Molecular Mechanisms Involved in Secondary Metabolite Production and Biocontrol of *Pseudomonas chlororaphis* PA23

By

Nicole Joanna Poritsanos

A Thesis

Submitted to the Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Department of Microbiology

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ABSTRACT

Sclerotinia sclerotiorum is a ubiquitous ascomycetous fungal pathogen that causes disease in over 400 crop species, specifically in soybean and canola plants, where stem rot is the most common disease symptom. *Pseudomonas chlororaphis* PA23 was previously isolated from the rhizosphere of soybean and has demonstrated excellent antifungal activity against *S. sclerotiorum in vitro*, greenhouse and field experiments. To elucidate the molecular mechanisms involved in PA23 biocontrol, random mutagenesis experiments were initiated. Several mutants were isolated that could be divided into three general classes.

Biocontrol activity of various *Pseudomonas* spp. is highly regulated by a GacS/GacA two-component global regulatory system. Class I PA23 mutants harboured Tn*5* insertions in the *gacS*-coding region, resulting in pleiotropic defects including deficiency in secondary metabolite production and biocontrol activity. Complementation with the wild type *gacS* allele *in trans* restored wild type phenotypes. These findings suggest that the ability of *P. chlororaphis* PA23 to suppress *S. sclerotiorum* causing stem rot in canola is dependent on a functional GacS/GacA global regulatory system. This is the first study assessing disease symptoms on canola (*Brassica napus* L.) plants inoculated with a *gacS* minus strain of *P. chlororaphis*.

Phenazine compounds are considered to be a key secondary metabolite contributing to the antagonistic and antifungal activity of *P. chlororaphis.* In *P. chlororaphis* PA23, mutations in phenazine biosynthetic genes exhibited equal or

more antifungal activity *in vitro*, compared to the wild type. To assess the effect of the deficiency in phenazine production, a Class II mutant , harbouring a Tn*5* insertion in *phzE* was tested for a number of biocontrol traits including secondary metabolite production, motility, and suppression of Sclerotinia pathogenic traits. Since no other traits were markedly affected beyond phenazine production, it was concluded that phenazine is not the major product contributing to *S. sclerotiorum* biocontrol.

A single Class III mutant was isolated harbouring a Tn*5* insertion in a gene encoding a transcriptional regulator of the LysR family. This mutant exhibited no antifungal activity on plate assays and was unable to protect against *S. sclerotiorum* in green house assays. A number of secondary metabolites were no longer produced by this mutant, suggesting that this LysR-type transcriptional regulator is either directly or indirectly involved in controlling several genes in *P. chlororaphis* PA23.

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1.0 INTRODUCTION

1.1 Biological Control

Biological control is defined as the mechanism involved in the management of disease symptoms and reduction of pathogen inoculum on plants, a treatment mediated through the use of organisms other than man (Cook *et al.*, 1983).

Currently the main biocontrol agents used on plants are chemical fungicides whose use raises a concern on the impact of human health and the development of fungal resistance. Biocontrol organisms belonging to *Pseudomonas* and *Bacillus* species have shown great antagonistic activity against soilborne pathogens of economically important crop plants (Savchuk and Fernando, 2004; Fernando *et al.*, 2004; Chin-A-Woeng *et al.*, 1998; Hoefnagels and Linderman, 1999; de Boer *et al.*, 1999; Ryder *et al.*, 1999; Hervas *et al.*, 1998).

In *Pseudomonas* spp. biocontrol activity is mediated through the production of antibiotics and lytic enzymes, as well as, through competitive exclusion (Pearson *et al.,* 1994*;* Elasri *et al*., 2001; Duffy and Defago, 1997; Schnider-Keel *et al.,* 2000). The global regulatory two-component system, GacS/GacA, is known to positively control secondary metabolite production which includes phenazine, pyrrolnitrin, 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin, hydrogen cyanide (HCN), exoprotease(s), and chitinase compounds (Chancey *et al.,* 1999; Chin-A-Woeng *et al.* 2000; Duffy and Defago, 2000;

Laville *et al.* 1992; Gaffney *et al.* 1994), as well as siderophore compounds pyochelin and pyoverdine (Duffy and Defago, 2000; Schmidli-Sachere, 1997).

Earlier studies on biocontrol activity focused on the ability of bacteria to compete for and sequester iron from the environment, thereby compromising the growth of the residing pathogen population (Bakker *et al.*, 1986; Loper and Henkers, 1999; O'Sullivan and O'Gara, 1992). Iron is important for RNA synthesis, oxidative stress and transcription of genes involved in iron transport systems (Braun, 1997; Braun and Mahren, 2005). *Pseudomonas* bacteria acquire their iron from the environment through the use of iron binding peptides termed siderophores (Neilands, 1995). These include pyoverdines or pseudobactins, which are produced and excreted into the surrounding environment under iron limiting conditions (Budzikiewicz, 1993; Meyer, 2000). In *Pseudomonas aeruginosa*, the uptake of pyoverdine-Fe(III) complex occurs through a TonB activated outer membrane protein, which functions as a receptor for the siderophore complex. Specificity will vary depending on the *Pseudomonas* species. Some findings indicate that receptors recognize heterologous pyoverdines produced by a different bacterium, while others have high specificity for their cognate pyoverdine molecules (Hohnadel and Meyer 1988; Cornelis *et al.*, 1989; Meyer *et al.*, 1999; Meyer *et al.*, 2002b). Strains of *Pseudomonas fluorescens* and *Pseudomonas putida* have exhibited the ability to use heterologous pyoverdines, which confers a competitive advantage in the rhizosphere (Raaijmakers *et al.*, 1995).

Competitive exclusion between bacteria and fungi is primarily dependent in the ability to produce, and have resistance to antibacterial or antifungal compounds (SavKa *et al.*, 2002). Antagonistic effects mediated by signals produced from other bacteria have also been documented in *Pseudomonas aureofaciens* 30-84. Morello *et al.* (2004) studied the interactions between bacteria from the same ecosystem, and they found a subpopulation of rhizobacteria with the ability to reduce phenazine gene expression and subsequently production of this antibiotic in *P. aureofaciens* 30-84. The investigators postulated that the inhibitory effect on phenazine is exerted at the transcriptional level (Morello *et al.*, 2004). Negative cross-communication has also been described between fungi and bacteria. The fungal metabolite fusaric acid produced by *Fusarium oxysporum* f. sp. *radicis lycopersici* interfered with the 2,4-DAPG autoregulatory mechanism in *P. fluorescence* CHA0 (Duffy and Defago, 1997; Schnider-Keel *et al.*, 2000) and phenazine-1-carboxamide (PCN) production in *P. chlororaphis* PCL1391 (van Rij *et al.*, 2005). This is a proof of secondary metabolism being antagonistic not only by biocontrol bacteria but also by fungal pathogens.

1.2 *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary belongs to the family Sclerotiniaceae and class Ascomycotina (Whetzel 1945; cited in Duncan 2003). This organism is a necrotic fungal pathogen to more than 400 species of plants (Purdy, 1979; Boland and Hall, 1994). In soybean and canola (Glycine max and

Brassica napus L.) plants, stem rot (stem blight) is the most important Sclerotinia disease symptom, causing significant losses in grain yield (Purdy, 1979). Sclerotinia pathogenicity is mediated mainly through the production of oxalic acid in infected tissues (Maxwell and Lumsden, 1970; Marciano *et al.*, 1983), where it chelates Ca^{2+} and acidifies the surrounding infection site. This ultimately results in cell wall degradation and susceptibility to other fungal pathogenicity factors (Godoy *et al.*, 1990; Bateman and Beer, 1965). Additionally, oxalic acid mediates its pathogenic effects by inhibiting oxygen burst, a plant defence mechanism involving the generation of oxygen radicals in response to invading pathogens (Ferrar and Walker, 1993; Cessna *et al.*, 2000).

Disease symptoms of Sclerotinia infection in soybean and canola plants resemble water-soaked lesions on the stem and leaves. Under humid conditions, infected plant sites exhibit lesions with white fluffy mycelial growth and the development of sclerotia (black seed-like reproductive and survival structures) on the stem surface, eventually leading to plant death (Grau, 1988; Martens *et al.*, 1994).

1.3. *Pseudomonas chlororaphis*

Pseudomonas spp. produce antibiotics exhibiting a broad spectrum of activity against fungal pathogens. The antibiotic mode of action may be synergistic or independent of other secondary metabolites (Keel *et al.*, 1996; Bender *et al.*, 1999). A variety of antibiotics are known to be responsible for the suppression of fungal diseases in plants. PA23 produces volatile and non-

volatile antibiotics (Fernando *et al.*, 2005; Fernando and Ramarathnam, 2005). Volatile antibiotics, like hydrogen cyanide (HCN), have the ability to diffuse into the soil and act as potent metabolic inhibitors towards fungal pathogens (Castric, 1981; Fernando and Linderman, 1994). PA23 produces several non-volatile antibiotics including phenazine-1-carboxylic acid (PCA), and 2-hydroxyphenazine (2-OH-PHZ) (Paulitz, de Kievit and Fernando, unpublished data). High-performance liquid chromatography (HPLC) performed by Paulitz and colleagues showed that PA23 also produces pyrrolnitrin (Tim Paulitz, personal communication). When tested for the antagonistic activity of *P. chlororaphis* PA23, findings showed that it inhibited ascospore germination of *S. sclerotiorum* and further controlled disease severity and stem rot incidence on canola plants (Savchuk and Fernando, 2004). A four-year study on field experiments proved that PA23 is a successful biocontrol agent of *S. sclerotiorum* canola stem rot (Savchuk S, 2002; Zhang Y, 2004). The inhibitory effects against *S. sclerotiorum* are hypothesized to be dependent primarily on phenazine production. Detailed discussion of antibiotics involved in biocontrol will be limited to phenazine and pyrrolnitrin antibiotics; since their presence is detected in cell free supernatants of *P. chlororaphis* strain PA23.

1.4. Phenazine

1.4.1 Mode of Action

Phenazines are hypothesised to function as redox-reactive molecules. A phenazine antibiotic termed methanophenazine produced by Archaea *M mazei*

Go1 was studied for its redox reactive properties in a membrane bound electron transport system (Abken *et al*., 1998). This is the first evidence linking phenazine as an electron carrier in the respiratory chain, specifically the electron transfer system $F_{420}H_2$ heterodisulfide oxidoreductase. Both methanophenazine and 2-hydroxyphenazine (2-OH-PHZ) were proven to function as electron acceptors from $F_{420}H_2$ dehydrogenase, as well as, electron donors to heterodisulfide reductase (Abken *et al*., 1998).

1.4.2 Phenazine Biosynthetic Cluster

P. fluorescens 2-79*, P. aureofaciens* 30-84, and *Pseudomonas aeruginosa* PAO1 have evolved a biosynthetic pathway for the generation of the phenazine nucleus that is evolutionarily conserved at the genetic level. Phenazine antibiotic production occurs during the late exponential phase or at the onset of the stationary phase, (Turner and Messenger, 1986; Messenger and Turner, 1983; Thomashow and Pierson, 1991). In *P. chlororaphis* PCL1391, the phenazine biosynthetic cluster consists of eight genes (*phzA*-*phzH*) (Figure 1) (Chin-A-Woeng *et al.*, 2001b; 2005). The phenazine biosynthetic genes *phzA*-*phzG,* in PCL1391, are arranged in the same order as those found in *P. fluorescens* 2-79 (Mavrodi *et al.,* 1998). In PCL1391 the open reading frames for genes *phzA* through *phzH* indicate that it is one transcriptional unit, with the absence of a promoter or terminator sequence between them. In *P. aureofaciens* 30-84, there is an overlap between genes *phzD* and *phzE*, as well

Figure 1: A model for the regulatory mechanism(s) involved in phenazine biosynthesis. Abbreviations: A to H, *phzA* to *phzH* genes; C6-HSL, *N*-hexanoyl-L-homoserine lactone; *N*-AHL, *N*-acyl-L-homoserine lactone; PCN, phenazine-1-caboxamide (Chin-A-Woeng *et al.*, MPMI, 2005).

as, between genes *phzF* and *phzG.* The *phzH* gene has only been identified in *P. chlororaphis* PCL1391 and the protein it encodes possesses a high degree of similarity with asparagine synthetases. As expected for this class of enzymes, the N-terminal domain of PhzH contains a motif characteristic of class II glutamine aminotrasferases, that functions in the conversion of PCA to phenazine-1-carboxamide (PCN) (Chin-A-Woeng *et al.*, 2001b). In *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 there is a terminator sequence downstream *phzG* which not only indicates that this is the last gene in the phenazine biosynthetic operon (Mavrodi *et al.*, 1998), but also shows that *phzH* is exclusively expressed in *P. chlororaphis* PCL1391.

In *P. fluorescens* 2-79 and *P. aureofaciens* 30-84, the gene products of *phzA* and *phzB* regulate the type and relative amounts of the phenazine being produced (Mavrodi *et al*, 1998). The functional role of PhzA and PhzB was proven by the heterologous expression of the entire *phz* operon (*phzABCDEFG*) resulting in PCA production, compared to the expression of only *phzCDEFG,* resulting in the production of a mixture of aromatic and heterocyclic nitrogen-containing compounds. Furthermore, it is postulated that PhzA and PhzB may also function to stabilize the phenazine biosynthetic complex. This was supported by the finding that, in the absence of PhzA and PhzB, the phenazine biosynthetic system is operational, although, at a less efficient mode. The function of PhzC was initially hypothesized to be involved in the conversion of PCA to 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA) (Pierson III *et al.*, 1995). According to Mavrodi *et al*, (1998), even though the *P. aureofaciens*

PhzC has 93% identity to the *P. fluorescens* 2-79 PhzF protein, its location in the biosynthetic cluster does not support PhzC mediated conversion of phenazine-1 -carboxylic acid (PCA) to the hydroxylated moiety. In *P. chlororaphis*, PhzC protein has homology with the 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthases, involved in the first enzymatic step of the shikimate pathway catalyzing the condensation reaction of phosphoenolpyruvate and erythrose-4-phosphate to yield DAHP and inorganic phosphate. PhzD is hypothesized to function as an isochorismatase to remove the pyruvate moiety from 2-amino-2-deoxyisochorismic acid (ADIC) to yield 3-hydroxy-anthranilic acid. Lastly, proteins PhzF and PhzG may function in the condensation reaction resulting in phenazine-1, 6-dicarboxylic acid, (Mavrodi *et al.*, 1998), which is a precursor of phenazine-1-carboxamide (PCN) and other phenazine antibiotics (Leisinger and Margraff, 1979). Furthermore, the investigators postulated that additional genes, other than the *phzABCDEF*, might be involved in the conversion of PCA into other phenazine derivatives (Mavrodi *et al*., 1998).

A newly identified phenazine gene, *phzO,* is located downstream from the *phzXYFABCD* operon in *P. aureofaciens* 30-84 (Delaney *et al.*, 2001). To date, *phzO* has only been identified as a unique gene in this *Pseudomonas* spp. It functions in hydroxylating PCA forming 2-OH-PCA and 2-OH-PHZ. PhzO has homology to a two-component nonheme flavin-diffusible bacterial aromatic monooxygenases, which are NADP(H) dependent flavoproteins. Flavin acts as a co-substrate during the oxidation of the aromatic compound, through the use of molecular oxygen (Delaney *et al.*, 2001). The conversion of 2-OH-PCA to 2-

hydroxyphenazine (2-OH-PHZ) is a spontaneous reaction (Flood *et al.*, 1972). This was proven when synthetic 2-OH-PCA was tested at various pH ranges (from pH 4, 6, 7, and 8) when there was nearly no 2-OH-PHZ formed (0.2%) at pH 4 compared to a significantly higher production at pH 7 (74%) (Delaney *et al.*, 2001).

In *P. aeruginosa* PAO1, PCN and 1-OH-PHZ are also produced. In addition to the presence of the *phzH* gene; *phzM* and *phzS* are located downstream the *phzABCDEFG* biosynthetic operon. Functional analysis revealed that the *phzM*-encoded protein is a methylase that is responsible for the production of pyocyanin, while the *phzS*-encoded protein is a hydroxylase whose catalytic reaction forms 1-hydroxyphenazine (Mavrodi *et al.*, 2001).

1.4.3 Shikimate Pathway

Phenazine biosynthesis branches off from the shikimate acid pathway (McDonald *et al.*, 2001). The shikimate pathway is responsible for the synthesis of the aromatic compounds tyrosine, phenylalanine, and tryptophan. The primary metabolites phosphoenolpyruvate and erythrose-4-phosphate are converted to 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) by a condensation reaction catalyzed by DAHP synthase (Herrmann, 1995a; 1995b). Dehydroquinate synthase is the second enzyme catalyzing the conversion of DAHP to 3-dehydroquinate, which is later converted to 3-dehydroshikimate. 3-dehydroshikimate is converted to shikimate, shikimate 3-phosphate, 5-enolpyruvoyl shikimate 3-phosphate, and lastly, chorismate. Chorismate is a

precursor for *p*-amino benzoate, tyrosine, phenylalanine, tryptophan and *p*-hydroxy benzoate (Herrmann, 1995a; 1995b).

McDonald *et al* (2001) showed that in *P. fluorescens*, ADIC is the branch point from the shikimate biosynthetic pathway that either forms tryptophan through the catalytic activity of TrpAa and TrpAb or forms phenazine compounds via a PhzE-catalyzed reaction (McDonald *et al*., 2001). It is interesting to know that PhzE has domains homologous to TrpAa (athranilate synthase) and TrpAb (ADIC synthase), which are involved in the formation of *L*-tryptophan (Xie *et al.*, 2003). *L*-tryptophan is the precursor for pyrrolnitrin, suggesting that there may be an evolutionary link between phenazine and pyrrolnitrin biosynthesis.

1.5 Pyrrolnitrin

Pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chloro-phenyl) pyrrole] is an antibiotic with broad spectrum of activity against fungal plant pathogens (Hammer *et al.*, 1999; Kirner *et al*., 1998; Hamill *et al.*, 1970; Burkhead *et al.*, 1994).

1.5.1 Mode of Action

It is an antibiotic previously used against dermatophytic fungi belonging to the genus of *Trichophyton* (Arima *et al.*, 1965). The mechanism of action of pyrrolnitrin was initially reported by Tripathi and Gottlieb (1969) who observed that pyrrolnitrin inhibited endogenous and exogenous respiration in *Saccharomices cerevisiae*. This was demonstrated by measuring oxygen uptake with sodium succinate as a substrate to assay for the activity of succinate

oxidase. In addition oxygen uptake was monitored with cytochrome *c* as a substrate to assay for cytochrome oxidase activity. These studies revealed that mitochondrial respiration was inhibited by pyrrolnitrin (Tripathi and Gottlieb, 1969). The target site was deduced to be before cytochrome *c* because it decreased in NADH- and succinate-cytochrome *c* reductase activity while having no effect on the activity of cytochrome *c* oxidase. In addition, pyrrolnitrin inhibited the metabolism of glucose. This was proven by the decrease in labelled $^{14}CO_2$ produced from the breakdown of labelled ^{14}C-glucose in response to increasing levels of pyrrolnitrin.

1.5.2 Pyrrolnitrin Biosynthetic Cluster

Four genes encode the enzymes for the biosynthesis of pyrrolnitrin (Figure 2) (Hammer *et al.*, 1997). The first open reading frame (ORF) encodes PrnA, an enzyme that catalyzes the chlorination of *L*-tryptophan to 7-chloro-*L*-tryptophan (7-CLT). The second gene product encoded, PrnB, catalyzes the conversion of the indole ring to a phenylpyrrole and the decarboxylation of 7-CLT to monodechloroaminopyrrolnitrin, a product that is later chlorinated by PrnC. PrnD is the final gene product of the pyrrolnitrin biosynthetic cluster, catalyzing the oxidation of aminopyrrolnitrin to form pyrrolnitrin (Kirner *et al.*, 1998).

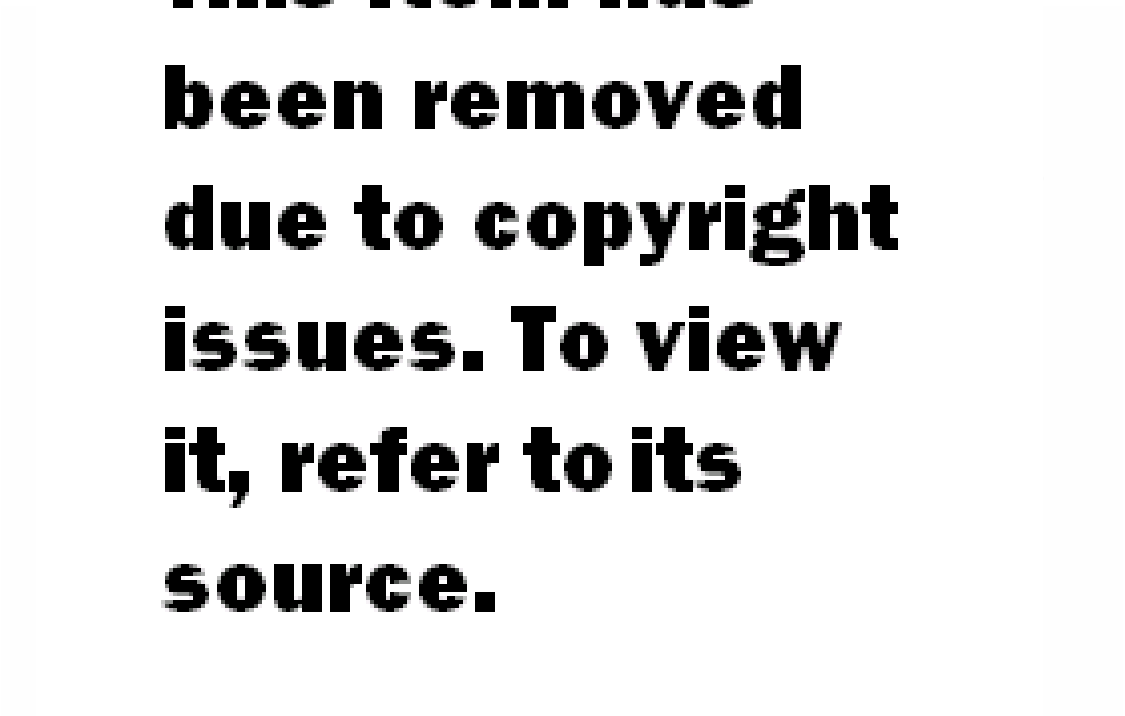

Figure 2. Pyrrolnitrin Biosynthesis (Kirner *et al.*, J Bact., 1998).

1.6 Regulation of Secondary Metabolite Production

1.6.1 Transcriptional Regulation

1.6.1.2 Sigma Factor Mediated

Fluorescent pseudomonads have been found to colonize a variety of habitats including plants and animals. Plant-associated *Pseudomonas* spp. encounter a multitude of environmental stress factors. In response to environmental conditions, the primary control of gene expression is mediated by the binding of specific sigma (σ) factors to the RNA polymerase core enzyme, necessary for promoter recognition. The expression of growth-related and housekeeping genes during exponential phase depends on RpoD (σ^{70}), whereas the expression of stationary phase genes is dependent on RpoS (σ^{s}). Throughout bacterial growth, σ factors are in competition for the binding of RNA polymerase (Maeda *et al.*, 2000; Jishage *et al.*, 1996). In plant-associated *Pseudomonas* spp., the levels of RpoD (σ^{70}) and RpoS (σ^{s}) influence the expression of secondary metabolites. For example, in *P. fluorescens* CHA0 an increase in RpoD (σ^{70}) increases the production of pyoluteorin and 2,4-DAPG both *in vitro* and in the rhizosphere, ultimately increasing the biocontrol ability of this bacterium (Maurhofer *et al.*, 1992; 1995; Schnider *et al.*, 1995). A mutation in *rpoS* increases the expression of *plt* (pyoluteorin) and *phl* (2,4-DAPG) biosynthetic genes. Conversely, an increase in the levels of RpoS (σ^{s}), functions to upregulate pyrrolnitrin, extracellular protease(s) and HCN production in the *P. fluorescens* strains CHA0 and Pf-5 (Sarniguet *et al.*, 1995; Whistler *et al.*, 1998;

Heeb *et al.,* 2005; Haas and Keel, 2003). These findings indicate that antibiotic production is influenced by direct competition between RpoD (σ^{70}) and RpoS (σ^{s}), mediating RNA polymerase promoter specificity. Currently, it is unknown whether the sigma factors exert their regulatory effects directly at antibiotic promoter regions or indirectly through the activation of additional regulators.

1.6.1.2 LysR family of Transcriptional Regulators

In prokaryotes, there are two large families of regulatory proteins. The first family consists of regulators designated LacI/GalR repressors. The second family is defined as the LysR family and it includes more than 50 autoregulatory transcriptional regulators (LTTR's), showing similarity in size, structure and function. Members of the LTTR family have a highly conserved amino terminus containing the helix-turn-helix DNA binding motif. Additional characteristics shared by LTTRs include: a) they are divergently transcribed from the promoters of their regulated target gene(s), b) their size ranges from 276-324 amino acid residues, and c) they can regulate the transcription of multiple and unlinked target genes (regulons) (Schell, 1993).

Henikoff *et al*. (1988) were the first to describe the LysR family of activator proteins. The transcriptional regulator LysR of *Esherichia coli* is the prototype of LTTR's, and it functions to activate diaminopimelate decarboxylase synthesis (lysine biosynthesis) in the presence of the coinducer molecule diaminopimelate (Stragier *et al*., 1983). Many LysR-type transcriptional regulators have been identified in plant-associated bacteria specifically in fluorescent *Pseudomonas*

spp. Their ability to survive environmental stress conditions in the plant environment is partially attributed to the diverse regulatory mechanisms that control the production of secondary metabolites. One of the most extensively characterized LTTRs is TrpI found in *P. aeruginosa*, *Pseudomonas putida*, and *Pseudomonas syringae*. TrpI transcriptionaly activates the expression of divergently transcribed *trpBA* genes, encoding TrpBA (tryptophan synthetase), an enzyme that catalyzes the last step in tryptophan biosynthesis (Crawford, 1989). The significance of TrpI in antibiotic production may be in that tryptophan is a precursor of pyrrolnitrin (Kirner *et al.*, 1998); a secondary metabolite produced by the biocontrol strain *P. fluorescens* Pf*5* (Rodriguez and Pfender, 1997). In another plant-associated bacterium, *P. aeruginosa* PA14, the LysR-type transcriptional regulator MvFR (multiple virulence factors regulator), positively activates the expression of phenazine biosynthetic operon *phnAB*, as well as the production of autoinducer signalling molecules and other secondary metabolites such as elastase and phospholipase (Cao *et al.*, 2001). Recently, Silby *et al.* (2005) identified a gene termed *finR* (fungal inhibition), encoding a LysR-type regulator that is involved in the antifungal activity in *P. aureofaciens* PA147-2 biocontrol strain. The investigators found that, compared to the wild type, an insertional inactivation of *finR* resulted in loss of antifungal metabolite production and subsequently an increase in competitive fitness in liquid culture under starvation growth conditions. These studies have shown that in response to environmental stress conditions, such as predation and nutrient availability, bacteria regulate the activity of LTTRs. *Pseudomonas* spp. can increase their

competitive fitness through the LTTR mediated production of biocontrol secondary metabolites. Conversely, repression of specific LTTRs can function in the downregulation of secondary metabolism in order to increase the energy resources available for primary metabolism or starvation response, ultimately aiding the bacterium to adapt and survive under specific environmental conditions.

