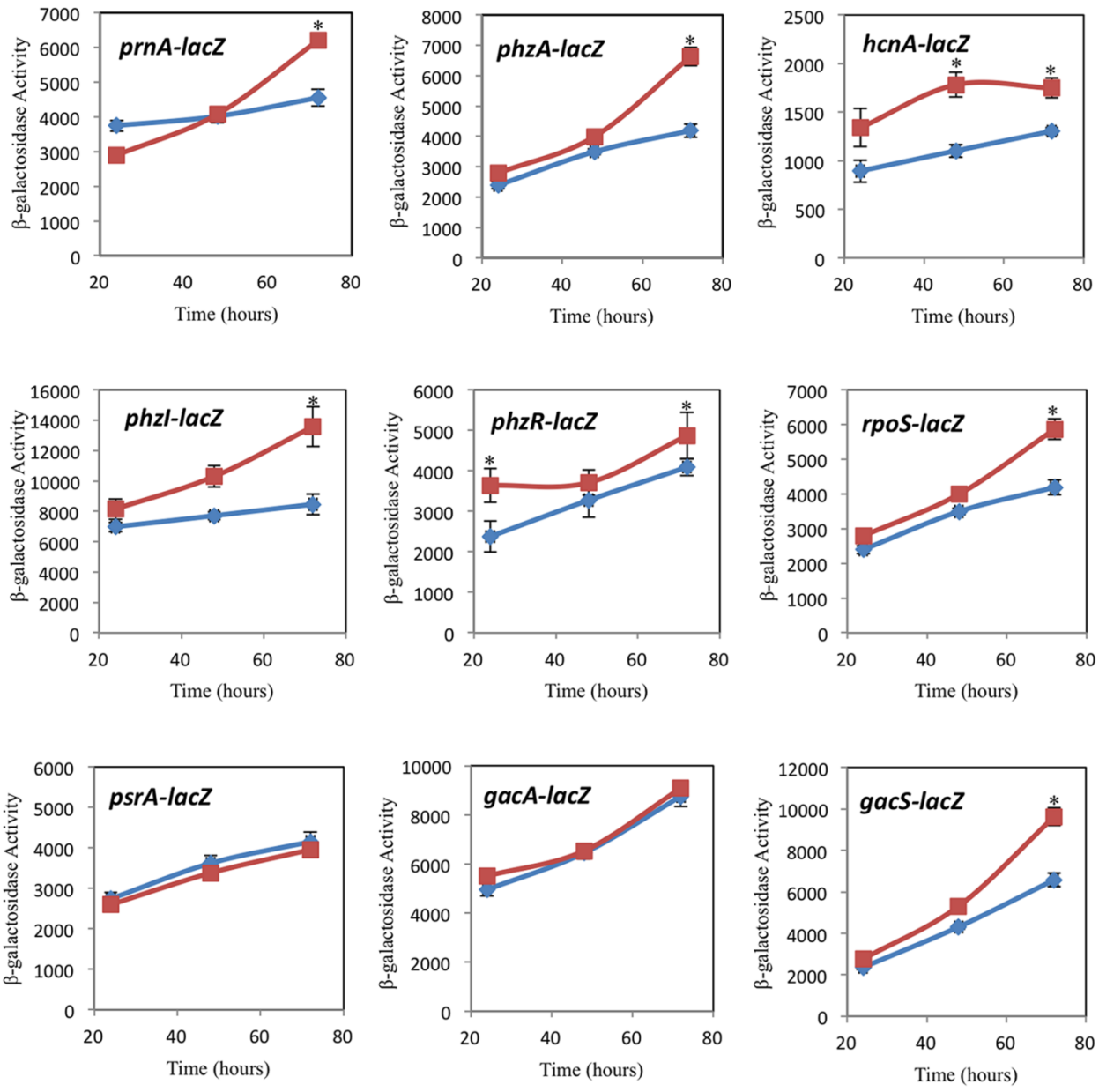


Figure 4.8. Co-culturing with *C. elegans* impacts *P. chlororaphis* PA23 gene expression. PA23 cultures harboring *prnA-lacZ*, *phzA-lacZ*, *hcnA-lacZ*, *phzI-lacZ*, *phzR-lacZ*, *rpoS-lacZ*, *psrA-lacZ*, *gacA-lacZ*, and *gacS-lacZ* fusions were grown in the presence (red squares) and absence (blue diamonds) of the nematodes. Cells were assayed for ß-galactosidase activity at 24, 48 and 72h. Each value represents the mean from three biological replicates ± standard error. Data points marked with an asterisk (*) are statistically significant. Experiments were performed three times; one representative data set is shown.

nematodes. We next examined key regulatory genes involved in PA23 biocontrol, including the QS genes *phzI* and *phzR*. We observed increasing levels of *phzI* activity in the presence of *C. elegans*, with statistically significant differences observed at 48 and 72 h; whereas *phzR* showed elevated gene expression at 24 and 72h (Figure 4.8). Increased expression of *rpoS* was found at 72 h; whereas *psrA*, which encodes an activator of *rpoS*, remained unchanged in the presence of *C. elegans* (Figure 4.8). A similar pattern was observed for *gacS* and *gacA* with the former showing elevated expression at 48 and 72 h in the presence of the nematodes while *gacA* exhibited no change in gene activity (Figure 4.8).

4.2.7. Analysis of the impact of nematode co-culture on PA23 phenotypic traits

Co-culturing with *C. elegans* lead to elevated *phzA* and *prnA* gene expression in PA23, suggesting that antibiotic production might be upregulated in a similar fashion. As outlined in Table 4.3, growth in the presence of the nematodes lead to increased levels of both PHZ and PRN; however, only the former showed a significant rise. *phzI* encodes an AHL synthase responsible for synthesizing AHL molecules. Employing a bioreporter assay, we discovered that PA23 cultures grown with *C. elegans* produce elevated levels of the QS signalling molecules (Table 4.3). Thus, our end-product analysis mirrored what was observed for *phzA*-, *prnA*- and *phzI-lacZ* expression. Other phenotypic traits including fungal inhibition, protease activity and swimming motility were unaffected by the nematodes (Table 4.3).

4.3. Discussion

The ability to avoid predation either through repulsive forces or reducing predator abundance is expected to improve the success of a biocontrol agent by increasing environmental

Table 4.3. Phenotypic characterization of *P. chlororaphis* PA23 grown in the presence and absence of *C. elegans*.

Organism	PRN[1] (µg/ml)	PHZ[1] (µg/ml)	AHL[1] (Miller units)	Antifungal[2] (mm)	Protease[2] (mm)	Motility[2] (mm)
PA23	3.9 (0.5)	27.7 (0.45)	1296.7 (63.4)	5.76 (0.18)	5.4 (0.5)	71.4 (2.3)
PA23 + *C.*	5.1 (0.5)	33.9 (1.16)[a]	1932.9 (81.6)[a]	5.9 (0.16)[a]	5.5 (0.7)	75.6 (1.5)
elegans						

[1]Mean (SD) from three replicates.

[2]Mean (SD) of zones of activity (mm) obtained from six replicates.

[a]Significantly different from the wild type (P<0.05).

doi:10.1371/journal.pone.0123184.t003

persistence. The focus of the current study was to investigate the interaction between biocontrol strain PA23 and the bacterivorous nematode *C. elegans*; in particular, we were interested to learn whether PA23 demonstrates nematicidal and or repellent activities.

On nutrient-rich BHI media, which supports rapid growth and production of high levels of secondary metabolites, strains deficient in HCN expression (*hcn* and *gacS* mutants) were unable to induce rapid death of *C. elegans* (Figure 4.2). The highest degree of nematicidal activity was observed for the PRN overproducing strains (Figure 4.2). Collectively, these findings indicate that HCN is the primary compound involved in *C. elegans* intoxication (Figure 4.2); however, at elevated levels, PRN also exhibits toxic effects. In slow-killing assays, which depend upon infection of the *C. elegans* intestine, a deficiency in either HCN or PRN production lead to decreased killing. While HCN is well established as inducing lethal paralysis in *C. elegans* (Tan *et al*., 1999), to the best of our knowledge this is the first study to report nematicidal activity associated with PRN. To further confirm its toxic effects, L4-stage adults were incubated in the presence of increasing concentrations of purified PRN. We observed that PRN exposure lead to reduced viability in a dose-dependent manner (Figure 4.5.A). In a study by Meyer and colleagues (2009), purified 2,4-diacylphloroglucinol (DAPG) exhibited toxic effects towards adults of the plant-parasitic nematode *Xiphinema americanum* but did not affect *C. elegans* J1 or adult-stage nematodes. Interestingly, 1- and 3-h incubation with DAPG actually stimulated *C. elegans* egg hatch (Meyer *et al*., 2009). In the current study, we observed a reduction in the frequency of egg hatching upon *C. elegans* propagation on the PRN-overproducing strains (Table 4.1) as well as when eggs were incubated in a PRN solution (Figure 4.5.B). At lower concentrations, egg hatching was merely delayed; however at higher, physiologically relevant concentrations (1, 5 and 10 μg/ml), hatching decreased to less than 50%

of wild type after 24h exposure (Figure 4.5.B). Collectively, these findings indicate that PRN affects many aspects of *C. elegans* physiology, acting as a nematicide and repellent for adult nematodes and reducing egg hatching.

Although PA23 produces two PHZ compounds, namely PCA and 2-OH-PHZ, they do not appear to be important for nematicidal activity. Under fast- and slow-killing conditions, there was no difference in lethality between the *prn* mutant and the *prn/phz* double mutant (Figures 4.2 and 4.4). If PHZ was contributing in some way to overall lethality, we would expect a reduction in mortality associated with the *phz* mutants, both of which are devoid of PHZ production (Selin *et al*., 2010). The fact that the single *phz* mutant exhibits the highest rate of killing in both the fast- and slow-killing assays supports the notion that PHZs are not involved in these two processes. Similar findings were reported for *P. aeruginosa* strain PAO1, wherein PHZs did not impact fast-killing by bacteria propagated on BHI media (Gallagher and Manoil, 2001). For *P. aeruginosa* PA14 grown on PGS agar, PCA was reportedly the primary compound underlying intoxication, with increased toxicity observed at lower pH ranges (Cezairliyan *et al*., 2013). Differences in media (PGS versus BHI) could account for the discrepancy in findings between these studies. It is also important to note that strain PA14 produces nearly twice as much PCA (52.7 μg/mL; Cezairliyan *et al*., 2013) as PA23 (28.5 μg/mL; Selin *et al*., 2010) which may have contributed to the observed differences as well. The notion that antibiotic concentration significantly impacts *C. elegans* viability is supported by our fast-killing assays, wherein the PRN overproducing strains exhibited elevated toxicity while the *prn*-null mutants were unaffected compared to PA23 (Figure 4.2).

The ability to avoid grazing-predator interactions all together would presumably benefit biocontrol bacteria to a greater extent than nematicidal activity. When we analyzed PA23

repellence of *C. elegans*, both HCN and PRN were able to act as powerful repellents (Figure 4.7). In a study by Burlinson *et al*. (2013), screening of a *Pseudomonas fluorescens* NZ17 transposon library revealed several genetic loci associated with *C. elegans* repellence. Among these were *gacS* and a newly-identified cluster of genes named EDB, for edible. While the EDB cluster was found to be under GacS control, the mechanism underlying EDB-mediated repellence is currently unknown (Burlinson *et al*., 2013). Analysis of the PA23 genome failed to reveal EDB homologues. Much like what was observed in the current study, the NZ17 *gacS* mutant exhibited the lowest repellence, while mutants deficient in the production of a single exoproduct (HCN, TOL, exoenzymes) retained some repellent activity (Burlinson *et al*., 2013).

Chemical signalling plays an important role in the interaction of an organism with its environment. Because bacterial exoproducts modulate the PA23-*C. elegans* interaction through their nematicidal and repellent effects, we were interested to learn whether the presence of *C. elegans* would elicit changes in PA23 gene activity. These changes could be mediated by either direct contact with the nematode or through perception of soluble chemical cues. Our analysis of regulatory genes revealed altered expression in some but not all cases. In terms of biosynthetic genes, co-culturing of the two organisms lead to increased *prnA*, *phzA* and *hcnA* gene expression at 72 h compared to growth in the absence of *C. elegans* (Figure 4.8). Exoproduct analysis showed that PHZ and AHL were significantly upregulated in the presence of *C. elegans*. PRN is the primary antibiotic responsible for PA23-mediated suppression of *S. sclerotiorum* (Selin *et al*., 2010); therefore, the unaltered change in antifungal activity upon co-culture with *C. elegans* was not surprising considering that PRN production was not significantly elevated. Jousset and coworkers (2010) reported similar findings wherein *P. fluorescens* CHA0 grown in the presence of cell-free supernatants of the amoeba *Acanthamoeba castellani* exhibited elevated *phlA*

(DAPG), *prnA* and *hcnA* gene expression and increased DAPG, PRN and HCN production. However, in direct contrast to our findings, these researchers observed that co-culturing with *A. castellani* decreased gene expression (Jousset *et al*., 2010). It was concluded that in response to soluble predator cues, CHA0 upregulates defense mechanisms; however, direct contact with bacteria enables the amoeba to repress bacterial toxicity (Jousset *et al*., 2010). In a second study, the cyclic lipopeptides massetolide and viscosin produced by *P. fluorescens* strains SS101 and SBW25, respectively, were found to protect bacteria from *Naegleria americana* protozoan grazing (Mazzola *et al*., 2009). When bacteria were either in direct contact with or in close proximity to *N. Americana,* increased *massABC* (massetolide) and *viscABC* (viscosinamide) expression were observed. Collectively these findings indicate that bacteria and bacterial-feeding eukaryotes are able to sense one another through soluble chemical cues and/or direct contact and this mutual perception modulates the predator-prey interaction. The idea of inter-kingdom signalling between bacteria and higher eukaryotes is not new. Much of the research to date has focused on quorum-sensing signals as the basis for this communication (Hughes and Sperandio, 2008). Recent findings suggest that the antibiotics serve multiple dose-dependent functions. At higher concentrations, they can inhibit or kill competing microbes, while at lower levels they act as intercellular signals capable of modulating bacterial gene expression (Hoffman *et al*., 2005; Linares *et al*., 2006). Our findings and those of others (Mazzola *et al*., 2009; Jousset *et al*., 2010) suggest that antibiotics may represent another "language" of communication between bacteria and eukaryotic organisms.

In summary, HCN and PRN are key compounds that affect the interaction of PA23 and *C. elegans*. HCN is well established as being toxic to *C. elegans*; however, our finding that PRN is a nematicidal agent is novel. Interestingly, PRN is the most important antibiotic for PA23-

mediated biocontrol of *S. sclerotiorum*. As environmental persistence is an essential feature of a successful biocontrol agent, PRN appears to play a role that extends beyond PA23-mediated pathogen suppression. Because synthesis of antifungal compounds is energetically costly, it makes sense for bacteria to limit production of these compounds to situations where they impart a fitness advantage. The presence of *C. elegans* leads to altered PA23 gene expression, indicating the bacteria are able to perceive soluble chemical cues and/or direct nematode contact, and this in turn modulates PA23 physiology. Studies are on-going to further define the mechanisms involved in sensing chemical signals in both *C. elegans* and PA23. Future work will seek to determine whether PA23 exhibits broad-spectrum nematicidal and repellent activities against a range of nematodes, including plant-pathogenic organisms, under both lab and field conditions.

4.4. Acknowledgments

I would like to thank Dr. Carrie Selin for her assistance with performing HPLC and creating pPSRA-*lacZ*. I would also like to thank Dr. Jerrylynn Manuel for assistance creating pGACS-*lacZ* and pGACA-*lacZ*.

Chapter 5

HCN production contributes to *Pseudomonas chlororaphis* strain PA23 biological control and is upregulated in the presence of ferric chloride and glycine

5.1. Introduction

For pseudomonads that are capable of antagonizing fungal pathogens, production of antimicrobial compounds is often the primary mode of pathogen suppression. Biocontrol bacteria secrete an arsenal of inhibitory compounds including degradative enzymes, siderophores and diffusible antibiotics (Haas and Keel, 2003). In addition, production of HCN has been found to contribute to fungal inhibition in a number of fluorescent pseudomonads (Voisard *et al*., 1989; Laville *et al*., 1998; Blumer and Haas, 2000; Michelsen and Stougaard, 2012).

HCN exerts its toxic effects through inhibiting cytochrome c oxidase, the final component of the aerobic respiratory chain, as well as other essential metalloenzymes (Knowles, 1976; Solomonson, 1981). In *Pseudomonas* spp., a membrane-bound HCN synthase is responsible for catalysis of glycine into HCN and CO_2 (Castric, 1977). A number of biotic and abiotic factors govern expression of the *hcnABC* operon, which encodes the HCN synthase (Pessi and Haas, 2000; Laville *et al*., 1998, Castric, 1983; Castric, 1994). In *P. fluorescens* and *P. aeruginosa*, for example, HCN expression is under control of ANR (anaerobic regulator of arginine deiminase and nitrate reductase), which is most active under low O_2 conditions (Laville *et al*., 1998; Zimmerman *et al*., 1991); as such, low O_2 concentrations favor cyanogenesis. ANR binds to a conserved sequence in the promoter of target genes, called the *anr* box, facilitating transcription initiation (Pessi and Haas, 2000). The Gac two-component signal transduction system, which was named in part because of its control over HCN synthesis (Global activator of antibiotic and cyanide synthesis), is another governing element (Lapouge *et al*., 2008). GacS and GacA work together with a second system, called Rsm, to modulate expression of secondary metabolites and extracellular enzymes during the transition from the logarithmic to stationary phase of growth (Heeb and Haas, 2001; Bejerano-Sagie and Xavier, 2007). In this manner, the

activity of the Gac-Rsm system is coordinated in a population-dependent fashion through production of unidentified signalling molecules (Lapouge *et al*., 2008). For pseudomonads that employ acyl-homoserine-lactone (AHL) based QS as part of their lifestyle, production of secondary metabolites, including HCN, is typically subject to QS control (Fuqua and Greenberg, 1998; Haas and Keel, 2003). Quorum sensing enables bacteria to coordinate gene expression according to population density through the production of small, diffusible AHL molecules. AHL levels increase concomitantly with cell number and at a threshold concentration they make a complex with the cognate LuxR transcriptional regulator. The LuxR-AHL complex then binds to a *lux* box sequence upstream of target genes (Fuqua and Greenberg, 1998). In *P. aeruginosa*, which utilizes two QS systems (Las and Rhl), cooperative binding of LasR, RhlR and ANR is required for maximal *hcnABC* transcription (Pessi and Haas, 2000). Intriguingly, for pseudomonads that do not have AHL-based QS systems, ANR and Gac are solely responsible for HCN expression, acting at the transcriptional and translational levels, respectively (Blumer *et al*., 1999; Blumer and Haas, 2000; Michelsen and Stougaard, 2012). Abiotic factors including oxygen and iron concentrations impact HCN production primarily through their effects on ANR (Laville *et al*., 1998; Blumer and Haas, 2000). Blumer and Haas (2000) proposed that the active form of ANR is a dimer containing two [4Fe-4S] clusters. In the presence of O_2, the [4Fe-4S] cluster is converted to [2Fe-2S] rendering ANR non-functional, similar to oxygen-mediated inactivation of FNR (Beinert and Kiley, 1999; Blumer and Haas, 2000). Not surprisingly, iron sufficiency favors [4Fe-4S] assembly and ultimately HCN production (Castric, 1975; Castric, 1983). Media composition also affects HCN production; for example glycine, the metabolic precursor for HCN biosynthesis, stimulates production of this volatile by *P. aeruginosa* (Castric, 1977). Propagation on nitrogen-rich media (King's B; Lysogeny broth) resulted in elevated HCN

levels compared to strains grown on the more carbohydrate-rich potato dextrose agar (Paulin *et al*., 2009; Michelsen and Stougaard, 2012).

