Functional characterization of two divergently transcribed genes: *ptrA*, encoding a LysR-type transcriptional regulator, and *scd*, encoding a short-chain dehydrogenase in *Pseudomonas chlororaphis* PA23

by

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ABSTRACT

Pseudomonas chlororaphis PA23 inhibits several root pathogens in both the greenhouse and field. A LysR-type transcriptional regulator (LTTR) called PtrA (Pseudomonas transcriptional regulator A) that is essential for *Sclerotinia sclerotiorum* antifungal activity was discovered through transposon mutagenesis. *P. chlororaphis* PA23 produces the antibiotics phenazine 1-carboxylic acid, 2-hydroxyphenazine and pyrrolnitrin, and several additional products that contribute to biocontrol. Phenotypic assays and proteomic analysis have revealed that production of these secondary metabolites are markedly reduced in a *ptrA* mutant. Most LTTRs regulate genes that are upstream of and divergently transcribed from the LTTR locus. A short chain dehydrogenase (*scd*) gene lies immediately upstream of *ptrA* in the opposite orientation. Characterization of an *scd* mutant, however, has revealed no significant changes in antifungal activity compared to wild-type PA23. Gene expression analysis of the *ptrA* mutant indicates that *ptrA* may exert its regulatory effects through the Gac-Rsm network, and may be controlling expression of the *scd* gene. Collectively these findings indicate that PtrA is an essential regulator of PA23 biocontrol and is connected to other regulators involved in fungal antagonism.

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A_{260} – absorbance at 260 nanometres
A_{280} – absorbance at 280 nanometres
AF – antifungal
$AgNO_3$ – silver nitrate
AHL – acyl-homoserine lactone
Amp - ampicillin
ATP – adenosine triphosphate
bp – base pairs
cAMP – cyclic adenosine monophosphate
CAS – Chrome Azurol S
cDNA – complementary DNA
Chl – chloramphenicol
COG – clusters of orthologous groups
dH_2O – distilled water
dTDP – thymidine diphosphate
DNA – deoxyribonucleic acid
DTT - dithiothreitol
EDTA – ethylenediaminetetraacetic acid
EPS - exopolysaccharide
g - gravity
Gm - gentamicin
H_2O_2 – hydrogen peroxide
HCl – hydrogen chloride
HCN – hydrogen cyanide
HPLC – high performance liquid chromatography
IAA – iodoacetamide
IMG – Integrated Microbial Genomes
iTRAQ – isobaric tag for relative and absolute quantification
LB – Luria-Bertani
LPS - lipopolysaccharide
LTTR – LysR-type transcriptional regulator
mA - milliamps
MS – mass spectometry
NADP(H) – nicotinamide adenine dinucleotide phosphate
NaCl – sodium chloride
NaOH – sodium hydroxide
NH_4OH – ammonium hydroxide
OD_{600} – optical density at 600 nanometres
2-OH-PHZ – 2-hydroxyphenazine
3-OH-PAME – 3-hydroxypalmitic acid methyl ester
PBS – phosphate buffered saline
PCA – phenazine-1-carboxylic acid
PCR – polymerase chain reaction

PDA – Potato Dextrose Agar
PHZ – phenazine
PIA – *Pseudomonas* Isolation Agar
Pip - piperacillin
PRN – pyrrolnitrin
PYO - pyocyanin
QS – quorum sensing
RBS – ribosomal-binding site
Rif - rifampicin
RNA – ribonucleic acid
SDR – short-chain dehydrogenase/reductase
SDS-PAGE – sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SR – stringent response
SSR – *Sclerotinia* stem rot
Tc – tetracycline
TEMED – N,N,N',N'-tetramethylethylenediamine
Tn – transposon
Tris – tris(hydroxymethyl)aminomethane
UV - ultraviolet
V - volts
V_{diff} – vector difference
v/v – volume per unit volume
w/v – weight per unit volume

1. INTRODUCTION

1.1. Plant disease in Canadian agriculture

A large part of the Canadian economy depends on the wealth generated from the sale and export of grain and oilseed crops. Therefore, plant disease suppression in these crops is of vital importance. As most commercially relevant crops are grown under monoculture conditions, the potential for plant pathogens to cause widespread disease is elevated. This in turn can cause massive economic losses resulting from partial to complete eradication of crops each year. Current methods implemented to control the spread of common plant pathogens include the application of petroleum-derived pesticides, crop rotation strategies and specific breeding for pathogen-resistant plant varieties. The use of chemical pesticides, however, raises concern over its impacts on human health, as well as the environmental impact caused by accumulation of these products in the surrounding ecosystems. An alternative approach to suppressing plant pathogens is the use of biocontrol measures, in which one or many organisms are used to control the growth of other, unwanted organisms in an ecosystem.

1.2. *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary is a soil-borne ascomycete fungus capable of infecting over 400 different plant species, including flaxseed, soybean, sunflower and canola (Hegedus and Rimmer, 2005). Sclerotinia disease is often referred to as stem rot or white mold, depending on the plant species it infects. *S. sclerotiorum* infection is characterized by the formation of special resting structures called sclerotia, which allow it

to survive in soil for several years under harsh environmental conditions (Hegedus and Rimmer, 2005). Sclerotia can then germinate to produce apothecia, which release ascospores into the air, causing widespread distribution of the pathogen over large crop areas.

1.3. Prevalence of *Sclerotinia* stem rot disease in canola

Stem rot disease development is favoured by cooler temperatures and increased precipitation levels prior to and during the canola flowering stage (Manitoba Agriculture 2011). The spores initially colonize senescent tissues, which provide most of the required nutrients for full infection of healthy leaf and stem tissues (Hegedus and Rimmer 2005). Disease is characterized by pale-gray or white lesions on the infected plant tissue (Manitoba Agriculture 2011).

In Manitoba, stem rot disease is one of the major causes of canola crop losses, with yield losses ranging from 5-100% in a growing season (Manitoba Agriculture 2011). Recent statistics for the 2012 growing season have revealed that in Saskatchewan, *Sclerotinia* stem rot (SSR) disease was found in 91% of the fields surveyed (233/253) and where SSR was present, the percentage of plants infected averaged 19% (Canadian Phytopathological Society 2013). In Manitoba, SSR was present in 65% (92/142) of the fields surveyed, with a mean incidence of 13.2% in infected fields (Canadian Phytopathological Society 2013).

Fungicidal applications are currently the most effective method for controlling and treating SSR disease. Synthetic fungicides approved for use to control stem rot of canola in Canada include Astound® (Syngenta), Quash® (Valent) and Vertisan®

(Dupont) (Saskatchewan Ministry of Agriculture 2013); all are considered to have broad-spectrum modes of action.

1.4. *Pseudomonas chlororaphis* strain PA23

Pseudomonas chlororaphis strain PA23 is a gram negative, fluorescent pseudomonad first isolated from soybean root tips (Savchuk and Fernando, 2004). PA23 has been shown to have antagonistic effects against *S. sclerotiorum* both under laboratory conditions and in field studies on *Brassica* spp. canola plants (Fernando *et al*., 2007).

The antifungal (AF) properties of PA23 are attributed to the production of metabolites, which include compounds such as phenazines (PHZ), pyrrolnitrin (PRN), proteases, lipases, hydrogen cyanide (HCN) and siderophores (Poritsanos *et al*., 2006). Studies have shown that the main compound critical for suppressing *S. sclerotiorum* is PRN (Selin *et al*., 2010). In PA23, production of secondary metabolites is controlled through various genetic regulatory cascades. The expression of PRN and PHZ in particular are under the control of the Gac-Rsm signal transduction pathway, the Phz quorum sensing (QS) network and the stationary phase sigma factor, RpoS (Manuel *et al.*, 2011, Poritsanos *et al*., 2006, Selin *et al*. 2012). Full elucidation of the genetic pathways involved in secondary metabolite production is key to understanding the molecular basis for the AF effects of PA23 in protecting canola from SSR.

1.5. Phenazines

A major class of antibiotic compounds produced by virtually all fluorescent pseudomonads is the pigmented PHZs. Over 6000 natural and synthetically derived PHZs now exist; however, the first PHZ compound, pyocyanin (PYO), was discovered in 1882 by Gessard and it is now known to be made by 90-95% of *P. aeruginosa* isolates (Mavrodi *et al*., 2006). Produced most abundantly by the gram-negative Proteobacteria, PHZs contribute to environmental competitiveness and virulence, as well as to the biocontrol abilities of the organism (Haas and Keel, 2003). Some common PHZs include phenazine-1-carboxylic acid (PCA), PYO, pyoverdin (also known as pseudobactin) and 2-hydroxyphenazine (2-OH-PHZ). All PHZs are characterized as having a central nitrogen ring, flanked by two benzene rings containing various substituted side chains. These compounds are formed through a branch point in the shikimic acid synthesis pathway (Haas and Keel, 2003; Mavrodi *et al*., 2006).

The PHZ operon is a well-studied system in many pseudomonads, with *phzABCDEFG* comprising the core biosynthetic locus (Mentel *et al*., 2009). PHZ production in *P. chlororaphis* is regulated through a two component QS regulated system (PhzR/PhzI) as well as the Gac-Rsm system, the TetR-like transcriptional regulator, PsrA and the alternative sigma factor, RpoS (Girard *et al*., 2006; Mavrodi *et al.* 2006; Poritsanos *et al*., 2006; Selin *et a*l., 2012). In some pseudomonads, genes downstream of the core biosynthetic operon have been identified that are required for modification of various PHZ products (Mavrodi *et al*., 2001; Delaney *et al*., 2001; Chin-A-Woeng *et al*., 2001). In *P. chlororaphis* 30-84, *phzO* is located immediately downstream of the core operon and encodes an aromatic hydroxylase. Further functional characterization

revealed that PhzO catalyzes the conversion of PCA to 2-OH-PHZ (Delaney *et al.*, 2001). More recently, in *P. chlororaphis* gp72, the *phzO* gene was discovered and its product was shown to convert PCA into 2-OH-PHZ through a 2-OH-PCA intermediate (Huang *et al*., 2011).

PHZs are believed to exert their effects through a redox-cycling mechanism which mediates the formation of radical oxidative species and hydrogen peroxide (H_2O_2) (Mavrodi *et al*., 2006).

1.6. Pyrrolnitrin

Pyrrolnitrin is another important AF secondary metabolite produced by *P. chlororaphis* PA23 that was first discovered in *Burkholderia* (*Pseudomonas*) *pyrrocinia* in 1964 by Arima *et al*. Later studies revealed that the compound is most effective against fungal pathogens (Tripathi and Gottlieb, 1969). It is believed that PRN inhibits electron transport chain intermediates, thereby blocking ATP synthesis, and subsequent DNA, RNA and protein biosynthesis (Tripathi and Gottlieb, 1969). Pyrrolnitrin also affects gram-positive bacteria by destabilizing cellular membrane phospholipids (Nose and Arima, 1969).

Pyrrolnitrin is derived from the amino acid tryptophan and its conversion is catalyzed by the biosynthetic operon *prnABCD* (Hammer *et al*., 1997). These genes are known to be regulated by the Gac two-component system in *Pseudomonas fluorescens* (Laville *et al*., 1992). As mentioned earlier, studies with *P. chlororaphis* PA23 have shown that PRN is the primary antibiotic compound responsible for suppression of *S. sclerotiorum* growth *in vitro* (Selin *et al*., 2010).

1.7. Gac-Rsm regulatory control

One of the most ubiquitous genetic systems governing secondary metabolite production in bacteria is the Gac-Rsm system (for global activator of antibiotic and cyanide; regulator of secondary metabolism) (Laville *et al*., 1992; Lapouge *et al*., 2008). Forming part of this regulatory circuit is the GacS/GacA two-component signal transduction system. GacS, a membrane-bound sensor kinase, responds to an unknown environmental signal, autophosphorylates, then phosphotransfers to GacA, the cytoplasmic response regulator. Phosphorylated GacA can then bind to regions of DNA to control transcription of various genes (Chancey *et al*., 1999). The activated form of GacA strongly activates the transcription of small non-coding RNA molecules, termed RsmX/Y/Z, which play further regulatory roles in the signal transduction cascade (Kay *et al*., 2006).

The RsmX/Y/Z RNAs contain special stem loop secondary structures that allow them to bind to and titrate out the RNA-binding proteins, RsmA and RsmE (Kay *et al*., 2006). RsmA and RsmE bind to ANGGAN (where N can be any nucleotide) sequence motifs of target mRNA, blocking the ribosome binding site (RBS) and subsequently translation (Kay *et al*., 2006; Lapouge *et al*., 2008). Translational repression can be relieved through the small RNAs, which bind multiple copies of RsmA and RsmE, allowing access to the RBS of target mRNAs (Reimmann *et al.*, 2005).

In *P. fluorescens* CHA0, single *rsmA* or *rsmE* mutants of the wild type or a Gac mutant resulted in only marginal increases in expression of *hcnA* (HCN), *aprA* (alkaline protease) and *phlA* (2,4-diacetylphloroglucinol) (Reimmann *et al*., 2005). When both *rsmA* and *rsmE* were inactivated, very strong increases in gene expression were seen,

indicating that both proteins are required for maximal repression of target mRNAs (Reimmann *et al*., 2005). Levels of both translational repressors vary throughout the growth of the organism; however, RsmA was found to be consistently present in greater amounts throughout all growth phases. RsmE was present in very low amounts at a low cell density and present in highest amounts at the end of growth, indicating that it may play a role in termination of Gac-controlled gene expression (Reimmann *et al*., 2005).

The Gac system can also been modulated by two orphan sensor-kinases, RetS and LadS. RetS is able to form heterodimers with GacS, which prevents its autophosphorylation, thus preventing phosphotransfer to GacA, and subsequent transcription of *rsmZ* (Goodman *et al*., 2009). However, upon detection of an unknown signal, RetS and GacS each form homodimers, allowing for proper phosphorylation of the circuit, downstream expression of *rsmZ* and titration of the RsmA and RsmE proteins. LadS seems to play an opposing role to RetS through an unknown mechanism (Goodman *et al*., 2009; Ventre *et al*., 2006). In studies with *P. aeruginosa*, *rsmZ* was shown to be necessary for proper biofilm development. Mutations in *retS* strongly enhanced the formation of biofilms, whereas mutations in *ladS* completely abolished biofilm formation (Kay *et al*., 2006).

In addition to regulation by the Gac system, evidence has shown that expression of *rsmX/Y/Z* may also be influenced by the RsmA and RsmE proteins. β-galactosidase assays have revealed that in *rsmA* and *rsmE* double mutants of *P. fluorescens* CHA0, transcriptional levels of *rsmX/Y/Z* are decreased (Kay *et al.*, 2005; Reimmann *et al*., 2005). The gene encoding RsmA was also found to be co-transcribed with an upstream

lysC (aspartokinase gene) and possibly *alaS* (alanyl-tRNA synthetase), further complicating the elucidation of its regulation (Reimmann *et al*., 2005).

A number of metabolites are regulated by the Gac system. For example, in *P. aeruginosa*, the Gac-Rsm system controls expression of genes required for extracellular virulence factors such as HCN, elastase and PYO, as well as the expression of the QS signal molecule *N*-butanoyl-homoserine lactone (Kay *et al*., 2006). Extensive studies with *P. fluorescens* CHA0 have shown that production of HCN, PRN, pyoluteorin, H_2O_2 resistance, motility and overall biocontrol in this strain are all governed by the Gac-Rsm system (Lapouge *et al*., 2008). In *P. chlororaphis*, this system regulates the production of acyl-homoserine lactones (AHLs), PHZs, HCN, surfactants, proteases and other biocontrol processes (Lapouge *et al*., 2008).

1.8. RpoS and PsrA regulatory control

Upon entry into stationary phase, the alternative sigma factor RpoS is a key player in the regulation of secondary metabolites in many pseudomonads. It was first identified in *Escherichia coli* as a regulator of general stress responses, but has been found in many non-enteric bacteria as well (Venturi *et al*., 2003). Once enough RpoS accumulates within the cell, it competes with other sigma factors for binding to the core RNA polymerase, thus altering its binding specificity to target promoter regions. In *Pseudomonas* spp., RpoS has a more unique function in controlling the production of secondary metabolites such as PYO, alginate, AHLs, HCN and PHZs (Oh *et al*., 2013; Venturi *et al*., 2003). Regulation of RpoS expression is tightly controlled by the Gac two-component system, QS systems and the TetR family regulator, PsrA (Venturi *et al*.,

2003). LacZ transcriptional fusion studies have revealed that upon entry into stationary phase, RpoS transcription exhibits a 3-fold induction in *P. fluorescens* Pf-5, and when the Gac system is absent in this same organism, RpoS transcription occurs more gradually (Venturi *et al*., 2003).

PsrA (Pseudomonas sigma regulator A) is also a positive regulator of RpoS transcription, binding directly to its promoter region to activate transcription (Venturi *et al*., 2003). In *P. putida* and *P. aeruginosa*, experiments have shown that in a *psrA*-mutant background, there is 50% less *rpoS* transcription compared to the wild type (Venturi *et al*., 2003). PsrA has been shown to negatively regulate its own expression in *P. chlororaphis* and *P. syringae* (Chatterjee *et al*., 2007; Girard *et al*., 2006).

1.9. Phz quorum sensing system

Quorum sensing is a cell-to-cell communication system that allows bacteria to regulate gene expression according to population density through the production of small, diffusible signalling molecules (Bassler, 2002). Most gram-negative bacteria use AHL-based QS systems consisting of a LuxR-type transcriptional activator and a LuxI-type AHL synthase (Bassler, 2002). After a threshold level of AHL has accumulated, it binds to the LuxR protein, allowing it to dimerize and regulate expression of target genes. In PHZ-producing pseudomonads, the PhzR/PhzI QS system controls expression of these antibiotics (Chin-A-Woeng *et al*., 2001; Khan *et al*., 2005; Mavrodi *et al*., 1998; Wood and Pierson, 1996). The PhzR AHL-complex activates the phz operon by binding to an upstream region called the "phz box" (Bassler, 2002). In PA23, *phzR* is located

immediately upstream of the PHZ operon, and *phzI* lies upstream of *phzR*, but in the opposite orientation (Selin et al., 2012).

