Investigation of the *Pseudomonas chlororaphis* PA23 - *Acanthamoeba castellanii* interaction and the role of polyhydroxyalkanoates in PA23 physiology

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Abstract

Pseudomonas chlororaphis PA23 is a biocontrol agent (BCA) that is able to protect canola against the pathogenic fungus *Sclerotinia sclerotiorum*. PA23 secretes a number of metabolites that contribute to fungal antagonism, including pyrrolnitrin (PRN), phenazine (PHZ), hydrogen cyanide (HCN) and degradative enzymes. Beyond pathogen suppression, the success of a BCA is dependent upon its ability to persist in the environment and avoid the threat of grazing predators, including protozoa. The first part of this thesis investigated whether PA23 is able to resist predation by *Acanthamoeba castellanii* (Ac) and defined the role of antifungal (AF) compounds in the bacterial-protozoan interaction. We discovered that PRN, PHZ and HCN contribute to PA23 toxicity towards Ac trophozoites. Ac preferentially migrated towards regulatory mutants devoid of AF metabolites as well as a PRN biosynthetic mutant, indicating that AF metabolites act to repel Ac. We also discovered that toxin-producing strains were able to survive inside trophozoites for up to 24 h. Collectively, our findings indicate that PRN, PHZ and HCN are involved in amoebicidal activity, and through the production of these molecules, PA23 is able to avoid the threat of predation.

PA23 accumulates polyhydroxyalkanoate (PHA) polymers as carbon and energy storage compounds. The second part of this thesis aimed to elucidate the role of PHAs in PA23 stress resistance and interaction with Ac. Three PHA biosynthesis mutants were created, PA23*phaC*, PA23*phaC1ZC2* and PA23*phaC1ZC2D*, which no longer accumulated PHA. We observed that PA23*phaC1ZC2D* produced less PHZ compared to wild type (WT). All three mutants exhibited enhanced sensitivity to UV radiation, starvation, heat and cold stress, and exposure to hydrogen peroxide. Moreover, a lack of PHA production resulted in increased motility, biofilm formation, exopolysaccharide production and root attachment. Interaction studies with the protozoan predator

Ac revealed that the WT, PA23*phaC1* and PA23*phaC1ZC2* mutants were less palatable compared to the PA23*phaC1ZC2D* mutant, which produced less PHZ. Taken together, the accumulation of PHA enhances bacterial resistance to various stress conditions, which could improve the environmental fitness of this bacterium in hostile environments.

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List of abbreviations

Ac	*Acanthamoeba castellanii*
AF	antifungal
AHL	acylhomoserine lactone
AI	autoinducer
Amp	ampicillin
ANR	anaerobic regulator of arginine deiminase and nitrate reductase
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
bp	base pair
Carb	carbenicillin
Chl	chloramphenicol
Csa	cell surface alterations
CV	crystal violet
DAPG	2,4-Diacetylphloroglucinol
EPS	Exopolysaccharides
FLA	Free-living amoeba
FNR	fumarate and nitrate reductase regulator
Gac	global activator of antibiotic and cyanide
GFP	green fluorescent protein
Gm	gentamicin
HCN	hydrogen cyanide
HPLC	High Performance Liquid Chromatography
ISR	induced systemic resistance
KCN	potassium cyanide
LB	lysogeny broth
LTTR	LysR-type transcriptional regulator
Mcl	Medium-chain length
MS	Murashige and Skoog media
NADH	Nicotinamide adenine dinucleotide
2-OH-PHZ	2-hydroxy-phenazine

PCA	phenazine-1-carboxylic acid
PCN	phenazine-1-carboxamide
PCR	polymerase chain reaction
PDA	potato dextrose agar
PGPR	plant growth promoting rhizobacteria
PHA	polyhydroxyalkanoates
PHB	polyhydroxybutyrate
PHZ	phenazine
PIA	Pseudomonas Isolation Agar
Pip	piperacillin
PLT	pyoluteorin
PYG	peptone yeast glucose
ppGpp	guanosine tetraphosphate
pppGpp	guanosine pentaphosphate
PRN	pyrrolnitrin
PsrA	Pseudomonas sigma regulator A
PtrA	Pseudomonas transcriptional regulator A
PVD	pyoverdin
PYO	pyocyanin
QS	quorum sensing
RBS	ribosome binding site
Rif	rifampicin
RMM	Ramsay’s minimal medium
RNAP	RNA polymerase
Rsm	regulator of secondary metabolism
Scl	short-chain length
S-layer	Surface layer
SR	stringent response
Tc	tetracycline
UV	ultraviolet
WT	Wild type

Chapter 1
Literature Review

1.1. Biological control of phytopathogens by *Pseudomonas* species

1.1.1. Biological control

Pathogenic microorganisms are able to infect a wide range of plants and they represent a major threat to agriculture production worldwide. As food production increased over the past few decades, farmers have become more reliant on chemical pesticides as a means of crop protection. However, the use of agrochemicals can cause adverse effects on the environment. For example agrochemicals are not always pathogen-specific and their impact on non-pest indigenous organisms can alter the ecosystem balance (Cook, 1993; Arias-Estèvez et al., 2008). Moreover, phytopathogens can develop resistance to applied pesticides. An environmentally safe alternative to pathogen management is urgently needed and one such approach is biological control.

Biological control can be defined as the utilization of living organisms to reduce or mitigate undesirable agricultural pests (Baker, 1987; Cook, 1993). This phenomenon was recognized through the discovery of naturally disease-suppressive soils, where the roots of crop plants were naturally protected from soil-borne pathogens. Interestingly, the transfer of less than 10% inoculum of suppressive soils to conductive soils was sufficient for pathogen inhibition. The phenomenon of disease control was attributed to the presence of soil microorganisms that antagonize pathogens (Shipton, 1973; Weller et al., 2002).

Numerous soil microorganisms, including members of the genus *Agrobacterium*, *Bacillus*, *Streptomyces*, *Pseudomonas* and *Burkholderia* have been recognized as biological control agents (BCAs) (Bloemberg & Lugtenberg, 2001; Weller, 2007). BCAs protect crops from pathogens via different modes of action including: i) hyperparasitism or antibiosis by direct antagonistic interaction with pathogens; ii) induced systemic resistance (ISR) to enhance the plant defence

response without direct interaction with the pathogen and iii) nutrient competition affecting the growth of the pathogen (Haas & Défago, 2005).

1.1.2. Biocontrol activity of *Pseudomonas* species

Bacteria belonging to the genus *Pseudomonas* are metabolically versatile and belong to the class Gammaproteobacteria. They are classified as Gram-negative, rod-shaped aerobes that have one or several polar flagella, providing motility (Stanier et al., 1966; Haas & Défago, 2005; Palleroni, 2008; Gomila et al., 2015). *Pseudomonas* species are ubiquitous in agricultural soils and are well adapted to survive in the rhizosphere and phyllosphere. Pseudomonads possess numerous attributes that contribute to their ability to function as plant growth-promoting rhizobacteria (PGPR) (Rovira, 1965; Glick, 1995). Such traits include rapid growth and rhizosphere colonization, plant growth stimulation, and the ability to effectively compete for nutrients. In addition, pseudomonads are recognized for their ability to secrete a wide range of compounds that inhibit pathogens. Thomashow and Weller (1988) were the first to demonstrate that the antibiotic phenazine-1-carboxylic acid (PCA) secreted by *Pseudomonas fluorescens* PF-5 was able to suppress the growth of the fungal pathogen *Gaeumannomyces graminis var. tritici* on wheat. Subsequently, *Pseudomonas protegens* CHA0 was reported to prevent the growth of the fungus *Thielaviopsis basicola* on tobacco (Voisard et al., 1989). Many pseudomonads have been characterized as BCAs and they produce a wide spectrum of metabolites responsible for pathogen inhibition (Haas & Keel, 2003; Haas & Défago, 2005).

1.2. *Pseudomonas chlororaphis* PA23

The biocontrol bacterium *Pseudomonas chlororaphis* strain PA23 was first isolated from soybean root tips (Savchuk & Fernando, 2004). PA23 is able to protect canola from stem rot caused by the fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary (Fernando et al., 2007). PA23 produces a number of exoproducts that contribute to fungal antagonism including phenazine (PHZ) and pyrrolnitrin (PRN) together with hydrogen cyanide (HCN), protease, lipase, and siderophores (Poritsanos et al., 2006; Zhang et al., 2006). In addition to directly suppressing fungal growth, this bacterium is capable of inducing the plant defense response (Duke et al., 2017). PA23 exoproducts are discussed in more detail below.

1.3. *P. chlororaphis* PA23 biocontrol compounds

1.3.1. Phenazines

Phenazines (PHZ) represent a large group of nitrogen-containing heterocyclic compounds produced by a diverse range of bacteria (Pierson & Pierson, 2010). The first compound to be isolated was Pyocyanin (PYO), by Fordos in 1859. Later, other PHZs were discovered including phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), 1-hydroxyphenazine (1-OH-PHZ), 2-hydroxyphenazine (2-OH-PHZ), pyoverdin or pseudobactin (PVD) (Wilson et al., 1987; Kerr et al., 1999; Delaney et al., 2001; Mavrodi et al., 2006). Over 100 different structural derivatives of PHZ have been identified, many of which exhibit antagonistic activity against bacteria, fungi and plants (Hernandez et al., 2004). PHZs are synthesized via the shikimic acid pathway through the action of enzymes encoded by the biosynthetic operon *phzABCDEFG*, which is conserved in fluorescent *Pseudomonas* species (Chin-A-Woeng et al., 2003; Mavrodi et al., 2006). PHZs have been reported to play an essential role in the disease-suppressive ability of

many biocontrol bacteria. For instance, a PHZ-deficient mutant of *P. fluorescens* 2–79 exhibited reduced suppression of take-all disease of wheat (Thomashow & Weller, 1988).

PHZs inhibit electron transport and catalyze the formation of hydroxyl radicals causing damage to lipids and other macromolecules (Britigan et al., 1992). *Pseudomonas chlororaphis* PA23 produces the PHZ derivatives 2-OH-PHZ and PCA (Zhang et al., 2006). A PHZ-deficient mutant exhibited enhanced AF activity, which was attributed to increased levels of a second antibiotic produced by PA23, called PRN (Selin et al., 2010). While PHZs play a lesser role in PA23 antagonism, they contribute to biofilm formation by this bacterium (Selin et al., 2010). As such, these compounds are expected to confer an ecological advantage by aiding colonization and persistence of bacterial cells in the rhizosphere and soil (Haas & Défago, 2005).

1.3.2. Pyrrolnitrin

Pyrrolnitrin [3-chloro-4-(3´-chloro-2´-nitrophenyl) pyrrole] (PRN) is an antibiotic that was first isolated in 1964 from *Pseudomonas pyrrocinia* (Gehmann et al., 1990). Subsequently, other *Pseudomonas* species, including *P. protegens* and *P. chlororaphis* were found to produce PRN (Sarniguet et al., 1995; Haas and Keel, 2003). This antibiotic exhibits broad-spectrum AF activity against a variety of Deuteromycete, Ascomycete, and Basidiomycete fungi (Warden & Edwards, 1976). PRN has been developed as an AF agent for clinical treatment of skin diseases (Tawara et al., 1989) and a structural derivative has been used as an agricultural fungicide (di Santo et al., 1998). This antibiotic has been implicated in the biological control of a number of fungal pathogens infecting plants. For example, a PRN mutant of *P. fluorescens* BL915 was unable to suppress cotton damping-off disease caused by *Rhizoctonia solani* (Hill et al., 1994). In PA23,

PRN is the primary AF compound that suppresses the growth of *S. sclerotiorum* (Selin et al., 2010).

The primary target of PRN is the cell membrane, where it hinders synthesis of protein, RNA and DNA as well as electron transport and ATP formation (Nose & Arima, 1969; Tripathi & Gottlieb, 1969, Hammer et al., 1997). PRN can cause severe damage to the electron transport system, accumulation of glycerol and synthesis of triacyl glycerol, all of which lead to cell membrane leakage (Lambowitz & Slayman, 1972; Tawara et al., 1989; van Pée & Ligon, 2000). This metabolite has demonstrated biological activity at low concentrations and can inhibit oxidative phosphorylation in *Neurospora crassa* (Lambowitz & Slayman, 1972).

The *prnABCD* biosynthetic operon encodes the enzymes required for conversion of tryptophan into PRN. Transfer of *prnABCD* into *E. coli* enables synthesis of PRN, demonstrating that these four genes are sufficient for antibiotic production (Hammer et al. 1997).

1.3.3. Hydrogen cyanide

HCN is a toxic, volatile compound produced by fluorescent pseudomonads, the proteobacterium *Chromobacterium violaceum*, and certain cyanobacteria (Knowles, 1976; Voisard et al., 1989; Laville et al., 1992). HCN inhibits the terminal cytochrome c oxidase in the respiratory chain (O'Sullivan & O'Gara, 1992). In bacteria, the precursor for HCN production is glycine, and HCN synthesis is enhanced by addition of glycine to the growth media (Castric, 1977; Askeland & Morrison, 1983; Laville et al., 1998). HCN is produced by the action of enzymes encoded by the *hcnABC* biosynthetic operon, encoding formate dehydrogenase (*hcnA*) and amino acid oxidases (*hcnB and hcnC*) (Laville et al., 1998). The *hcn* operon is conserved across the fluorescent pseudomonads (Knowles, 1976; Blumer & Haas, 2000).

HCN has been shown to have a beneficial effect on plant health and is known for its role in disease suppression (Castric, 1977; Nandi et al., 2017). For example, production of HCN by fluorescent pseudomonads plays a vital role in controlling diseases caused by phytopathogenic fungi, such as *Thielaviopsis basicola* and *Septoria tritici* on tobacco and wheat, respectively (Voisard et al., 1989; Flaishman et al., 1999). In PA23, HCN displays nematocidal activities and contributes to suppression of *S. sclerotiorum* infection (Nandi et al., 2015; Nandi et al., 2017).

1.3.4. Siderophores

Siderophores are small, high-affinity metal-chelating molecules secreted by microorganisms such as bacteria and fungi (Haas & Défago, 2005). Siderophores bind to ferric iron in the soil or rhizosphere and transport it across cell membranes using outer membrane receptors (Kloepper et al., 1980; Neilands, 1995). Bacteria that produce siderophores are able to compete for the limited supply of iron in the rhizosphere, thereby suppressing the growth of microbes with less robust iron-uptake systems (Bakker et al., 1986). These compounds were first isolated from *P. putida* B10 after researchers observed that the transfer of this strain to disease-conductive soil resulted in suppression of take-all and Fusarium-wilt (Kloepper et al., 1980). Siderophores have also been found to contribute to inhibition of phythopathogens like *P. ultimu*, and *Gaeumannomyces graminis var. tritici* (Cook & Weller, 1987). In another study, a siderophore mutant of *Pseudomonas* strain WCS358 lost the ability to enhance the growth of potato plants (Bakker et al., 1986). Interestingly, siderophore-mediated iron competition by *P. fluorescens* was found to prevent growth of the human pathogen *E. coli* O157: H7 on food products (McKellar, 2007).

1.3.5. Lytic enzymes

Pseudomonads are capable of producing degradative enzymes such as chitinases, lipases, proteases and cellulases (Pal & Gardener, 2006). These enzymes cause severe damage to fungi by degrading cell wall constituents such as glucans and chitins (Chin-A-Woeng et al., 2003). Lytic enzymes enable biocontrol bacteria to suppress plant pathogens, for example, chitinase and glucanase released by *Pseudomonas stutzeri* resulted in lysis of *Fusarium solani* mycelia (Lim et al., 1991). Studies on *P. protegens* CHA0 revealed that extracellular protease induced mortality of the pathogenic nematode *Meloidogyne incognita* during tomato and soybean infection (Siddiqui et al., 2005). Several reports indicate that cell wall-degrading enzymes and other AF compounds act synergistically to suppress plant diseases (Di Pietro et al. 1993; Duffy et al. 1996; Fogliano et al. 2002).

1.4. Regulatory elements governing biocontrol activity in *P. chlororaphis* PA23

A complex regulatory network that functions at the transcriptional and post-transcriptional levels governs production of biocontrol factors by PA23. Regulatory elements include the GacA/GacS two-component system which functions together with Rsm, the Phz QS system, RpoS, the transcriptional regulators PsrA, PtrA and ANR, and the stringent response (SR). A model of the cascade regulating production of PA23 AF compounds is depicted in Figure 1.1.

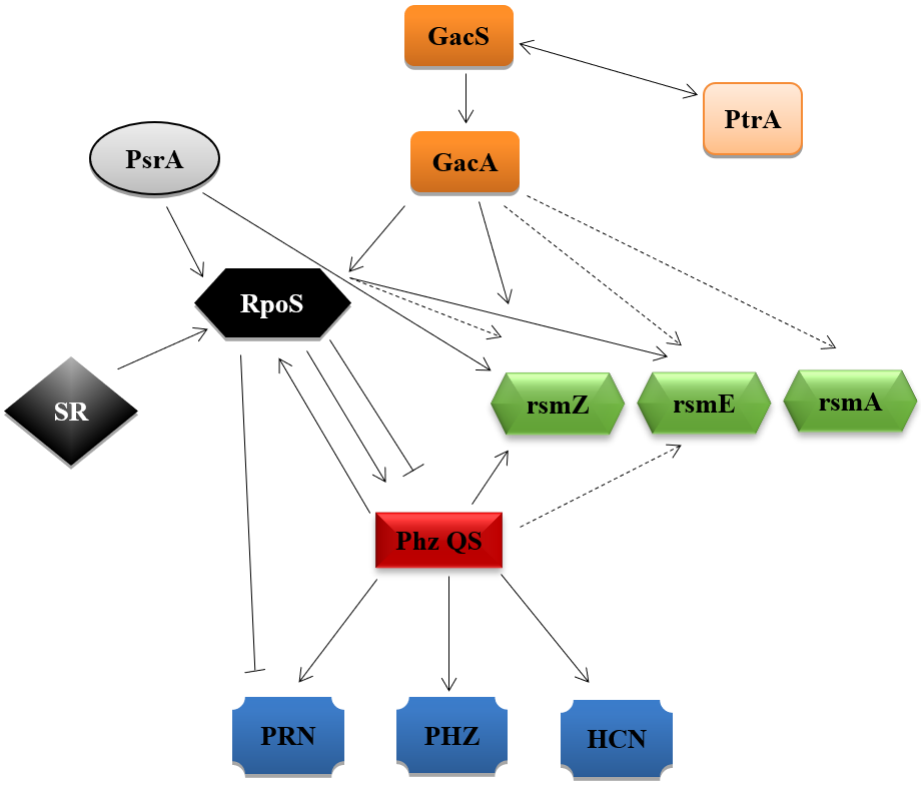

Figure 1.1. The regulatory cascade overseeing production of secondary metabolites involved in *Pseudomonas chlororaphis* PA23-mediated antagonism. An unknown signal causes GacS autophosphorylation and phosphotransfer to GacA, which can then activate transcription of the small noncoding RNAs RsmZ (and RsmY and X, not shown). These sRNAs titrate out the translational repressor proteins RsmA and RsmE, allowing expression of mRNAs required for biosynthesis of secondary metabolites. PtrA is a LysR type regulator that is functionally intertwined with GacS through an unknown mechanism. PsrA and the Stringent Response (SR) positively modulate RpoS. RpoS inhibits expression of PRN and PhzR, while activating PhzI. The PHZ QS system positively regulates expression of RpoS, RsmZ, RsmE as well as the production of PRN and PHZ. Symbols: ↑, positive effect; ⊥, negative effect; solid lines, direct effect; dashed lines, indirect effect (Poritsanos et al., 2006; Selin et al., 2012; Manuel et al., 2012; Selin et al., 2014; Klaponski et al., 2014; Nandi et al., 2017; Shah et al., 2017).

1.4.1. Gac-Rsm signal transduction system

The Gac-Rsm (Global activator of Antibiotic and Cyanide; Regulator of Secondary Metabolism) system serves to regulate the expression of secondary metabolites and extracellular enzymes in response to environmental signals (Laville et al., 1992; Lapouge et al., 2007). This network is highly conserved among Gram-negative bacteria. It has been intensively studied in *Pseudomonas* species, particularly in *P. aeruginosa* for its role in virulence (Zolfaghar et al., 2005; Ventre et al., 2006) and *P. protegens* for controlling expression of factors that protect plants from pathogenic fungi (Haas & Défago, 2005).

The GacA/GacS two-component system consists of GacS, a membrane-bound sensor kinase, and its cognate response regulator GacA (Laville et al., 1992). Both GacA and GacS are essential for synthesis of secreted extracellular products. As such, mutations in either *gacA* or *gacS* have pleiotropic effects, eliminating the production of secondary metabolites in several pseudomonads (Chancey et al., 1999; Heeb et al., 2002; Chin-A-Woeng et al., 2003).

Activation of the GacS-GacA cascade occurs when an unknown signal binds to GacS resulting in autophosphorylation and subsequent phosphotransfer to GacA. Upon activation, the phosphorylated GacA initiates transcription of small non-coding RNA molecules, RsmX, RsmY and RsmZ by binding to a conserved sequence present in the promoter regions of *rsmX/Y/Z* (Heeb et al., 2002; Haas & Défago, 2005). These small non-coding RNAs work in concert with RsmA and RsmE to control the expression of biocontrol metabolites at the post-transcriptional level. The RsmA/E proteins function as repressors by binding to the ribosome-binding site (RBS) in target mRNA, preventing translation (Heeb et al., 2002; Kay et al., 2006). The activity of RsmA/E is antagonized by the action of RsmZ, RsmY and RsmX, thereby alleviating translational repression of target mRNAs (Heeb et al., 2002; Haas & Défago, 2005; Kay et al., 2006). Studies on *P.*

protegens revealed that mutations in either *rsmA* or *rsmE* led to increased expression of *aprA*- (alkaline protease), *hcnA*- (HCN), and *phlA-lacZ* (2,4-diacetylphloroglucinol) translational fusions (Reimmann et al., 2005). By contrast, an *rsmA/rsmE* double mutation resulted in dramatically increased expression of the reporter fusions (Reimmann et al., 2005). As such, both RsmA and RsmE function as post-transcriptional repressors of the targeted genes.

Two orphan sensor kinases, RetS and LadS also interact with the GacS/GacA system to modulate the expression of secondary metabolites (Goodman et al., 2004; Records & Gross, 2010). In *P. aeruginosa*, the RetS and LadS regulons encompass factors associated with virulence, biofilm formation, motility and secretion (Ventre et al., 2006; Records & Gross, 2010). RetS forms heterodimers with GacS, blocking GacS autophosphorylation and subsequently reducing transcription of the small RNA molecules. RetS is believed to promote transcription of genes required for acute virulence factors, while LadS functions as an activator of RsmZ and RsmY (Goodman et al., 2004, 2009; Ventre et al., 2006).

In PA23, *gacS* and *gacA* mutants exhibit decreased production of secondary metabolites such as PHZ, PRN, HCN and proteases. This results in a loss of AF activity both *in vitro* and in greenhouse analyses (Poritsanos et al., 2006; Selin et al., 2014). The Gac system works synergistically with other regulatory elements including QS, RpoS, PsrA and the SR, to control expression of AF compounds (Selin et al., 2014). While homologs of *retS* and *ladS* have been identified in PA23 (Shah et al., 2016), their involvement in fungal suppression has yet to be revealed.

1.4.2. *Pseudomonas* transcription regulator A

In PA23, Pseudomonas transcription regulator A **(**PtrA) is a protein belonging to the LysR-type transcriptional regulator (LTTR) family, a well conserved group of regulars that is ubiquitous among prokaryotes (Maddocks & Oyston, 2008). LTTRs can functions as either activators or repressors to control a wide range of metabolic activity involved in cell division, QS, motility, virulence, and oxidative stress (Maddocks & Oyston, 2008). PtrA was identified through transposon mutagenesis, in which a mutation in the *ptrA* gene resulted in a complete loss of AF activity (Klaponski et al., 2014), similar to the *gacS* mutant phenotype (Poritsanos et al., 2006; Selin et al., 2014). Proteomic analysis of the *ptrA* mutant revealed differential expression of essential proteins involved in PA23 biocontrol including PHZ, PRN and degradative enzymes (Klaponski et al., 2014). In subsequent transcriptomic analysis, decreased expression of genes required for PA23 AF activity was observed in the *ptrA* mutant background (Shah et al., 2016). Providing *gacS* in trans partially restored the *ptrA* mutant phenotype to that of WT, suggesting a regulatory link between the sensor kinase GacS and PtrA (Shah et al., 2016).

1.4.3. Quorum sensing (QS)

Many bacteria regulate secondary metabolite synthesis through a cell-cell communication process known as QS (Miller & Bassler, 2001). QS involves the synthesis and accumulation of small diffusible signaling molecules called autoinducers (AIs) that enable bacteria to modulate gene expression in response to their population density (Fuqua et al., 2001; Juhas et al., 2005; Venturi, 2006). These systems were first discovered in the marine bacterium *Vibrio fischeri* wherein which the regulation of the luciferase operon (*luxCDABE*) used for light emission is under QS control (Miyamoto et al., 2000).

The most common AI signals utilized by Gram-negative bacteria to communicate are N-acyl homoserine lactones (AHLs) (Venturi, 2006). AHL molecules are synthesized by an autoinducer synthase enzyme, called a LuxI-type protein (Bassler, 2002). These AHLs accumulate intra- and extracellularly and passively diffuse through the cell membrane in accordance with population density (Mavrodi et al., 2006). After a threshold level of AHL has been reached, it binds to a cognate LuxR-type transcriptional regulator. The LuxR-AHL complex recognizes and binds to a specific "lux box" sequence in the promoter region of QS-controlled genes leading to changes in gene transcription (Fuqua et al., 1994; Miller & Bassler, 2001, Haas & Keel, 2003).