P. chlororaphis strain PA23 is a biocontrol bacterium capable of protecting canola from Sclerotinia stem rot caused by *S. sclerotiorum* (Fernando *et al*., 2007; Savchuk & Fernando, 2004). A number of secondary metabolites are believed to contribute to PA23-mediated fungal suppression including PHZ, PRN, proteases, lipases and siderophores (Poritsanos *et al*., 2006; Zhang *et al*., 2006). Production of these compounds is tightly regulated at both the transcriptional and translation level by the Gac-Rsm system, PhzI/PhzR QS, the stationary phase sigma factor RpoS, a positive activator of *rpoS* transcription, called PsrA, and the stringent response (Poritsanos *et al*. 2006; Manuel *et al*. 2012; Selin *et al*. 2012). In addition to these other compounds, strain PA23 produces HCN; however, the role of HCN in fungal antagonism is currently unknown.

The aim of the current study was to generate an *hcn* mutant to elucidate whether this volatile compound contributes to PA23 fungal suppression. Moreover, we were interested in determining the impact of $FeCl_3$ and glycine addition on the production of HCN as well as other metabolites produced by PA23.

5.2. Results

5.2.1. HCN contributes to PA23 AF activity *in vitro*

To assess the role of HCN in PA23 fungal suppression, an *hcn* mutant was created. Radial diffusion assays on 1/5 PDA revealed a significant decrease in the zone of inhibition surrounding the *hcn* mutant compared to that of the wild type (Figure 5.1; Table 5.1). No

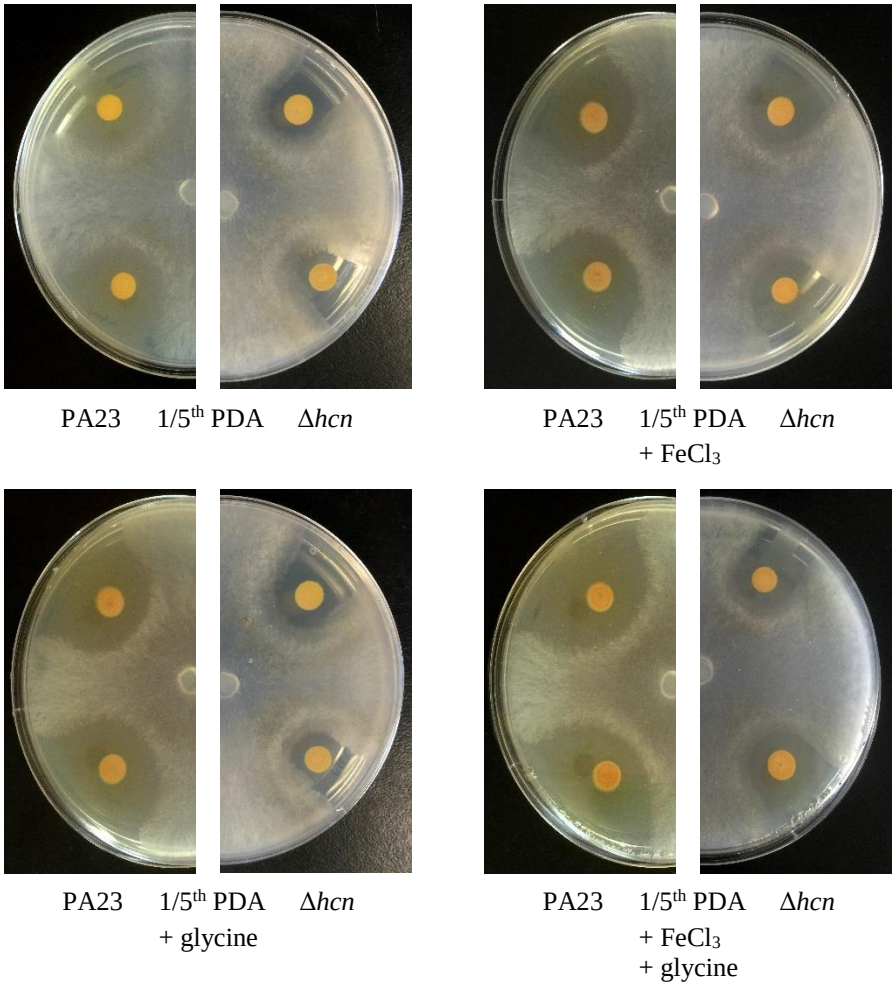


Figure 5.1. Antifungal activity of PA23 and Δ*hcn* strains against *Sclerotinia sclerotiorum* on $1/5^{th}$ PDA alone, supplemented with 100 µM $FeCl_3$ and 20 mM glycine separately or in combination.

Table 5.1. Results of antifungal radial diffusion assays of PA23 and Δ*hcn* strain against *Sclerotinia sclerotiorum* on 1/5th PDA alone, supplemented with 100 µM $FeCl_3$ and 20 mM glycine separately or in combination.

	Zone of clearing[a]			
Strains	**PDA**	**PDA + $FeCl_3$**	**PDA + glycine**	**PDA + $FeCl_3$ + glycine**
PA23	3.8 (0.3)	5.1 (0.6)[b]	5.3 (0.4)[b]	5.7 (0.7)[b]
Δ*hcn*	1.2 (0.4)[c]	2.4 (0.3)[c]	2.1 (0.4)[c]	2.5 (0.3)[c]

[a]Mean (SD) of zone of clearing between edge of bacterial colony and fungal growth front (mm) from ten replicates.
[b]Significantly different from wild type on 1/5th PDA (P<0.001).
[c]Significantly different from wild type grown on respective media (P<0.0001).

difference in AF activity was observed when strains were plated on full-strength PDA (data not shown).

5.2.2. Fungal antagonism is elevated in the presence of $FeCl_3$ and glycine

Previous studies have revealed that addition of $FeCl_3$ is able to elevate HCN production through its affect on ANR (Michelsen and Stougaard, 2012). Moreover as the metabolic precursor of the HCN synthase, glycine amendment leads to enhanced synthesis of this volatile (Castric, 1977). Thus, we were interested to determine the impact of these two compounds on PA23 AF activity. In the presence of $FeCl_3$ and glycine, alone or in combination, PA23-mediated fungal suppression was markedly increased (Figure 5.1, Table 5.1). Intriguingly, these same effects were observed with the *hcn* mutant (Figure 5.1, Table 5.1), indicating that these compounds could be affecting other aspects of PA23 physiology. One apparent difference we did notice was growth. As depicted in Figure 5.2, cultures grown in different media conditions entered the stationary phase around the same time (approximately 24 h). However, in the presence of $FeCl_3$, PA23 reached a 1.5-fold higher OD_{600} prior to entering into the stationary phase compared to cells grown in M9 alone or with glycine.

5.2.3. Glycine increases HCN production in PA23

To establish the impact of $FeCl_3$ and glycine on PA23 HCN levels, cyantesmo paper was used to qualitatively assess production of this volatile. As indicated in Figure 5.3a, elevated HCN production was observed when PA23 was cultured on glycine-supplemented media; whereas $FeCl_3$ had no effect. Analysis of an *hcnA-lacZ* transcriptional fusion revealed similar findings; gene expression was significantly elevated in the presence of glycine but not $FeCl_3$ (Figure 5.3b).

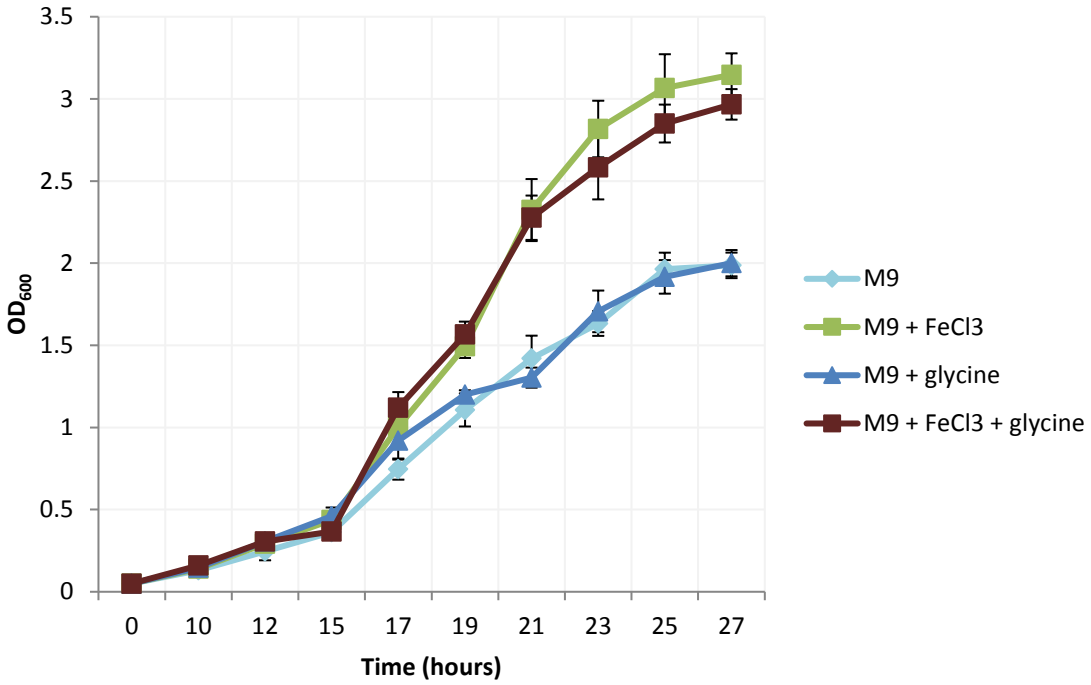
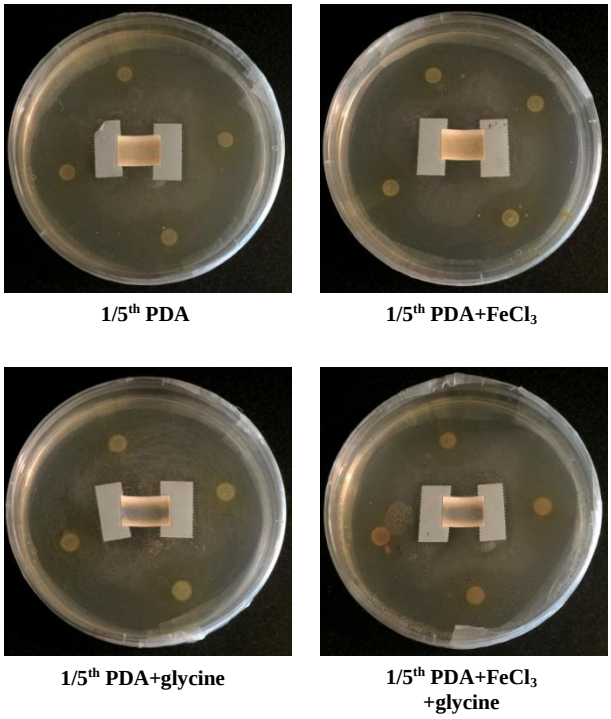


Figure 5.2. Growth curve analysis of the wild type PA23 grown in M9 minimal media (M9 + 0.2% glucose + 1 mM $MgSO_4$) alone, supplemented with 100 µM $FeCl_3$ and 20 mM glycine separately or in combination. Spectrophotometric values for optical densities (ODs) were taken at 600 nm. Each data point represents the mean from three biological replicates ± standard error.

a



b

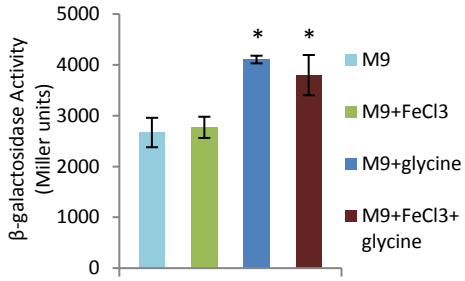


Figure 5.3. (a) Assessment of HCN production by PA23 wild type using cyantesmo paper. Cultures were spotted on 1/5th PDA alone, supplemented with 100 µM $FeCl_3$ and 20 mM glycine separately or in combination. Pictures were taken at 48 h. (b) PA23 culture harboring *hcnA-lacZ* fusion was grown in the presence and absence of media additions. Cells were assayed for β-galactosidase activity at 24 h. Each value represents the mean from three biological replicates ± standard error. Data points marked with an asterisk (*) are statistically significant ($P<0.05$).

The impact of glycine on *hcnA-lacZ* activity was somewhat surprising. As the metabolic precursor for the HCN synthase, glycine is expected to enhance HCN production but not at the level of transcription. These findings suggested that glycine might be indirectly affecting *hcnA-lacZ* expression through other transcriptional regulators, the most obvious being the PhzRI QS system. To address this possibility, we monitored *phzI* and *phzR* expression in PA23 grown in the presence and absence of $FeCl_3$ and glycine. Similar to what was observed for *hcnA-lacZ* activity, both genes were significantly upregulated when glycine was added to the media, whereas $FeCl_3$ had no effect on gene expression (Figure 5.4a). Qualitative analysis of AHL production using CVO26-seeded agar revealed significantly higher production by PA23 grown in the presence of glycine (Table 5.2). Similarly when we quantified the amount of AHL present in culture extracts using *P. aeruginosa* QSC105 (pEAL01), a strain capable of detecting a broad range of AHLs (Ling *et al*., 2009), 1.6-fold higher AHL levels were achieved through glycine amendment (Figure 5.4b). Therefore, our endproduct analysis supports glycine-mediated upregulation of *phzI* expression. Collectively, these findings suggest that the impact of glycine on PA23 HCN expression is mediated through QS.

5.2.4. QS control of HCN production and relationship to media amendments

Expression of an *hcnA-lacZ* transcriptional fusion was analyzed in the QS-deficient strains. The transcription of *hcnA* was markedly reduced in the Δ*phzR* and AI-deficient (PA23-6863) strains compared to the wild type under all media conditions, indicating that QS positively regulates *hcnA* expression (Figure 5.5). Moreover, increased *hcnA* expression in the QS mutants was observed in the presence of $FeCl_3$, although the expression was below the level of wild type. Glycine, on the other hand, was unable to upregulate *hcnA* expression in the QS mutants (Figure

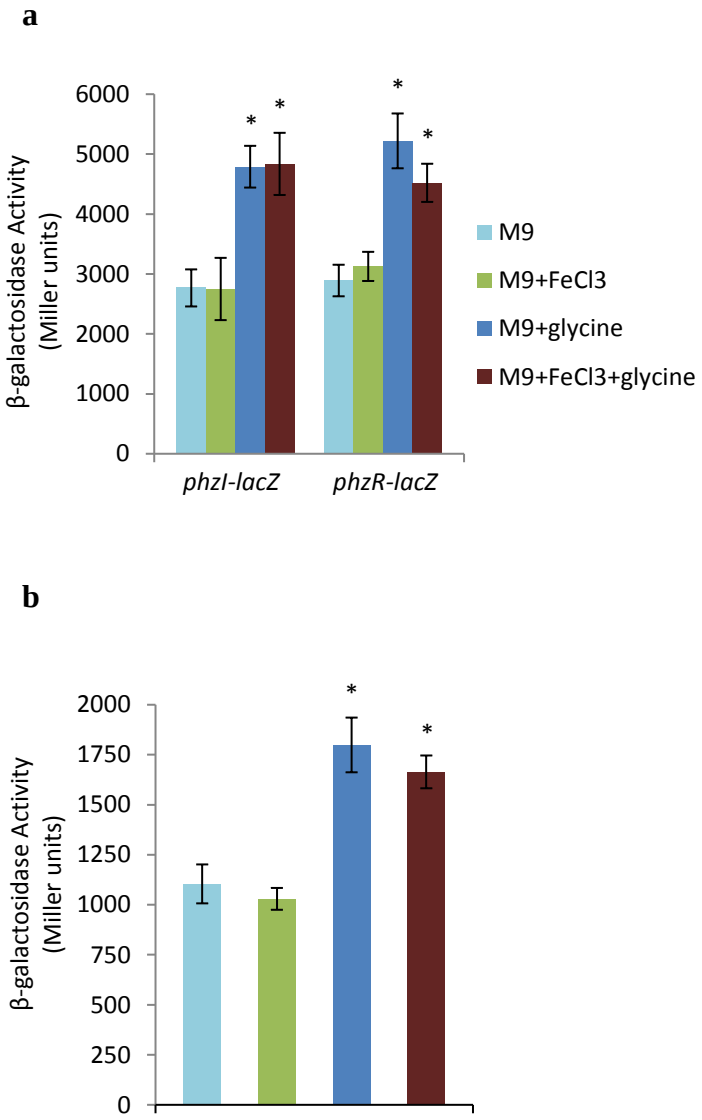


Figure 5.4. (a) PA23 cultures harboring *phzI*- and *phzR-lacZ* fusions were grown in the presence and absence of media additions. Cells were assayed for β-galactosidase activity at 24 h. (b) PA23 cultures grown in the presence and absence of media additions were subjected to AHL extraction. AHLs were added to the cultures of *P. aeruginosa* QSC105 (pEAL01), followed by β-galactosidase assay at 18 h. Colors indicate the same media conditions as mentioned in (a). For both (a) and (b), each value represents the mean from three biological replicates ± standard error. Data points marked with an asterisk (*) are statistically significant within each group ($P<0.05$).

Table 5.2. Quantification of phenazines (PHZs) and pyrrolnitrin (PRN) present in the cultures of PA23 grown in M9 minimal media (M9 + 0.2% glucose + 1 mM $MgSO_4$) alone, supplemented with 100 µM $FeCl_3$ and 20 mM glycine separately or in combination.

Media	PRN (µg/ml)[a]	PHZ (µg/ml)[a]	Autoinducer[b]
M9	2.72 (0.02)	43.57 (5.38)	0.67 (0.29)
M9 + $FeCl_3$	0.56 (0.08)[c]	19.33 (4.82)[d]	1.17 (0.28)[f]
M9 + glycine	3.94 (0.06)[d]	60.61 (2.04)[d]	2.0 (0.5)[e]
M9 + $FeCl_3$ + glycine	2.47 (0.36)[f]	36.47 (4.31)[f]	2.0 (0.5)[e]

[a]Mean (SD) from three triplicates.

[b]Mean (SD) of the zones of activity (mm) from six replicates.

[c]Significantly different from M9 ($P<0.001$).

[d]Significantly different from M9 ($P<0.01$).

[e]Significantly different from M9 ($P<0.05$).

[f]Not significantly different from M9.

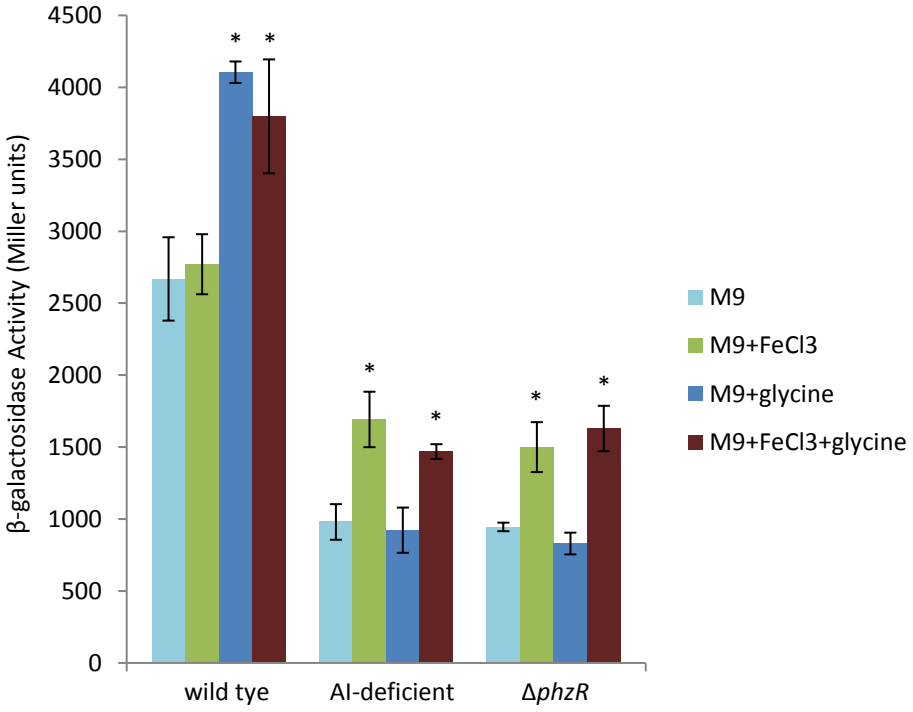


Figure 5.5. Cultures of PA23, AI-deficient and Δ*phzR* strains harboring *hcnA-lacZ* fusion were grown in the presence and absence of media additions. Cells were assayed for β-galactosidase activity at 24 h. Each value represents the mean from three biological replicates ± standard error. Data points marked with an asterisk (*) are statistically significant within each group ($P<0.05$).