1.10. Regulation of antifungal factors in PA23

In PA23, the production of secondary metabolites is regulated through numerous factors. In the absence of a functional *gacS* or *gacA* gene, PA23 exhibits a dramatic decrease in AF activity, primarily attributed to a loss of HCN, protease, PHZ, PRN and autoinducer production (Poritsanos *et al*., 2006; Selin *et al*., 2014). In addition, *rpoS* transcription was found to be decreased in the *gacS* mutant compared to wild-type PA23 (Poritsanos *et al*., 2006).

Another positive regulator of PA23 biocontrol is the Phz QS system. Analysis of a *phzR* mutant and an AHL-deficient strain revealed a decrease in protease, PHZ, PRN and a loss of AF activity (Selin *et al.*, 2012). Both *phzA* and *prnA* expression were found to be under QS control. Further analysis also revealed cross-regulation between QS and RpoS, as an *rpoS*-deficient mutant exhibits decreased *phzI* expression, while *phzR* is upregulated in this strain (Selin *et al*., 2012). Furthermore, *rpoS* is under positive control of the Phz QS system (Selin *et al*., 2012).

The stringent response (SR) is a mechanism used by bacteria to adapt to nutrient stress through the production of the alarmone (p)ppGpp (Potrykus and Cashel, 2008). In SR mutants of PA23, an overall increase in AF activity was observed, likely due to increased protease, lipase and PRN production (Manuel *et al*., 2011). The SR effects were found to be mediated through the alternative sigma factor RpoS, as *lacZ* transcriptional fusions indicated a decrease in *rpoS* expression in the SR mutant

backgrounds (Manuel *et al*., 2011). These results were not surprising since RpoS has a repressive effect on PA23 AF activity. Characterization of an *rpoS* mutant revealed an overall increase in AF activity due to increased PRN and protease production (Manuel *et al*., 2011).

PsrA, a positive regulator of RpoS, also negatively regulates PA23 AF activity. A PsrA mutant showed enhanced AF activity, with increased protease and PRN production, but decreased PHZ production compared to the wild type PA23, similar to the *rpoS*-mutant phenotype (Manuel *et al*., 2011; Selin *et al*., 2014).

From previous studies conducted with PA23, it is evident that significant cross-regulation occurs with respect to secondary metabolite production (Manuel *et al.,* 2011; Poritsanos *et al.,* 2006; Selin *et al.*, 2010; Selin *et al.*, 2012). For example, *lacZ*-transcriptional fusion studies revealed that in a *gacA* mutant, expression of *rsmA/E/Z* is significantly reduced, and addition of *phzR* and autoinducer *in trans* is able to partially restore *rsmZ* expression (Selin *et al*., 2014).

Many of these cross-regulatory interactions are outlined in Figure 1, which depicts our current model of the regulatory cascade governing expression of secondary metabolites.

1.11. LysR-type transcriptional regulators

The LysR-type transcriptional regulators (LTTRs) represent another group of proteins that regulate secondary metabolites within bacteria. This family of proteins is the next most common type of regulators, apart from two-component signal transduction systems (Maddocks and Oyston, 2008).

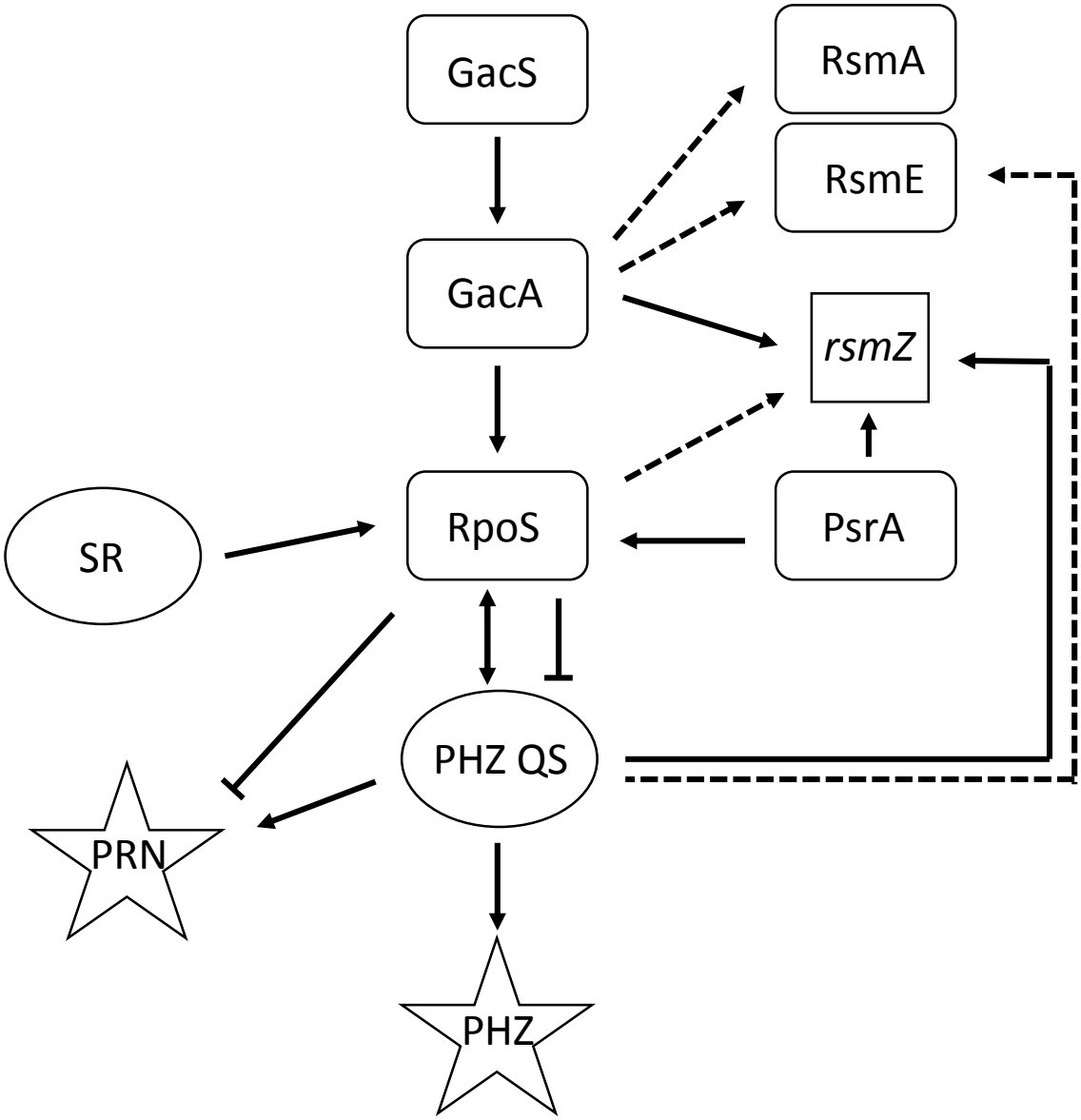

Figure 1. Current model of the genetic regulatory cascade governing secondary metabolite production in *Pseudomonas chlororaphis* PA23. An unknown signal causes autophosphorylation of GacS and subsequent phosphotransfer to GacA. GacA induces expression of the small non-coding RNA, RsmZ, the translational repressors, RsmA and RsmE and the RpoS alternative sigma factor. RpoS is positively regulated through PsrA and the SR. RpoS negatively regulates expression of PRN and PhzR and positively regulates expression of PhzI. The PHZ QS system positively regulates expression of RpoS, RsmZ, RsmE and production of PRN and PHZ, Symbols: ↑, positive effect; ⊤, negative effect; solid lines, direct effect; dashed lines, indirect effect. Abbreviations are those used in the text. Modified from Selin *et al.* (2014).

These regulators are named after the well-studied transcriptional activator, LysR, of the enzyme LysA, which catalyzes the conversion of diaminopimelate to lysine (Stragier, 1983). LTTRs act as global transcriptional regulators, either through activation or repression of target genes and they are often autoregulatory in nature (Maddocks and Oyston, 2008). The genes under LTTR control are frequently upstream of and divergently transcribed from the LTTR gene; however, they may be located elsewhere within the genome. These regulators often require co-inducer molecules to facilitate DNA binding through conformational changes (Maddocks and Oyston, 2008).

The LTTRs are well-conserved throughout bacterial evolution, and they regulate a wide range of physiological traits, including QS, motility, attachment and biofilm formation, metabolism and virulence (Maddocks and Oyston, 2008). Genes encoding LTTRs have been found in many species of bacteria, and as numerous paralogous copies throughout a single bacterial genome. Although they are diverse, specific structural regions within the LTTR protein remain highly conserved (Maddocks and Oyston, 2008). Most LTTRs average 330 amino acids in length, with the C-terminus being the co-factor-binding domain, and the N-terminus being the helix-turn-helix (HTH) DNA-binding domain. The latter is a common motif found in bacterial DNA-binding proteins (Maddocks and Oyston, 2008). The LTTR gene sequence consists of a high G + C content (Maddocks and Oyston 2008).

This grouping of proteins can be further divided into transcriptional activators and repressors, either by dimerically binding at the regulatory binding sites (RBS) or the activation binding sites (ABS), both of which contain LTTR-boxes. These dimers can then interact as tetramers, which bend DNA to physically interact with RNA polymerase,

allowing transcription of target genes or operons (Figure 2) (Maddocks and Oyston, 2008).

LTTRs have been shown to play significant regulatory roles affecting a myriad of physiological traits. For example, in gram-negative bacteria, the LTTR OxyR is a H_2O_2-responsive activator of defensive oxidative stress genes, the QS regulated *rsaL* gene and genes involved in iron homeostasis (Schell *et al.,* 1994; Wei *et al.,* 2012).

1.12. *Pseudomonas* transcriptional regulator A

Through Tn*5* insertional mutation studies of *P. chlororaphis* PA23, an isolate was discovered, called PA23-443, that exhibited a complete loss of AF activity. Sequence analysis revealed that the transposon had interrupted a gene encoding an LTTR. This gene was designated *ptrA* for *Pseudomonas* transcriptional regulator A. Preliminary phenotypic analysis revealed that the *ptrA* mutant was devoid of PHZ production (loss of orange pigmentation) and it no longer produced protease and AHL molecules (Poritsanos, 2005). Greenhouse assays showed that the *ptrA* mutant was unable to protect canola from SSR; thus, PtrA is a novel regulator that is essential for PA23 biocontrol. Exactly how PtrA controls expression of genes involved in biocontrol, however, has yet to be elucidated.

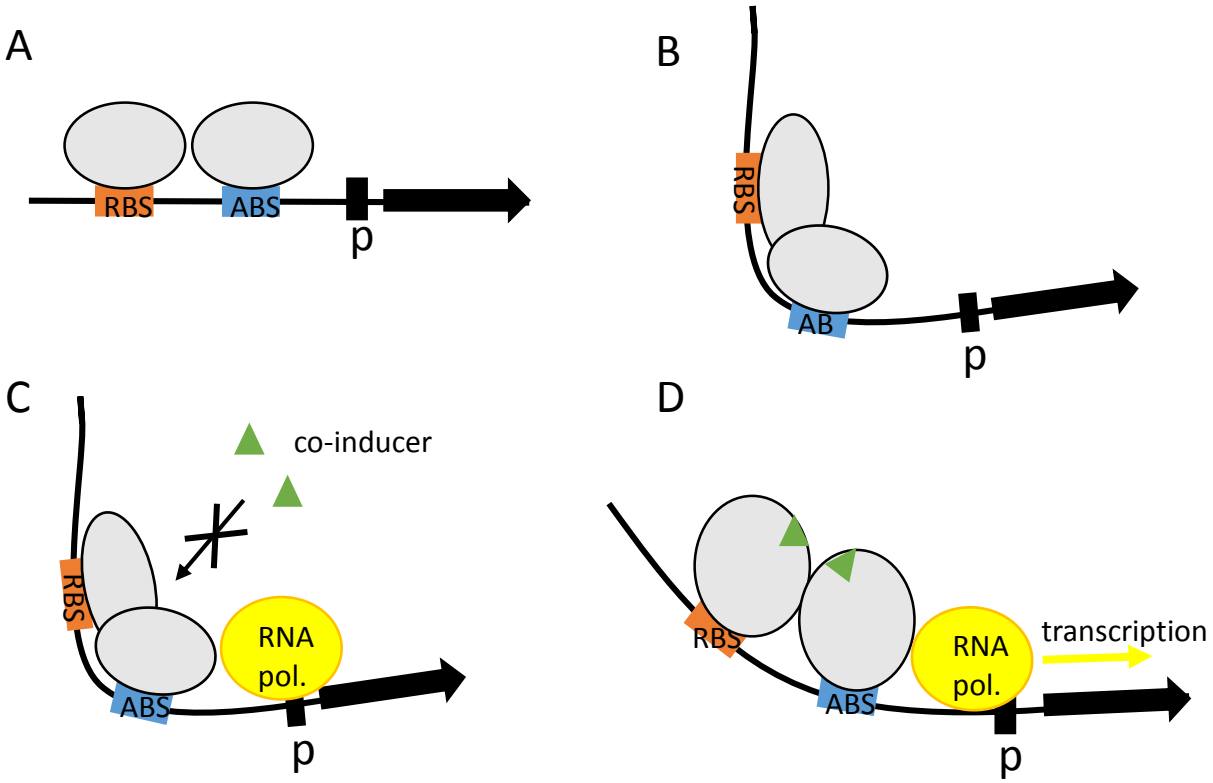

Figure 2. Schematic representation of LTTR-dependent transcriptional activation. A, LTTR dimers (grey oval) bind regulatory binding sites (RBS) and activation binding sites (ABS). B, Two LTTR dimer protein-protein interactions form a tetrameric protein and cause the DNA to bend. C, RNA polymerase binds at the promoter region but no transcriptional activation occurs in the absence of co-inducer binding. D, Co-inducers bind the LTTR tetramer and the DNA relaxes. RNA polymerase makes contact with the LTTR tetramer to initiate transcription of target gene. Modified from Maddocks and Oyston (2008).

1.13. Short-chain dehydrogenase

Immediately upstream of *ptrA*, in the opposite orientation, lies a gene encoding a short-chain dehydrogenase (*scd*). Short-chain dehydrogenases are part of a superfamily of enzymes designated as the NAD(H)- or NADP(H)-dependent short-chain dehydrogenases/reductases (SDRs). The SDRs comprise a very large grouping of biologically important proteins found in virtually all forms of life (Jornvall *et al.*, 1999). These enzymes display a wide range of substrate specificities, including, but not limited to, steroids, alcohols, carbohydrates, aromatic compounds and even xenobiotics (Kramm *et al*., 2012). Currently, there are over 47,000 members of the SDR super family and over 30 full 3-dimensional (3D) structures have been reported (Oppermann *et al*., 2003). Most SDRs exhibit highly similar α and β-folding patterns with a characteristic "Rossman-fold" motif, a β-pleated sheet flanked by α-helices. These enzymes may contain N- and C-terminal transmembrane domains or form part of larger multi-enzyme complexes (Oppermann *et al*., 2003).

The physical proximity of the *scd* gene to *ptrA* suggests that it may be involved in PtrA regulation. What role, if any, *scd* has in PtrA-mediated biocontrol has yet to be determined.

1.14. Thesis Objectives

PtrA is a newly-identified member of the LTTR family that is essential for PA23 biocontrol. Exactly how PtrA controls expression of genes encoding secondary metabolites is unknown. Therefore, the specific objectives of this thesis are:

1. To fully characterize the phenotype of the *ptrA* mutant.
2. To determine where PtrA fits in the regulatory cascade overseeing production of AF compounds.
3. To determine whether *scd* is regulated by PtrA.
4. To determine whether *scd* is involved in the biocontrol capability of PA23.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

All bacterial strains used in this study are outlined in Table 1. *E. coli* strains were cultured on Lennox Luria Bertani (LB) media (Difco Laboratories, Detroit, MI) at 37°C. *P. chlororaphis* PA23 and *C. violaceum* CV026 were cultured and maintained on LB media (Difco) at 28°C. For selection of *P. chlororaphis* PA23 from *E. coli* strains, cells were grown on Pseudomonas Isolation Agar (PIA; Difco). *S. sclerotiorum* was cultured and maintained on Potato Dextrose Agar (PDA; Difco) at 23°C. All antibiotics used were from Research Products International Corp. (Prospect, IL) and supplemented at the following concentrations: piperacillin (Pip; 40 µg/mL), gentamicin (Gm; 20 µg/mL), tetracycline (Tc; 15 µg/mL), rifampicin (Rif; 100 µg/mL), ampicillin (Amp; 100 µg/mL) and chloramphenicol (Chl; 30 µg/mL). For all phenotypic assays, strains were grown in M9 Minimal Salts Media (M9; Difco) supplemented with 1 mM $MgSO_4$ and 0.2% glucose.

2.2. Nucleic acid manipulations

Isolation, purification, endonuclease digestion, ligation and all other manipulation of DNA was performed according to the techniques outlined in Sambrook *et al*. (1989). Polymerase Chain Reaction (PCR) was performed following standard conditions recommended by Invitrogen Life Technologies data sheets supplied with their buffer system and *Taq* polymerase.

Table 1. Bacterial strains, plasmids and oligonucleotide sequences.