1.6.2 Quorum Sensing

Gram-negative, (Swift *et al.,* 1993; Fuqua *et al.,* 1996), and gram-positive, (Kleerebezem *et al.,* 1997), bacteria have evolved regulatory mechanisms to control gene expression and metabolism in response to environmental conditions. This regulation is defined as quorum sensing and is based on intercellular communication through the use of a diffusible signal. Quorum sensing was initially described in the marine luminescent bacterium *Vibrio fischeri* (Nealson, 1977). In *V. fischeri,* environmental sensing of population density is achieved through a diffusible signal termed autoinducer that later binds to the transcriptional activator LuxR. As a complex they activate the transcription of luminescence genes (Nealson, 1977; Eberhard, 1981; Kaplan and Greenberg, 1985).

In *Pseudomonas* spp., quorum sensing is involved in regulating secondary metabolism, including the production of metal chelators (pyoverdines), lytic enzymes (protease, chitinazes), volatiles (HCN), and antifungal antibiotics. Pseudomonas bacteria producing autoinducer signal molecules known as

acylhomoserine lactones (AHL) include: *P. aeruginosa*, *P. chlororaphis*, *P. fluorescence*, *P. putida*, *Pseudomonas syringae*, *Pseudomonas cichorii*, and *Pseudomonas corrugata* (Pearson *et al.,* 1994; Elasri *et al*., 2001). A study conducted on *Pseudomonas* strains isolated from various regions of the world, found that quorum sensing and AHL production is more common among plant-associated *Pseudomonas* spp. compared to soilborne *Pseudomonas* spp (Pierson *et al.*, 1998). These findings indicated that quorum sensing and the regulation of secondary metabolite production occurs under environmental conditions involving bacteria-host interactions. Pierson *et al.* (1998) were the first to identify that the PhzR/PhzI quorum sensing system regulates phenazine production in *P. aureofaciens* 30-84. PhzI is an AHL synthase responsible for generating the signal molecule *N*-hexanoyl-homoserine lactone (C_6-HSL) (Wood *et al.*, 1997). PhzR is a transcriptional activator that when complexed with C_6-HSL regulates expression of phenazine biosynthetic genes (Pierson *et al.* 1994; 1995). Activation is mediated through the direct binding of PhzR-AHL complex at the *phz*-box, located upstream of the phenazine biosynthetic genes, or through the activation of an intermediate factor that, in turn, regulates the phenazine biosynthetic cluster (Figure 1).

In response to environmental conditions, rhizosphere competence of the total population is achieved through bacterial communication. The outcome of cross-communication may have an antagonistic or a supporting role on secondary metabolite production. Wood *et al.* (1997) demonstrated that bacteria in the wheat rhizosphere are capable of positively influencing the expression of

genes in a bacterial population inhabiting the same niche. They tested the ability of a bacterial population to restore phenazine expression in an autoinducer-deficient (*phzI*) mutant of *P. aureofaciens* 30-84. Their findings indicated that AHL could function as an interpopulation signal and that different bacterial species within the same rhizosphere can have an additive effect on antibiotic production (Wood *et al*., 1997). Furthermore, positive cross-communication was proven to be a common phenomenon among bacteria in the wheat rhizosphere. A study performed on non-isogenic bacterial isolates from different geographic regions, showed that they were able to induce phenazine expression in a test strain. These bacterial isolates produced AHL(s) that activated *phzI-inaZ* reporter fusion *in situ* in *P. aureofaciens* 30-84 (Pierson III *et al*., 1998).

1.6.3 Positive Autoregulation

Schnider-Keel *et al.* (2000) explained the importance of positive autoinduction. From an ecological aspect, autoinduction may be essential in combating fungal diseases through a rapid increase in antibiotic production, only when there is a sufficient bacterial population to efficiently support the antifungal effect. This biocontrol effect was proven to be amplified when positive autoregulation functions as a cross-communication mechanism among 2,4-DAPG producing bacteria (Maurhofer *et al*., 2004). It is possible that these regulatory mechanisms serve as an evolutionary adaptation, especially when the production of antifungal compounds can induce fungal defense mechanism(s) (Morrissey and Osbourn, 1999). Overall, the biocontrol effect will be more

efficient through the use of cross-communication among homogenous or mixed bacterial populations.

Autoregulation of 2,4-DAPG biosynthesis occurs at the transcriptional level (Bangera and Thomashow 1999). The mechanism postulated is as follows; 2,4-DAPG forms a complex with a repressor protein, PhlF, thereby lifting repression of the *phlA* promoter region. Similarly, positive autoregulation of pyoluteorin has been identified in *P. fluorescence* strains Pf-5 and CHA0 (Brodhagen *et al.,* 2004; Haas and Keel, 2003). Positive autoregulation has also been demonstrated in *P. aeruginosa* siderophore biosynthesis. This was apparent because exogenous pyochelin siderophore induced the expression of *pchEF,* encoding the pyochelin synthetases (Reimmann *et al.,* 1998). Gutterson also postulated that the production of the antifungal antibiotic oomycin A may be under a positive feedback mechanism in *P. fluorescence* Hv37a (Gutterson, 1990).

1.6.4 Negative Regulation

The ability of one antibiotic to negatively regulate expression of a different antibiotic has been previously demonstrated. In *P. fluorescens* Pf-5, pyoluteorin and 2,4-DAPG inhibit each other's biosynthesis at the transcriptional level. This was proven when pyoluteorin-deficient mutants were found to produce more 2,4-DAPG than the wild type; consistent with the idea that pyoluteorin represses 2,4-DAPG expression (Brodhagen *et al.*, 2004). Additionally, pyoluteorin, 2,4-DAPG, and salicylate affect the production of other antibiotics produced by *P.*

fluorescence (Haas and Keel, 2003), suggesting that depending on the environmental conditions, certain antibiotics may be preferentially favoured for mediating biocontrol.

Similarly, fungal toxins can negatively regulate antibiotic production. *F. oxysporum* f. sp. *radicis-lycopersici* produces fucaric acid, that functions as a phytotoxin, as well as an inhibitor of PCN production, by repressing *phzR* expression in *P. chlororaphis* PCL1391 (van Rij *et al.*, 2004; 2005), and 2,4-DAPG production in *P. fluorescens* CHA0 (Duffy and Defago, 1997).

1.6.5 Global Regulatory Mechanisms

1.6.5.1 Global antibiotics and cyanide control (Gac) System

GacS/GacA is a two-component signal transduction system present in many gram-negative bacteria including *Pseudomonas* spp and *Escherichia coli.* GacS (LemA) is a transmembrane sensor kinase first identified in *Pseudomonas syringae* pv. *syringae* B728a (Willis *et al.*, 1990). In response to an environmental signal, GacS is autophosphorylated and, subsequently, it transfers a phosphoryl group to its cognate cytoplasmic response regulator GacA. GacA was first identified in *P. fluorescens,* (Laville *et al.*, 1992), and upon activation by phosphorylation, it controls its target genes either by a direct or indirect mechanism. For many *Pseudomonas* rhizobacteria, GacS/GacA controls transcription of secondary metabolites. Unlike most two–component systems in bacteria, the genes encoding GacS and GacA are unlinked (Nam *et al.*, 2003; Kinscherf and Willis, 1999).

The GacS/GacA system has been found in both pathogenic and nonpathogenic *Pseudomonas* spp. The non-pathogenic bacteria investigated include: *P. fluorescens* Pf-5, CHA0, BL915, *P. chlororaphis* PCL1391, and *P. aureofaciens* strain 30-84. The pathogenic bacteria for which GacS/GacA has been studied include *P. syrigae* pv. *syringae* and *P. aeruginosa*, which are pathogens for plants and animals, respectively. Since both pathogenic and nonpathogenic *Pseudomonas* spp. have GacS/GacA two-component regulatory systems, it is of importance to identify what signal(s) is commonly present and responsible for virulence or protection, depending on the bacteria-host association (Goodier and Ahmer, 2001). Currently, the environmental signal responsible for GacS activation has not been identified.

GacS/GacA regulation of target gene expression occurs by positively regulating AHL synthesis (Chancey *et al.*, 1999; Kinscherf & Willis, 1999; Reimmann *et al.*, 1997), as well as acting independently of the AHL transduction pathway (Chancey *et al.*, 1999; Kinscherf and Willis, 1999; Pessi and Haas, 2001). In *P. fluorescens*, no AHL signals have been detected (Heeb *et al.*, 2002); however the GacS/GacA system positively regulates secondary metabolite and enzyme production in this genetic background.

1.6.5.2 GacS/GacA and Secondary Metabolite Production

In *Pseudomonas* spp., a mutation in either gacS or gacA impairs their biocontrol or pathogenic abilities. The biocontrol strains of *P. chlororaphis* and *P. aureofaciens*, harboring a mutation in the *gacS* region, lost its ability to protect

plants against fungal pathogens (Schmidt-Eisenlohr *et al.*, 2003; Chancey *et al*., 2002). *In vitro* assays showed that the lack in biocontrol activity was attributed to the deficiency in secondary metabolite production. In these genetic backgrounds, a *gacS* or *gacA* mutation impaired the ability to produce antifungal metabolites PCN, PCA, 2-OH-PCA, protease, and AHL(s) (Schmidt-Eisenlohr *et al.*, 2003; Chancey *et al*., 2002). Likewise, in the plant pathogenic bacterium *P. syrigae* pv. *syringae* B728a, GacS/GacA is required for the production of the toxin syringomycin, the extracellular polysaccharide alginate, and exoprotease (Hirano and Upper, 2000; Willis *et al.*,. 2001).

1.6.5.3 GacS/GacA and Growth Conditions

In *P. fluorescens* CHA0, *gacS* and *gacA* spontaneous mutations occur at high frequencies under nutrient-rich growth conditions and during stationary growth phase (Duffy and Defago, 2000). This suggests that there is a selective pressure towards a *gacS* or *gacA* mutation that may impact a growth advantage phenotype. In *P. chlororaphis* SPR044 and *P. aureofaciens*, a *gacS* mutation caused bacteria to have a reduced lag phase and an earlier entrance into the exponential growth phase compared to the wild type (Schmidt-Eisenlohr *et al*., 2003; Chancey *et al.*, 2002). It has been suggested that mutations in *gacS* or *gacA* may be beneficial for the entire bacterial population. Studies have shown that *gacS* mutants produce higher levels of iron chelators, (Duffy and Defago, 2000; Chancey *et al.*, 2002), indicating that in addition to a growth advantage provided by a *gacS* mutant population, the biocontrol activity mediated

predominantly by the wild-type population may be improved by limiting the levels of iron in the environment at an earlier time point.

1.6.6 Abiotic Factors Influencing Secondary Metabolite Production in *Pseudomonas* spp.

Environmental factors influencing antibiotic production include carbon sources and minerals. For example, phenazine antibiotic production is stimulated by certain carbon sources. Glucose and glycerol exhibit a stimulatory effect in PCN production by *P. chlororaphis* PCL1391 (van Rij *et al.*, 2004) and *P. aeruginosa* (Kanner *et al.*, 1978), as well as PCA production by *P. fluorescens* 2-79 (Slininger and Shea-Wilbur, 1995). Other antibiotics influenced by carbon source include pyoluteorin and 2,4-DAPG, which show increased production in the presence of glycerol and glucose, respectively (Duffy and Defago, 1999). Dilution of growth media amended with glucose or glycerol had a stimulatory effect on 2,4-DAPG but not on pyoluteorin (Duffy and Defago, 1999). This differential effect on antibiotic production within the same organism may reflect the antibiotic specificity required under environmental changes in the ecosystem. Pyrrolnitrin production was stimulated by mannitol and fructose, and to a lesser magnitude, by glucose and glycerol (Duffy and Defago, 1999),

In *P. chlororaphis* PCL1391, PCN production is stimulated by aromatic amino acids; tyrosine, phenylalanine, and to a smaller magnitude, tryptophan. Likewise, nitrogen sources like ammonia exhibit a stimulatory effect on PCN

production by *P. aeruginosa* (Kanner *et al*., 1978) and *P. chlororaphis* PCL1391 (Slininger and Shea-Wilbur, 1995).

Mineral-mediated regulation of antibiotic production is evident in *Pseudomonas* spp. In *P. fluorescens,* PCA production is increased in the presence of iron or magnesium (Slininger and Jackson, 1992; Slininger and Shea-Wilbur, 1995). The presence of inorganic phosphate has inhibitory effects on pyoluteorin, 2,4-DAPG in *P. fluorescens* (Duffy and Defago, 1999) and phenazine production in *Pseudomonas* spp. (Martin *et al.*, 1994; Turner and Messenger, 1986). Conversely, inorganic phosphate induced pyocyanine phenazine production in *P. aeruginosa* (Turner and Messenger, 1986).

Abiotic factors such as pH, temperature, and oxygen have been shown to significantly influence antibiotic production. The pH optimum for PCN production in *P. chlororaphis* PCL1391 is in the alkaline range (pH 7-8) (Duffy and Defago, 1999), while in *P. aeruginosa* PCN production is optimal only at pH 7 and it is reduced at pH 8 (Slininger and Shea-Wilbur 1995). In *P. chlororaphis* PCL1391, PCN production is inhibited by salt stress (0.1 M NaCl), low temperature (16°C), and oxygen (10-20%). Under low oxygen conditions, PCN production increased and occurred at an earlier point in growth (van Rij *et al.* 2004). Since abiotic environmental factors influence the type of antibiotic being produced, biocontrol activity may vary under different environmental conditions.

1.7 Motility

Bacterial motility is essential for biocontrol activity in *Pseudomonas* spp. Motility mutants of *P. fluorescens* WCS374 (de Weger *et al.*, 1987), and *P. putida* WCS358, (Simons *et al.*, 1996), were impaired in their ability to colonize the tomato and potato rhizosphere. *P. chlororaphis* PCL1391 motility mutants were deficient in root colonization and biocontrol activity despite their ability to produce PCN (Chin-A-Woeng *et al.*, 2000). These studies suggest that efficient biocontrol activity requires the ability to move across a plant surface in addition to having a functional circuitry of secondary metabolism.

Three types of motility have been shown to be important for the biocontrol ability of *Pseudomonas* strains, specifically swimming, swarming and twitching motility. Swimming motility involves the movement of bacterial cells across water channels through the use of polar flagella. In *P. aeruginosa* and *P. fluorescens*, flagellar motility is important for stable attachment and the formation of a monolayer of bacterial cells on abiotic surfaces (Lawrence *et al.*, 1987; O'Toole and Kolter, 1998). Swarming motility is based on flagellar-mediated translocation across a surface in the presence of biosurfactants, carbohydrates, peptides, and proteins. In contrast to swimming, swarming motlity is a social phenotype where bacteria migrate in groups by making cell-cell contact along their longitudinal axis, ultimately colonizing a greater surface area (Fraser and Hughes, 1999). Twitching motility is essential for adherence and surface-associated movement by extension and retraction of the pilus filament (O'Toole and Kolter, 1998 b)

1.8 Biofilms

In nature, bacteria cells are found predominantly as a biofilm, rather than in a planktonic (free living) state (Costerton *et al.*, 1995). Bacteria initiate biofilm development by adhering to surfaces that provide a growth advantage, through the presence of organic macromolecules (proteins, polysaccharides and lipids). Following the initial adhesion, bacteria produce extracellular macromolecules leading to cell-cell attachment and microcolony formation. Biofilms consist of micro-colonies (15% total volume) embedded in an exopolysaccharide matrix (85% total volume) with intervening fluid-filled channels that support nutrient and waste transport (de Beer *et al.*, 1994).

1.8.1 Biofilms Associated with Plant Surfaces

On plant surfaces, biofilms provide the innate bacterial population with tolerance to many environmental stress factors such as UV radiation, dryness, limited nutrition and exposure to antibiotics that are otherwise detrimental to their planktonic counterparts. Several forms of bacterial growth have been detected on plant surfaces. These include individual cells, microcolony aggregates and biofilms, where the bacteria are embedded in an extracellular polysaccharide matrix. The main plant surfaces supporting bacterial growth include the rhizosphere (root surfaces) and the phyllosphere (leaf surfaces) (Fuqua and Matthysse, 2001).

Biofilms in the rhizosphere are generally larger and more stable due to the hydrophilic surface of the roots. This is due to two main factors, first the soil

environment supports the development of a water film on the roots and second nutrient availability is higher due to nutrients provided by the soil, as well as the roots, which secrete carbohydrates and amino acids. All together, the rhizosphere provides the plant-associated bacteria with favourable growth conditions that lead to biofilm formation. Conversely, in the phyllosphere, surfaces are covered by a waxy cuticle, making them relatively dry and nutrient-limited. The levels of hydration depend on the rainfall and the humidity in the atmosphere. Biofilm formation by leaf-associated bacteria, is an adaptation mechanism for dealing with dryness and UV radiation (Beattie and Lindow, 1995; Fuqua). Studies have shown that the leaf surfaces enable biocontrol strains of *P. fluorescens* and *P. syringae* to develop biofilms (Boureau *et al.*, 2003; Beattie and Lindow, 1995; Takahashi and Doke, 1984).

Molecular analyses of mechanisms involved in biofilm formation have revealed that several factors including flagella, type IV pili and biosurfactant production are involved in different stages of biofilm development (O'Toole and Kolter, 1998a; 1998b; Pratt and Kolter, 1998; Walker *et al.*, 2004; Lawrence *et al.*, 1987; Whitchurch *et al*., 2004; Bradley, 1980). During the initial stages of biofilm formation on a solid surface, flagella are involved in cell adhesion and monolayer formation, while type IV pili function in micro-colony formation. In later stages of biofilm development, biosurfactant facilitates cell-cell attachment (O'Toole and Kolter, 1998; Parkins *et al.*, 2001). To our knowledge this is the first study analyzing the molecular mechanisms of biofilm formation involved in *P.*

chlororaphis. It is expected that many factors involved in biofilm development in other *Pseudomonas* species will also be important for *P. chlororaphis* PA23.

1.9 Thesis Objectives

The main focus of this thesis is to investigate the molecular mechanisms mediating biocontrol in *P. chlororaphis* strain PA23. The specific objectives are i) to isolate PA23 mutants deficient in biocontrol activity towards *S. sclerotiorum*; ii) to characterize these mutants with respect to secondary metabolite production, growth rate, motility and biofilm formation; and iii) to assess the ability of these mutants to persist in the phytosphere.

2.0 MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids, Media and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultured at 37°C on Lennox LB agar (Difco Laboratories, Detroit, MI, USA). *P. chlororaphis* PA23 was cultured at 28°C on LB agar, M9 minimal media (Difco), Minimal M9 casamino acid (M9CA; Difco), Bacto-Terrific broth (TB; Appendix 1) or Peptone Tryptic soy broth (PTSB; Appendix 1). For antifungal activity, protease, lipase and chrome azurol S (CAS) assays, cultures were grown in LB broth at 28°C. For long-term storage, bacterial cultures were maintained in 15% glycerol at -70ºC.

2.2 DNA Manipulations

For plasmid propagation and selection of mutants, media were supplemented with the following antibiotics: For *E. coli*, tetracycline (Tc; 15 µg/mL) (Sigma-Aldrich, Canada), gentamicin (Gm; 15 µg/mL) (Research Products International Corp. USA), ampicilin (Amp; 100 µg/mL), and for *P. chlororaphis*, rifampicin (Rif; 25 µg/mL) (Sigma), Tc; 15 or 100 µg/mL, Gm 25 µg/mL, tobramycin (Tb; Bayer Leverkuse, Germany) and ciprofloxacin (Cipro; Bayer).

Genomic DNA was isolated according to the procedure described by Meabe *et al*. (1982), with the following modifications. Five mL of an overnight

Table 1. Bacterial strains and plasmids

Strains	Genotype	Source or Reference
Pseudomonas chlororaphis		
PA23	Phz^+Rif^R wild type (soy bean plant isolate)	Fernando, 1996
PA23-314	Phz^- Rif^R *gacS*::Tn*5*-OT182 genomic fusion	This study
PA23-314 (pUCP23-gacS)	Phz^- Rif^R *gacS*::Tn*5*-OT182 genomic fusion, *gacS* complemented strain	This study
PA23-2490	Phz^- Rif^R *gacS*::Tn*5*-OT182 genomic fusion	This study
PA23-2490(pUCP23-*gacS*)	Phz^- Rif^R *gacS*::Tn*5*-OT182 genomic fusion, *gacS* complemented strain	This study
PA23-63	Phz^- Rif^R *phzE*::Tn*5*-OT182 genomic fusion	This study
PA23-754	Phz^- Rif^R *phzC*::Tn*5*-OT182 genomic fusion	This study
PA23-443	Phz^- Rif^R Tn*5* in LysR family transcriptional regulator	This study
PA23-443(pUCP23-*gacS*)	Phz^- Rif^R Tn*5* in LysR family transcriptional regulator, *gacS in trans*	This study
***Escherichia coli* strains**		
DH5α	*supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	
SM10	Mobilizing strain; RP4 *tra* genes integrated in chromosome; Km^R Tc^R	
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB* lac^qZ*ΔM15* Tn*10* (Tc^R)]	
Plasmids		
pOTI82	pSUP102(GM)::Tn*5*-OT182 Cm^R Gm^R Amp^R Tc^R	
pOT182-314 (*Xho*I)	pOT182 containing *gacS*::Tn*5*-OT182 genomic fusion	This study
pOT182-443 (*Xho*I)	pOT182 containing LysR type transcriptional regulator::Tn*5*-OT182 genomic fusion	This study
pOT182-63 (*Xho*I)	pOT182 containing *phzE*::Tn*5*-OT182 genomic fusion	This study
pUCP23	Broad-host-range vector; IncP OriT, Amp^R Gm^R	Olsen, 1982
pUCP23-*gacS*	pUCP23 containing *gacS* from *P. chlororaphis* PA23	This study
pCR^R2.1TOPO	Cloning vector for PCR products	Invitrogen
pME3219	pME6010 with a 410-bp *hcnA* promoter fragment and an *hcn*A'-*'lacZ* translational fusion at *P*stI site in *hcnA*	Laville, 1998

culture ($OD_{600\ nm}$ = 2.0) was sedimented by centrifugation (10,000 x g) and washed with an equal volume of cold Phosphate Buffered Saline (1X PBS) followed by a second wash with an equal volume of $T_{10}E_{25}S_{150}$ (TES) buffer (Appendix 1). Cells were resuspended in 2.5 mL of $T_{10}E_{25}$ buffer (Appendix 1) and a 0.25 mL aliquot of lysozyme (2 mg/mL in TE) was added and incubated for 15 min. at 37°C. Following cell lysis, 0.6 mL of Sarcosyl-protease solution (10% Sarcosyl, 5-mg/mL protease in TE) was added and incubated for an additional 1 h at 37°C. Lysed cells were extracted twice with 5 mL of chloroform. The aqueous phase was treated with ammonium acetate (0.3M) and precipitated with 0.54 volumes of isopropanol at -20°C. Following centrifugation, the precipitated DNA was sequentially washed with 95% ethanol and 70% ethanol. The pelleted DNA was air dried at 28°C for ~15 min and resuspended in 50 µL TE or sdH_2O. The DNA preparation was treated with 1 µg/mL of RNase at 37°C for 30 min.

Small- and large-scale plasmid preparations were performed by alkaline lysis as described by Sambrook *et al.* 1989 and the Qiagen midi plasmid preparation kit (Qiagen Inc.), respectively.

2.3 Standard DNA Procedures

DNA was digested with appropriate restriction endonucleases (Invitrogen) following manufacturer's instructions. For cloning experiments, LB agar was supplemented when required with 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (IPTG; 20 mM; Invitrogen) and 5-bromo-4-chloro-3-indolyl-

β-D-galactopyranoside (X-gal; 40 µg/mL; Invitrogen). DNA fragments were separated on 0.7 to 1.0% agarose (Invitrogen) gels in Tris-acetate buffer and gel purified with the UltraClean^{TM}15 DNA purification kit (MOL BIO Laboratories, Inc.).

2.4 PCR

DNA template of 1 µL (20-60 ng) was amplified with 1.25 µL (2 pmol) of each primer. The PCR master mix consisted of 2.5 µL of 10X *Taq* PCR buffer minus Mg [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 1.5 µL $MgCl_2$ (3 mM) and 0.2 mM of each deoxyribonucleotide (dATP, dCTP, dGTP and dTTP). Two U of *Taq* DNA polymerase was added (0.4 µL) in a total reaction volume of 25 µL. All PCR reagents and primers were purchased from Invitrogen Life Technologies (Canada). For each PCR experiment, a negative control (DNA free) and a corresponding positive control were screened at the same time as the test samples. PCR reactions were performed in a Techgene thermocycler (Fisher Scientific). The PCR conditions were as follows: the template was denatured at 95°C for 3 min., followed by 30 cycles of denaturation at 95°C for 1 min., annealing at 55-68°C for 1 min. and extension at 72°C for 2 min. Final extension was set at 72°C for 10 min. Samples were held at 4°C prior to gel electrophoresis.

Digoxigenin (DIG) (Roche Diagnostics, Canada) labelling of probes was performed by PCR amplification using a DIG DNA labelling mix (Roche)

containing dUTP conjugated to digoxigenin (DIG) following manufacturer's instructions.