In *P. chlororaphis*, the PhzI/PhzR QS system consists of the transcriptional regulator PhzR and the AHL synthase PhzI, which controls expression of the PHZ biosynthetic operon (Miller & Bassler, 2001; Selin et al., 2012). The *phzI* and *phzR* genes are adjacent to one another with *phzR* located immediately upstream of but in the opposite orientation to the *phzABCDEF* biosynthetic operon. Complexing with AHL is believed to induce PhzR homodimerization, enabling binding to a "phz box" sequence located in the promoter region of target genes (Miller & Bassler, 2001). In PA23, both *phzR*- and AI-deficient strains exhibit reduced AF activity, which is attributed to decreased production of PHZ, PRN and proteases (Selin et al., 2012). Additionally, the Phz QS system is involved in PA23 biofilm formation (Selin et al., 2012).

Another LuxR-LuxI-type QS system called CsaR/CsaI (Cell Surface Alteration) was identified in the related strain *P. chlororaphis* 30-84. This QS system does not control expression of PHZ; instead, it is involved in regulating cell surface components (Zhang & Pierson, 2001). The CsaR/CsaI QS system also plays a role in the rhizosphere competence of *P. chlororaphis* 30-84 (Juhas et al., 2005; Zhang & Pierson, 2001). PA23 harbors copies of the *csaI* and *csaR* genes (Loewen et al., 2014) and their role in PA23 physiology is currently under investigation.

1.4.4. RpoS and PsrA regulatory control

The RpoS (σ^{38}/ σ^{S}) sigma factor is a well-known global regulator that controls expression of many genes at the onset of the stationary phase or in response to environmental stress (Battesti et al., 2011). This sigma factor was first discovered in *E. coli*, and subsequently it has been identified in a variety of Gram-negative bacteria, including *Pseudomonas* spp. (Loewen et al., 1998; Venturi, 2003; Heeb et al., 2005). In *E. coli*, RpoS was found to directly and indirectly regulate 10% of its genome, mostly related to stress resistance (Weber et al., 2005), whereas in *Pseudomonas* spp. it plays a more significant role in regulating secondary metabolites (Sarniguet et al., 1995; Venturi, 2003). At early stationary phase, RpoS accumulates within the cell and competes with other sigma factors for interaction with the core RNA polymerase (RNAP). This in turn alters the affinity of RNAP for specific promoter sequences, resulting in a global change in gene expression (Venturi, 2003).

In *Pseudomonas* spp., a mutation in *rpoS* leads to altered production of secondary metabolites, including PHZ, PRN, PLT, HCN, and AHLs (Heeb et al., 2005; Oh et al., 2013) as well as an aberrant stress response (Sarniguet et al., 1995; Heeb et al., 2005). Several studies have demonstrated that the regulatory effect of RpoS is species dependent. For instance, a mutation in *rpoS* in *P. fluorescens* Pf-5 and *P. protegens* CHA0 leads to reduced PRN levels (Venturi, 2003; Haas & Défago, 2005; Heeb et al., 2005), whereas the same mutation in PA23 enhances PRN production (Manuel et al., 2012; Selin et al., 2012).

RpoS is positively regulated by a transcriptional regulator called PsrA (Pseudomonas sigma regulator A) (Kojic and Venturi, 2001). PsrA was first identified in *P. putida* and *P. aeruginosa* for its role in *rpoS* transcriptional activation. A *psrA* mutant in both strains exhibits a 50% decrease in *rpoS* transcription compared to the WT (Venturi, 2003). PsrA is negatively

autoregulated through binding to recognition sites in its promoter region and in some *Pseudomonas* spp., it controls AHL production (Venturi, 2003; Chin-A-Woeng et al., 2005).

A global stress response system known as the SR also controls RpoS expression. The SR is a regulatory mechanism that enables bacteria to adapt to nutrient limitation. This response involves intracellular accumulation of the alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively known as (p)ppGpp (Potrykus and Cashel, 2008). (p)ppGpp binds to RNAP resulting in increased transcription of genes that are involved in amino acid biosynthesis and decreased transcription at other loci. In PA23, SR mutants were found to exhibit enhanced AF activity due to elevated production of PRN, protease and lipase. The observation that *rpoS* transcription was dramatically reduced in the SR mutant background and providing *rpoS in trans* restored the WT phenotype, lead to the conclusion that the SR primarily impacts biocontrol through RpoS (Manuel et al., 2012).

1.4.5. ANR regulator

Although aerobic respiration is a key mechanism of energy generation in many *Pseudomonas* species, anaerobic pathways enable bacteria to adapt to different environments, including the rhizosphere (Arai, 2011). The ANR regulator (anaerobic regulator of arginine deiminase and nitrate reductase) is crucial for metabolic activity in reduced oxygen conditions for many bacteria. This regulator was first discovered in *E. coli* as a homolog of the FNR (Fumarate and Nitrate Reductase) family of transcriptional regulators that can be converted to its active form under low oxygen concentration (Sawers, 1991). In *P. aeruginosa* and *P. fluorescens*, ANR dimerizes under low oxygen concentrations and binds to conserved DNA binding sites in the promoter of target genes, called *anr*-boxes, enabling regulation of transcription (Sawers, 1991; Laville et al., 1998).

ANR enables energy generation under reduced oxygen conditions (Trunk et al., 2010) and it controls maintenance of redox state, cytochrome biosynthesis, oxidative stress resistance and QS (Eschbach et al., 2004; Galimand et al., 1991; Hammond et al., 2015; Trunk et al., 2010).

In *P. chlororaphis* PA23, ANR is a crucial regulator overseeing fungal antagonism under atmospheric oxygen conditions (Nandi et al., 2016). ANR is required for AF and protease activity, PRN, PHZ and HCN production. In addition, a connection exists between ANR and QS with ANR positively regulating *phzI*/*phzR*, and PhzR negatively controlling *anr* transcription (Nandi et al., 2016).

1.5. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are a group of polyesters synthesized by a wide range of bacterial strains as intracellular storage granules in the cytoplasm (Anjum et al, 2016; Zou et al., 2017). Since its discovery in 1926, PHA has gained interest from the research community due to its biodegradability and biocompatibility as well as similarities with petrochemical polymers such as polypropylene and polystyrene (Anderson & Dawes, 1990; Kumar et al., 2014, 2018; Tsuge et al., 2015). PHA accumulation has been reported for many microorganisms, including Gram-negative and Gram-positive bacteria (Laycock et al., 2014). Bacteria synthesize and accumulate PHA as a carbon and energy storage reservoir when carbon is in excess and other nutrients such as nitrogen, phosphorus or iron are depleted (Schlegel et al., 1961; Ciesielski et al., 2010). Under carbon-limiting conditions, PHA polymers are degraded by depolymerases and utilized for energy generation (Anderson & Dawes, 1990; Ciesielski et al., 2010).

PHA molecules are composed of 600 to 35,000 (*R*)-hydroxy fatty acid monomer units (Khanna & Srivastava, 2005). The monomer units contain a side chain *R* group, which is usually a

saturated alkyl group, but it can also contain unsaturated alkyl or branched alkyl groups (Huijberts et al., 1992; Khanna & Srivastava, 2005; Lu et al., 2009). Most microorganisms accumulating PHA synthesize short-chain length PHAs or medium-chain length PHAs. Short-chain length PHA (Scl-PHA) consisting of 3–5 carbon atom monomers is accumulated by several bacterial species (Byrom, 1987; Anderson et al., 1990a). Synthesis of medium-chain length PHA (Mcl-PHA) composed of 6 to 14 carbon atom monomers is limited to *Pseudomonas* rRNA homology group I including *P. putida*, *P. syringae*, *P. fluorescens*, *P. chlororaphis* and *P. aeruginosa* (Anderson et al., 1990a; Madison & Huisman, 1999; Diard et al., 2002). PHA chain length is usually related to the carbon source provided. For instance, when fatty acids such as coconut oil and oleic acid are provided as a carbon source, the monomer chain structure becomes more complex (Taniguchi et al., 2003; Thakor et al., 2005).

1.5.1. PHA biosynthesis pathways

PHA biosynthesis is linked with central metabolic pathways such as β-oxidation, de novo fatty acids synthesis and the Krebs cycle (Rothermich et al., 2000; Yamane, 1993; Kadouri et al., 2005; Peplinski et al., 2010). PHA polymers can be synthesized from both structurally related carbon sources, such as fatty acids, aliphatic alkanes and alkenes (Kim et al., 1995; Hazenberg & Witholt, 1997; Durner et al., 2000; Sun et al., 2007b) and non-related carbon substrates, including glucose, gluconate and sucrose (Timm & Steinbüchel, 1990; Sánchez et al., 2003). Structurally related carbon sources induce higher PHA production (Sun et al., 2007a). Biosynthesis of PHA occurs via central pathways, such as the β-oxidation and fatty acid de novo pathway for (*R*)-3-hydroxyacyl-CoA generation, and a specific pathway encoded by the *pha* cluster (de Eugenio et al., 2007; Huisman et al., 1991; Ren et al., 2009). The β-oxidation pathway uses structurally

similar carbon substrates such as alkanoic acids and fatty acids; whereas, the fatty acid de novo synthesis pathway converts non-related carbon compounds, such as glucose, gluconate or acetate, to (*R*)-3-hydroxyacyl-CoA (Huijberts et al., 1992).

The intermediates involved in PHA biosynthesis are usually shared among the central pathways, with acetyl-CoA being the most essential intermediate. Acetyl-CoA is converted into PHA by three enzymes namely 3-ketothiolase (PhaA), Acetoacetyl-CoA reductase (PhaB) and PHA synthase (PhaC) (Tsuge et al., 2015). In PHA-producing bacteria, the flux of acetyl-CoA to PHA synthesis is dependent on nutrient availability. During normal growth, the high amount of coenzyme A produced from the Krebs cycle inhibits the enzymatic activity of 3-ketothiolase, shunting acetyl CoA back to the Krebs cycle for cell growth and energy production (Madison & Huisman, 1999; Steinbüchel & Hein, 2001; Kadouri et al., 2005). When growth is limited but carbon is in excess, acetyl-CoA is channelled towards PHA biosynthesis as coenzyme A exhibits a less inhibitory effect on 3-ketothiolase under these conditions (Madison & Huisman, 1999; Jung & Lee, 2000).

In pseudomonads, the PHA biosynthetic locus is highly conserved and arranged as two divergently transcribed gene clusters: *phaC1ZC2D* and *phaFI*. The *phaC1ZC2D* cluster encodes two polymerases (PhaC1 and PhaC2), a depolymerase (PhaZ) and a TetR-like transcriptional regulator PhaD. The *phaFI* genes encode the phasins PhaF and PhaI that are involved in the PHA synthesis and stability (Huisman et al., 1991; Steinbüchel & Hein, 2001; Sharma et al., 2017). PhaC1 and PhaC2, which are members of the α/β hydrolase subfamily, catalyze the polymerization of several (*R*)-3-hydroxy-acyl-CoA derivatives into PHA (Lageveen et al., 1988; García et al., 1999; Steinbüchel & Hein, 2001). The depolymerase PhaZ is a hydrolytic enzyme that degrades the PHA polymer so that the released hydroxyacyl-CoA can be used as a source of

carbon and energy (Zinn et al., 2001; de Eugenio et al., 2007, 2010). The phasins PhaF and PhaI have a stabilizing function as they cover the PHA granule surface. These proteins provide an amphiphilic interface between the hydrophobic core and the aqueous cytoplasm of the granules (Obruca et al., 2018). PhaF also acts a regulator repressing *phaC1ZC2D* and *phaI* expression (Prieto et al., 1999). A model depicting the metabolic pathways for mcl-PHA biosynthesis in PA23 is presented in Figure 1.2 (Mohanan et al., 2019).

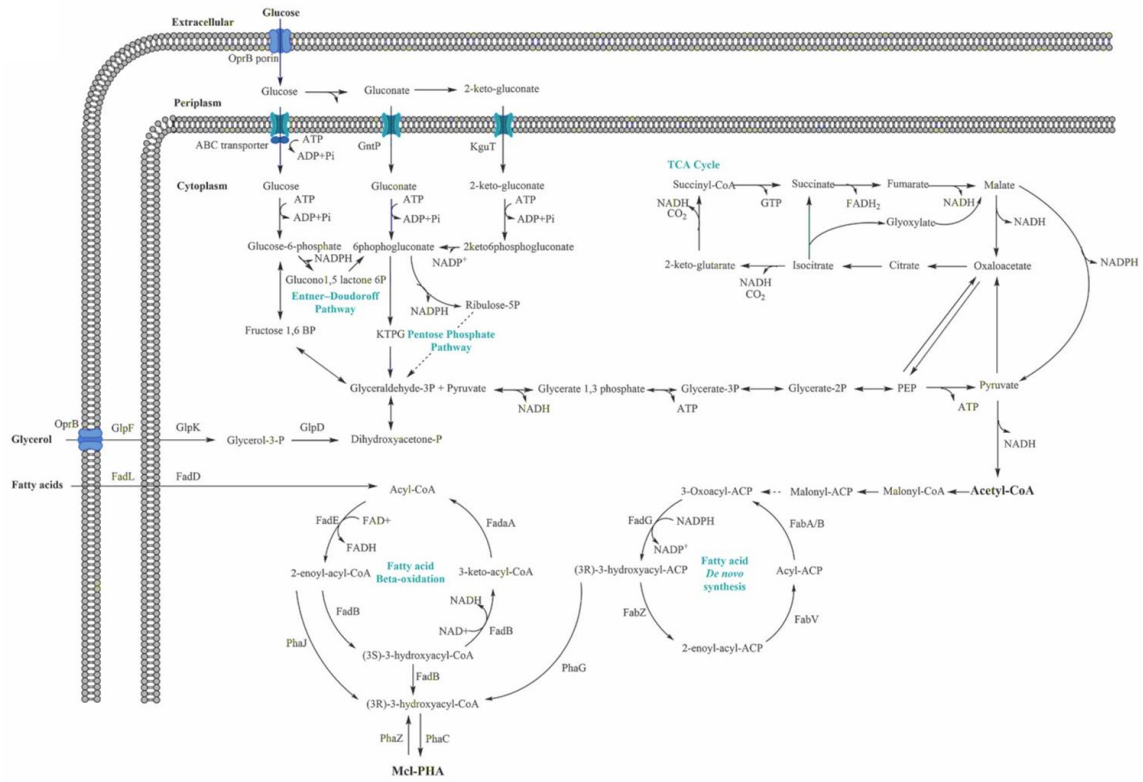

Figure 1.2. Metabolic pathways for the biosynthesis of medium-chain length PHA in PA23. Reprinted with permission from BLACKWELL PUBLISHING LTD. (Oxford University Press Journals-*FEMS microbiology letters*), 05 November 2019. doi.org/10.1093/femsle/fnaa033. License number: 1028769-1.

1.5.2. The role of PHA in bacterial endurance against stress

In bacteria, PHA is stored in the cytoplasm as discrete spherical granules that can account for up to 90% of the cellular dry weight (Johnson et al., 2009; Tan et al., 2014). These granules are attached and surrounded by granule-associated proteins, which include PHA synthase, depolymerase, regulatory and other structural proteins (Bresan et al., 2016). PHA accumulation enables bacteria to survive nutrient deprivation, extreme temperatures, UV irradiation, osmotic and oxidative stress, and desiccation (Kadouri et al., 2005; Obruca et al., 2016). In *Azospirillum brasilense*, PHA-accumulating strains were more resistant to UV irradiation, osmotic pressure and desiccation compared with PHA synthase- and PHA depolymerase mutants that were unable to synthesize and degrade PHA, respectively (Kadouri et al., 2002, 2003a, 2003b). Similarly, PHA-production by *Aeromonas hydrophila* was linked to increased survival upon exposure to high and low temperatures, ethanol, H_2O_2, UV radiation, and osmotic shock (Zhao et al., 2007).

PHA granules have biological and biophysical properties that act synergistically to enhance the survival and stress tolerance of bacteria. For instance, PHA degradation induces expression of RpoS, which activates genes that protect against high temperature, ethanol, H_2O_2 and other cellular stresses (Ruiz et al., 2001). Apart for their primary role in covering the surface PHA granules, phasins exhibit a chaperone-like function, which contributes to the overall protective mechanism of this polymer (Mezzina et al., 2015).

1.5.3. PHA in host-bacterial symbiosis

Some bacteria that live in close association with eukaryotic hosts are able to produce PHA granules. For example, PHA accumulation within *Burkholderia* is required for effective symbiosis with the insect *Riptortus pedestris* (Kim et al., 2013). It was observed that PHA-deficient strains

exhibited reduced cell viability in the midgut of *R. pedestris* compared to the WT, suggesting that PHA accumulation plays a role in this symbiotic association (Kim et al., 2013). Another intracellular bacterium, *Legionella pneumophila,* is able to produce the related compound polyhydroxybutyrate (PHB). This bacterium was able to survive in a culturable state for at least 600 days in tap water at 24°C, suggesting that PHB plays a major role in promoting long term persistence of *Legionella* in low-nutrient environments outside of the amoeba host (James et al., 1999). PHA production has also been identified in rhizobia including *Sinorhizobium, Azorhizobium* and *Bradyrhizobium* in both free-living isolates and symbionts (Van-Rhijn & Vanderleyden, 1995). Analysis of PHA-deficient strains of *Sinorhizobium meliloti* revealed that PHA is required for root nodule symbiosis (Aneja et al., 2005; Willis & Walker, 1998).

1.6. Free-living amoebae

Free-living amoebae (FLA) are protozoa ubiquitously distributed in soil and water (Rodríguez-Zaragoza, 1994). This group encompasses genera such as *Acanthamoeba*, *Hartmannella*, *Dictyostelium*, *Naegleria* and *Vahlkampfia* (Fields et al., 1990; Marciano-Cabral & Cabral, 2003; Schuster, 2002). Some FLA species can be pathogens of humans and animals. In humans, FLA causes diseases such as encephalitis (*Naegleria fowleri*, *Acanthamoeba*, *Balamuthia*) and keratitis (*Acanthamoeba*) (Schuster & Visvesvara, 2004), but their prevalence is usually low. FLA also play an essential role as predators in the environment, significantly impacting bacterial density, community structure, and nutrient cycling (Kreuzer et al., 2006; Schuster & Visvesvara, 2004). Their small size and flexible body enables amoebae to track and feed on bacteria in narrow soil crevices, where larger bacterivores are precluded from

entering (Ekelund et al., 1994). Work presented in this thesis employed the model protozoan predator *Acanthamoeba castellanii* (Ac), which is described in more detail below.

1.6.1. *Acanthamoeba castellanii*

Ac is a FLA that is ubiquitously found in soil and water (de Jonckheere, 1991; Schuster, 2002). The Ac life cycle consists of two phases: a vegetative trophozoite stage and a dormant cyst stage. The trophozoite represents the active form wherein amoebae replicate by binary fission. Trophozoites range in size from 15 to 50 µm in diameter. The trophozoite cell structure includes a nucleus with a centrally large, densely staining nucleolus, several food vacuoles, free ribosomes, mitochondria and a contractile vacuole. Ac has three different types of cytoplasmic vacuoles: contractile vacuoles, involved in osmoregulation; secretory vacuoles, containing digestive enzymes; and digestive vacuoles, for food degradation (Bowers & Korn, 1968; Allen et al., 2002; Khan, 2006). A unique feature of the trophozoite is the presence of spine-like structures that arise from the body surface called acanthopodia (pseudopodia). Acanthopodia are essential for cellular movement, adhesion to surfaces, and capturing prey (Khan, 2006; Gómez-Couso et al., 2007). Ac actively grazes on bacteria, algae, yeast, and small nutrient particles (Allen & Dawidowicz, 1990; Weekers et al., 1993). Food uptake can occur by phagocytosis and pinocytosis. Phagocytosis (cellular eating) involves intake of solid particles, giving rise to an internal compartment called a phagosome (Haas, 2007). Pinocytosis (cellular drinking) involves intake of liquid particles with the formation of vacuoles called pinosomes (Khunkitti et al., 1998; khan, 2006).

Under adverse environmental conditions, such as food deprivation, the trophozoite transforms into a metabolically inactive cyst. The cyst is composed of an outer ectocyst and an inner endocyst, which together form a wrinkled, double-walled cell structure. Ac cysts range in size from 10 to 20 µm in diameter (Tomlinson & Jones, 1962; Lloyd et al., 2001, 2014). Cysts are

extremely resistant to adverse environmental conditions such as high temperature, freezing and UV irradiation (Chatterjee, 1968; Brown & Barker, 1999; Turner et al., 2000; Lloyd et al., 2001;). Studies have shown that the cysts can remain viable for 24 years in water at 4°C (Mazur et al., 1995; Sriram et al., 2008). Under favourable conditions, amoeba trophozoites can emerge from the cyst, leaving the outer shell behind (Bowers & Korn, 1969; Schuster, 2002; Khan, 2006).

1.6.1.1. How intracellular bacteria survive in Ac

Acanthamoeba actively feed on bacteria, but many bacterial species are able to survive and multiply within phagosomes or the cytoplasm of these organisms. The ability to survive intracellularly can lead to enhanced growth, increased virulence and resistance to antibiotics (Casadevall, 2008; Ray et al., 2009). Many Gram-negative and Gram-positive bacteria, including *Mycobacterium*, *Vibrio*, *Legionella*, *Salmonella*, *E. coli*, and *Pseudomonas* are able to establish an endosymbiotic association with *Acanthamoeba* (Greub & Raoult, 2004; Hilbi et al., 2007). The term endosymbiosis is defined as a regulated cohabitation of two nonrelated organisms, where one organism survives inside the other (Greub & Raoult, 2004).

Amoebae typically engulf bacteria through phagocytosis and once inside the phagosome, bacteria are exposed to harsh acidification and oxidative stress conditions (Cosson & Lima, 2014;). However, many bacteria have evolved strategies to cope with the phagosome environment, enabling them to benefit from host cell resources (Ray et al., 2009). The most common strategy employed to avoid phagosomal killing involves escape into the cytosol. The cytosol is considered a favourable environment for bacteria because it provides a rich source of nutrients (Ray et al., 2009). For example, *M. tuberculosis* escapes vacuoles via a process that requires the mycobacterial type VII secretion system (Mittal et al., 2018). Additionally, this

bacterium can exit the cell via an F-actin structure called an ejectosome enabling it to infect another cell (Hagedorn et al., 2009; Gerstenmaier et al., 2015). Some intracellular bacteria are able to invade the eukaryotic nucleus; for example, *Candidatus* Nucleicultrix amoepiphila was found in the nuclei of *Hartmannella* sp. (Schulz et al., 2014). Another strategy that intracellular bacteria deploy is to remain viable inside phagosomes. This tactic is achieved by subverting antimicrobial mechanisms such as preventing phagosome-lysosome fusion, damaging phagosomal membranes and modulating phagosomal pH (Berger et al., 1994; Brand et al., 1994). For instance, *L. pneumophila* uses a multiprotein nanomachine known as the Icm/Dot type IV secretion system to avoid degradation (Hubber & Roy, 2010). This secretion system allows bacteria to hijack the phagocytic vacuole (Isberg et al., 2009; Hoffmann et al., 2014), enabling *L. pneumophila* to hide and replicate in a safe intracellular niche called the *Legionella*-containing vacuole (Horwitz & Silverstein, 1980; Casadevall, 2008). After replication, *L. pneumophila* lyses and exits the amoeba, and goes on to re-infect new cells (Steinert, 2011). Numerous other intracellular bacteria utilize similar strategies (Garcia-del Portillo & Finlay, 1995; Casadevall, 2008; Ray et al., 2009). Figure 1.3 provides an overview of different mechanisms employed by bacteri to survive inside protozoa and macrophages.

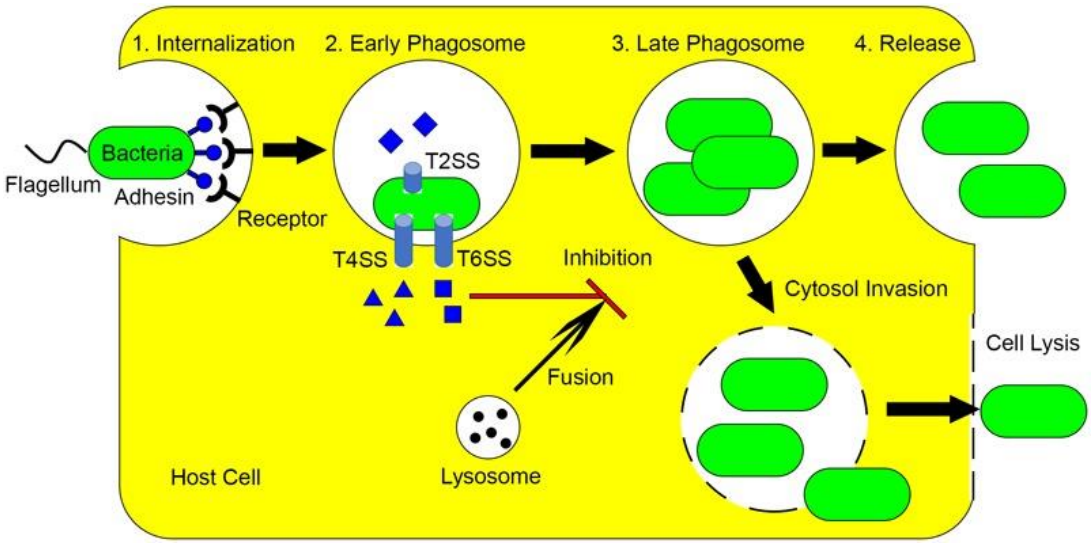

Figure 1.3. Mechanisms of persistence utilized by bacteria to escape intracellular degradation by protozoa and macrophage. (1) Entry into cells through adhering to host cell receptors. (2) Phagosome invasion and inhibiting lysosome-phagosome fusion via the release of effector proteins. (3) Replication inside the phagosome or escape into the cytosol. (4) Exit through exocytosis or host cell lysis. Figure has been sourced from Sun et al. (2018). *Frontiers in Microbiology* authorizes republishing of the requested material without permission.