Strains	Relevant characteristics	Source or reference
Pseudomonas chlororaphis		
PA23	$Phz^{+}Rif^{R}$ wild type (soy bean plant isolate)	Savchuk and Fernando, 2004
PA23-443	$Phz^{-}Rif^{R}$ *ptrA*::Tn*5*-OT182 genomic fusion	This study
PA23-314	$Phz^{-}Rif^{R}$ *gacS*::Tn*5*-OT182 genomic fusion	Poritsanos *et al*., 2006
PA23*gacA*	Gm^{R} marker inserted into *gacA* gene	Selin *et al*., 2014
PA23*scd*	Tc^{R} marker inserted into *scd* gene	This study
Escherichia coli		
DH5α	*supE44 ΔU169 (φ80lacZΔM15) hadR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco
DH5αλpir	DH5λpir 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		
pUCP23	Broad-host range vector; Amp^{R}, Gm^{R}	West *et al*., 1994
pUCP22-*gacA*	1.65-kb fragment containing *gacA* and *uvrC* from *P. fluorescens* CHA0 in pUCP22	This study
pUCP23-*gacS*	3.1-kb fragment containing *gacS* in pUCP23	Poritsanos *et al*., 2006
pUCP22-*ptrA*	2.2-kb fragment containing *ptrA* in pUCP22	This study
pUCP22-*scd*	1.25-kb fragment containing *scd* in pUCP22	This study
pUCP22-*rpoS*	1.3-kb fragment containing *rpoS* in pUCP22	Poritsanos *et al*., 2006
pUCP22-*psrA*	950-bp fragment containing *psrA* in pUCP22	Selin, 2012
pUCP22-*rsmA*	190-bp fragment containing *rsmA* in pUCP22	Selin, 2012
pUCP22-*rsmE*	600-bp fragment containing *rsmE* in pUCP22	Selin, 2012
pUCP23-*rsmZ*	400-bp fragment containing *rsmZ* in pUCP23	Selin, 2012
pUCP23-*phzR*	1.68-kb fragment containing *phzR* in pUCP23	Selin *et al*., 2012
pRK600	Mobilization plasmid containing *tra* genes, Chl^{R}	Finan *et al*., 1986

pCR2.1	Cloning vector for PCR products	Invitrogen
pCR2.1-*scd*	1.25-kb fragment containing the *scd* gene in pCR2.1	This study
pCR2.1-*ptrA*Tn5	804-bp fragment containing portion of *ptrA* and Tn*5*-OT182	This study
pKNOCK-Tc	Suicide vector designed for insertional mutagenesis; R6K ori; RP4 oriT; Tc^R	Alexeyev, 1999
pKNOCK-*scd*	542-bp internal *scd* fragment cloned into pKNOCK-Tc	This study
Oligonucleotide sequences		
scd-pUCP FWD	5′-GCAT**AAGCTT**AGCGTTCGACGCGATACAACT-3′†	This study
scd-pUCP REV	5′-GAAC**TCTAGA**AGGTCATCCATCTCGCCCA-3′†	This study
tet FWD	5′- ACCCGTCCTGTGGATTCTCTA-3′	This study
tet REV	5′- CAACCCGTTCCATGTGCTC-3′	This study
MCS FWD	5′-TTTCCCAGTCACGACGTTGTAAAAC-3′	This study
MCS REV	5′- ACCTACAGCGTGAGCTTTGAGAAA-3′	This study
scd-pKNOCK FWD	5′-TATT**GGATCC**TTCCACGCTCTTGGCGTA-3′†	This study
scd-pKNOCK REV	5′-TATT**CTCGAG**CCAACGGCACCATAGGTTCA-3′†	This study
gacS RT-PCR FWD	5′- TGGTCAGCCTGGTGTATC-3′	This study
gacS RT-PCR REV	5′- TGTCTTCGTGTTCTTCTTCG-3′	This study
rpoS RT-PCR FWD	5′- TGGCTTTCCGAATTGACC-3′	This study
rpoS RT-PCR REV	5′- CAGACGCTTGAGACCTTC-3′	This study
prnA RT-PCR FWD	5′- CTGTCGTCGTGCTTTCTG-3′	This study
prnA RT-PCR REV	5′- GATCTCGGCGTTGAATGC-3′	This study
ptrA RT-PCR FWD	5′- CTGGCACGAGATGACCTG-3′	This study
ptrA RT-PCR REV	5′- CCGCTGTAATGACTGTTGAG-3′	This study
phzI RT-PCR FWD	5′- GCGATGCCGTTGTTCTGG-3′	This study
phzI RT-PCR REV	5′- AGCCGTTCGTAGTGGACTC-3′	This study
phzR RT-PCR FWD	5′- GAATCCTTGGCTTCAGACC-3′	This study
phzR RT-PCR REV	5′- ATCAGGCGGCTAACTACG-3′	This study
psrA RT-PCR FWD	5′- CCATCTTCATGCGTCTTCTG-3′	This study
psrA RT-PCR REV	5′- ATGTAGCGGCGGAATACC-3′	This study
rsmZ RT-PCR FWD	5′- TGCGGTATGAAAGTTGTCTATTTG-3′	This study
rsmZ RT-PCR REV	5′- ATCCTTGATGGTTGTGTCTATCC-3′	This study

rsmE RT-PCR FWD	5′- GAAAGCATAAATATCGGTGAC-3′	This study
rsmE RT-PCR REV	5′- CGTTGGTAGATTTCTTCGC-3′	This study
phzA RT-PCR FWD	5′- GACTGGCAATGGCACAAC-3′	This study
phzA RT-PCR REV	5′- GCAATAACCTTCGGGATAACC-3′	This study
gacA RT-PCR FWD	5′- CTGGTGTTCAAGTCATTCC-3′	This study
gacA RT-PCR REV	5′- AAGATACGGTAACGGTAGG-3′	This study
rpoD RT-PCR FWD*	5′- GCCAGTGACGACGAAGAAG-3′	This study
rpoD RT-PCR REV*	5′- GCCTTGCGGGTGATTTCC-3′	This study
rsmA RT-PCR FWD	5′- ATGCTGATTCTGACTCGTC-3′	This study
rsmA RT-PCR REV	5′- GCACCGCTACCTCTTTAG-3′	This study
R6Kori-3 FWD	5′- GCTCTCATGTTTCACGTACTAAGC-3′	This study
scdBamHI-3 REV	5′- GTGGCTATAAGCCGGTTCC-3′	This study
new ptrA TL start FWD	5′- GCAAGCAAGCTTCGACGCGATACAACTGGC- 3′	This study
IS50 REV	5′- CACGATGAAGAGCAGAAG-3′	This study
ptrA 2.2 REV	5′- ATCCAGTTGCTGGAGCGTATT-3′	This study
M13 FWD	5′- GTAAAACGACGGCCAG-3′	Invitrogen
M13 REV	5′- CAGGAAACAGTATGAC-3′	Invitrogen

*, sequence information obtained from *P. chlororaphis* subsp. *aureofaciens* 30-84 (GenBank accession number JN397563.1).

†, restriction sites indicated in bold.

2.3. Sequence analysis of DNA flanking the Tn*5*-OT182 insertion in mutant PA23-443

The site of Tn*5*-OT182 insertion within the *ptrA* gene of PA23 was determined through sequence analysis. Briefly, a 804-bp fragment containing one end of the Tn*5* transposon, the *ptrA* region and downstream sequence was amplified using primers IS50 REV and ptrA 2.2 REV (Table 1) and cloned into pCR2.1 to yield pCR2.1-*ptrA*Tn5. The plasmid was sequenced using the M13 FWD and REV primers (Table 1) and an Applied Biosystems 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) with supplied software.

Each reaction mixture contained 150-300 ng of DNA, 3.2 pM of sequencing primer, 0.5 µL of BigDye™ Terminator (Applied Biosystems) and 2 µL of the supplied 5X sequencing buffer (Applied Biosystems) brought to a final volume of 20 µL with water. PCR reaction conditions included an initial denaturation at 96°C for one minute, followed by 25 cycles of: 96°C for 10 seconds, 50°C for 5 seconds and 64°C for 4 minutes. Following PCR amplification, DNA was treated with 2 µL of EDTA (125 mM) at pH 8.0, 2 µL of sodium acetate (3 M) at pH 4.8 and 60 µL of ice-cold 95% ethanol. Following 15 minutes incubation at room temperature (RT), DNA was pelleted and washed twice with ice-cold 70% ethanol and dried. DNA was denatured with 20 µL Hi-Di™ formamide (Applied Biosystems) for 5 minutes at 94°C and 19-µL aliquots were used for subsequent sequence analysis.

2.4. Generation of an *scd* mutant

To generate PA23*Δscd*, a 542-bp internal fragment of the *scd* gene was PCR amplified from PA23 genomic DNA using primers scd-pKNOCK FWD and scd-pKNOCK REV. The PCR product was gel purified and digested with *Bam*HI and *Xho*I and cloned into the same sites of pKNOCK-Tc. The pKNOCK-*scd* plasmid was then mobilized into PA23 through triparental mating with the donor strain *E. coli* DH5αλpir containing pKNOCK-*scd* and the helper strain DH5α (pRK600). PIA supplemented with Tc (150µg/mL) was used to screen for transconjugants. To verify that pKNOCK-*scd* had correctly inserted into the *scd* gene, PCR analysis was performed using primers tet FWD and new ptrA TL start FWD. Sequence analysis of this fragment was performed to verify the correct fragment had been amplified.

2.5. Plasmid construction

To observe any phenotypic changes resulting from *scd* overexpression, pUCP23-*scd* was generated as follows: a 1.25-kb *scd* PCR fragment was amplified from PA23 genomic DNA using primers scd-pUCP FWD and scd-pUCP REV. The 1.25-kb fragment was cloned into pCR2.1 to yield pCR-*scd*. The pCR-*scd* plasmid was digested with *Hin*dIII and *Xba*I and the 1.25-kb fragment was cloned into the same sites of pUCP23 to yield pUCP23-*scd*. This overexpression plasmid was then electroporated into the PA23 wild type and derivative strains.

2.6. Antifungal assays

To study the AF activity of bacterial strains against *S. sclerotiorum*, radial diffusion assays were performed. Overnight cultures (5 µL) were spotted onto PDA plates and incubated at 28°C for 24 hours prior to the placement of a fungal mycelial plug in the centre of the agar. Plates were then incubated for 5 days at RT to allow for growth of *S. sclerotiorum*. Fungal inhibition was assessed by measuring the zone of clearing between the edge of the bacterial colony and the fungal growth front. Five replicates of each strain were analyzed and experiments were repeated three times.

2.7. Extracellular protease assays

To assess the ability of strains to produce extracellular proteases, 5 µL of an overnight culture was spotted onto 1.5% agar plates containing 2% skim milk. Following 24, 48 and 72 hours of incubation at 28°C, zones of proteolysis were measured from the edge of the bacterial colony to the edge of the zone of clearing. Three replicates of each strain were analyzed in triplicate experiments.

2.8. Quantitative analysis of phenazine

Production of PCA and 2-OH-PHZ was quantified according to the methods outlined by Chancey *et al*. (1999). Overnight cultures (5 mL) were grown in M9 minimal media and cell supernatants were collected and combined with 5 mL of benzene and one drop of concentrated HCl. Following one hour of shaking at RT, the top organic layer was removed and dried under air. Dried extracts were resuspended in 1 mL 0.1 M NaOH

and filtered to remove particulate matter. Spectrophotometric quantification was performed at 367nm and 490nm for PCA and 2-OH-PHZ, respectively, according to Maddula *et al.* (2008). To determine the relative amounts of PHZ present, absorption maxima were divided by their standard extinction coefficients (PCA: 3019 $M^{-1}cm^{-1}$; 2-OH-PHZ: 7943 $M^{-1}cm^{-1}$). Each strain was tested in triplicate.

2.9. High performance liquid chromatography pyrrolnitrin quantification analysis

Production of the antibiotic PRN was quantified according to the methods outlined in Selin *et al.* (2010). Briefly, 20 mL cultures of PA23 and its derivatives were grown for 5 days in M9 minimal media and PRN was extracted with an equal volume of ethyl acetate. Before extraction, toluene (5 mL) was added to each sample as an internal control. Toluene and PRN UV absorption maxima were recorded at 225nm with a Varian 335 diode array detector. PRN peaks were detected at 4.7 mins. Samples were analyzed in duplicate.

2.10. Motility analysis

Strains were assessed for flagellar (swimming) motility by inoculating 5 µL of an overnight culture grown in M9 minimal media into the center of a 0.3% M9 agar plate. Each strain was inoculated into five separate plates and the swim zone diameters were measured at 24 and 48 hours. The experiment was repeated three times.

2.11. Autoinducer analysis

The production of AHL was analyzed by spotting 5 µL of an overnight culture onto LB agar plates seeded with *C. violaceum* CV026. This strain is able to detect exogenous AHLs with carbon chain length structures ranging from C4 to C8, resulting in a purple halo surrounding the colonies (Latifi *et al*., 1995). The diameter of the purple zones was measured at 24 hours.

2.12. Chitinase analysis

Bacterial strains were analyzed for their ability to produce chitinase during early stationary and late stationary phases following the methods outlined by Wirth and Wolf (1990). Briefly, cultures were grown to the desired growth phase in M9 minimal media. A 250-µL aliquot of each of cell-free supernatant, 0.1 M NaOAc, pH 5.2 and carboxymethyl-chitin-Remazol brilliant violet aqueous solution (Blue Substrates, Göttingen, Germany) were incubated for 1 hour at 37°C. The reaction was stopped by the addition of 250 µL of 1 M HCl. Reaction mixtures were cooled on ice for 10 mins, spun at 20,000×g for 10 min, and the absorbances at 550nm were recorded. Each experiment was performed in triplicate.

2.13. Siderophore analysis

To analyze siderophore production, overnight cultures grown in M9 minimal media were spotted onto Chrome Azurol S (CAS) media according to the methods

outlined in Schwyn and Neilands (1987). The diameter of the yellow zone surrounding the colonies was measured following 24 hours incubation at 28°C.

2.14. Growth rate analysis

Cultures of wild-type PA23 and mutant PA23-443 were inoculated at a starting OD_{600} of 0.01 in M9 minimal media. OD_{600} readings were taken at 1 hour, 5 hours and 9 hours, followed by readings every 2 hours until 27 hours of growth had occurred. Triplicate samples were analyzed.

2.15. Semi-quantitative reverse transcriptase-PCR

To monitor expression of several genes involved in biocontrol, semi-quantitative reverse-transcriptase (RT) PCR was used. PA23 and its derivatives were grown to early stationary phase and total RNA was extracted using a RNeasy Mini Kit (QIAGEN, Valencia, USA). Residual genomic DNA was removed by treatment with TURBO RNAase-free DNAse I (Ambion, Carlsbad, USA) during the RNA isolation procedure. RNA concentrations were measured at 260 and 280 nm and only RNA samples with A_{260}/A_{280} between 1.8-2.0 were used in subsequent steps. cDNA was generated by reverse transcription using the Maxima First Strand cDNA Synthesis Kit (ThermoScientific, Rockford, USA) and random hexamer primers in a 20 µL total reaction volume. The following conditions were employed: initial heating at 25°C for 10 min, 50°C for 15 min for reverse transcription and 85°C for 5 min for enzyme

denaturation. Sequences for the genes of interest from PA23 were obtained from GenBank. The primer sequences are listed in Table 1. PCR was performed using a StepOnePlus Lightcycler system (Applied Biosystems, Carlsbad, USA) and its recommended SYBR® Green master mix (Applied Biosystems, Carlsbad, USA). The final 25-µL volume mixture contained 0.5 µL of both forward and reverse primers (12 µM), 2 µL of 1:40 diluted cDNA, 12.5 µL 2X SYBR® Green master mix (Applied Biosystems, Carlsbad, USA) and 9.5 µL nuclease-free water. PCR reaction conditions included an initial denaturation at 95°C for 3 min, followed by 45 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 seconds. Melt-curve analysis was performed to evaluate the formation of primer dimers and other artefacts to validate results. Each reaction was performed in triplicate and experiments were repeated three times with three biological replicates. Relative gene expression was calculated using the ΔΔCt method as described by Livak and Schmittgen (2001).

2.16. iTRAQ proteomic analysis

Wild-type PA23 and mutant PA23-443 cells were grown in duplicate samples to early stationary phase. Cultures were centrifuged at 10,000×g for 10 minutes at 4°C, and pellets were washed three times in PBS buffer and frozen at -80°C. Further sample preparation and isobaric tag for relative and absolute quantification (iTRAQ) labelling was carried out at the Manitoba Centre for Proteomics and Systems Biology. Briefly, 100 µg protein samples were mixed with 100 mM ammonium bicarbonate, reduced with 10 mM dithiothreitol (DTT) and incubated at 56°C for 40 min. Samples were then alkylated

with 50 mM iodoacetamide (IAA) for 30 min at RT in the dark. Addition of 17 mM DTT was used to quench excess IAA, and proteins were digested overnight with sequencing-grade trypsin (Promega, Madison, WI, USA). Dried samples were then desalted with 0.1% trifluoroacetic acid and subjected to two-dimensional high-performance liquid chromatography (2D-HPLC) - mass spectrometry (MS) according to the methods described in Rydzak *et al*. (2012).

2D-HPLC-MS/MS spectra data from three independent runs were analyzed using ProteinPilot (v2.0.1, Applied Biosystems/MDS Sciex, Concord, ON, Canada) which employs the Paragon™ algorithm. Searches were performed against the *P. chlororaphis* strain gp72 reference genome. Reporter ion iTRAQ tags were labelled as follows: tags 114 and 115 to replicates of wild-type PA23 grown to early stationary phase, and tags 116 and 117 to replicates of mutant PA23-443 grown to early stationary phase. Results were reported as Z-scores, the log2 of the ratio among replicates ($Z0=tag_{116}/tag_{114}$; $Z1=tag_{117}/tag_{115}$; $Z2=tag_{115}/tag_{114}$; $Z3=tag_{117}/tag_{116}$). Peptide Z-scores values were histogrammed (Z0, Z1) to determine the overall population distribution. Further statistical analysis was performed according to the methods outlined in Rydzak *et al*., (2012). Vector difference (V_{diff}) scores were assigned to allow the determination of statistical significance of protein expression ratios between both the wild-type and mutant samples while also taking into account the variation between biological replicates. Plotted Z-scores were transformed into vector values, allowing comparison between points (Z0,Z1) and (Z2,Z3). Differences between magnitudes of the vector values from the origin to points (Z0,Z1) and (Z2,Z3) were adjusted to the widths of the peptide population distributions. Direction of the vector values (+ or -) were assigned based on the angle

subtended by the vector value from the origin to point (Z0,Z1). A V_{diff} value greater than or equal to +1.65 and less than or equal to -1.65 corresponds to proteins expressed in the upper or lower 10% of the population distribution. $V_{diff} \geq +1.96$ or $V_{diff} \leq -1.96$ corresponds to proteins expressed in the upper or lower 5% of total population distribution. $V_{diff} \geq +2.58$ or $V_{diff} \leq -2.58$ corresponds to proteins expressed in the upper or lower 1% of total population distribution (Rydzak *et al*., 2012). Functional classification of proteins was carried out using the Integrated Microbial Genomes (IMG) database (http://img.jgi.doe.gov/cgi-bin/w/main.cgi) against the *P. chlororaphis* strain gp72 genome.