2.5 Tn*5*-OT182 Transposon Mutagenesis

Bacterial conjugations were performed to introduce Tn*5*-OT182 into *P. chlororaphis* PA23 by biparental mating. The recipient PA23 and the donor *E. coli* SM10 harbouring pOT182 were separately propagated overnight in LB medium to stationary phase (~$OD_{600\ nm}$ = 2.0), sedimented by centrifugation (5,000 x g) and resuspended in an equal volume of LB medium. Equal volumes of donor and recipient were mixed to a total of 1.5 mL and pelleted by centrifugation (5,000 x g). The cells were resuspended in 0.1 mL of LB and spotted onto sterile 0.45 µm-pore-size nitrocellulose filters placed on LB agar plates supplemented with 10 mM $MgSO_4$. The donor and recipient were spotted on separate filters as negative controls. After incubation for 8-10 h at 28ºC, the cells were washed from the filters with 1 mL LB medium and a 100 µL aliquot was plated onto prewarmed LB agar plates amended with Tc 15 µg/mL and Rif 25 µg/mL. Transconjugants were isolated after incubation for 30-36 h at 28°C and plated onto Pseudomonas isolation agar (PIA; Difco) supplemented with Tc 50 µg/mL. For each mating, 5-10 Tc^R colonies were screened by PCR to ensure that transconjugants contained a Tn5 insertion. For the Tn*5* PCR, primers TNP*5*-FORWARD (5'-ACCATTTCAACGGGGTCTCAC-3') and TNP*5*-REVERSE (5'-TGACTTCCATGTGACCTCCTA-3') were designed from the Tn*5*-OT182 transposase *tnp* region. To determine the site of Tn*5*-OT182 chromosomal

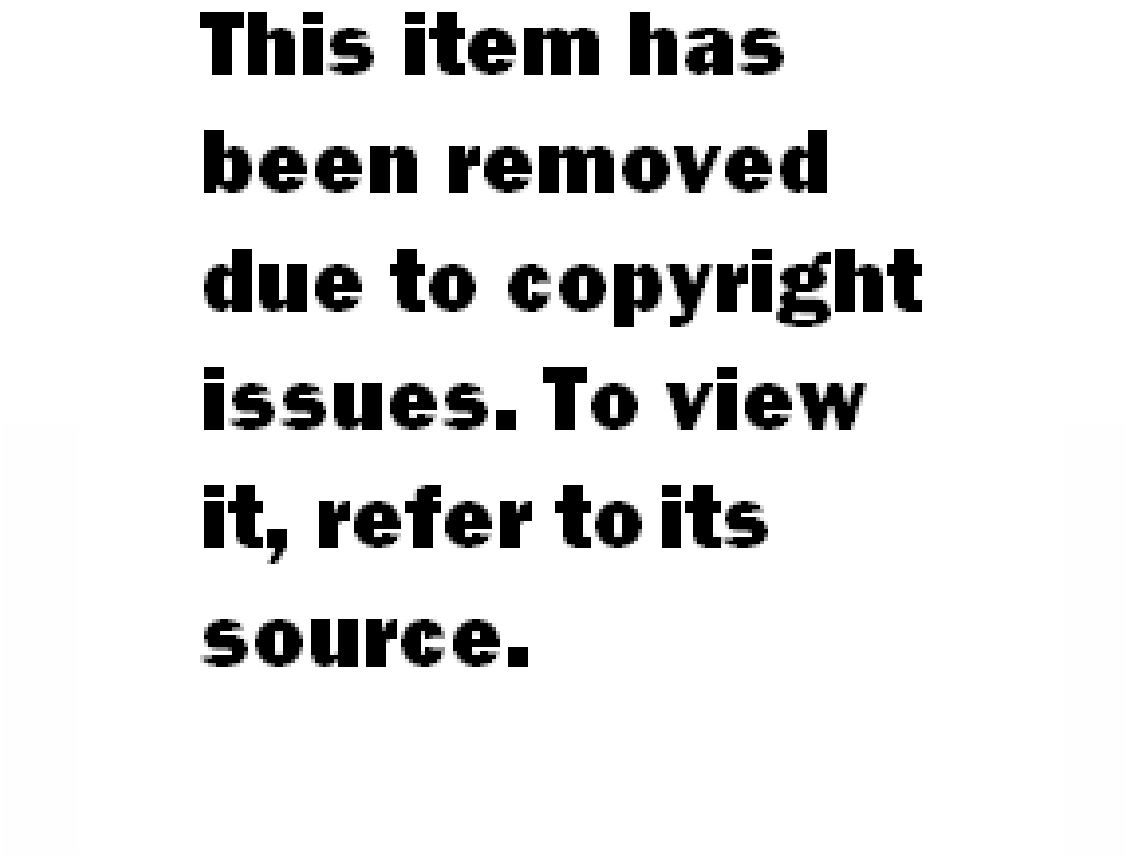

Figure 3. Physical and genetic map of Tn*5*-OT182. Abbreviations: *lacZ*, promoterless gene encoding β-gal; *bla*, β-lactamase; ori, pBR325 origin of replication; B, *BamH*I; C, *Cla*I; Ss, *Sst*I; Bs, *Bsi*WI; E, *EcoR*I; Cs, *Csp*451; X, *Xma*I; Sc, *Sca*I; V, *Vsp*I; Sa, *Sal*I; Ap, *Apa*I; S, *Stu*I; A, *Avr*II; H, *Hind*III; Nh, *Nhe*I; N, *Not*I; and Xh, *Xho*I. (Merriman TR, and Lamont IL. Gene. 1993).

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insertion, the self-replicating transposon and the flanking DNA was cleaved with *Xho*I, *BamH*I or *Cla*I restriction endonuclease. Plasmid rescue (ligation) reactions containing 2 µg of DNA in a total volume of 20 µL were incubated at 15°C for 16 h. The ligations were precipitated in two volumes of 95% ethanol, centrifuged for 30 min. (12,000 x g), resuspended in 10 µL sdH_2O and then electroporated into DH*5*α competent cells. After electroporation, cells were resuspended in 1 mL of LB broth and incubated for 6-8 h at 37°C with no aeration. Cells were sedimented by centrifugation (2,000 x g) and plated on LB agar supplemented with Tc 15 µg/mL, to select for *E. coli* harbouring rescue clones.

2.6 Sequence Analysis

Plasmids isolated from Tc^R *Xho*I clones were sent for sequencing using oligonucleotide primer Tn*5*-ON82 (5'GATCCTGGAAAACGGGAAAGG-3') which anneals upstream the 5' end of the Tn5-OT182 *lacZ* coding region. *BamH*I or *Cla*I rescue plasmids were sequenced using primer Tn*5*-OT182 RIGHT SIDE (5'-ATGTTAGGAGGTCACATG-3') which anneals to the 3' end of Tn*5*-OT182. All sequencing was performed at the University of Calgary Core DNA Services. Sequences were analyzed using blastn and blastx databases.

2.7 *prnD* PCR amplification

Oligonucleotide primers for *prnD* PCR were designed from *P. fluorescens* Pf5 (de Souza and Raajimakers, 2003). PRND1 ('5-GGGGCGGGCCGTGGTGATGGA-3') and PRND2 ('5-YCCCGCSGCCTGYCTGGTCTG-3') primers were used to create a DIG labeled *prnD* probe for Southern analysis. The PCR program conditions for *prnD*-DIG amplification were as follows: Denaturation of the template occurred at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 1 min., annealing at 68°C for 1 min. and extension at 72°C for 1 min. Final extension was set at 72°C for 5 min. Samples were held at 4°C prior to gel electrophoresis.

2.8 Isolation and Cloning of the PA23 *gacS* Gene

To clone the PA23 *gacS* gene, oligonucleotide primers NEW GacS-F (5'-GGGATTCATTAGCTTCTGCAA-3') and NEW GacS-R (5'-TGGCTGCTGAAGAGAATCGT-3') were designed from the *P. chlororaphis* PCL1391 *gacS* gene sequence (GeneBank accession no. AF192795). *P. chlororaphis* PA23 DNA template (1 µL; 20 ng) was amplified with 1.25 µL (2 pmol) of each primer. The PCR master mix consisted of 2.5 µL of 10X High Fidelity PCR buffer minus Mg [60 mM Tris-SO_4 (pH 8.9), 18 mM Ammonium Sulfate], 1.5 µL $MgSO_4$ (1.5 mM) and 0.2 mM of each deoxyribonucleotide (dATP, dCTP, dGTP and dTTP). Two U of Platinum^R *Taq* DNA Polymerase High Fidelity (Invitrogen) was added (0.4 µL) in a total reaction volume of 50 µL. The PCR conditions for *gacS* are the same as those mentioned above with the

following modifications: initial denaturation at 94°C for 2 minutes, 30 cycles of denaturation at 94°C for 30 sec., annealing at 55°C for 30 sec. and extension at 68°C for 3 min. Final extension was set at 68°C for 5 min.

The *gacS* PCR amplified product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). Addition of 3' A-overhangs to a 3 µL aliquot of the *gacS* purified PCR product (2 µg) was performed by adding 2 µL of 10XTaq PCR buffer minus Mg [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 1.5 µL $MgCl_2$ (3 mM), 0.5 µL of 10 mM dATP and 0.5 µL of *Taq* polymerase (2.5 U) in a total reaction volume of 20 µL. The reaction was incubated for 15 min. at 72°C. A TOPO cloning kit was used to clone the *gacS* PCR product into pCR2.1-TOPO vector according to the manufacturer's instructions. Briefly, the cloning reaction was performed with 4 µL of *gacS* PCR product (0.384 µg), 1 µL pCR2.1-TOPO vector and 1 µL TOPO salt. The reaction was mixed by gently inverting the tube at room temperature for 30 min. These conditions were designed to favour cloning of large PCR products (1>kb). Transformation of the TOPO cloned *gacS* PCR product was performed by adding 2 µL of the *gacS* TOPO cloning reaction into a vial of TOP10F' chemically competent cells, gently mixing the tube and incubating on ice for 5 min. Following heat-shock, the cells were incubated at 37°C for 1 h with aeration (150 RPM). Selection of *gacS* clones was performed by isolating white or light blue colonies on LB agar plates supplemented with IPTG, X-gal and Amp. The orientation of the *gacS* insert was determined by sequencing using M13 Forward Primer (5'-CTGGCCGTCGTTTTAC-3').

2.9 Isolation and Cloning of the PA23-443 Mutated Gene Encoding the Transcriptional Regulator of the LysR Family

To clone the gene harbouring the Tn*5* insertion in mutant PA23-443, oligonucleotide primers 443 FORWARD NEW (5'-AAGTACGGGGCGTAACTGTC3') and 443 2.2 REVERSE (5'-ATCCAGTTGCTGGAGCGTATT-3') were designed from the *P. chlororaphis* PA23 sequence flanking the Tn*5* insertion in mutant PA23-443. The PCR master mix was the same as the one mentioned above with the following modifications: 2.5 µL of 10X PCR buffer (BioLabs), two U of *Taq* DNA Polymerase (BioLabs) (0.4 µL) and 1 µL of dimethyl sulfoxide (DMSO) was added in a total reaction volume of 50 µL. The PCR program conditions were the same as those mentioned for the cloning of the *gacS* gene. The 2.2 kb PCR amplified product was purified and cloned into pCR2.1-TOPO vector with the same conditions mentioned above. The orientation of the gene encoding the LysR transcriptional regulatior was determined by sequencing (University of Toronto) using M13 Forward Primer (5'-CTGGCCGTCGTTTTAC-3') and M13 Reverse Primer (5'-CAGGAAACAGCTATGAC '3).

2.10 *gacS* Subcloning into pUCP23

To express *gacS* in *P. chlororaphis*, the 3.0-kb *gacS* insert was excised from pCR2.1-TOPO vector using *EcoR*V and *Hin*dIII. The 3-kb band corresponding to *gacS* was gel purified, using the UltraClean^{TM}15 DNA Purification kit, and ligated into *Sma*I- and *Hin*dIII-cleaved pUCP23, resulting in pUCP23-*gacS*.

2.11 Southern Hybridization and Generation of DIG-labelled Probe

For the detection of single chromosomal Tn*5*-OT182 insertions in *P. chlororaphis* PA23, Southern analysis was performed using a *tnp*-DIG labelled probe. For Southern blot analysis, the DNA was depurinated by treating the gel with 0.25M HCl for 10 min., followed by a denaturation treatment for 45 min. with 0.5M NaOH and 1.5M NaCl and a neutralization treatment for 45 min. with a solution consisting of 1.5M NaCl and 1.0M Tris-HCl (pH 7.5). Genomic DNA was transferred from the gel to Hybond-N nylon membrane (Amersham Biosciences) by alkaline capillary transfer overnight in 10X SSC (Appendix 1). The blot was washed in 5X SSC for 10 min. with gentle shaking and the nucleic acids were immobilized by UV induced cross-linking for 5 min. Prehybridization solution (Appendix 1) was added to the blot and incubated overnight at 65°C in a ProBlot hybridization oven (Mandel, Canada). The blot was hybridized for 16-24 h with a DIG-labelled DNA probe. Unbound probe was removed by washing twice with a salt detergent mix (2X SSC, 0.1% SDS) for 15 min at 22°C and then with 0.5X SSC, 0.1% SDS for 30 min. at 65°C. The blot was rinsed with washing buffer (Appendix 1) for 5 min. and blocking solution was added (Appendix 1) for 30 min. Following the blocking step, the blot was incubated for 60 min. with anti-DIG-polyclonal antibody conjugated to alkaline phosphatase (diluted 1:10,000 in 1X blocking solution). The blot was rinsed three times with sdH_2O before and after the treatment with the washing buffer (Appendix 1) for 15 min. Subsequently, the blot was equilibrated with 10 mL of detection buffer (Appendix 1) for 5 min.

Chemiluminescent detection reagent CDP-star (Roche) (2 μL CDP-Star in 200 μL Detection buffer) was added to the blot, covered with plastic wrap and exposed to X-ray film (Kodak BioMax XAR Film, Kodak, Rochester, NY, USA) for 1-4 h at room temperature (21°C).

2.12 Biocontrol Assays

Fungal inhibition *in vitro* was detected by spotting 5 μL of an overnight culture on PDA (Difco) 0.5 cm away from the edge of the plate. After overnight incubation at 28°C, a 0.6 cm fungal plug was inoculated onto the center of the plate, which was then incubated at room temperature. Antifungal activity was assessed after 3-4 days by measuring the distance between the edges of the colony and the fungal mycelium. Each plate contained PA23 wild type and three test strains.

2.13 Phenazine Extraction

Total phenazine production was analyzed by benzene extraction (1:1, vol/vol), as describe by Pierson III and Thomashow (Pierson III and Thomashow, 1992). Briefly, 6 mL of an overnight culture was centrifuged (5,000 x g) and cell-free supernatants were treated with 2 drops of 12N HCl and 6 mL of benzene and then mixed on a rocking table at room temperature. Following 1 h incubation, samples were centrifuged to separate the benzene phase (organic layer) from the aqueous phase. Approximately, 8 mL of the benzene phase was

evaporated to dryness, followed by resuspension with 1.5 mL NaOH. Total phenazine production was assessed by diluting the samples 1/10 in NaOH and measuring the absorbance spectrophotometrically (221-600 nm). Total phenazine is detected at 367 nm.

2.14 Autoinducer Production

Homoserine lactone production was assessed qualitatively by spotting 5 μL of an overnight culture on *Chromobacterium violaceum* CV026 indicator plates. The production of C4-, C6- or C8-HSL was indicated by the production of violacein, a purple pigment surrounding the growth of the bacterial colony.

2.15 Protease Activity

Extracellular protease activity was determined qualitatively by inoculating 5 μL of overnight culture on a 1.5% agar plate containing 2% skim milk. Proteolysis was indicated by a zone of lysis around the colony after 24-36 h, 28°C.

2.16 Lipase Activity

Lipase activity was detected by using the protocol of Lonon *et al.* (1988) with the following modifications. Lipolytic activity was assayed by spotting 5 μl of an overnight culture on LB agar plates supplemented with 2% Tween 80 and

2mM $CaCl_2$. Lipase activity is indicated by the zone of fatty acid precipitation around the colony after 24 to 72 h incubation at 28°C.

2.17 Siderophore Production

Siderophore production was assayed by spotting 5 µL of overnight culture on Chrome azurol S (CAS) agar (Schwyn and Neilands, 1987) plates followed by incubation for 16 h at 28°C. Qualitative analysis of siderophore production and iron (III) chelating activity is detected by the CAS-iron complex converting from blue to an orange halo around the colony.

2.18 Expression of *hcn* Genes

Expression of an *hcnA-lacZ* translational fusion was used to monitor hydrogen cyanide activity. Plasmid pME3219 (from D. Haas), which contains an *hcnA-lacZ* translational fusion, was transformed into PA23 and PA23-314 strains. The level of *hcnA* expression was assessed by β-galactosidase activity. For β-galactosidase assays, cultures were grown in PTSB at 28°C for 16 h. Cultures were adjusted to an OD of 0.5 and incubated until they reached early stationary phase ($OD_{600\ nm}$= 2.0). β-Galactosidase was determined by measuring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) as described by Miller (1972). Samples were assayed in duplicate, from three separate cultures.

2.19 Hydrogen Peroxide Sensitivity-Broth Assay

PA23 (pUCP23), PA23-314 (pUCP23) and PA23-314 (pUCP23-*gacS*) strains were grown to stationary phase in LB broth supplemented with Gm 25. Cultures were adjusted to $OD_{600\ nm}$ 0.1 followed by a 1:100 dilution in warm LB broth supplemented with Gm 25 μg/mL. Bacteria were grown in sterile flasks with aeration (200 rpm) at 28°C. At mid log-phase (~$OD_{600\ nm}$ = 1.0) and stationary phase (~$OD_{600\ nm}$ = 2.5-4.0) cultures were diluted 1:10 in 10 mL prewarmed LB broth supplemented with Gm and incubated with 9 mM or 45 mM H_2O_2 for 60 and 120 min. The suspensions were serially diluted with 1X PBS and plated on LB agar Gm. CFU/mL were enumerated to determine sensitivity to oxidative stress.

2.20 Growth Curve Experiments

Growth rate analysis of wild-type strain PA23 (pUCP23), PA23-314 (pUCP23) and PA23-314 (pUCP23-*gacS*), was performed in rich and minimal media using a Bioscreen^R C automated turbidometer. A single colony was inoculated into 2 mL of a rich or minimal media and grown at 28°C for 16 h. The cultures were adjusted to an OD_{600} of 0.1 by diluting with the same medium (rich or minimal) and 100 μL of culture was inoculated into each well of a Bioscreen^R C microtiter plate. Control wells contained an equal volume of sterile media. Cultures were monitored for 72 h at 28°C. Microbial growth was measured by an automated turbidometer at an $OD_{wideband}$ at 15 min. intervals. Wideband filter provides OD readings that are indicative of the turbidity and not the change in

colour of the growth medium. All samples were done in triplicate and assays were repeated four times.

2.21 Flagellar Motility

Flagellar (swimming) motility was assayed by inoculating 5 μL of an overnight bacterial culture into a 25 mL 0.3% LB agar plate. Five replicates of each culture were performed. Motility was measured at two time points.

2.22 Swarming Motility

Swarming motility was assayed by inoculating bacterial cells with an applicator stick on the surface of a 30 mL 0.5% SWM plate (0.5% Peptone, 0.3% Yeast extract and 0.5% agar) (Difco) previously air dried for 2 h. Overall results were obtained between 16-30 h at 28°C.

2.23 Twitching Motility

Plates consisting 20 mL LB medium solidified with 1% agar (Bacto-Difco) were stab-inoculated with an inoculation stick containing bacteria from an overnight culture. Twitching motility was assessed by measuring the zones of growth after 24 h at 28°C for *P. chlororaphis* or 37°C for *P. aeruginosa*.

2.24 Minimum Inhibitory Concentrations (MICs)

The MIC assays were performed by testing the presence and absence of planktonic bacterial growth in M9CA medium, supplemented with the antibiotic to be tested. To determine the antibiotic susceptibility for *P. chlororaphis*, overnight cultures were set to an $OD_{600\ nm}$ = 0.1 (10^8 CFU/mL) into M9CA (0.2% glucose, 1mM $MgSO_4$) containing various antibiotic concentrations. Antibiotic sensitivity was tested at the following concentrations: (i) ciprofloxacin, 0.125, 0.250, 0.5, 0.8, 1.5, 3.0, 6.125, 12.5, 25.0 and 50.0 µg/mL; and (ii) trobramycin, 0.125, 0.25, 0.5, 1.5, 3.0, 6.125, 12.5, 25.0, 50.0 and 100.0 µg/mL. The MIC was defined as the lowest concentration of antibiotic with no visible growth after 24 h incubation.

2.25 Biofilm Development

To assess biofilm formation capability, the wild type and Tn*5*-OT182 mutant strains were tested for the ability to adhere on the wells of polyvinylchloride plastic (Becton Dickinson Labware). Strains were grown overnight in M9CA supplemented with Gm. The cultures were diluted 1:100 in M9CA (0.2% glucose, 1 mM $MgSO_4$) and 200 µL was added to each well of a 96-well microtitre plate. After 16 h incubation at 28°C, cells were stained by the addition of 25 µL of a 1% Crystal Violet (CV) solution to each well for 15 min. Wells were rinsed repeatedly with H_2O to remove any unbound cells. Biofilm formation was quantitated with the addition of 200 µL of 95% ethanol to each CV-stained well and transferring the entire ethanol suspension into a 1.5 mL eppendorf tube. The volume was raised to 1 mL by the addition of 800 µL

ethanol and the absorbance was measured spectrophotometrically at an optical density of 600_{nm}. Biofilm formation was determined by an increase in optical density compared to the media-only control.

2.26 Biofilm Formation and Antibiotic Susceptibility

Biofilm antibiotic susceptibility was tested according to the procedure described by Parkins et al. (2001) with the following modifications. Strains were inoculated into M9CA (0.2% glucose, 1mM MgSO4) supplemented with Gm and grown to an OD600 nm > 1.0 at 28°C. The cultures were diluted 100-fold (1X107 CFU/mL) in M9CA and 200 μL was added to each well of a 96-well (Nunc) plate. A special lid containing 96 pegs (Nunc) was used for establishing biofilms on the surface of the pegs. This biofilm plate was incubated at 28°C on a rocking table at a speed 4.5 rpm, to generate shear force for the development of biofilms. Following 16 h incubation, the biofilm pegs were rinsed twice with 1X PBS to eliminate residual planktonic cells. The 96-peg lid was transferred to a microtitre challenge plate containing different concentrations of a single antibiotic. Biofilm MIC (BMIC) was determined by testing at the following antibiotic concentrations (μg/mL): (i) ciprofloxacin at 0.0125 (0.1XMIC); 0.0625 (0.5XMIC); 0.125 (1XMIC); 0.625 (5XMIC); 1.25 (10XMIC); 12.5 (100XMIC); 18.75 (150XMIC); 25.0 (200XMIC) and 31.25 μg/mL (250XMIC); (ii) trobramycin at 0.025 (0.1XMIC); 0.125 (0.5XMIC); 0.250 (1XMIC); 1.25 (5XMIC); 2.5 (10XMIC); 25.0 (100XMIC); 100.0 (400XMIC); 112.5 (450XMIC); 125.0 (500XMIC); 137.5 (550XMIC); 150.0 (600XMIC) and 162.5 μg/mL (650XMIC). The challenge plate had a sterility

control lane and a positive growth control lane. Biofilms were challenged with antibiotics for 20 h at 28°C, with constant shear force. The biofilm lid was rinsed twice with 1X PBS, transferred to a recovery plate containing 200 μL of M9CA and incubated for 16 h at 28°C. The BMIC was defined as the lowest concentration of antibiotic resulting in no bacterial growth in the recovery plate.

2.27 Petal Colonization

In order to determine the efficiency of the bacteria to colonize the plant, the population of PA23 and its Tn*5*-OT182 mutants present on canola petals was analyzed. For each test sample, 10 yellow petals were resuspended in 10 mL of 1X PBS and mixed using a vortex mixer for 30 sec. The bacterial suspension was serially diluted to 10^{-6} and 1 mL of each dilution was plated onto LB agar plates supplemented with selective antibiotics. Plates were air-dried in the flowhood for 10 min. and incubated for 30 h at 28°C to determine CFU/mL. Each sample was analyzed in triplicate.

3.0 RESULTS

3.1 Isolation of *P. chlororaphis* PA23 Mutants Affected in Biocontrol Activity

To identify genes responsible for biocontrol activity in *P. chlororaphis* strain PA23, random mutagenesis was performed using transposon Tn*5*-OT182 (Tc^R). The Tc^R transconjugants were screened for antifungal activity against *S. sclerotiorum*. This lead to the isolation and characterization of several mutants exhibiting increased or decreased antifungal activity.

3.2 Characterization of the *P. chlororaphis* PA23 Mutated Sequences

The presence of Tn*5*-OT182 insertion was identified by sequencing using Tn*5*-OT182 specific primers (TNP*5*-FORWARD and TNP*5*-REVERSE). From 3,000 transconjugants, several mutants were identified which could be divided into three different classes depending on the site of Tn*5* insertion. Class I mutations occurred in genes encoding a two-component sensor kinase, Class II mutations occurred in genes involved in phenazine biosynthesis, and a Class III mutation affected a gene encoding a transcriptional regulator of the LysR family (Appendix 2).

As outlined in Table 2, Class I mutants, consisted of strains PA23-314 and PA23-2490. Sequence analysis of the rescue clone from PA23-314 revealed that a gene showing 97% identity to the *gacS* gene from *P. chlororaphis* 06 (GeneBank accession no. AF192795) had been interrupted. Similarly, sequence

Table 2. Phenotypic characterization of *P. chlororaphis* PA23 and its Tn*5* mutants.

Strains	Tn*5* Insertion/ Genotype	Antifungal zone radius [cm (SD)][1]	Secondary Metabolite Activity			
			Autoinducer Production	Protease zone radius [cm (SD)][1]	Siderophore zone radius [cm (SD)][1]	Lipase
P. chlororaphis PA23	Wild type	0.8 (0.05)	1.2	0.5 (0.0)	0.6 (0.0)	++
Class I						
PA23-314	*gacS*	0.0 (0.0)	0.0	0.0 (0.0)	1.6 (0.35)	-/+[3]
PA23-314*gacS*	*gacS*::Tn*5* (pUCP23-*gacS*)	0.8 (0.05)	1.2	0.5 (0.0)	0.8 (0.0)	++
PA23-2490	*gacS*	0.0 (0.0)	0.0	0.0 (0.0)	1.4 (0.0)	-/+[3]
PA23-2490*gacS*	*gacS*::Tn*5* (pUCP23-*gacS*)	0.8 (0.0)	1.2	0.38 (0.0)	0.6 (0.0)	++
Class II						
PA23-63	*phzE*	1.15 (0.1)	0.9	0.63 (0.0)	0.4 (0.0)	+++
PA23-754	*phzC*	1.2 (0.0)	1.2	0.5 (0.0)	0.5 (0.0)	+++
Class III						
PA23-443	Tn*5* in the coding region of a LysR-type transcriptional regulator,	0.0 (0.0)	0-0.43[2]	0.0/0.4[2](0.0)	0.8 (0.0)	+
PA23-443*gacS*	Transcriptional regulator gene::Tn*5* (pUCP23-*gacS*)	0.7 (0.07)	1.2	0.4 (0.0)	0.6 (0.0)	+++

[1] The values are the mean and standard deviation from four replicates performed in the same experiment.
[2] No activity at 24 h, activity at 72 h.
[3]lipase activity at 24 h = -, at 72 h = +.

flanking the Tn*5* insertion in PA23-2490 was 99% identical to the *gacS* gene from *P. chlororaphis* 06 (GeneBank accession no. AF192795).

Class II mutants included strains PA23-63 and PA23-754, having Tn*5* insertions in *phzE* and *phzC*, respectively. Sequence analysis of a DNA flanking the Tn*5* insertion in mutant PA23-63 showed 92% identity to the *phzE* gene of *P. chlororaphis* PCL1391 (GeneBank accession no. AF195615). The deduced nucleotide sequence of Tn*5* flanking DNA in PA23-754, showed 96% identity to *phzC* gene of *P. chlororaphis* PCL1391 (GeneBank accession no. AF195615). The proteins PhzC and PhzE have been previously identified in *P. chlororaphis* as enzymes involved in phenazine biosynthesis. The PhzC protein has homology with the DAHP synthases, involved in the first enzymatic step of the shikimate pathway catalyzing the condensation reaction of phosphoenolpyruvate and erythrose-4-phosphate to yield DAHP and inorganic phosphate. The PhzE protein has fused domains homologous to anthranilate and ADIC synthetases, responsible for the conversion of chorismic acid to ADIC. In *P. fluorescens*, ADIC is the branch point in the shikimate pathway that either forms tryptophan, the pyrrolnitrin precursor, or phenazine antibiotics (McDonald *et al.*, 2001).

A single Class III mutant, PA23-443, was isolated and further examined. Sequence analysis revealed an insertion in a gene exhibiting 85% identity to a genetic locus encoding a LysR-type transcriptional regulator in *P. fluorescence* Pf5 (GeneBank accession no. CP000076).

One mutant from Class I (PA23-314), Class II (PA23-63), and Class III (PA23-443) was characterized. The *gacS* mutant PA23-314 was studied in the greatest detail.

3.3 Verification of a Single Transposon Insertion using Southern Analysis

A single Tn*5*-OT182 chromosomal insertion in the PA23 mutants was confirmed using Southern analysis. Total genomic DNA from PA23 and its Tn*5*-OT182 mutants, as well as plasmid DNA from pOT182 was digested with restriction endonuclease *BamH*I. Hybridization with a Tn*5*-DIG labelled probe showed a single probe-reactive band for pOT182 and the PA23 Tn*5* mutants (Figure 4).