1.7. What makes a successful biocontrol agent?

The rhizosphere is an ecological niche that is home to highly diverse groups of organisms, including bacteria, fungi, protozoa, nematodes, and arthropods (Buée et al., 2009; Mendes et al., 2013; Philippot et al., 2013). The elevated number of microorganisms in the rhizosphere usually leads to an increase in the size of the predator population (Taylor, 1977). Free-living nematodes and protozoa are well known bacterial predators that can have a significant impact on bacterial community structure (Bonkowski & Brandt, 2002; Rønn et al., 2002). In fact, consumption by these grazing predators is considered to be a major source of bacterial mortality in ecosystems (Girlanda et al., 2001; Johansen et al., 2002). Introduced biocontrol bacteria must evince not only efficient control of plant pathogens but also persistence at levels significant for pathogen suppression (Bouwman & Zwart, 1994; Rodriguez-Zaragoza et al., 2005). There are two factors challenging rhizobacterial fitness in the environment: i) competition for nutrients (bottom-up regulation) and ii) predation pressure by bacterial feeders (top-down regulation).

1.7.1. Bottom-up regulation

Competition for nutrients and other resources is considered a major force impacting survival of a particular bacterial species in the rhizosphere (Hibbing et al., 2010). In soil, the majority of bacteria are nutrient-limited and therefore live in a starved state (Ramos et al., 2000). Studies have revealed that the ribosomal RNA content of bacterial cells colonizing the rhizosphere of barley seedlings is similar that of starved cells (Ramos et al., 2000). Furthermore, the competitive success of rhizobacteria is attributed to certain traits, such as growth rate. Fast-growing bacteria are often successful competitors that increase in density by exploiting existing resources (Taylor, 1977; Amarasekare, 2003;). However, nutrient availability can also determine competitive success of a species. For instance, slow-growing bacteria are able to survive and exploit low

concentrations of nutrients (Aerts, 1999; Harpole & Tilman, 2006). Thus, both growth rate and nutrient availability play an essential role in the competition for nutrients, impacting bacterial abundance in the community (Aerts, 1999).

Rhizosphere bacteria must compete for nutrients with other heterotrophic organisms such as fungi (Marschner et al., 1997). Studies have revealed that plants infected with mycorrhizae exhibit diminished root exudation, thereby reducing the colonization density of rhizobacteria (Marschner et al., 1997). Traits that promote rhizobacterial establishment include motility, which facilitates the search for nutrients (Czaban et al., 2007), production of antimicrobial compounds that inhibit competitors (Dubuis et al., 2007a), and adhesion to remain in favorable environments (Rudrappa et al., 2008).

1.7.2. Top-down regulation

As discussed above, root exudates increase nutrient availability thereby enhancing bacterial population density in the rhizosphere. In turn, the increased number of bacteria serves to attract organisms that prey upon them (Pernthaler et al., 1996; Folman et al., 2001; Achouak et al., 2004). Microfaunal predators, including bacterivorous protozoa and nematodes, are found in the rhizosphere at levels 30-fold higher than that of bulk soil and they can consume up to 60% of the bacterial population (Persson, 1983; Griffiths, 1990; Foissner, 1999). Predation by microfaunal predators is considered the leading cause of bacterial mortality in the environment (Rønn et al., 2001; Rosenberg et al., 2009); therefore, the ability to cope with top-down control is crucial for bacterial persistence in the environment (Queck et al., 2006; Dubuis et al., 2007). A number of factors affect predation including the size, metabolic state, surface properties and habitat structure of the prey (Postma et al., 1990; Posch et al., 2001; Wootton et al., 2007). For example, amoebae can search for prey in the narrowest pores in soil, whereas, ciliates and flagellates are limited to

water pellicles (Coûteaux & Darbyshire, 1998). Bacterivores play a vital role in nutrient cycling by releasing nutrients from consumed bacteria, such as nitrogen, which become available for uptake by plant roots (Uikman et al., 1991; Ferris et al., 2004). Increased turnover of bacterial biomass is termed the "microbial loop" and it is believed to be a major contributor to the beneficial effects of protozoa on plant health (Ekelund et al., 1994; Bonkowski, 2004; Ferris et al., 2004).

1.8. Bacterial defence against predators

Biotic interaction is a significant parameter shaping the structure of bacterial communities in aquatic and terrestrial habitats, including the rhizosphere (Rønn et al., 2002; Bonkowski, 2004; Mao et al., 2007). Bacteria and their predators perceive and respond to chemical cues and such interplay serves to modulate their interaction (Matz & Kjelleberg, 2005). To cope with the threat of predation, bacteria have developed sophisticated defence strategies, which are described in detail in the following sections.

1.8.1. Defence against predators: the effect of bacterial secondary metabolites

One of the most efficient defence strategies bacteria employ to circumvent predation is production of unpalatable or toxic metabolites, which act to repel or kill predators (Jousset, 2012). Secretion of toxins can also lead to elevated predation pressure on competitors, providing producers with increased access to nutrients (Heeb et al., 2002; Jousset et al., 2006). For example, *P. protegens* CHA0, which produces 2,4-DAPG, pyoluteorin, PRN and HCN, exhibited toxicity towards the ciliate *Colpoda steinii* and the amoeba *Vahlkampfia*, resulting in rapid lysis of both protists (Jousset et al., 2010). Additionally, PRN, PLT and protease were found to induce encystation of these protists (Jousset et al., 2010). The authors reported elevated expression of the

toxin-encoding *phlA*, *prnA* and *hcnA* genes when bacteria were co-incubated with *A. castellanii* cell-free supernatant. These findings indicate that bacteria are able to respond to predator-specific cues and adjust their gene expression accordingly. In contrast, co-incubation *P. protegens* CHA0 with amoeba resulted in reduced expression of these genes; as such direct contact with bacteria appears to enable the amoeba to repress bacterial toxicity (Jousset et al., 2010).

Certain bacterial species are able to produce antibacterial compounds with surfactant properties known as cyclic lipopeptides, which can have a detrimental effect on soil amoeba. For example, the bacterium *P. protegens* DR54 is able to inhibit the growth of *Hartmanella vermiformis* through the production of the biosurfactant viscosinamide (Mazzola et al., 2009). Other *P. protegens* strains, like SS101 and SBW25, that produce the biosurfactants massetolide and viscosin, respectively, can hinder the growth of the predator *Naegleria americana* (Song et al., 2015). Transcriptomic analyses of SS101 and SBW25 revealed that a total of 55 genes in strain SS101 and 65 genes in strain SBW25 were upregulated upon interaction with *N. americana*. Moreover, biosynthesis of putrescine by SS101 was increased in response to predation (Song et al., 2015). Interestingly, this compound was found to induce trophozoite encystment and affect cyst viability (Song et al., 2015).

1.8.2. Morphological adaptation

Morphological adaptations are considered to be one of the major defence strategies employed by bacteria to cope with predation pressure (Shikano et al., 1990; Hahn & Höfle, 1999). These morphological responses involve formation of inedible filaments, microaggregates or biofilms (Hahn et al., 1999, 2004; Tarao et al., 2009). Bacterial biofilms are defined as an assemblage of microbial cells embedded within a hydrated extra-polymeric matrix adhered to a

biotic or abiotic surface (Costerton et al., 1995; Queck et al., 2006). Many bacterial species form different biofilm structures, which mediate protection from protozoan grazing. A microcolony-type biofilm protected *Serratia marcescens* from the flagellate *Bodo saltans*, while a filamentous biofilm sheltered this bacterium from *Acanthamoeba polyphaga* (Queck et al., 2006). Moreover *P. aeruginosa* strain PAO1 is able to form grazing-resistant microcolonies in response to the presence of the flagellate *Rhynchomonas nasuta* (Matz et al., 2004). Formation of long, inedible filaments is another response of bacteria to grazing. This morphotype constitutes a large portion of the bacterial biomass under intense predation pressure (Hahn et al., 1999). For example, filament-forming *Flectobacillus* spp. exhibited resistance against the flagellate *Ochromonas* spp. (Hahn et al., 1999). Another adaptive strategy is to increase bacterial motility. A study revealed that swimming speeds exceeding 30µm s^{-1} successfully inhibited prey capture by bacterivorous flagellates (Matz & Jürgens, 2005).

1.8.3. Biochemical surface properties

Bacterial surface properties can provide protection against microfaunal grazing and some protozoa can distinguish between prey using membrane-bound receptors (Monger et al., 1999; Tarao et al., 2009). For example, *Salmonella enterica* serovars are consumed by intestinal amoebae at different rates based on variability of the lipopolysaccharide O antigen (Wildschutte et al., 2004). Formation of an S-layer (surface layer) is another protective mechanism shielding bacteria from predators (Sára & Sleytr, 2000). Intracellular resistance of *Synechococcus* cells in the ciliate *Tetrahymena* was correlated to the presence of protective S-layers (Koval, 1993). Additionally, the S-layer of Actinobacteria has been reported to affect both ingestion and digestion by the nanoflagellate *Poterioochromonas* (Tarao et al., 2009).

1.9. Thesis objectives

Beyond pathogen suppression, the success of a BCA depends on its ability to persist in the environment and to avoid the threat of grazing predators. Predation can have a significant impact on the structure of bacterial communities in the environment. At present, it is not known whether the BCA *P. chlororaphis* PA23 can avoid consumption by the protozoan predator Ac. Therefore, PA23-Ac interaction studies were undertaken to address this knowledge gap.

In addition to producing diffusible antibiotics and degradative enzymes that mediate biocontrol, PA23 synthesizes PHA polymers as a carbon and energy sink. Exactly how PHA accumulation affects PA23 physiology is currently unknown. In the second half of the thesis, PHA mutants were generated and phenotypically characterized. Moreover, the role of this storage polymer in the interaction of PA23 with Ac was investigated.

The specific objectives of this thesis are as follows:

1. To investigate the role of PA23 exoproducts in defence against the predator Ac and to determine whether the presence of amoeba alters PA23 gene expression.
2. To determine how PHA accumulation affects PA23 physiology including AF activity, PHZ production, biofilm formation, and stress endurance.
3. To examine whether the ability to accumulate PHAs influences the PA23-Ac interaction.

Chapter 2

Materials and Methods

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was cultured at 37°C on Lysogeny Broth (LB) agar (Difco Laboratories, Detroit, MI, USA). *P. chlororaphis* strains were routinely cultured on LB or in M9 minimal salts medium supplemented with 0.2% glucose and 1mM magnesium sulphate (M9-glc) at 28°C. Ramsay's Minimal Medium (RMM) supplemented with 20g/L glucose (RMM-glc) was used to induce the accumulation of PHA in PA23 strains (Sharma et al., 2017). Media were supplemented with the following antibiotics: ampicillin (Amp; 100 μg/ml), gentamicin (Gm; 15 μg/ml) for *E. coli*, and piperacillin (Pip; 40 μg/ml), Gm (20 μg/ml), tetracycline (Tc; 15 μg/ml) for PA23. All antibiotics were obtained from Research Products International Corp. (Mt. Prospect, IL, USA).

2.2. *Acanthamoeba* strain and culture conditions

Acanthamoeba castellanii (ATCC 30234) was grown axenically without shaking in 20 ml of PYG medium (proteose peptone 10 g/L, yeast extract 5 g/L, glucose 10 g/L and the additives: 4 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $CaCl_2$, 0.05 mM $Fe(NH_4)2(SO_4)2 \cdot 6H_2O$, 2.5 mM $Na_2HPO_4 \cdot 7H_2O$, 2.5 mM KH_2PO_4, 0.1 M glucose and 3.4 mM sodium citrate$\cdot 2H_2O$) in T75 tissue culture flasks (Sarstedt, Saint-Leonard, QC, Canada) in a humidified incubator at 25°C. Before the experiment, cultures were washed three times with Ac buffer (PYG medium lacking proteose peptone, yeast extract and glucose) (Moffat & Tompkins, 1992) to remove non-adherent cells or cysts. Amoeba trophozoite density was measured using a Neubauer cell counting chamber. To obtain amoeba cell-free supernatant, cells were grown in Ac buffer for three days at 25°C and then filtered using 0.22μm filters (Sarstedt). Supernatants were stored at -80°C.

2.2.1. Reviving *A. castellanii* trophozoites from frozen stocks

An ampule containing 1 ml of frozen Ac trophozoites was thawed in a 35ºC water bath for approximately 2 to 3 minutes as outlined by the American Type Culture Collection (ATCC) protocols. After thawing, the contents of the ampule were immediately transferred to a 5-ml volume of PYG medium and centrifuged at 600 x g for 5 minutes at room temperature. Supernatant was discarded, and the pellet was resuspended in 5 ml PYG medium and transferred to a T25 tissue culture flask (Sarstedt). The flask was incubated at 25°C and when the culture was at or near peak density, cells were passaged to a T75 flask.

2.3. Nucleic acid manipulation

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

Table 2.1. Bacterial strains and plasmids used in the study.

Strains, plasmids & primers	Relevant genotype, phenotype or sequence	Reference or source
Strains		
Acanthamoeba castellanii	Wild-type ATCC 30324	ATCC Manassas, VA, USA
Pseudomonas 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	Nandi et al. (2015)
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*rpoS*	PA23 with pKNOCK-Tc vector inserted into *rpoS* gene	Selin et al. (2012)
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*phaC1*	PA23 with a Tc^R marker inserted into *phaC1*	This study
PA23*phaC1ZC2*	Unmarked deletion mutant missing internal portions of *phaC1* and *phaC2* and the entire *phaZ* gene	This study
PA23*phaC1ZC2D*	Unmarked deletion mutant missing internal portions of *phaC1* and *phaD* and the entire *phaZ* and *phaC2* genes	This study
PA23*phaC1*(C)	pUCP23-*phaC1* in PA23*phaC1* mutant	This study
PA23*phaC1ZC2*(C)	pUCP23-*phaC1ZC2* in PA23*phaC1ZC2* mutant	This study

PA23*phaC1ZC2D*(C)	pUCP23-*phaC1ZC2D* in PA23*phaC1ZC2D*	This study
PA23-*gfp*	PA23 containing GFP expressed from pTDK-GFP	This study
PA23-8-*gfp*	PA23-8 containing GFP expressed from pTDK-GFP	This study
PA23-63-*gfp*	PA23-63 containing GFP expressed from pTDK-GFP	This study
PA23-63-1-*gfp*	PA23-63-1 containing GFP expressed from pTDK-GFP	This study
PA23*hcn-gfp*	PA23*hcn* containing GFP expressed from pTDK-GFP	This study
PA23-6863-*gfp*	PA23-6863 containing GFP expressed from pTDK-GFP	This study
PA23*phzR-gfp*	PA23*phzR* containing GFP expressed from pTDK-GFP	This study
PA23*rpoS-gfp*	PA23*rpoS* containing GFP expressed from pTDK-GFP	This study
PA23*gacS-gfp*	PA23*gacS* containing GFP expressed from pTDK-GFP	This study
E. coli		
DH5α	*supE44 ΔU169* (ϕ80*lacZ*ΔM15)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco
DH5α λpir	λpir lysogen of DH5α	House et al. (2004)
Chromobacterium violaceum		
CVO26	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31532 autoinducer biosensor	Latifi et al. (1995)
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)
pEX18Ap	Suicide plasmid, Amp^R	Hoang et al. (1998)
pRK600	Contains *tra* genes for mobilization, Chl^R	Finan et al. (1986)
pCR2.1*phaC1*	610 bp fragment containing *phaC1* gene in pCR2.1	This study
pKNOCK-*phaC1*	610 bp fragment containing *phaC1* in pKNOCK-Tc	This study
*phaC1C2*pEX18Ap	1.3 kb internal fragment missing portion from *phaC1* and *phaC2* and the entire *phaZ* gene inserted in pEX18Ap	This study

*phaC1D*pEX18Ap	1 kb internal fragment missing *phaC2* and *phaZ* genes and a portion from *phaC1* gene inserted in pEX18Ap	This study
pUCP23	Broad-host-range vector, Amp^R Gm^R	West et al. (1994)
pUCP23-*phaC1*	The entire *phaC1* gene in pUCP23	This study
pUCP23-*phaC1ZC2*	*phaC1ZC2* in pUCP23	This study
pUCP23-*phaC1ZC2D*	*phaC1ZC2D* in pUCP23	This study
pTdK-GFP	GFPmut3.1 gene under control of the lac promoter, contains an origin of replication for both *P. aeruginosa* and *E. coli*, Amp^R	de Kievit et al. (2001)
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)
pPHZI-*lacZ*	*phzI* promoter in pLP170	Selin et al. (2012)
pPHZR-lacZ	*phzR* promoter in pLP170	Selin et al. (2012)
pRPOS-*lacZ*	*rpoS* promoter in pLP170	Poritsanos et al. (2006)
pGACS-*lacZ*	*gacS* promoter in pLP170	Nandi et al. (2015)
pGACA-*lacZ*	*gacA* promoter in pLP170	Nandi et al. (2015)
Primers		
phaC1-FOR	5′-atcggcaagaaacctgggcaccagtgaaggc-3′	This study
phaC1-REV	5′- tcgccagcggattgagctcccagcaggtagttgt-3′	This study
phaC2-FOR	5′-gagctcaatccgctggcgatcaaggagatcttca-3′	This study
phaC2-REV	5′-accttggccatgtcgcgaccgt-3′	This study
phaD-FOR	5′-gagctcaatccgctggcgaagttcgagcgtttcc-3′	This study
phaD-REV	5′-gtgttcgtcgaggtaacgcagggccatctgc-3′	This study
phaC1(C)-FOR	5′- atctgcaattctttgaatgctgggctgc-3′	This study
phaC1(C)-REV	5′-tcgaagcttcggctgttcttc-3′	This study
phaC2(C)-REV	5′- gagttctagaatccggtcgcgggttt-3′	This study
phaD(C)-REV	5′- ccaacaagcttcagccagtcctgagttt-3′	This study

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

2.4. Generation of PA23*phaC1*, PA23*phaC1ZC2*, PA23*phaC1ZC2D* mutants

All primers and plasmids utilized for the construction of mutant strains are listed in Table 1. To generate the PA23*phaC1* mutant, a 610-bp internal region of the *phaC1* gene was PCR amplified using primers *phaC1*-FOR and *phaC1*-REV. Primers were designed based on the sequence of the *phaC1* gene obtained from *P. chlororaphis* PA23 (GenBank accession no. NZ_CP008696). A TOPO® kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to clone the 610-bp PCR amplicon into the pCR®2.1-TOPO® vector, generating pCR2.1-*phaC1*. To liberate the insert, pCR2.1-*phaC1* was digested with *Xba*I and *Hin*dIII and was subcloned into the same sites of the pKNOCK-Tc suicide vector. Triparental mating between the donor *E. coli* DH5α λpir (pKNOCK-*phaC1*), *E. coli* DH5α (pRK600) and PA23 was performed. Pseudomonas Isolation Agar (PIA; Difco) supplemented with Tc (50 µg/ml) was used to screen for transconjugants. To verify that pKNOCK-*phaC1* had correctly inserted into the *phaC1* gene, PCR analysis was performed using primers *phaC1*-FOR and *phaC1*(C)-REV.

The PA23*phaC1ZC2* mutant was created by first amplifying two fragments from *phaC1* and *phaC2*. To accomplish this, an internal 610-bp segment from the *phaC1* gene was amplified using *phaC1*-FOR and *phaC1*-REV, and 680-bp from the *phaC2* gene was amplified using *phaC2*-FOR and *phaC2*-REV. The *phaC1*-REV primer was designed such that it has a 5′ overhang complementary to the end of the 5′ end of the *phaC2* fragment. Splicing by overhang extension (SOE) PCR was used to ligate the two products using *phaC1*-FOR and *phaC2*-REV. The 1.3-kb fragment was then digested with *Eco*RI and *Hin*dIII and subcloned into the same sites of pEX18Ap.

The *phaC1ZC2D* mutant was generated using the same steps utilized for creating a *phaC1ZC2* mutant except that a 450-bp internal region from the *phaD* gene was amplified using

phaD-FOR and *phaD*-REV. SOE-PCR was used to ligate the two products using *phaC1*-FOR and *phaD*-REV. The 1-kb fragment was then digested with *Eco*RI and *Hin*dIII and subcloned into the same sites of pEX18Ap. Triparental mating between *E. coli* DH5α containing *phaC1ZC2*pEX18Ap or *phaC1ZC2D*pEX18Ap, *E. coli* DH5α (pRK600) and PA23 was performed. Transconjugants were screened on PIA. To select bacteria that had undergone a double cross-over event, colonies were streaked onto LA supplemented with sucrose (10%). PCR was performed to confirm that the WT allele(s) had been successfully replaced with mutated derivatives.

2.5. Plasmid construction

For complementation analysis, pUCP23-*phaC1*, pUCP23-*phaC1ZC2* and pUCP23-*phaC1ZC2D* were created. The inserts were amplified by PCR using PA23 genomic DNA as a template and primers listed in Table 1. To complement PA23*phaC1*, a 1.9-kb PCR fragment containing the entire *phaC1* gene was obtained using *phaC1*(C)-FOR and *phaC1*(C)-REV and cloned into pCR2.1-TOPO to create *phaC1*-pCR2.1. The *phaC1*-pCR2.1 was then excised as an *Xba*l-*Hin*dIII fragment and subcloned into pUCP23 digested with the same enzymes yielding pUCP23-*phaC1*. The entire *phaC1ZC2* was amplified using primers *phaC1*(C)-FOR and *phaC2*(C)-REV. The 4.7-kb *phaC1ZC2*-containing fragment was cloned into pCR2.1 (*phaC1ZC2*-pCR2.1) and then excised with *Sca*I and *Xba*I and subcloned into the same sites of pUCP23, generating pUCP23-*phaC1ZC2*. To complement *phaC1ZC2D*, a 5.4-kb fragment containing the *phaC1ZC2D* cluster was amplified using *phaC1*(C)-FOR and *phaD*(C)-REV and cloned into pCR2.1-TOPO to yield *phaC1ZC2D*-pCR2.1. The 5.4-kb *Sma*I-*Hin*dIII fragment was excised from *phaC1ZC2D*-pCR2.1 and subcloned into the same sites of pUCP23, creating pUCP23-*phaC1ZC2D*.

2.6. Growth rate analysis

Overnight cultures of PA23 WT and derivative strains grown in RRM-glc were adjusted to an OD_{600} of 0.1 by diluting with the same media. One hundred µL of diluted culture was dispensed into the wells of a sterile 96-well polystyrene plate (Sarstedt, Montreal, QC, Canada) and growth was monitored for 48 h using a SpectraMax M2 microplate reader (Molecular Devices, CA, USA) with continuous shaking at 28ºC. Five replicates were tested for each strain and the growth rate analysis was repeated three times.

2.7. *Acanthamoeba* assays

2.7.1. *P. chlororaphis* PA23 – *A. castellanii* co-culture assays

To study amoeba-bacterial interactions, Ac trophozoites were washed three times with Ac buffer. Amoebae were then adjusted to 10^6 cells/ml, and 1-ml aliquots were transferred into wells of a 24-well plate and incubated at 28ºC for 1 h to allow adherence. Wells were washed three times with Ac buffer to remove non-adherent amoebae. Next, bacterial suspensions grown in M9-glc or RMM-glc were adjusted to 10^8 CFU/ml, and a 1-ml volume of bacteria was added to each well and allowed to incubate at 28ºC for 15 days. Growth and viability of amoeba trophozoites was determined by microscopic visualization of eosin-stained cells (dead cells stain red; live cells remain unstained; cysts are morphologically distinct). Amoebae grown in the absence of bacteria were used as a negative control. The number of bacteria residing in the co-culture samples was determined through viable plate counting.

2.7.2. Effect of PRN, PHZ and KCN on Ac trophozoite viability

Cultures of PA23 strains were incubated for 3 days at 28ºC, and then cells were pelleted, and the supernatant passed through a 0.2-µm filter to remove all bacterial cells. The experimental setup was the same as described above (**2.7.1**), except that bacterial supernatant was added to the wells. The morphological changes exhibited by amoeba were monitored using an inverted microscope (Zeiss Observer Z1 inverted microscope, Carl Zeiss Microscopy GmbH, Göttingen, Germany). To determine the effect of purified PRN on Ac, amoebae were adjusted to 10^6 cells/ml in Ac buffer containing commercially purified PRN (Sigma, St. Louis, MO, USA) at the following concentrations: 0 µg/ml (control), 0.1, 0.5, 1, 5 and 10 µg/ml. For PHZ analysis, an overnight culture of the PRN- derivative (PA23-8) grown in LB medium was used to extract PHZ following the method of Chancey et al. (1999). PHZ extractions were quantified with UV-visible spectroscopy (Biochrom Ltd. Cambridge, England), and the absorption maxima for PCA and 2-OH-PHZ were measured at 367 and 490 nm, respectively. Amoeba cells were incubated with extracted PHZ at the following concentrations: 0, 10, 20, 35, and 50 µg/ml. Ac subcultures containing various concentrations of PRN and PHZ were incubated in 24-well plates at 28ºC, and amoeba viability was monitored at 1, 3, 6, 12, 18, 24, 48 and 72 h. For KCN analysis, Ac trophozoites were incubated with commercial KCN (Sigma, St. Louis, MO, USA) at the following concentrations: 0 µg/ml (control), 50, 100, 200, 400 and 800 µg/ml and Ac counts were monitored at 1, 6, 12, 18, 24, 48 and 72 h. Five replicates were included per assay, and the experiment was repeated three times.