2.17. Lipopolysaccharide analysis

To analyze differences in outer membrane composition, the lipopolysaccharide (LPS) of PA23 and derivative strains was extracted according to the methods outlined in Hitchcock and Brown (1983). Briefly, 1 mL of overnight bacterial cultures was collected and centrifuged at 3,000×g for 5 minutes, and washed twice with 1 mL phosphate buffered saline (PBS). Cultures were diluted to an OD_{600} of 0.5 in 1 mL PBS, centrifuged at 3,000×g for 5 minutes and resuspended in 250 µL of Hitchcock and Brown lysis buffer (Appendix A). Samples were heated to 100°C for 30 minutes, and cooled to RT. A 1.5-µL aliquot of proteinase K (20 mg/mL) was added to the samples, which were incubated at 56°C for 2 hours to remove any proteins present. LPS was analyzed by SDS-PAGE according to the method of Hancock *et al*. (1983). Prepared LPS samples were loaded

into the stacking gel (Appendix A) and separated by the resolving gel (Appendix A) at a constant voltage of 100 V and 35 mA with 0.1% SDS-Tris-glycine buffer (pH 8.3).

Separated LPS components were visualized by the silver stain method outlined in Hitchcock and Brown (1983) with the following modifications: gels were fixed overnight in 60% (v/v) methanol and 10% (v/v) acetic acid, followed by soaking in 7.5% (v/v) acetic acid for 30 minutes, and washing with 0.2% periodic acid for 30 minutes. Gels were immersed in staining solution (Appendix A) for 20 minutes, washed several times with distilled water, followed by immersion in developer solution (Appendix A) until brown LPS bands appeared. The reaction was stopped with several washes of distilled water.

2.18. Bioinformatic analysis of *scd*

To determine potential function(s) of the *scd* gene, which is divergently transcribed from the *ptrA* promoter, predictive bioinformatics analysis was performed. The Scd amino acid sequence in *P. chlororaphis* PA23 was analyzed using ProtScale (ExPASY server) to predict highly hydrophobic and hydrophilic regions within the protein sequence. The ProSite (ExPASY server) program was also used to predict common signatures of short sequences of amino acids within the larger protein sequence. The reference sequence was subjected to additional analysis using the ExPASY template identification InterProScan function (EMBL-EBI Swiss Model Workspace server), allowing a putative function and secondary structure to be ascribed to the Scd amino acid sequence.

3. RESULTS AND DISCUSSION

3.1. Isolation of a PA23 mutant devoid of antifungal activity

In a previous Tn*5* mutagenesis screen one mutant was isolated, designated PA23-443, that had lost its ability to inhibit *S. sclerotiorum* (Poritsanos, 2005). Sequence analysis revealed the interrupted gene encoded an LTTR that was subsequently named PtrA (Pseudomonas transcriptional regulator A). In greenhouse assays, the *ptrA* mutant was unable to protect canola from SSR confirming that this regulator is essential for PA23 biocontrol (Poritsanos, 2005). The PA23 *ptrA* gene was PCR amplified and cloned into pUCP22 for PA23-443 complementation. The presence of *ptrA in trans* restored AF activity to that of the wild type, confirming that the mutant phenotype results from *ptrA* inactivation. Elucidating exactly how PtrA governs secondary metabolite production represents one of the major objectives of this thesis. In strain PA23, expression of AF factors is controlled by a complex network involving transcriptional and post-transcriptional regulation. To better understand how PtrA fits into this cascade, the *ptrA* mutant harbouring plasmid-borne copies of these other regulatory genes (*gacS, gacA, rpoS, rsmA, rsmE, rsmZ, psrA, phzR*) is included in many of the phenotypic assays described below.

Sequence analysis has revealed that the Tn*5*-OT182 insertion occurred 803 bp from the translational start (Figure 3). This insertion disrupts the co-inducer recognition/response domain of PtrA, a region of amino acid residues shown to be least conserved among LTTRs (Schell, 1993). Previous studies of the LTTRs NodD and NahR

5'-
GCGCACTTGGACRCRACTGCTGTACGCAGTCAGTCAGGTAGTAGACASTCGASACTYTTGCGCTCGTACGGCCTGACAG
GTGAGTCGRCAGTCTTSCTGSGATGCCAGSCTGCAGATGCAGCGCAGTCTCATCGATGTCGATCGCAGCTGAGTCGTGA
TCGTACATGACAGGTCGTCGCGGACGCACCATGCGTCAGGCGGTTTACAACGCGATATAGCGCAAGCCTGGACCCGTGA
GGTTAAACGGTGCCGTCGCTCAGTCGCTGCGCGAGCACCGAAATAGSGCAACCGCGCTGGACCTGGCCATCGACCGCAG
TTCCGCGGTCAGGTGCTGCACGCTGCCGGTCTTGCTGTCACGCAGCGCTGCGGCGCGACCCAGAGTTCGACATGGCGGT
TGTTGGCTTCGCTGCTGAGGTTGAAGCGGCCGTCGCCCAGTTGCTCTTCCAGGTAAGGCAGCGCCAGTGCCGGCTGGCC
GGCGCGTTCGATGCGCATGCCCTTGGCGCTGACATCGACGTTCCAGTCCGGAGCCTTGCCACTGGCCCGCAGGATCAGG
CGCGGGAAGTCGGGATCGCCGCAGGCATCGTTCGAGCGTTCGACGCGATACAACTGGCTCAGCGCCACTTCACTGTCGG
CGCCGCCGGCCTTGCTCGGGGAAAAGTGCCCGCGCAGGTCGGCGAACAGCGTGCCCTGCTGGTCGGCCAGGCTGGCCGC
TTCCTGGAGGATGCTGGTGCCGCCGTTGTCCTTGACCACATAACGCTGCTCGCCGACGCAAGGCTTGAACAGCAACTGG
CCGTCGGCGGCGGTCAGCGTGCCCTGCATGCGGGTCTGGCCGGCCTGGGACTGCGTCCGGGGCTGGCCATCGAACACCG
ACAGCATCTGGCAACCGGCAAACAGGGGGAGCAGGGCAATACAAACAAGGGAACGGGCGGCACGCATCGTCGGGTCTCC
AATCAAGTGCCGCCACGTTACGCAGCCTGACCGTTCATCACAACCCCGGATCCAATCGCGCCTCTGTAGGAGCCAGGCT
TGCCGGCGATGCAGGTACTGCGGTGTGACTGGCGTACCGCTATCGCGAGCAATCGAGCGTCGACCGGCTGCTCCTACAG
AAGATCCATGTGGGTCAGCCGACGATATAGGKCTGGCCGGTCTGCAGGCCTTCCACGCTCTTGGCGTAGGCCAGCGCCA
CGTCGGCGCCGGGAACCGGCTTATAGCCACGGAAGTACGGGGCGTAACTGTCCATGGCCTCCACCAGCACGGTCGGGCT
TACCGAGTTCACCCGCAGGCCGCGCGGCAACTCGATGGCGGCGGCGCGGACGAAACCGTCGATGGCGCCGTTGACCAGG
GCTGCCGAGGCGCCGCTGCGGATCGGGTCGCGGTTGAGGATGCCGGAGGTGAAGGTGAACGAGGCGCCGTCGTTGGCGA
ACTCGCGACCGATCAGCAGCAGGTTGACCTGGCCCATCAGCTTGTCCTGCAGGCCCGGGGCGAAGTGCTGTTCGCTCAT
CTCGCCCAGTGGCGCGAAGGTGACATTGCCGGCGGCGCAGATCAGCGCGTCGAATGTGCCGGTTTGCTCGAAGAGCGCG
CGGGTCGAGGCGCTGTCGCTGATATCCACTTGGAAATCACCGCTGGTACGGCCAATGCGGATGATTTCATGACGCTGCG
ACAGCTCTTTGTCGACCGCTGAACCTATGGTGCCGTTGGCGCCGATCAGAAGAATTTTCATGGGGCTGTTCCTCGCGTG
GGTTGAACGAGGTTGCAGTCTAGTGCTGGTTTTTCCCGTTGATTAGCGCACTAATAGGCAACCTTTGGTTTTCAAATGG
AAACAATCCGTGGGCGAGATGGATGACCTGGCGGCGTTCGCCGTGCTGATCGAGGCCGGCAGTTTCACCCTGGCCGCCC
AGCAATTGGGTTGCAGCAAGGGGCAATTGTCCAAGCGCATCAGCGCCCTGGAGGCGCAGTTCTCGGTCGTGCTGTTGCA
GCGCACCACGCGCCGCTTGAGCCTGACCGCGGCGGGCGCCGCCTTGTTGCCCCAGGCCCAGGCGCTGCTGGTCCAGGTC
GAGCGGGCGCGCCAGGCGCTGGCGCGGCTCAAGGACGACATGGCCGGGCCGGTGCGGCTGACGGTGCCGGTGTCCCTGG
GGGAGACCTTTTTCGAGGGCCTGTTGCTGGAGTTTTCCCGGCAGTATCCCGAGGTGCAGATCGAGCTGGAGCTGAACAA
CAACTACCGCGACCTGACCCGGGACGGCTTCGACCTGGCGATCCGCTCGGAGGTGGCCAACGACCAGCGGCTGGTGGCC
AGGCCGCTGCTGGCCTGGCACGAGATGACCTGCGCCAGCCCGGCTTACCTGGAGCAATATGGCGAGCCGCAGACGCCCA
GGGACCTGGCCGAGCATCGCTGCCTGCTCAACAGTCATTACAGCGGTCGTGAGGAGTGGCTCTATCACCAGCAGCACGA
GCTGTTGCGGGTGCGGGTGTCGGGGCCCTTCGCCAGCAATCACTACAACCTGCTGAAAAAGGCCGCGCTGGTGGGCGCC
GGGATCGCCCGACTGCCGTCCTACCTGTTGCAGGCGGAACTGGCCGATGGGCGTTTGCGCGGGCTGCTGCGCGACTACC
AGACCCGCAGCATGCCGATGTACCTGGTGCA{Tn5}CCCGTATCAGTATCAGGGCGGGCTGCCCAGGCGCACCCAGGTC
CTGGCCGATTACCTGATCGGCTGGTTCAAGCGCAGCGGCGAAGCGCTGGATCGCCTCTGACTTGATGCTGCGATGGACC
TTGTAGCCGCTGCGGCAGGCTGCGCAGGGCCCGAAGGGCCTCGCGATCTTGAGACCTTGCGCGTCCTTCGGACCCGATC
GCAGCCTGCGGCAGCGGCCACAGCTACAGCGGTGGGCGATCAGATCTGTAGGAGCGAAGCTTGCTCGCGATGGCGGCAC
CGCGGGCTGTCTGAAAAACCGCGTTATCGTTCATCGCGAGCAAGTAGCGCTCCTACAGGGACAGAGGGTCAAGGTCGCT
GGTAGCGGCGGGCGATCAGGTGATCGATGGAGAGCTTGCCGGGGCCGCGGGCCATCAGGTACAGCAGGACCGCCGCCCA
GGTGCCGTGGGTCGGGTAGGCATCGGGGTAGACGAACAGCTGGATGGTCAAGGTCATGCCCAGCAGCGCCAGGGCCGAG
AAGCGCGTGGCGAAACCGACCAGGAGCAGCAGCGGGAACAGATGCTCGGCGAATGCCGCCAGGTGCGCGGCCAGTTCGG
GGGAGAGCAGCGGTACCCGGTACTCGCTCTGGAACAAGGGAATGGTCGAGTCGGCCAGGCGCGGCATGCCGAGCTCGAA
CGTCCCGGAGAACAGATCGATGGCCAGGCCCTCGACCTTGGTCTGGCCGGATTTCCAGAACACCGCGGCAATCGACAAG
CGCGCGATAAAGGCGATCAGGCTGTGGGGAATACGCTCCAGCAACTGGATGAGTCGGTG-3'

Figure 3. DNA sequence of the *ptrA* region (grey) and *scd* region (green). The PtrA translational start and stop codons are underlined sequences. The Tn*5* insertion occurred 803 bp from the translational start (highlighted in blue).

revealed that mutations in this region result in a co-inducer-independent phenotype that affects DNA binding and thus the activation/repression properties of the proteins (Maddocks and Oyston, 2008; Schell, 1993).

Antifungal assays have revealed that the PA23-443 mutant no longer exhibits AF activity, but providing *ptrA in trans* results in full complementation of AF activity (Table 2). The only other regulatory gene capable of partially restoring AF activity was *gacS* (Table 2), indicating a regulatory link between PtrA and the Gac system.

3.2. Identification of a gene encoding a short-chain dehydrogenase upstream of *ptrA*

As mentioned earlier, LTTR proteins frequently regulate genes upstream of and divergently transcribed from the LTTR gene itself. Sequence analysis revealed an open reading frame immediately upstream of *ptrA* but in the opposite orientation (Figure 3). Because this gene is predicted to encode a short-chain dehydrogenase it was designated *scd*. To determine if *scd* plays a role in PtrA-mediated regulation, an *scd* mutant was generated through plasmid insertion using the pKNOCK system (Figure 4), generating PA23*Δscd*. Sequencing confirmed that the scd-KNOCK suicide vector inserted into the *scd* coding region. Figure 4 depicts a schematic representation of the insertion event.

Phenotypic assays were also conducted with PA23*Δscd* to determine the role of Scd, if any, in regulating production of AF metabolites. Antifungal assays revealed that the PA23*Δscd* mutant showed no change in AF activity from wild-type PA23 (Table 2).

Table 2. Results of antifungal radial diffusion assays of *Pseudomonas chlororaphis* PA23 and mutants PA23-443 and PA23*Δscd* harbouring various overexpression plasmids grown in LB media or M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose following 48 hours incubation at room temperature with *Sclerotinia sclerotiorum*.

Strain	**Zone of clearing[a]**	
	LB	**M9**
PA23 (pUCP22)	8.4 (0.6)	8.2 (0.4)
PA23-443 (pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23-443 (*rsmA*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23-443 (*rsmE*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23-443 (*rsmZ*-pUCP23)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23-443 (*rpoS*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23-443 (*psrA*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23-443 (*ptrA*-pUCP22)	8.8 (0.8)[d]	6.8 (0.4)[c]
PA23-443 (*phzR*-pUCP23)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23-443 (*gacA*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23-443 (*gacS*-pUCP23)	4.9 (0.7)[b]	4.8 (0.4)[b]
PA23-443 (*scd*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23*Δscd* (pUCP22)	7.9 (0.3)[d]	7.6 (0.3)[d]
PA23*Δscd* (*scd*-pUCP22)	7.8 (0.4)[d]	7.9 (0.2)[d]

[a]Mean (standard deviation) of zone of clearing between edge of bacterial colony and fungal growth front (mm) from five replicates.

[b]Significantly different from wild type ($P<0.0001$).

[c]Significantly different from wild type ($P<0.01$).

[d]Not significantly from wild type.

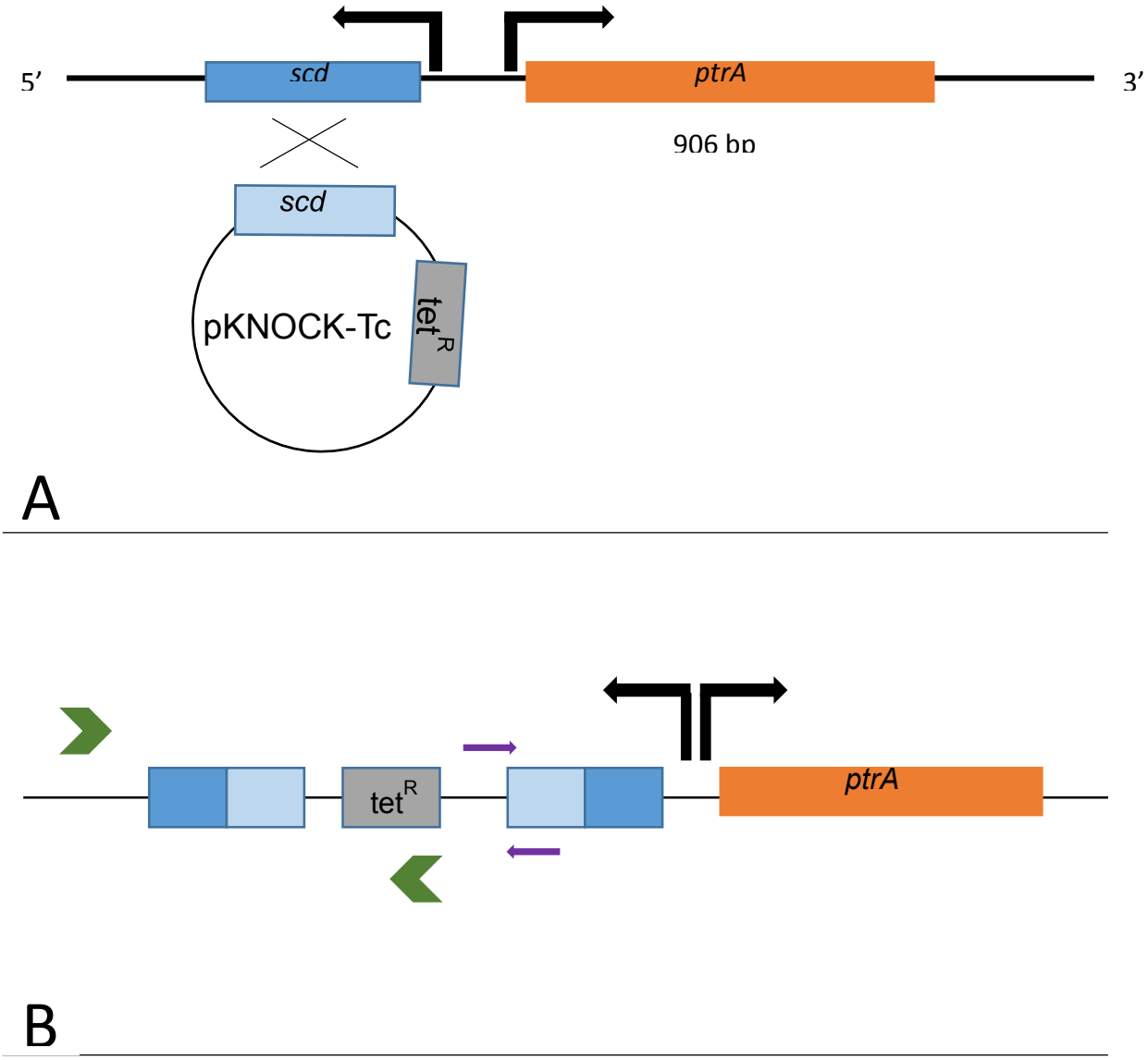

Figure 4. A, schematic representation of *scd*-pKNOCK-Tc allelic exchange into the *scd* region of the PA23 genome. B, *scd* genetic region of genome amplified by primers tet FWD and new ptrA TL start FWD (indicated by green arrows) used to verify the correct insertion. Purple arrows indicate primers R6Kori-3 FWD and scdBamHI-3 REV (Table 1) used in qRT-PCR analysis of *scd* gene expression in PA23*Δscd*. Black arrows indicate predicted transcriptional start sites of genes.