3.4 Detailed Characterization of Class I Mutant PA23-314 (*gacS*::Tn*5*-OT182)

PA23 produces a number of extracellular compounds that are likely to contribute to its excellent biocontrol activity against *S. sclerotiorum* (Savchuk and Fernando, 2004). These compounds include homoserine lactone signal(s), and secondary metabolites such as phenazine(s), pyrrolnitrin, siderophores, extracellular protease(s), and lipase. Mutant PA23-314 has no antifungal activity *in vitro* against *S. sclerotiorum*, and is deficient in the production of many secondary metabolites as described below.

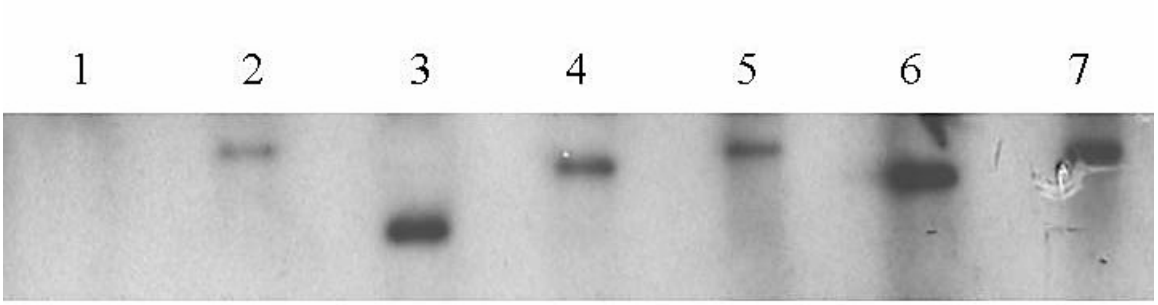


Figure 4: Southern analysis of PA23 chromosomal DNA digested with *BamH*I. Autoradiogram showing the fragment of Tn*5*-OT182 that hybridized with the Tn*5*-specific probe. Lane 1, PA23; lane 2, PA23-314; lane 3, PA23-2490; lane 4, PA23-63; lane 5, PA23-754, lane 6, PA23-443, and lane 7, PA23-2461.

3.4.1 Analysis of Secondary Metabolite Production

Phenazines belong to a class of antibiotics produced by *Pseudomonas* and *Streptomyces* species (MacDonald, 1967; Turner and Messenger, 1986; Budzikiewicz, 1993) known to contribute to antifungal activity through the disruption of membrane redox potential (Adken *et al.,* 1998). *P. chlororaphis* PA23 produces PCA and 2-OH-PHZ (Zhang Y, 2004), resulting in an orange pigment. In contrast, PA23-314 is white in colour suggesting that phenazines are not being produced. Spectral analysis confirmed that this is the case (Figure 5A and 5B). While compounds 2-OH-PHZ and PCA, were detected in the PA23 cultures, (collectively at a peak of 367 nm) no phenazines were produced by PA23-314.

The PhzR/PhzI quorum sensing system regulates the production of phenazines in other *P. chlororaphis* strains (Chin-A-Woeng *et al*., 2001; Kang *et al*., 2004). When tested against a *C. violaceum* AHL biosensor strain CV026, PA23-314 was deficient in AHL production, as noted by the absence of a purple ring around the growth of the colony. Conversely, in plate assays the wild type activated the bioreporter strain CV026 (Figure 6A).

The ability to degrade intracellular proteins has been attributed as an antifungal property (Whistler *et al.*, 2000). In *P. aureofaciens* 30-84, protease production is under the regulation of CsaR/CsaI (cell surface alterations) quorum sensing system, as well as GacS/GacA two-component system (Zhang and Pierson, 2001). The *gacS* mutant PA23-314 was deficient in extracellular

protease(s) activity compared to the wild type, as indicated by the absence of a clearing zone on 2% skim milk plate assays (Figure 6B).

Lipases, (triacylglycerol acylhydrolases), are known to catalyze the hydrolysis of phospholipids, which are major constituents of cell membranes. Lipolytic activity has been proven to contribute to bacterial mediated pathogenesis by *P. aeruginosa* and *Burkholderia cepacia* (Jaeger *et al*., 1994*).* In the *P. chlororaphis* mutant PA23-314, there was a delay in onset of lipase activity (72 h) compared to the wild type (Figure 6C and 6D, Table 2).

Competition for iron has been documented to be a contributing factor towards biocontrol activity (Kloepper *et al.*, 1980). The ability of fluorescent *Pseudomonas* spp. to sequester iron from the rhizosphere functions as an inhibitory mechanism against the growth of fungal pathogens by depriving them from an essential cofactor required for many redox-dependent enzymatic activities. The *gacS* mutant strain PA23-314 had a 2-fold increase in siderophore activity compared to the wild type when tested on a CAS plate assay (Figure 6E), suggesting that GacS may be negatively regulating siderophore production.

Hydrogen cyanide is another antifungal metabolite produced by *Pseudomonas* spp. To assess the regulatory function of GacS in hydrogen cyanide expression, an *hcnA*'-'*lacZ* translational fusion was tested in the wild type and the *gacS* mutant backgrounds. As a positive control, the *hcnA* fusion was also tested in *P. fluorescens* Pf-5, an organism that has high degree of homology to *P. fluorescens* CHA0; the strain from which the *hcnA* gene was isolated. The

expression levels of *hcnA* were equivalent in both PA23 and Pf-5 genetic backgrounds. Mutant PA23-314 had expression levels near the detection limit and significantly lower than the wild type ($P < 0.05$) (Figure 7). The production of HCN in this organism may be beneficial by contributing to its ecological significance as a biocontrol strain of fungal pathogens. thus further substantiating the significance of a functional GacS regulatory system.

For all of the secondary metabolite assays described above, complementation with a functional *gacS* allele (carried on pUCP23-*gacS*) restored the mutant phenotype to that of the wild type.

3.4.2 Sensitivity to H_2O_2

It was hypothesized that a mutation in *gacS* would render higher sensitivity to H_2O_2 stress. As shown in Figure 8A, for the logarithmic growth phase this is not the case since logarithmic cultures of the *gacS* mutant had a significant increase in 9 mM H_2O_2 tolerance ($P < 0.005$) compared to the wild type. However, during the stationary phase the *gacS* mutant had increased sensitivity ($P < 0.005$) to 9 mM H_2O_2 treatments compared to the wild type (Figure 8B). These trends hold true for both 1 h and 2 h exposure, with the greatest difference seen at 1 h.

Under extreme oxidative stress conditions (45 mM H_2O_2) resistance was demonstrated only by stationary phase cultures where the wild type had significant tolerance ($P < 0.005$) compared to the *gacS* mutant strain PA23-314 (Figure 8C). During logarithmic growth, the wild-type and the *gacS* mutants

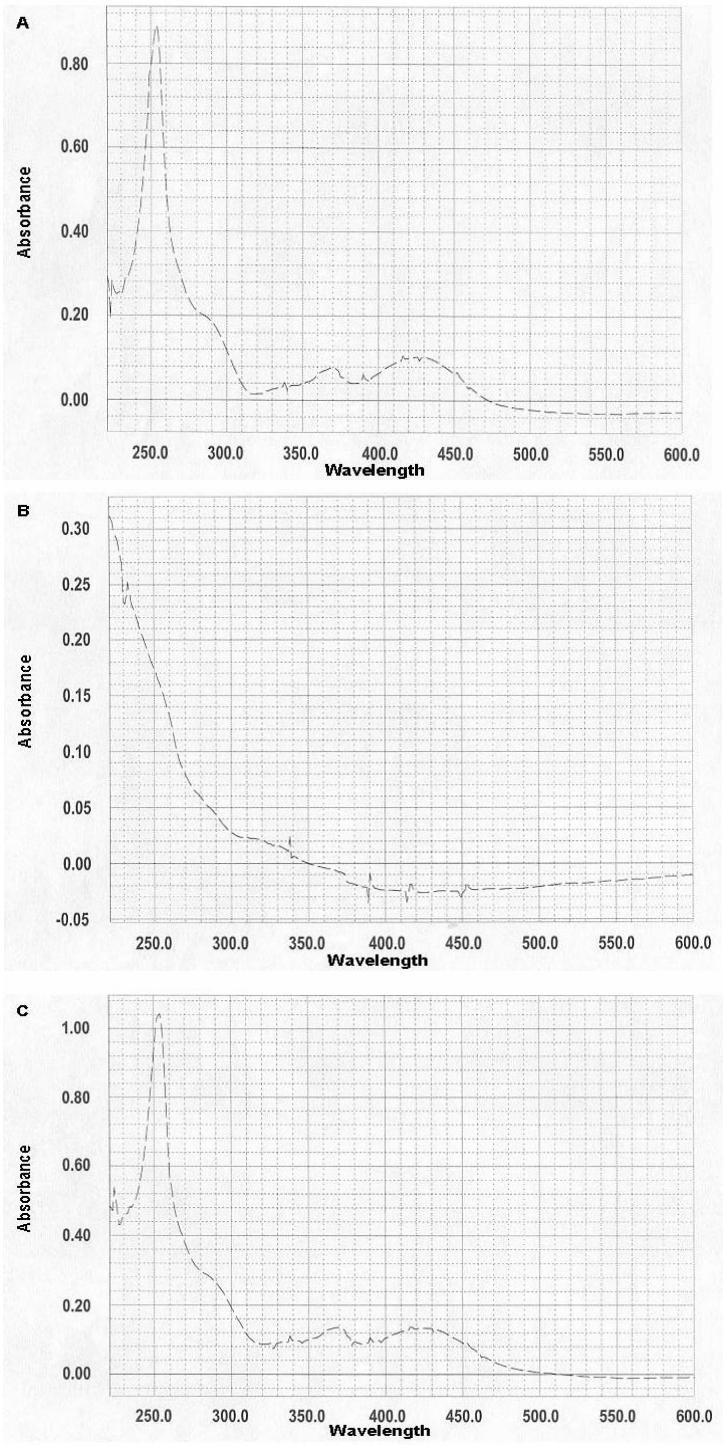


Figure 5: Ultra-violet spectral analysis of total phenazine production (1/10 dilution) by *P. chlororaphis* PA23 and its Tn*5* mutants (OD $_{221\text{-}600\ nm}$). **Panel A:** PA23; **Panel B:** PA23-314; and **Panel C:** PA23-314 (pUCP23-*gacS*).

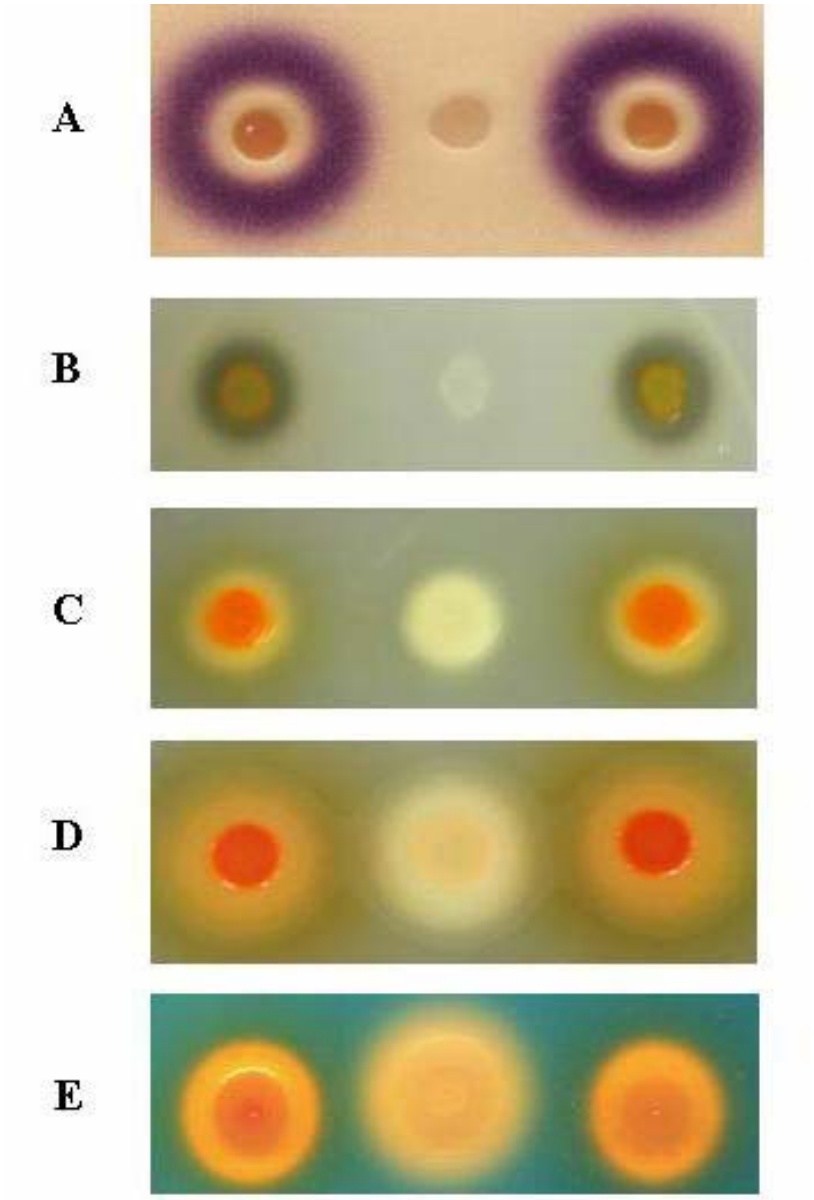


Figure 6: Panel A: Homoserine lactone (HSL) production in PA23, PA23-314 and *gacS* complemented PA23-314 strains. HSL activity was tested on an indicator media containing *C. violaceum* CV026 (*cvi*I::Tn*5*) reporter strain. **Panel B:** Extracellular protease production in PA23, PA23-314 and *gacS* complemented PA23-314 strains. Protease hydrolyzing activity was tested on 2% skim milk agar. **Panels C and D:** Lipase hydrolysis of 2% Tween 80 by PA23, PA23-314 and *gacS* complemented PA23-314 strains at 24 h and 72 h, respectively. **Panel E:** Siderophore production in PA23, PA23-314 and *gacS* complemented PA23-314 strains. Siderophore chelating activity of iron (III) was tested on Chromo azurol S agar.

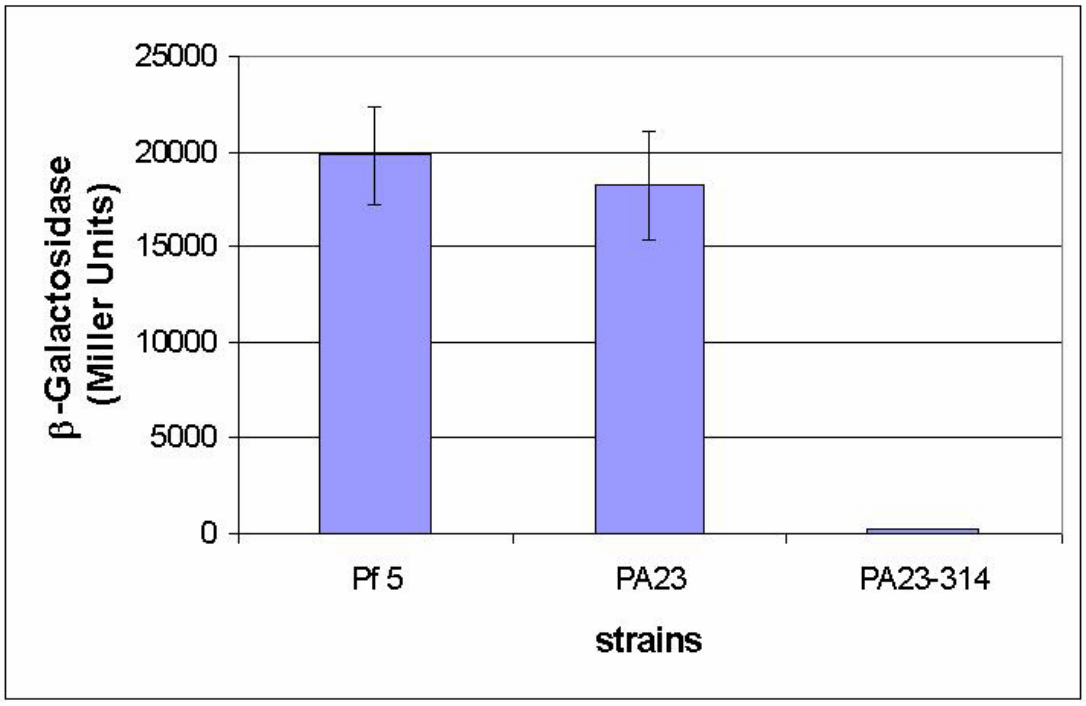


Figure 7: β-Galactosidase activity of *P. fluorescens* Pf5, *P. chlororaphis* PA23, and *gacS* mutant PA23-314, harboring an *hcnA'-'lacZ* translational fusion (pME3219). β-Galactosidase activity is expressed in Miller units and represents the average of three independent cultures (these bars are representative of the standard error).

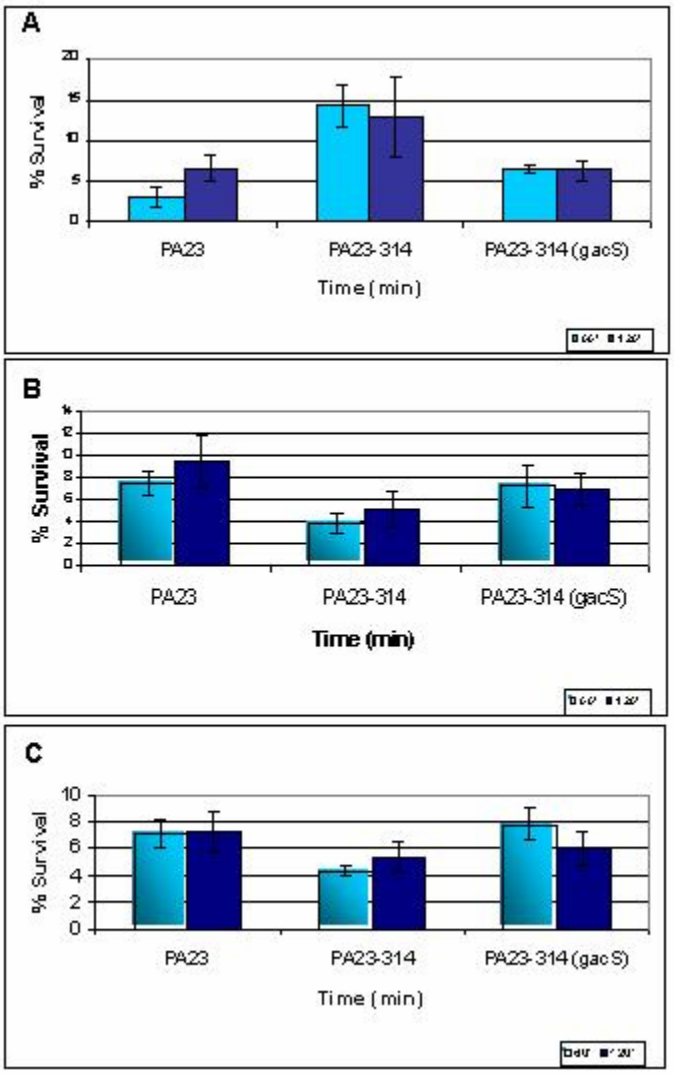


Figure 8: Hydrogen Peroxide Sensitivity-Broth Assay**.** Effect of *gacS* mutation on stress response was tested using PA23, *gacS* mutant PA23-314 and *gacS* complemented strain PA23-314 (pUCP23-*gacS*). **Panel A:** Stress response to H_2O_2 was measured on cell cultures grown to logarithmic phase ($OD_{600\ nm}$ = 1-1.5) and exposed to (**A**) 9 mM H_2O_2; **Panels B and C**: Stress response to H_2O_2 measured on cell cultures grown to stationary phase ($OD_{600\ nm}$ = 4) and treated with either (**B**) 9 mM H_2O_2 or (**C**) 45 mM H_2O_2. Cultures were treated for 60 and 120 min. CFU/mL were enumerated after 24 h incubation at 28°C.

were highly sensitive to 45 mM H_2O_2, as observed by less than 0.001% survival (data not shown).

3.4.3 Growth Rate Analysis of Class I Planktonic Cultures

To determine the fitness of a GacS mutant under minimal and rich nutrient conditions, growth rate analysis was performed for *P. chlororaphis* strains PA23 (pUCP23), PA23-314 (pUCP23), and PA23-314 (pUCP23-*gacS*).

3.4.3.1 Minimal Media

In M9 media supplemented with 0.2% glucose, the *gacS* mutant entered logarithmic phase earlier than either the wild type or the *gacS* complemented strain (3 h versus 6.25 h) at an $OD_{wideband}$ = 0.14 (Figure 9A). At 10 h, the *gacS* mutant had a 33% increase in growth compared to the wild type and the *gacS* complemented strain ($OD_{wideband}$ = 0.255 versus 0.17). At 22 h, all three strains reached equal growth levels of $OD_{wideband}$ = 0.39. (Figure 9A). In M9 media supplemented with 0.5% glucose, the growth rate over time had the same trend as M9 plus 0.2% glucose (data not shown).

In M9 media supplemented with 0.2% glycerol, the *gacS* mutant entered the logarithmic growth phase 4 h earlier than the wild type and the *gacS* complemented strain $OD_{wideband}$ = 0.14 (6 h versus 10 h) (Figure 9B). At 8 h, the *gacS* mutant strain exhibited nearly 30% higher population density compared to the other strains carrying a functional *gacS* gene. At 25 h the wild type and mutant PA23-314 reached equal growth levels ($OD_{wideband}$ = 0.3). At 45 h the

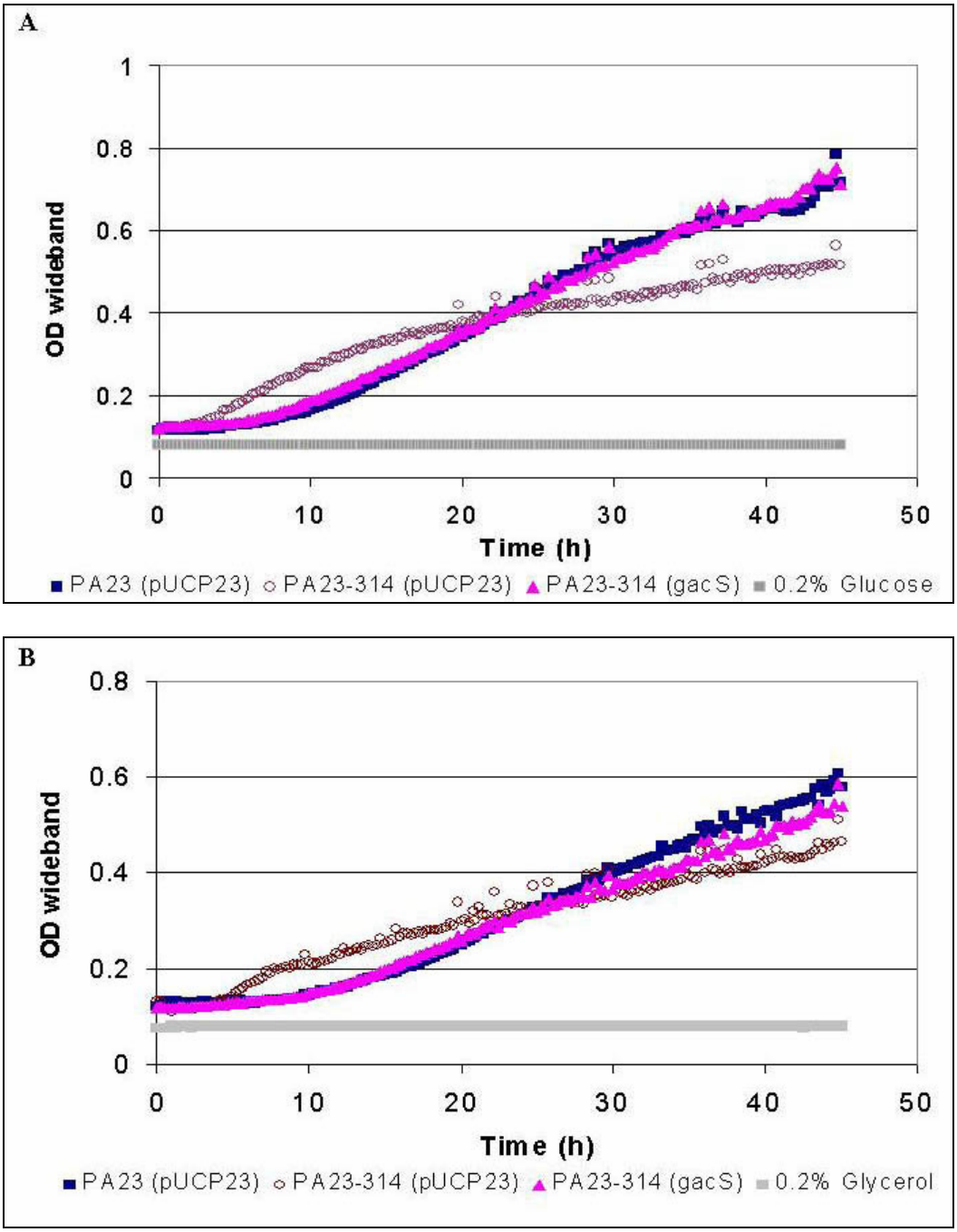


Figure 9: Growth of *P. chlororaphis* PA23 and its Tn*5* mutants in liquid culture. Strains PA23 (wild type, pUCP23), PA23-314 (*gacS*::Tn*5*, pUCP23) and *gacS* complemented PA23-314 (*gacS*::Tn*5*, pUCP23-*gacS*) were grown in minimal media. **Panel A:** 0.2% glucose; and **Panel B:** 0.2% glycerol.

population density of the wild type and the *gacS* complemented strain continued to increase, whereas the *gacS* mutant plateaued and did not reach an OD over 0.6. In M9 media supplemented with 0.5% glycerol, the growth curves were almost identical to that observed for M9 0.2% glycerol (data not shown).

The results show that minimal media supplemented with either glucose or glycerol support earlier entry into the logarithmic growth phase by 3-4 h in a *gacS* mutant background, followed by a 30% growth increase compared to the wild type. By 45 h however, the *gacS*-sufficient strains had a 30% higher population density, compared to the *gacS* mutant PA23-314. These results indicate that the *gacS*$^-$ population seems to stop increasing in number at an earlier time point under low nutrient conditions.

3.4.3.2 Rich Media

In LB medium, the wild type, *gacS* mutant, and *gacS* complemented mutant entered the exponential growth phase at the same time point (2 h; $OD_{wideband}$ = 0.2) (Figure 10A). At 22 h, the *gacS* mutant entered the stationary phase depicted by the plateau of growth at an $OD_{wideband}$ = 1.0. The wild type and *gacS* complemented strain entered the stationary phase at 40 h when their growths reached a plateau at an $OD_{wideband}$ = 1.2. Compared to *gacS* mutant PA23-314, the wild type had a 22.5% increase in growth at the end of the 45 h incubation.