2.7.3. Intracellular survival assay

For analysis of intracellular survival, the experiment was carried out as described above except that bacteria and amoeba co-cultures were incubated at 28ºC for 3 h according to Bui et al. (2012). Wells were then washed three times with Ac buffer to remove extracellular bacteria and resuspended in 1ml Ac buffer supplemented with gentamicin (750 µg/ml). After 1 h incubation at 28ºC the gentamicin buffer was removed, and the wells were washed three times with Ac buffer to eliminate residual antibiotic. A 100-µl aliquot from the last wash was plated on LA to determine the number of extracellular bacteria remaining after gentamicin treatment. Intracellular survivors were quantified as follows: amoeba cells were lysed by passing 10 times through a 27-gauge needle, resulting in total cell lysis as shown by microscopy. Ten-fold serial dilutions of the lysate were plated to reveal the number of viable internalized bacteria. Three replicates were included for each assay, and the experiment was repeated twice.

2.7.4. Chemotaxis assays

Bacteria were grown in M9-glc medium at 28ºC for 24 h. Axenically grown Ac cultures were prepared as described above. Petri dishes (60 x 15 mm) containing 5 ml of 1.5% water agar had three wells created 20 mm apart. An aliquot of 50 µl of Ac (10^6 cells/ml) was transferred into the centre well. One of the two outside wells contained 50 µl of "test" bacterial inoculum, while the second well contained a 50 µl suspension of PA23 WT, the *gacS* mutant, or saline as the "control" sample. A hand-crafted grid coverslip was placed underneath the plate for counting amoebae that had migrated from the centre well towards the outside wells. The chemotactic index was calculated based on the formula: number of amoebae migrating towards the test sample/number of amoebae moving towards the control sample.

2.7.5. Microscopic imaging of Ac internalized bacteria

Bacterial strains harbouring constitutively expressed green fluorescent protein (GFP) were generated by electroporating plasmid pTDK-GFP (de Kievit et al., 2001) into the PA23 WT, *gacS*$^-$ PRN$^-$, PHZ$^-$ and PRN/PHZ$^-$ strains. Amoeba and bacterial co-cultures were incubated in 24-well plates at 28°C for 0, 5, and 24 h. For microscopic examination, a 5-µl volume of the co-culture was applied to a 2% agarose pad on a glass microscope slide. Cells were examined with a Zeiss LSM 700 scanning confocal laser microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany). GFP-tagged bacteria were visualized using 485 nm excitation and 509 nm emission wavelengths.

2.8. Analysis of transcriptional fusions in the presence and absence of Ac

The activity of *prnA-*, *phzA-*, *phzI-*, *phzR-*, *rpoS-*, and *gacS-lacZ* transcriptional fusions was determined in PA23 cultured in the presence and absence of Ac. Active amoebae were adjusted to 10^6 cells/ml in Ac buffer, as described above. Overnight bacterial cultures grown in M9-glc were adjusted to an optical density of 0.1 (2 x 10^8 CFU/ml), prior to co-culture with Ac or amoeba-free supernatant. Samples were grown for 24, 48, and 72 h in M9-glc at 28°C. The effect of Ac cells or cell-free supernatant on PA23 gene activity was determined by β-galactosidase assays (Miller, 1972).

2.9. Stress tolerance

To assess the role of PHA in stress tolerance, the WT, *pha*-mutants and complemented strains were exposed to high and low temperature, hydrogen peroxide, UV irradiation, and starvation conditions as described in detail in the following sections. For these experiments, 3-ml aliquots of overnight cultures were used to inoculate flasks containing 30 ml of RMM-glc. Bacterial cultures

were incubated with shaking (150 rpm) at 28°C for 48 h. Cells were washed and resuspended in saline before exposure to the various stress conditions. The same starting inoculum was used for all experiments (OD_{600nm} = 0.1, which corresponds to 2.0 x 10^8 CFU/ml). Bacterial survival was assessed by plate counting (CFU/ml) at various time points and expressed as percentage of the initial population. Three replicates were included for each strain and assays were repeated three times.

2.9.1. Heat exposure

A 10-ml volume of bacterial suspension was incubated in a water bath at 50°C for 60 min (Kadouri et al., 2003). Viable bacterial counts (CFU/ml) were measured every 10 min by serial dilution plating.

2.9.2. Cold temperature exposure

To assay tolerance to cold and freezing conditions, 3 ml of bacterial cells resuspended in saline were incubated at both 4°C and −20°C following the method of Ayub et al. (2009). Bacterial viability was determined every 10 h for 30 h. The frozen suspensions were thawed and then resuspended using a vortex mixer before plating on LB agar.

2.9.3. Oxidative stress exposure

Sensitivity to hydrogen peroxide (H_2O_2) was assessed using a disk assay (Kadouri et al., 2003a). Whatman filter discs impregnated with 0.3% H_2O_2 were placed in the center of an LB agar plate covered with a lawn of bacteria. After 24h growth at 28°C, the zone of inhibition surrounding the discs was measured.

2.9.4. UV exposure

Bacteria were exposed to UV light in a Biological Safety Cabinet (Thermo Fisher Scientific). A 20-ml volume of bacterial suspension was placed in a 90-mm plastic Petri dish and the lids were removed before the exposure to UV radiation. Bacterial viability was determined every 10 seconds for 1 min.

2.9.5. Starvation experiments

Bacteria were collected and washed three times by centrifugation at 5,000 rpm for 5 min in saline solution. Bacteria were adjusted to OD_{600nm} of 0.1 in saline and incubated with shaking (150 rpm) at 28°C for 12 days according to Tal & Okon (1985).

2.10. Confocal microscopy imaging of PHA accumulation

Cultures of PA23 WT and PHA derivative strains were inoculated into RMM-glc to an OD_{600nm} of 0.1 and incubated at 28°C with shaking (150 rpm) for 48 h. To detect the presence of PHA granules, a 100-µL aliquot of culture was stained with 0.1% Nile blue (Sigma), incubated at 60°C for 15 minutes and washed once with LB broth to remove the unbound stain. Finally, 10 µL of the bacterial suspension was deposited onto a glass slide containing a 2% agarose pad. Images were taken using a Zeiss LSM 700 confocal laser-scanning microscope under 63× magnification. PHA granules stained with Nile blue were visualized using 555 nm excitation and 580 nm emission wavelengths. Images are representative of 10 frames from each slide.

2.11. Phenotypic assays

2.11.1. Antifungal activity

To assess the ability of PA23 and derivative strains to inhibit the growth of *S. sclerotiorum in vitro*, radial diffusion assays were performed. A 5-µL volume of an overnight culture was spotted onto 1/5th strength potato dextrose agar plates (PDA; Difco) and allowed to grow for 24 h at 28°C. A plug of *S. sclerotiorum* mycelia was then placed in the center of the plate. Plates were incubated at 22°C for up to 3 days to allow mycelium growth. Fungal inhibition was assessed by measuring the zone of clearing surrounding the bacterial colony as described by Poritsanos et al. (2006). To determine whether growth in the presence of Ac impacts AF activity, bacteria were grown in M9-glc in the presence and absence of trophozoites for 72 h at 28°C. Bacteria were spotted onto PDA plates and assessed for fungal inhibition as described above. Five replicates were analyzed for each strain and experiments were repeated three times.

2.11.2. Autoinducer detection assay

For qualitative analysis of AHL production, cultures were spotted onto LB agar seeded with *C. violaceum* CVO26. Strain CVO26 generates neither AHL nor the QS controlled pigment violacein (Latifi et al., 1996). However, this strain is able to detect exogenous AHLs with carbon chain lengths ranging from C4-C8, resulting in a deep purple halo surrounding the bacterial colony. A 5-µL volume of overnight culture was spotted onto CVO26-seeded agar and incubated for 24-48 hours at 28°C. The diameter of purple pigment surrounding the bacterial colonies was measured (Poritsanos et al., 2006). Five replicates were analysed for each strain, and the experiment was repeated three times.

Quantitative analysis of AHL production was performed according to Ling et al. (2009) with the following modifications. PA23 was grown in the presence or absence of Ac trophozoites in 30 ml M9-glc for 72 h at 28ºC. 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 dried under a stream of air. An overnight culture of *P. aeruginosa* QSC105 (pEAL01) grown in PTSB supplemented with carbenicillin (200 µg/mL) was diluted to a final OD_{600} of 0.1. A 1-mL aliquot of culture was added to each of the dried extracts. Cultures were grown for 18 hours at 37°C and then analyzed for β-galactosidase activity (Miller, 1972). Strains were analyzed in triplicate, and the experiment was repeated twice.

2.11.3. Protease analysis

Extracellular protease production was determined qualitatively by inoculating a 5-µL volume of bacterial culture onto a 1.5% agar plate containing 2% skim milk (Difco). Protease activity was indicated by a zone of lysis surrounding the colony after 36 – 48 h growth at 28ºC (Poritsanos et al., 2006). Zones of clearing were measured for each strain. Data represent the average of five replicates, and the experiment was repeated three times.

2.11.4. Motility analysis

Flagellar (swimming) motility was monitored by inoculating a 5-µl volume of an overnight bacterial culture below the surface of 0.3% LB agar plate (Poritsanos et al., 2006). To assess the impact of amoeba on PA23 swimming motility, bacteria were grown in the presence and absence of Ac trophozoites in M9-glc for 72 h at 28ºC. The diameter of swim zones was measured at 24,

48 and 72 h. For the assays, five replicates were analyzed, and the experiment was repeated three times.

2.11.5. Quantitative analysis of PRN

Production of PRN was quantified by HPLC as described by Selin et al. (2010) with the following modifications. Cultures of PA23 were grown in the presence and absence of the amoeba at 28°C in 30 ml M9-glc and PRN was extracted and quantified after 96 h of growth. Toluene was added to the culture supernatants as an internal control. Peaks corresponding to toluene and PRN were analyzed by UV absorption at 225 nm using a Varian 335 diode array detector. Samples were analyzed in triplicate, and the experiments were repeated three times.

2.11.6. Quantitative analysis of PHZ

Production of PHZ was quantified according to the method described by Selin et al. (2010). Briefly, a 5-mL volume of overnight culture was grown in M9-glc at 28°C. Cultures were centrifuged at 6000 rpm for 10 min and cell supernatants were collected and combined with an equal volume of benzene and one drop of concentrated HCl. The mixture was shaken for 1 hour at room temperature and the top organic layer was collected and dried under air. The dried extracts were resuspended in 1 mL 0.1 M NaOH and absorption maxima for PCA and 2-OH-PHZ was measured at 367 nm and 490 nm, respectively, using UV-visible spectroscopy according to Maddula et al. (2008). To determine the relative amounts of PCA and 2-OH-PHZ in the cultures, absorption maxima were divided by their standard extinction coefficients (PCA: 3019; 2-OH-PHZ: 7943; Olson & Richards, 1967). PHZ quantification was performed in triplicate and experiments were repeated twice.

2.12. Biofilm formation

To assess the ability of PA23 WT, PA23*phaC1*, PA23*phaC1ZC2*, PA23*phaC1ZC2D*, and complemented strains to form biofilms, a 96-well plate assay was conducted as described by Berry et al. (2010). Briefly, bacterial cultures grown in RMM-glc were adjusted to an OD of 1.0 and then diluted 1 in 100 in fresh media. A 200 µl aliquot of the diluted culture was added to each well of the 96-well plates (Becton-Dickenson, Oakville, ON). After 24 h incubation at 28°C, 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. A 200-µL aliquot of 95% ethanol was added 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. Five replicates of each strain were tested, and experiments were repeated twice.

2.13. Root colonization assay

For this assay, surface-sterilized seeds of *Arabidopsis thaliana* (Columbia-0) were germinated on Petri dishes containing half-strength Murashiga and Shoog (MS) medium (Sigma, St. Louis, MO, USA) supplemented with 2% glucose for 7 days. The ability of the PA23 WT and derivative strains to colonize root surfaces was analyzed following the protocol of Kadouri et al. (2002). An overnight culture of bacterial inoculum (OD_{600nm} of 0.1), resuspended in 5 ml saline solution, was used to inoculate flasks containing 0.4 g of freshly harvested 7-day old roots of *A. thaliana*. After 2 h of incubation with shaking (150 rpm) at 28°C, the roots were gently washed three times in saline solution, followed by vigorous washing using a vortex mixer for 2 min to detach bacteria from the roots. A 100-µl aliquot was plated on LA agar plates to determine the

percentage of viable bacteria attached to the roots. Strains were tested in triplicate and experiments were repeated twice.

2.14. Extracellular polysaccharide quantification

Exopolysaccharides (EPS) were extracted from PA23 WT, the *pha* mutants and complemented stains, according to Burdman et al. (2000). Briefly, overnight cultures grown in RMM-glc were centrifuged at 4000×g for 20 min at 4°C. The supernatant was collected, filtered through a 0.22-µm membrane, and incubated overnight at 4°C with three volumes of cold ethanol to precipitate EPS. The anthrone-sulfuric acid colorimetric assay was used to quantitate EPS in 96-well microtitration plates (Laurentin & Edwards, 2003). A 40-µL volume of water (blank), standard (0.05 - 0.4 g/L glucose), or sample was added to each of the wells and incubated at 4ºC for 15 min. A 0.1-mL aliquot of freshly prepared anthrone solution was added to each well, plates were sealed with parafilm and then incubated in a water bath at 92ºC for 3 minutes to allow colour development. Absorption was measured at 630 nm in SpectraMax M2 microplate reader. Five replicates were included for each strain and the experiment was repeated twice.

2.15. Statistical analysis

An unpaired Student's t-test was used for statistical analysis of PRN, PHZ, AHL production, swimming motility, AF activity and protease production. The Tukey test was applied to determine the chemotactic preference of amoeba for each of the bacterial strains. One-way ANOVA was used for statistical analysis of biofilm formation, root attachment and H_2O_2 assays. The two-way ANOVA test was applied for amoeba-bacterial co-culture assays, gene expression analysis, UV radiation, heat, cold and freezing exposure experiments.

Chapter 3

Pseudomonas chlororaphis PA23 metabolites provide protection against grazing and facilitate survival within intracellular vacuoles of *Acanthamoeba castellanii*

3.1. Introduction

Pseudomonas chlororaphis strain PA23 is a biocontrol agent capable of suppressing disease caused by the fungal pathogen *S. sclerotiorum* (Fernando et al., 2007; Savchuk & Fernando, 2004). This bacterium produces an arsenal of secondary metabolites, which contribute to biocontrol. Secreted compounds include the diffusible antibiotics phenazine (PHZ) and pyrrolnitrin (PRN) together with hydrogen cyanide (HCN), protease, chitinase, and lipase (Poritsanos et al., 2006; Zhang et al., 2006). Mutants deficient in either PRN or HCN production exhibit reduced fungal antagonism, indicating that these two products are important for PA23 biocontrol (Selin et al., 2010; Nandi et al., 2017). While PHZ plays a more minor role in pathogen suppression, it does contribute to biofilm formation by this bacterium (Selin et al., 2010). A complex regulatory network that functions at the transcriptional and post-transcriptional level governs the expression of these metabolites. For example, the GacS-GacA two-component system, which works in concert with a second system called Rsm, acts as a positive regulator of PA23 biocontrol (Poritsanos et al., 2006; Selin et al., 2014). Similarly, the PhzRI quorum-sensing (QS) system activates expression of biocontrol genes; while RpoS and the sigma regulator PsrA function as repressors through downregulation of PRN (Manuel et al., 2012; Selin et al., 2012, 2014).

Beyond its ability to suppress disease-causing pathogens, the success of a biocontrol agent is contingent upon successful colonization of a given environment. One of the primary threats to environmental persistence is consumption by microfaunal predators, including protozoa and nematodes that feed upon bacteria. In response, bacteria have evolved strategies to help resist predation. One such antipredator defence tactic is the production of compounds with toxic and or repellent activities (Ekelund & Ronn, 1994; Jousset, 2012; Philippot et al., 2013). We have

previously demonstrated that PA23 produces PRN and HCN that exhibit nematocidal and repellent activities towards the nematode *Caenorhabditis elegans* (Nandi et al., 2015). Moreover, co-culturing leads to increased expression of genes and products associated with biocontrol, indicating that PA23 is able to sense and respond to the presence of *C. elegans* (Nandi et al., 2015).

In the soil, naked amoebae are key drivers of microbial community structure and activity due to their ability to access small pores (Ekelund & Ronn, 1994). Ac is one such example that has been used as a model organism to explore bacteria-amoebae interactions. The life cycle of Ac is comprised of two stages: a vegetative trophozoite and a dormant cyst form. Trophozoites are covered with spindle-like surface projections known as acanthopodia, which are believed to facilitate prey capture, adhesion to surfaces, and cell motility. Under harsh conditions, trophozoites can differentiate into non-dividing, highly resistant cysts (Marciano-Cabrabal & Cabral, 2003; Khan, 2006).

To date, the fate of strain PA23 in the presence of the grazing predator Ac has yet to be explored. The focus of the current study was to ascertain whether PA23 is able to persist in the presence of this amoeba and to define the role of exoproducts in the predator-prey interaction. Our findings revealed that PRN, PHZ and HCN have detrimental effects on trophozoite viability and therefore provide protection against protozoan grazing *in vitro*. Moreover, the production of exoproducts enabled PA23 to survive inside Ac vacuoles, thus protecting bacteria from intracellular degradation. Co-culturing with amoeba led to enhanced expression of secondary metabolite genes and products, suggesting that PA23 is able to detect the presence of amoeba and adjust its physiology accordingly.

3.2. Results

3.2.1. PA23 affects Ac trophozoite viability

To determine the impact of PA23 on trophozoite viability and cyst formation, the PA23 WT and derivative strains, including regulatory (*rpoS*$^-$, *gacS*$^-$, *phzR*$^-$ and AI-deficient) and biosynthetic mutants (PRN$^-$, PHZ$^-$, and HCN$^-$) were offered to Ac as prey. Previous phenotypic analysis showed that the *gacS*$^-$, *phzR*$^-$ and AI-deficient strains produce little to no antibiotics and degradative enzymes (Poritsanos et al., 2006; Selin et al., 2012). Conversely, the *rpoS*$^-$ and PHZ$^-$ mutants secrete elevated levels of PRN relative to WT (Selin et al., 2010; Manuel et al., 2012). As illustrated in Figure 3.1, in the presence of the *gacS*$^-$, *phzR*$^-$, and AI-deficient strains, Ac trophozoite numbers increased 2.2-, 1.7-, and 1.9-fold, respectively by day 5, after which the amoebae remained active but declined slowly due to food shortage. When co-incubated with PA23 WT, PHZ$^-$, PRN$^-$, HCN$^-$ and *rpoS*$^-$ cells, the number of trophozoites decreased over time (Fig. 3.1) either through transforming into dormant cysts or undergoing cell lysis (data not shown). At day 15, there were less trophozoites present in co-cultures with the PHZ$^-$ strain, which produces 2-fold more PRN compared to WT. Conversely, trophozoite numbers were significantly increased when grown on the PRN$^-$ and HCN$^-$ strains (Fig. 3.1). Collectively, these results indicate that PA23 exoproducts play a role in the inhibition of Ac growth.

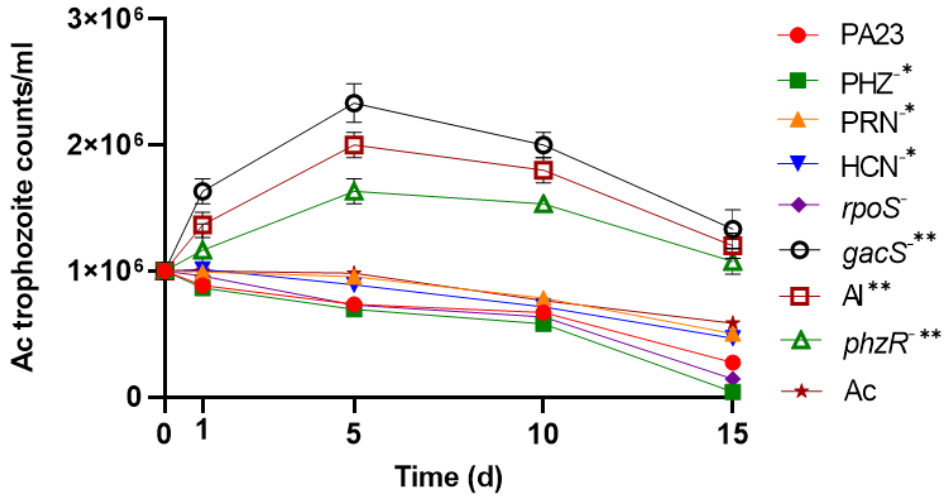

Figure 3.1. Growth of Ac trophozoites on PA23 and derivative strains in M9-glc. Amoeba growth and viability were monitored for 15 days. Asterisks indicate significant difference from the PA23 WT as determined by two-way ANOVA (*, $P < 0.001$; **, $P < 0.0001$). Note: PHZ$^-$, PRN$^-$ and HCN$^-$ mutants are statistically significant at day 15 only, whereas the *gacS*$^-$, AI-deficient, and *phzR*$^-$ strains are statistically significant at days 1, 5, 10 and 15. Experiments were performed three times; one representative data set is shown.

3.2.2. Bacterial persistence upon co-culturing with Ac trophozoites

To investigate whether bacterial growth was affected by the presence of amoeba, bacteria were co-cultured with Ac and viability was assessed over time. The number of PA23 WT, *rpoS*$^-$ and PHZ$^-$ cells increased from 1 x 10^8 CFU/ml on day 0 to between 9.7 x 10^8 and 9.8 x 10^8 CFU/ml on day 1 (Fig. 3.2). Similarly, the HCN$^-$ and PRN$^-$ strains increased from 1 x 10^8 CFU/ml to 7.5 x 10^8 and 7.6 x 10^8 CFU/ml, respectively. In the case of the QS-deficient *phzR*$^-$ and AI$^-$ strains, both showed a slight increase on day 1 from 1 x 10^8 CFU/ml to 5.1 x 10^8 and 3.2 x10^8, respectively. The *gacS* mutant, on the other hand, was the only strain that did not increase in abundance; instead, numbers declined to 3.1x10^7 CFU/ml. On day 5, the PA23 WT and the PRN over-producing PHZ$^-$ and *rpoS*$^-$ strains continued to increase in abundance. The PRN$^-$ and HCN$^-$ populations also increased but to a lesser degree. For the *gacS*$^-$ mutant, there were no viable bacteria detected on day 5, while the number of QS-deficient cells was dramatically reduced. Bacteria populations continued to decrease by day 10, with the largest number of cells remaining for the PA23 WT, PHZ$^-$ and *rpoS*$^-$ strains (Fig. 3.2). There were no viable cells recovered on day15 (data not shown). In the absence of Ac, there were no observable differences in bacterial viability between strains over time (Fig. 3.3).

3.2.3. The effect of PA23 metabolites on Ac viability

To further explore the impact of PA23 exoproducts on Ac trophozoites, amoebae were challenged with PA23 cell-free supernatant. One h after incubation with supernatant, amoeba cells started to swell, and this continued until they began to burst at 2 h (Fig. 3.4a). Incubation with supernatant from the *gacS*$^-$ mutant did not affect amoeba morphology (Fig. 3.4b). In this case trophozoites were virtually identical to those suspended in Ac buffer (Fig. 3.4d).

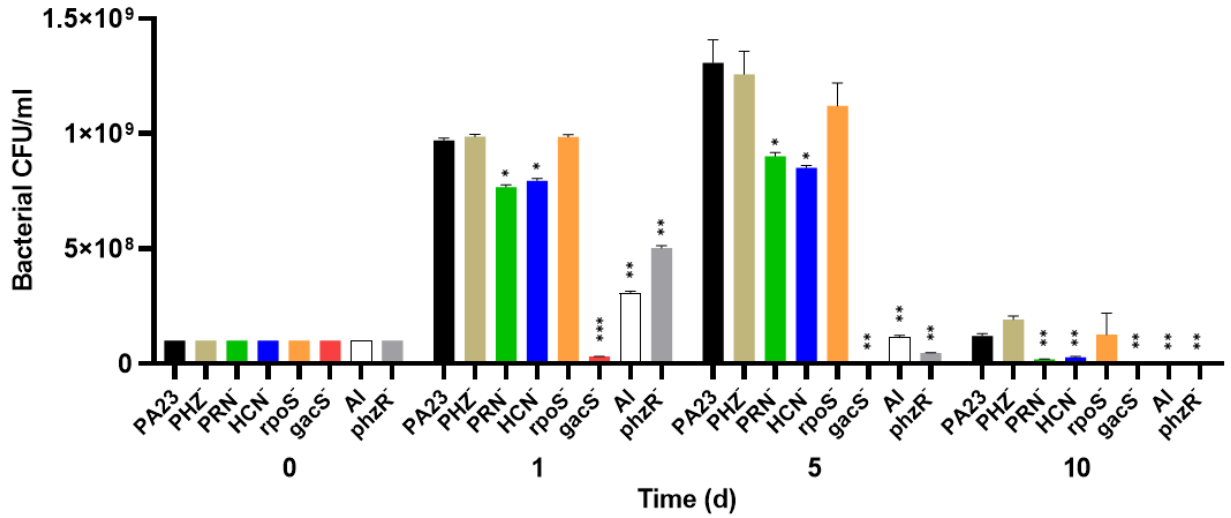

Figure 3.2. Effect of Ac trophozoites on the growth of PA23 and derivative strains in M9-glc. Bacteria and amoeba were co-cultured for 15 days, and bacteria were enumerated on days 1, 5, 10 and 15. By day 15 there were no viable bacteria remaining. Asterisks indicate statistical significance of difference using two-way ANOVA (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$). Experiments were performed three times; one representative data set is shown.