3.3. Differential protein expression between the PA23 wild type and the *ptrA* mutant

PtrA belongs to the LTTR family, which is the largest known family of prokaryotic DNA binding proteins (Maddocks and Oyston, 2008). LTTRs can function as either repressors or activators for single or operonic genes. Furthermore, these regulators may be divergently transcribed from their target genes or may control expression of numerous genes scattered about the chromosome (Maddocks and Oyston, 2008). In PA23 expression of AF metabolites is governed by a complex network of regulatory elements and substantial interaction occurs between the regulators themselves (Poritsanos *et al*. 2006, Manuel *et al*., 2011; Selin *et al*., 2012). We were interested, therefore, in determining the global impact of the *ptrA* mutation on PA23 physiology. iTRAQ proteomic analysis was carried out to highlight proteins that were differentially expressed in the PA23 wild type and *ptrA* mutant. A total of 771 proteins were matched to proteins found within the *P. chlororaphis* gp72 reference genome; 59 of which showed significant differences in expression between the two strains (Table 3). The 59 proteins could be classified into 16 clusters of orthologous groups (COGs) based on their predicted function. Figure 5 summarizes classification of the identified proteins, indicating significant up- or downregulation of protein expression. The largest COG category was the unknown function group, suggesting that many yet-to-be-identified proteins play a role in the loss of biocontrol exhibited by PA23-443.

Table 3. List of significant differentially expressed proteins in mutant PA23-443, $V_{diff} \geq +1.65$; $V_{diff} \leq -1.65$.

COG Category	Locus Tag	Predicted Function	Fold Change[a]	V_{diff} Score
Amino acid transport and metabolism	MOK_00491	4-aminobutyrate aminotransferase and related aminotransferases	1.59	2.24
	MOK_03651	Monoamine oxidase	-2.39	-2.7
	MOK_04019	ornithine carbamoyltransferase	-1.48	-1.67
Nucleotide transport and metabolism	MOK_04929	hypothetical protein	-3.13	-2.54
Carbohydrate transport and metabolism	MOK_03378	Chitinase	-3.30	-3.76
	MOK_05029	Glucose/sorbosone dehydrogenases	-1.68	-2.04
	MOK_05478	Chitinase	-2.61	-1.66
Lipid transport and metabolism	MOK_04573	Acyl dehydratase	-2.16	-2.42
Translation, ribosomal structure and biogenesis	MOK_00565	Translation elongation factor P (EF-P)/translation initiation factor 5A (eIF-5A)	1.61	1.94
	MOK_01324	ribosomal protein L32	2.33	2.77
	MOK_02337	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase, C subunit	2.09	1.7
	MOK_04471	ribosomal protein S19, bacterial/organelle	1.49	1.7
Transcription	MOK_02056	cold shock domain protein CspD	-2.31	-1.81

	MOK_02888	Cold shock proteins	2.30	2.44
	MOK_03359	Cold shock proteins	1.26	1.65
Replication, recombination and repair	MOK_00606	competence protein ComEA helix-hairpin-helix repeat region	-2.78	-3.04
Cell wall, membrane and envelope biogenesis	MOK_05137	Outer membrane protein and related peptidoglycan-associated (lipo)proteins	-1.65	-1.79
Cell motility	MOK_01499	Flagellin and related hook-associated proteins	2.71	3.26
Post-translational modification, protein turnover and chaperones	MOK_00750	monothiol glutaredoxin, Grx4 family	1.20	1.81
	MOK_01830	peroxiredoxin, OsmC subfamily	-2.61	-2.69
	MOK_05742	Peroxiredoxin	-1.84	-1.78
	MOK_05953	Peptidyl-prolyl cis-trans isomerase (rotamase) - cyclophilin family	2.00	1.73
Inorganic ion transport and metabolism	MOK_05447	Predicted periplasmic lipoprotein involved in iron transport	1.42	1.73
Secondary metabolites biosynthesis, transport and catabolism	MOK_01048	Phenazine biosynthesis protein A/B.	-4.22	-4.58
	MOK_01049	Phenazine biosynthesis protein A/B.	-3.25	-4.26

	MOK_01053	phenazine biosynthesis protein PhzF family	-1.19	-2.1
	MOK_01054	Pyridoxamine-phosphate oxidase	-1.25	-2.18
	MOK_01055	Aromatic ring hydroxylase	-2.45	-2.43
General function prediction only	MOK_01152	Predicted periplasmic or secreted lipoprotein	-2.29	-2.42
	MOK_02985	intracellular protease, PfpI family	1.67	1.93
	MOK_03813	Predicted O-methyltransferase	-2.12	-1.73
	MOK_05714	Serine protease inhibitor ecotin	-1.33	-1.65
Function unknown	MOK_00258	Protein of unknown function (DUF3313).	-1.81	-2.03
	MOK_00808	hypothetical protein	-8.28	-7.73
	MOK_01097	hypothetical protein	-2.10	-2.22
	MOK_01302	hypothetical protein	-1.32	-2.08
	MOK_01398	hypothetical protein	-2.04	-2.19
	MOK_01832	Protein of unknown function (DUF1161).	-1.14	-1.94
	MOK_02425	Sigma 54 modulation protein / S30EA ribosomal protein.	1.36	2.22
	MOK_02468	poly(hydroxyalkanoate) granule-associated protein	-2.70	-3.66
	MOK_02469	poly(hydroxyalkanoate) granule-associated protein	-1.75	-2.32
	MOK_03057	Uncharacterized protein conserved in bacteria	-1.86	-2.29
	MOK_03064	type VI secretion protein, VC_A0107 family	-2.87	-3.14

	MOK_03065	type VI secretion protein, EvpB/VC_A0108 family	-2.72	-3.02
	MOK_03231	outer membrane porin, OprD family.	1.49	1.8
	MOK_03379	Uncharacterized protein conserved in bacteria	-4.52	-5.06
	MOK_03717	hypothetical protein	-5.36	-6.81
	MOK_03859	hypothetical protein	-2.60	-2.27
	MOK_04005	Protein of unknown function (DUF3613).	-2.39	-2.06
	MOK_04318	Predicted integral membrane protein	-1.80	-2.21
	MOK_04378	Putative phospholipid-binding domain./LysM domain.	-2.22	-3.47
	MOK_04746	hypothetical protein	-2.29	-2.71
	MOK_04755	hypothetical protein	-3.36	-3.84
	MOK_05477	Uncharacterized protein conserved in bacteria	-2.09	-1.41
	MOK_05648	hypothetical protein	-4.51	-4.7
	MOK_05758	hypothetical protein	-4.00	-4.19
	MOK_06084	Iron-sulfur cluster assembly accessory protein	1.72	1.73
	MOK_06136	hypothetical protein	-5.20	-5.37
Signal transduction mechanisms	MOK_04087	Putative Ser protein kinase	-1.38	-2.06

[a] $\log_2(tag_{115}/tag_{117})$

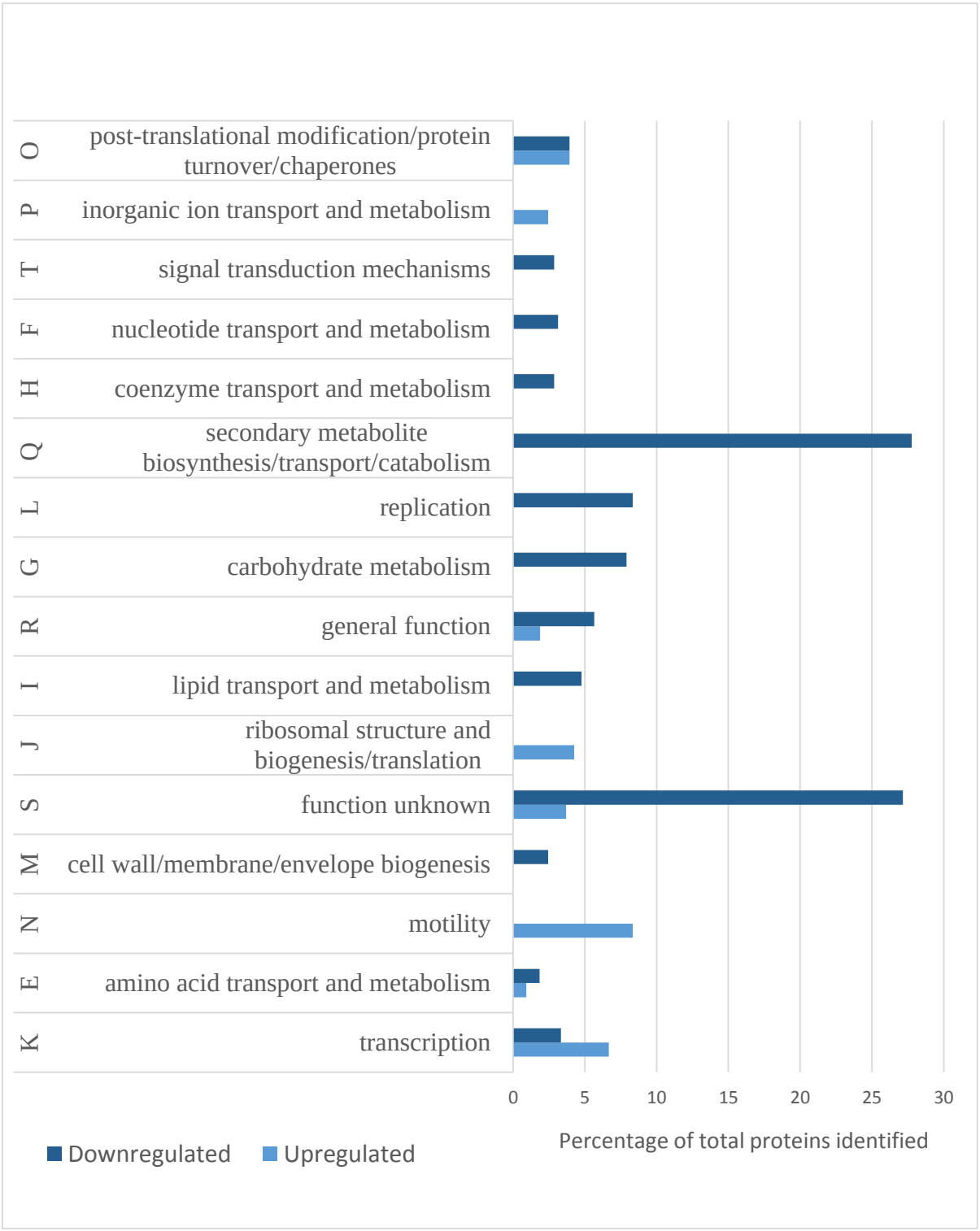

Figure 5. Differentially expressed proteins in mutant PA23-443 compared to the wild type. Fifty-nine proteins were found to be differentially regulated and they were classified into 16 clusters of orthologous groups based on their predicted function.

3.4. PtrA regulates phenazine production in PA23

The secondary metabolite biosynthesis, transport and catabolism COG category represented the next largest grouping (Table 3). Initially, two of the proteins (MOK_01048, MOK_01053) were classified under the general function category and one protein (MOK_01054) was categorized under the transport and metabolism category. Upon further investigation, the locus tags indicated that they are part of the PHZ biosynthetic operon, leading to their reclassification into the secondary metabolite biosynthesis COG category.

The PHZ operon has been well characterized in many pseudomonads, with *phzABCDEFG* comprising the core biosynthetic locus (Mentel *et al*., 2009). In this study, proteins with locus tags MOK_01048 and MOK_01049, identified as PHZ biosynthesis protein A/B, were significantly downregulated, as indicated in Table 3. All PHZ-producing pseudomonads have an adjacent and nearly identical copy of the *phzB* gene, termed *phzA* (Mentel *et al*., 2009). PhzA catalyzes the condensation reaction of two ketone molecules in the PHZ biosynthesis pathway (Mentel *et al*., 2009). PhzF (identified as MOK_01053 in this study) works as an isomerase, converting *trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHHA) into 6-amino-5-oxocyclohex-2-ene-1-carboxylic acid prior to the condensation reaction catalyzed by the PhzA/B proteins (Mentel *et al*., 2009). *phzG* encodes an FMN-dependent pyridoxamine oxidase (identified as MOK_01054 in this study). PhzG is hypothesized to catalyze the conversion of DHHA to 5,10-Dihydro-PCA (Pierson et al., 1995). In some pseudomonads, genes downstream of the core biosynthetic operon are required for

generation of PHZ derivatives (Mavrodi et al., 2001; Delaney et al., 2001; Chin-A-Woeng et al., 2001). In *P. chlororaphis* 30-84, for example, *phzO* lies downstream of the core operon; PhzO is an aromatic hydroxylase that catalyzes the conversion of PCA into 2-OH-PHZ (Delaney et al., 2001). More recently, in *P. chlororaphis* gp72, the *phzO* gene was shown to convert PCA into 2-OH-PHZ through a 2-OH-PCA intermediate (Huang et al. 2010). Like other *P. chlororaphis* strains, PA23 produces 2-OH-PHZ and we believe the downregulated aromatic ring hydroxylase (MOK_01055) is PhzO. Therefore, in the absence of a functional *ptrA* gene, four of the core PHZ biosynthetic enzymes (PhzA, PhzB, PhzF, PhzG) and one aromatic ring hydroxylase (PhzO) are significantly downregulated. The fact that PtrA plays a critical role in regulating *phz* expression was not surprising considering the lack of orange pigment produced by the *ptrA* mutant (Figure 6). Reduced PHZ expression was further substantiated by quantitative assays. Table 4 indicates that there is a 15-fold decrease in PHZ production in the mutant PA23-443 compared to PA23 wild-type. When *ptrA* was expressed *in trans*, some restoration of PHZ production was achieved. Moreover, the presence of *gacS* but not *gacA* leads to a 2-fold increase in PHZ production in PA23-443 (Table 4). Both plate and broth cultures showed similar results; only *gacS* was able to partially restore PHZ production by the mutant (Figure 6 and Table 4). Collectively, our results show that the sensor kinase GacS is able to complement the PtrA deficiency for PHZ production to some degree. Table 4 indicates that when *rpoS* and *psrA* were overexpressed in the PA23-443 mutant background, a further 2.2- and 1.5-fold decrease in total PHZ production, respectively, was observed when compared to PA23-443 (empty vector). As RpoS and PsrA both have negative effects on PA23 AF activity, these

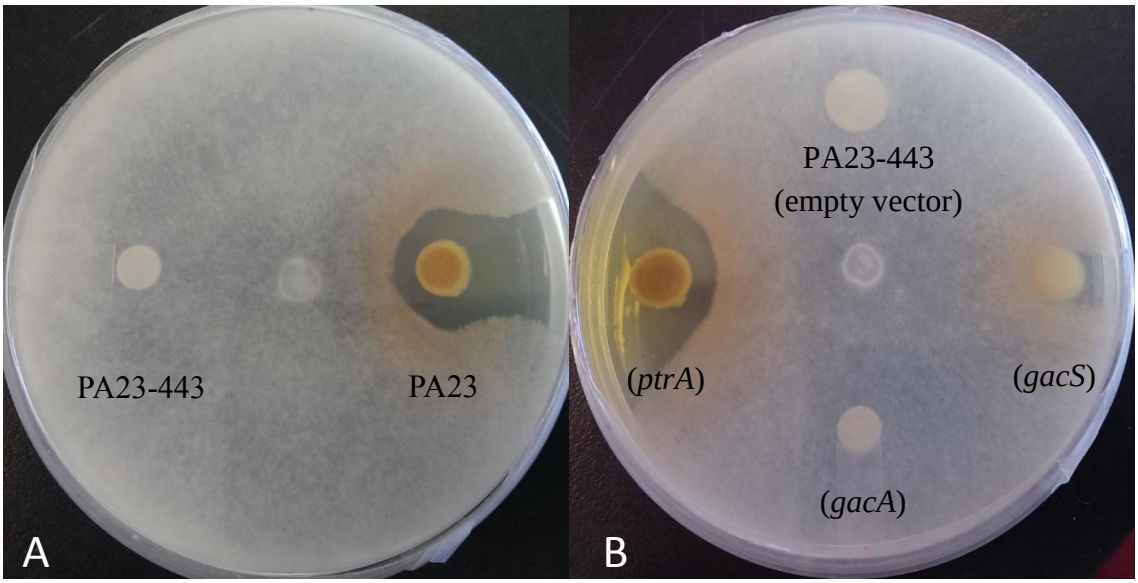

Figure 6. Antifungal activity of PA23 and derivative strains against *Sclerotinia sclerotiorum*. Panel A: the PA23-443 mutant and PA23 wild type are shown. Panel B: Antifungal activity of PA23-443 harbouring either empty vector, *ptrA*, *gacS* or *gacA in trans*. Note that the presence of plasmid-borne *ptrA* and *gacS* is able to fully and partially restore, respectively, antifungal activity in PA23-443.