5.5). Next, we searched for the presence of a *phz* box element in the promoter region of *hcnA*. An 18 nucleotide sequence was identified upstream of *hcnA* (133 bases from the ATG start) that has 13/18 nucleotides in common with the lux box consensus of *hcnA* in *P. aeruginosa* (Pessi and Haas, 2000) (Figure 5.7).

5.2.5. Effect of media amendments in the regulation of PRN and PHZ

In PA23, both PHZ and PRN are under QS control (Selin *et al*., 2012); as such, we were interested in analyzing whether media additions affect expression of these antibiotics. Glycine was found to upregulate both *phzA*- and *prnA-lacZ* transcriptional fusions; whereas $FeCl_3$ downregulated expression (Figure 5.6). Next we addressed whether $FeCl_3$ and glycine affect antibiotic production. PHZ and PRN production was elevated 1.4 fold in the presence of glycine. Conversely, $FeCl_3$ was found to decrease PHZ and PRN production by more than 2 fold and 4 fold, respectively. With both supplements, antibiotic levels were at or below that from media without $FeCl_3$ or glycine addition (Table 5.2).

5.3. Discussion

Fluorescent pseudomonads functioning as biocontrol agents produce a myriad of secondary metabolites, some of which are essential for pathogen suppression while others are expendable (Haas and Keel, 2003). Adding to this complexity is the fact that conditions favoring production of one compound may be vastly different from those that stimulate expression of another. Accordingly, it is essential to dissect out which compounds are involved in pathogen suppression as well as conditions promoting maximal expression. *P. chlororaphis* PA23 is an effective biocontrol strain that inhibits the growth of ascomycetes, such as *S.*

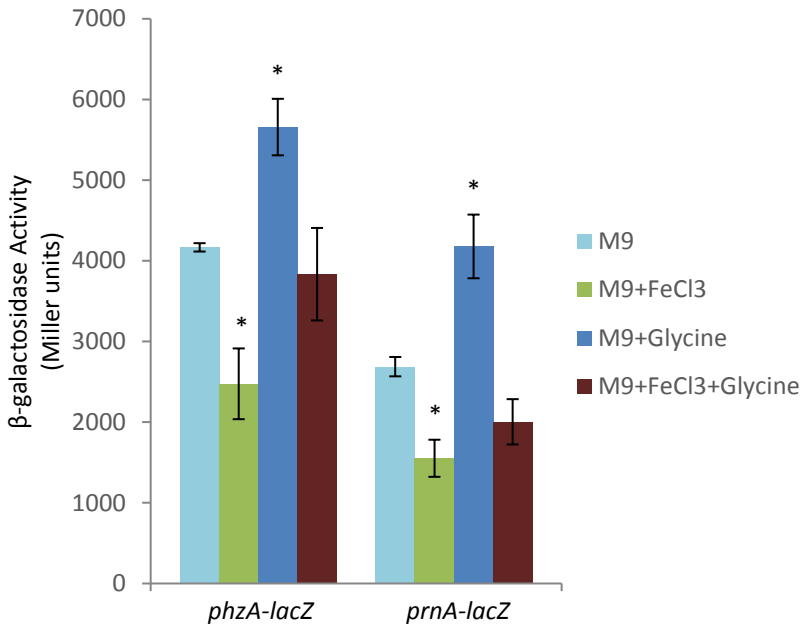


Figure 5.6. PA23 cultures harboring *phzA*- and *prnA-lacZ* fusions were grown in the presence and absence of media additions. Cells were assayed for β-galactosidase activity at 24 h. Each value represents the mean from three biological replicates ± standard error. Data points marked with an asterisk (*) are statistically significant within each group ($P<0.05$).

5´-
GGCAGGGCGGCGGGAGGCGCATTCCAGGGCCTTGTGCTTTGCTTGGCCCAGATCAAT
GTTCTTGTTCGACAAACCTACTAGATTGGCCTGGTGTTCAGCTCCGAGGAGCTGGCG
TCGCGCAACCCTGAACCGGGTCGAAGTCCAGGTTCAGGGTGATGCACAGCAGGACG
CTGGCATTCCTCTTCACTCAAGGACGAATTTACTG**ATG**CGCCATACCGAACGAACTT
TCGATATCCAGCCGCTGCAACAGGCGGATATGACCATCAGCCTCAATGGCCAGCCG
GTGACGGCGGCGATTGGTGAAACCGTACTCAGCGTCATCCAGTCCCTCGGGCTGCGT
CAGGTGGCGCGCAACGACCACGGCCAGCTGGCCGGCGCCTATTGCGGCATGGGCGT
ATGCCATTGCTGCCTGGTGCAGATCGACGGCCGGCACAAGCGCCGGGCCTGCCAGA
CCCAGGTGCGGCCGGGCATGCAGGTGCAGACCGAGGTCAACCGCATCGTCGAGGCG
GAGGAGGTGCTATGAGCCTGAATCCACTGATCGTCGGCGGCGGCCCCGCGGGCATG
GCCGCGGCCATCGAACTGGCCGAGCACGGGGTGCGCAGCACCCTGATCGATGAAGC
GTCGCGCCTGGGCGGCGTGGTCTATCGCGGGCCGCTGCGCGACGGCGTGCAGCTGG
ATTATCTGGGGCCGCGTTATTGCGAAGCCCTGGCGAAACTGCACGGCGCCTTTTCCG
ACTGTGAACAGATGATCGACGTGCGCCTCAACCACCGGGTGGTGGGGGCCGAAGGC
AAGGGCAGCCTGATCCTGCTGGATG-3´

P. aeruginosa lux box	C C T A C C A G A A T T G G C A G G…….ATG (*hcnA*)
PA23 *phz* box	C C T A C T A G A T T G G C C T G G…….ATG (*hcnA*)

P. protegens CHA0 *anr* box	T T G G C C C A G A T C A A…….ATG (*hcnA*)
PA23 *anr* box	T T G G C C C A G A T C A A…….ATG (*hcnA*)

Figure 5.7. Identification of *phz* (green) and *anr* (blue) box sequences in the *hcnA* promoter region of PA23. The *hcnA* translational start site is the bold and underlined sequence. Alignment of the *phz* box in the *hcnA* promoter of PA23 with the *lux* box in the *hcnA* promoter of *P. aeruginosa* is shown (Top). Alignment of the *anr* box sequences in the *hcnA* promoter of PA23 and *P. protegens* CHA0 is shown (bottom). The conserved nucleotides within the *phz* and *anr* boxes are shaded in grey.

sclerotiorum and *Leptosphaeria maculans* (Poritsanos *et al*., 2005; Ramarathnam *et al*., 2011). Among the arsenal of weaponry released by PA23, the antibiotic PRN is essential for controlling both fungal pathogens (Selin *et al*., 2010). While PHZs play only a modest role in AF activity, they do contribute to the biofilm-forming ability of this bacterium (Selin *et al*., 2010). It has been known for over a decade that PA23 produces HCN (Poritsanos *et al*., 2006) and it was recently discovered that synthesis of this volatile together with PRN enables PA23 to resist predation by the bacterivorous nematode *C. elegans* (Nandi *et al*., 2015). HCN in particular was shown to have strong nematicidal effects; thus, we expect this compound to play an important role in facilitating environmental persistence by enabling PA23 to escape predation. Understanding how HCN contributes to the biocontrol capabilities of PA23 is the focus of the current study.

We began our investigation by creating an *hcn* mutant. Characterization of PA23*hcn* revealed that HCN contributes to AF activity under some but not all conditions. For example compared to the *hcn* mutant, the wild type exhibited significantly increased fungal suppression on 1/5 PDA (Figure 5.1) media but not full strength PDA (data not shown). As media composition has a profound effect on expression of biocontrol compounds, including HCN, variable degrees of pathogen inhibition are not surprising. Michelsen and Stougaard (2012) reported similar findings for *P. fluorescens* In5 wherein HCN was found to contribute to suppression of *Rhizoctonia solani* and *Pythium aphanidermatum* when propagated on nitrogen-rich LB and KB. Conversely, cultivation on carbohydrate-rich PDA resulted in a lack of hyphal inhibition by either strain. Inconsistent findings regarding the contribution of HCN to the suppression of plant-pathogenic fungi (Flaishman *et al*., 1996; Pal *et al*., 2000; Nagarajkumar *et al*., 2004; Rezzonico *et al*., 2007) is expected to result, at least in part, from differences in the experimental conditions employed.

In the current study, we explored how two compounds previously reported to increase HCN production, namely glycine and $FeCl_3$, affect PA23 fungal suppression. We discovered that glycine supplementation increased both HCN production and AF activity (Table 5.1; Figure 5.3a). Glycine is the metabolic precursor of HCN and in *P. aeruginosa* glycine (20 mM) elevates production of this volatile five to six fold (Castric, 1977). Somewhat unexpectedly, we observed a 1.5-fold increase in *hcnA-lacZ* expression in the presence of glycine. These effects were mediated through the Phz QS system, as *phzR* and *phzI* transcription were both elevated in the presence of glycine (Figure 5.4a). In a study examining environmental conditions impacting phenazine-1-carboxamide (PCN) synthesis, glycine supplementation resulted in a 7-fold increase in PCN production by *P. chlororaphis* PCL1391 (van Rij *et al*., 2004). Conditions that increased PCN levels also increased AHL production, leading to the conclusion that the effects were being channeled through QS (van Rij *et al*., 2004), consistent with our results. With respect to iron supplementation, $FeCl_3$ is expected to increase *hcn* transcription through ANR. Previously it was found that iron favors formation of the [4Fe-4S] cluster, a cofactor for ANR, which is required for HCN synthesis in pseudomonads (Blumer and Haas, 2000). Based on *in vitro* studies, it has been predicted that the binding of ANR with its cofactor occurs at less than 5 µM O_2 concentration (Jordan *et al*., 1997). Since our assays were carried out under aerobic conditions, it is not surprising that $FeCl_3$ did not lead to upregulated *hcnA* transcription and HCN production (Figure 5.3). Inspection of the *hcnA* promoter sequence revealed the presence of a 14-nucleotide *anr* box, which is 100% identical to that upstream of *P. protegens* CHA0 *hcnA* (Figure 5.7). This binding sequence suggests a role for ANR in controlling HCN expression, although additional work is required to verify its involvement. Increased AF activity was observed for Δ*hcn* in the presence of $FeCl_3$ (Table 5.1), suggesting elevated production of AF compound(s) other than

HCN. Through transcriptional and endproduct analysis we discovered that $FeCl_3$ has a repressive effect on PHZ and PRN (Figure 5.6, Table 5.2); consequently, upregulation of these antibiotics cannot account for the increase in fungal inhibition.

Previously we found reduced HCN production by the QS-deficient strains compared to PA23 wild type (Nandi *et al*., 2015). In this study, *hcnA* expression was significantly reduced in the QS-deficient strains compared to the wild type (Figure 5.5). The identification of a *phz* box upstream of *hcnA* (Figure 5.7) further substantiates the role of QS in HCN production. In the PA23 wild type, glycine upregulated expression of *phzI* and *phzR*, together with the QS-controlled *phzA*, *prnA*, *hcnA* and their encoded products (Figure 5.3, 5.4, 5.6, Table 5.2). In the QS-deficient strains, however, glycine showed no effect on *hcnA* expression and in this background, $FeCl_3$ actually increased *hcnA-lacZ* activity (Figure 5.5). One explanation for these findings is that $FeCl_3$ favors co-factor assembly and subsequently ANR binding upstream of *hcnA*, leading to increased transcription. This is observed solely in the QS-minus background because of the short 30-bp separation between the *anr* box and the *phz* box (Figure 5.7). We hypothesize that when present, PhzR occludes binding of ANR to the *anr* box. Accordingly in the wild-type background, PhzR bound to the *hcn* promoter masks the stimulatory effects of $FeCl_3$ on ANR. As mentioned earlier, ANR is generally thought to function as an anaerobic regulator under low oxygen conditions. In a recent study, *Pseudomonas putida* KT2440 was found to contain three ANR homologs, one of which was highly responsive to low concentrations of oxygen (Ibrahim *et al*., 2015). The remaining two ANR proteins showed reduced oxygen sensitivity leading to the conclusion that these proteins had evolved to fulfill different roles (Ibrahim *et al*., 2015). Clearly more work is required to determine whether ANR controls expression of the PA23 *hcn* operon under different oxygen conditions. If ANR is

involved, exactly how it functions with the Phz QS system, acting independent of or working cooperatively with PhzR, will be the subject of future studies.

In conclusion, PA23 produces various secondary metabolites that play a role in plant disease suppression. Now we have determined that HCN plays an important role in PA23 AF activity and we have identified conditions that promote production of this volatile compound. Overall, the results of this study will provide insight into how to improve biocontrol inoculants in order to maximize disease suppression in the field.

5.4. Acknowledgments

I would like to thank our project student Gabriel Brawerman and summer student Biobelemoye Irabor for their contribution in this project. I would like to thank Dr. Carrie Selin for her assistance with performing HPLC.

Chapter 6

Characterization of the role of the anaerobic regulator ANR in *Pseudomonas chlororaphis* strain PA23 fungal suppression

The work presented in this chapter has been recently submitted to the *Microbiology* journal (article # MIC-D-16-00315).

6.1. Introduction

The anaerobic regulator ANR (anaerobic regulator of arginine deiminase and nitrate reductase) is classified as a FNR-like (fumarate and nitrate reductase regulator) transcriptional regulator which under low oxygen (O_2) concentrations can convert to its active form (Spiro, 1994). FNR belongs to the FNR-CRP (cAMP receptor protein) superfamily of transcriptional regulators first discovered in the enterobacterium *E. coli* (Körner *et al*., 2003; Lambden and Guest, 1976). These proteins can respond to various stimuli, such as oxygen, nitric oxide, carbon monoxide and cyclic nucleotides (nt), and in turn regulate expression of target genes (Körner *et al*., 2003). In *P. aeruginosa*, ANR is necessary for activation of pathways providing energy under low O_2 involving the machinery required for denitrification, high-affinity cytochrome oxidases and arginine fermentation enzymes (Zimmermann *et al*., 1991; Ye *et al*., 1995).

Another role for ANR was revealed with the discovery that in *P. aeruginosa* and *P. protegens* transcription of the *hcnABC* locus required for HCN production is dependent upon this regulator (Pessi and Haas, 2000; Laville *et al*., 1998). In *P. protegens* strain CHA0, cyanogenesis is dependent upon activation by ANR at the level of transcription, followed by components of the Gac-Rsm system at the level of translation (Blumer and Haas, 2000). This is consistent with the fact that an *anr* mutant produces very little HCN compared to the CHA0 wild type, while a *gacA* mutant is totally deficient in HCN synthesis (Laville *et al*., 1992; Laville *et al*., 1998). Moreover, a CHA0 *anr* mutant exhibited a 30% decrease in disease suppression highlighting the importance of ANR in this aerobic biocontrol strain (Laville *et al*., 1998). In *P. aeruginosa*, ANR functions together with its two QS systems, Las and Rhl, to control *hcnABC* expression (Pessi and Haas, 2000). However, in *P. protegens* CHA0 which lacks AHL-based QS, HCN production is solely dependent upon Gac and ANR (Blumer and Haas, 2000; Laville *et al*., 1998).

Studies of ANR regulation have revealed that below a critical threshold concentration of O_2, ANR dimerizes via a dimerization helix (Sawers, 1991; Yoon *et al*., 2007). This dimeric form carries a $4[4Fe\text{-}4S]^{2+}$ cluster which is a cofactor for ANR (Spiro, 1994). Three N-terminal cysteine residues and one internal cysteine residue (Cys-20, Cys-23, Cys-29 and Cys-122) are predicted to bind a $4[4Fe\text{-}4S]^{2+}$ cluster at less than 5 µM O_2 concentration. At high O_2 concentrations, the $4[4Fe\text{-}4S]^{2+}$ cluster is converted to $[2Fe\text{-}2S]^{2+}$ which leads to ANR inactivation (Jordan *et* al., 1997; Zimmermann *et al*., 1991). In its active form, ANR binds to a conserved sequence (5´-TTGATNNNNATCAA-3´) referred to as *anr* box in the promoter region enabling it to regulate transcription of target genes (Winteler and Haas, 1996; Yoon *et al*., 2007).

P. chlororaphis strain PA23 is a BCA, that is able to protect canola from sclerotinia stem rot caused by the phytopathogen *S. sclerotiorum* (Lib.) de Bary (Savchuk and Fernando, 2004). This bacterium produces a number of exoproducts including PCA, 2OH-PHZ, PRN, HCN, protease, lipase, chitinase and siderophores (Zhang *et al*., 2006; Poritsanos *et al*., 2006). Multiple regulatory elements oversee production of these secondary metabolites, including the Gac-Rsm regulatory cascade, the PhzI/PhzR QS system, the stationary phase sigma factor RpoS, a positive regulator of RpoS, called PsrA and the SR system (Poritsanos *et al*., 2006; Selin *et al*., 2012; Manuel *et al*., 2012; Selin *et al*., 2014). At present, with the exception of *P. protegens* CHA0 (Laville *et al*., 1998), there is little known about the impact of ANR on the AF activity of pseudomonads. Therefore, the focus of the current study was to discover whether ANR is involved in PA23 antagonism of *S. sclerotiorum*. A PA23 *anr* mutant was created and characterized with respect to antibiotic and degradative enzyme production. Finally, we explored whether there is a link between ANR and QS in this bacterium.

6.2. Results

6.2.1. Phenotypic characterization of PA23 *anr*

A PA23 *anr* mutant was generated and assessed for its ability to inhibit *S. sclerotiorum in vitro*. PA23*anr* exhibited a complete loss of fungal antagonism; as expected, complementation with *anr*-pUCP23 restored AF activity to near wild-type levels (Figure 6.1; Table 6.1). PRN is the primary antibiotic responsible for PA23-mediated fungal antagonism and production of the antibiotic PHZ imparts an orange phenotype to this bacterium (Selin *et al*., 2010). The lack of AF activity along with reduced pigmentation suggested that the *anr* mutant was producing decreased levels of both antibiotics (Figure 6.1). Quantification of PRN and PHZ revealed this to be the case (Table 6.2). In PA23, both PHZ and PRN are under QS control (Selin *et al*., 2012); thus, we were interested in determining whether the absence of *anr* affects QS. In qualitative AHL assays, the Δ*anr* strain showed a lack of QS signal production (Figure 6.2; Table 6.1). In all cases, PA23*anr* carrying a plasmid-borne copy of *anr* exhibited near wild-type levels of PRN, PHZ and AHL production (Table 6.1, 6.2).