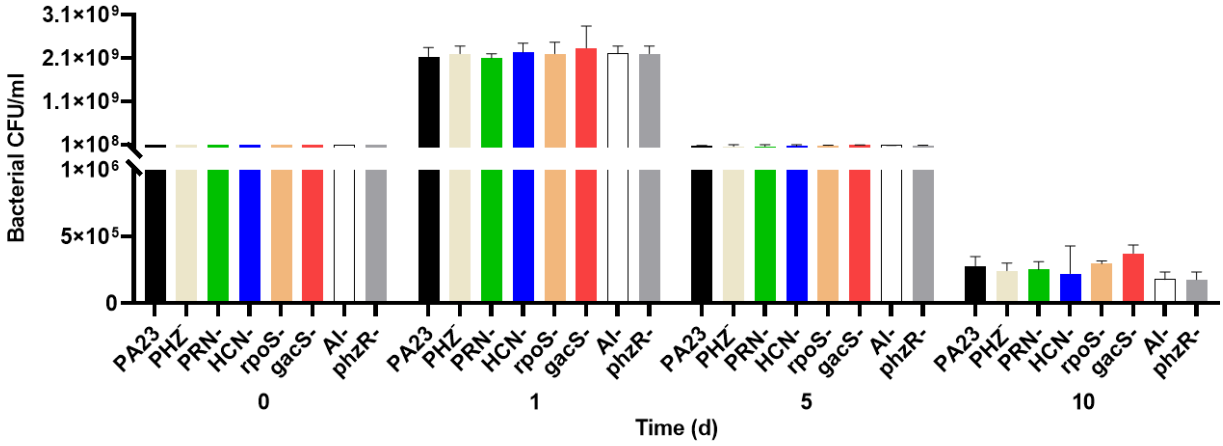

Figure 3.3. Growth of PA23 and derivative strains in the absence of Ac trophozoites in M9-glc for 15 days. Bacteria were enumerated on days 1, 5, 10 and 15. No viable bacteria were recovered on day 15. Experiments were performed three times; one representative data set is shown.

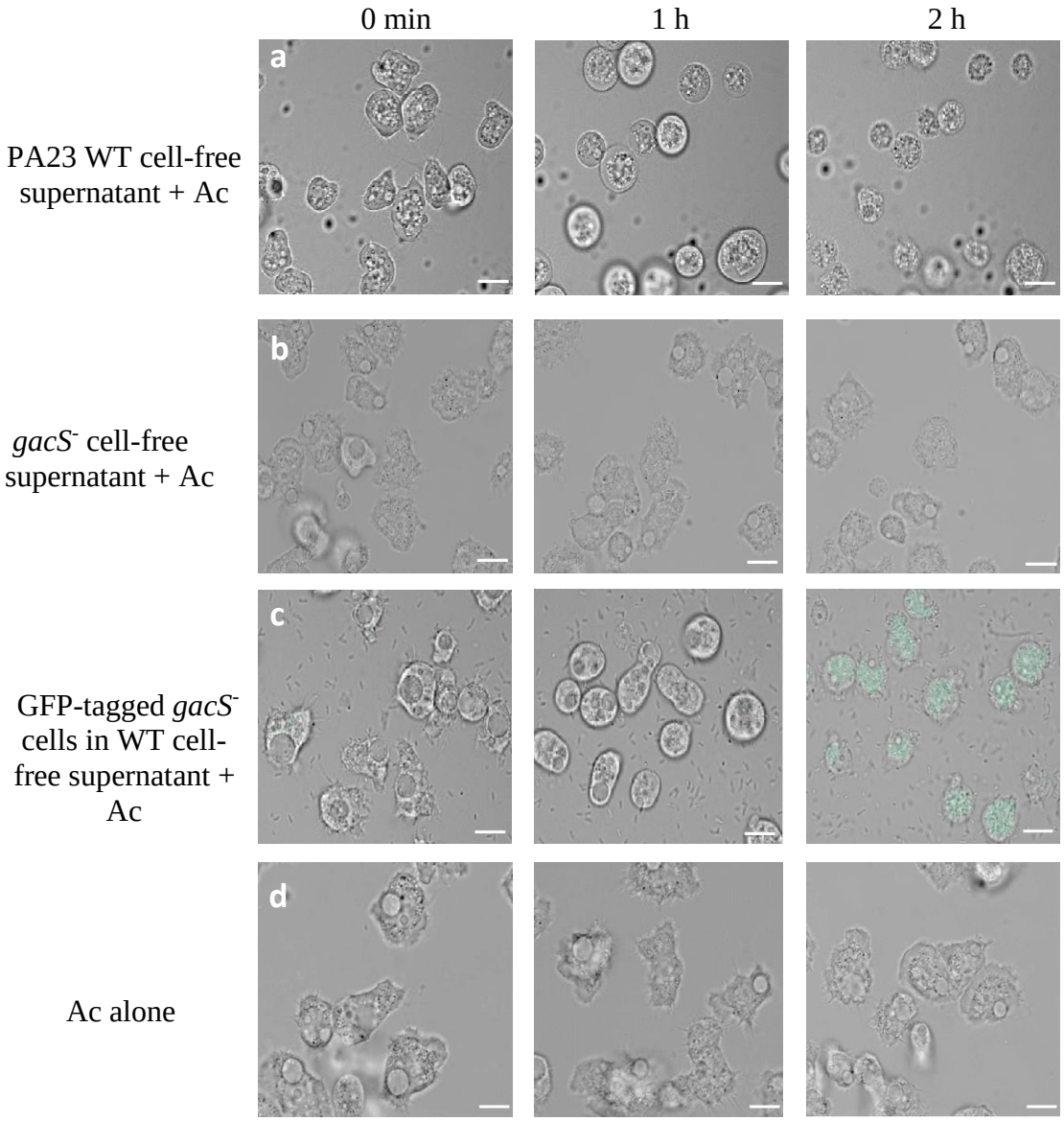

Figure 3.4. Incubation of Ac trophozoites with PA23 WT cell-free supernatant (a), *gacS*$^{-}$ cell-free supernatant (b), GFP-tagged *gacS*$^{-}$ cells containing WT cell-free supernatant (c), and trophozoites in Ac buffer (d). The swelling of the Ac trophozoites was observed at 1 h and the final burst was noticed at 2 h of incubation. Images were captured using a Zeiss Observer Z1 inverted microscope under 40× magnification. Scale bar = 10 μm.

To investigate how feeding on nontoxic strains in the presence of toxic metabolites impacts amoeba, Ac trophozoites were co-cultured with GFP-tagged *gacS*$^{-}$ mutant cells resuspended in PA23 cell-free supernatant. As depicted in Figure 3.4c, at 1h, amoeba had lost their amoebic shape. After 2 h incubation, trophozoites were fluorescing green consistent with uptake of the *gacS*$^{-}$ cells. Despite the fact that the trophozoites were actively feeding, they underwent the same morphological changes as when challenged with PA23 WT supernatant alone, including cell lysis. Collectively these finding indicate that secreted PA23 metabolites exert deleterious effects on Ac trophozoites.

To explore whether purified compounds would exhibit the same toxicity, trophozoites were challenged with PRN (0-10 μg/ml), PHZ (0-50 μg/ml) and KCN (0-800 μg/ml). As illustrated in Figure 3.5, when exposed to PRN at a concentration of 1 μg/ml or lower, there was no impact on amoeba viability. However, at higher concentrations, the number of Ac trophozoites declined in a dose-dependent fashion (Fig. 3.5a). PHZ was also found to exhibit toxic effects on the amoebae. At concentrations of 20 μg/ml and lower, PHZ had little effect on protozoan survival. When challenged with higher concentrations (35-50 μg/ml), amoeba viability decreased to less than 50% after 24 h (Fig. 3.5b). Exposure to KCN led to a reduction in the number of Ac trophozoite at concentrations of 400 μg/ml and above (Fig. 3.5c).

3.2.4. PA23 exoproducts affect the chemotactic response of Ac

Bacterial exoproducts can exhibit either attractant or repellent effects, and this can ultimately impact predator grazing. To study the chemotactic response of Ac towards bacteria, binary choice assays were undertaken, as depicted in Figure 3.6a. Compared to saline control, trophozoites were more attracted to the *gacS*$^{-}$, QS-deficient, and PRN^{-} strains, all of which are deficient in PRN production (Fig. 3.6b).

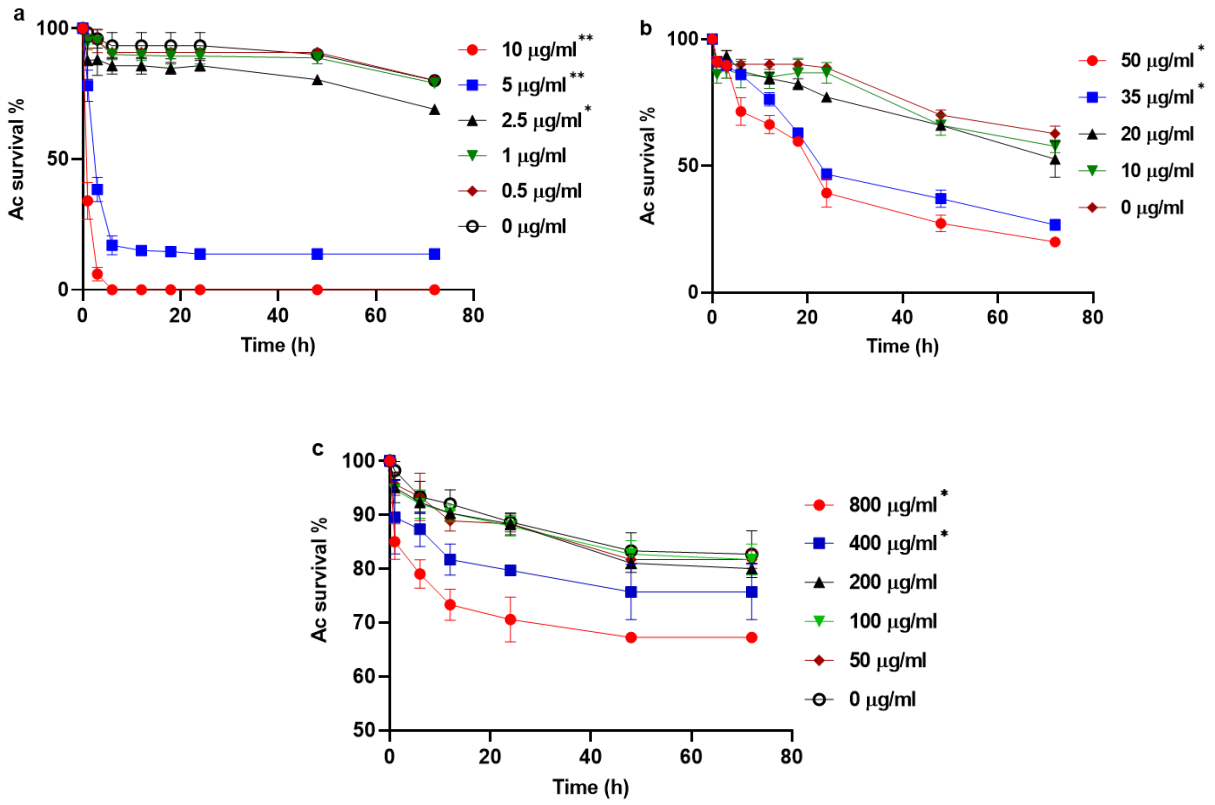

Figure 3.5. Ac trophozoites were challenged with PRN (0-10 µg/ml) (a), PHZ (0-50 µg/ml) (b) and KCN (0-800 µg/ml) (c). Asterisks indicate statistical significance of difference using two-way ANOVA (*, $P < 0.01$; **, $P < 0.001$). Three replicates were used per trial, and the experiment was repeated three times. One representative data set is shown.

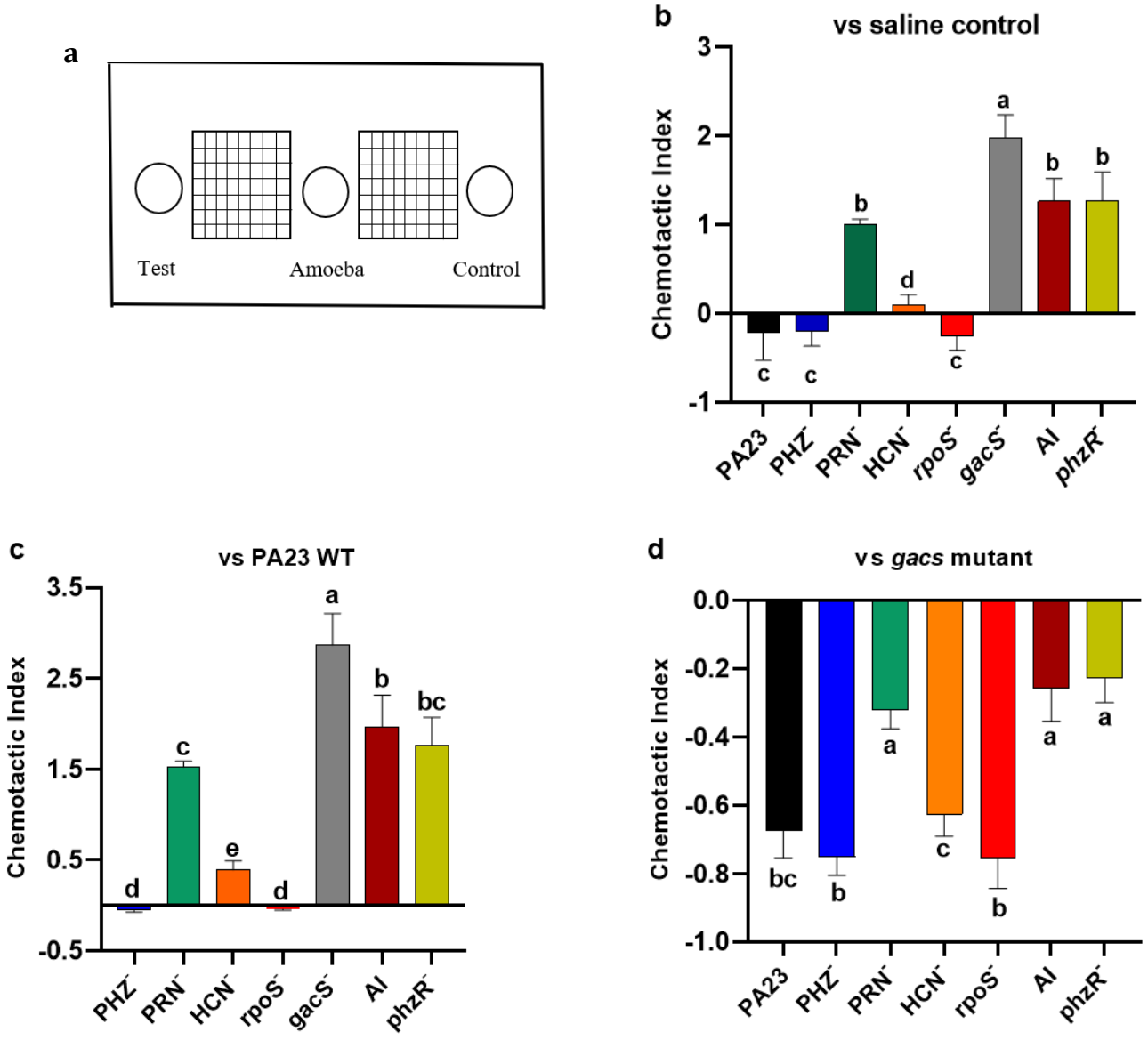

Figure 3.6. Chemotactic response of Ac towards PA23 WT and derivative strains. (**a**) Schematic diagram illustrating Petri plate set up. Active amoebae were placed in the center well; the test bacterium was placed in the test well, and PA23 WT, the *gacS* mutant or saline was added to the control well. Chemotactic preference assays were carried out against saline control (**b**), PA23 WT (**c**), and the *gacS* mutant (**d**). The chemotactic response was determined as follows: the number of amoeba migrating towards the test well / the number of amoebae migrating towards the control well. Assays were performed in triplicate and the experiment was repeated three times. Error bars indicate ± SD; columns labelled with the same letter do not differ significantly by the Tukey test ($P > 0.05$).

Whereas the PA23 WT, and PRN hyper-producing PHZ$^-$ and *rpoS*$^-$ mutants exhibited a repellent effect. Amoebae were only marginally attracted to the HCN$^-$ strain (Fig. 3.6b). Employing PA23 as the control, amoeba preferentially migrated towards all of the strains except for the PRN overproducers (PHZ$^-$ and *rpoS*$^-$ mutants; Fig. 3.6c). Trophozoites had a strong preference for the *gacS*$^-$ derivative because Ac consistently migrated towards this bacterium when it was included as the control (Fig. 3.6d). Again, the PRN-producers (PHZ$^-$, *rpoS*$^-$, HCN$^-$ and PA23 WT) exhibited the strongest repellent activity (Fig. 3.6d).

3.2.5. Growth in the presence of Ac affects PA23 gene expression

To determine whether bacteria can sense the presence of the predator, PA23 was grown together with amoeba or cell-free supernatants and monitored for changes in gene expression. For this assay, biosynthetic (*prnA* and *phzA*) and regulatory genes (*phzI*, *phzR*, *rpoS*, *gacS*) were analyzed. No changes in gene expression were observed in bacteria incubated with Ac cell-free supernatants. Co-incubation of PA23 with trophozoites led to increased expression of *phzA* and *prnA* at both 48 h and 72 h (Fig. 3.7). For the QS genes *phzI* and *phzR*, direct contact with amoeba resulted in a significant increase in *phzI-lacZ* activity at all time points tested, in contrast, *phzR* activity was elevated at only 48 h (Fig. 3.7). Growth with amoeba led to an increase in the *rpoS-lacZ* activity at 72 h, while no change in *gacS* expression was observed at any of the time points (Fig. 3.7).

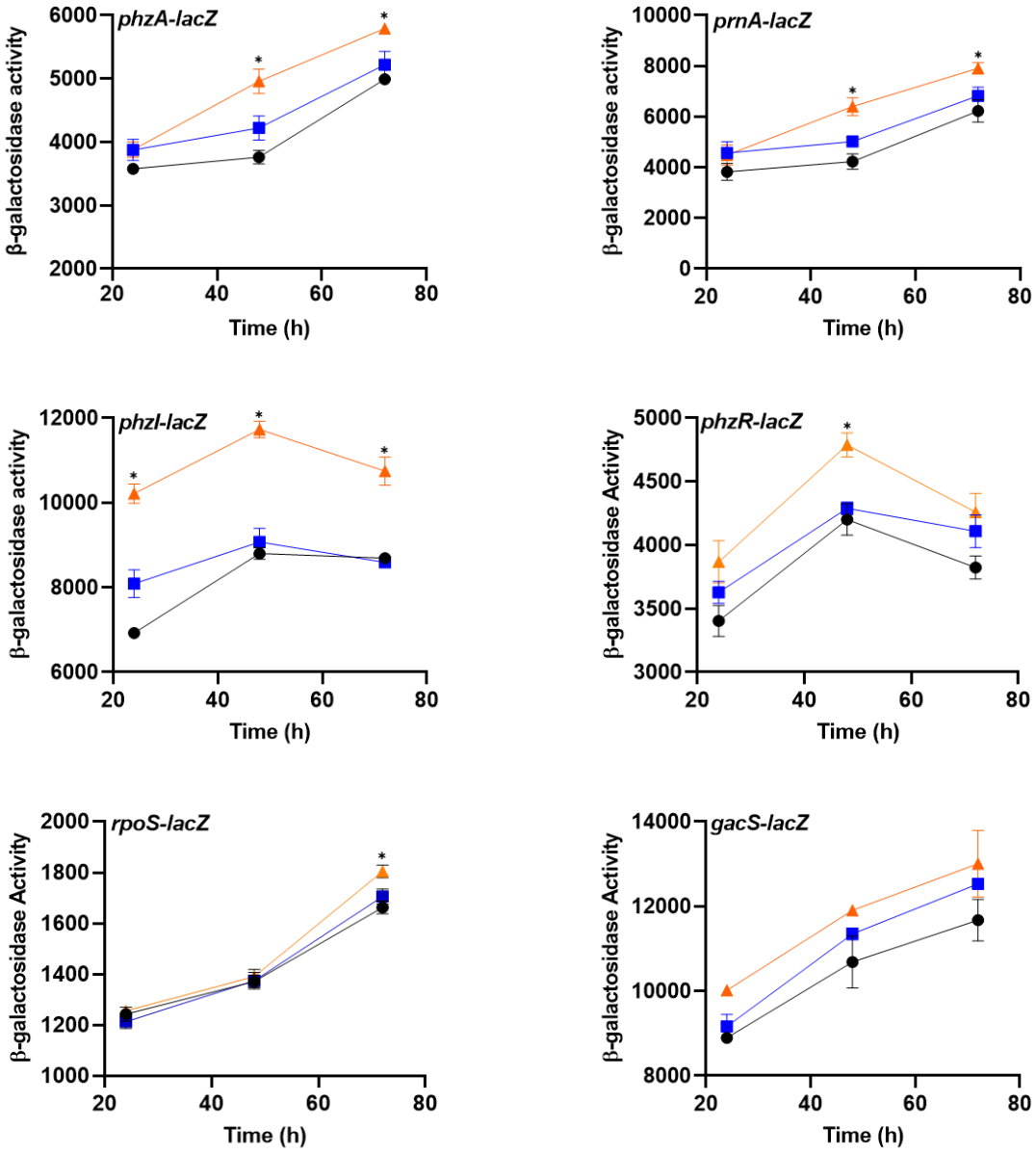

Figure 3.7. The impact of *A. castellanii* cells and cell-free supernatant on *phzA*, *prnA*, *phzR*, *phzI*, *rpoS* and *gacS* expression in *P. chlororaphis* PA23. Co-cultures with Ac trophozoites (▲), Ac cell-free supernatant (■) and bacteria alone (●) were analyzed for β-galactosidase activity at 24, 48 and 72 h. Asterisks indicate statistical significance of difference using two-way ANOVA (*, $P < 0.01$). Experiments were performed three times; one representative data set is shown.

3.2.6. Impact of Ac on PA23 phenotypic traits

Phenotypic analysis was undertaken to determine whether changes in secondary metabolite production or other traits were brought on by growth in the presence of Ac. As outlined in Table 3.1, co-incubation with amoeba led to increased PRN and PHZ production, consistent with the elevated *phzA* and *prnA* gene activity. Other phenotypic traits, including fungal inhibition, protease activity, and swimming motility, were unaffected by Ac (Table 3.1).

3.2.7. Survival of PA23 inside Ac

To investigate whether PA23 can tolerate the acidic environment of amoeba vacuoles, we carried out intracellular survival assays with the PA23 WT *gacS*$^-$, PRN$^-$, PHZ$^-$, and PRN/PHZ$^-$ strains. Amoeba were infected for 3 h and then treated with gentamicin to kill extracellular bacteria. The number of recovered bacteria was less than 250 CFU/ml, confirming the effectiveness of antibiotic killing. Amoeba cells were lysed (time 0h) and the number of bacteria recovered for the PA23 WT, PRN$^-$ and PHZ$^-$ strains was found to be ~1% of the original inoculum. For the PRN/PHZ$^-$ and *gacS*$^-$ strains, significantly fewer cells were recovered (Fig. 3.8). All five strains could be recovered at 5 h; however, by 24 h only the PA23 WT, PRN$^-$ and PHZ$^-$ remained (Fig. 3.8). These findings suggest that exoproduct-producing strains of PA23 are able to remain viable and resist degradation in the acidic vacuoles of amoeba over the course of 24 h. At 48 h, no growth was detected for any of the strains (data not shown).

Confocal laser scanning microscopy (CLSM) was utilized to visualize GFP-tagged bacteria inside of trophozoites (Fig. 3.9). The panel of strains examined in the intracellular survival assays (Fig. 3.8) were analyzed microscopically.

Table 3.1. Phenotypic characterization of PA23 grown in the presence and absence of Ac

Organism	PHZ (µg/ml)[a]	PRN (µg/ml)[a]	Antifungal (mm)[b]	Protease (mm)[b]	AHL (mm)[b]	Motility (cm)[b]
PA23 alone	32.8 (1.4)	3.4 (0.3)	5.12 (0.6)	4.87 (0.2)	4.62 (0.4)	59.6 (1.2)
PA23 + Ac	38.16 (0.9)*	4.4 (0.3)*	5.25 (0.5)	5.25 (0.5)	4.62 (0.4)	62 (0.8)

[a] Mean ± SD obtained from five replicates.

[b] Mean ± SD of zones of activity obtained from five replicates.

* Significantly different from PA23 WT ($P < 0.05$).

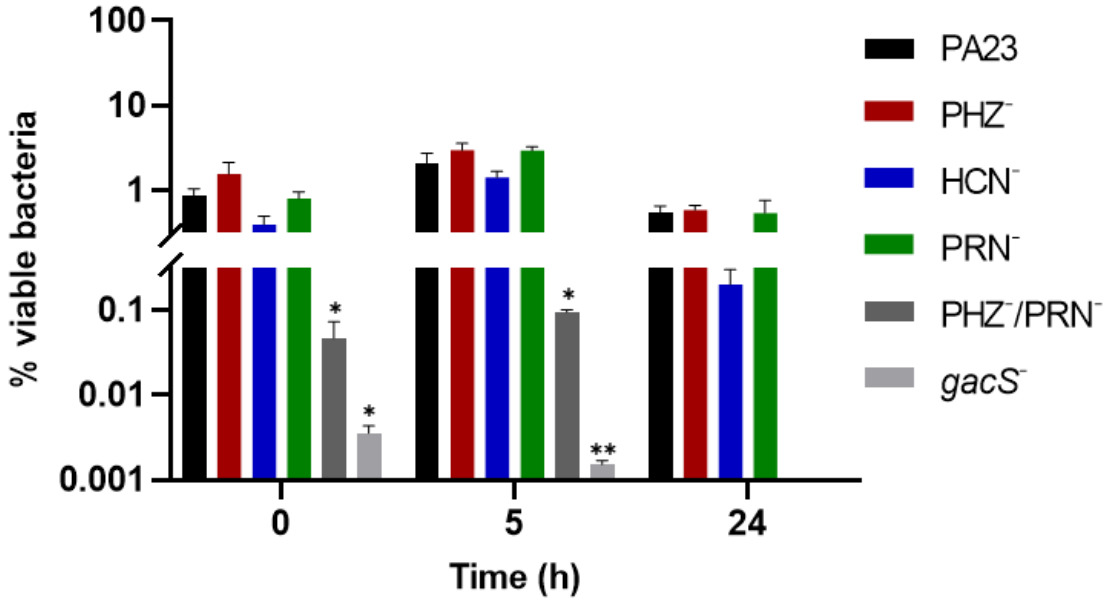

Figure 3.8. Intracellular survival of PA23 strains in Ac trophozoites. Bacteria and amoeba were co-incubated for 3 h at a multiplicity of infection (MOI) of 100. Bars represent bacterial CFU/ml recovered from amoeba lysates after gentamicin treatment. Data with asterisks indicate statistical significance from PA23 WT using two-way ANOVA (*, $P < 0.05$; **, $P < 0.01$). Experiments were performed three times; one representative data set is shown.