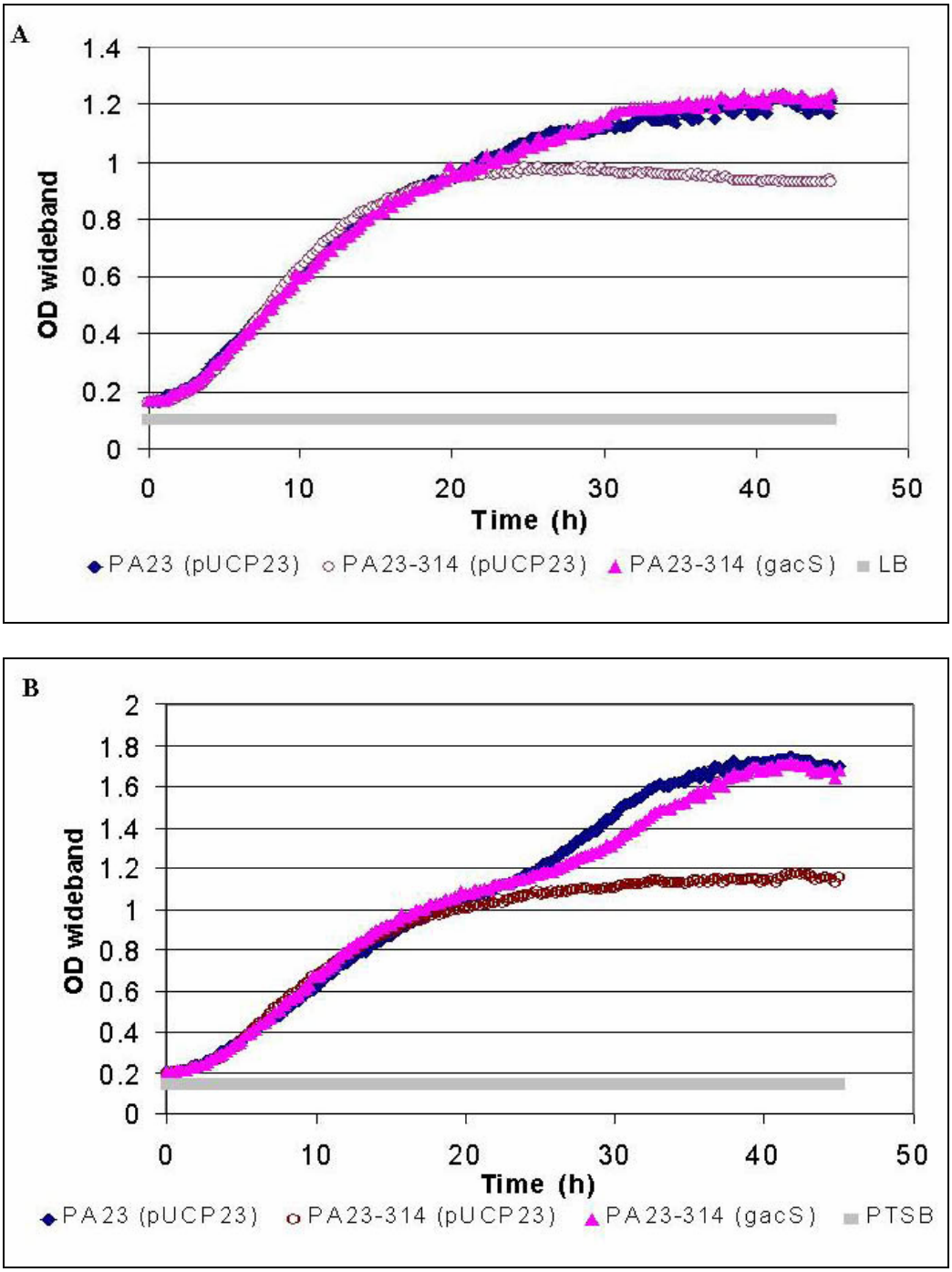


Figure 10: Growth of *P. chlororaphis* PA23 and its Tn*5* mutants in liquid culture. Strains PA23 (wild type, pUCP23), PA23-314 (*gacS*::Tn*5*, pUCP23) and *gacS* complemented PA23-314 (*gacS*::Tn*5*, pUCP23-*gacS*) were grown in rich media. **Panel A:** LB; and **Panel B:** PTSB.

Growth in PTSB followed the same trend as that shown in LB medium with the exception that at the end of the 45 h incubation, the wild type had a 41% increase in growth compared to the *gacS* mutant (Figure 10B).

In TB medium, the wild type and the *gacS* complemented strain entered into the exponential growth phase 4 h earlier than the *gacS* mutant. At 20 h the wild type had a 60% increase in growth compared to *gacS* mutant PA23-314, which persisted up to the end of the 45 h incubation (Figure 11). Thus it appears that a *gacS* mutation leads to dramatically decreased population under highly rich nutrient conditions.

3.4.4 Motility

To determine the regulatory role of GacS in motility, the wild type, the *gacS* mutant, and the *gacS* complemented strain were tested for flagellar, swarming, and twitching motilities.

3.4.4.1 Flagellar Motility

Both the wild type and the *gacS* mutant exhibited flagellar motility; however, the *gacS* mutant had a significantly lower rate of flagellar motility compared to the wild type and the *gacS* complemented strain (Figure 12). These findings show that in the PA23-314 mutant background, there is a functional flagella system but motility is reduced.

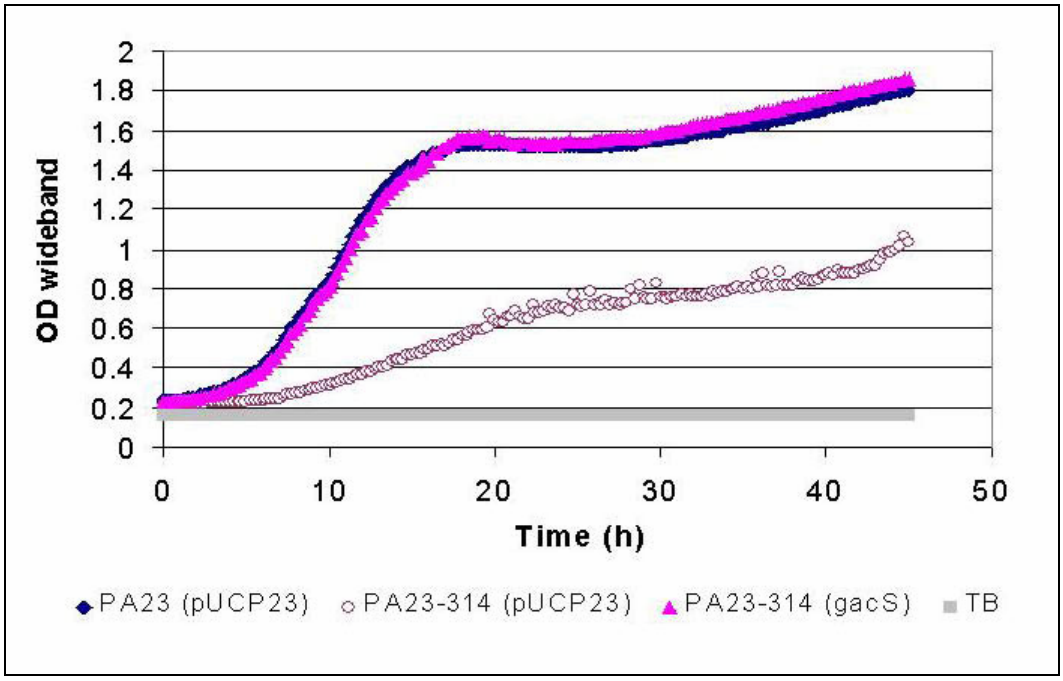


Figure 11: Growth of *P. chlororaphis* PA23 and its Tn*5* mutants in liquid culture. Strains PA23 (wild type, pUCP23), PA23-314 (*gacS*::Tn*5*, pUCP23) and *gacS* complemented PA23-314 (*gacS*::Tn*5*, pUCP23-*gacS*) were grown in the ultra-rich media: TB.

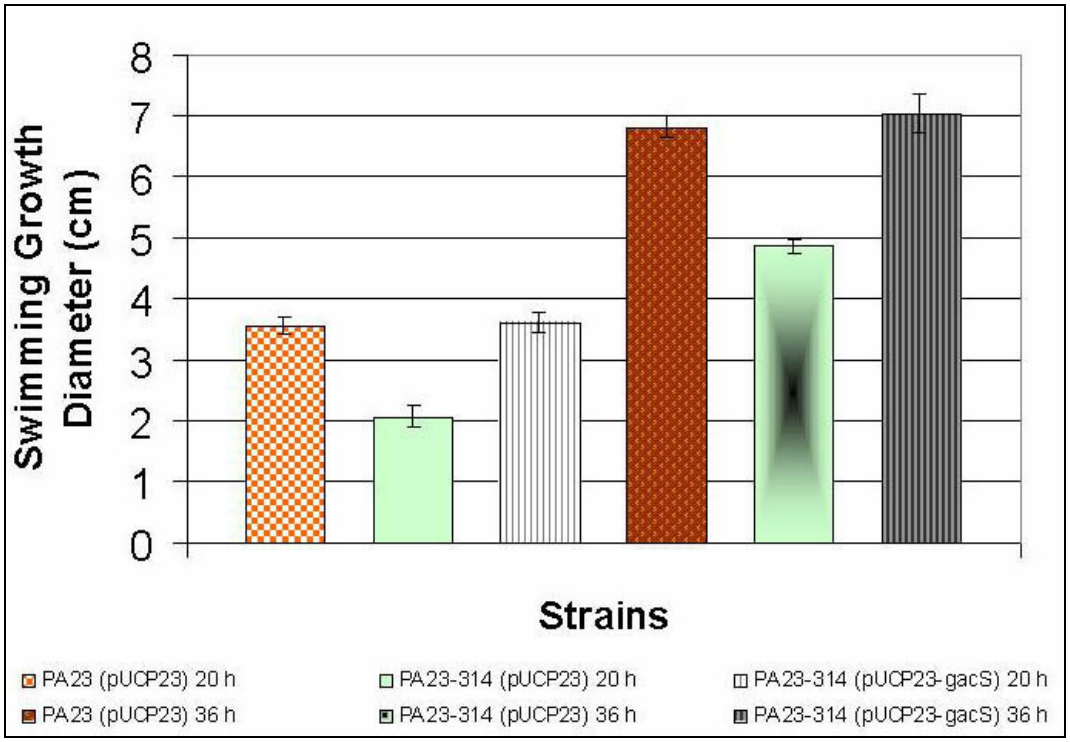


Figure 12: The swim zones of *P. chlororaphis* flagellar motility in 0.3% agar. Strains PA23 (wild type, pUCP23), PA23-314 (*gacS*::Tn*5*, pUCP23) and *gacS* complemented PA23-314 (*gacS*::Tn*5*, pUCP23-*gacS*) were measured at 20 h (open bars) and 36 h (filled bars).

3.4.4.2 Swarming Motility

The role of GacS in swarming motility is not significant. Both the wild type and the *gacS* complemented strain had a swarming phenotype represented by a branching of liquid streams that resembled a dendritic outward growth (data not shown). The *gacS* mutant was consistently delayed in swarming initiation, as well its swarming phenotype was slightly altered compared to the wild type, where certain region(s) of motility resembled flagellar more than swarming motility (data not shown). These results indicate that GacS does not play a major in regulating swarming motility in PA23.

3.4.4.3 Twitching Motility

P. chlororaphis PA23 is deficient in twitching motility as shown by the absence of a zone of twitching in 1% agar plates (data not shown). For a positive control, PA01 was included and it showed the presence of a subsurface zone of twitching motility (data not shown). The results indicated that under the conditions tested, PA23 does not exhibit type IV pili-mediated motility.

3.4.5 Biofilm Formation

To determine the role of GacS in biofilm formation, mutant PA23-314, was compared to the wild type and the *gacS* complemented strain for the ability to form biofilms in a 96-well PVC microtitre plate. Mutant PA23-314 had an 87% decrease in biofilm formation compared to the wild type (Figure 13). There was

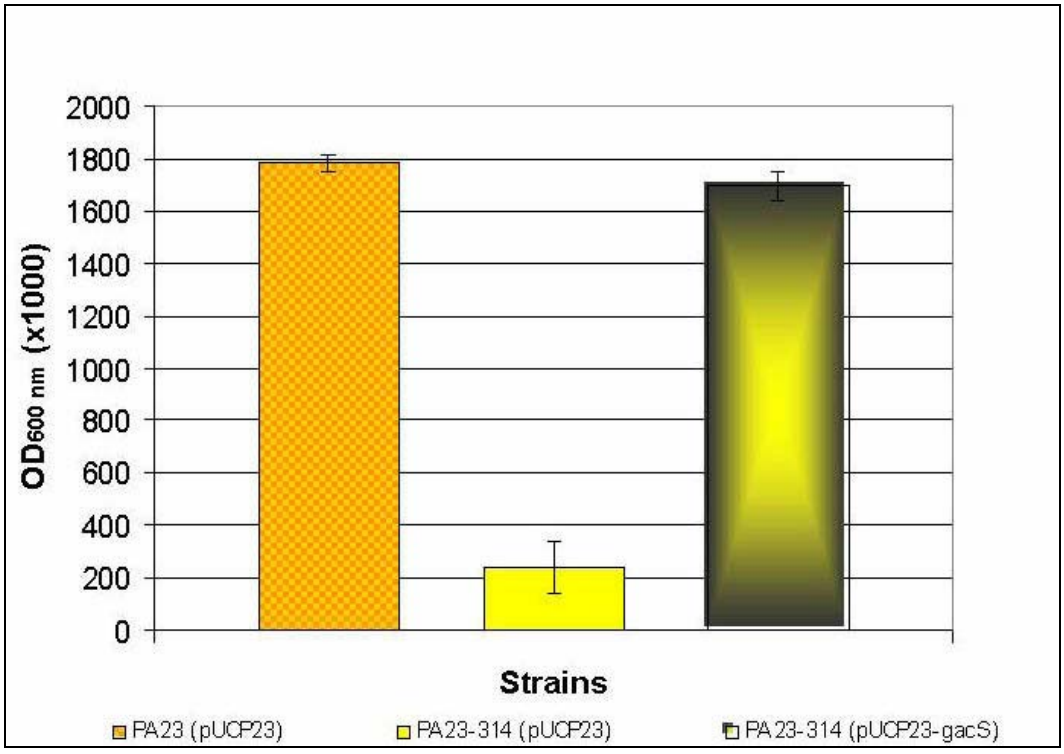


Figure 13: Biofilm growth of *P. chlororaphis* in M9CA (0.2% glucose, 1mM $MgSO_4$). Strains PA23 (wild type, pUCP23), PA23-314 (*gacS*::Tn*5*, pUCP23) and *gacS* complemented PA23-314 were tested for their ability to form biofilms on a PVC plastic surface.

no significant difference in biofilm formation between the wild type and the *gacS* complemented strain. The results indicate that a functional GacS system is required for optimal biofilm formation on abiotic surfaces.

3.4.6 Biofilm Development and Antibiotic Challenge

To determine whether a biofilm defect in a *gacS* mutant background would yield an increase in antibiotic susceptibility, strains PA23, PA23-314 (*gacS*::Tn*5*-OT182), and PA23-314 (pUCP23-*gacS*) were tested for their biofilm minimum inhibitory concentration (BMIC) in 96-well microtitre plates. Among the three genetic backgrounds, the trend in planktonic MIC and BMIC values was the same. Ciprofloxacin is readily diffusible across the biofilm matrix. The BMIC values for ciprofloxacin were 100X MIC (12.5 μg/mL) indicating that the biofilm population is significantly more resistant than planktonic cells (Table 3). Tobramycin is a positively charged, bacteriocidal antibiotic effective against non-growing cells. Diffusion into the biofilm is inhibited mainly by its large molecular size, as well as its binding affinity with the negatively charged exopolysaccharide matrix (Davis, 1987; McKenney and Allison, 1997). For tobramycin challenge, the BMIC values were 650X MIC (162.5 μg/mL) (Table 3), indicating that this antibiotic is not effective in penetrating through the biofilm. The increased resistance to tobramycin observed in the *gacS* mutant biofilm indicates that even with considerably reduced levels of biofilm formation, the presence of exopolysaccharide matrix is enough to delay the bacteriocidal effects of this antibiotic.

Table 3. Antibiotic susceptibility of planktonic and biofilm cells of *P. chlororaphis* strains

Strains	Antibiotic Treatment			
	Ciprofloxacin (µg/mL)		Tobramycin (µg/mL)	
	MIC	BMIC	MIC	BMIC
P. chlororaphis PA23 (pUCP23)	0.125	12.5	0.250	162.5
PA23-314 (pUCP23)	0.125	12.5	0.250	162.5
PA23-314 (pUCP23-*gacS*)	0.125	12.5	0.250	162.5

3.4.7 Plant Assays

Analysis of the biocontrol ability of PA23, PA23-314, and PA23-314 (pUCP23-*gacS*) was conducted in greenhouse studies performed by Dr. Nakkeeran in the laboratory of Dr. D. Fernando (Department of Plant Science, University of Manitoba). To determine the importance of GacS in managing *S. sclerotiorum* infection in canola plants, the wild type PA23, mutant PA23-314, and PA23-314 *gacS* complemented strain were tested for their ability to reduce stem rot incidence and incidence of leaf infection.

3.4.7.1 Disease Severity

After inoculation of *S. sclerotiorum* ascospores, development of symptoms was observed and recorded on a scale of 0-7 (0: no lesions on the stem; 2: 1-20cm lesion on the stem; 3: 21-40 cm lesion; 4: 41-60 cm lesion; 5: 61-80 cm lesion; 6: 81-100cm lesion, 7: >100 cm lesion or plant death). In PA23-314-inoculated plants, the disease severity was significantly higher (4.62) ($P < 0.0001$) compared to the wild-type treatments (1.37) (Figure 14); signifying the importance of a functional GacS/GacA system in the management and suppression of *S. sclerotiorum* disease severity on canola plants.

3.4.7.2 Incidence of Stem Rot and Leaf Infection

In soybean and canola (Glycine max and *Brassica napus* L.) plants, stem rot is the most important *Sclerotinia* disease symptom, causing significant losses in grain yield (Purdy, 1979). Stem rot incidence achieved by *S. sclerotiorum* on

canola was 5% on plants treated with both *P. chlororaphis* PA23 and *S. sclerotiorum* ascospores; which was significantly lower ($P < 0.005$) than the pathogen-only control, demonstrating the efficient biocontrol activity of PA23. Conversely, a mutation in *gacS* did not afford protection as indicated by 48% stem rot incidence on PA23-314-inoculated plants, which was similar to the pathogen control (Figure 15A). Incidence of leaf infection with PA23-inoculated plants was 10%, providing significant protection ($P < 0.005$) compared to pathogen control (75%) (Figure 15B). On the contrary, disease incidence on leaves of PA23-314-inoculated plants was 60%, signifying the importance of GacS in disease protection.

Complementation with a functional *gacS* allele restored disease protection to wild type levels for both stem and leaf infection.

3.4.7.3 Bacterial Population Dynamics of Class II Mutant on Canola

PA23, PA23-314, and PA23-314 (pUCP23-*gacS*) strains were analyzed for their ability to colonize canola petals over time. All three strains tested had similar colonization patterns. Mutant PA23-314 was able to become established and to maintain a population size sufficient for biocontrol activity in the

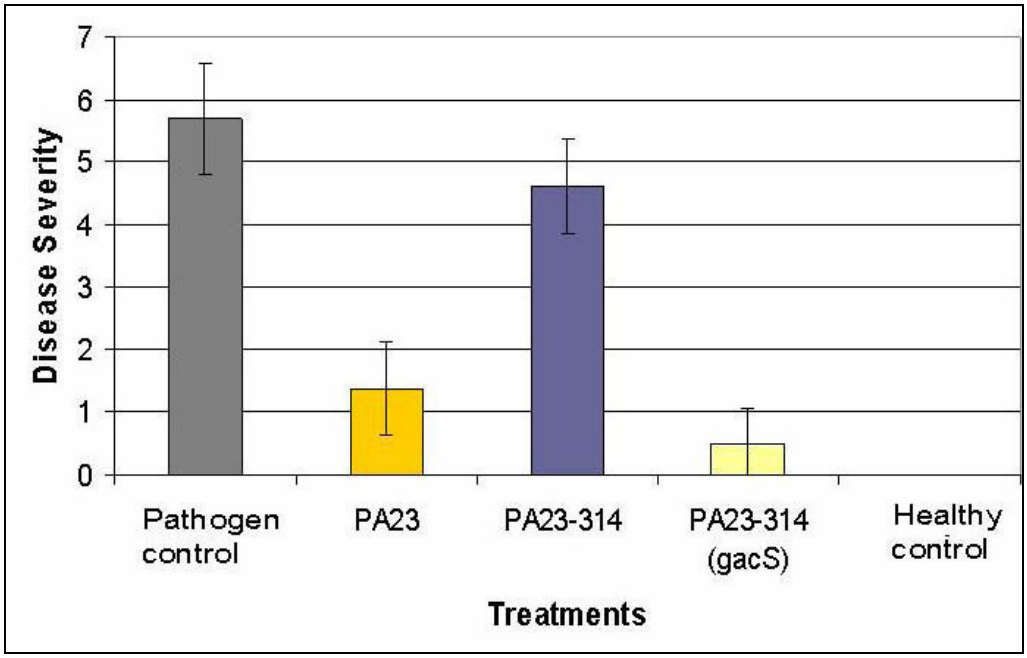
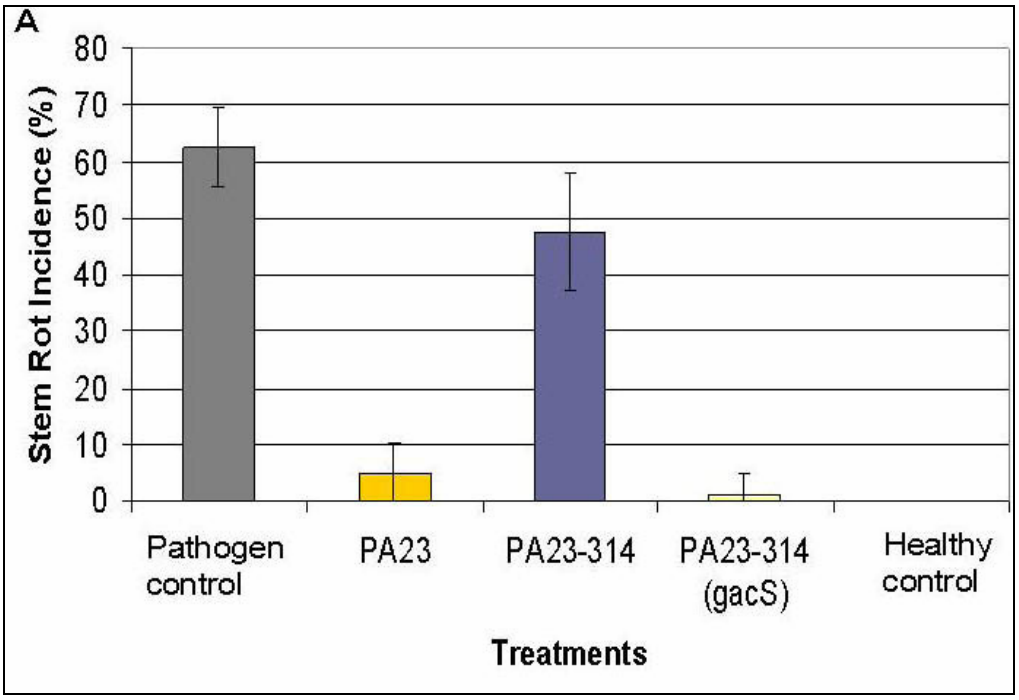


Figure 14: Biocontrol of *S. sclerotiorum* by *P. chlororaphis* PA23 (wild type, pUCP23), PA23-314 (*gacS*::Tn5, pUCP23) and *gacS* complemented strain PA23-314 (pUCP23-*gacS*).



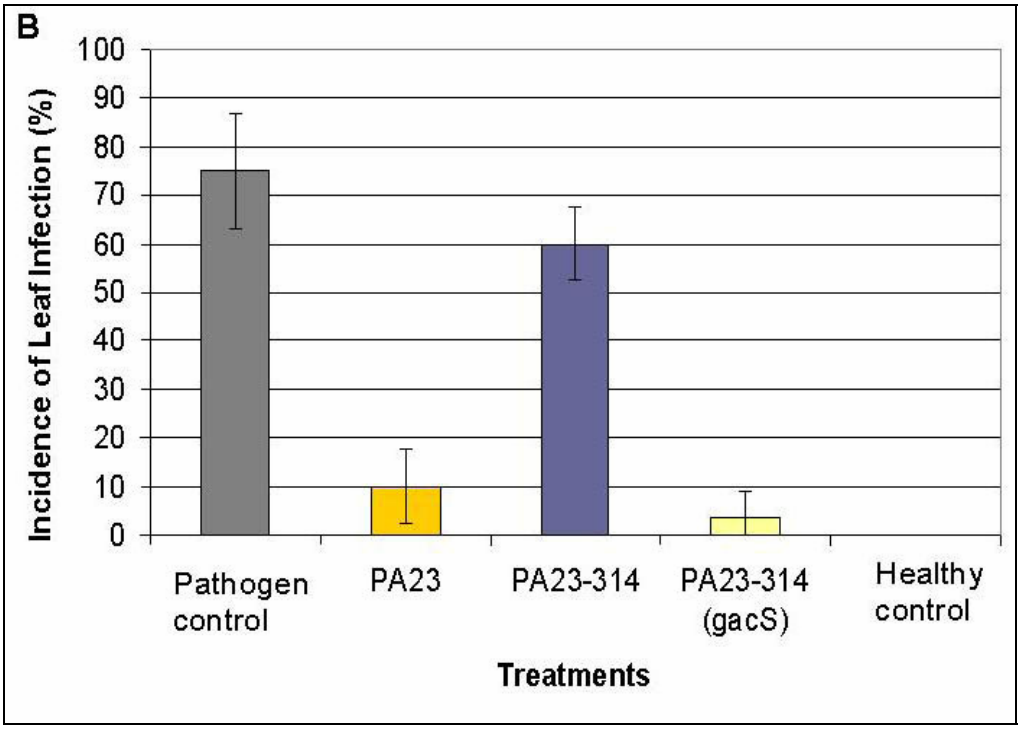


Figure 15: Efficiency of *P. chlororaphis* PA23 (wild type, pUCP23), *gacS* mutant PA23-314 (pUCP23) and *gacS* complemented strain PA23-314 (pUCP23-*gacS*) in managing *S. sclerotiorum* infection on canola plants. **Panel A:** Percent stem incidence; and **Panel B:** Percent Incidence of leaf infection.

phyllosphere (Figure 16). These findings show that the deficiency in suppressiveness of *S. sclerotiorum* disease observed in mutant PA23-314, is due not to poor population sustainability in the phyllosphere, but is mediated by the deficiency in secondary metabolite production.