As PA23 produces other exoproducts, such as protease and HCN that may contribute to fungal antagonism, we investigated whether these exoproducts were also controlled by ANR. The *anr* mutant exhibited a complete loss of extracellular protease activity (Figure 6.3; Table 6.1). Moreover using cyantesmo paper, we found that this strain produced very little HCN compared to the wild type (Table 6.1). Both protease activity and HCN production were restored to that of the wild type when *anr* was provided in *trans* (Figure 6.3; Table 6.1).

a **b**

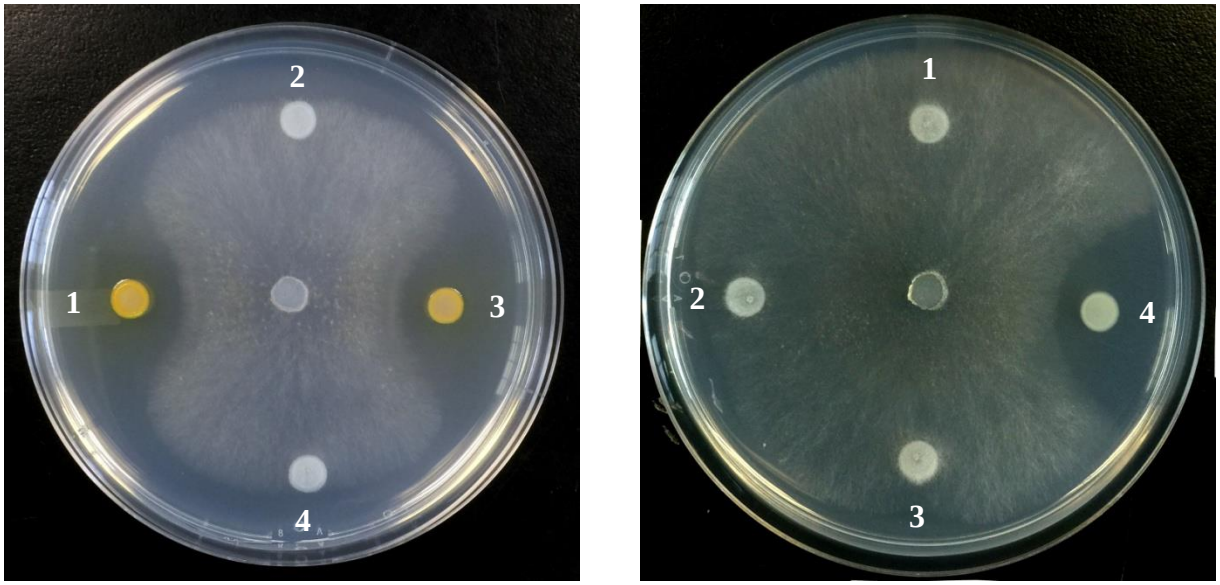


Figure 6.1. Antifungal activity of *P. chlororaphis* PA23 and derivatives against *S. sclerotiorum*. (a) sample 1: PA23 (wild type); sample 2: Δ*anr*; sample 3: Δ*anr* (*anr*-pUCP23); sample 4: Δ*phzR*. (b) sample 1: Δ*anr* (*phzR*-pUCP23); sample 2: Δ*anr* + C_6-HSL; sample 3: Δ*anr* (*phzR*-pUCP23) + C_6-HSL; sample 4: Δ*phzR* (*anr*-pUCP23). Note that the presence of plasmid-borne *anr* is able to restore the AF activity of the Δ*phzR* strain.

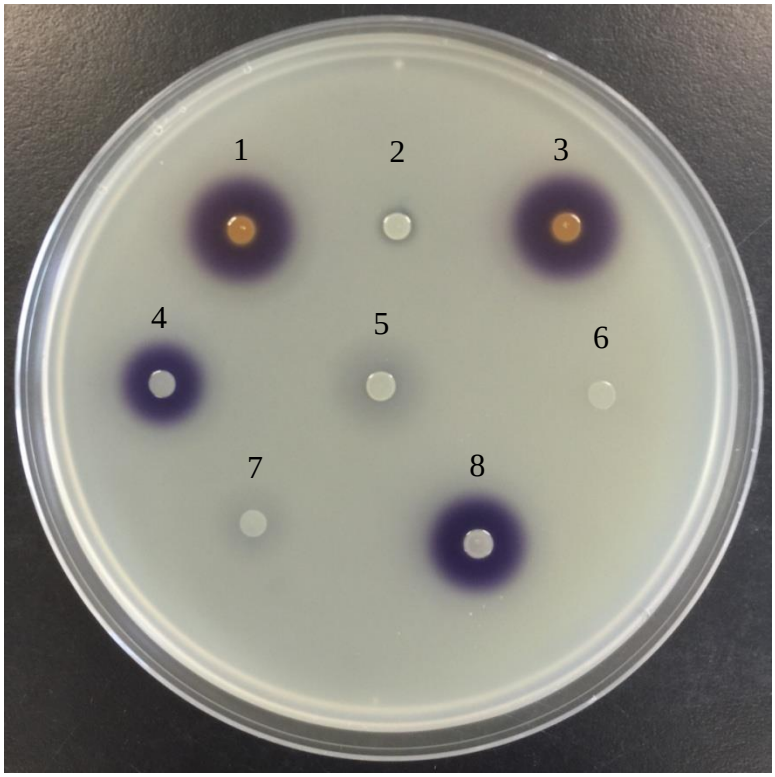


Figure 6.2. Autoinducer assay of *P. chlororaphis* PA23 and derivatives on CVO26-seeded agar. Sample 1: PA23; sample 2: Δ*anr*; sample 3: Δ*anr* (*anr*-pUCP23); sample 4: Δ*phzR*; sample 5: Δ*anr* + AHL; sample 6: Δ*anr* (*phzR*-pUCP23); sample 7: Δ*anr* (*phzR*-pUCP23) + AHL; sample 8: Δ*phzR* (*anr*-pUCP23). Note that the presence of *anr* in *trans* restores AHL production by the Δ*phzR* strain. The modest purple zones surrounding samples 5 and 7 are believed to result from the addition of exogenous AHL to the liquid cultures.

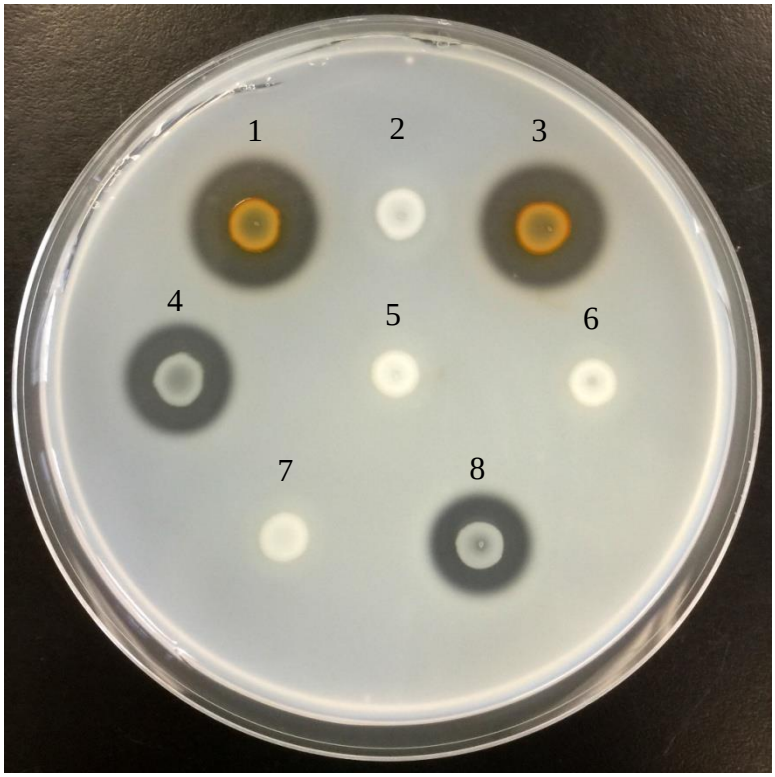


Figure 6.3. Protease plate assay of *P. chlororaphis* PA23 and derivatives on 2% skim milk agar. Sample 1: PA23; sample 2: Δ*anr*; sample 3: Δ*anr* (*anr*-pUCP23); sample 4: Δ*phzR*; sample 5: Δ*anr* + AHL; sample 6: Δ*anr* (*phzR*-pUCP23); sample 7: Δ*anr* (*phzR*-pUCP23) + AHL; sample 8: Δ*phzR* (*anr*-pUCP23). Note that providing *anr* in *trans* is not able to complement the Δ*phzR* mutant with respect to protease activity.

Table 6.1. Phenotypic characteristics of PA23 and its derivatives.

Strains	Extracellular metabolite activity			
	Antifungal[*]	Protease[*]	Autoinducer[*]	HCN[§]
PA23	5.2 (0.3)	6.1 (0.2)	6.9 (0.4)	+++
Δ*anr*	0[a]	0[a]	0[a]	+
Δ*anr* (*anr*-pUCP23)	4.1 (0.2)[b]	5.4 (0.4)[c]	6.3 (0.4)[c]	+++
Δ*anr* + C_6-HSL	0[a]	0[a]	1.6 (0.3)[a]	+
Δ*anr* (*phzR*-pUCP23)	0[a]	0[a]	0[a]	+
Δ*anr* (*phzR*-pUCP23) + C_6-HSL	0[a]	0[a]	1.3 (0.2)[a]	+
Δ*phzR*	0[a]	3.8 (0.3)[d]	3.3 (0.4)[d]	+
Δ*phzR* (*anr*-pUCP23)	3.0 (0.6)[d]	3.7 (0.3)[d]	5.1 (0.4)[e]	++

[*]Mean (SD) of the zones of activity (mm) obtained from five replicates.
[§]Determined using cyantesmo paper. +, low; ++, intermediate; +++, high.
[a]Significantly different from wild type ($P<0.0001$).
[b]Significantly different from wild type ($P<0.05$).
[c]Not significantly different from wild type.
[d]Significantly different from wild type ($P<0.001$).
[e]Significantly different from wild type ($P<0.01$).

Table 6.2. Quantification of PRN and PHZ from PA23 and its derivatives.

Strains	PRN (µg/ml)[*]	PHZ (µg/ml)[*]
PA23	2.80 (0.36)	37.16 (3.59)
Δ*anr*	0[a]	8.67 (3.87)[a]
Δ*anr* (*anr*-pUCP23)	2.70 (0.30)[b]	33.34 (4.06)[b]
Δ*anr* + C_6-HSL	0.05 (0.05)[a]	7.94 (1.56)[a]
Δ*anr* (*phzR*-pUCP23)	0.06 (0.05)[a]	7.14 (2.80)[a]
Δ*anr* (*phzR*-pUCP23) + C_6-HSL	0.06 (0.05)[a]	8.33 (2.64)[a]
Δ*phzR*	0[a]	6.37 (1.51)[a]
Δ*phzR* (*anr*-pUCP23)	0.95 (0.18)[c]	8.02 (1.98)[a]

[*]Mean (SD) obtained from a triplicate set.
[a]Significantly different from wild type (P<0.0001).
[b]Not significantly different from wild type.
[c]Significantly different from wild type (P<0.01).

6.2.2. ANR positively regulates QS, *rpoS* and antibiotic gene expression in PA23

To assess the effect of an *anr* mutation on PA23 gene expression, qRT-PCR was employed. Expression of *phzA*, *prnA*, *hcnA*, *phzI*, *phzR*, *rpoS* and *anr* were analyzed in PA23 and the Δ*anr* strain. In the *anr* mutant, all of the aforementioned genes showed reduced expression indicating that ANR positively regulates these biosynthetic and regulatory genes (Figure 6.4). This data correlate well with the phenotypic assays wherein a significant reduction in PRN, PHZ, HCN and AHL production was observed for the Δ*anr* strain compared to the wild type (Table 6.1, 6.2).

Typically, ANR binds to a conserved 14-nt sequence, known as the *anr* box, located in the promoter region of the target genes (Winteler and Haas, 1996; Yoon *et al*., 2007). Inspection of the PA23 *hcnA* promoter region revealed the presence of a 14-nt *anr* box (163 bp from the ATG start) which is 100% identical to that found upstream of *P. protegens* CHA0 *hcnA* (Laville *et al*., 1998) (Figure 6.5.b). We next looked for putative *anr* boxes upstream of *phzA*, *prnA*, *phzI*, *phzR*, *rpoS* and *anr*. Sequences having eight to ten nt in common with the *anr* box consensus of *hcnA* were identified upstream of the *phzI* (99 bp from the ATG start), *phzR* (45 bp from the ATG start), *phzA* (69 bp from the ATG start) and *prnA* (264 bp from the ATG start) (Figure 6.5.b). Similarly, an *anr* consensus sequence was identified in the promoter regions of *anr* (268 bp from the ATG start; 10/14 bp match) and *rpoS* (74 bp from the ATG start; 8/14 bp match) (Figure 6.5.b). The presence of this sequence upstream of *anr* together with the fact that *anr* expression was markedly reduced in the Δ*anr* strain suggests that ANR is subject to positive autoregulation in PA23.

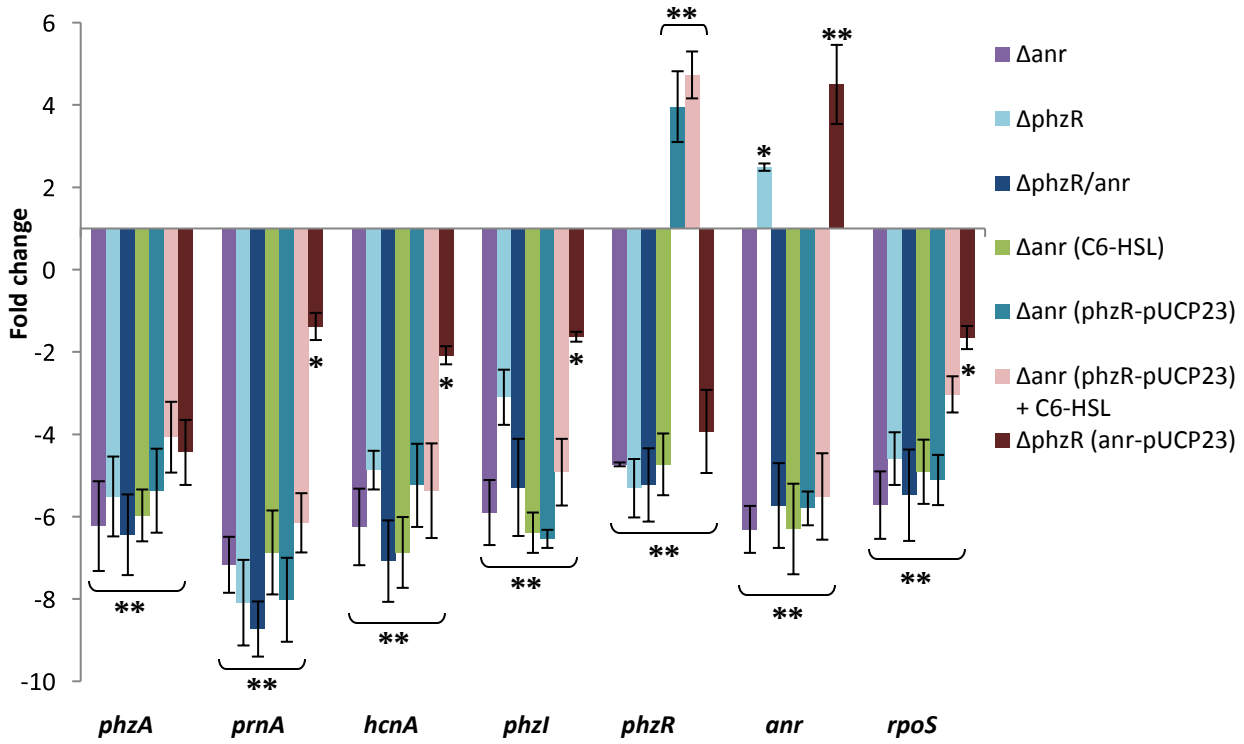


Figure 6.4. qRT-PCR fold change in gene expression in the Δ*anr*, Δ*phzR*, Δ*phzR/anr*, Δ*anr* (C_6-HSL), Δ*anr* (*phzR*-pUCP23), Δ*anr* (*phzR*-pUCP23) + C_6-HSL and Δ*phzR* (*anr*-pUCP23) strains compared to the wild type. Expression levels in the wild type were normalized to 1; differentially expressed genes are indicated with an asterisk (*, P<0.01; **, P<0.001).

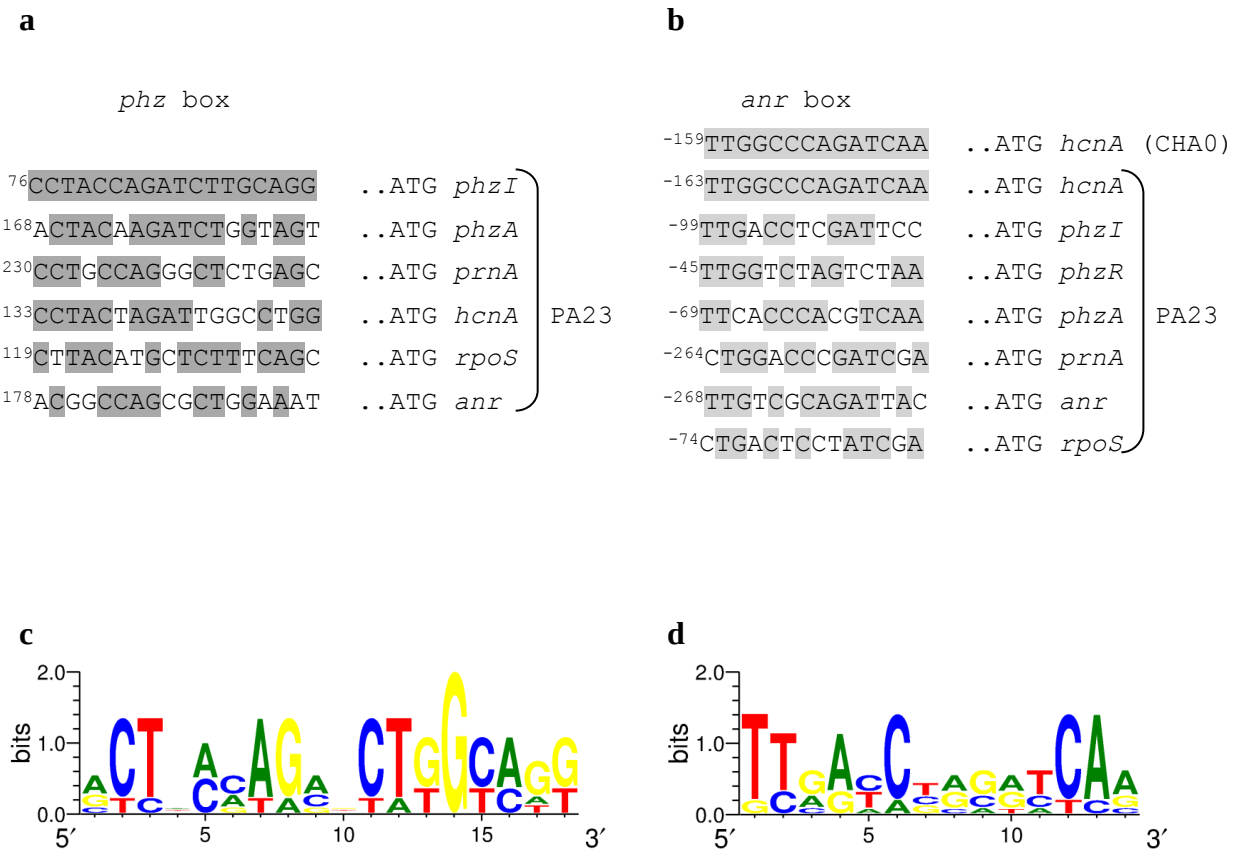


Figure 6.5. Identification of *phz* and *anr* box sequences in the *hcnA*, *phzI*, *phzR*, *phzA*, *prnA*, *anr* and *rpoS* promoter regions of *P. chlororaphis* PA23. (a) The location of *phz* box in the *phzI*, *phzA* and *prnA* promoter regions was previously reported by Selin *et al*. (2012). The putative *phz* box sequences of *hcnA*, *rpoS* and *anr* are aligned with the *phz* box consensus upstream of *phzI* and the conserved nucleotides are shaded in dark grey. (b) The *anr* box identified in the *hcnA* promoter region of PA23 is 100% identical to the *anr* box of *hcnA* found in *P. protegens* CHA0 (Laville *et al*., 1998). The putative *anr* box sequences of *phzI*, *phzR*, *phzA*, *prnA*, *anr* and *rpoS* genes are aligned with the *anr* box consensus upstream of *hcnA*; the conserved nucleotides are shaded in light grey. (c), (d) Sequences of the six *phz* boxes and seven *anr* boxes identified in *P. chlororaphis* PA23, were used to generate the shown (c) *phz* and (d) *anr* position weight matrix sequences, respectively.