Table 4. Quantification of phenazines present in overnight cultures of *Pseudomonas chlororaphis* PA23 and mutants PA23-443 and PA23*Δscd* harbouring various overexpression plasmids grown in M9 minimal media supplemented with 1mM $MgSO_4$ and 0.2% glucose at 28°C.

Strain	**[PCA][a]**	**[2-OH-PHZ][a]**	**[Total Phenazine][a]**
PA23 (pUCP22)	65.46 (10.30)	11.04 (2.18)	76.49 (12.47)
PA23-443 (pUCP22)	11.04 (2.30)[b]	0.98 (0.20)[c]	12.02 (2.49)[b]
PA23-443 (*ptrA*-pUCP22)	38.24 (4.73)[d]	5.11 (0.91)[d]	43.36 (5.60)[d]
PA23-443 (*gacA*-pUCP22)	3.42 (3.25)[b]	0.45 (0.44)[b]	3.86 (5.52)[b]
PA23-443 (*gacS*-pUCP23)	25.85 (5.91)[b]	3.92 (1.07)[b]	29.71 (6.78)[b]
PA23-443 (*psrA*-pUCP22)	6.81 (1.83)[b]	0.70 (0.29)[c]	7.52 (2.10)[b]
PA23-443 (*rpoS*-pUCP22)	4.98 (1.36)[b]	0.50 (0.21)[c]	5.48 (1.56)[b]
PA23-443 (*phzR*-pUCP23)	10.42 (3.77)[b]	1.03 (0.48)[c]	11.46 (4.24)[b]
PA23-443 (*rsmA*-pUCP22)	0.94 (0.82)[b]	0.02 (0.03)[b]	0.96 (0.79)[b]
PA23-443 (*rsmE*-pUCP22)	11.42 (2.80)[b]	1.33 (0.42)[c]	12.75 (3.22)[b]
PA23-443 (*rsmZ*-pUCP23)	11.70 (2.92)[b]	1.07 (0.34)[c]	12.77 (3.25)[b]
PA23-443 (*scd*-pUCP22)	10.10 (5.91)[c]	0.80 (0.35)[c]	10.90 (6.24)[c]
PA23 *Δscd* (pUCP22)	77.47 (5.66)[e]	13.89 (0.60)[e]	91.35 (5.02)[e]
PA23 *Δscd* (*scd*-pUCP22)	32.25 (8.06)[c]	4.41 (1.26)[c]	36.70 (9.18)[c]

[a]Mean (standard deviation) of concentrations of PCA, 2-OH-PHZ and total phenazine (μg/mL) from three replicates.

[b]Significantly different from wild type ($P<0.001$).

[c]Significantly different from wild type ($P<0.01$).

[d]Significantly different from wild type ($P<0.05$).

[e]Not significantly different from wild type.

findings are not surprising (Manuel *et al.*, 2011; Selin *et al*., 2014). When *rsmA* was overexpressed in the PA23-443 mutant background, a 12.5-fold decrease in PHZ production was noted (Table 4) compared to PA23-443 (empty vector). RsmA acts as a translational repressor; therefore, overexpression of RsmA likely decreases expression of the PHZ operon, resulting in a further decrease in PHZ compared to the the *ptrA* mutant alone. Interestingly, in the PA23*Δscd* mutant background, slight increases in PCA, 2-OH-PHZ and total PHZ was seen compared to PA23 wild type. When *scd* was expressed *in trans* in the same background, PHZ production decreased by approximately 2-fold compared to wild-type PA23 (empty vector).

3.5. Chitinase production is under PtrA control

Our iTRAQ proteomic results showed that two chitinase enzymes (MOK_03378 and MOK_05478) were significantly downregulated in the PA23-443 mutant (Table 2). These results were supported by chitinase assays conducted *in vitro*, which clearly indicated no enzyme activity detectable in the *ptrA* mutant (Table 5). Mutant PA23-443 harbouring plasmid-borne *ptrA* exhibited slightly lower chitinase activity compared to the wild type during early and late stationary phases. For PA23-443 expressing *gacS in trans*, chitinase activity was restored to 50% that of the wild type by late stationary phase. Conversely, no change in chitinase activity resulted from addition of *gacA* (Table 5). These results indicate that *ptrA* is necessary for proper production of chitinase, and *gacS* is able to partially complement the mutant for chitinase production. As chitin composes a large part of fungal cell walls, the dramatic decrease in chitinase activity may be

Table 5. Chitinase activity of *Pseudomonas chlororaphis* PA23, Tn*5* mutant PA23-443 and Tn*5* mutant harbouring *ptrA*, *gacA* and *gacS in trans* grown in M9 minimal media supplemented with 1mM $MgSO_4$ and 0.2% glucose at 28°C.

Strain	Chitinase Activity (A_{550}*min^{-1}*mg total $protein^{-1}$)	
	Early stationary phase[a]	Late stationary phase[a]
PA23 (pUCP22)	0.11 (0.03)	0.12 (0.004)
PA23-443 (pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[c]
PA23-443 (*ptrA*-pUCP22)	0.10 (0.03)[d]	0.11 (0.01)[e]
PA23-443 (*gacA*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[c]
PA23-443 (*gacS*-pUCP23)	0.03 (0.02)[e]	0.06 (0.01)[f]

[a]Mean (standard deviation) of enzyme activity of three replicates.
[b]Significantly different from wild type ($P<0.005$).
[c]Significantly different from wild type ($P<0.0001$).
[d]Not significantly different from wild type.
[e]Significantly different from wild type ($P<0.05$).
[f]Significantly different from wild type ($P<0.0005$).

contributing to a loss of antifungal activity in the PA23-443 mutant. LTTR regulation of chitinases has been previously shown, where ChiR indirectly regulated all chitinases produced in *Serratia marcescens* 2170 (Suzuki et al., 2001). Proteomic analysis of a *P. aeruginosa gacA* mutant revealed that chitinase (ChiC) and a chitin-binding protein (CbpD) were decreased 8-fold and 2.2-fold respectively, as compared to the wild type (Kay *et al*., 2006).

3.6. Siderophore production is upregulated in PA23-443 compared to the PA23 wild type

In the *ptrA* mutant, a lipoprotein involved in iron transport (MOK_05447) was found to be significantly upregulated (Table 3). This finding prompted us to explore whether the mutant exhibited elevated siderophore expression. Siderophores are believed to contribute to biocontrol by sequestering iron, thereby restricting pathogen growth. Following 24 hours growth on CAS agar plates, mutant PA23-443 showed a 3-fold increase in siderophore production compared to the wild type (Table 6). Overexpression of *ptrA* and *gacS* restored the wild-type phenotype, whereas *gacA* overexpression modestly decreased the size of the halo. Siderophore production has been shown to be under the negative control of the Gac system in many fluorescent pseudomonads including PA23 (Chancey *et al*., 1999; Hassan *et al*., 2010; Poritsanos *et al*., 2006). A PA23 *gacS* mutant exhibited a halo 2.5 times larger than the wild type on CAS agar plates (Poritsanos et al., 2006). Since both *ptrA* and *gacS* mutants express significantly increased levels of siderophore but exhibit a complete loss of AF activity, it is clear that

Table 6. Siderophore production in *Pseudomonas chlororaphis* PA23, Tn*5* mutant PA23-443 and Tn*5* mutant harbouring *ptrA*, *gacA* and *gacS in trans* grown in M9 minimal media supplemented with 1mM $MgSO_4$ and 0.2% glucose following 24 hour incubation on CAS agar at 28°C.

Strain	**Zone of orange halo[a]**
PA23 (pUCP22)	0.5 (0.0)
PA23-443 (pUCP22)	1.6 (0.2)[b]
PA23-443 (*ptrA*-pUCP22)	0.6 (0.2)[c]
PA23-443 *(gacA*-pUCP22)	0.8 (0.3)[d]
PA23-443 (*gacS*-pUCP23)	0.5 (0.0)[c]

[a]Mean (standard deviation) of size of orange haloes (mm) surrounding colonies from five replicates.

[b]Significantly different from wild type ($P<0.0001$).

[c]Not significantly different from wild type.

[d]Significantly different from wild type ($P<0.05$).

elevated siderophore expression alone is not sufficient for *S. sclerotiorum* control (Poritsanos *et al*., 2006).

3.7. Loss of *ptrA* results in early entry into stationary phase

We observed significant upregulation of proteins involved in translation, ribosomal structure and biogenesis in the *ptrA* mutant (Table 3). These proteins include a translation elongation factor (MOK_00565), a tRNA amidotransferase (MOK_02337) and ribosomal proteins L32 and S19 (MOK_01324 and MOK_04471, respectively), which make up structural components of both the large and small ribosomal subunits of the 70S ribonucleoprotein complex (Lecompte et al., 2002) (Table 3). To determine whether PA23-443 exhibited an altered pattern of growth compared to the wild type, growth rate analysis was undertaken. As depicted in Figure 7, the mutant begins the logarithmic (log) growth phase around hour 8 with growth starting to plateau around hour 13. Conversely, the PA23 wild type does not enter log phase until hour 11, ending with entrance into early stationary phase after 19 hours of growth. Another interesting difference observed was the maximum population density achieved. The PA23 wild type consistently reaches a higher OD_{600} in stationary phase compared to PA23-443 (Figure 7). LTTRs have previously been implicated in the regulation of cellular growth factors. For example, the well-studied LTTR OxyR has been shown to be involved in regulating the expression of various metabolic genes such as tRNA nucleotidyl transferases and synthetases, ribosomal proteins in addition to QS regulated targets (Wei et al., 2012). We have previously reported that a PA23 *gacS* mutant has a shorter lag phase and earlier

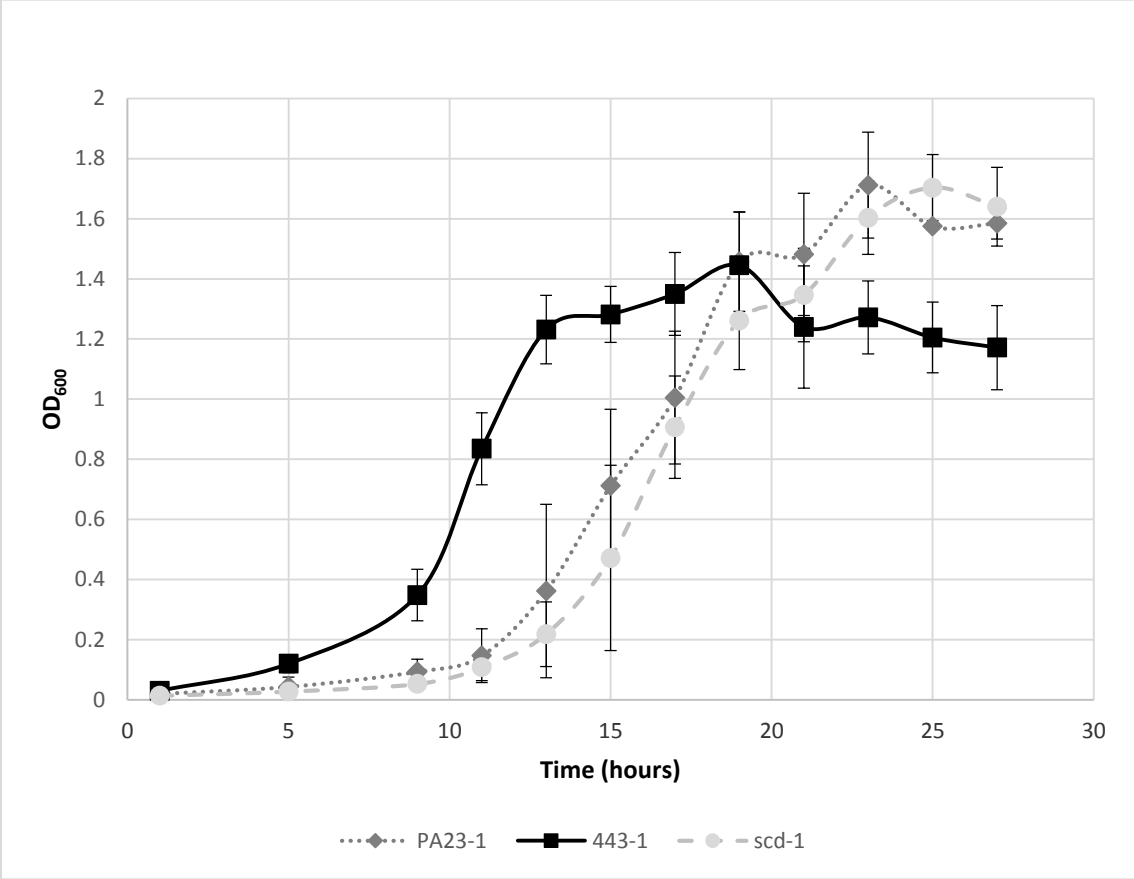

Figure 7. Growth curve analysis of wild-type PA23, mutant PA23-443 and PA23-Δ*scd*. Cells were grown in M9 minimal media supplemented with 1mM $MgSO_4$ and 0.2% glucose. Spectrophotometric optical densities were taken at 600 nm from three replicates.

entry into logarithmic growth phase (Poritsanos et al., 2006), similar to *P. chlororaphis* 30-84 (Driscoll et al., 2011).

3.8 PtrA negatively affects motility

To verify the iTRAQ proteomic data indicating upregulation of flagellin and related hook-associated protein (MOK_01499) in PA23-443, motility assays were conducted. The data revealed an increase in swimming (flagellar) motility in the mutant PA23-443 strain in comparison to the wild type, indicating that PtrA may be acting as a repressor of motility (Table 7). The Gac/Rsm system has previously been shown to negatively regulate swimming motility in *E. coli* and some fluorescent pseudomonads (Martínez-Granero et al., 2006; Martínez-Granero et al., 2012; Lapouge et al., 2008; Wei et al., 2001); however the exact regulatory pathway is still unclear. Proteomic analysis of a *P. aeruginosa gacA* mutant revealed a 7.5-fold and 8.8-fold increase in expression of a flagellin (FliC) and flagellar-capping protein (FliD), respectively (Kay *et al*., 2006). Overexpression of *gacA* in the *ptrA* mutant background almost doubled the motility, further substantiating a role for *gacA* in swimming motility. Surprisingly, *ptrA* expression *in trans* did not restore the wild-type phenotype, as expected. This may be due to the fact that multiple copies of the *ptrA* gene are expressed, rather than one single chromosomal copy. As LTTRs bind both activation binding sites (ABS) and regulatory binding sites (RBS) upstream of target genes (Maddocks and Oyston, 2008), the number of copies of the transcriptional regulator present within the cell may be of critical importance for proper binding, bending and subsequent regulation of target genes. This observation was also noted with complementation studies of the LTTR, OxyR, in restoration of

Table 7. Motility analysis of *Pseudomonas chlororaphis* PA23 and mutants PA23-443 and PA23*Δscd* harbouring various overexpression plasmids grown in M9 minimal media supplemented with 1mM $MgSO_4$ and 0.2% glucose following 24 and 48 hours incubation at 28°C.

Strain	**Motility zone diameter[a]**	
	24h	**48h**
PA23 (pUCP22)	10.3 (0.5)	17.0 (0.0)
PA23-443 (pUCP22)	15.3 (0.4)[b]	47.5 (0.5)[b]
PA23-443 (*rsmA*-pUCP22)	9.8 (0.4)[c]	31.5 (3.2)[b]
PA23-443 (*rsmE*-pUCP22)	8.3 (0.4)[d]	29.5 (1.8)[b]
PA23-443 (*rsmZ*-pUCP23)	10.3 (0.4)[c]	23.5 (3.5)[e]
PA23-443 (*rpoS*-pUCP22)	5.8 (0.4)[b]	19.7 (2.1)[c]
PA23-443 (*psrA*-pUCP22)	7.0 (0.7)[d]	25.5 (1.5)[b]
PA23-443 (*ptrA*-pUCP22)	25.3 (0.8)[b]	43.8 (1.6)[b]
PA23-443 (*phzR*-pUCP23)	19.0 (0.0)[b]	45.8 (0.8)[b]
PA23-443 (*gacA*-pUCP22)	47.8 (0.8)[b]	83.0 (0.0)[b]
PA23-443 (*gacS*-pUCP23)	18.8 (0.4)[b]	41.3 (0.8)[b]
PA23-443 (*scd*-pUCP22)	22.8 (0.4)[b]	51.0 (0.7)[b]
PA23*Δscd* (pUCP22)	10.2 (0.2)[c]	17.4 (0.3)[c]
PA23*Δscd* (*scd*-pUCP22)	10.5 (0.5)[c]	18.0 (0.8)[c]

[a]Mean (standard deviation) of swim zone (mm) from five replicates.

[b]Significantly different from wild type ($P<0.0001$).

[c]Not significantly different from wild type.

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

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

rhamnolipid and PYO production in *P. aeruginosa* (Vinckx *et al.,* 2010). When *oxyR* was present in multiple copies in the cell, restoration of the wild-type phenotype was not observed, whereas when one single chromosomal copy of the LTTR was re-inserted into the genome, full restoration of the wild-type phenotype was achieved (Vinckx *et al.,* 2010). In the absence of a functional *ptrA* gene, multiple copies of *gacA* further upregulate flagellar motility in the PA23-443 mutant background, rather than restoring motility closer to wild-type levels, as expected of a repressor of motility. Together, these data indicate there may be some regulatory interaction between PtrA and GacA.

Overexpression of *rpoS* and *psrA* resulted in full and partial complementation of the wild-type phenotype in the PA23-443 mutant background, respectively. Expression of *rpoS in trans* resulted in a decrease in flagellar motility to 1.2-fold that of wild-type PA23 (Table 7) and expression of *psrA in trans* resulted in an overall decrease in motility to 1.5-fold that of the wild type (Table 7). When *rsmA*, *rsmE* and *rsmZ* were overexpressed in the mutant background, partial complementation was also observed, with decreases in motility measured to 1.9-, 1.7-, and 1.4-fold that of the wild type, respectively (Table 7). As *gacA* is a positive regulator of *rsmZ* expression, and indirectly regulates expression of *rsmA* and *rsmE* (Selin *et al*., 2014), it is somewhat surprising that overexpression of these Rsm system components and not *gacA* decreased motility closer to that of the wild type. Nonetheless, these results further substantiate a regulatory link between PtrA and the Gac-Rsm system. The PA23*Δscd* mutant showed no change in motility from wild-type PA23.