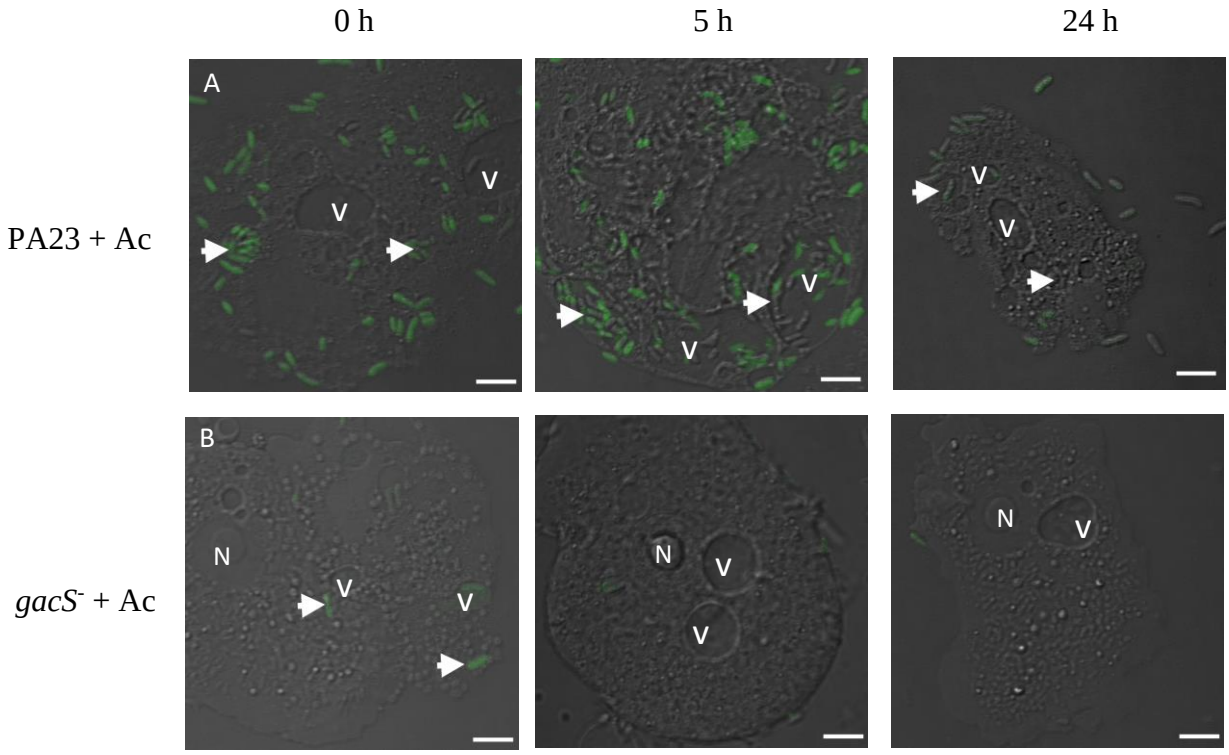

Figure 3.9. Confocal microscopy images of Ac trophozoites infected with PA23 WT and *gacS*$^{-}$ strains expressing green fluorescent protein (GFP) at 0, 5, and 24 h post gentamicin treatment. Images were taken using a Zeiss LSM 700 confocal laser scanning microscope under 63× magnification. Arrows point to intracellular bacteria. N, Nucleus; V, vacuoles. Scale bar represents 5 µm.

No differences were observed between the PHZ$^-$, HCN$^-$, PRN$^-$ strains and the PA23 WT; similarly, the PHZ$^-$/PRN$^-$ strain closely resembled the *gacS*$^-$ mutant (data not shown). Accordingly, the Ac-PA23 WT and Ac-*gacS* images presented in Figure 3.9 are representative of all 6 strains. Immediately after gentamicin treatment (time 0), bacterial strains were detected in amoeba vacuoles. However, amoebae infected with the PA23 WT, PHZ$^-$, HCN$^-$, and PRN$^-$ strains contained vacuoles filled with motile bacteria. Conversely, coincubation with the *gacS*$^-$ and PHZ$^-$/PRN$^-$ strains revealed fewer bacteria and they were nonmotile (Fig. 3.9). After 5 h of incubation, amoeba vacuoles increased in numbers, and were filled with highly motile PA23 WT, PHZ$^-$, HCN$^-$, and PRN$^-$ cells. In contrast, there was very little change in the number of *gacS*$^-$ and PHZ$^-$/PRN$^-$ cells present. At 24 h, PA23 WT, PHZ$^-$, HCN$^-$, and PRN$^-$ cells were still visible inside of amoeba, although numbers had declined dramatically; very few *gacS*$^-$ and PHZ$^-$/PRN$^-$ cells were detectable at this time point (Fig 3.9). Based on these data, we conclude that the production of secondary metabolites contributes to PA23 survival inside of Ac trophozoites.

3.3. Discussion

The ability of bacteria to persist in the soil is profoundly affected by grazing predators, including protozoa. In response, bacteria have developed a number of different defence mechanisms to avoid predation, such as toxin production (Jousset, 2012). The current study aimed to investigate the interaction between PA23 and the model protozoan predator Ac. Specifically, we were interested in whether PA23 AF metabolites facilitate survival in the presence of this predator and their impact on Ac viability. Additionally, we explored bacterial survival within vacuoles of amoeba trophozoites.

PA23 synthesizes an arsenal of metabolites such as PRN, PHZ, and HCN and strains deficient in these compounds exhibit altered AF activity. We have previously demonstrated that mutations in QS and GacS-GacA two-component systems abolished exoproduct formation, which in turn led to a decrease in AF activity (Poritsanos et al., 2006; Selin et al., 2012, 2014). Our prey-predator co-culture assay revealed that the PA23 WT, and the PHZ$^-$ and *rpoS*$^-$ mutants caused a dramatic reduction in the number of Ac trophozoites either by transforming into dormant cysts or causing cell death (Figure 4.9). PRN production is elevated 2.2-and 1.6-fold in PHZ$^-$ and *rpoS*$^-$ backgrounds, respectively (Manuel et al., 2012; Selin et al., 2012). The increased mortality of Ac trophozoites co-cultured with these PRN hyper-producing strains led us to speculate that this antibiotic is involved in PA23 toxicity towards the predator (Fig. 3.1). When Ac trophozoites were challenged with different concentrations of purified PRN, we observed a significant decline in the percentage of viable amoeba in a dose-dependent fashion (Fig. 3.4a). Consistent with these findings, Jousset and coworkers (2010) reported that purified PRN and 2,4-DAPG exhibited toxic effects towards Ac trophozoites and caused rapid cell death after 6h of incubation. In another study, the antibiotics 2,4-DAPG, pyoluteorin (PLT) and PRN induced cyst formation in the

amoeba *Vahlkampfia*, while the growth of amoeba was enhanced when co-cultured with toxin-deficient strains (Jousset et al., 2006). The toxicity associated with PRN is not surprising as it is known to affect a wide range of microorganisms, including fungi and protists (Chernin et al., 1996). This compound interferes with cellular processes such as respiratory pathways and osmotic regulation (Okada et al., 2005; Tripathi & Gottlieb, 1969).

PA23 also produces the volatile compound HCN that plays a role in AF activity (Nandi et al., 2017) and contributes to its nematicidal effects on *C. elegans* (Nandi et al., 2015). For that reason, we were interested to understand whether HCN exerts toxic effects on Ac trophozoites. We observed that Ac preferentially consumed the HCN^- strain and this bacterium supported slightly higher trophozoite numbers compared to PA23 WT (Fig. 3.1 & 3.2). When amoebae were incubated with purified KCN, a significant decline in the number of Ac was detected at concentrations of 400 μg/ml and higher (Fig. 3.4c). HCN is a broad-spectrum toxin that affects a wide range of organisms, such as fungi and nematodes (Blumer & Haas, 2000) and it also appears to inhibit Ac growth, albeit modestly.

PA23 produces two diffusible PHZ compounds, namely PCA and 2-OH-PHZ. In co-cultures, the PHZ-producing strains (WT, PRN^-, HCN^-, *rpoS*$^-$) were less palatable than several of the PHZ-deficient bacteria (*gacS, phzR-,* AI-deficient) (Fig. 3.2). The PHZ^- mutant was not highly consumed, which we believe was due to elevated PRN production in this background (Selin et al., 2010). PHZ toxicity was further demonstrated by the fact that exposure to this compound resulted in a dose-dependent decrease in Ac viability (Fig. 3.4b). To the best of our knowledge, this is the first report of PHZ having amoebicidal activity. A study by Matz et al. (2004) reported that the purple pigment violacein produced by *Janthinobacterium lividum* and *Chromobacterium violaceum* is acutely toxic for the bacterivorous nanoflagellates *Bodp saltans Ochromonas* sp. and

Spumella sp. Ingestion of WT bacteria induced rapid cell lysis whereas non-pigmented mutants supported protozoan growth. In addition, purified violacein was found to be highly toxic for the flagellates (Matz et al., 2004).

Beyond their cidal effects, secondary metabolites are highly beneficial if they deter predators, enabling bacteria to avoid consumption all together. To determine whether PA23 exoproducts exhibit repellent or attractant properties, chemotactic response assays were performed. Our results revealed that in all cases amoeba had a strong preference for the toxin-deficient *gacS*$^{-}$, *phzR*$^{-}$, and AI-deficient strains (Fig. 3.6). Moreover, there was very little difference between these three bacteria and the PRN^{-} strain, suggesting that PRN acts as a strong repellent (Fig. 3.6). PRN was previously reported to repel *C. elegans* (Nandi et al., 2015). The HCN^{-} mutant, on the other hand, closely resembled PA23 WT (Fig; 3.6); therefore, HCN does not significantly impact Ac chemotaxis. Because the PHZ- strain produces twice as much PRN as WT, it was not possible to assess whether PHZ affects Ac migration. While PA23 AF metabolites served to repel Ac, bacterial chemicals can in some cases act as attractants. Gaines and coworkers (2019) reported that the model protozoa *Eglena gracilis* showed a positive chemotactic response towards *Listeria monocytogenes* cells. The authors suggested that the small molecules released from *L. monocytogenes* such as volatile organic compounds exhibited chemoattractant activity and were responsible for attracting *Euglena* (Gaines et al., 2019). Collectively, our findings suggest that Ac trophozoites were able to sense and respond to PA23 chemical cues. Ac was only attracted to toxin-deficient strains, in particular those lacking PRN; as such, this antibiotic may facilitate PA23 survival in the soil.

Although the production of toxic metabolites by bacteria is an effective defensive strategy for reducing predator population biosynthesis of these compounds is energetically costly (Jousset,

2012). Clearly, the ability to optimize toxin production according to predation risk is beneficial for bacteria (Steiner, 2007). Therefore, we were interested to determine whether co-culturing with Ac alters expression of PA23 genes and AF products. Increased expression of *phzA* and *prnA* occurred in the presence of amoeba at 48 h and 72 h; whereas no change was observed when bacteria were incubated with Ac supernatants. Our PHZ and PRN analysis confirmed elevated production of these antibiotics (Table 3.1). It is interesting that the *phzI* and *phzR* QS genes were also upregulated in the presence of Ac, because the Phz QS system positively regulates *phz* and *prn* gene expression (Selin et al., 2012). At present it is unclear whether the effects of Ac on PHZ and PRN production are mediated directly or indirectly through QS. We have previously shown that co-culturing PA23 with *C. elegans* led to increased *prnA* and *phzA* gene expression, while cell-free supernatants had no effect (Nandi et al., 2015). Similarly, Mazzola and coworkers (2009) reported that production of the cyclic lipopeptides massetolide and viscosin by *Pseudomonas protegens* SS101 and SBW25, respectively, were essential for protecting bacteria from predation by *Naegleria americana*. Moreover, the authors observed an upregulation of *massABC* (massetolide) and *viscABC* (viscosinamide) when bacteria were challenged with protozoa (Mazzola et al., 2009). In contrast to our findings, *P. protegens* CHA0 grown in the presence of Ac cell-free supernatants exhibited elevated *phlA* (DAPG) and *prnA* gene expression and increased DAPG and PRN production. However, direct contact with the predator resulted in a reduction in gene expression (Jousset et al., 2010). Collectively these findings indicate that predators and prey can sense and respond to one another, either through direct contact or soluble chemical cues.

Certain bacterial species are able to survive Ac grazing due to the inability of these microfaunal predators to digest internalized bacteria. Intracellular persistence is achieved by

defence mechanisms such as production of toxic metabolites and bacterial outer-membrane structures (Casadevall, 2008; Ray et al., 2009; Thomas et al., 2010). Survival inside of a protozoan host can be beneficial for the bacteria as it provides protection from fluctuations in temperature, pH, and exposure to oxidative stress (Thomas et al., 2010; Thomas & McDonnell, 2007). Therefore, we were interested to explore whether PA23 can survive inside Ac trophozoites, as this may lead to increased environmental fitness. We discovered that the WT, PRN^-, HCN^- and PHZ^- strains remained viable inside of Ac trophozoites for 24 h; whereas, the PRN/PHZ^- and *gacS* mutants died (Fig. 3.8). The inedible bacteria located within food vacuoles were hyper-motile, consistent with them being metabolically active (Fig. 3.9). Thus, it appears that PRN, PHZ, HCN and possibly other PA23 metabolites facilitate bacterial persistence within Ac vacuoles. We hypothesize that these compounds interfere with the cellular degradation process by yet to be discovered mechanisms.

In summary, findings presented herein demonstrate that PRN, PHZ and HCN all contribute to PA23-mediated inhibition of Ac *in vitro*. PA23 is able to sense the presence of amoeba and upregulate expression of genes and antipredator compounds accordingly. We have previously shown that PHZ is not essential for PA23-mediated biocontrol of the plant pathogen *S. sclerotiorum* but it is involved in biofilm formation. Intriguingly, PHZ also has amoebicidal properties. We discovered that AF metabolites contribute to PA23 survival within amoeba vacuoles, which may increase fitness of this bacterium in the environment. Taken together, antipredator toxins produced by PA23 exhibit broad-spectrum antagonism, not only towards fungal phytopathogens and *C. elegans,* but also Ac. Future studies on the interplay between bacteria and predators in the rhizosphere using different protists will provide additional insight into PA23 persistence in the environment.

Chapter 4

Exploring the role of polyhydroxyalkanoates (PHA) in PA23 biofilm formation, stress endurance and interaction with the protozoan predator *Acanthamoeba castellanii*

4.1. Introduction

Pseudomonas chlororaphis strain PA23 produces an arsenal of metabolites that enable it to function as a BCA (Poritsanos et al., 2006; Zhang et al., 2006). In addition to these antifungal (AF) compounds, PA23 can synthesize a group of carbon and energy compounds known as polyhydroxyalkanoates (PHA) that are deposited as discrete granules within the cytoplasm (Sharma et al., 2017). Accumulation of these polymers frequently occurs during unbalanced growth conditions; therefore, PHA is utilized as a sink for carbon and energy when other nutrients are depleted (Madison & Huisman, 1999).

In *Pseudomonas* species, PHA biosynthesis occurs through central pathways, such as the β-oxidation and de novo fatty acid synthesis pathways, to convert fatty acids or carbohydrate intermediates into different (*R*)-3-hydroxyalkanoyl-CoAs (Ren et al., 2009; de Eugenio et al., 2010; de Prieto et al., 2016). Pathway-specific enzymes are responsible for directing the carbon flux towards PHA accumulation (de Eugenio et al., 2010; de Prieto et al., 2016). *P. chlororaphis* synthesizes medium-chain-length PHAs containing 6-14 carbon-chain monomers that are produced from renewable resources like glucose, gluconate and fatty acids as a sole carbon source (Madison & Huisman, 1999; Prieto et al., 2007; Sharma et al., 2017). The PA23 genome harbours a PHA biosynthetic gene cluster consisting of six *pha* genes, which are highly conserved among *Pseudomonas* species (Prieto et al., 2007; Sharma et al., 2017). This locus is arranged as two clusters: *phaC1ZC2D* encodes two polymerases (PhaC1 and PhaC2), a depolymerase (PhaZ) and the TetR-like transcriptional regulator PhaD. The divergently transcribed cluster (*phaFI*) encodes the phasins PhaF and PhaI, involved in PHA organization (Prieto et al., 2007; Sharma et al., 2017).

Studies have demonstrated that PHA production enhances rhizobacterial fitness, especially in competitive environments where carbon and energy resources are limited (Ruiz et al., 2001; Kadouri et al., 2003b; Pham et al., 2004). In addition to acting as a carbon and energy sink, PHA can increase bacterial stress resistance. For instance, this compound acts as a chaperone protecting enzymes and other biological molecules from high and low temperatures, oxidative stress, and UV radiation (Kadouri et al., 2003a; Ayub et al., 2009; Obruca et al., 2016; Slaninova et al., 2018). In addition to these abiotic stresses, bacteria must cope with the threat of grazing predators, such as protozoa. In response, bacteria have developed defensive strategies to resist predation including production of unpalatable compounds or toxic metabolites, which act to repel or kill would-be predators (Jousset, 2012). This in turn, can elevate predation on neighbouring competitors, increasing nutrient availability for the producer (Rønn et al., 2002; Pedersen et al., 2009).

The focus of the current study was to investigate how PHA production impacts PA23 physiology, looking at the role of this compound in AF activity, biofilm formation and survival under stressful conditions. We also explored how accumulation of PHA affects PA23 interaction with the model protozoan predator Ac.

4.2. Results

4.2.1. Generation of *phaC1*, *phaC1ZC2*, and *phaC1ZC2D* mutant strains

In *Pseudomonas* species, the ability to accumulate and degrade PHA depends upon the activity of polymerases (PhaC1 and PhaC2) and a depolymerase (PhaZ), encoded by the *phaC1ZC2D* cluster. Isogenic *phaC1*, *phaC1ZC2*, and *phaC1ZC2D* mutants were generated together with their corresponding complemented derivatives harbouring the WT gene(s) in trans. This battery of strains was used to explore the role of PHA in PA23 phenotypic traits, stress tolerance and survival inside *A. castellanii* trophozoites, as described below.

4.2.2. Visualization of PHA accumulation through confocal imaging

To monitor PHA accumulation in PA23 WT and derivative strains, cells were stained with 3% Nile blue and subjected to confocal microscopy. As depicted in Figure 4.1, the PA23 WT is visible as brightly stained rods that are filled with PHA granules. In contrast, the PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* mutants exhibited markedly reduced PHA accumulation. The complemented strains appeared much brighter than PA23 WT, suggesting increased PHA accumulation, which we attribute to a gene dosage effect (Fig. 4.1).

4.2.3. The role of *phaC1*, *phaC1ZC2*, and *phaC1ZC2D* in fungal suppression and PHZ production

PA23 WT is able to inhibit growth of the fungal pathogen *S. sclerotiorum* through production of AF metabolites. When we tested the PHA mutants and complemented derivatives for their ability to inhibit *S. sclerotiorum in vitro*, zones of inhibition were equivalent for PA23 WT (5.2 +/- 1.2 mm), PA23*phaC1* (4.9 +/- 0.4 mm), PA23*phaC1ZC2* (5.5 +/- 1.1 mm), PA23*phaC1ZC2D* (5.1 +/- 0.9 mm), PA23*phaC1*(C) (5.3 +/- 0.2 mm), PA23*phaC1ZC2*(C) (4.7 +/- 1.7 mm) and

PA23*phaC1ZC2D*(C) (4.9 +/- 0.6 mm). Therefore, PHA accumulation does not appear to impact PA23-mediated fungal antagonism. This BCA produces two soluble antibiotics, PRN and PHZ. PRN is primarily responsible for fungal antagonism, while PHZ production imparts an orange pigment to PA23 cells (Selin et al., 2010). We observed that PA23*phaC1ZC2D* is a paler orange colour compared to WT, suggesting that PHZ levels are reduced in this background (Fig. 4.2a). Quantitative analysis revealed that this strain produced 2.6-fold less PHZ, whereas PHZ was unchanged in PA23*phaC1* and PA23*phaC1ZC2*. As expected, providing *phaC1ZC2D* in trans restored PHZ production to those of PA23 WT (Fig. 4.2b).

4.2.4. The role of PHAs in surface attachment, extracellular polysaccharide production and motility.

Analysis of the ability of the PA23 WT and derivative strains to adhere to the surface of a 96-well plate revealed a link between PHA production and biofilm formation. Adherent biomass increased 2.2-, 1.8-, and 1.5-fold for PA23*phaC1*, PA23*phaC1ZC2*, and PA23*phaC1ZC2D*, respectively, compared to PA23 WT (Fig. 4.3). No differences were observed between the complemented strains and PA23 (Fig. 4.3). Next, we sought to determine whether PHA accumulation would impact PA23 attachment to plants roots, specifically those of *A. thaliana*. All three PHA mutants exhibited significantly enhanced root adherence, which was returned to WT levels in the complemented strains (Fig. 4.4).

Flagellar motility is known to play an essential role during the early stages of biofilm development (Davey & O'Toole, 2000); as such, we examined whether swimming motility was

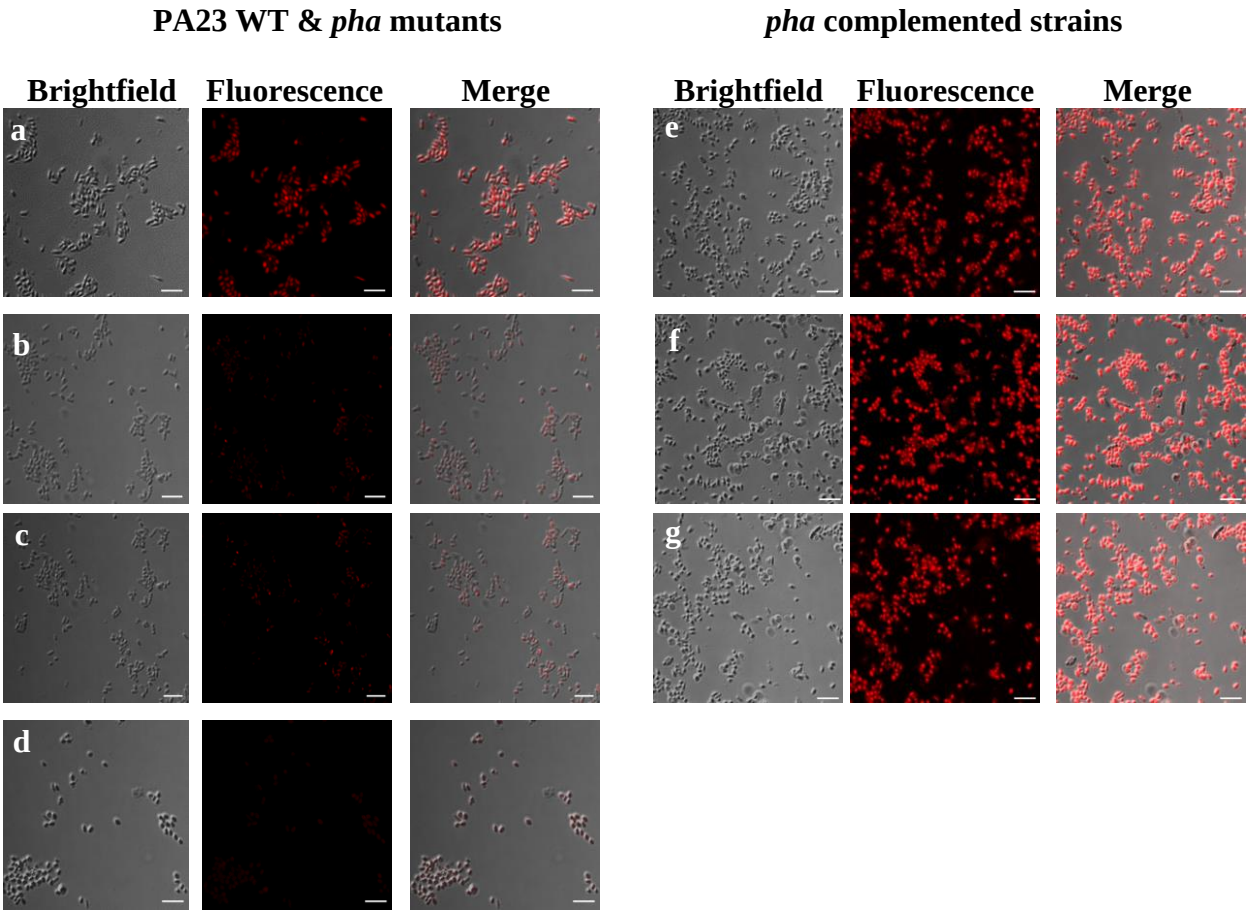

Figure 4.1. Confocal microscopy imaging of PHA accumulation in PA23 WT (a), PA23*phaC1* (b), PA23*phaC1ZC2* (c), PA23*phaC1ZC2D* (d), PA23*phaC1*(C) (e), PA23*phaC1ZC2*(C) (f) and PA23*phaC1ZC2D*(C) (g) strains grown in RMM-glc. Images were taken using a Zeiss LSM 700 confocal laser scanning microscope under 63× magnification. PHA granules stained with Nile blue were visualized using 555 nm excitation and 580 nm emission wavelengths. Scale bar represents 5 µm.