6.2.3. PhzR negatively regulates *anr* expression in PA23

Since ANR positively regulates both the *phzI* and *phzR* QS genes, we were interested to see whether there is reciprocal cross-regulation of *anr* by QS. qRT-PCR analysis was conducted to monitor *anr*, as well as *phzA*, *prnA*, *hcnA*, *phzI*, *phzR*, and *rpoS* transcription in the Δ*phzR* strain. With the exception of *anr*, transcription of all of the genes was markedly reduced in the *phzR* mutant background (Figure 6.4). Interestingly, a greater than 2-fold increase in *anr* transcription was observed in the *phzR* mutant, suggesting that PhzR might repress *anr* expression (Figure 6.4). When expression of the aforementioned genes was monitored in a *phzR/anr* double mutant, they were all significantly downregulated as expected (Figure 6.4). Analysis of the *anr* promoter region revealed an 18-bp region (178 bp upstream of the *anr* ATG start) that has 9/18 nt in common with the *phz* box consensus of *phzI* (Selin *et al*., 2012) (Figure 6.5.a). The presence of a *phz* box suggests that the *anr* promoter is recognized and bound by the PhzR-AHL complex, which governs transcription of QS-controlled genes.

6.2.4. QS does not complement an *anr* mutant

Next we addressed whether constitutively expressed *phzR*, addition of 1 µM C_6-HSL or both would complement the *anr* mutant. Upon mobilizing *phzR*-pUCP23 into the Δ*anr* strain, extracellular metabolite activity remained the same as that of the *anr* mutant (Table 6.1, 6.2). Similar results were observed for the Δ*anr* or Δ*anr* (*phzR*-pUCP23) strains grown in the presence of C_6-HSL (Table 6.1, 6.2). Thus, providing one or both of the missing QS components did not restore the *anr* mutant phenotype to that of the wild type. Based on qRT-PCR analysis, we observed that expression of the biosynthetic (*phz*, *prn*, *hcn*) and regulatory genes (*phzI*, *phzR*, *anr*, *rpoS*) in the aforementioned strains was significantly downregulated similar to the

expression profiles of the Δ*anr* strain (Figure 6.4). Only *phzR* expression was found to be upregulated in strains harboring *phzR*-pUCP23 (Figure 6.4).

6.2.5. ANR partially complements a QS mutant

Next we sought to determine whether providing constitutively expressed *anr* in *trans* would complement the *phzR* mutant. When *anr*-pUCP23 was mobilized into the *phzR* mutant, AF activity, as well as AHL, HCN and PRN production was partially restored (Tables 6.1, 6.2). Conversely, this plasmid was unable to restore PHZ production and protease activity (Tables 6.1, 6.2). qRT-PCR analysis was conducted to assess gene expression in the Δ*phzR* strain harboring *anr*-pUCP23. Expression of *prn*, *hcn*, *phzI* and *rpoS* were restored to near wild-type levels (Figure 6.4), consistent with our phenotypic findings (Tables 6.1, 6.2). However, the *phzR* mutant harboring *anr*-pUCP23 showed no change in *phzA* expression which correlates with the decreased PHZ levels observed for this strain (Figure 6.4, Table 6.2).

6.3. Discussion

In the BCA *P. chlororaphis* PA23, multiple regulators oversee production of metabolites essential for biocontrol (Poritsanos *et al*., 2006; Selin *et al*., 2012; Manuel *et al*., 2012; Selin *et al*., 2014). In the present study, characterization of an *anr* mutant revealed a complete loss of fungal antagonism (Figure 6.1), establishing that ANR is essential for PA23 biocontrol. Based on studies in other *Pseudomonas* spp., ANR is conserved and required for expression of compounds that play a role either in biocontrol or pathogenicity (Laville *et al*., 1998; Hammond *et al*., 2015). Additionally, cross-regulation between ANR and QS in *P. aeruginosa* PAO1 and PA14 has been reported (Hammond *et al*., 2015). Therefore, the aim of the current study was to analyze how

ANR affects production of AF compounds in *P. chlororaphis* PA23 and to investigate whether cross-regulation occurs between ANR and QS.

It has been shown that ANR is important for cyanide synthesis in *P. protegens* CHA0 (Laville *et al*., 1998). Based on phenotypic characteristics exhibited by PA23*anr*, we learned that in addition to HCN, this regulator is responsible for PRN, PHZ production, protease activity, and QS in PA23 (Table 6.1, 6.2). These data correlate well with the gene expression profiles showing significant downregulation of all of the biosynthetic and regulatory genes analyzed in the *anr* mutant background (Figure 6.4). Similarly, QS has a major role in PA23 biocontrol (Selin *et al*., 2012). In accordance with previous data, we found that the PA23 *phzR* mutant exhibited a complete loss of fungal antagonism and reduced exometabolite production (Tables 6.1, 6.2). In addition to *prn*, *phz* and *rpoS* (Selin *et al*., 2012), PhzR positively regulates *hcn* (Figure 6.4). The phenotypic assays together with gene expression analysis indicate that both ANR and QS are important for the production of secondary metabolites in PA23. Schuster and Greenberg (2006) reported similar findings for *P. aeruginosa* wherein an *anr* box sequence was identified in the promoter regions of 25% of QS-controlled virulence genes. These findings suggest that both ANR and QS are important for expression of those virulence factors depending on whether aerobic or anaerobic conditions prevail. In another study, ANR was found to control expression of multiple QS-associated genes in *P. aeruginosa* PAO1 and PA14 (Hammond *et al*., 2015). Thus in *P. aeruginosa*, there appears to be significant overlap between the QS and ANR regulons.

Through gene expression analysis we discovered the existence of cross-regulation between ANR and QS. ANR was shown to positively regulate *phzR*, while PhzR has a repressive effect on *anr* (Figure 6.4). Similarly Hammond and coworkers (2015) reported that for *P.*

aeruginosa strains PAO1 and PA14, the LasRI QS system acts as a repressor for ANR. To further investigate cross-regulation between ANR and QS, we conducted studies using an *anr* mutant complemented with *phzR*-pUCP23 and C_6-HSL, either alone or in combination, and a *phzR* mutant complemented with *anr*-pUCP23. Complementing PA23*anr* with either *phzR*-pUCP23, C_6-HSL, or both yielded no change in gene expression or exoproduct formation, indicating that QS alone is insufficient to activate the transcription of target genes in an *anr* mutant background (Table 6.1, 6.2; Figure 6.4). On the other hand, complementing a *phzR* mutant with *anr*-pUCP23, resulted in partial restoration of *phzI*, *prn*, *hcn*, and *rpoS* expression, as well as AF activity, AHL, HCN and PRN production (Table 6.1, 6.2; Figure 6.4). However, *phz* expression and protease activity remained unchanged (Table 6.1; Figure 6.4). Our data indicate that providing *anr* in *trans* can partially complement the QS-deficient phenotype in terms of some but not all biocontrol genes and products. It has been previously reported that ANR can partially substitute for a loss of QS with respect to regulating specific virulence factors in *P. aeruginosa*, consistent with our results (Hammond *et al*., 2015).

The role of ANR in the activation of the *hcn* and *arcDABC* (encoding enzymes for arginine deiminase pathway) promoters in *P. aeruginosa* PAO1 has been extensively studied (Pessi and Hass, 2000; Lu *et al*., 1999). Although the QS regulators (LasR, RhlR) and ANR can synergistically activate *hcn* transcription, without LasR and RhlR, ANR cannot bind upstream of *hcn* to activate transcription (Pessi and Hass, 2000). Conversely, ANR can independently activate the *arcDABC* promoter even in the absence of the arginine regulator ArgR (Lu *et al*., 1999). In PA23, the tandem arrangement of the *phz* and *anr* box sequences upstream of *phzI*, *phzA*, *prnA*, *hcnA*, and *rpoS* suggests that transcription is dependent upon binding of both QS and ANR (Figure 6.5). However, variations were found with respect to the arrangement of the *phz*

and *anr* boxes in the promoters of the target genes. Upstream of *phzI*, *prnA*, *hcnA* and *rpoS*, the *phz* and *anr* boxes are separated by only 23 to 45 bp; in the case of *phzA*, there is a 99-bp separation between the two (Figure 6.5). The presence of the *anr* box in close proximity to the *phz* binding region and the fact that *anr* in *trans* partially complements the *phzR* mutant suggests that ANR can activate *phzI*, *prnA*, *hcnA* and *rpoS* in the absence of QS. Conversely, the absence of complementation for PHZ production and *phzA* transcription together with the larger intervening region between the *phz* and *anr* boxes implies that both regulators are required to activate the *phz* biosynthetic operon. In the future, DNA binding assays, such as gel shift and DNase footprinting assays will help to clarify whether ANR and PhzR-AHL bind separately or cooperatively at each of these promoter regions.

It is generally believed that the FNR-like transcriptional regulators function optimally under low O_2 concentrations; however, there are reports showing that their role is not restricted to anaerobic conditions. *P. protegens* CHA0 is a strict aerobe and studies have shown that ANR does not allow this bacterium to grow anaerobically (Laville *et al*., 1998). Even so, ANR controls expression of an arginine deiminase pathway in this strain (Laville *et al*., 1998). Whereas in *P. aeruginosa* PAO1, ANR is essential for anoxic growth mediated by both denitrification and arginine deiminase pathways (Galimand *et* al., 1991; Alvarez-Ortega and Harwood, 2007). Interestingly, a recent study focused on *P. putida* KT2440 revealed the presence of three FNR proteins having different sensitivities to O_2 (Ibrahim *et al*., 2015). Comparison with the FNR of *E. coli*, revealed deviations in the amino acid residues close to four conserved cysteines in the *P. putida* FNR proteins. Two out of the three FNRs had higher mismatches and were found to be less sensitive to O_2 (Ibrahim *et al*., 2015). Previous studies of *E. coli* FNR have shown that replacing specific amino acids adjacent to the cysteine residues can

Figure 6.6. Alignment of *E. coli* FNR and *P. chlororaphis* PA23 ANR protein sequences using the Clustal Omega alignment program. The conserved cysteine residues are shaded in green. The mismatches in the residues adjacent to the N-terminal cysteine residues are shaded in grey. (*) indicates residues that are identical in both proteins; (:) indicates residues with strongly similar properties; (.) indicates residues with weakly similar properties.

increase the stability of the [4Fe-4S] cluster as well as FNR activity in the presence of O_2 (Bates *et al*., 2000; Jervis *et al*., 2009). Based on sequence alignment with *E. coli* FNR, the PA23 ANR protein is 53% identical (173/226 similar amino acid residues), with three N-terminal and one internal cysteine residues conserved between them (Figure 6.6). However, we found mismatches in the PA23 ANR adjacent and close to the N-terminal cysteine residues which may ultimately affect O_2 sensitivity (Figure 6.6).

In summary, ANR is a key regulator governing *P. chlororaphis* PA23 secondary metabolite production. We have demonstrated that ANR is required for AF, protease activity, QS, PRN, PHZ and HCN production in PA23. In addition, ANR and QS are subject to cross-regulation, with ANR positively regulating *phzI*/*phzR* and PhzR having a repressive effect on *anr*. Based on complementation studies, both ANR and QS are required for PHZ and protease production by PA23. Moreover, we discovered that expressing *anr* in *trans* can override a QS-deficiency to restore expression of *phzI*, *prn*, *hcn* and *rpoS*. Our results indicate that ANR is a global regulator, vital for exoproduct formation by PA23. Future studies are required to understand how ANR functions at different O_2 levels and how this impacts PA23 growth, metabolism and ultimately biocontrol under different conditions.

6.4. Acknowledgments

I would like to thank Dr. Carrie Selin for her assistance with performing HPLC. I would also like to thank Gabriel Brawerman for helping me in generating pKNOCK*anr*-23.

Chapter 7

Conclusions and future directions

7.1. Conclusions and future directions

Beyond pathogen control, a successful BCA should be able to persist in the environment and avoid predation by microfaunal predators. In the present study, biocontrol agents *P. brassicacearum* DF41 and *P. chlororaphis* PA23 were investigated for their ability to resist predation by the bacterivorous nematode *C. elegans*. We found that DF41 and PA23 are capable of killing the nematodes through production of toxic metabolites such as HCN, which is well established for having lethal effects on *C. elegans*. In addition one of the PA23 exoproducts, namely PRN, was revealed to be a potent nematicidal agent. We discovered that DF41 is also capable of killing the nematodes through biofilm formation on the nematode anterior, which is unique for a pseudomonad as it has only been reported for *Yersinia* spp. and *X. nematophila* (Darby *et al*., 2002; Drace and Darby, 2008). DF41 biofilm formation was found to depend upon the Gac two-component system as well as the concentration of NaCl in the media. DF41 co-cultured in the presence of nematodes lead to increased expression of the *pdfRI-rfiA* quorum-sensing genes and *hcnA*. Consistent with our gene expression analysis, we observed that HCN and AHL signal production by DF41 were upregulated. Additionally, co-culturing PA23 in the presence of nematodes increased *phzA*, *hcnA*, *phzR*, *phzI*, *rpoS* and *gacS* expression; while the production of PHZ and AHL signals were also elevated. Our findings suggest that both DF41 and PA23 can sense the presence of *C. elegans* and respond to nematode cues by upregulating expression of toxin-related genes and products. These antipredator strategies are expected to allow BCAs to sustain higher populations in the rhizosphere, which is important for biocontrol.

Our study of bacterial defense against predation opens up a number of areas for future study. For example, the phenomenon of biofilm formation by DF41 on the nematode surface should be explored in more detail. Both *Yersinia* spp. and *X. nematophila* require the

hmsHFRS gene cluster to be able to adhere biofilms to the nematode anterior (Tan and Darby, 2004; Drace and Darby, 2008). The DF41 genome revealed a homologue of the *hmsHFRS* operon which shares high amino acid sequence homology (91-99%) with PgaABCD (HmsHFRS) of *P. fluorescens.* These gene products are responsible for synthesis of a linear homopolymer of poly-β-1,6-*N*-acetyl-D-glucosamine (Itoh *et al*., 2008). *PgaABCD*-mediated biofilm formation occurs in different species, including *E. coli* and *S. epidermidis* (Itoh *et al*., 2008; Itoh *et al*., 2005). Hence, it will be important to generate a DF41 *hmsHFRS* mutant in order to characterize its role in biofilm development. It would also be interesting to elucidate the chemical composition of the DF41 biofilm. Lectins, such as *Limax flavus* agglutinin (LFA) and wheat-germ agglutinin (WGA) are proteins that can bind to specific carbohydrate structures (Berg, 2002; Tan and Darby, 2004). According to Drace and Darby (2008), biofilm formed by *Yersinia* spp. on *C. elegans* binds strongly with WGA, suggesting that the attached extracellular matrix contains *N*-acetyl-D-glucosamine or *N*-acetylneuraminic acid. Lectins can be used in our study as a probe not only to discern the components of DF41 biofilm, but also to detect biofilm on nematodes as well as plant surfaces. Mass spectrometry (MS) or infrared (IR) spectroscopy represents alternative strategies for elucidating the chemical composition of the DF41 biofilm. The effect of various parameters, such as temperature, pH, humidity and root exudates on DF41 biofilm formation should also be studied.

Studying predator-prey interaction allows an understanding of factors driving the fitness of a biocontrol species. In our study, we identified exoproducts, including PRN and HCN that are expected to increase the persistence of BCAs in the presence of *C. elegans*. Whole genome transcriptomic analysis of PA23 and DF41 cultured together with *C. elegans* might reveal unknown factors involved in predation-mediated responses. Analyzing interactions between

these BCAs and other predators, such as the free-living amoeba *Acanthamoeba castellani* will shed light on their anti-protozoan characteristics. Additionally, the effects of PA23 and DF41 on plant-pathogenic nematodes, like *Meloidogyne incognita*, could reveal biocontrol potential that extends beyond fungal pathogens.

Balanced metabolite production by biocontrol pseudomonads is essential in terms of fitness in its natural environment as well as pathogen suppression. Production of certain secondary metabolites, such as 2,4-DAPG, PLT, PRN, HCN not only help *Pseudomonas* spp. resist predation, but they also contribute to fungal inhibition (Neidig *et al*., 2011; Nandi *et al*., 2015; Gallagher and Manoil, 2001; de Souza *et al*., 2003; Maurhofer *et al*., 1994; Selin *et al*., 2010; Michelsen and Stougaard, 2012). The role of antibiotics in PA23 fungal antagonism has been characterized for both PRN and PHZ, with PRN being the primary antibiotic underlying AF activity (Selin *et al*., 2010). While PHZ plays a more minor role in fungal suppression, it is involved in PA23 biofilm formation (Selin *et al.*, 2010). In addition, PA23 produces HCN (Poritsanos *et al*., 2006); however, its role in biocontrol had not previously been explored. In the second part of the thesis, the contribution of this volatile to PA23 fungal suppression was revealed through characterization of an *hcn* mutant. Based on *in vitro* assays, we observed that HCN contributes to PA23 AF activity. We found that media amendment, particularly glycine (20 mM) increased the AF activity as well as HCN production. In PA23, both PHZ and PRN are under QS control (Selin *et al*., 2012) and HCN was found to be positively regulated by QS. Through gene expression and endproduct analysis, we discovered that glycine-mediated upregulation of HCN, as well as PHZ and PRN occurs though QS. Conversely, $FeCl_3$ (100 µM) supplementation did not elevate HCN production and was found to have a repressive effect on PHZ and PRN production in the wild type. While $FeCl_3$ increased *hcnA* expression in the QS-

deficient strains, transcription was below wild-type levels. In addition to a *phz* box, an *anr* box was identified in the *hcnA* promoter region, suggesting a role for ANR in regulating *hcnA*. Previous studies have established that ANR controls HCN synthesis in *Pseudomonas* spp. (Laville *et al*., 1998; Pessi and Haas, 2000). To better understand the role of ANR in expression of HCN and other metabolites produced by PA23, an *anr* mutant was generated. Our studies revealed that ANR is crucial for AF and protease activity, as well as QS, PRN, PHZ and HCN production. Moreover, ANR positively regulates *phzI*/*phzR*, while PhzR negatively regulates *anr*, establishing that cross-regulation occurs between them. We showed that expressing *anr* in *trans* partially complements a *phzR* mutant in terms of *phzI*, *prn*, *hcn* and *rpoS* expression, indicating that ANR can activate these genes in the absence of QS. Conversely, *phzA* transcription is dependent upon QS and is not activated by ANR alone.

Understanding production of secondary metabolites and the regulators controlling expression should help in optimizing the yield of biocontrol factors, thereby making a BCA more effective for disease suppression. We have shown the effects of both abiotic (glycine, $FeCl_3$) and biotic (ANR, QS) factors on HCN production by PA23. Different abiotic factors, such as carbon and nitrogen sources, including amino acids should be tested for their influence on HCN production and PA23-mediated biocontrol. According to an earlier study, salts such as sodium chloride (NaCl), potassium chloride (KCl), and sodium sulfate (Na_2SO_4) decreased production of the antibiotic phenazine-1-carboxamide (PCN), whereas the presence of low concentrations of magnesium ions increased PCN production in *P. chlororaphis* PCL1391 (van Rij *et al*., 2004). Moreover, osmoprotectants, like trehalose, betaine, choline and L-proline can promote bacterial growth and metabolism in a high osmolality environment (Kempf and Bremer, 1998). As environmental factors can have both a positive and negative impact on the production of

exometabolites, analyzing several factors controlling production of HCN as well as other AF compounds might help to improve the biocontrol potential of PA23 in the field.

Besides Gac-Rsm, PhzI/PhzR, RpoS, characterizing the transcriptional regulator ANR in PA23 adds another level of intricacy to the regulatory cascade. Even though it is generally believed to be an anaerobic regulator, ANR was essential for PA23 secondary metabolite production under aerobic conditions. Protein sequence alignment with FNR from *E. coli* revealed amino acid differences in close proximity to the N-terminal cysteine residues, which might affect the O_2 sensitivity of the PA23 ANR, as suggested previously (Bates *et al*., 2000; Jervis *et al*., 2009; Ibrahim *et al*., 2015). Subsequent analysis should focus on analyzing the role of ANR in exoproduct formation by PA23 under O_2 limiting and anaerobic conditions. Additionally, *phz* and *anr* box sequences were identified in the promoter regions of biosynthetic and regulatory genes, suggesting a significant overlap between ANR- and QS-controlled genes in PA23. In the future, DNA binding assays are required to determine whether ANR and PhzR-AHL bind independently or synergistically at the promoter regions of target genes. Using RNA-Seq to compare the gene expression patterns of the wild type, Δ*anr*, Δ*phzR* and Δ*anr*/*phzR* double mutant will reveal the composition of the ANR and QS regulons. Additionally, transcriptomic comparisons of these strains grown under aerobic versus O_2-limiting conditions will shed light on how O_2 availability influences PA23 gene expression.