3.9. PtrA positively affects protease activity

To study other phenotypic changes that may be occurring as a result of a *ptrA* mutation, extracellular protease assays were conducted. The PA23-443 mutant exhibited a complete loss of extracellular protease activity at 24 and 48 hours compared to the wild type in both LB and M9 media (Figure 8; Table 8). When *ptrA* was expressed *in trans*, protease activity was restored to 1.1-fold that of the wild type in LB media, and 1.8-fold that of the wild type in M9 media. When *gacS* was expressed *in trans* in the same strain, partial restoration of protease activity was observed in both media types, but only following 48 hour incubation. Overexpression of *gacA* following 48 hours incubation on M9 media also partially restored protease activity. These findings indicate that PtrA is required for extracellular protease production in PA23, and *gacS* and *gacA* expression *in trans* are able to partially complement the PtrA mutation. All other overexpression constructs were unable to restore protease activity in the PA23 *ptrA* mutant. The PA23*Δscd* mutant and PA23*Δscd* complemented strain showed no significant change in extracellular protease activity from the wild-type PA23 (Figure 8).

The loss of exoprotease production has been previously shown to reduce the biocontrol abilities of other pseudomonads. For example, in *P. fluorescens* CHA0, *aprA* and *gacA* mutants were defective for exoprotease production, and resulted in a loss of biocontrol against the Knot-Root nematode, *Meloidogyne incognita* (Blumer *et al*., 1999; Siddiqui *et al*., 2005).

Table 8. Protease activity of overnight cultures of *Pseudomonas chlororaphis* PA23 and mutants PA23-443 and PA23*Δscd* harbouring various overexpression plasmids grown in LB media or M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose following incubation at 28°C for 24 and 48 hours.

Strain	**Extracellular protease activity[a]**			
	LB		**M9**	
	24h	**48h**	**24h**	**48h**
PA23 (pUCP22)	1.9 (0.2)	4.5 (0.0)	0.5 (0.0)	2.3 (0.2)
PA23-443 (pUCP22)	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)[b]
PA23-443 (*rsmA*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)[b]
PA23-443 (*rsmE*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)[b]
PA23-443 (*rsmZ*-pUCP23)	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)[b]
PA23-443 (*rpoS*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)[b]
PA23-443 (*psrA*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)[b]
PA23-443 (*ptrA*-pUCP22)	2.3 (0.2)[c]	5.2 (0.2)[d]	1.4 (0.2)[b]	4.2 (0.2)[b]
PA23-443 (*phzR*-pUCP23)	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)[b]
PA23-443 (*gacA*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	1.0 (0.3)[d]
PA23-443 (*gacS*-pUCP23)	0.1 (0.2)[b]	1.1 (0.2)[b]	0.0 (0.0)	1.3 (0.2)[d]
PA23-443 (*scd*-pUCP22)	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)[b]
PA23*Δscd* (pUCP22)	1.8 (0.2)[e]	4.5 (0.3)[e]	0.5 (0.0)	2.5 (0.3)
PA23*Δscd* (*scd*-pUCP22)	2.0 (0.3)[e]	4.5 (0.4)[e]	0.5 (0.0)	2.5 (0.0)

[a]Mean (standard deviation) of proteolytic zone (mm) from five replicates.

[b]Significantly different from wild type ($P<0.0001$).

[c]Significantly different from wild type ($P<0.05$).

[d]Significantly different from wild type ($P<0.001$).

[e]Not significantly different from wild type.

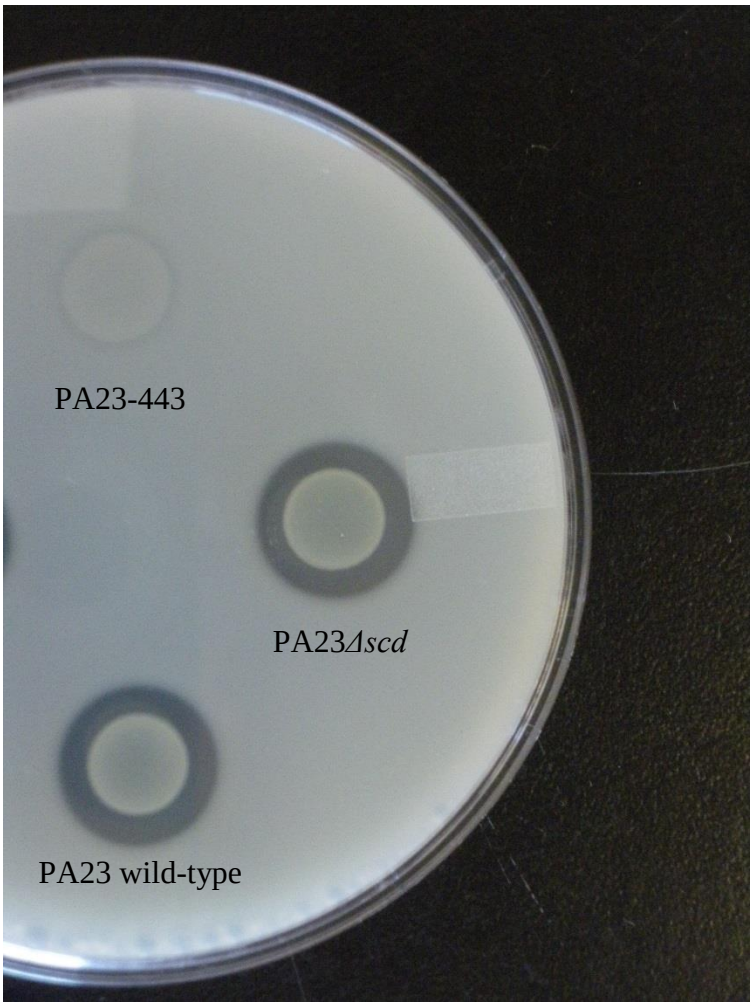

Figure 8. Protease plate assay of overnight culture of PA23, PA23-443 and PA23*Δscd* grown in M9 minimal media supplemented with 1mM $MgSO_4$ and 0.2% glucose.

3.10. PtrA positively affects autoinducer production

The QS regulatory mechanism is based on the production of small, diffusible autoinducer molecules. In PA23-443, autoinducer is no longer produced, indicating that the *ptrA* mutation affects cell-cell communication and subsequent production of AF metabolites (Table 9). Interestingly, when all overexpression plasmids were introduced individually into the PA23-443 background, varying degrees of complementation were observed in both LB and M9 media (Table 9). Overexpression of *ptrA* and *gacA* achieved full complementation, whereas all other overexpression plasmids resulted in partial complementation.

In *Ralsonia* (*Pseudomonas*) *solanacearum*, expression of AHL requires the *solR* and *solI* LuxR/I homologues, which were shown to be regulated by the LTTR, *phcA* (Flavier *et al*., 1997). Both *solR*/*I* and *phcA* expression exhibit cell-density associated expression patterns (Flavier *et al*., 1997). PhcA expression itself is negatively regulated by its own two-component signal transduction system, PhcR/S, and some PhcA-controlled products also require the global two- component system VsrA/D (GacA/S) (Clough *et al*., 1997; Huang *et al.,* 1995). A similar system may be in play in *P. chlororaphis* PA23 regarding PtrA regulation of autoinducer production. The fact that all of the regulatory genes tested (*rpoS, phzR, gacA, gacS*, etc.) were able to complement AHL production in the *ptrA* mutant to some degree is surprising. Although the molecular mechanisms involved are unknown, these findings suggest that regulation of AHL production in PA23 is extremely complex.

Table 9. Autoinducer assay of *Pseudomonas chlororaphis* PA23 and mutants PA23-443 and PA23*Δscd* harbouring various overexpression plasmids grown in LB media or M9 minimal media supplemented with 1mM $MgSO_4$ and 0.2% glucose following 24 hour incubation on *C. violaceum* CVO26-seeded LB agar at 28°C.

Strain	**Zone of autoinducer diffusion[a]**	
	LB	**M9**
PA23 (pUCP22)	6.3 (0.4)	4.0 (0.3)
PA23-443 (pUCP22)	0.0 (0.0)[b]	0.0 (0.0)[b]
PA23-443 (*rsmA*-pUCP22)	6.1 (0.2)[c]	1.4 (0.2)[b]
PA23-443 (*rsmE*-pUCP22)	3.9 (0.4)[b]	0.9 (0.2)[b]
PA23-443 (*rsmZ*-pUCP23)	3.2 (0.5)[b]	0.7 (0.4)[b]
PA23-443 (*rpoS*-pUCP22)	3.5 (0.3)[b]	0.2 (0.4)[b]
PA23-443 (*psrA*-pUCP22)	6.0 (0.4)[c]	1.3 (0.2)[b]
PA23-443 (*ptrA*-pUCP22)	6.0 (0.0)[c]	4.0 (0.5)[c]
PA23-443 (*phzR*-pUCP23)	4.1 (0.2)[b]	1.0 (1.3)[d]
PA23-443 (*gacA*-pUCP22)	5.6 (0.4)[e]	3.5 (0.7)[e]
PA23-443 (*gacS*-pUCP23)	6.5 (0.3)[c]	2.5 (1.0)[e]
PA23-443 (*scd*-pUCP22)	2.9 (0.5)[b]	0.1 (0.2)[b]
PA23*Δscd* (pUCP22)	6.5 (0.5)[c]	3.5 (0.4)[c]
PA23*Δscd* (*scd*-pUCP22)	6.0 (0.4)[c]	3.6 (0.3)[c]

[a]Mean (standard deviation) of purple autoinducer zone of diffusion (mm) from five replicates.

[b]Significantly different from wild type ($P<0.0001$).

[c]Not significantly different from wild type.

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

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

3.11. PtrA positively affects PRN production

HPLC analysis has revealed complete loss of PRN production in the PA23-443 mutant (Table 10). Full complementation was achieved when *ptrA* was expressed *in trans* in the PA23-443 mutant background and partial complementation occurred with *gacS* expression *in trans* in the same mutant background. No significant changes in PRN production occurred in the PA23*Δscd* mutant compared to wild-type PA23. Blocked biosynthesis of PRN has been previously reported in Gac mutants of *P. fluorescens* CHA0 (Duffy and Défago, 2000). In PA23, PRN production is negatively regulated by the alternative sigma factor, RpoS and positively regulated by the Phz QS system (Selin et al., 2014). PRN regulation by LTTRs has not previously been reported.

3.12. PtrA mutation affects gene regulation in PA23

qRT-PCR analysis was conducted to assess the effect of the *ptrA* and *scd* mutations on gene transcription in PA23. In PA23-443, expression of *gacA* was significantly downregulated, whereas expression of *rsmA* and *scd* were upregulated compared to wild-type PA23 (Figure 9). As previously mentioned, the phenotype of GacS/A mutants of PA23 is similar to that of the *ptrA* mutant with a loss of AF activity and PHZ and PRN production. Therefore, qRT-PCR results indicating *gacA* transcription is decreased in the *ptrA* mutant is consistent with our phenotypic analysis. RsmA is a post-transcriptional repressor which binds to target mRNA to block the ribosome binding site and thus translation (Kay *et al*., 2006; Lapouge *et al*., 2008). Increased expression of RsmA is consistent with the loss of AF activity observed in PA23-443 because AF

Table 10. Quantification of pyrrolnitrin present in 120 hour cultures (20mL) of *Pseudomonas chlororaphis* PA23 and mutants PA23-443 and PA23*Δscd* harboring various overexpression plasmids grown in M9 minimal media supplemented with 1mM $MgSO_4$ and 0.2% glucose at 28°C.

Strain	**[Pyrrolnitrin][a]**
PA23 (pUCP22)	3.48 (0.45)
PA23-443 (pUCP22)	N.D.[b]
PA23*Δscd* (pUCP22)	3.74 (0.32)[c]
PA23-443 (*ptrA*-pUCP22)	3.90 (0.20)[c]
PA23-443 (*gacA*-pUCP22)	N.D. [b]
PA23-443 (*gacS*-pUCP23)	2.56 (0.28)[c]
PA23-443 (*psrA*-pUCP22)	N.D. [b]
PA23-443 (*rpoS*-pUCP22)	N.D. [b]
PA23-443 (*phzR*-pUCP23)	N.D. [b]
PA23-443 (*rsmA*-pUCP22)	N.D. [b]
PA23-443 (*rsmE*-pUCP22)	N.D. [b]
PA23-443 (*rsmZ*-pUCP23)	N.D. [b]
PA23-443 (*scd*-pUCP22)	N.D. [b]

[a]Mean (standard deviation) of amounts of pyrrolnitrin (µg) from two replicates.
[b]Significantly different from wild type ($P<0.01$).
[c]Not significantly different from wild type.
N.D., not detected.

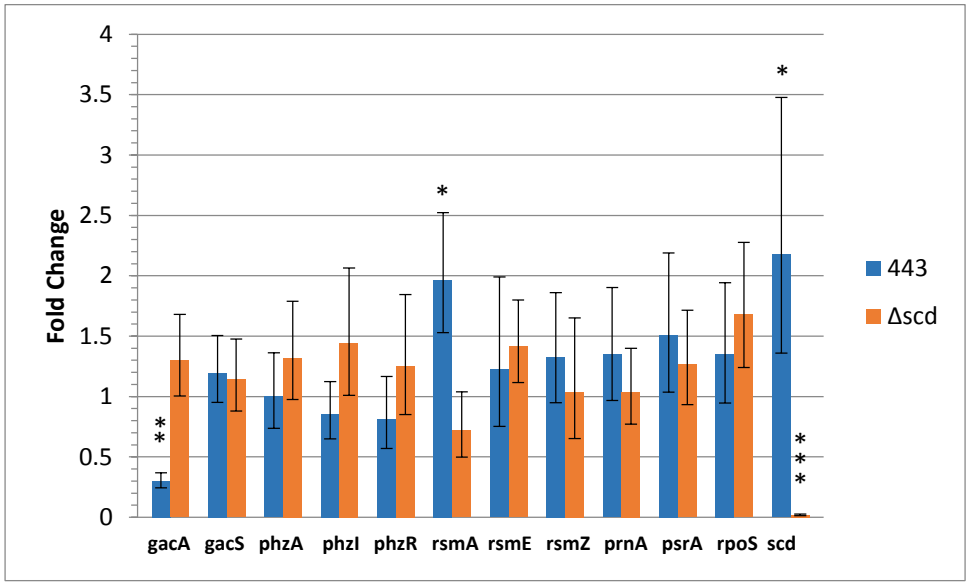

Figure 9. qRT-PCR fold change in gene expression in Tn*5* mutant PA23-443 and PA23*Δscd* compared to wild-type PA23. Analyzed genes were compared against *rpoD* reference gene. For strains that differ significantly from the wild type, columns are labeled with asterisks (*, P<0.05; **, P< 0.001; ***, P<0.0001).

metabolites of other pseudomonads are also translationally repressed by RsmA (Kay *et al*., 2006; Lapouge *et al*., 2008; Reimmann *et al*., 2005).

Another regulatory link appears to exist between PtrA and *scd* expression. In PA23-443, *scd* expression increased greater than 2-fold, indicating the PtrA may be acting as a negative regulator of the divergently transcribed *scd*. Whether or not this apparent increase in *scd* expression is contributing to the loss of AF activity associated with PA23-443 is not yet clear. In PA23*Δscd*, *scd* transcription appears to be significantly downregulated, but different primer pairs were used to measure *scd* expression than those used in the PA23-443 mutant and PA23 wild type. As a result of the pKNOCK-*scd* plasmid insertion, two truncated, non-functional copies of the *scd* gene were generated (Figure 4). One copy is located directly downstream of the original *scd* promoter, whereas the second copy is located downstream of the tet promoter. The *scd* primers used for qRT-PCR analysis in the PA23-443 mutant amplify a 121-bp internal fragment of the *scd* gene. These primers showed a 4-fold increase in *scd* gene expression in the PA23*Δscd* strain. The significant increase in *scd* expression in PA23*Δscd* was most likely due to strong expression from the tet promoter (Figure 4), as opposed to its native promoter. To bypass this possibility, new primers were generated (R6Kori-3 FWD and scdBamHI-3 REV; Table 1) to measure expression solely from the native promoter, which had to include portions of the pKNOCK plasmid backbone (Figure 4). These primers amplified a slightly larger product than the amplicon from the PA23-443 mutant, which did not contain a pKNOCK insertion (250-bp fragment compared to a 121-bp fragment). Results from qRT-PCR analysis using this set of primers showed a drastic decrease in the production of this *scd* transcript fragment compared to wild-type PA23.

Thus, it is still unclear how Scd is regulated and further analysis is needed to verify these findings.

3.13. Putative reductase function of the Scd protein

Predictive bioinformatics analysis of the Scd amino acid sequence hydrophobicity implies that Scd is a membrane-bound protein, due to the elevated number of hydrophobic residues present. ProtScale analysis to determine hydrophobic and hydrophilic regions within the Scd protein were reported on a Kyte and Doolittle hydrophobicity plot (Figure 10). Seven hydrophobic regions were assigned to the secondary protein structure, and one distinct hydrophilic region at residue 20 was predicted. In some cases, the gene upstream of the LTTR encodes a co-inducer molecule that modulates LTTR activity (Schell, 1993). Although *scd* is upstream of *ptrA*, ProtScale analysis suggests that Scd is membrane-bound and therefore not the co-inducer molecule of PtrA.

The InterProScan results attribute the putative function of the protein to be that of a dTDP-4-dehydrorhamnose reductase that catalyzes the last four steps in making dTDP-rhamnose, a precursor of lipopolysaccharide core and O antigens. The predicted substrate is dTDP-6-deoxy-L-mannose. ProSite analysis (Table 11) indicates common signatures of short amino acid sequences within the larger Scd sequence. Two N-glycosylation sites, two casein kinase II phosphorylation sites and two protein kinase C phosphorylation sites were predicted within the Scd protein sequence.