3.5 Phenotypic Characterization of Class II Mutant PA23-63 (*phzE*::Tn*5*-OT182)

3.5.1 Analysis of Secondary Metabolite Production

Strain PA23-63 had a white colony phenotype, indicative of a deficiency in phenazine antibiotic production compared to the wild-type strain which is orange. Spectral analysis of PA23-63 supernatants showed no production of phenazines (Figure 17). Further, analysis of mutant PA23-63 showed that it had a decrease in HSL (25%) and siderophore (30%) activity, while it had a higher protease (20%) activity compared to the wild type. Mutant PA23-63 had a 30% increase in antifungal activity compared to the wild type (Table 2). These findings indicated that there is a secondary metabolite being produced other than phenazine that is responsible for providing efficient antifungal activity. Previous studies have shown that pyrrolnitrin exhibits antifungal activity against *Sclerotinia homoeocarpa* (Rodriguez and Pfender, 1997). It is possible that, this secondary metabolite may be a contributing factor in biocontrol of *S. sclerotiorum* as well. The pyrrolnitrin biosynthetic genes, *prnABCD*, have been identified in PA23 by PCR (Zhang Y., 2004) and Southern analysis (Figure 18). Quantification of pyrrolnitrin production by high-performance liquid chromatography (HPLC)

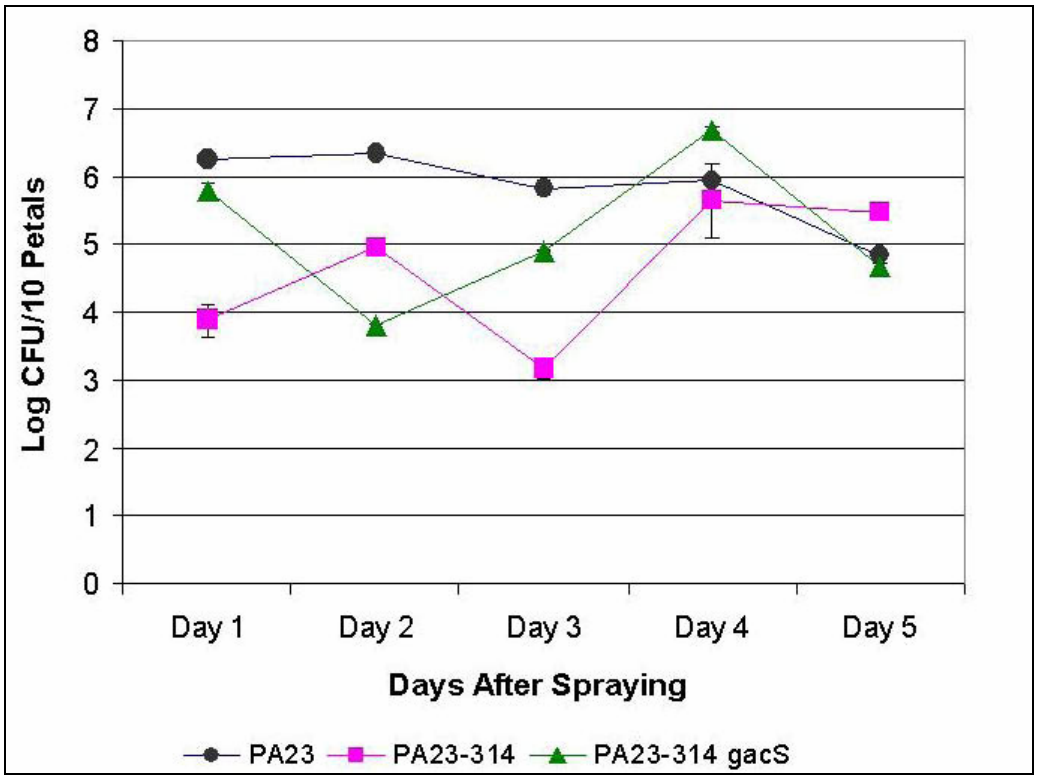


Figure 16: Sustainability of *P. chlororaphis* strains PA23, PA23-315 (*gacS*::Tn*5*) and PA23-314 (*gacS*::Tn*5*, pUCP23-*gacS*) on canola petals.

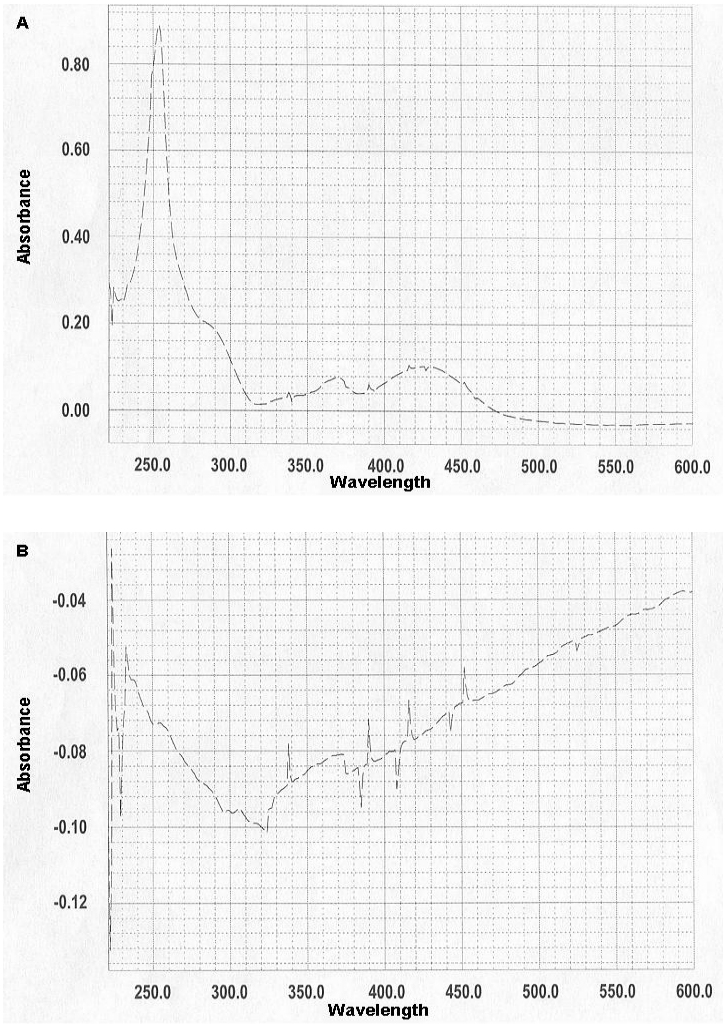


Figure 17: Ultra-violet spectral analysis of total phenazine antibiotic production (1/10 dilution) by *P. chlororaphis* (OD $_{221\text{-}600\ nm}$). **Panel A**: wild type PA23 and **Panel B**: Tn*5* mutant PA23-63.

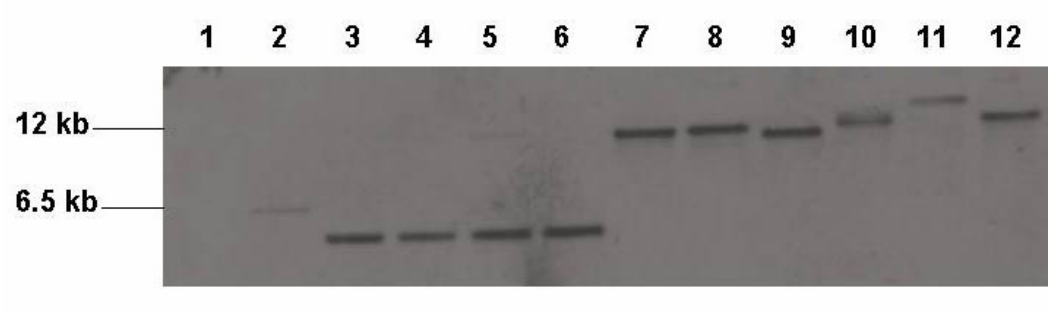


Figure 18: Southern analysis of pyrrolnitrin biosynthetic genes in PA23 chromosomal DNA. Autoradiogram showing probe-reactive *prnD*-containing fragment hybridized with the *prnD*-DIG. **Lane 1**, negative control; **lane 2**, Pf*5* restriction digested with *Kpn*I (positive control for 6.5 kb *prnABCD* cluster); **lane 3**, PA23 *Kpn*I; **lane 4**, PA23 *Kpn*I-*BamH*I; **lane 5**, PA23 *Kpn*I-*Bgl*I; **lane 6**, PA23 *Kpn*I-*EcoR*I; **lane 7**, PA23 *EcoR*I; **lane 8**, PA23 *EcoR*I-*BamH*I; **lane 9**, PA23 *EcoR*I- *Bgl*I; **lane 10**, PA23 *Bgl*I; **lane 11**, PA23 *BamH*I; and **lane 12**, PA23 *Bgl*I-*BamH*I.

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analysis revealed that PA23-63 produces pyrrolnitrin at levels similar to the wild type (Paulitz T., personal communication). Accordingly, pyrrolnitrin is most likely the secondary metabolite produced by PA23 that is responsible for the *S. sclerotiorum* antifungal activity.

To assess whether a deficiency in phenazine production will alter hydrogen cyanide expression, a *hcnA*'-'*lacZ* translational fusion was tested in the wild type and PA23-63 mutant. The expression levels of *hcnA* were equivalent in both PA23 and Pf-5 genetic backgrounds. Mutant PA23-63 had ~30% expression of the *hcnA'-'lacZ* fusion compared to the wild-type PA23 strain (Figure 19). These findings suggest that a deficiency in phenazine production correlates with a deficiency in cyanogenesis. Currently, the regulatory mechanisms linking phenazine and cyanide production in PA23 are not known.

3.5.2 Growth Analysis of Class II Planktonic Cultures

Growth of mutant PA23-63 was compared with the wild type in minimal media M9 supplemented with either 0.2% glucose (Figure 20A) or 0.2% glycerol (Figure 20B) and rich media LB, PTSB, and TB (Figure 21A, 21B and 22). Growth analysis in M9 0.2% glucose showed PA23-63 having a longer lag phase compared to the wild type (Figure 20A), indicating that a mutation in *phzE* may result in a delayed entrance into the exponential phase under minimal nutrient conditions. There was no difference in the duration of the lag and exponential growth phase between PA23-63 and the wild type when tested under rich growth conditions in LB, PTSB, and TB media (Figures 21A, 21B, and 22).

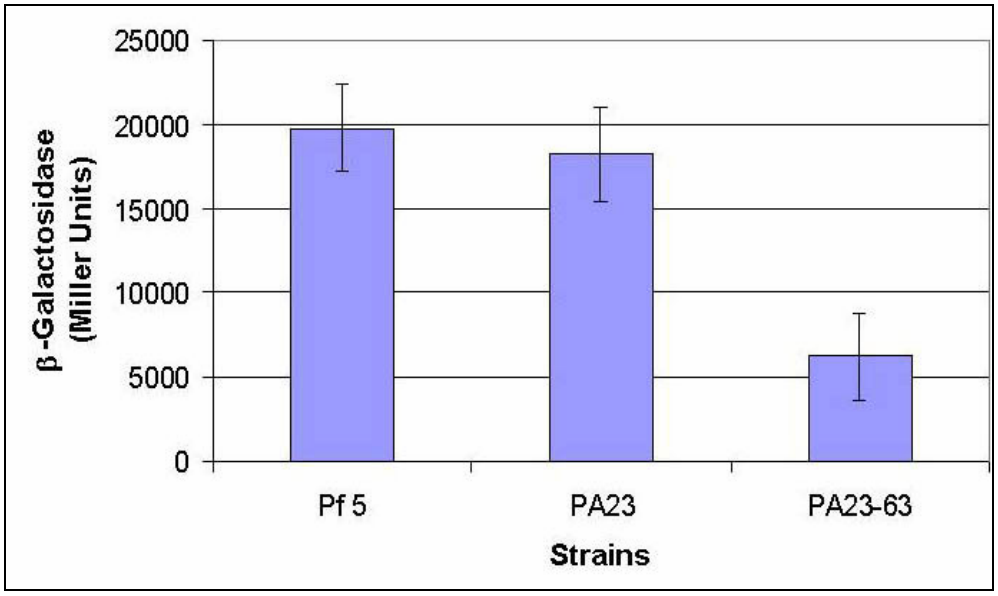

Figure 19: β-Galactosidase activity of *P. fluorescens* Pf5, *P. chlororaphis* PA23, and PA23-63, harboring an *hcnA'-'lacZ* translational fusion (pME3219). β-Galactosidase activity is expressed in Miller units and represents the average of three independent cultures.

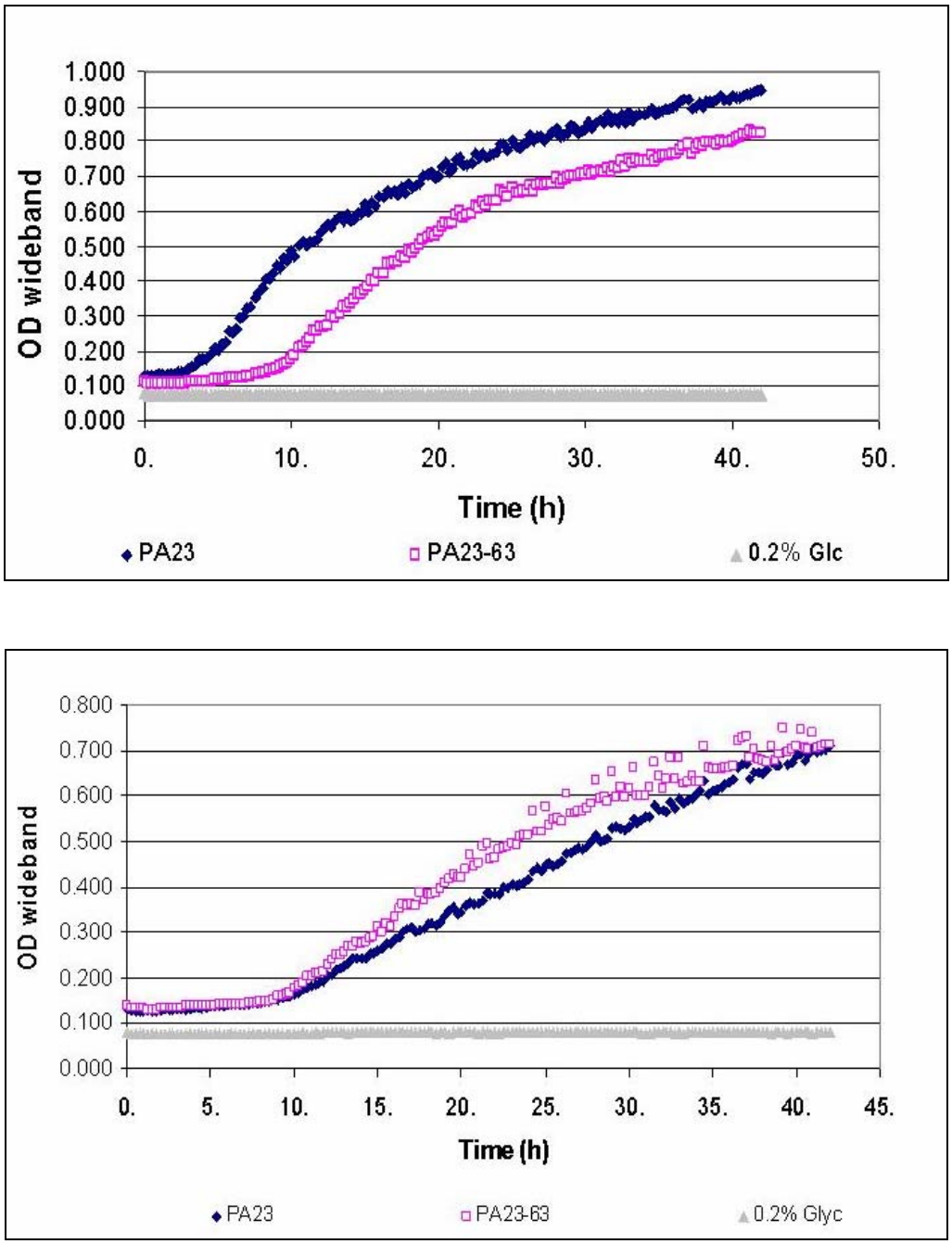

Figure 20: Growth of *P. chlororaphis* PA23 and Tn*5* mutant PA23-63 in liquid culture. Strains PA23 (wild type, pUCP23) and PA23-63 (*phzE*::Tn*5*, pUCP23) were grown in minimal media. **Panel A:** 0.2% glucose; and **Panel B:** 0.2% glycerol.

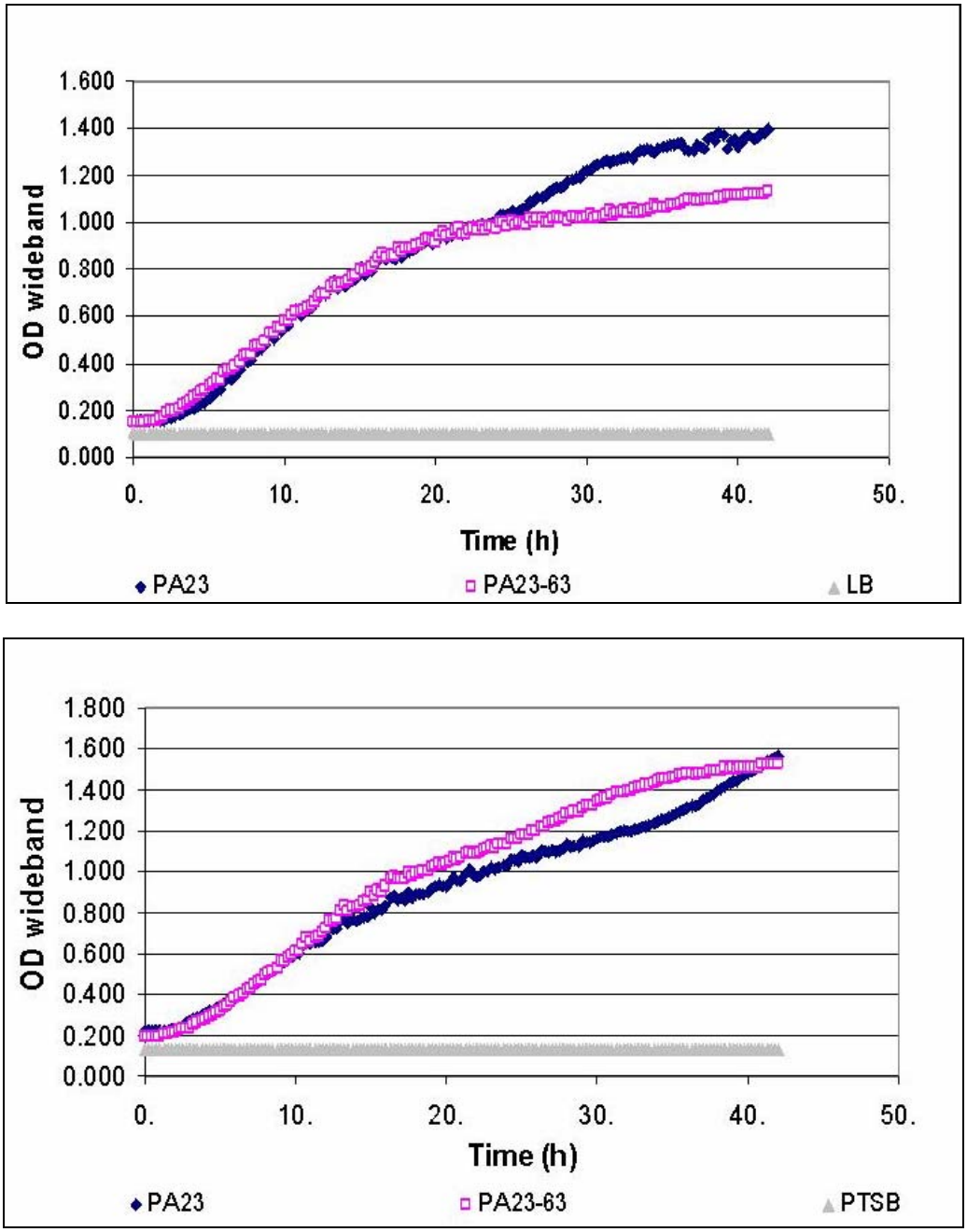

Figure 21: Growth of *P. chlororaphis* PA23 and its Tn*5* mutant PA23-63 in liquid culture. Strains PA23 (wild type, pUCP23) and PA23-63 (*phzE*::Tn*5*, pUCP23) were grown in rich media. **Panel A:** LB; and **Panel B:** PTSB.

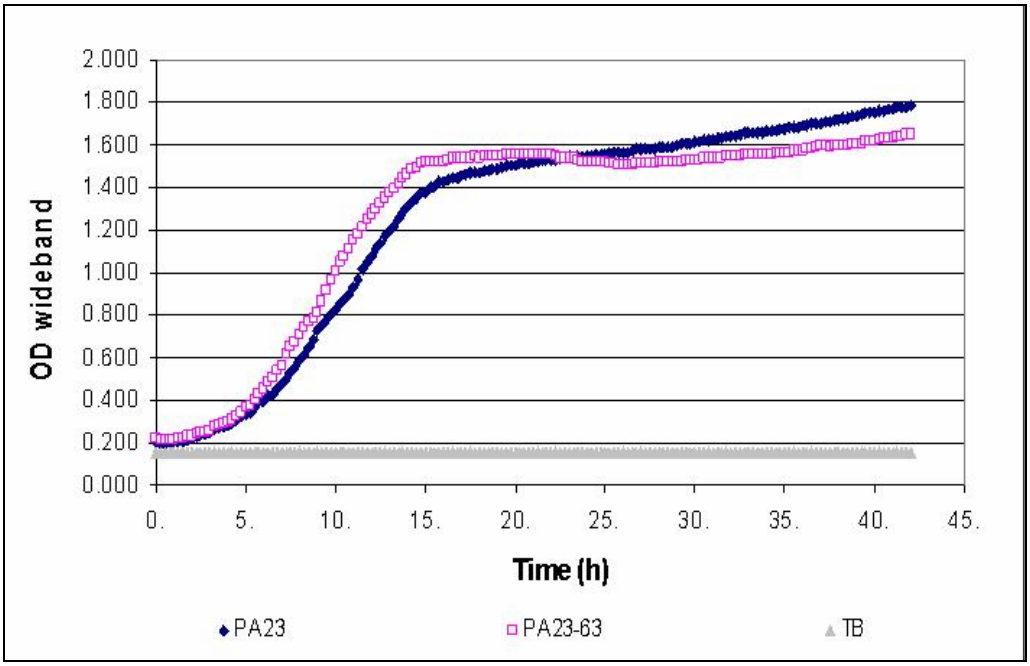

Figure 22: Growth of *P. chlororaphis* PA23 and its Tn*5* mutant PA23-63 in liquid culture. Strains PA23 (wild type, pUCP23) and PA23-63 (*phzE*::Tn*5*, pUCP23) were grown in ultra-rich media TB.

3.5.3 Motility Analysis

The PA23-63 mutant had a significantly higher rate of flagellar motility compared to the wild type (Figure 23), suggesting that in a *phzE* mutant background there is functional flagellar system. Furthermore, the wild type and the mutant PA23-63 strain were able to propagate across the surface of swarm plates at equivalent rates (data not shown) indicating that, in addition to a functional flagella system, there is biosurfactant production.

3.5.4 Plant Assays

The following plant studies were performed by Dr. Nakkeeran in the laboratory of Dr. D. Fernando (Department of Plant Science, University of Manitoba). In plants inoculated with PA23-63 and *S. sclerotiorum* ascospores, stem rot incidence caused by the fungus was 2.8%. Therefore, PA23-63 provided a slightly higher ($P < 0.5$) disease protection compared to the 5% stem rot incidence of the wild type (Figure 24A). Analysis of percent leaf infection caused by *S. sclerotiorum* showed that the wild type and mutant PA23-63 reduced the disease incidence by 90% and 91.25%, respectively (Figure 24B). Overall pathogenesis was represented as disease severity. In PA23-63-inoculated plants the disease severity was significantly lower ($P < 0.0005$) compared to the pathogen inoculated control. Therefore, an additional secondary metabolite(s), presumably pyrrolnitrin, is responsible for providing efficient plant protection (Figure 25).

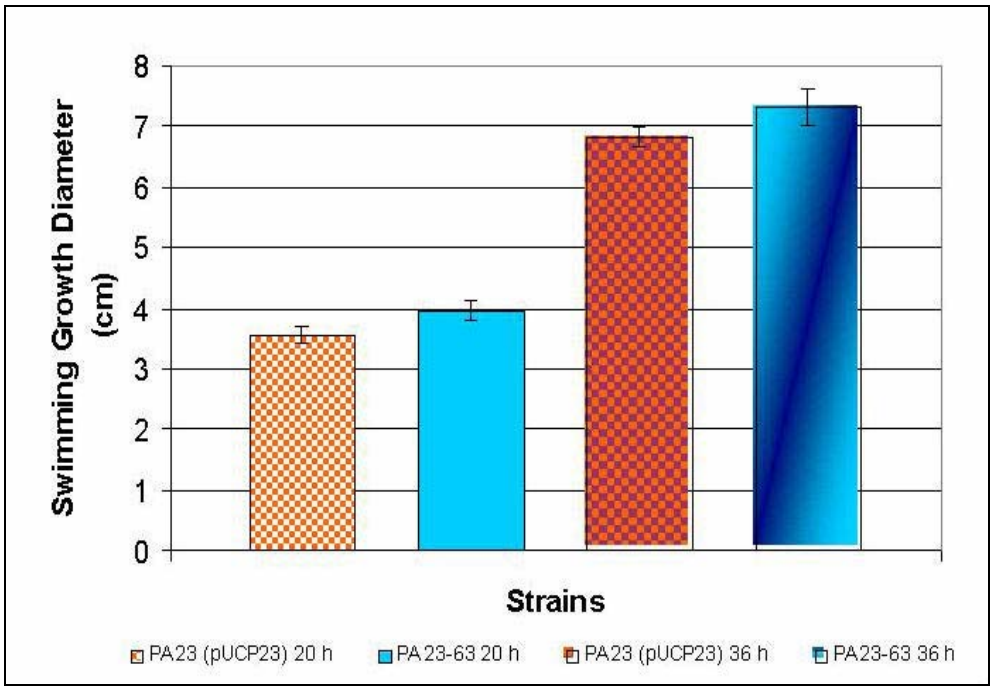

Figure 23: The swim zones of *P. chlororaphis* flagellar motility in 0.3% agar. Strains PA23 (wild type), PA23-63 (*phzE*::Tn*5*) were measured at 20 h (open bars) and 36 h (filled bars).