Although research on biocontrol bacteria as a means to inhibit fungal pathogens has been gaining momentum during the last few decades, it has not reached its full potential. The commercial application of these beneficial bacteria is often limited by their low persistence in the rhizosphere and fluctuations in expression of antagonistic compounds. Research on bacterial defense against predators, together with unveiling the role of exometabolites and regulators

involved in biocontrol will facilitate development of new strategies for successful establishment of biocontrol bacteria in the environment.

References

Aballay, A., Drenkard, E., Hilbun, L.R. and Ausubel, F.M. 2003. *Caenorhabditis elegans* innate immune response triggered by *Salmonella enterica* requires intact LPS and is mediated by a MAPK signaling pathway. Curr Biol. **13**(1): 47-52.

Adams, P.B. and Ayers, W.A. 1979. Ecology of *Sclerotinia* species. Phytopathology **69**: 896-899.

Alegado, R.A., Campbell, M.C., Chen, W.C., Slutz, S.S., Tan, M.W. 2003. Characterization of mediators of microbial virulence and innate immunity using *Caenorhabditis elegans* host-pathogen model. Cell Microbiol. **5**: 435-444.

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**: 824-826.

Alvarez-Ortega, C. and Harwood, C.S. 2007. Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. Mol Microbiol. **65**(1): 153-65.

Anderson, K.S. and Winding, A. 2004. Non-target effects of bacterial biological control agents on soil protozoa. Biol Fertil Soils**. 40**: 230-236.

Arima, K., Imanaka, H., Kousaka, M., Fukuda, A., and Tamura, G. 1964. Pyrrolnitrin, a new antibiotic substance produced by *Pseudomonas*. Agr Biol Chem. **28**: 575-576.

Babalola, O.O. 2010. Beneficial bacteria of agricultural importance. Biotechnology letters. **32**(11): 1559-1570.

Bailey, D., Johnson, R.E. and Salvador, U.J. 1973. Pyrrole antibacterial agents. 1. Compounds related to pyoluteorin. J Med Chem. **16:** 1298-1300.

Baker, K.F. and Snyder, W.C. 1965. Ecology of soil-borne plant pathogens: prelude to biological control. University of California Press, Berkeley, CA, USA.

Baltz, R.H. 2009. Chapter 20. Biosynthesis and genetic engineering of lipopeptides in *Streptomyces roseosporus*. Methods Enzymol. **458**: 511-31.

Bangera, M.G. and Thomashow, L.S. 1999. Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. J Bacteriol. **181**: 3155-3163.

Bargmann, C.I., Hartwieg, E. and Horvitz, H.R. 1993. Odorant-selective genes and neurons mediate olfaction in *C. elegans*. Cell **74**: 515-527.

Bargmann, C.I. 2006. Chemosensation in *C. elegans*. WormBook. **25**: 1-29.

Bassler, B.L. 2002. Small talk: cell-to-cell communication in bacteria. Cell. **109**(4): 421-424.

Bates, D.M., Popescu, C.V., Khoroshilova, N., Vogt, K., Beinert, H., Münck, E. and Kiley P.J. 2000. Substitution of leucine 28 with histidine in the *Escherichia coli* transcription factor FNR results in increased stability of the $[4Fe\text{-}4S]^{2+}$ cluster to oxygen. J Biol Chem. **275**: 6234-6240.

Beale, E., Li, G., Tan, M.W. and Rumbaugh, K.P. 2006. *Caenorhabditis elegans* senses bacterial autoinducers. Appl Environ Microbiol. **72**(7): 5135-5137.

Becker, J.O. and Cook, R.J. 1988. Role of siderophores in suppression of *Pythium* species and production of increased growth response of wheat by fluorescent pseudomonads. Phytopathology. **78**: 778-782.

Beinert, H. and Kiley, P.J. 1999. Fe-S proteins in sensing and regulatory functions. Curr Opin Chem Biol. **3**(2): 152-7.

Bejerano-Sagie, M. and Xavier, K.B. 2007. The role of small RNAs in quorum sensing. Curr Opin Microbiol. **10**(2): 189-98.

Bender, C.L., Alarcon-Chaidez, F. and Gross, D.C. 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol Mol Biol Rev. **63**(2): 266-292.

Berg, J.M. 2002. Lectins Are Specific Carbohydrate-Binding Proteins. In Biochemistry 5th Edition.

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.

Berry, C.L., Brassinga, A.K.C., Donald, L.J., Fernando, W.G.D., Loewen, P.C. and de Kievit, T.R. 2012. Chemical and biological characterization of sclerosin, an antifungal lipopeptide. Can J Microbiol. **58**: 1027-1034.

Berry, C.L., Nandi, M., Manuel, J., Brassinga, A.K.C., Fernando, W.G.D., Loewen, P.C. and de Kievit, T.R. 2013. Characterization of the *Pseudomonas* sp DF41 quorum sensing locus and its role in fungal antagonism. Biological Control. **69**: 82-89.

Blumer, C., Heeb, S., Pessi, G. and Haas, D. 1999. Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc Natl Acad Sci. USA. **96**: 14073-14078.

Blumer, C. and Haas, D. 2000. Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch Microbiol. **173**(3): 170-177.

Brenner, S. 1973. The genetics of behaviour. British Medical Bulletin. **29**(3): 269-271.

Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. Genetics **77**: 71-94.

Britigan, B.E., Roeder, T.L., Rasmussen, G.T., Shasby, D.M., McCormick, M.L. and Cox CD. 1992. Interaction of the *Pseudomonas aeruginosa* secretory products pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to endothelial cells. Implications for *Pseudomonas* associated tissue injury. J Clin Invest **90**: 2187-2196.

Brodhagen, M., Henkels, M.D. and Loper, J.E. 2004. Positive autoregulation and signaling properties of pyoluteorin, an antibiotic produced by the biological control organism *Pseudomonas fluorescens* Pf-5. Applied and Environmental Microbiology. **70**(3): 1758-1766.

Burlinson, P., Studholme, D., Cambray-Young, J., Heavens, D., Rathjen, J., Hodgkin, J. *et al*. 2013. *Pseudomonas fluorescens* NZ17 repels grazing by C. elegans, a natural predator. ISME J. **7**: 1126-1138.

Canadian Phytopathological Society. 2013. Canadian Plant Disease Survey: Disease Highlights.

Carmi, R., Carmeli, S., Levy, E. and Gough, F.J. 1994. (+)-(S)-Dihydroaeruginoic acid, an inhibitor of *Septoria tritici* and other phytopathogenic fungi and bacteria, produced by *Pseudomonas fluorescens*. Journal of natural products. **57**(9): 1200-1205.

Castric, P.A. 1975. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. Can J Microbiol. **21**(5): 613-18.

Castric, P.A. 1977. Glycine metabolism by *Pseudomonas aeruginosa*: hydrogen cyanide biosynthesis. J Bacteriol. **130:** 826-831.

Castric, P.A. 1983. Hydrogen cyanide production by *Pseudomonas aeruginosa* at reduced oxygen levels. Can J Microbiol. **29**: 1344-1349.

Castric, P.A. 1994. Influence of oxygen on the *Pseudomonas aeruginosa* hydrogen cyanide synthase. Curr Microbiol. **29**: 19-21.

Cezairliyan, B., Vinayavekhin, N., Grenfell-Lee, D., Yuen, G.J., Saghatelian, A. and Ausubel, F.M. 2013. Identification of *Pseudomonas aeruginosa* phenazines that kill *Caenorhabditis elegans*. PloS Pathogens **9**(1): e1003101.

Chancey, S.T., Wood, D.W. and Pierson, L.S.,3rd. 1999. Two-component transcriptional regulation of N-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. Appl Environ Microbiol. **65**(6): 2294-2299.

Chatterjee, A., Cui, Y., Hasegawa, H. and 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. Appl Environ Microbiol. **73**(11): 3684-3694.

Chin-A-Woeng, T.F., Thomas-Oates, J.E., Lugtenberg, B.J. and Bloemberg, G.V. 2001. Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. Mol Plant Microbe Interact. **14**(8): 1006-1015.

Chin-A-Woeng, T.F., Bloemberg, G.V. and Lugtenberg, B.J.J. 2003. Phenazines and their role in biological control by *Pseudomonas* bacteria. New Phytologist **157**: 503-523.

Chin-A-Woeng, T.F., van den Broek, D., Lugtenberg, B.J.J. and Bloemberg, G.V. 2005. The *Pseudomonas chlororaphis* PCL1391 sigma regulator PsrA represses the production of the antifungal metabolite phenazine-1-carboxamide. Mol Plant-Microbe Interact. **18**: 244-253.

Cook, R. J. and Weller, D.M. 1987. Management of take-all in consecutive crops of wheat and barley, innovative approaches to plant disease control. Chet, I. (ed). Wiley, New York. pp 41-76.

Corbell, N. and Loper, J. 1995. A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. Journal of Bacteriology. **177**(21): 6230-6236.

Corno, G. and Jurgens, K. 2006. Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. Appl Environ Microbiol. **72**: 78-86.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. 1995. Microbial biofilms. Annu Rev Microbiol. **49**: 711-745.

Couillault, C. and Ewbank, J.J. 2012. Diverse bacteria are pathogens of *Caenorhabditis elegans*. Infect Immun. **70**: 4705-4707.

Cox, C.D., Rinehart, K.L., Moore, M.L. and Cook, J.C. 1981. Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. Proc Natl Acad Sci. **78**: 4256.

Cronin, D., Moenne-Loccoz, Y., Fenton, A., Dunne, C., Dowling, D.N. and O'Gara, F. 1997. Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol pseudomonad strain F113 with the potato cyst nematode *Globodera rostochiensis*. Appl Environ Microbiol. **63**: 1357-1361.

Dapa, T., Leuzzi, R., Ng, Y.K., Baban, S.T., Adamo, R., Kuehne, S.A. *et al*. 2013. Multiple factors modulate biofilm formation by the anaerobic pathogen *Clostridium difficile*. J Bacteriol. **195**: 545-555.

Darby, C., Cosma C.I., Thomas, J.H. and Manoil, C. 1999. Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. Prot Natl Acad Sci USA. **96**: 15202-7.

Darby, C., Hsu, J.W., Ghori, N. and Falkow, S. 2002. *Caenorhabditis elegans*: plague bacteria biofilm blocks food intake. Nature. **417**: 243-244.

Darby, C., Chakrabarti, A., Politz, S.M., Daniels, C.C., Tan, L., Drace, K. 2007. *Caenorhabditis elegans* mutants resistant to Yersinia biofilms. Genetics. **176**: 221-230.

Davey, M.E. and O'Toole, G.A. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev. **64**: 847-867.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. and Greenberg, E.P. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. **280**: 295-298.

Dawei, G., Liping, W. and Chengping, L. 2012. In vitro biofilm forming potential of *Streptococcus suis* isolated from human and swine in China. Braz J Microbiol. **43**: 993-1004.

de Bruijn, I. and Raaijmakers, J.M. 2009. Diversity and functional analysis of LuxR-type transcriptional regulators of cyclic lipopeptide biosynthesis in *Pseudomonas fluorescens*. Appl Environ Microbiol. **75**: 4753-4761.

de Kievit, T.R., Gillis, R.J., Marx, S., Brown, C. and Iglewski, B.H. 2001. Quorum sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. Appl Environ Microbiol. **67**: 1865-1873.

de Mesel, I., Derycke, S., Moens, T., Van Der Gucht, K., Vincx, M. and Swings, J. 2004. Top-down impact of bacterivorous nematodes on the bacterial community structure: a microcosm study. Environ Microbiol. **6**: 733-744.

de Souza, J.T., Arnould, C., Deulvot, C., Lemanceau, P., Gianinazzi-Pearson, V. and Raaijmakers, J.M. 2003. Effect of 2,4-diacetylphloroglucinol on *Pythium*: Cellular responses and variation in sensitivity among propagules and species. Biological Control. **93**(8): 966-975.

Debabrata, S. and Naik, P. 2000. Phloroglucinol enhances growth and rate of axillary shoot proliferation in potato shoot tip cultures in vitro. Plant Cell Tissue Organ Cult. **60**: 139-149.

Delaney, S.M., Mavrodi, D.V., Bonsall, R.F. and Thomashow, L.S. 2001. *phzO*, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30–84. J Bacteriol. **183**(1): 318-327.

Dietrich, L.E., Price-Whelan, A., Petersen, A., Whiteley, M. and Newman, D.K. 2006. The phenazine pyocyanin is a terminal signaling factor in the quorum sensing network of *Pseudomonas aeruginosa*. Molecular Microbiology. **61**: 1308-1321.

Drace, K. and Darby, C. 2008. The hmsHFRS operon of *Xenorhabdus nematophila* is required for biofilm attachment to *Caenorhabditis elegans*. Appl Environ Microbiol. **74**(14): 4509-15.

Drago, L. 2009. Bacteria and biofilm in respiratory tract infections. Infect Med. **17**(Suppl 2): 3-9.

Duffy, B.K. and Défago, G. 1997. Zinc improves biocontrol of *Fusarium* crown and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. Phytopathology. **87**: 1250-2157.

Fenton, A.M., Stephens, P.M., Crowley, J., O'Callaghan, M. and O'Gara, R. 1992. Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl Environ Microbiol. **58**: 3873-3878.

Fernando, W.G.D., Zhang, Y., Nakkeeran, S. and Savchuk, S. 2007. Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Prot. **26**: 100-107.

Finan, T.M., Kunkel, B., Vos, G.F.D. and Signer, E.R. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J Bacteriol. **167**: 66-72.

Flaishman, M.A., Eyal, Z., Zilberstein, A., Voisard, C. and Haas, D. 1996. Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producing strains of *Pseudomonas putida*. Mol Plant-Microbe Interact. **9**(7): 642-645.

Friman, V.P., Hiltunen, T., Laakso, J. and Kaitala, V. 2008. Availability of prey resources drives evolution of predator-prey interaction. Proc R Soc Lond B Biol Sci. **275**: 1625-1633.

Fuqua, W.C., Winans, S.C. and Greenberg, E.P. 1994. Quorum sensing in bacteria – the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol. **176**: 269-275.

Fuqua, C. and Greenberg, E.P. 1998. Self perception in bacteria: quorum sensing with acylated homoserine lactones. Curr Opin Microbiol. **1**(2): 183-9.

Fuqua, W.C. and Eberhard, A. 1999. Signal generation in autoinduction systems: synthesis of acylated homoserine lactones by LuxI-type proteins. G. Dunny, S.C. Winans (Eds.), Cell-Cell Communication in Bacteria, AMS Press, Washington. pp 211-230.

Galimand, M., Gamper, M., Zimmermann, A. and Haas, D. 1991. Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. J Bacteriol. **173**: 1598-1606.

Gallagher, L.A. and Manoil, C. 2001. *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. J Bacteriol. **183**(21): 6207-6214.

Girard, L.R., Fiedler, T.J., Harris, T.W., Carvalho, F., Antoshechkin, I., Han, M., *et al.* 2007. NematodeBook: the online review of *Caenorhabditis elegans* biology. Nucleic Acids Res. **35**: D472-D475.

Golden, J.W. and Riddle, D.L. 1984. The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature. Dev Biol. **102**: 368-378.

Goller, C., Wang, X., Itoh, Y. and Romeo, T. 2006. The cation-responsive protein NhaR of *Escherichia coli* activates *pgaABCD* transcription, required for production of the biofilm adhesion poly-β-1,6-N-acetyl-D-glucosamine. J Bacteriol. **188**: 8022-8032.

Gomez, W., Buela, L., Castro, L.T., Chaparro, V., Ball, M.M. and Yarzabal, L.A. 2010. Evidence for gluconic acid production by *Enterobacter intermedium* as an efficient strategy to avoid protozoan grazing. Soil Biol Biochem. **42**: 822-830.

González, J.M, Sherr, E.B. and Sherr, B.F. 1993. Differential feeding by marine flagellates on growing versus starving, and on motile versus nonmotile, bacterial prey. Mar Ecol Prog Ser. **102:** 257-267.

Griffiths, B.S. 1990. A comparison of microbial-feeding nematodes and protozoa in the rhizosphere of different plants. Biol Fertil Soils. **9**: 83-88.

Griffiths, B.S., Bonkowski, M., Dobson, G. and Caul, S. 1999. Changes in soil microbial community structure in the presence of microbial-feeding nematodes and protozoa. Pedobiologia. **43**: 297-304.

Haas, D. and Défago, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Review Microbiology, **4**: 307-319.

Haas, D. and Keel, C. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annu Rev Phytopathology. **41**: 117-153.

Hammer, P., Hill, D.S., Lam, S., van Pée, K-H. and Ligon, J. 1997. Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. Appl Environ Microbiol. **63**: 2147-2154.

Hammond, J.H., Dolben, E.F., Smith, T.J., Bhuju, S. and Hogan, D.A. 2015. Links between Anr and Quorum Sensing in *Pseudomonas aeruginosa* Biofilms. J Bacteriol. **197**(17): 2810-2820.

Hartmann, A., Gantner, S., Schuhegger, R., Steidle, A., Dürr, C., Schmid, M., *et al*. 2004. *N*-acyl-homoserine lactones of rhizosphere bacteria trigger systemic resistance in tomato plants. In Biology of Molecular Plant-Microbe Interactions. eds Lugtenberg, B., Tikhonovich, I. and Provorov, N. St. Paul, Minnesota: MPMI-Press, **4**: 554-556.

Hassan, H.M. and Fridovich, I. 1980. Mechanism of action of pyocyanin. J Bacteriol, **141**: 156-163.

Hausmann, K. 1978. Extrusive organelles in protists. Int Rev Cytol. **52**: 197-276.

Heeb, D. and Haas, D. 2001. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. Mol Plant Microbe Interact**. 14**: 1351-1363.

Heeb, D., Blumer, C. and Hass, D. 2002. Regulatory RNA as mediator in GacA/RsmA dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. J Bacteriol. **184**: 1046-1056.

Heeb, S., Valverde, C., Gigot-Bonnefoy, C. and Haas, D. 2005. Role of the stress sigma factor RpoS in GacA/RsmA-controlled secondary metabolism and resistance to oxidative stress in *Pseudomonas fluorescens* CHA0. FEMS Microbiology Letters. **243**: 251-258.

Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev. **66**: 373-395.

Herbert, R.B. and Holiman, F.G. 1969. Pigments of *Pseudomonas* spp. Part II. Structure of aeruginosin B. J Chem Soc C. **19**: 2517-2520.

Hernandez, M.E., Kappler, A. and Newman, D.K. 2004. Phenazines and other redox-active antibiotics promote microbial mineral reduction. Applied and Environmental Microbiology. **70**: 921-928.

Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A. and Miller, S.I. 2005. Animoglycoside antibiotics induce bacterial biofilm formation. Nature. **436**: 1171-1175.

Hope, I.A. 1999. *C. elegans*: A Practical Approach. Hames, B.D., ed., Oxford University Press, Oxford.

Hornby, D. 1983. Suppressive soils. Annual Review Phytopathology, **21**: 65-85.

House, B.L., Mortimer, M.W. and Kahn, M.L. 2004. New recombination methods for *Sinorhizobium meliloti* genetics. Appl Environ Microbiol. **70**: 2806-2815.

Howard, L.O. 1930. A history of applied entomology. Smithsonian Misc. Coll., **84**: 1-564.

Howell, C. and Stipanovic, R. 1980. Suspension of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology. **70**(8): 712-715.

Hrabak, E.M. and Willis, D.K. 1992. The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. J Bacteriol. **174**: 3011-3020.

Hu, H.B., Xu, Y.Q., Chen, F., Zhang, X.H. and HUR, B.K.I. 2005. Isolation and characterization of a new fluorescent *Pseudomonas* strain that produces both phenazine 1-carboxylic acid and pyoluteorin. Journal of microbiology and biotechnology. **15**(1): 86-90.

Hughes, D.T. and Sperandio, V. 2008. Inter-kingdom signalling: communication between bacteria and their hosts. Nat Rev Microbiol. **6**: 111-120.

Ibrahim, S.A., Crack, J.C., Rolfe, M.D., Borrero-de Acuña, J.M., Thomson, A.J., Le Brun, N.E., Schobert, M., Stapleton, M.R. and Green, J. 2015. Three Pseudomonas putida FNR Family Proteins with Different Sensitivities to O_2. J Biol Chem. **290**(27): 16812-23.