The secondary structure predictions based on InterProScan results (Figure 11) indicate that there are five distinct alpha-helical regions within the amino acid sequence,

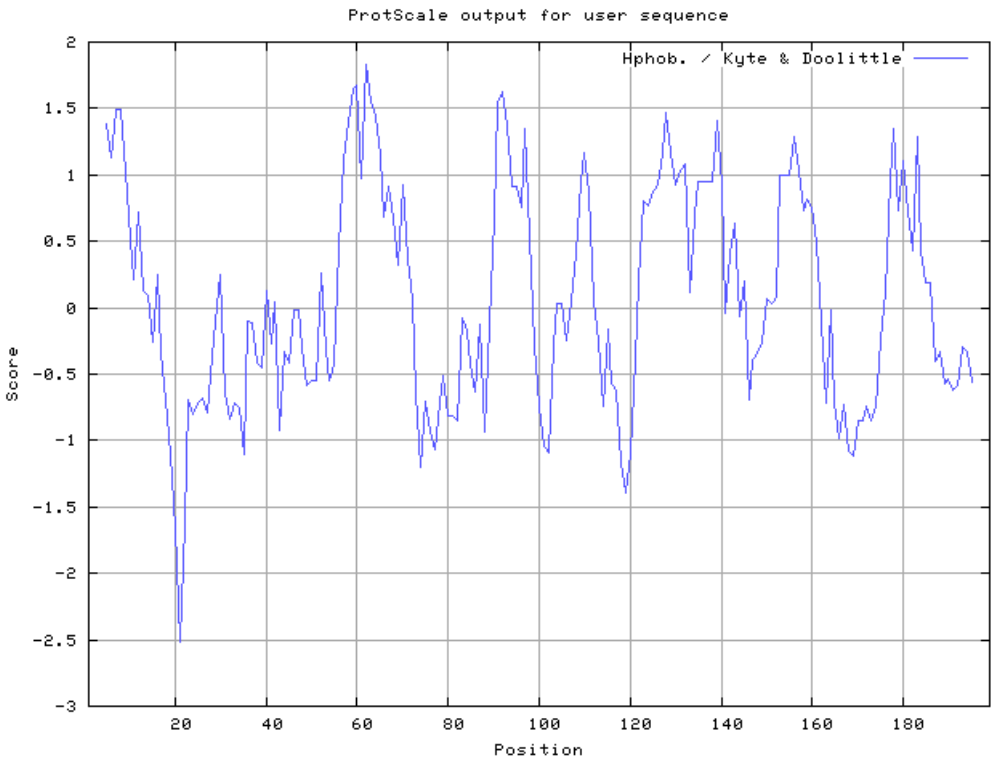

Figure 10. Kyte and Doolittle hydrophobicity plot predicting highly hydrophobic and hydrophilic domains within the 199 amino acid sequence of a short-chain dehydrogenase in *Pseudomonas chlororaphis* PA23. +scores indicate hydrophobic regions; - scores indicate hydrophilic regions.

Table 11. ProSite analysis summary indicating highly probable amino acid signatures within a 199 amino acid sequence of Scd in *Pseudomonas chlororaphis* PA23.

Amino Acid Residue(s)	**Predicted Signature**
9-12; 65-68	N-glycosylation sites
14-17(S) ; 32-35(T)	Casein kinase II phosphorylation sites
21-23(S) ; 45-47(S)	Protein kinase C phosphorylation sites

S, phosphoserine; T, phosphothreonine.

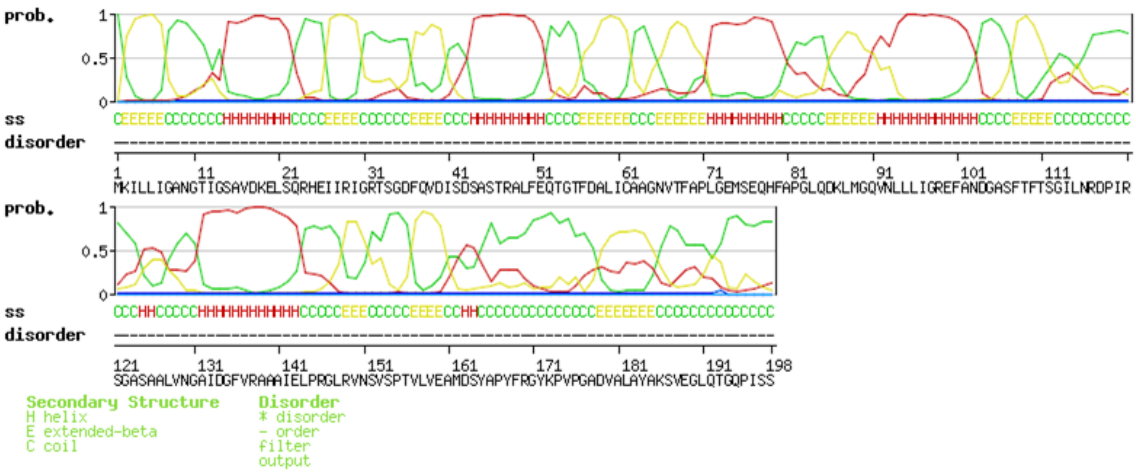

Figure 11. InterProtScan results showing possible secondary structures within a 199 amino acid sequence of a short-chain dehydrogenase in *Pseudomonas chlororaphis* PA23. H, helices structures; E, beta sheet structures; C, coil structures.

and several beta-pleated sheet domains. These results confirm the presence of a Rossmann-fold domain within this short-chain dehydrogenase sequence, which typically includes five alpha-helices surrounding a beta-pleated sheet. Together these secondary structures form the NAD^+ binding domain, which is necessary for the proper functioning of a SDR protein (Bashton and Chothia, 2002).

3.14. LPS structures of PA23-443 and PA23Δscd are unchanged from wild-type PA23

Because of the putative role of Scd in LPS synthesis, we sought to determine whether any changes in LPS expression occur in the *ptrA* and *scd* mutants. SDS-PAGE analysis revealed that the LPS profiles of the aforementioned mutants were identical to that of the wild type (Figure 12). LPS structural changes have been previously shown to affect bacterial interactions with plant hosts. In studies with *Rhizobium leguminosarum* biovar *viciae* 3841, mutations in a 27- hydroxyoctacosanoic acid within the lipid A of LPS resulted in a flagellar-deficient, non-motile phenotype, as well as altered biofilm formation (Vanderlinde *et al.,* 2009). An LPS mutant of *P. syringae* pv. *syringae* 61 demonstrated reduced biofilm formation, decreased motility and reduced plant pathogenesis (Deng *et al.,* 2010). Based on our results, however, the predicted function of Scd (dTDP-4-dehydrorhamnose reductase) appears to have no significant effect on the LPS composition of PA23 when mutated.

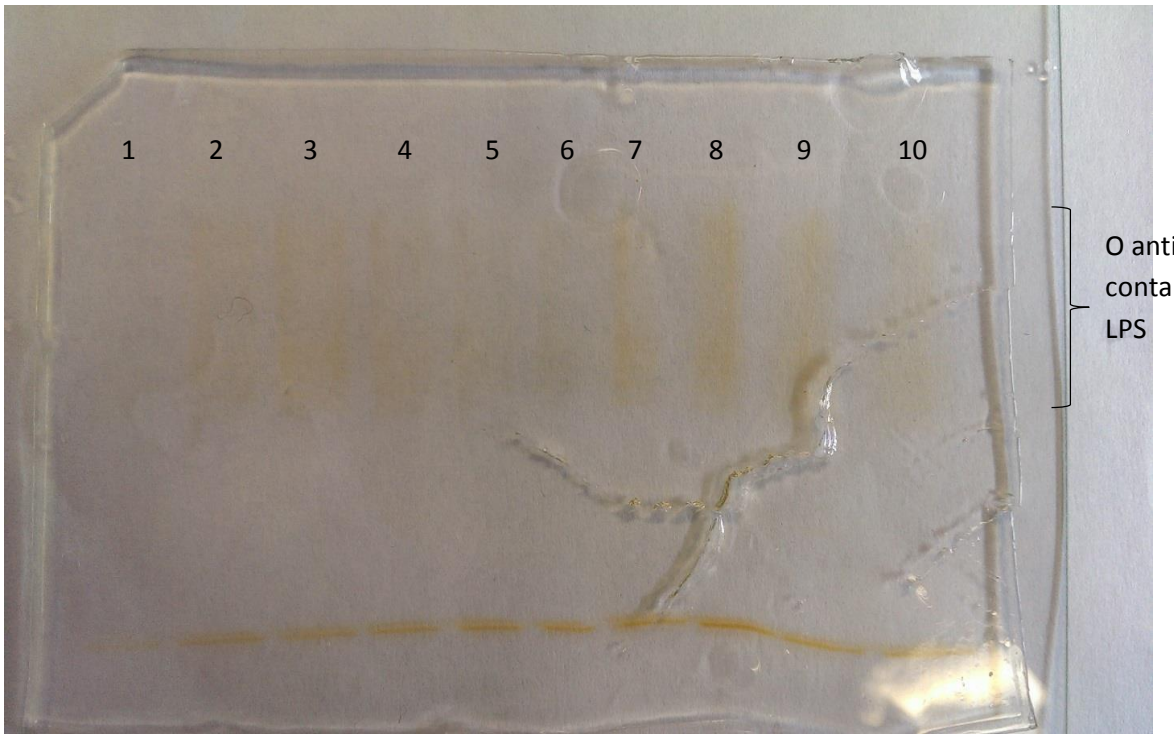

Figure 12. LPS analysis of PA23 and derivative strains. LPS was prepared from overnight cultures grown in M9 minimal media (1mM $MgSO_4$; 0.2% glucose) run on SDS-PAGE gel and visualized by silver staining. 1-2; PA23 wild type, 3-4; PA23-443, 5-6; PA23-443 (*ptrA*-pUCP22), 7-8; PA23*Δscd*, 9; PA23*ΔgacA,* 10; PA23-314.

4. CONCLUSIONS AND FUTURE DIRECTIONS

In the present study, characterization of a novel LTTR, designated PtrA, in *P. chlororaphis* PA23 was investigated. A mutation in the coding region of the *ptrA* gene in mutant PA23-443 results in a loss of AF activity, indicating that PtrA is essential for PA23 biocontrol of *S. sclerotiorum*. Interestingly, PA23-443 exhibits a similar phenotype to Gac mutants of PA23. Sequence analysis indicated an open reading frame upstream of and divergently transcribed from *ptrA*, which we termed Scd. Predictive bioinformatics analysis of the *scd* gene revealed a putative function in LPS production; however, our findings revealed no change in LPS expression in either the *ptrA* of *scd* mutants. The role of *scd* in PA23, therefore, remains unknown.

Specific physiological changes that were observed in the *ptrA* mutant include differential protein expression profiles across 16 different COG categories and a loss of PHZ, PRN, autoinducer, chitinase and extracellular protease production. In addition, PA23-443 exhibited altered flagellar motility, growth rate and siderophore production. qRT-PCR data revealed that *gacA* expression is decreased in the *ptrA* mutant, whereas *rsmA* and *scd* expression is increased. Surprisingly, *gacS* was the only gene that appeared to partially complement the PtrA mutant in terms of PHZ, PRN, autoinducer, protease, siderophore, chitinase and overall AF activity even though no changes in *gacS* transcription were seen in the PA23-443 background. Collectively, these findings suggest that there may be a regulatory connection between PtrA and the Gac system. No significant physiological differences were observed in the *scd* mutant compared to wild-type PA23.

The GacS/GacA two-component signal transduction system consists of GacS, a membrane-bound sensor kinase, which, upon binding to an unknown environmental signal, leads to autophosphorylation and phosphotransfer to GacA, a cytoplasmic response regulator. Phosphorylated GacA binds to DNA to control transcription of target genes (Lapouge *et al*., 2008). In many other pseudomonads, two additional orphan sensor kinases, RetS and LadS, modulate the Gac regulatory system. RetS is able to form heterodimers with GacS, which prevents its autophosphorylation, thus preventing phosphotransfer to GacA, and subsequent transcription of *rsmZ* (Goodman *et al*., 2009). However, upon detection of an unknown signal, RetS and GacS each form homodimers, allowing for proper phosphorylation of the circuit, downstream expression of *rsmZ* and titration of the RsmA and RsmE proteins. LadS seems to play an opposing role to RetS through an unknown mechanism (Goodman *et al*., 2009; Ventre *et al*., 2006). In studies with *P. aeruginosa*, mutations in *retS* strongly enhanced the formation of biofilms, whereas mutations in *ladS* completely abolished biofilm formation (Kay *et al*., 2006). Although it is still not completely apparent how overexpression of GacS restores the wild-type phenotype in PA23-443, one theory is that additional copies of the GacS sensor kinase in the outer membrane may act by saturating, through phosphorylation, any available GacA response regulator. This would act to alleviate transcriptional repression of downstream regulatory genes since *gacA* transcript levels are decreased in the *ptrA* mutant.

The Gac regulatory system is made up of complex signalling networks that control multiple physiological traits in bacteria. Our findings suggest that PtrA forms another layer of complexity in this system overseeing production of biocontrol factors in

PA23. As *gacA* expression was decreased in the *ptrA* mutant, it is possible that *ptrA* binds directly to the promoter region of *gacA* to stimulate its expression. Subsequent DNA-protein interaction studies, such as gel shift assays and DNAseI footprinting studies could potentially identify binding targets of PtrA, including *gacA.*

iTRAQ analysis of *ptrA*-directed protein expression may benefit from complete genome information of PA23. As the analysis was directly compared to the *P. chlororaphis* gp72 genome, many proteins that were differentially expressed in the *ptrA* mutant compared to wild type PA23 were identified as unknown proteins (Table 3). Full sequencing of the PA23 genome may allow for identification of some of these currently unknown proteins.

An LTTR regulatory network in *R. solanacearum* has been previously studied which appears to regulate virulence factors in a manner similar to the regulation of AF metabolites in PA23. In this system, the LTTR PhcA controls several phenotypic traits along with the two-component signal transduction system, VsrA/D, which has been shown to be homologous to the GacS/GacA system in fluorescent pseudomonads (Schell *et al*., 1994; Schell, 1996). *R. solanaceanum* is a plant pathogen that causes lethal wilt disease in over 200 plant species. Several two-component signal transduction systems are in play in this organism to coordinate gene expression in response to environmental signals (Huang et al., 1995). In *phcA* and *vsrA/D* mutants, hypermotility and hyper siderophore production is observed, together with decreased production of an exopolysaccharide (EPS) and cell wall degrading enzymes, and overall virulence in plants (Bhatt et al., 2004; Brumbley and Denny, 1990; Schneider et al., 2009). Both PhcA and the VsrA/D system increase expression of the transcriptional activator, XpsR, which

directly binds to the EPS promoter to regulate expression of this virulence factor (Schell 1996). When either PhcA or the Vsr system are absent in the cell, an overall decrease in virulence factors is observed, indicating that both systems need to be active for proper expression of EPS and cell wall degrading enzymes (Huang et al., 1995). An additional two-component signal transduction system, PhcS/R, was also discovered in *R. solanacearum* that negatively regulates expression of PhcA (Clough et al., 1997; Huang et al., 1995). The intermediate factor, XpsR appears to link the Phc system to the Vsr system in the regulation of certain virulence factors in this bacterium (Huang et al., 1995). A great deal remains to be discovered regarding PtrA regulation of AF compounds in PA23. Because this bacterium employs a complex network of biocontrol factors, it seems likely that one or more of these regulators interact with PtrA, similar to the interactions revealed in the *R. solanacearum* system.

LTTRs are frequently autoregulated and co-inducer molecules profoundly impact binding specificity (Schell, 1993). The PhcA inducer molecule was isolated through liquid chromatography and identified as 3-hydroxypalmitic acid methyl ester (3-OH-PAME) via gas chromatography and mass spectroscopy (Flavier et al., 1997). In *phcB* mutants, this molecule was no longer produced, resulting in dramatically decreased production of EPS and cell-wall degrading enzymes, but when exogenous 3-OH-PAME was added to cultures, virulence factor production was restored (Clough *et al*., 1997; Flavier *et al.,* 1997). Currently, the inducer molecule of PtrA has not yet been identified. Gas chromatography and mass-spectroscopy studies may shed light into the nature of the co-inducing molecule required for proper functioning of this LTTR. Previous studies attempting to solve the full-length structure and effector-binding domain of PtrA were

unsuccessful as PtrA exhibits low stability in solution (Chan, 2012). When *ptrA* was overexpressed in *E. coli*, it was consistently found to be bound to DNA, resulting in even greater insolubility and precipitation (Chan, 2012).

Further studies into the nature of PtrA regulation will undoubtedly shed light on how PA23 controls production of secondary metabolites. Understanding the complex regulatory genetic hierarchy present in *P. chlororaphis* PA23 will help to optimize the production of biocontrol factors, making it a more suitable alternative in disease suppression.

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APPENDIX A

LPS outer membrane analysis

Hitchcock and Brown Lysis Buffer
2% SDS
4% β-mercaptoethanol
10% glycerol
1M Tris-HCl, pH 6.8
0.002% bromophenol blue

Stacking Gel
0.5 mL acrylamide:bisacrylamide (30:0.8)
0.12 mL ammonium persulfate (10mg/mL)
0.05 mL 10% SDS
1.25 mL 1 M Tris base, pH 6.8
3.08 mL dH_2O
10 µL TEMED (N,N,N',N'-tetramethylethylenediamine)

Resolving Gel
4.29 mL acrylamide: bisacrylamide (44:0.8)
0.30 mL ammonium persulfate (10mg/mL)
0.25 mL 10% SDS
0.21 mL 5M NaCl
3.12 mL 1.5M Tris base, pH 8.8
4.5 mL dH_2O
20 µL TEMED

Staining Solution
42 mL 0.36%(w/v) NaOH
2.8 mL conc. NH_4OH
8.0 mL 19.4%(w/v) $AgNO_3$
148 mL dH_2O

Developer Solution
10 mg citric acid salt
103 µL 37% formaldehyde
20 mL methanol
180 mL dH_2O