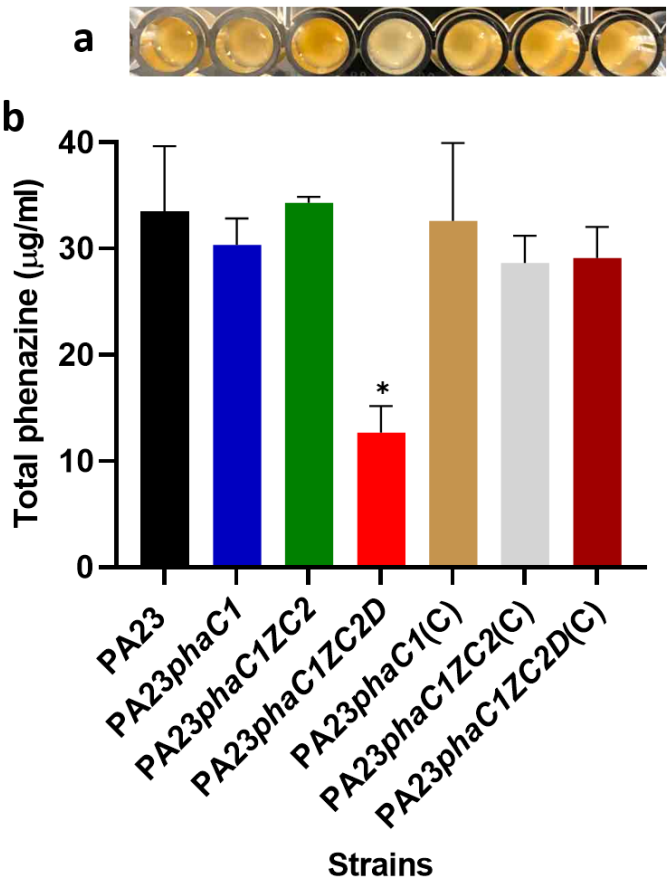

Figure 4.2. Phenazine production in PA23 WT, PA23*phaC1*, PA23*phaC1ZC2*, PA23*phaC1ZC2D* and their respective complemented strains. Panel (a): Color development of overnight cultures grown in LB media; wells coincide with strains labelled below. Panel (b): Quantitative PHZ analysis of cells grown in LB media. Asterisk indicates significant difference from PA23 WT as determined by one-way ANOVA (*; $P < 0.0001$). PA23 WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* contain empty vector.

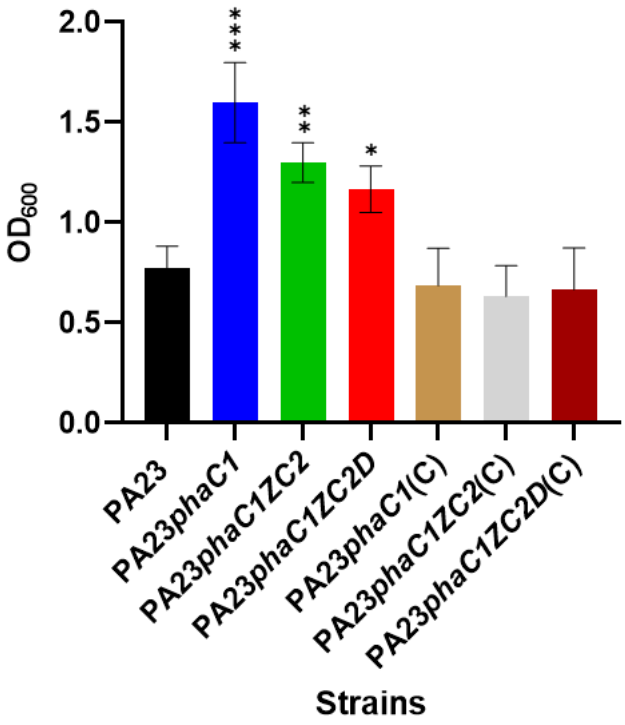

Figure 4.3: Biofilm formation in PA23 WT, *pha* mutants and complemented strains. Cultures were grown in 96-well microtiter plates containing RMM-glc for 48 h at 28°C. Biofilm formation, indicated by crystal violet staining, was measured at A_{600}. Values shown are the means of five replicates; error bars, SD. Asterisks indicate significant difference from PA23 WT as determined by one-way ANOVA test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$). PA23 WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* contain empty vector.

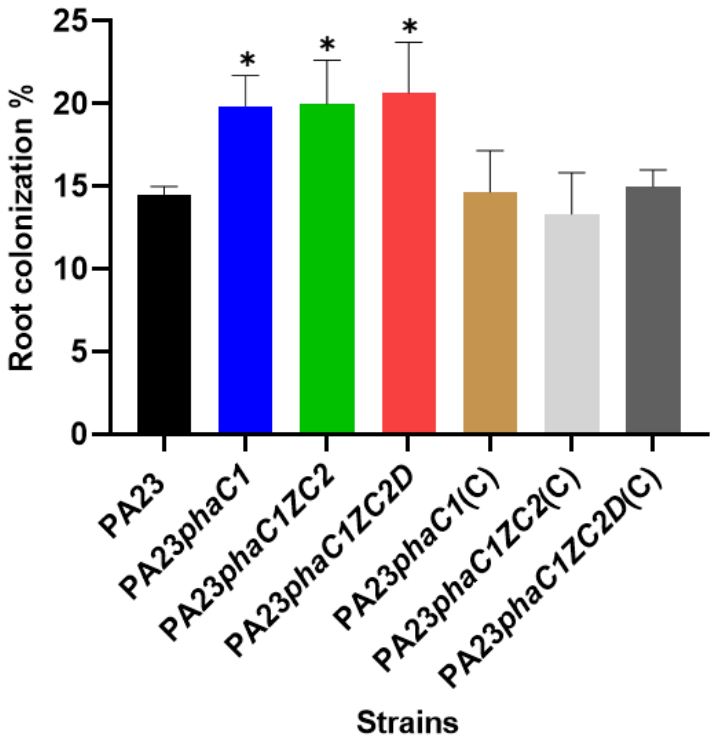

Figure 4.4. Percentage adhesion of PA23 WT, *pha* mutants and complemented strains to *Arabidopsis thaliana* roots. Values represent the mean (SD) from three replicates. Asterisks indicate significant difference from the PA23 WT as determined by one-way ANOVA (*, $P < 0.05$). PA23 WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* contain empty vector.

altered in the PHA-deficient strains. We discovered that the *phaC1*, *phaC1ZC2*, and *phaC1ZC2D* mutants exhibited enhanced motility at 24, 48 and 72 h compared to the PA23 WT and complemented strains (Table 4.1).

In some bacterial species, production of extracellular polysaccharides (EPS) is involved not only in biofilm formation but also during plant colonization (Burdman *et al*., 2000). Therefore, we were interested to compare EPS production by PA23 and the PHA derivatives. The amount of EPS extracted from the *phaC1* mutant was 3-fold higher than WT. For PA23*phaC1ZC2* and PA23*phaC1ZC2D*, EPS levels were nearly 5 times those of PA23 and the complemented derivatives (Table 4.2). Elevated EPS production is consistent with the increased surface attachment to both abiotic and biotic surfaces (Figs. 4.3 & 4.4).

4.2.5. PHA production is involved in resistance to starvation and sub-optimal temperatures *in vitro*.

To investigate whether PHA synthesis is involved in protection against environmental stress, the WT, PA23*phaC1*, PA23*phaC1ZC2*, and PA23*phaC1ZC2D* were examined for survival *in vitro* under sub-optimal temperature conditions. When grown in RMM-glc at 28°C, no differences in growth were detected between PA23 WT and the PHA derivatives (Fig. 4.5). However, when cells were cultured under starvation conditions (no carbon source), by day 4 the viability of the *pha* mutants decreased significantly to less than 0.5% of the original inoculum compared to 51.6 % for PA23 WT (Fig. 4.6). At day 8, PA23*phaC1ZC2*(C) and PA23*phaC1ZC2D*(C) showed increased survival compared to WT, likely due to multiple copies of the genes being provided in trans. By day 12, no differences were observed between strains (Fig. 4.6).

Table 4.1. Flagellar motility of PA23 WT, *pha* mutants and complemented strains.

Strain	Motility* (mm)		
	24 h	48 h	72 h
PA23ø	16 (1.0)	22.2 (2.0)	40.6 (1.1)
PA23*phaC1*ø	27 (2.6)‡	40 (3.5)†	60 (0.4)†
PA23*phaC1ZC2*ø	29 (2.1)‖	36.7 (4.0)†	58 (1.0)†
PA23*phaC1ZC2D*ø	26 (4.1)‡	37.2 (3.0)†	61 (0.5)†
PA23*phaC1*(C)	18 (0.5)	24 (1.0)	38.2 (0.7)
PA23*phaC1ZC2*(C)	17 (1.2)	25 (0.6)	43 (0.8)
PA23*phaC1ZC2D*(C)	14 (0.6)	20 (1.1)	42 (1.3)

* Mean (SD) of swim zones from three replicates.
ø Contain empty vector.
† Significantly different from PA23 WT ($P < 0.001$).
‡ Significantly different from PA23 WT ($P < 0.05$).
‖ Significantly different from PA23 WT ($P < 0.01$).

Table 4.2. Exopolysaccharide production by PA23 WT and PHA derivatives

Strain	**EPS mg/l**
PA23 ø	30.04
PA23*phaC1* ø	90.67*
PA23*phaC1ZC2* ø	146.56*
PA23*phaC1ZC2D* ø	140.70*
PA23*phaC1*(C)	29.37
PA23*phaC1ZC2*(C)	26.31
PA23*phaC1ZC2D*(C)	28.02

* Significantly different from PA23 WT ($P < 0.01$)

ø Contain empty vector.

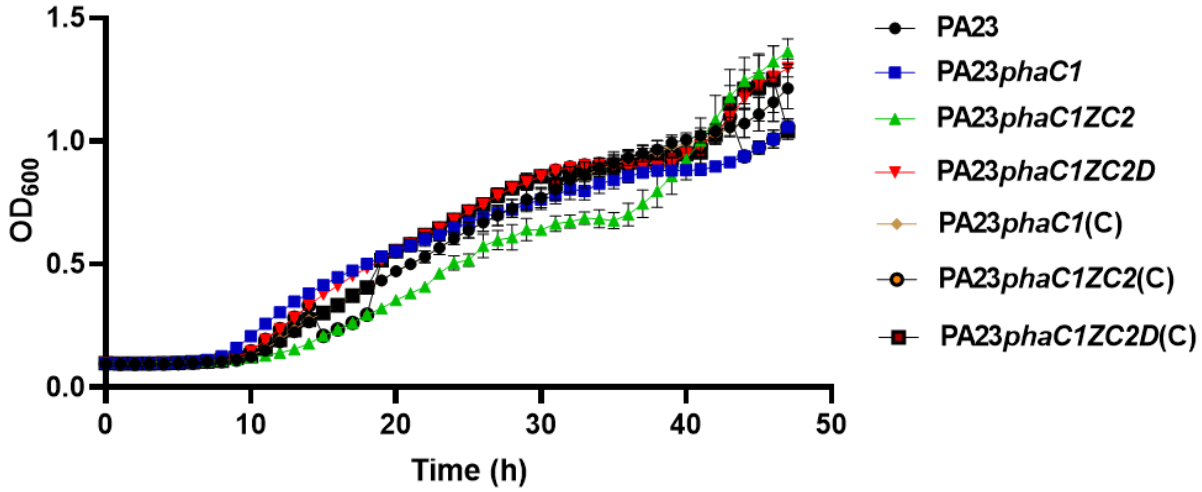

Figure 4.5. Growth analysis of the PA23 WT, *pha* mutants, and complemented strains in RMM-glc. Growth was analyzed using a SpectraMax M2 microplate reader for 48 h at 28°C with continuous shaking. Five replicates were tested for each strain. Experiments were repeated three times and one representative data set is shown.

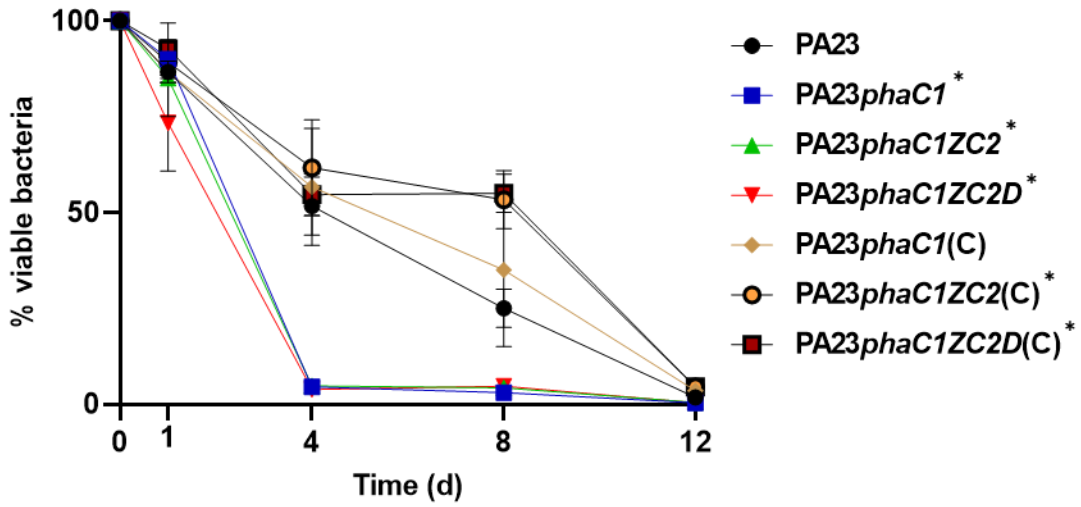

Figure 4.6. Survival of PA23 WT and derivative strains under starvation conditions. Cells were grown in RMM-glc for 48 h and then resuspended in saline. Bacterial viability was monitored for 12 days. PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* are significantly different from PA23 WT at 4h and 8h; while PA23*phaC1ZC2*(C) and PA23*phaC1ZC2D*(C) are significantly different from PA23 WT at 8h, as determined by two-way ANOVA (*, $P < 0.01$). The assays were performed three times, and one representative dataset is shown. PA23 WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* contain empty vector.

When bacteria were heat-shocked at 50°C for 60 min, the PHA-deficient strains died rapidly. After 10 min exposure, only 1% of the mutant population remained viable compared to 47% for the PA23 WT (Fig. 4.7a). After 30 min heat exposure, no viable cells were recovered. To investigate if PHA accumulation influences bacterial survival at lower temperatures, we assessed PA23 tolerance to 4°C cold exposure. Under these conditions, cell viability declined at a similar rate for all seven strains tested (Fig. 4.7b). When the cells were incubated at -20°C, cell viability decreased to the same degree at 10h; however, by 20h, all *pha* mutants showed a dramatic loss in viability compared to the PA23 WT and complemented strains. After 30h, there was no difference in survival between strains (Fig. 4.7c). These findings indicate that PHA protects PA23 from heat and freezing conditions.

4.2.6. Protective effect of PHA against UV radiation and oxidative damage

Upon exposure to UV radiation, the *pha* mutants showed increased sensitivity compared to PA23 WT (Fig. 4.8a). This difference was most dramatic at 10-second exposure, where less than 7% of the mutant population was recovered compared to a 40% recovery rate for PA23 and the complemented derivatives. At 20-second exposure, a significant decline in viability was observed for all strains and after 60 seconds, no growth was detected. Next, we examined whether PHA has a role in PA23 resistance to oxidative stress. Zones of clearing surrounding H_2O_2-impregnated filter discs illustrated that the *pha* mutants were much more sensitive to oxidative stress compared to the WT. As expected, there was no difference between the complemented derivatives and the parental strain (Fig. 4.8b). Thus, it appears that PHA accumulation protects PA23 against UV radiation and H_2O_2 *in vitro*.

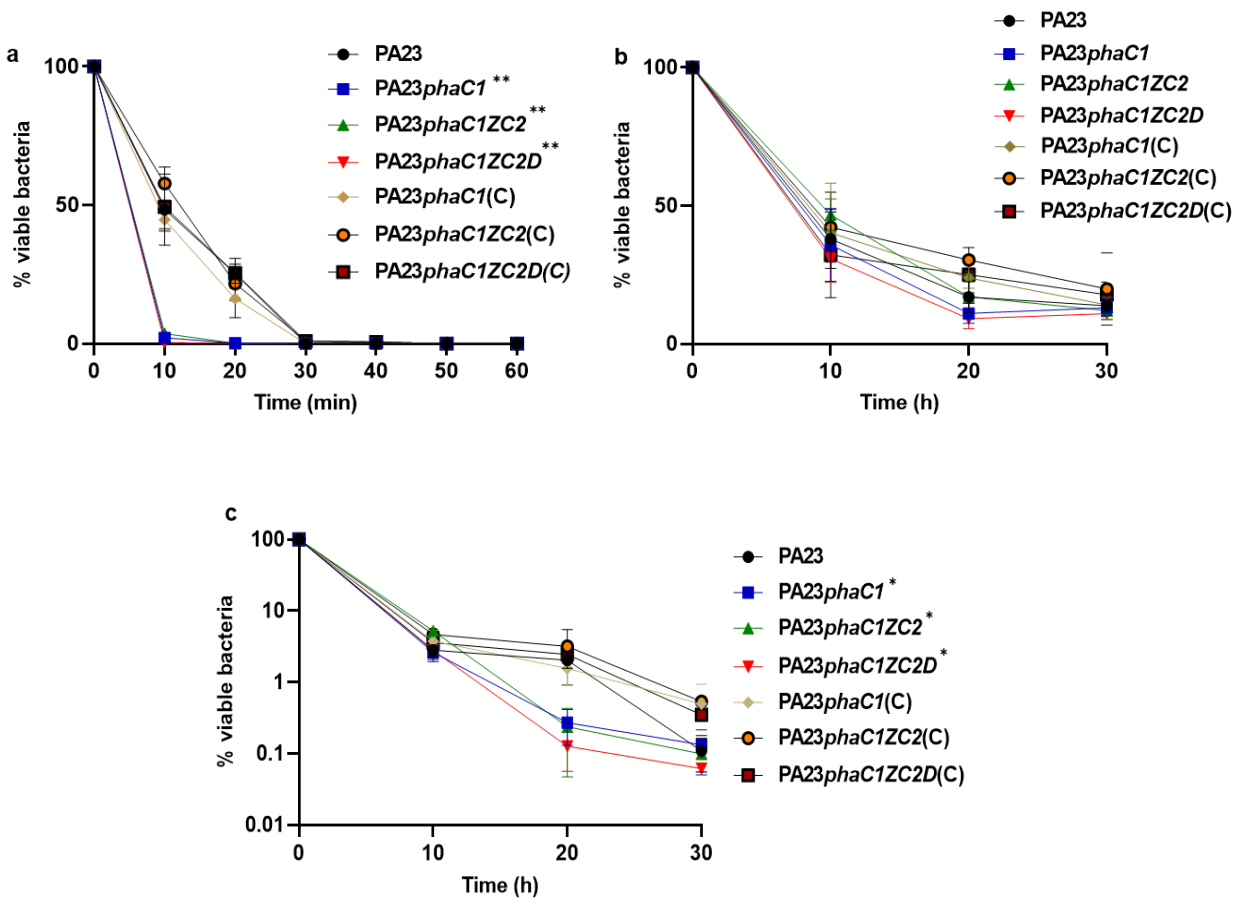

Figure 4.7. Survival of PA23 WT and *pha* derivatives at 50ºC (a), 4ºC (b) and -20ºC (c). Cells were grown in RMM-glc medium for 48 h, resuspended in saline and then exposed to sub-optimal temperature conditions. PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* are significantly different from PA23 WT at 10 min and 20 min when challenged with heat. When exposed to freezing, these mutants are statistically significant at 20h. Significance was determined using the two-way ANOVA test (*, $P < 0.01$; **, $P < 0.001$). Experiments were performed three times and one representative data set is shown. PA23 WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* contain empty vector.

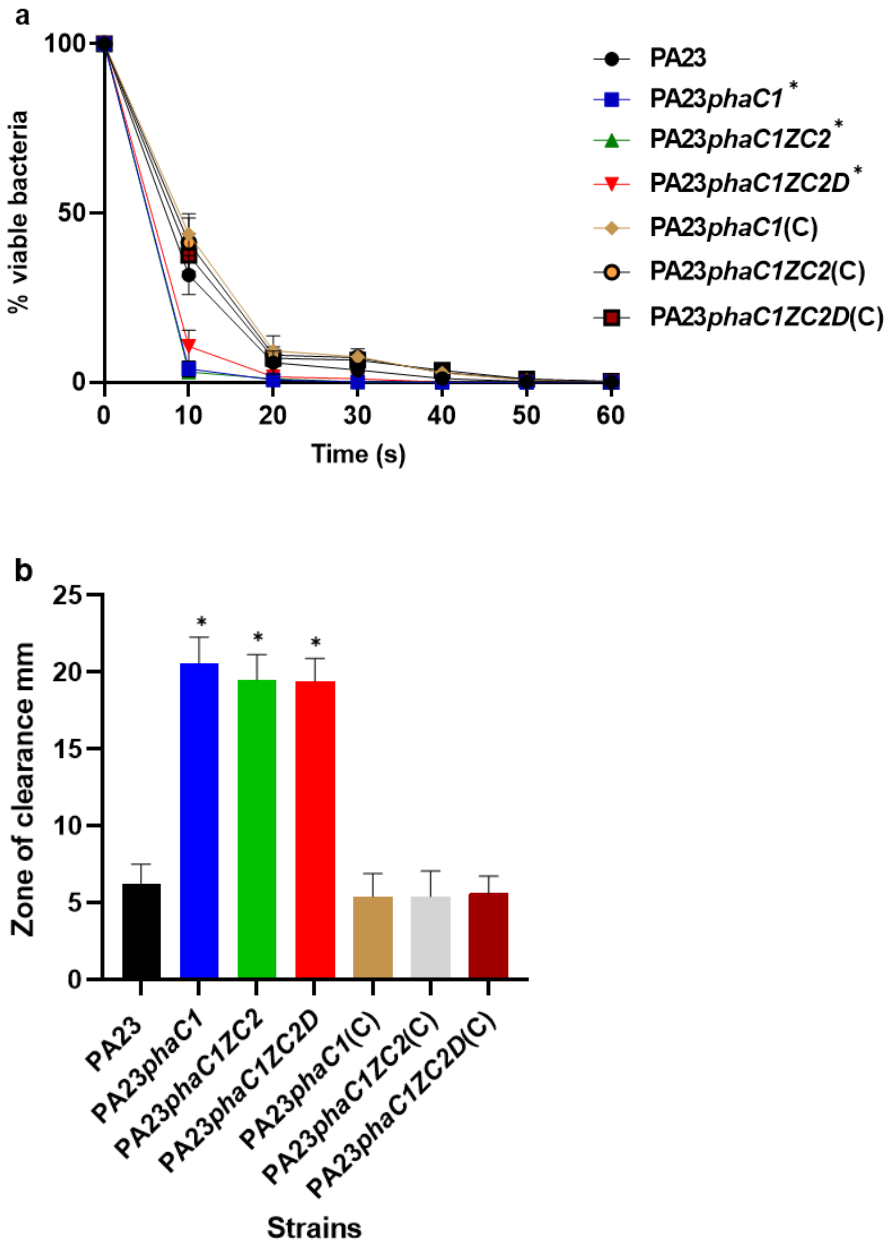

Figure 4.8. The sensitivity of PA23 WT, *pha* derivatives and complemented strains to UV radiation (a) and hydrogen peroxide (b). Asterisks indicate a significant difference from PA23 WT as determined by one-way ANOVA (*, $P < 0.001$). In Panel (a): PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* are statistically different from PA23 WT at 10 seconds. PA23 WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* contain empty vector. Experiments were performed three times; one representative data set is shown.

4.2.7. The impact of PHA accumulation on Ac trophozoite viability

To determine whether the PA23-Ac interaction is affected by PHA production, the WT, PHA-deficient and complemented strains were offered to Ac as prey. Both trophozoites and bacteria were enumerated in the co-cultures. While the number of Ac trophozoites declined over time in the presence of all seven strains, significantly more trophozoites were present in PA23*phaC1ZC2D* co-cultures (Fig. 4.9). When we analyzed bacterial survival in the presence of Ac, we found no difference between any of the strains except PA23*phaC1ZC2D*. In this case, there were drastically fewer bacteria recovered on days 1 through 10 (Fig. 4.10). Collectively, these results suggest that the *phaC1ZC2D* mutant is more palatable for Ac compared to any of the other strains.

4.2.8. Role of PHA accumulation in survival of PA23 inside Ac

Next, the role of PHA in PA23 survival within the acidic vacuoles of Ac was explored. Gentamicin was used to kill bacteria not taken up by amoeba, which proved to be very effective with less than 100 CFU/ml recovered. Following antibiotic treatment, amoebae were lysed, and bacterial survivors enumerated. At 1h, the number of recovered WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* decreased to 10, 10.3, 9 and 8.5% of the original inoculum, respectively (Fig. 4.11). Conversely, the PHA-complemented strains, PA23*phaC1*(C), PA23*phaC1ZC2*(C) and PA23*phaC1ZC2D*(C) showed significantly higher survival rates. At 5h, there was little change in the number of recovered bacteria. By 24 h, bacterial numbers decreased to <0.5% of the original inoculum for all strains, with WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* exhibiting less than 0.2% survival (Fig. 4.11). Collectively these findings indicate that, while PHA is not essential for intracellular survival, at elevated levels it increases PA23 persistence.