Insunza, V., Alstrom, S. and Eriksson, K.B. 2002. Root bacteria from nematicidal plants and their biocontrol potential against trichodorid nematodes in potato. Plant Soil. **24**1(2): 271-278.

Iriberri, J., Azúa, I., Labirua-Iturburu, A., Artolozaga, I. and Barcina, I. 1994. Differential elimination of enteric bacteria by protists in a freshwater system. J Appl Microbiol. **77**(5): 476-483.

Itoh, Y., Wang, X., Hinnebusch, B.J., Preston, J.F., 3rd, Romeo, T. 2005. Depolymerization of beta-1,6-N-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. J Bacteriol. **187**: 382-387.

Itoh, Y., Rice, J.D., Goller, C., Pannuri, A., Taylor, J., Meisner, J. *et al*. 2008. Roles of *pgaABCD* genes in synthesis, modification, and export of the *Escherichia coli* biofilm adhesion poly-β-1,6-*N*-acetyl-D-glucosamine. J Bacteriol. **190**: 3670-3680.

Jackson, D.W., Suzuki, K., Oakford, L., Simecka, J.W., Hart, M.E. and Romeo, T. 2002. Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. J Bacteriol. **184**: 290-301.

Jervis, A.J., Crack, J.C., White, G., Artymiuk, P.J., Cheesman, M.R., Thomson, A.J., Le Brun, N.E. and Green, J. 2009. The O_2 sensitivity of the transcription factor FNR is controlled by Ser24 modulating the kinetics of the [4Fe-4S] to [2Fe-2S] cluster conversion. Proc Natl Acad Sci. USA. **106**: 4659-4664.

Jeschke, J.M., Kopp, M. and Tollrian, R. 2002. Predator functional responses: discriminating between handling and digesting prey. Ecol Monogr. **72**: 95-112.

Jordan, P.A., Thomson, A.J., Ralph, E.T., Guest, J.R. and Green, J. 1997. FNR is a direct oxygen sensor having a biphasic response curve. FEBS Letter. **416:** 349-352.

Joshua, G.W., Karlyshev, A.V., Smith, M.P., Isherwood, K.E., Titball, R.W. and Wren, B.W. 2003. A *Caenorhabditis elegans* model of Yersinia infection: Biofilm formation on a biotic surface. Microbiology. **149**: 3221-3229.

Jousset, A., Lara, E., Wall, L.G. and Valverde, C. 2006. Secondary metabolites help biocontrol strain *Pseudomonas fluorescens* CHA0 to escape protozoan grazing. Appl Environ Microbiol. **72**: 7083-7090.

Jousset, A., Rochat, L., Péchy-Tarr, M., Keel, C., Scheu, S. and Bonkowski, M. 2009. Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters. ISME J. **3**: 666-674.

Jousset, A., Rochat, L., Scheu, S., Bonkowski, M. and Keel, C. 2010. Predator–prey chemical warfare determines the expression of antifungal genes by rhizosphere pseudomonads. Appl Environ Microbiol. **76**: 5263-5268.

Junaid, J.M., Dar, N.A., Bhat, T.A., Bhat, A.H. and Bhat, M.A. 2013. Commercial biocontrol agents and their mechanism of action in the management of plant pathogens. International Journal of Modern Plant & Animal Sciences, **1**(2): 39-57.

Kay, E., Dubuis, C. and Haas, D. 2005. Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0. Proc Natl Acad Sci USA. **102**: 17136-17141.

Kay, E., Humair, B., Dénervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C. and Haas, D. 2006. Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. J Bacteriol. **188**: 6026-6033.

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D. and Défago, G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Mol Plant-Microbe Interact, **5**: 4-13.

Kempf, B. and Bremer, E. 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch Microbiol. **170**: 319-330.

Kerr, J.R., Taylor, G.W., Rutman, A., Høiby, N, Cole, P.J. and Wilson, R. 1999. Pseudomonas aeruginosa pyocyanin and 1-hydroxyphenazine inhibit fungal growth. J Clin Pathol. **52**(5): 385-387.

King, E.O., Ward, M.K. and De, R. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Med. **44**(2): 301-307.

Kippert, F., Saunders, D.S. and Blaxter, M.L. 2002. *Caenorhabditis elegans* has a circadian clock. Curr Biol. **12**(2): R47-9.

Kloepper, J.W. and Schroth, M.N. Plant growth-promoting rhizobacteria in radish. 1978. In: Proc 4th International conference on plant pathogenic bacteria. Gilbert-Clarey, Tours, France, pp 879-882.

Kloepper, J.W., Leong, J., Teintze, M. and Schroth, M.N. 1980. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature. **286**: 885-886.

Knowles, C.J. 1976. Microorganisms and cyanide. Bacteriol Rev. **40**(3): 652-80.

Koch, B., Nielson, T.H., Sorenson, P., Anderson, J.B., Christopherson, C., Molin, S., Givskov, M., Sorenson, J. and Nybroe, O. 2002. Lipopeptide production in *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudates via the Gac two-component regulatory system. Appl Environ Microbiol. **68**: 4509-4516.

Kojic, M. and Venturi, V. 2001. Regulation of *rpoS* gene expression in *Pseudomonas:* involvement of a TetR family regulator. J Bacteriol. **183**: 3712-3720.

Kojic, M., Aguilar, C. and Venturi, V. 2002. TetR family member PsrA directly binds the *Pseudomonas rpoS* and *psrA* promoters. J Bacteriol. **184**: 2324-2330.

Körner, H., Sofia, H.J. and Zumft, W.G. 2003. Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. FEMS Microbiol Rev. **27**: 559-592.

Kumar, N.R., Arasu, V.T. and Gunasekaran, P. 2005. Genetic diversity of plant growth promoting fluorescent pseudomonads: PCR-RAPD, ARDRA and rDNA sequence analysis. Indian J experimental Biol. **43**: 84-89.

Kutcher, H.R., Dokken-Bouchard, F., Turkington, T.K., Fernando, W.G.D., Bullock, P., McLaren, D.L., Boyetchko, S., Buchwaldt, L., Hegedus, D. and Parkin, I. In: Managing sclerotinia stem rot in canola, Canola Research Summit, April 12-13, 2011. Winnipeg, MB, Canada.

Lainhart, W., Stolfa, G. and Koudelka, G.B. 2009. Shiga toxin as a bacterial defense against a eukaryotic predator*, Tetrahymena thermophila*. J Bacteriol**. 191**: 5116-5122.

Lambden, P.R. and Guest, J.R. 1976. Mutants of *Escherichia coli* K12 unable to use fumarate as an anaerobic electron acceptor. J Gen Microbiol. **97**(2): 145-60.

Lapouge, K., Schubert, M., Allain, F.H. and Haas, D. 2008. Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behaviour. Mol Microbiol. **67**(2): 241-253.

Latifi, A., Winson, M.K., Foglino, M., Bycroft, B.W., Stewart, G.S., Lazdunski, A. and Williams, P. 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Mol Microbiol. **17**(2): 333-343.

Latifi, A., Foglino, M., Tanaka, M., Williams, P. and Ladzdunski, A. 1996. A hierarchial quorum-sensing cascade in *Peudomonas aeruginosa* links the transcriptional activators LasR and

RhlR (VsmR) to expression of the stationary phase sigma factor RpoS. Mol Microbiol. **21**: 1137-1146.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G. and Haas, D. 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc Natl Acad Sci. USA. **89**(5): 1562-1566.

Laville, J., Blumer, C., Von Schroetter, C., Gaia, V., Défago, G., Keel, C. and Haas, D. 1998. Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. J Bacteriol. **180**(12): 3187-96.

Lee, J.H., Ma, K.C., Ko, S.J., Kang, B.R., Kim, I.S. and Kim, Y.C. 2011. Nematicidal activity of a nonpathogenic biocontrol bacterium, *Pseudomonas chlororaphis* O6. Curr Microbiol. 2011 **62**(3): 746-51.

Levy, E., Gough, F.J., Berlin, K.D., Guiana, P.W. and Smith, J.T. 1992. Inhibition of *Septoria tritici* and other phytopathogenic fungi and bacteria by *Pseudomonas fluorescens* and its antibiotics. Plant Pathol. **41**: 335-341.

Licciardello, G., Bertani, I., Steindler, L., Bella, P., Venturi, V. and Catara, V. 2009. The transcriptional activator *rfiA* is quorum-sensing regulated by cotranscription with the *luxI* homolog *pcoI* and is essential for plant virulence in *Pseudomonas corrugata*. Mol Plant Microbe Interact. **22**(12): 1514-22.

Ligon, J.M., Hill, D.S., Hammer, P.E., Torkewitz, N.R., Hofmann, D., Kempf, H.J. and van Pée, K.H. 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. Pest Manage Sci. **56**: 688-695.

Lim, H.S., Kim, Y.S. and Kim, S.D. 1991. *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism against *Fusarium solani*, an agent of plant root rot. Appl Environ Microbiol. **57**(2): 510-516.

Linares, J.F., Gustafsson, I., Baquero, F. and Martinez, J.L. 2006. Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci USA. **103**: 19484-19489.

Lindblom, T.H. and Dodd, A.K. 2006. Xenobiotic detoxification in the nematode *Caenorhabditis elegans*. J Exp Zool A Comp Exp Biol. **305**(9): 720-30.

Ling, E.A, Ellison, M.L. and Pesci, E.C. 2009. A novel plasmid for detection of *N*-acyl homoserine lactones. Plasmid. **62**: 16-21.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. **25**(4): 402-408.

Lively, D.H., Gorman, M., Haney, M. E. and Mabe, J.A. 1967. Metabolism of tryptophans by *Pseudomonas aurofaciens*. I. Biosynthesis of pyrrolnitrin. Antimicrobial Agents and Chemotherapy-1966, p. 462-469.

Loewen, P.C., Hu, B., Strutinsky, J. and Sparling, R. 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. Can J Microbiol. **44**: 707-717.

Lorck, H. 1948. Production of hydrocyanic acid by bacteria. Physiol Plant. **1**: 142-146.

Lu, C.D., Winteler, H., Abdelal, A. and Haas, D. 1999. The ArgR regulatory protein, a helper to the anaerobic regulator ANR during transcriptional activation of the *arcD* promoter in *Pseudomonas aeruginosa*. J Bacteriol. **181**: 2459-2464.

Lugtenberg, B.J.J, Dekkers, L. and Bloemberg, G.V. 2001. Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annual Review Phytopathology, **39**: 461-490.

Maddula, V.S.R.K., Pierson, E.A. and Pierson, L.S. III. (2008). Altering the ratio of phenazines in *Pseudomonas chlororaphis* (*aureofaciens*) strain 30-84, effects on biofilm formation and pathogen inhibition. J Bacteriol. **190**: 2759-2766.

Magazin, M.D., Moores, J.C. and Leong, J. 1986. Cloning of the gene coding for the outer membrane receptor protein for ferric pseudobactin, a siderophore from a plant growth-promoting *Pseudomonas* strain. J Biol Chem. **261**: 795-799.

Manitoba Agriculture, FARI. 2002. Pest Management Plant Disease. *Sclerotinia*. From http://www.gov.mb.ca/agrigulture/crops/diseases/fac07s00.html

Manuel, J., Berry, C., Selin, C., Fernando, W.G.D. and de Kievit, T.R. 2011. Repression of the antifungal activity of *Pseudomonas* sp. strain DF41 by the stringent response. Appl Environ Microbiol. **77**(16): 5635-5642.

Manuel, J., Selin, C., Fernando, W.G.D. and de Kievit, T.R. 2012. Stringent response mutants of *Pseudomonas chlororaphis* PA23 exhibit enhanced antifungal activity against *Sclerotinia sclerotiorum* in vitro. Microbiology. **158**(Pt 1): 207-216.

Maraheil, M.A., Stachelhaus, T. and Mootz, H.D. 1997. Modular Peptide Synthetases Involved in Nonribosomal Peptide Synthesis. Chem Rev. **97**(7): 2651-2674.

Marketon, M.M., Gronquist, M.R., Eberhard, A. and González, J.E. 2002. Characterization of the *Sinorhizobium meliloti sinR*/*sinI* locus and the production of novel N-acyl homoserine lactones. J Bacteriol. **184**(20): 5686-95.

Matz, C., Bergfeld, T., Rice, S.A. and Kjelleberg, S. 2004. Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environ Microbiol. **6**: 218-226.

Matz, C. and Jurgens, K. 2001. Effects of hydrophobic and electrostatic cell surface properties of bacteria on feeding rates of heterotrophic nanoflagellates. Appl Environ Microbiol. **67**(2): 814-20.

Matz, C. and Jurgens, K. 2005. High motility reduces grazing mortality of planktonic bacteria. Appl Environ Microbiol. **71**: 921-929.

Maurhofer, M., Keel, C., Schnider, U., Voisard, C., Haas, D. and Defago, G. 1992. Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. Phytopathology. **82**(2): 190-195.

Maurhofer, M., Keel, C., Haas, D. and Defago, G. 1994. Pyoluteorin production by *Pseudomonas fluorescens* strain CHA0 is involved in the suppression of *Pythium* damping-off of cress but not of cucumber. European Journal of Plant Pathology. **100**(3): 221-232.

Mavrodi, D.V., Blankenfeldt, W., Thomashow, L.S. and Mentel, M. 2006. Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. Annual review of Phytopathology. **44**: 417-445.

Mazzola, M., de Bruijn, I., Cohen, M.F. and Raaijmakers, J.M. 2009. Protozoan-induced regulation of cyclic lipopeptide biosynthesis is an effective predation defense mechanism for *Pseudomonas fluorescens*. Appl Environ Microbiol. **75**: 6804-6811.

Meyer, J.M. and Abdallah, M.A. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. J Gen Microbiol. **107**: 319-328.

Meyer, J.M., Stintzi, A., Coulanges, V., Shivaji, S., Voss, J.A., Taraz, K. and Budzikiewic, H. 1998. Siderotyping of fluorescent pseudomonads: characterization of pyoverdines of *Pseudomonas fluorescens* and *Pseudomonas putida* strains from Antarctica. Microbiology. **144**: 3119-3126.

Meyer, S.L., Halbrendt J.M., Carta, L.K., Skantar, A.M., Liu, T., Abdelnabby, H.M.E., *et al*. 2009. Toxicity of 2,4-diacetylphloroglucinol (DAPG) to plant-parasitic and bacterial-feeding nematodes. J Nematodol. **41**: 274-280.

Michelsen, C.F. and Stougaard, P. 2012. Hydrogen cyanide synthesis and antifungal activity of the biocontrol strain *Pseudomonas fluorescens* In5 from Greenland is highly dependent on growth medium. Can J Microbiol. **58**: 381-390.

Miller, J.H. 1972. Assay of β-galactosidase. Experiments in Molecular Genetics, pp. 352 355. Cold Spring Harbor NY, Cold Spring, Harbor Laboratory Press.

Mohapatra, B.R. and Fukami, K. 2007. Chemical detection of prey bacteria by the marine heterotrophic nanoflagellate *Jakoba libera*. Basic Appl Ecol. **8**: 475-481.

Müller, M.S., Scheu, S. and Jousset, A. 2013. Protozoa drive the dynamics of culturable biocontrol bacterial communities. PLOS ONE. **8**(6): e66200.

Nagarajkumar, M., Bhaskaran, R. and Velazhahan, R. 2004. Involvement of secondary metabolites and extracellular lytic enzymes produced by Pseudomonas fluorescens in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. Microbiol Res. **159**(1): 73-81.

Nandi, M., Selin, C., Brassinga, A.K.C., Belmonte, M.F., Fernando, W.G.D., Loewen, P.C. and de Kievit, T.R. 2015. Pyrrolnitrin and hydrogen cyanide production by *Pseudomonas chlororaphis* strain PA23 exhibits nematicidal and repellent activity against *Caenorhabditis elegans*. PloSOne/doi:10.1371/Journal.poine.0123184.

Neidig, N., Paul, R., Scheu, S. and Jousset, A. 2011. Secondary metabolites of *Pseudomonas fluorescens* CHA0 drive complex non-trophic interactions with bacterivorous nematodes. Microb Ecol. **61**: 853-859.

Neilands, J.B. 1981. Microbial iron compounds. Annu Rev Biochem. **50**: 715-731.

Nelson, B. 1998. Biology of *Sclerotinia*. In: Proceedings of the 10th International Sclerotinia workshop, 21 January 1998. North Dakota State University, Department of Plant Pathology, Fargo, ND, USA. pp. 1-5.

Nielsen, T.H., Thrane, C., Christophersen, C., Anthoni, U. and Sørensen, J. 2000. Structure, production characteristics and fungal antagonism of tensin - a new antifungal cyclic lipopeptide from *Pseudomonas fluorescens* strain 96.578. J Appl Microbiol. **89**(6): 992-1001.

Nowak-Thompson, B., Chaney, N., Wing, J.S., Gould, S.J. and Loper, J.E. 1999. Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. Journal of Bacteriology. **181**(7): 2166-2174.

Nybroe, O. and Sorensen, J. 2004. Production of cyclic lipopeptides by fluorescent pseudomonads. *Pseudomonas*, Biosynthesis of Macromolecules and Molecular Metabolism. J.-L. Ramos. New York, U.S.A., Kluwer Academic/Plenum Publishers**:** 147-172.

Oh, S.A., Kim, J.S., Han, S.H., Park, J.Y., Dimkpa, C., Edlund, C., Anderson, A.J. and Kim, Y.C. 2013. The GacS-regulated sigma factor RpoS governs production of several factors involved in biocontrol activity of the rhizobium *Pseudomonas chlororaphis* O6. Can J Microbiol. **59**: 556-562.

O'Loughlin, C.T., Miller L.C., Siryaporn, A., Drescher, K., Semmelhack, M.F. and Bassler, B.L. 2013. A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. Proc Natl Acad Sci U S A. **110**(44): 17981-6.

Paciello, I., Silipo, A., Lembo-Fazio, L., Curcurù, L., Zumsteg, A., Noël, G., Ciancarella, V., Sturiale, L., Molinaro, A. and Bernardini, M.L. 2013. Intracellular Shigella remodels its LPS to

dampen the innate immune recognition and evade inflammasome activation. Proc Natl Acad Sci. U S A. **110**(46): E4345-54.

Pal, K.K., Tilak, K.V.B.R., Saxena, A.K., Dey, R. and Singh, C.S. 2000. Antifungal characteristics of a fluorescent Pseudomonas strain involved in the biological control of *Rhizoctonia solani*. Microbiol Res. **155**(3): 233-242.

Pal, K.K. and Gardener, B.M.S. 2006. Biological control of plant pathogens. The Plant Health Instructor. **2**: 1117-1142.

Palleroni, N.J. 2008. The road to the taxonomy of *Pseudomonas.* In: *Pseudomonas*: Genomics and Molecular Biology, pp. 1–18. ed. Cornelis, P. Caister Acad Press, Hethersett, UK.

Paulin, M.M., Novinscak, A., St-Arnaud, M., Goyer, C., DeCoste, N.J., Privé, J.-P., *et al*. 2009. Transcriptional activity of antifungal metabolite-encoding genes *phlD* and *hcnBC* in *Pseudomonas* spp. using qRT-PCR. FEMS Microbiol Ecol. **68**(2): 212-222.

Pauwelyn, E, Huang, C.J., Ongena, M., Leclère, V., Jacques, P., Bleyaert, P., Budzikiewicz, H., Schäfer, M. and Höfte, M. 2013. New linear lipopeptides produced by *Pseudomonas cichorii* SF1-54 are involved in virulence, swarming motility, and biofilm formation. Mol Plant Microbe Interact. **26**(5): 585-98.

Pearson, J.P., Van Delden, C. and Iglewski, B.H. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J Bacteriol. **181**: 1203-1210.

Pedersen, A., Nybroe, O., Winding, A., Ekelund, F. and Bjørnlund, L. 2009. Bacterial feeders, the nematode *Caenorhabditis elegans* and the flagellate Cercomonas longicauda, have different effects on outcome of competition among the *Pseudomonas* biocontrol strains CHA0 and DSS73. Microb Ecol. **57**: 501-509.