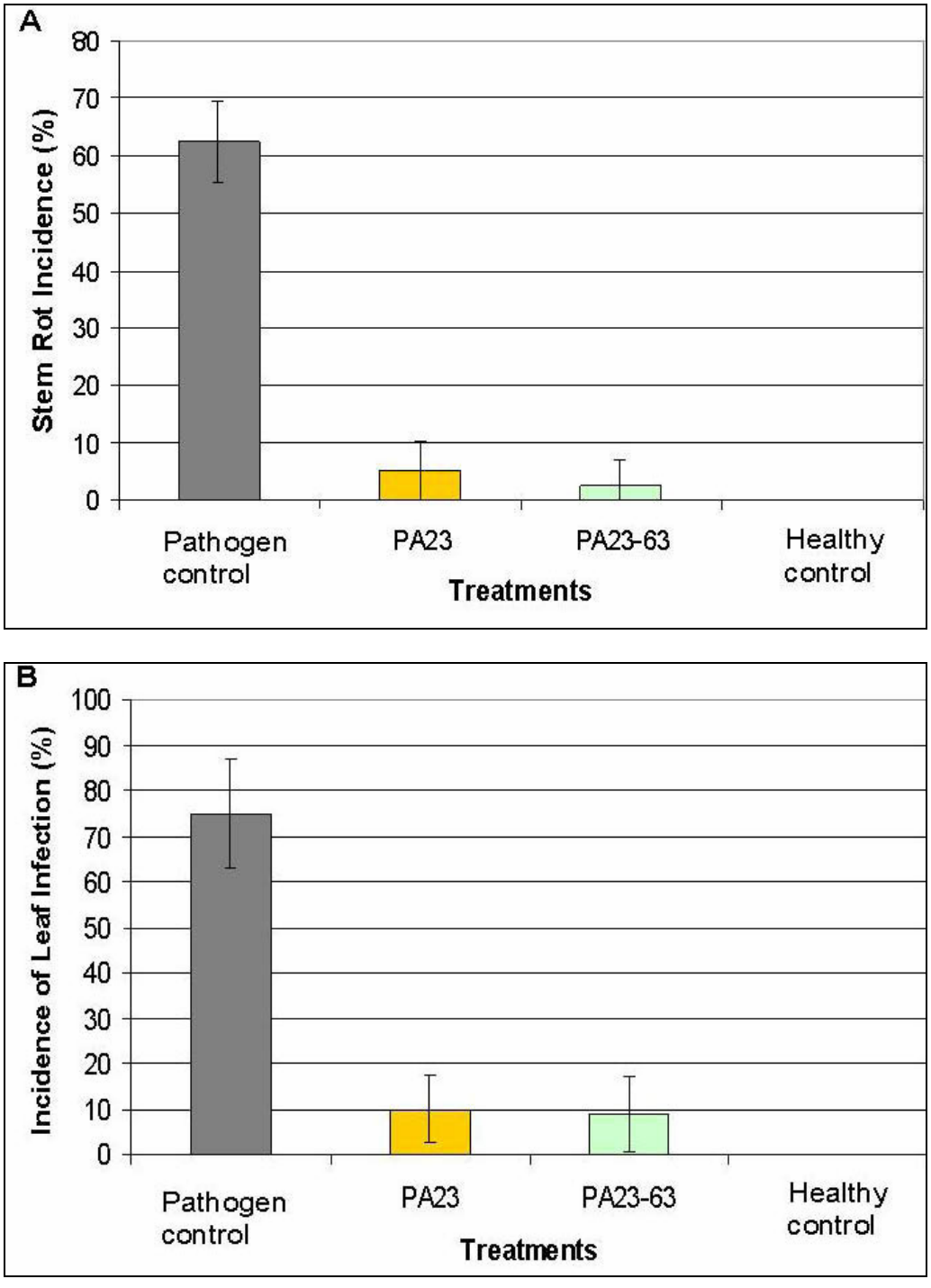

Figure 24: Efficiency of *P. chlororaphis* PA23 (wild type) and PA23-63 (*phzE*::Tn*5*) in managing *S. sclerotiorum* infection on canola plants. **Panel A:** Percent stem rot incidence; and **Panel B:** Percent incidence of leaf infection.

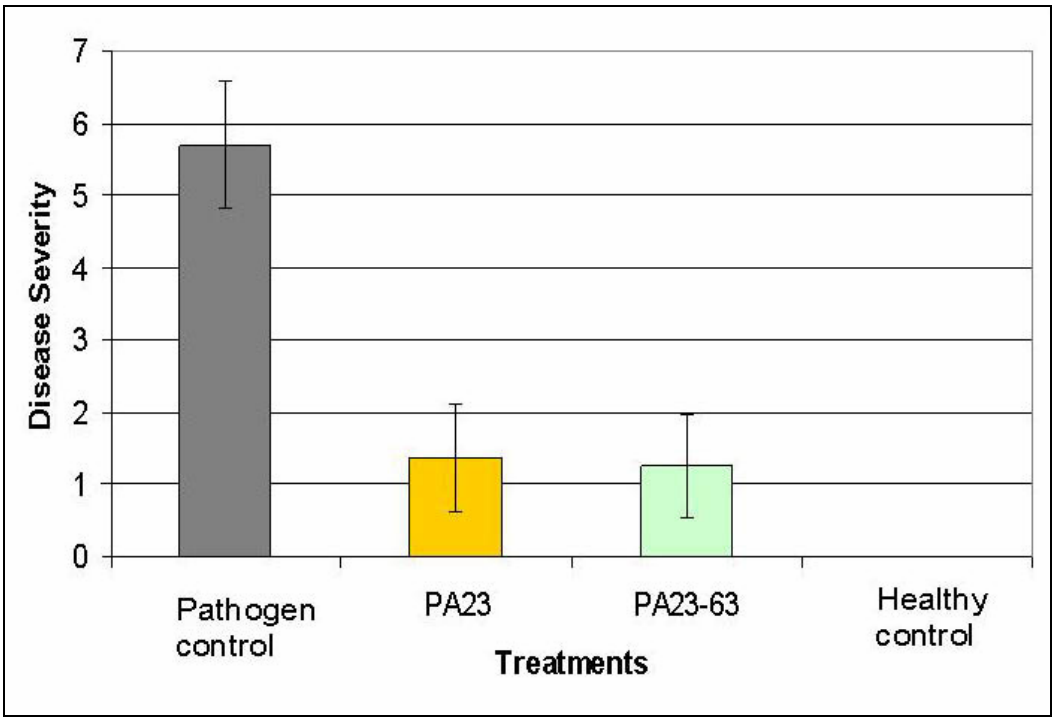

Figure 25: Biocontrol of *S. sclerotiorum* by *P. chlororaphis* PA23 (wild type) and PA23-63 (*phzE*::Tn*5*).

3.5.5 Bacterial Sustainability of Class II Mutant PA23-63 on Canola

Strains PA23 and PA23-63 showed similar colonization patterns over a 5-day period. Mutant PA23-63 colonization on the canola petals was 20% lower than the wild type on day 1 (Figure 26). Both the wild type and mutant had equivalent populations on the canola petals by day 5, indicating that PA23-63 is able to colonize and sustain itself in the canola phyllosphere.

3.6 Phenotypic Characterization of Class III Mutant PA23-443 (Tn*5*-OT182 in a transcriptional regulator of the LysR family)

3.6.1 Analysis of Secondary Metabolite Production

Mutant PA23-443 has a unique phenotype in that it has a white pigment at 24 h but it turns orange at ~48 h, indicating that as the culture matures, there is a latent production of phenazine. Spectral analysis revealed that there was no phenazine production in late logarithmic cultures (20 h growth) (Figure 27). Strain PA23-443 antifungal activity was tested with cultures grown for 16 h, 24 h, and 48 h. All three cultures exhibited no antifungal activity against *S. sclerotiorum*. At 24 h, mutant PA23-443 produces no AHL or protease activity (Table 2). At 72 h, strain PA23-443 showed 36% AHL production and 80% protease activity, indicative of a latent expression of *phzI* and protease genes, respectively. Addition of high

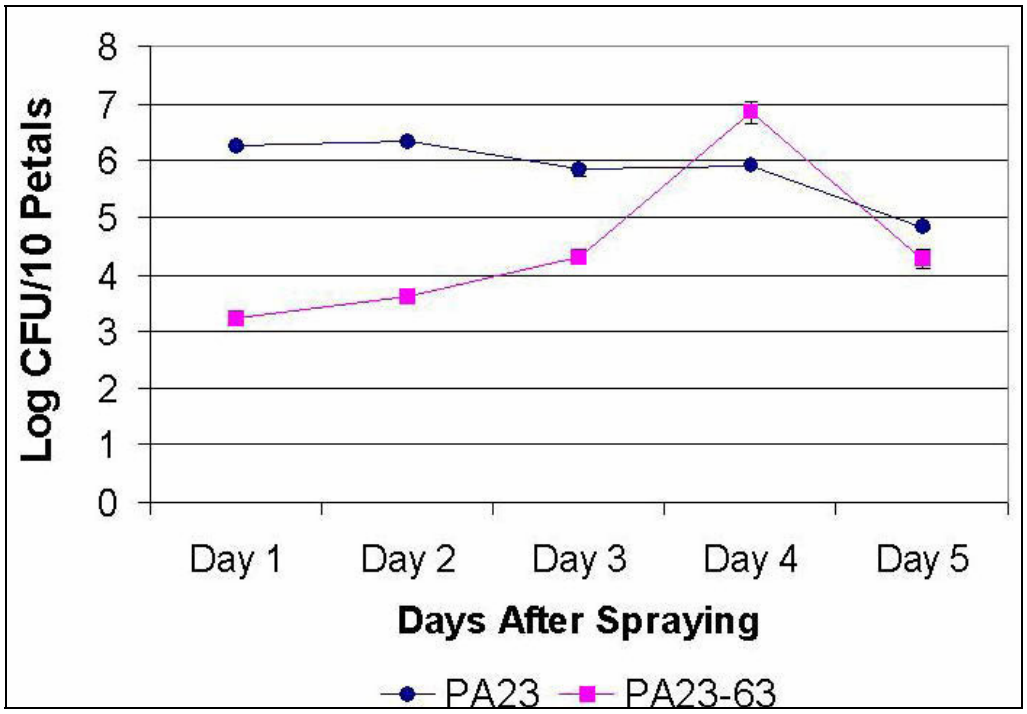

Figure 26: Sustainability of *P. chlororaphis* strains PA23 (wild type) and PA23-63 (*phzE*::Tn*5*) on canola petals.

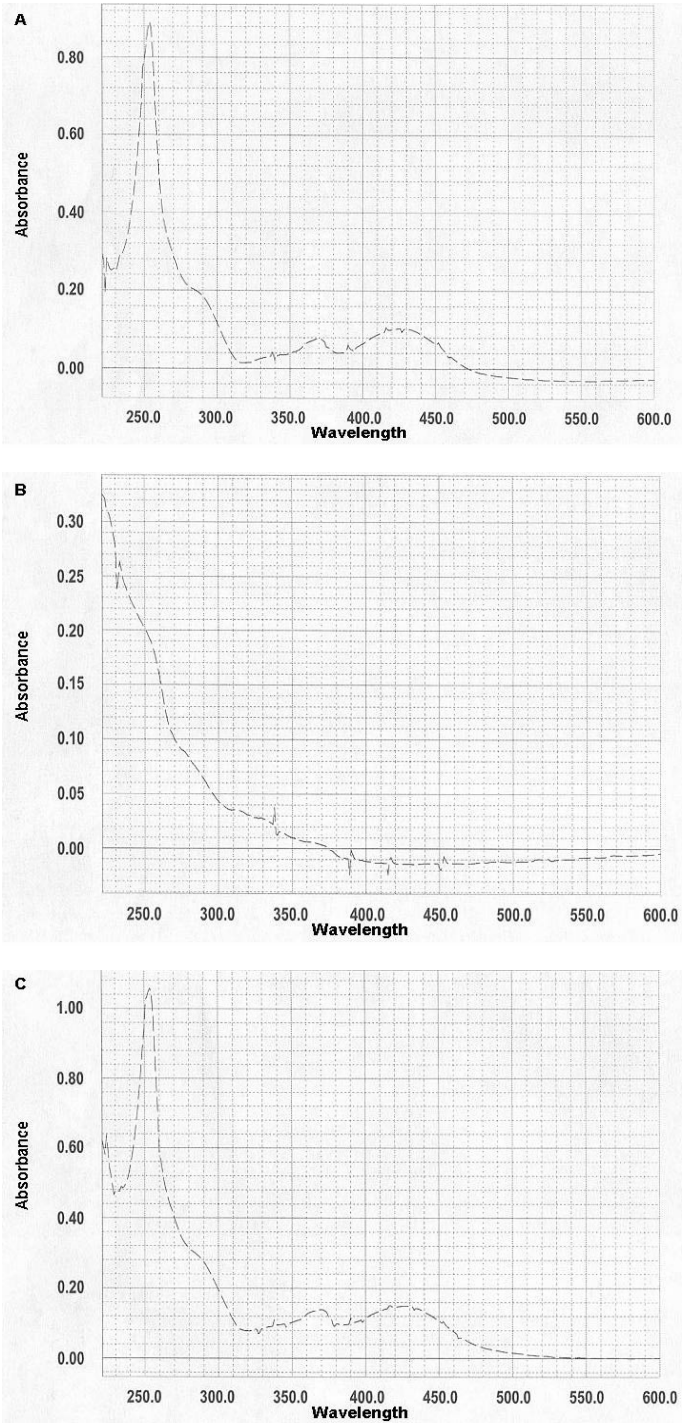

Figure 27: Ultra-violet spectral analysis of total phenazine antibiotic production (1/10 dilution) by P. chlororaphis PA23 and its Tn5 mutant PA23-443 (OD $_{221\text{-}600\ nm}$). **Panel A:** PA23 (pUCP23); **Panel B:** PA23-443 (pUCP23); and **Panel C:** PA23-443(pUCP23-*gacS*).

copy numbers of *gacS* on pUCP23-*gacS* restored all wild-type phenotypes (Table 2). Thus, it appears that multiple copies of *gacS* somehow compensate for the PA23-443 mutation.

To assess the effect of this regulator on hydrogen cyanide expression, an *hcnA*'-'*lacZ* translational fusion was tested in the wild type and the PA23-443 mutant backgrounds. The expression levels of *hcnA* were equivalent in both PA23 and Pf-5 genetic backgrounds. Mutant PA23-443 had expression levels near the detection limit, significantly lower than the wild type ($P < 0.05$) (Figure 28).

3.6.2 Growth Analysis of Class III Planktonic Cultures

The growth profile of LysR mutant PA23-443 was compared with the wild-type PA23 and PA23-443 (pUCP23-*gacS*) strains, harbouring a functional *gacS* allele, tested in minimal media M9 supplemented with 0.2% glucose (Figure 28A) and rich media LB, PTSB and TB (Figure 28B, 29A and 29B respectively).

In M9 minimal media, the PA23-443 entered the logarithmic growth phase 5 h earlier than the wild type. The presence of extra copies of *gacS* in PA23-443 did not result in a longer lag phase as that seen by the wild-type strain, suggesting that the rapid increase in population density is due to the inactivation of this LysR-type transcriptional regulator. Over prolonged growth (20 h), the PA23-443 population density plateaued compared to the steady increase observed with the wild-type and PA23-443 (pUCP23-*gacS*) strains. At 45 h the PA23-443 mutant exhibited a sudden increase in population density at a time when the wild type and PA23-443 (pUCP23-*gacS*) strains had plateaued.

In LB medium (Figure 28B), mutant PA23-443 had the similar growth profile as that of the wild type, with the exception of the presence of a plateau in population density at 20-40 h. As in the minimal growth conditions, mutant PA23-443 exhibited a an increase in population density (60 h) that reached the levels of the wild type. Under ultra rich growth conditions (Figure 29A, 29B) PA23-443 had a rapid decrease in the rate of population growth over time. However, over a prolonged period of growth (85 h) mutant PA23-443 established a population density similar to the wild type. The presence of multiple copies of *gacS* in PA23-443 restored the growth profile to that of the wild type (Figure 28B, 29A, 29B)

3.6.2 Motility Analysis

The PA23-443 mutant had a significantly lower rate of flagellar motility compared to the wild type. Furthermore, addition of a functional *gacS* gene complemented the flagellar motility rate to wild-type levels (Figure 30). The PA23-443 swarming phenotype consisted of a shoot of growth from one region of the colony that later expanded to resemble flagellar motility (data not shown). In contrast, the wild type and strain PA23-443 (pUCP23-*gacS*), exhibited a swarming phenotype resembling a dendritic pattern of growth (data not shown). These results indicate that mutant PA23-443 exhibits an altered pattern of swarming motility and high copies of *gacS* seems to compensate for these anomalies.

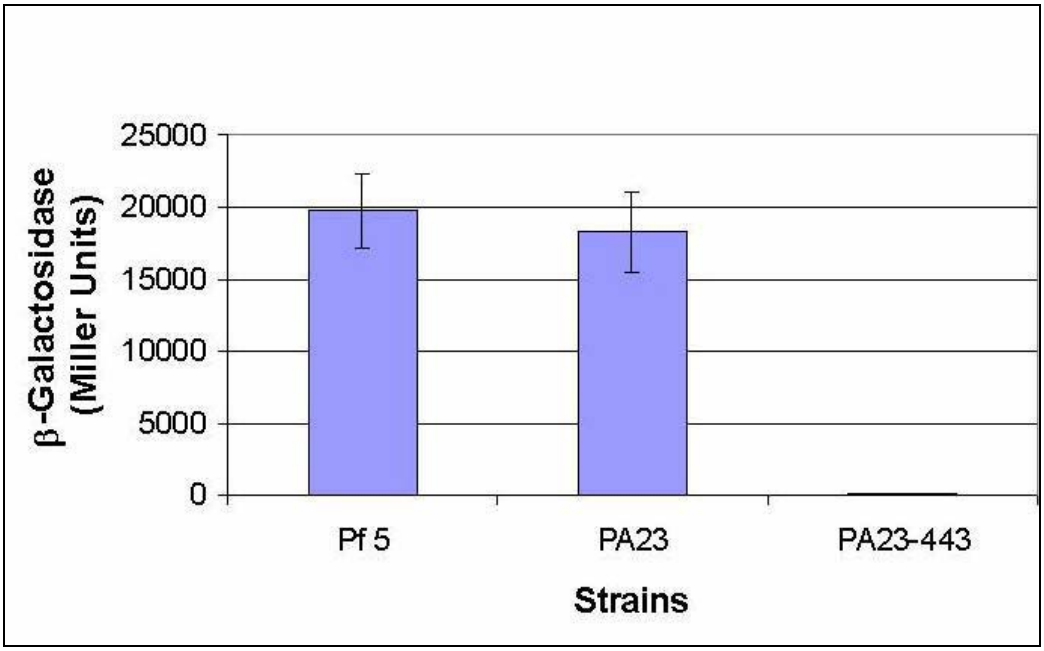

Figure 28: β-Galactosidase activity of *P. fluorescens* Pf5, *P. chlororaphis* PA23, and PA23-443, harboring an *hcnA'-'lacZ* translational fusion (pME3219). β-Galactosidase activity is expressed in Miller units and represents the average of three independent cultures.

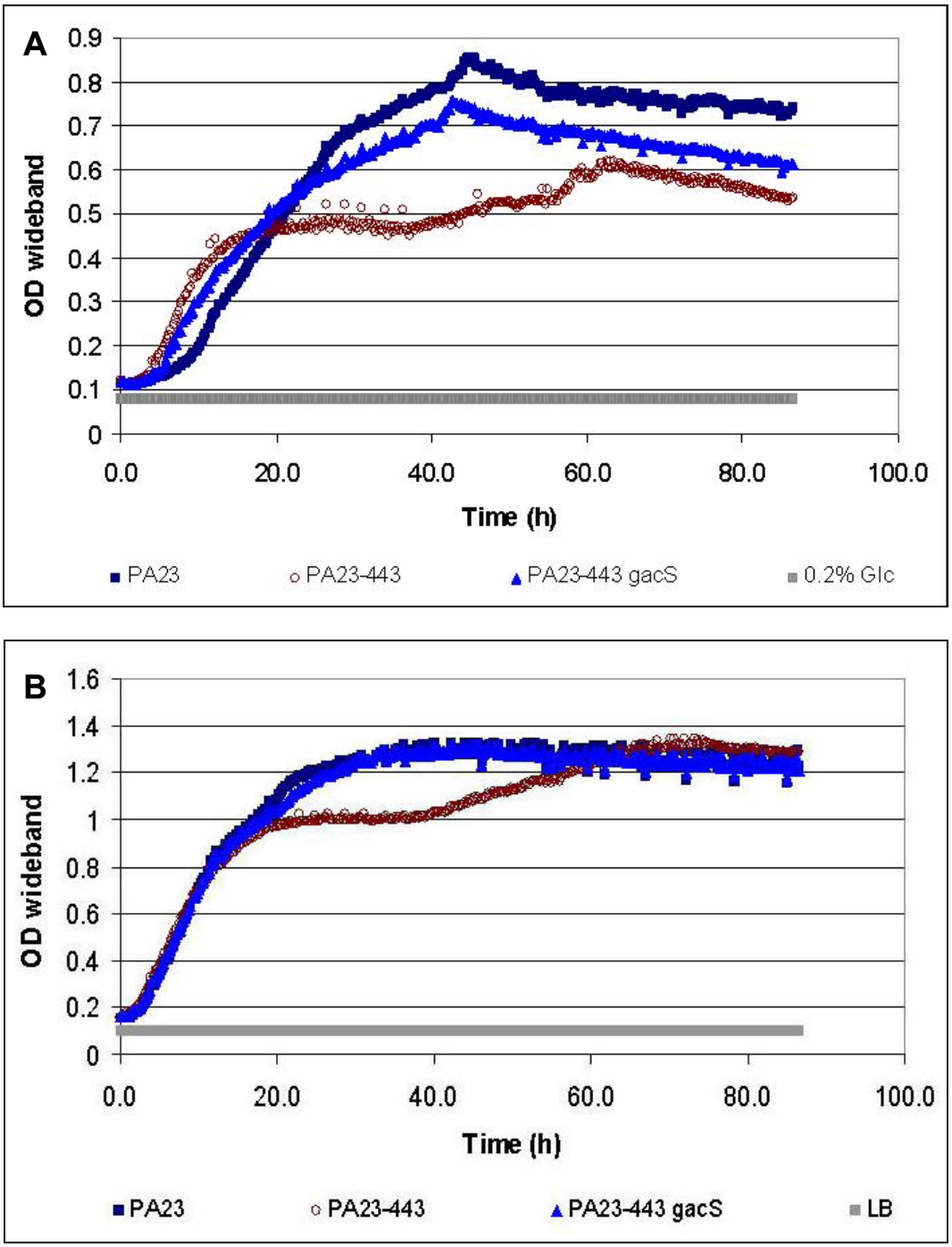

Figure 29: Growth of *P. chlororaphis* PA23 and Tn*5* mutant PA23-443 in liquid culture. Strains PA23 (wild type, pUCP23), PA23-443 (*lysR*::Tn*5*, pUCP23) and PA23-443 *gacS* (pUCP23-*gacS*) were grown in minimal and rich media. **Panel A:** 0.2% glucose; and **Panel B:** LB.

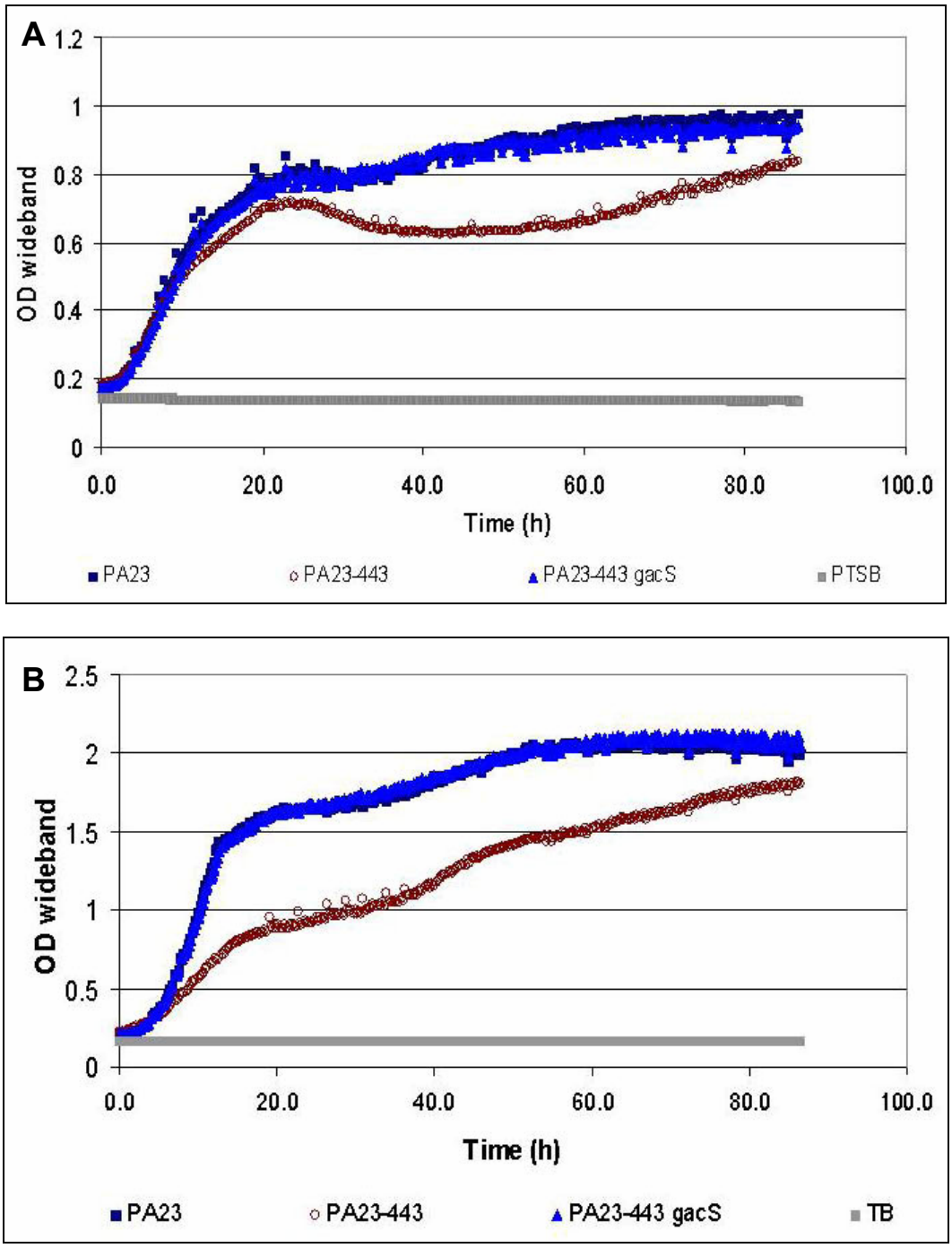

Figure 30: Growth of *P. chlororaphis* PA23 and Tn*5* mutant PA23-443 in liquid culture. Strains PA23 (wild type, pUCP23), PA23-443 (*lysR*::Tn*5*, pUCP23) and PA23-443 *gacS* (pUCP23-*gacS*) were grown in rich media. **Panel A**: PTSB; and **Panel B**: TB.

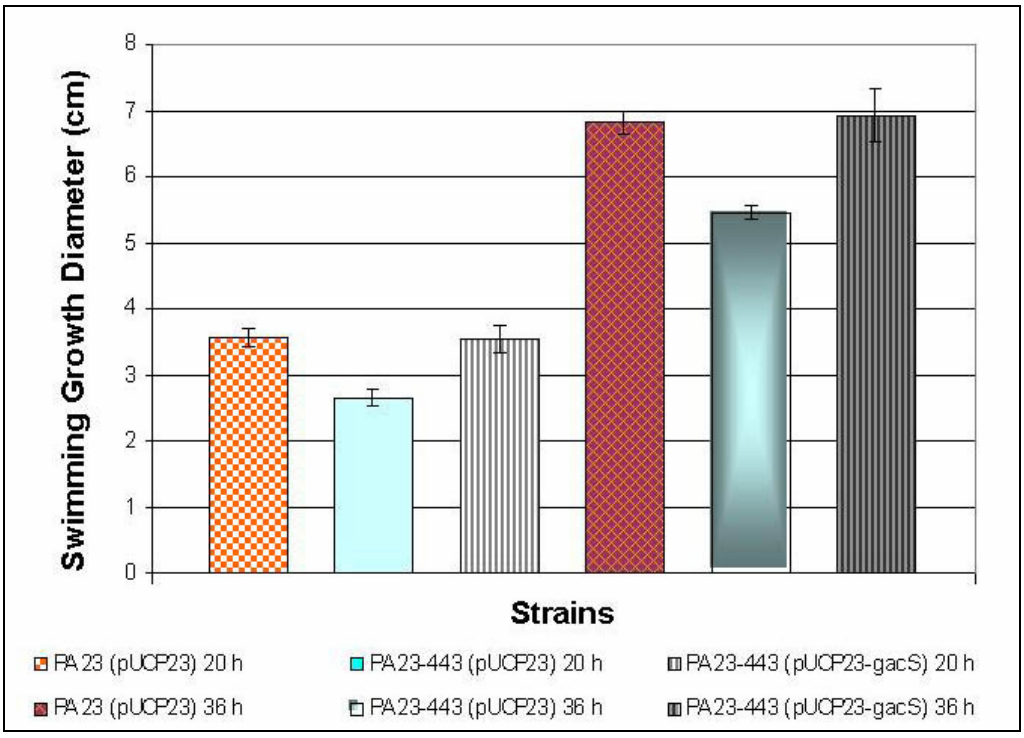

Figure 31: The swim zones of *P. chlororaphis* flagellar motility in 0.3% agar. Strains PA23 (wild type, pUCP23), PA23-443 (Tn*5* in the coding region of a LysR-type transcriptional regulator, pUCP23) and PA23-443 (pUCP23-*gacS*) with multiple *gacS* copies, were measured at 20 h (open bars) and 36 h (filled bars).