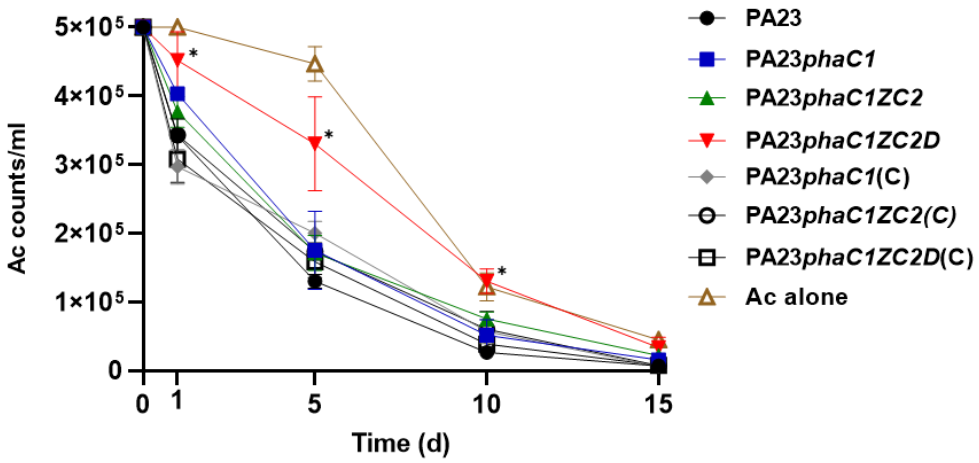

Figure 4.9. Growth of Ac trophozoites on PA23 WT, *pha* mutants and complemented strains in Ac buffer. Amoeba growth and viability were monitored for 15 days. Experiments were performed three times; one representative data set is shown. Asterisks indicate a significant difference from PA23 WT as determined by a two-way ANOVA test (*, $P < 0.001$). PA23 WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* contain empty vector.

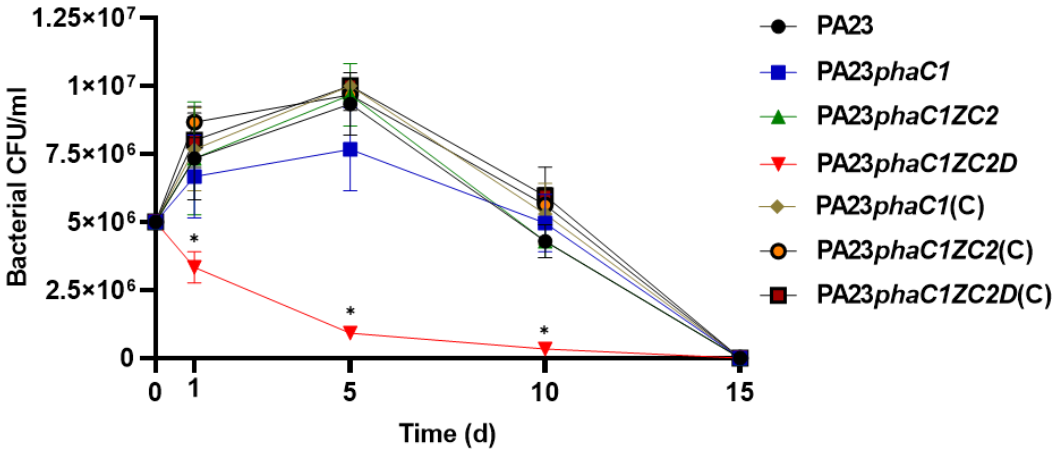

Figure 4.10. Effect of Ac trophozoites on the growth of PA23, *pha* mutants and complemented strains. Bacteria and amoeba were co-cultured in Ac buffer for 15 days. Asterisks indicate a significant difference from PA23 WT as determined by a two-way ANOVA test (*, $P < 0.01$). PA23 WT, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* contain empty vector. Experiments were performed three times; one representative data set is shown.

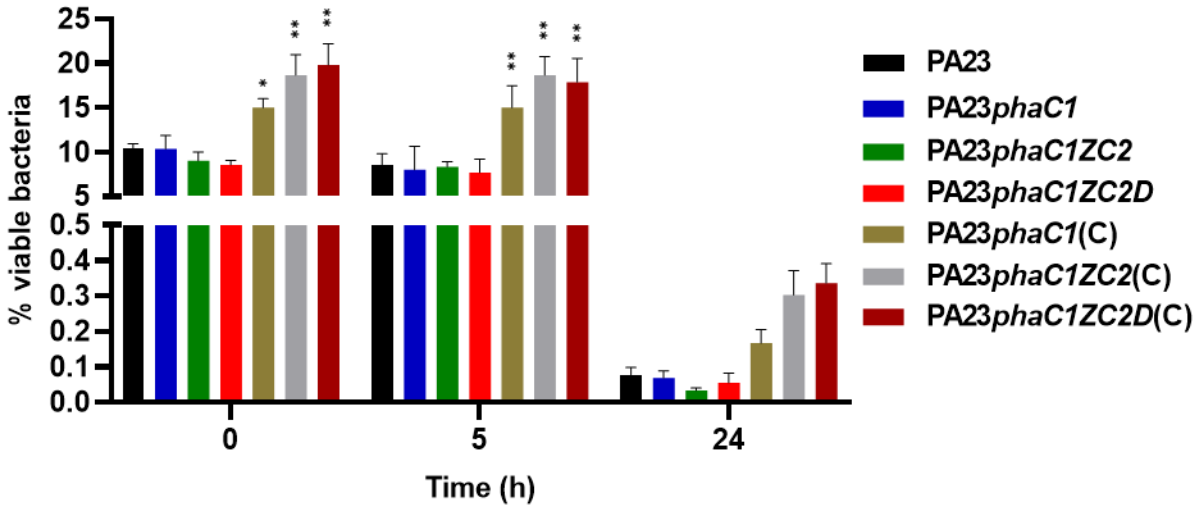

Figure 4.11. The survival of PA23 strains inside Ac trophozoites. Bacteria and amoeba were co-incubated for 3 hours at a multiplicity of infection (MOI) of 100. Amoebae were lysed and intracellular bacteria enumerated by viable plate counting. Time zero represents bacteria recovered 1 h after gentamicin treatment. Asterisks indicate a significant difference from PA23 WT as determined by a two-way ANOVA test (*, $P < 0.01$; **, $P < 0.001$). PA23, PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D* contain empty vector. Experiments were performed three times; one representative data set is shown

4.3. Discussion

Pseudomonas chlororaphis strain PA23 is an effective biocontrol agent capable of supressing fungal pathogens through the production of secondary metabolites, including antibiotics, enzymes and siderophores (Savchuk & Fernando, 2004; Poritsanos et al., 2006; Zhang et al., 2006). Additionally, PA23 synthesizes PHA polymers that serve as a carbon and energy sink when carbon is in excess, and other nutrients including nitrogen, phosphorous and oxygen are limited (Sharma et al., 2017). To understand the role of PHAs in PA23 physiology three different mutants were created, namely PA23*phaC1*, PA23*phaC1ZC2*, and PA23*phaC1ZC2D*. These mutants together with their complemented derivatives were then subject to phenotypic analysis.

Microscopic analysis of Nile Blue-stained cells revealed that PHA production was reduced in all three mutant backgrounds (Fig. 4.1). Thus, the absence of the PhaC1 synthase alone notably reduces PHA accumulation. PA23*phaC1ZC2D* exhibited a paler orange color compared to the PA23 WT and two other PHA mutants, which was confirmed in quantitative PHZ analysis (Fig. 4.2). We have previously demonstrated that PHZ is not a major contributor to PA23 biocontrol (Selin et al., 2010) and we saw no difference in the ability of PA23*phaC1ZC2D*, or PA23*phaC1ZC2* and PA23*phaC1* to inhibit *S. sclerotiorum*. The fact that the *phaC1ZC2* mutant, which has a functional copy of *phaD*, showed no change in PHZ production suggests that PhaD is impacting PHZ expression. In *P. putida* KT2442, the *pha* synthetic locus consists of five functional promoters upstream of the *phaC1*, *phaZ*, *phaC2*, *phaF* and *phaI* genes, namely PC1, PZ, PC2, PF and PI respectively. PC1 and PI are the most active promoters, controlling the expression of *phaC1ZC2D* and *phaIF*. In this strain, the transcriptional regulator PhaD controls the transcripition of the *phaC1ZC2D* and *phaIF* clusters by binding to consensus sequences called OPRcI and OPRi, upstream of *phaC1* and *phaI*, respectively (De Eugenio et al., 2010). Inspection

of the genomic landscape surrounding the PA23 *phz* operon revealed a lack of these consensus sequences (data not shown). As such, we believe that the impact of PhaD on PHZ production is likely indirect.

Like many other pseudomonads, strain PA23 is capable of adhering to solid surfaces and forming biofilms (Poritsanos et al., 2006). The ability to establish biofilms offers protection from environmental assaults and enhances growth under unfavourable conditions (Jefferson, 2004). Exploration of the role of PHA in binding to abiotic surfaces revealed that the PHA-deficient strains produced more adherent biomass compared to PA23 WT (Fig. 4.3). For many bacteria, flagella-mediated motility is required for attachment to and/or movement over surfaces during the early stages of biofilm formation (Davey & O'toole, 2000). Consequently, we examined swimming motility and discovered that all three PHA mutants were hypermotile (Table 4.1). These results are consistent with those reported by Kadouri et al. (2002), wherein a *phbC* mutant of *A. brasilense* showed a three-fold increase in motility compared to PA23 WT. The authors proposed that impaired PHB biosynthesis leads to excess reducing power through the TCA cycle resulting in increased motility (Kadouri et al., 2002). Another trait known to impact biofilm formation is EPS, which forms part of the matrix encasing the microbial community (Davey & O'Toole, 2000). Our analysis revealed that EPS levels were elevated for the PA23*phaC1*, PA23*phaC1ZC2*, and PA23*phaC1ZC2D* mutants compared to the parent strain (Table 4.2). Consistent with these findings, Pham et al. (2004) reported that a PHA-negative strain of *P. aeruginosa* PAO1 formed a more differentiated biofilm, due to overproduction of alginate.

In rhizobia, both EPS production and motility play an essential role in biofilm formation and attachment to root surfaces (Burdman et al., 2000; Wingender & Flemming, 2001). These findings prompted us to explore whether PHA impacts attachment to *A. thaliana* roots. The PHA-

deficient strains all exhibited enhanced colonization of roots (Fig. 4.4). Collectively these results indicate that reduced PHA production enhances the ability of PA23 to establish biofilms on biotic and abiotic surfaces, a trait likely mediated through hyper-motility and elevated EPS production.

In order to be an effective BCA, bacteria must be able to persist in the environment. Being able to tolerate exposure to high and low temperatures, oxidative stress, UV radiation and starvation conditions are clearly beneficial traits. PHA accumulation has been reported to increase bacterial survival in deleterious environments (Goh & Tan, 2012; Kadouri et al., 2003b; Pavez et al., 2009; Iustman et al., 2015); thus, we were interested to learn how PHA affects PA23 survival under sub-optimal conditions. Our starvation assays revealed a significant reduction in the survival of PHA mutants compared to PA23 WT (Fig. 4.6). This is consistent with Kadouri and coworkers (2002) who observed that under starvation conditions, *A. brasilense* rich in PHB exhibited enhanced survival compared to cells with lower PHB levels. Similarly, intracellular PHB accumulation enabled long-term growth (>600 days) of *Legionella pneumophila* under low-nutrient conditions (James et al., 1999).

Our heat shock experiments showed that the PHA mutants were more sensitive to heat than the parental strain (Fig. 4.7a). For many bacteria, PHA granules serve as a site for binding of heat-resistant proteins, with the granules acting as potent chaperones preventing enzyme denaturation (Obruca et al., 2016). In *Pseudomonas extremaustralis*, for example, heat shock proteins bind to PHA granules, facilitating survival in extreme temperatures (Catone et al., 2014). Soto and colleagues (2012) demonstrated that PHA accumulation in *Pseudomonas* sp. CT13 prevented protein aggregation, which usually occurs upon heat exposure. And in *Pseudomonas oleovorans*, a PHA depolymerase-deficient mutant exhibited increased sensitivity to heat challenge (Ruiz et al., 2001). When we looked at cold tolerance, we found that PHA did not

contribute to PA23 survival at 4°C (Fig. 4.7b); however, at -20°C, the PA23 WT exhibited increased viability compared to the PHA mutants at 20h exposure (Fig. 4.7c). PHA-mediated cold temperature survival has been reported for a number of bacteria including *Cupriavidus necator* H16 (Obruca et al., 2016). In this bacterium, PHA enhanced the rate of water transport outside of cells, which in turn, prevented formation of intracellular ice crystals (Obruca et al., 2016). Similarly, in bacterial isolates from Antarctica, PHA granules prevented cellular damage caused by intracellular ice formation, as well as dehydration and oxidative stress (Goh & Tan, 2012; Ciesielski et al., 2014). In *Pseudomonas* sp. 14-3, cold shock led to rapid degradation of PHA enabling the NADH/NAD ratio to be maintained; whereas, PHA mutants experienced a redox imbalance (Ayub et al., 2009). Collectively, these findings indicate that PHA facilitates suboptimal temperature survival for a number of bacteria, including PA23.

In bacteria, oxidative damage to enzymes is one of the major causes of protein degradation, which can be accelerated by exposure to reactive oxygen species (ROS) (Nguyen & Sok, 2003). To further explore the protective efficacy of PHA accumulation in stress tolerance, we assessed the ability of PA23 and the PHA mutants to endure H_2O_2 exposure. We observed that the PA23 WT was less sensitive to H_2O_2 compared to non-PHA accumulating strains (Fig. 4.7b). Consistent with these findings, a *phbC* mutant of *A. brasilense* exhibited greater sensitivity to H_2O_2 (Kadouri et al., 2002). Obruca and coworkers (2016) reported that accumulation of PHB in *C. necator* H16 protected lipase against oxidative stress caused by both H_2O_2 and metals. The authors suggested that PHA granules physically protect proteins against oxidative damage, thereby functioning as antioxidants. When we looked at UV resistance, we discovered that the PA23*phaC1*, PA23*phaC1ZC2*, and PA23*phaC1ZC2D* mutants showed significantly reduced viability after 10-second UV exposure compared to PA23 WT (Fig. 4.8a). Similar findings were

reported by Slaninova et al. (2018) wherein *C. necator* PHA granules decreased ROS accumulation by scattering UV light, thereby, shielding cells from radiation-induced damage.

Another major factor impacting persistence of bacteria in the environment is the threat of grazing predators. Protozoa in particular, profoundly influence the microbial community structure with their preference for certain bacterial strains over others (Jousset, 2012). In co-culturing assays, there was no difference in trophozoite population size when feeding on any of the bacterial strains except for PA23*phaC1ZC2D,* which supported higher Ac numbers (Fig. 4.9). Consistent with these findings, the PA23*phaC1ZC2D* population was much lower when grown with Ac compared to the other strains (Fig. 4.10). This bacterium produces reduced levels of PHZ (Fig. 4.2), and as detailed in Chapter 3, PHZ is toxic to Ac. It seems likely that reduced PHZ production increases the palatability of PA23*phaC1ZC2D,* thereby supporting higher trophozoite populations. Next, we investigated whether PHA production would contribute to the survival of this bacterium inside of Ac vacuoles. We observed that there was no significant difference between the PHA-deficient strains and WT; however, the PHA-complemented strains showed enhanced intracellular survival (Fig. 4.11). We hypothesize that the higher PHA content in the complemented strains provides increased protection against the acidic environment of the Ac vacuole. PHA has been found to play an important role in bacteria that live in association with eukaryotic hosts. For example, Kim and coworkers (2013) demonstrated that PHA accumulation in *Burkholderia* was required for the survival inside the bean bug *Riptortus pedestris* host. The authors observed that PHA-deficient strains of the *Burkholderia* symbiont exhibited a significantly reduced viability in the midgut of the *R. pedestris* host compared to the WT, suggesting that accumulation of PHA plays a vital role in *Burkholderia* stress tolerance under symbiotic conditions.

In summary, we have demonstrated that the accumulation of PHA enhances PA23 tolerance to a number of stresses *in vitro* including starvation, exposure to high and low temperatures, oxidative stress and UV radiation. With respect to the biocontrol potential of this bacterium, PHA production did not affect fungal suppression *in vitro*. However, it could increase the shelf life of bioinoculant formulations and overall plant beneficial effects in the field, which has been reported for *A. brasilense* (Kadouri et al., 2005). Additional studies are required to determine whether PHA production increases PA23 fitness in the environment. It is interesting that PHA non-producers adhere better to root surfaces, providing access to amino acids and sugars in the form of root exudates. We propose that PHA-mediated fitness may be context dependent; with accumulation of this carbon and energy sink benefiting cells in more harsh environments. In the nutrient-replete environment of the rhizosphere, a PHA-deficiency might be advantageous, as it appears to increase PA23 association with plant roots.

Chapter 5

Conclusions and Future Directions

5.1. Conclusions and future directions

In soil ecosystems, several bacteria known as BCAs are able to inhibit the growth of soilborne plant pathogens. As such, these bacteria can be utilized as environmentally safe alternatives to replace chemical pesticides for the control of crop diseases (Lugtenberg & Dekkers, 1999; Compant et al., 2005). The application of BCAs in a particular environment, however, is often limited due to a lack of consistency in their fitness and fluctuations in their antagonistic activity (Thomashow, 1996; Köhl et al., 2019). *Pseudomonas chlororaphis* strain PA23 is a BCA capable of inhibiting growth of the fungal pathogen *S. sclerotiorum* (Savchuk & Fernando, 2004). This BCA produces an arsenal of toxic metabolites, including PHZ, PRN, HCN, proteases, lipases and siderophores, all of which contribute to PA23-mediated fungal antagonism (Poritsanos et al., 2006; Zhang et al., 2006; Selin et al., 2010). In addition, PA23 can synthesize and accumulate a group of polyesters known as polyhydroxyalkanoates (PHA) as carbon and energy storage compounds (Sharma et al., 2017). PHA accumulation enables bacteria to survive under certain environmental stressors such as nutrient deprivation, extreme temperatures, UV irradiation, osmotic and oxidative stress, and desiccation (Kadouri et al., 2005; Obruca et al., 2016). The ability of PA23 to resist predation by microfaunal predators and to adapt to fluctuating environmental conditions is expected to enhance the ecological fitness of this BCA in the environment. Therefore, the overall goal of this thesis was to investigate the role of PA23 exoproducts in defence against the predator Ac and to explore the role of PHA in bacterial resistance against stress conditions.

The successful establishment of an introduced BCA depends upon its ability to colonize a given environment and resist grazing predators, including protozoa and nematodes that feed upon bacteria. Protozoan predation on bacteria can have a profound impact on microbial communities

in the environment (Bonkowski, 2004; Pernthaler, 2005; Jürgens & Matz, 2002; Steiner, 2007; Jousset, 2012). Since bacterial metabolites play a major role in antagonistic activity against predators (Jousset, 2012), studies on predator-prey interaction may uncover pathways regulating bacterial defence mechanisms. We have previously shown that PA23 is able to kill the nematode *C. elegans* through production of toxic metabolites, including HCN and PRN (Nandi et al., 2015). Additionally, these metabolites act as repellents to deter *C. elegans in vitro* (Nandi et al., 2015). In this study, we discovered that PRN, PHZ and HCN exhibit toxic effects on Ac trophozoites *in vitro* by either killing or inducing cyst formation. Although we have previously shown that PHZ plays a minor role in PA23-mediated fungal antagonism and it is more involved in PA23 biofilm formation (Selin et al., 2010), here we revealed that PHZ has a detrimental effect on Ac viability. Incubation of Ac trophozoites in the presence of PHZ led to a significant decrease in amoeba counts over time. To the best of our knowledge, this is the first report of PHZ exhibiting amoebicidal activity. Apart from their cidal effects, bacterial metabolites can also act as repellents to deter potential predators (Jezbera et al., 2006; Neidig et al., 2011). The ability of bacteria to avoid direct contact with protozoan predators would be highly beneficial for maintaining their population size. Our chemotactic results revealed that Ac is able to sense and respond to the presence of bacteria. The trophozoites preferentially migrated towards PA23 mutants deficient in PRN production (*gacS*$^{-}$, QS-deficient and PRN^{-}), while the PRN-producers (PHZ^{-}, *rpoS*$^{-}$, HCN^{-} and PA23 WT) exhibited the strongest repellent activity. These findings suggest that PRN production affects the chemotactic behaviour of amoeba. In a similar fashion, other studies have reported that protozoa are able to distinguish between bacterial species and exert higher predation pressure on less toxic strains in diverse bacterial communities (Pedersen et al., 2009).

The interplay between PA23 and amoeba led to enhanced transcription of secondary metabolite genes involved in biocontrol. Specifically, we observed increased expression of the *prn* and *phz* biosynthetic genes in the presence of Ac trophozoites. Elevated PRN and PHZ levels in PA23-Ac co-cultures further supported these findings. The *phzI* and *phzR* QS genes also showed increased transcriptional activity in PA23 grown together with trophozoites. Since the *phz* and *prn* genes are under control of the PhzRI QS system, it would be of interest to explore whether contact with amoeba affects expression of these genes directly or indirectly through QS. No changes in gene expression or antibiotic production were observed upon co-culturing with amoeba cell-free supernatant, suggesting that soluble chemical cues were unable to elicit the same response as trophozoites. Future studies analyzing the global transcriptomic response of PA23 to Ac would provide a better understanding of molecular mechanisms involved in PA23-mediated antagonism against amoeba.

Biotic interaction studies have established that most microfaunal predators selectively feed on less toxic bacteria, which in turn promotes the survival of toxin producers. Toxic compound production, therefore, facilitates bacterial survival in highly competitive environments (Matz & Kjelleberg, 2005; Pedersen et al., 2009). We have demonstrated that PA23 metabolites exhibit amoebicidal and repellent activity against Ac in vitro. Future studies should be focused on determining whether AF metabolites affect Ac predation in soil environments. Moreover, it would be interesting to utilize metagenomic analysis to investigate how Ac grazing impacts PA23 fitness in the presence of diverse bacterial communities.

In the rhizosphere, predation plays a vital role in nutrient recycling by releasing nutrients locked in bacterial biomass, which then become available for plants (Bonkowski, 2004; Bais et al., 2004). Plant-bacterial interaction studies revealed that rhizobacteria secrete compounds that

stimulate root elongation, thereby improving nutrient uptake by the plant (van Rhijn & Vanderleyden, 1995; Flores-Gallegos & Nava-Reyna, 2018). We have reported that PA23 is able to enhance canola defence against the pathogenic fungus *S. sclerotiorum* through the induction of local and systemic resistance networks (Duke et al., 2017). In the future it would be interesting to study whether interaction of PA23 with Ac in the rhizosphere affects the root architecture of canola in the presence and absence of *S. sclerotiorum*. Moreover, global transcriptomic analysis could reveal how Ac grazing impacts the response of canola to PA23 as well as attack by *S. sclerotiorum*.

Several bacteria are able to survive inside protozoan hosts. As such, these bacteria have evolved strategies to cope with the intracellular environment and benefit from host cell resources (Garcia-del Portillo & Finlay, 1995; Casadevall, 2008; Ray et al., 2009; Thomas et al., 2010). Here, we discovered that PA23 could remain viable inside of Ac trophozoite vacuoles for up to 24 h. The production of PRN, PHZ and HCN appears to be involved in intracellular survival since the *gacS*$^{-}$ and PRN/PHZ mutants exhibited reduced viability and died rapidly. At present, it is unknown if the aforementioned metabolites affect cellular degradation mechanisms of the amoeba. Future work on Ac gene expression during phagocytosis of both toxic and non-toxic strains would be important to reveal possible mechanisms used by PA23 to survive within Ac vacuoles. Additionally, it would be interesting to study the gene expression profile of PA23 inside of Ac vacuoles, to reveal molecular factors facilitating intracellular survival.

PA23 is capable of accumulating PHA as a carbon and energy sink. The ability to accumulate and degrade PHA has been shown to enhance bacterial endurance against various environmental stressors (Zinn et al., 2001; Kadouri et al., 2005; Lu et al., 2009; Obruca et al., 2018). As such PHA may enhance survival of bacteria in harsh environments and promote

colonization and persistence in the rhizosphere. We observed that PHA-producing strains of PA23 (WT and *pha* complemented strains) were more resistant to various stress conditions, including high temperatures, freezing, UV irradiation and exposure to H_2O_2 compared to *pha*-deficient strains (PA23*phaC1*, PA23*phaC1ZC2* and PA23*phaC1ZC2D*). Additionally, PHA producers survived better under starvation conditions compared to their respective mutants. The connection between PHA synthesis and bacterial stress resistance has many practical applications. For instance, PHA accumulation enhances the fitness of bacteria under non-optimal conditions, which can be advantageous when bacteria are utilized as agricultural inoculants (Kadouri et al., 2005; Obruca et al., 2018). In this context, introduced bacteria are expected to be exposed to various stressors and starvation conditions. Accordingly, robust PHA-producing strains could be prioritized for creating bioinoculants. Future studies on the application of PHA-producing strains of PA23 in the canola phyllosphere and rhizosphere would help to determine the role of PHA in bacterial fitness, especially in competitive environments where carbon and energy are limited. It would also be interesting to examine whether PHA accumulation increases the shelf-life of commercial formulations based on PA23 and other BCAs.

Use of bacteria as an alternative to replace chemical pesticides is dependent upon the ability of the introduced microbe to persist in the environment and resist the threat of grazing predators. Research on biotic interactions between bacteria and their predators has gained more attention during the last few decades. Despite this, our understanding of how predator-prey interactions affect bacterial fitness in the environment is still limited. Research on bacterial defense is essential to gain deeper insights into traits that affect the outcome of predator-prey interactions. Ultimately, understanding PA23 responses to Ac could open new perspectives for the improvement of its potential to control crop diseases in agriculture.

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