Pernthaler, J. 2005. Predation on prokaryotes in the water column and its ecological implications. Nat Rev Microbiol. **3**: 537-546.

Pertot, I. How to develop a biofungicide based on a bacterial strain: the main steps for turning your discover into a plant protection product. In: 10th International PGPR Workshop, June 16 – 19, 2015. Liege, Belgium, Europe.

Pertot, I., Alabouvette, C., Esteve, E.H. and Franca, S. 2015. Mini-paper: The use of microbial biocontrol agents against soil-borne diseases. eip-agri Agriculture & Innovation, funded by European Commission.

Pessi, G. and 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*. J Bacteriol. **182:** 6940-6949.

Pessi, G. and Haas, D. 2004. Cyanogenesis, p. 671-687. In J. L. Ramos (ed.), *Pseudomonas*: biosynthesis of macromolecules and molecular metabolism, **3**: Kluwer Academic Publishers, Dordrecht, Netherlands.

Pfender W.F., Kraus, J. and Loper, J.E. 1993. A genomic region from *Pseudomonas fluorescens* Pf-5 required for pyrrolnitrin production and inhibition of *Pyrenophora tritici-repentis* in wheat straw. Phytopathology. **83**: 1223-1228.

Pierce-Shimomura, J.T., Morse, T.M. and Lockery, S.R. 1999. The fundamental role of pirouettes in *Caenorhabditis elegans* chemotaxis. J Neurosci. **19**: 9557-9569.

Pierson, L.S. III and Pierson, E.A. 2010. Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. Appl Microbiol Biotechnol, **86**: 1659-1670.

Poritsanos, N.J. 2005. 'Molecular Mechanisms Involved in the Secondary Metabolite Production and Biocontrol of Pseudomonas chlororaphis PA23', M.Sc. Thesis, University of Manitoba.

Poritsanos, N., Selin, C., Fernando, W.G.D., Nakkeeran, S. and 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. Can J Microbiol. **52**(12): 1177-1188.

Potrykus, K. and Cashel, M. 2008. (p)ppGpp: still magical? Annu Rev Microbiol. **62**: 35-51.

Pradel, E., Zhang, Y., Pujol, N., Matsuyama, T., Bargmann, C.I. and Ewbank, J.J. 2007. Detection and avoidance of a natural product from the pathogenic bacterium *Serratia marcescens* by *Caenorhabditis elegans*. Proc Natl Acad Sci USA**. 104**: 2295-2300.

Preston, M.J., Seed, P.C., Toder, D.S., Iglewski, B.H., Ohman, D.E., Gustin, J.K., Goldberg, J.B. and Pier, G.B. 1997. Contribution of proteases and LasR to the virulence of *Pseudomonas aeruginosa* during corneal infections. Infect Immun. **65**: 3086-3090.

Purdy, L.H. 1979. *Sclerotinia sclerotiorum*: history, disease and symptomatology, host range, geographic distribution and impact. Phytopathology **69**: 875-880.

Quadri, L.E., Keating, T.A., Patel, H.M. and Walsh, C.T. 1999. Assembly of the *Pseudomonas aeruginosa* nonribosomal peptide siderophore pyochelin: *In vitro* reconstitution of aryl-4,2-bisthiazoline synthetase activity from PchD, PchE, and PchF. Biochemistry**. 38**: 14941-14954.

Queck, S.Y., Weitere, M., Moreno, A.M., Rice, S.A. and Kjelleberg, S. 2006. The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. Environ Microbiol. **8**: 1017-1025.

Raaijmakers, J.M., Leeman, M., van Oorschot, M.M.P, Sluis, I.V, Shchipper, B. and Bakker, P.A.H.M. 1995. Dose response relationship in biological control of *Fusarium* wild of radish by *Pseudomonas* spp. Phytopathology. **85**: 1075-1081.

Raaijmakers, J.M., Weller, D.M. and Thomashow, L.S. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl Environ Microbiol, **63**: 881–887.
Ran, H., Hassett, D.J. and Lau, G.W. Human targets of *Pseudomonas aeruginosa* pyocyanin. Proc Natl Acad. Sci USA. **100**: 14315-14320.

Raaijmakers, J.M. and Weller, D.M. 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. Mol Plant-Microbe Interact. **11**:144-152.

Raaijmakers, J.M., de Bruijn, I. and de Kock, M.J. 2006. Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. Mol Plant Microbe Interact. **19**(7): 699-710.

Raaijmakers, J.M., de Bruijn, I., Nybroe, O. and Ongena, M. 2010. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. Fems Microbiol Rev. **34**(6): 1037-62.

Ramarathnam, R., de Kievit, T.R. and Fernando, W.G.D. 2011. The role of antibiosis and induced systemic resistance, mediated by strains of *Pseudomonas chlororaphis*, *Bacillus cereus* and *B. amyloliquefaciens*, in controlling blackleg disease of canola. Biocontrol. **56**: 225-235.

Ramette, A., Frapolli, M., Defago, G. and Moenne-Loccoz, Y. 2003. Phylogeny of HCN synthase-encoding hcnBC genes in biocontrol fluorescent pseudomonads and its relationship with host plant species and HCN synthesis ability. Mol Plant-Microbe Interact. **16**(6): 525-535.

Ra'oof, W.M. and Latif, I.A.R. 2010. *In vitro* study of the swarming phenomena and antimicrobial activity of pyocyanin produced by *Pseudomonas aeruginosa* isolated from different human infections. Eur J Sci Res. **47**: 405-421.

Reimmann, C., Ginet, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., Zala, M., Heurlier, K., Triandafillu, K., Harms, H, Défago, G. and Haas, D. 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. Microbiology. **148**: 923-932.

Reimmann, C., Valverde, C., Kay, E. and Haas, D. 2005. Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHA0. J Bacteriol. **187**: 276-285.

Rezzonico, F., Zala, M., Keel, C., Duffy, B., Moënne-Loccoz, Y. and Défago, G. 2007. Is the ability of biocontrol fluorescent pseudomonads to produce the antifungal metabolite 2,4-diacetylphloroglucinol really synonymous with higher plant protection? New Phytol. **173**(4): 861-872.

Romanowski, A., Migliori, M.L., Valverde, C. and Golombek, D.A. 2011. Circadian variation in *Pseudomonas fluorescens* (CHA0)-mediated paralysis of *Caenorhabditis elegans*. Microb Pathog. **50**: 23-30.

Rosenberg, K., Bertaux, J., Scheu, S. and Bonkowski, M. 2009. Soil amoeba rapidly change bacterial community composition in *Arabidopsis thaliana* rhizosphere. ISME J. **3**: 675-684.

Saigusa, T., Ishizaki, S., Watabiki, S., Ishii, N., Tanakadate, A., Tamai, Y. and Hasegawa, K. 2002. Circadian behavioural rhythm in *Caenorhabditis elegans*. Curr Biol. **12(**2): R46-7.

Sakaguchi, M., Murakami, H. and Suzaki, T. 2001. Involvement of a 40-kDa glycoprotein in food recognition, prey capture, and induction of phagocytosis in the protozoon *Actinophrys sol*. Protist. 152(1): 33-41.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Press, New York.

Sára, M. and Sleytr, U.B. 2000. S-Layer proteins. J Bacteriol. **182**(4): 859-68.

Sarniguet, A., Kraus, J., Henkels, M.D., Muehlchen, A.M. and Loper, J.E. 1995. The sigma factor σ^s affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. Proc Natl Acad Sci. USA **92**: 12255-1259.

Saskatchewan Ministry of Agriculture. 2013. Guide to Crop Protection: weeds, plant diseases, insects. Saskatchewan Ministry of Agriculture, Saskatchewan.

Savanat, T. and Pavillard, E.R. 1964. The ability of *Amoeba proteus* to kill *Salmonella enteritidis* introduced by micro-injection, and the influence of opsonins on intracellular killing. Aust J Exp Biol Med Sci. **42**: 615-24.

Savchuk, S. and Fernando, W.G.D. 2004. Effect of timing of application and population dynamics on the degree of biological control of *Sclerotinia sclerotiorum* by bacterial antagonists. FEMS Microbiol Ecol. **49**(3): 379-388.

Sawers, R.G. 1991. Identification and molecular characterization of a transcriptional regulator from *Pseudomonas aeruginosa* PAO1 exhibiting structural and functional similarity to the FNR protein of *Escherichia coli.* Mol Microbiol. **5:** 1469-1481.

Schroth, M.N. and Hancock, J.G. 1982. Selected topics in biological control. Annual Review Microbiology, **35**: 453-467.

Schuster, M. and Greenberg, E.P. 2006. A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. Int J Med Microbiol. **296(**2-3): 73-81.

Selin, C., Habibian, R., Poritsanos, N., Athukorala, S.N., Fernando, W.G.D. and 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 Microbiol Ecol. **71**(1): 73-83.

Selin, C., Fernando, W.G.D. and 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**: 896-907.

Selin, C., Manuel, J., Fernando, W.G.D. and de Kievit, T.R. 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.

Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N., Palmer, A.E. and Tsien, R.Y. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. Nat Biotechnol. **22**(12): 1567-1572.

Shanmugaiah, V., Mathivanan, N. and Varghese, B. 2010. Purification, crystal structure and antimicrobial activity of phenazine-1-carboxamide produced by a growth-promoting biocontrol bacterium, *Pseudomonas aeruginosa* MML2212. J Appl Microbiol. **108**(2): 703-11.

Sharifi-Tehrani, A., Zala, M., Natsch, A., Moenne-Loccoz, Y. and Defago, G. 1998. Biocontrol of soil-borne fungal plant diseases by 2,4- diacetylphloroglucinol-producing fluorescent pseudomonads with different restriction profiles of amplified 16S rDNA. Eur J Plant Pathol. **104**: 631-643.

Shen, X., Chen, M., Hu, H., Wang, W., Peng, H., Xu, P. and Zhang, X. 2012. Genome sequence of *Pseudomonas chlororaphis* GP72, a root-colonizing biocontrol strain. J Bacteriol. **194**(5): 1269-1270.

Shrout, J.D., Chopp, D.L., Just, C.L., Hentzer, M., Givskov, M. and Parsek, M.R. 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* is nutritionally conditional. Mol Microbiol. **62**: 1264-1277.

Siddiqui, I.A., Haas, D. and Heeb, S. 2005. Extracellular protease of *Pseudomonas fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode *Meloidogyne incognita*. Appl Environ Microbiol. **71**(9): 5646-5649.

Solomonson, L.P. 1981. Cyanide as a metabolic inhibitor. p. 11–28. *In* Vennesland, B., Conn, E. E., Knowles, C. J., Westley, J. and Wissing, F. (ed.), Cyanide in biology. Academic Press Ltd., London, England.

Song, C., Mazzola, M., Cheng, X., Oetjen, J., Alexandrov, T., Dorrestein, P., Watrous, J., van der Voort, M. and Raaijmakers, J.M. 2015. Molecular and chemical dialogues in bacteria-protozoa interactions. Sci Rep. **5**: 12837.

Spencer, M., Ryu, C.-M., Yang, K.-Y., Kim, Y.C., Kloepper, J.W. and Anderson, A.J. 2003. Induced defence in tobacco by *Pseudomonas chlororaphis* strain O6 involves at least the ethylene pathway. Physiol Mol Plant Pathol. **63**(1): 27-34.

Spiro, S. 1994. The FNR family of transcriptional regulators. Antonie Leeuwenhoek. **66**: 23–36.
Steiner, U.K. 2007. Investment in defense and cost of predator-induced defense along a resource gradient. Oecologia. **152**: 201-210.

Stintzi, A., Cornelis, P., Hohnadel, D., Meyer, J.M., Dean, C., Poole, K., Kourambas, S. and Krishnapillai, V. 1996. Novel pyoverdine biosynthesis gene(s) of *Pseudomonas aeruginosa* PAO. Microbiology**. 142**: 1181-1190.

Stupp, G.S., von Reuss, S.H., Izrayelit, Y., Ajredini, R., Schroeder, F.C. and Edison, A.S. 2013. Chemical detoxification of small molecules by *C. elegans*. ACS Chem Biol. **8**(2): 309-13.

Stutz, E., Défago, G. and Kern, H. 1986. Naturally occurring fluorescent pseudomonad involved in suppression of black root rot of tobacco. Phytopathology. **76**: 181-185.

Suh, S.-J., Silo-Suh, L., Woods, D.E., Hasset, D.J., West, S.E.H. and Ohman, D.E. 1999. Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. J Bacteriol. **181**: 3890-3897.

Tan M.W., Mahajan-Miklos, S. and Ausubel, F.M. 1999. Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc Natl Acad Sci USA. **96**: 715-720.

Tan, L. and Darby, C. 2004. A moveable surface: formation of *Yersinia* sp. biofilms on motile *Caenorhabditis elegans*. J Bacteriol. **186**: 5087-5092.

Tarao, M., Jezbera, J. and Hahn, M.W. 2009. Involvement of cell surface structures in size-independent grazing resistance of freshwater actinobacteria. Appl Environ Microbiol. **75**: 4720-4726.

Tavakol-Norabadi, M., Sahebani, N. and Etebarian, H.R. 2013. Biological control of root-knot nematode (*Meloidogyne javanica*) disease by *Pseudomonas fluorescens* (CHA0). Archive of Phytopathology and Plant Protection, **47**: 615-621.

Tawara, S., Matsumoto, S., Hirose, T., Matsumoto, Y., Nakamoto, S., Mitsuno, M. and Kamimura, T. 1989. In vitro antifungal synergism between pyrrolnitrin and clotrimazole. Jpn J Med Mycol. **30**: 202-210.

Taylor, W.D. 1978. Growth responses of ciliate protozoa to the abundance of their bacterial prey. Microb Ecol. **4**: 207-214.

Tekeda, R. 1958. *Pseudomonas* pigments: I. Pyoluteorin, a new chlorine-containing pigment produced by Pseudomonas aeruginosa. Hakko Kgaku Zasshi. **36**: 281-290.

Tribelli, P.M., Hay, A.G. and López, N.I. 2013. The Global Anaerobic Regulator Anr, Is Involved in Cell Attachment and Aggregation Influencing the First Stages of Biofilm Development in *Pseudomonas extremaustralis*. PLoS One. **8**(10): e76685.

Tripathi, R.K. and Gottlieb, D. 1969. Mechanism of action of the antifungal antibiotic pyrrolnitrin. J Bacteriol. **100**(1): 310-318.

Troemel, E.R., Kimmel, B.E. and Bargmann, C.I. 1997. Reprogramming chemotaxis responses: sensory neurons define olfactory preferences in *C. elegans*. Cell **91**: 161-169.

Trunk, K., Benkert, B., Quäck, N., Münch, R., Scheer, M., Garbe, J., *et al*. 2010. Anaerobic adaptation in *Pseudomonas aeruginosa*: definition of the Anr and Dnr regulons. Environmental Microbiology. **12**(6): 1719-1733.

Vaitkevicius, K., Lindmark, B., Ou, G., Song, T., Toma, C., Iwanaga, M., *et al*. 2006. A *Vibrio cholerae* protease needed for killing of *Caenorhabditis elegans* has a role in protection from natural predator grazing. Proc Natl Acad Sci USA. **103**: 9280-9285.

Van den Broek, D., Chin-A-Woeng, T.F., Eijkemans, K., Mulders, I.H., Bloemberg, G.V. and Lugtenberg, B.J. 2003. Biocontrol traits of *Pseudomonas* spp. are regulated by phase variation. Mol Plant Microbe Interact. **16**(11): 1003-12.

van Rij, E.T., Wesselink, M., Chin-A-Woeng, T.F., Bloemberg, G.V. and Lugtenberg, B.J.J. 2004. Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. Mol Plant-Microbe Interact. **5**: 557-566.

Venette, J. 1998. *Sclerotinia* spore formation, transport and infection. In: Proceedings of the Sclerotinia Workshop, 21 January 1998. North Dakota State University, Department of Plant Pathology, Fargo, ND, USA.

Venturi, V. 2003. Control of rpoS transcription in *Escherichia coli* and *Pseudomonas*: why so different? Mol Microbiol. **49**(1): 1-9.

Venturi, V. 2006. Regulation of quorum sensing in *Pseudomonas*. FEMS Microbiol Rev. **30**: 274-291.

Voisard, C., Keel, C., Haas, D. and Defago, G. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root-rot of tobacco under gnotobiotic conditions. EMBO J. **8**(2): 351-358.

Wang, W., Lu, X., Qin, X.J., Zhang, X.H. and Xu, Y.Q. 2008. Solubility of pyoluteorin in water, dichloromethane, chloroform, and carbon tetrachloride from (278.2 to 333.2) K. Journal of Chemical & Engineering Data. **53**(9): 2241-2243.

Wang, X., Preston, III J.F. and Romeo, T. 2004. The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesion required for biofilm formation. J Bacteriol. **186**: 2724-2734.

Wang, X., Dubey, A.K., Suzuki, K., Baker, C.S., Babitzke, P. and Romeo, T. 2005. CsrA post-transcriptionally represses *pgaABCD*, responsible for synthesis of a biofilm polysaccharide adhesion of *Escherichia coli*. Mol Microbiol. **56**: 1648-1663.

Ward, S. 1973. Chemotaxis by the nematode *Caenorhabditis elegans*: identification of attractants and analysis of the response by use of mutants. Proc Natl Acad Sci USA. **70**: 817–821.

Ward, A., Liu, J., Feng, Z. and Xu, X.Z. 2008. Light sensitive neurons and channels mediate phototaxis in *C. elegans*. Nat Neurosci. **11**(8): 916-22.

Weller, D.M . 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annual Review Phytopathology, **26**: 379-407.

Werner, K.M., Perez, L.J., Ghosh, R., Semmelhack, M.F. and Bassler, B.L. 2014. *Caenorhabditis elegans* recognizes a bacterial quorum-sensing signal molecule through the AWCON neuron. J Biol Chem. **289**(38): 26566-73.

West, S.E., Schweizer, H.P., Dall, C., Sample, A.K. and Runyen-Janecky, L.J. 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their application in *Pseudomonas aeruginosa.* Gene. 1**48**: 81-86.

Willard, S.S. and Devreotes**,** P.N. 2006. Signaling pathways mediating chemotaxis in the social amoeba, *Dictyostelium discoideum*. Eur J Cell Biol. **85**(9-10): 897-904.

Winteler, H.V. and Haas, D. 1996. The homologous regulators ANR of *Pseudomonas aeruginosa* and FNR of *Escherichia coli* have overlapping but distinct specificities for anaerobically inducible promoters. Microbiology **142**: 685-693.

Wood, W.B. 1988. The Nematode *Caenorhabditis elegans*. Cold Spring Harbor Monograph Series, Cold Spring Harbor Laboratory, New York, NY, USA.

Ye, R.W., Haas, D., Ka, J.O., Krishnapillai, V., Zimmermann, A., Baird, C. and Tiedje, J.M. 1995. Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. J Bacteriol. **177**: 3606-3609.

Yoon, S.S., Karabulut, A.C., Lipscomb, J.D., Hennigan, R.F., Lymar, S.V., Groce, S.L., *et al*. 2007. Two-pronged survival strategy for the major cystic fibrosis pathogen, *Pseudomonas aeruginosa*, lacking the capacity to degrade nitric oxide during anaerobic respiration. EMBO J. **26:** 3662-3672.

Youn, M., Lee, K.M., Kim, S.H., Lim, J., Yoon, J.W. and Park, S. 2013. *Escherichia coli* O157:H7 LPS O-side chains and pO157 are required for killing *Caenorhabditis elegans*. Biochem Biophys Res Commun. **436**(3): 388-93.

Zhang, R. and Hou, A. 2013. Host-microbe interactions in *Caenorhabditis elegans*. ISRN Microbiology. Article ID 356451.

Zhang, Z. and Pierson, L.S.III. 2001. A second quorum sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. Appl Environ Microbiol. **67**: 4305-4315.

Zhang, Y., Fernando, W.G.D., de Kievit, T.R., Berry, C., Daayf, F. and Paulitz, T.C. 2006. Detection of antibiotic-related genes from bacterial biocontrol agents using polymerase chain reaction. Can J Microbiol. **52**: 476-481.

Zimmermann, A., Reimmann, C., Galimand, M. and Haas, D. 1991. Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. Mol Microbiol. **5:** 1483-1490.