3.6.3 Biofilm Formation in Class III Mutant PA23-443

PA23-443 had a 92% reduction in biofilm formation on a PVC abiotic surface compared to the wild type (Figure 32), suggesting that the initial stages of biofilm establishment may be negatively affected in this mutant background. Once again, multiple copies of *gacS* restored the phenotype to that of PA23 wild type.

3.6.4 Plant assays

The following plant studies were performed by Dr. Nakkeeran in the laboratory of Dr. D. Fernando (Department of Plant Science, University of Manitoba). To determine the importance of the PA23 putative transcriptional regulator in managing *S. sclerotiorum* infection in canola plants, the wild type PA23 and PA23-443 mutant were tested in greenhouse studies. Incidence of stem rot caused by *S. sclerotiorum* in canola plants inoculated with PA23-443 and Sclerotinia ascospores was 41%, compared to the wild type (5%) and the pathogen-only (62.5%) control (Figure 33A). PA23-443 inoculated plants resulted in 53.75% incidence of leaf infection compared to 10% for the wild type and 75% for the pathogen-only control (Figure 33B). Overall the disease severity of PA23-443 inoculated plants was a factor of 4 (in a scale 0-7), representing a significantly decreased ($P < 0.005$) disease suppressive ability compared to the wild type (1.37) (Figure 34). These findings imply that in a PA23 background,

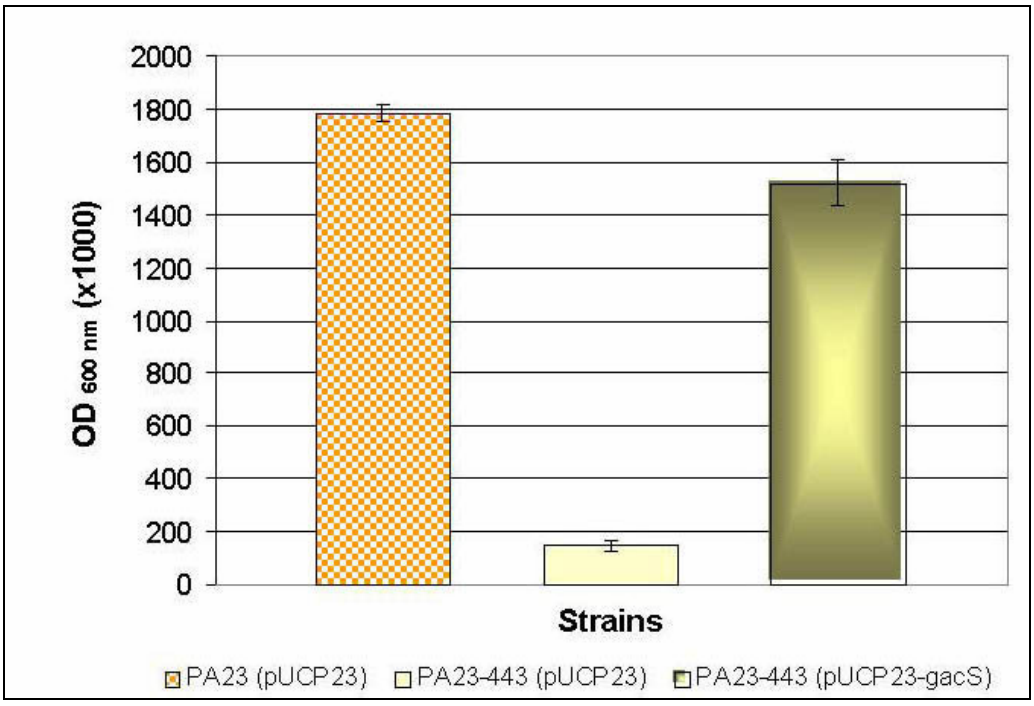

Figure 32: Biofilm growth of *P. chlororaphis* in M9CA (0.2% glucose, 1mM $MgSO_4$). Strains PA23 (wild type, pUCP23), PA23-443 (Tn*5* insertion in the coding region for a LysR-type transcriptional regulator, pUCP23) and PA23-443 (pUCP23-*gacS*) were tested for their ability to form biofilms on PVC plastic surface.

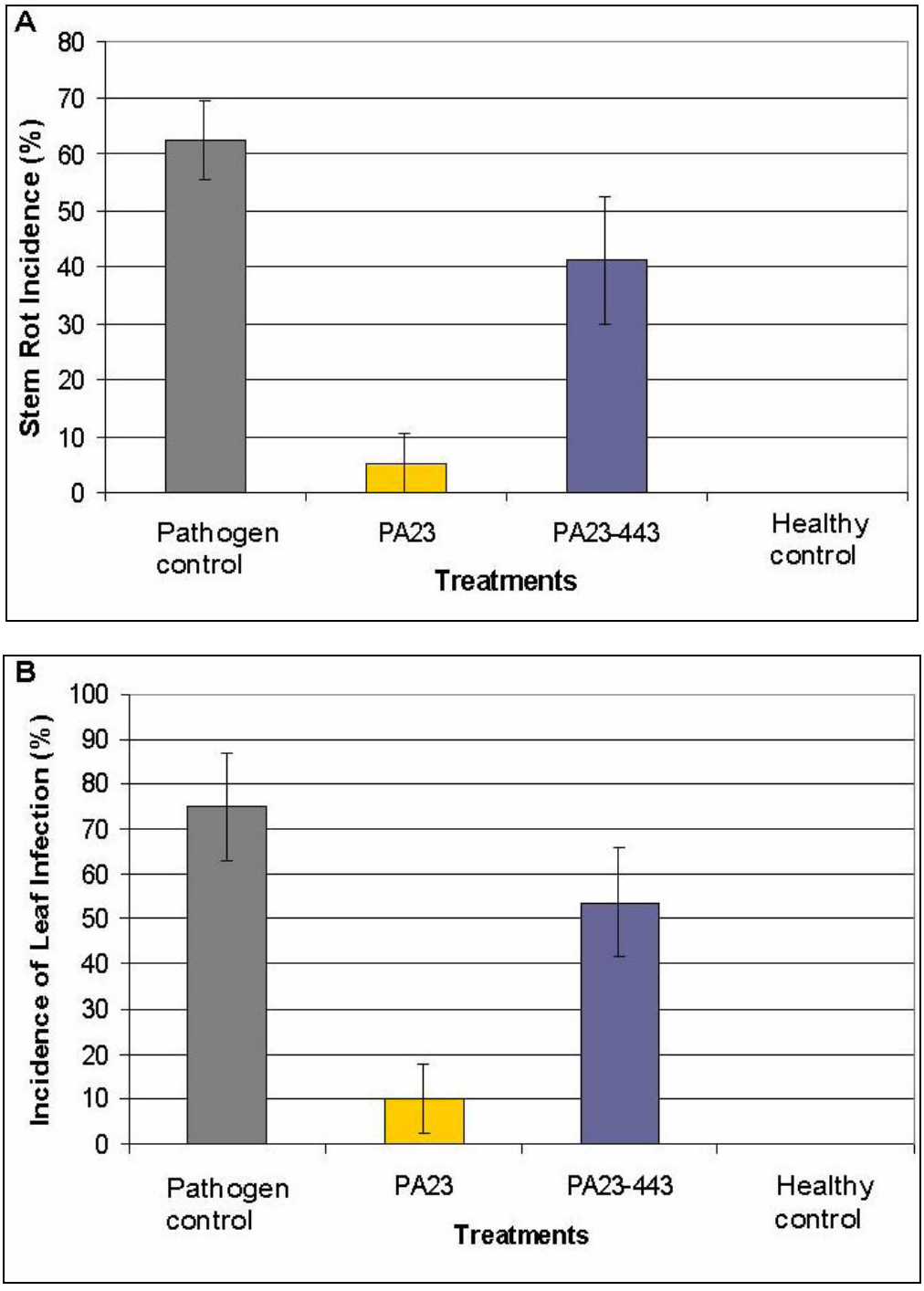

Figure 33: Efficiency of *P. chlororaphis* PA23 and PA23-443 (Tn*5* insertion in the coding region for a LysR-type transcriptional regulator) in managing *S. sclerotiorum* infection in canola plants. **Panel A:** Percent stem rot incidence; and **Panel B:** Percent incidence of leaf infection.

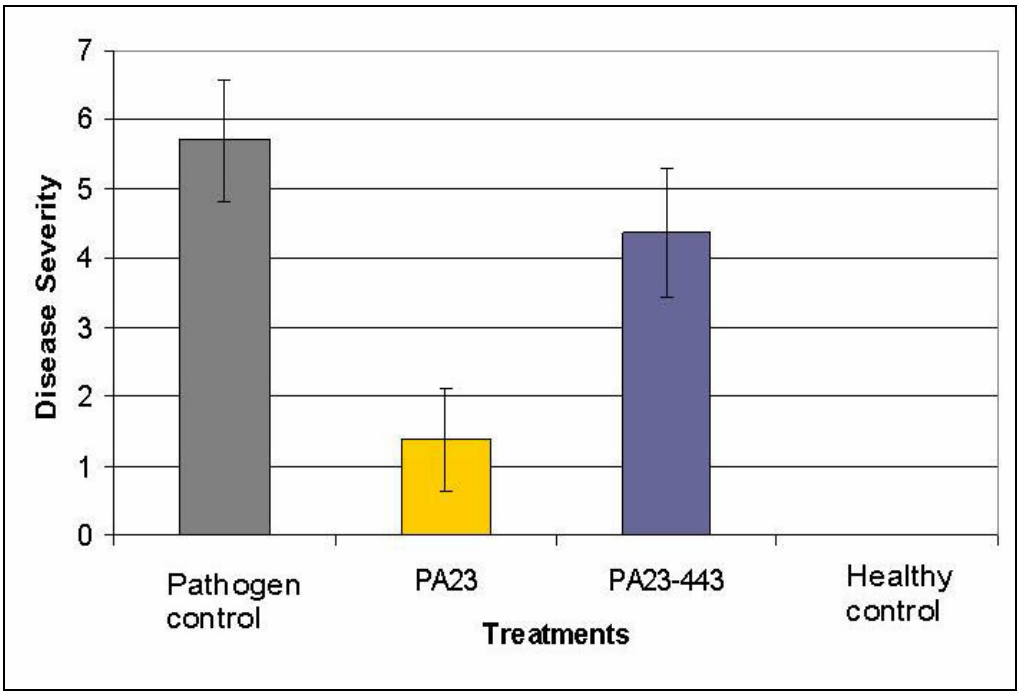

Figure 34: Biocontrol of *S. sclerotiorum* by *P. chlororaphis* PA23 (wild type) and PA23-443 (Tn*5* insertion in the coding region for a LysR-type transcriptional regulator).

mutation in the gene encoding this LysR-type transcriptional regulator significantly reduces the ability to protect canola plants from the pathogenic effects of *S. sclerotiorum*.

3.6.5 Bacterial Population Dynamics of Class III Mutant PA23-443 on Canola

Analysis of the colonization patterns of PA23 and PA23-443 over a 5-day period revealed the size of the mutant PA23-443 population was 50% lower compared to the wild type on day 1 (Figure 35). By day 3 the two populations were equivalent and on day 5, mutant PA23-443 had a 17% higher population size than the wild type (Figure 35). These data indicate that mutant PA23-443 was able to establish itself and to maintain a population sufficient for biocontrol in the phyllosphere. Therefore, the deficiency in biocontrol activity observed in PA23-443 can be attributed to a delayed or deficient production of secondary metabolites and not in the inability to colonize the canola plant.

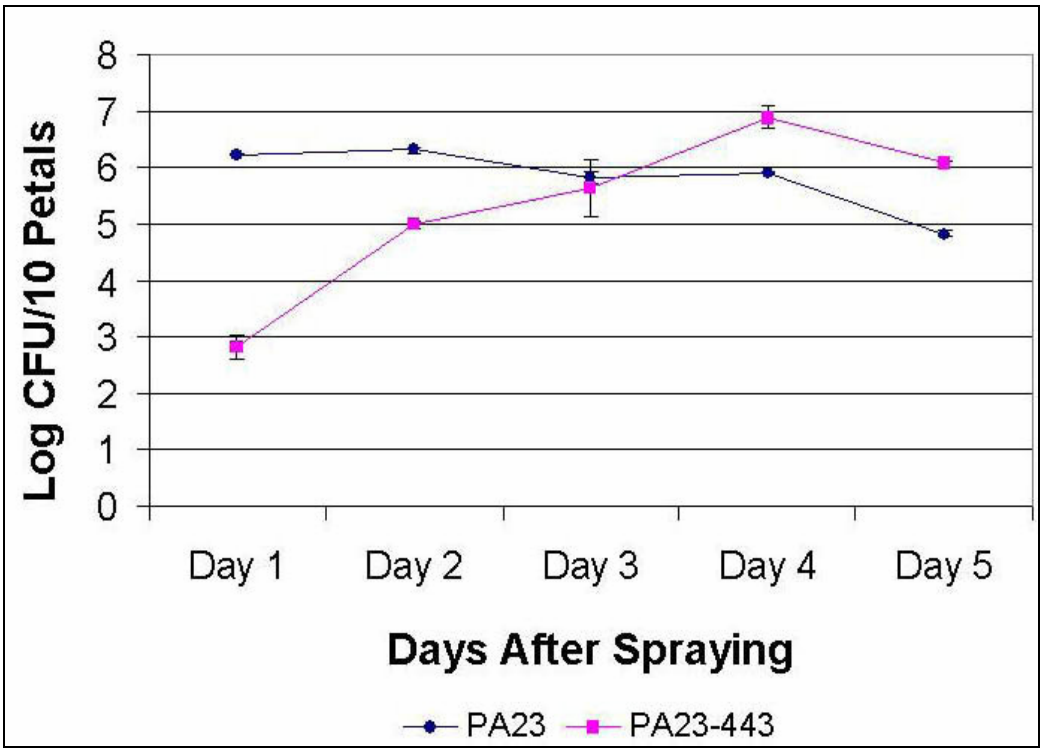

Figure 35: Sustainability of *P. chlororaphis* strains PA23 (wild type) and PA23-443 (Tn*5* insertion in the coding region of a LysR-type transcriptional regulator) on canola petals

4.0 DISCUSSION

Biocontrol activity mediated by antibiotic production has been proven to be one of the main mechanisms of *S. sclerotiorum* disease suppression by *P. chlororaphis* PA23, both in the fields and in green house studies (Zhang Y. 2004). To elucidate molecular mechanisms underlying the biocontrol activity of *P. chlororaphis* PA23, transposon mutagenesis was performed. Three classes of PA23 mutants exhibiting significantly altered biocontrol activity were isolated and characterized. Mutants in class I had insertions in the *gacS* gene, encoding a transmembrane sensor kinase of the GacS/GacA two-component global regulatory system. Class II mutants contained Tn*5* insertions in phenazine biosynthetic genes. A single class III mutant was isolated with a mutation in a gene encoding a putative transcriptional regulator of the LysR family. When tested *in vitro* against *S. sclerotiorum*, Class I and III mutants lost biocontrol activity*,* whereas Class II mutants showed equal or increased biocontrol activity compared to the wild type.

The *gacS* mutant PA23-314, has multiple defects including an inability to produce AHL quorum sensing signals, phenazine antibiotics, HCN, and extracellular lytic enzymes, such as proteases and lipases (Table 2). Unlike the other secondary metabolites, siderophore production is upregulated in this mutant background (Table 2). This is characteristic of other *Pseudomonas* spp. having a *gacS* mutation, displaying a hyperfluorescent phenotype due to the increased levels of siderophore production (Chancey *et al.*, 1999; 2002; Duffy and Defago, 2000; Sanchez-Contreras *et al.*, 2002).

Earlier studies analyzing factors contributing to disease suppression by *Pseudomonas* spp. indicated that siderophore activity, involving the uptake of iron from the environment, was a biocontrol factor. Siderophores limit the iron availability in the environment with the outcome of compromising the growth of pathogens inhabiting the same niche (Bakker *et al.*, 1986; Loper and Henkers, 1999; O'Sullivan and O'Gara, 1992). In PA23-314, the increase in siderophore production was not sufficient for biocontrol of *S. sclerotiorum* when tested in plate assays and green house experiments.

Colonization of the plant surface has been proven to be an essential factor for biocontrol activity in *P. chlororaphis* (Schmidt-Eisenlohr *et al.*, 2003; Chin-A-Woeng *et al*., 2000; Dekkers *et al.*, 2000). When tested in green house assays, the *gacS* mutant PA23-314 was efficient in establishing a population density required for biocontrol over a 5-day period compared to the wild-type strain PA23 (Figure 16). The increase (days 2 and 4) and decrease (day 3) in population density observed by the *gacS* mutant PA23-314, may be explained by a deficiency in the majority of the PA23-314 population to survive in the phyllosphere over a prolonged period of time. Thus, after day 2, the majority of the PA23-314 population dies and only a small portion remains viable that is responsible for the rapid increase in population growth observed towards day 4 (Figure 16). Previous work by Schmidt-Eisenlohr and coworkers (2003) showed that a *gacS* mutant strain of *P. chlororaphis* SPR044 exhibited a deficiency in rhizosphere colonization (Schmidt-Eisenlohr *et al.*, 2003). Therefore, depending

on the environmental setting, rhizosphere or phyllosphere, and on the genetic background, a *gacS* mutation may have different effects.

To our knowledge this is the first study assessing disease symptoms on canola plants inoculated with a *gacS* minus strain of *P. chlororaphis.* Total biocontrol activity was assessed based on the disease severity observed and recorded on a scale of 0-7 (0: no lesions; and 7: lesions of more than 100 cm, or plant death). In PA23-314-inoculated plants, the disease severity was 4.7 (~60 cm lesion), representing significantly lower plant protection ($P < 0.0001$) compared to the wild type PA23 inoculated plants (Figure 14). Canola stem rot incidence and incidence of leaf infection was significantly lower in the wild type-inoculated plants compared to the fungal-inoculated control (Figures 15A and 15B). In contrast, the PA23-314-inoculated plants closely resembled the pathogen only control, indicating that GacS is essential for disease protection against *S. sclerotiorum*.

It was previously shown that GacS influences growth rate of *P. aureofaciens* 30-84 in liquid culture (Chancey *et al.*, 2002). Growth analysis of PA23-314 indicated that under minimal growth conditions, the *gacS* mutant population enters the exponential phase more rapidly than the wild type (Figures 9A and 9B). Competition for nutrients and iron is known to be part of the biocontrol activity in *Pseudomonas* spp. by limiting the ability of the fungal pathogen to propagate and survive in that same environment (Fernando *et al.*, 1996; Handelsman and Stabb, 1996). Considering the earlier entrance into exponential growth phase and presumably faster acquisition of carbon sources

from the environment, as well as, the ability to scavenge higher levels of iron in the PA23-314 *gacS* mutant background, it may be advantageous to include PA23-314 as part of the total biocontrol population in *P. chlororaphis* PA23 coinoculation experiments.

When grown in rich nutrient conditions (LB and PTSB) and especially ultra-rich conditions (TB), the *gacS* mutant had an earlier entry into the stationary phase compared to the wild type (Figures 10A, 10B and 11). Presently, it is not clear why the *gacS* mutant population density plateaus earlier than does the wild type. Possibly the *gacS* mutant is unable to acquire readily utilizable carbon sources from a complex nutrient medium. In support of this, Schmidt-Eisenlohr *et al.* (2003) proposed that reduced colonization of a *P. chlororaphis gacS* mutant might be attributed to decreased nutrient utilization due to a lack of extracellular enzymes. The decrease in colonization was reversed when the *gacS* mutant was co-inoculated with the wild type in the rhizosphere (Schmidt-Eisenlohr *et al.*, 2003).

GacS positively regulates the stationary sigma factor RpoS, which induces expression of genes encoding catalase and peroxidase in response to oxidative stress (Heeb *et al.*, 2005; Kang *et al.*, 2004; Whistler *et al.*, 1998). Sensitivity to 9 mM H_2O_2 in the *gacS* mutant background was lower ($P < 0.005$) than the wild type. In *P. fluorescens* Pf-5, a *gacS* mutation yields no detectable RpoS levels in exponential growth (Whistler *et al.*, 1998). These findings suggest that in *P. chlororaphis* PA23-314, the observed resistance may not be due to an RpoS mediated catalase or peroxidase activity. It is possible that in response to H_2O_2

stress, there is superoxide activity that is independent of RpoS regulation. In *E. coli,* superoxide dismutase (SOD), Fe-SOD (iron-cofactored), and Mn-SOD (manganese-cofactored), are known to catalyze the conversion of superoxide (O_2^-) into H_2O_2 and O_2 (McCord and Fridovich, 1969). Both Fe-SOD and Mn-SOD are found in *P. aeruginosa* (Hassett *et al.,* 1992; Hassett *et al.* 1993). In the presence of iron, Fe-SOD is the predominant SOD enzyme that is expressed during the exponential and stationary phase (Hassett *et al.,* 1992). In light of this, the ability of *gacS* mutant strain PA23-314 to acquire high levels of iron may elevate Fe-SOD activity and, subsequently, activate the catalase mediated response to H_2O_2 oxidative stress. This hypothetical cascade mechanism may be responsible for providing the *gacS* mutant, during logarithmic growth, with an increase in tolerance to 9 mM H_2O_2 stress compared to the wild type (Figure 8A). In stationary phase, stress tolerance to 45 mM H_2O_2 was highest in the wild type and *gacS* complemented strain (Figure 8C), suggesting that a *gacS* mutation reduces the oxidative stress response during the stationary growth phase. These results are consistent with those observed by Kang and colleagues (2004), where *P. chlororaphis* O6 tolerance to oxidative stress (45 mM H_2O_2) by the wild type and *gacS* complemented cultures increased as they entered the stationary phase, while the *gacS* mutant lost culturability from the logarithmic phase.

In natural environments, bacterial cells are found mostly in a biofilm state (Potera C., 1996; Costerton *et al*., 1995). Bacterial biofilms render resistance to antimicrobials, desiccation, oxidative and pH stress (Beattie and Lindow, 1995;

Wilson *et al.*, 1965). Two main hypotheses explain the reduced susceptibility of biofilm populations to antimicrobials. The first hypothesis suggests that there is a restricted penetration to the site of action. The second hypothesis proposes that there are physiological and genetic changes in the microorganisms residing in the biofilm that render them more resistant. These changes may include decreased growth rate and the expression of stress coping factors. The *gacS* mutant PA23-314 was tested for the ability to establish biofilms on a PVC surface. Results showed that PA23-314 had a dramatic decrease in biofilm production compared to the wild type. Flagella mediated swimming and Type IV pili-mediated attachment have been shown to be involved in the initial stages of biofilm formation in *P. aeruginosa* and *P. fluorescens* (O'Toole and Kolter, 1998a; O'Toole and Kolter 1998b). To investigate factors involved in the PA23-314 biofilm deficiency, three forms of motility were tested; flagellar, swarming, and twitching motilities. In PA23-314, flagellar motility was reduced compared to the wild type (Figure 12). As well, the *gacS* mutant strain was capable of delayed swarming motility that differed in phenotype compared to the wild type. Collectively, the decreased swimming and aberrant swarming ability may contribute to the PA23-314 biofilm defect. Twitching was not detected in the wild-type PA23 strain; therefore it cannot contribute to the decreased biofilm development observed for PA23-314. Considering the complexities of biofilm development and the pleiotropic nature of *gacS* mutations, it would not be surprising to find multiple factors contributing to the *gacS* biofilm defect.

In the plant environment, *P. chlororaphis* PA23 would likely be subjected to microbial competition and the detrimental effects of antimicrobial products. To test the effect of a *gacS* mutation on antibiotic resistance, biofilm cultures were exposed to two antibiotics. Ciprofloxacin and tobramycin were chosen, each having a different molecular structure and biofilm penetrating ability. Ciprofloxacin is a synthetic quinolone that readily penetrates across the biofilm barrier of *P. aeruginosa* (Vrany *et al.*, 1997). Biofilm antibiotic susceptibility to ciprofloxacin was equivalent between the wild type and the *gacS* mutant strain PA23-314. Both bacterial populations had 100-fold increase in ciprofloxacin resistance compared to the planktonic cultures (Table 3). Because the biofilm does not retard Ciprofloxacin, these findings suggest that both the wild-type and *gacS*$^-$ biofilm populations developed intrinsic resistance.

Biofilm cultures of *P. chlororaphis* PA23 and *gacS*$^-$ strains were treated with tobramycin, an aminoglycoside antibiotic produced by *Streptomyces tenebrarius* (Higgens and Kastner, 1963). Biofilm resistance to tobramycin is attributed to its multiple cationic groups that are responsible for the increased binding affinity to the negatively charged exopolysaccharide matrix (Davis, 1987; McKenney and Allison, 1997). Biofilm analysis showed that resistance to tobramycin was equivalent between the wild type and the *gacS* mutant, reaching 650-fold higher resistance compared to planktonic MICs (Table 3). The increased resistance in antibiotic susceptibility observed with the *gacS* mutant, indicates that even though it did not establish a biofilm to the level of the wild

type, a sufficient diffusion barrier was present to prevent the bacteriocidal effects of tobramycin.

The ability of PA23 to form protective biofilms bodes well for its biocontrol capacity in the plant environment. In soybean and canola plants, *S. sclerotiorum* mediates its disease symptoms primarily through the production of oxalic acid (Maxwell and Lumsden, 1970; Marciano *et al.*, 1983). It is possible that *P. chlororaphis* PA23 will be resistant to potentially detrimental plant or microbial exoproducts, such as oxalic acid (*S. sclerotiorum*) or tobramycin (*S. tenebrarius*), through biofilm establishment. These traits should help to support bacterial sustainability and biocontrol ability of PA23 in the plant environment.

Class II mutant PA23-63 had a Tn*5* insertion in a phenazine biosynthetic gene *phzE* making this strain deficient in phenazine production (Figure 17). PA23-63 has a significant reduction in *hcnA* expression (Figure 19). In *P. aeruginosa*, under anaerobic conditions, phenazine methosulfate functioned as an electron acceptor in the oxidation of glycine to cyanide and CO_2 (Castric *et al.*, 1981). In this regard, the deficiency in phenazine production in PA23-63 may be responsible for the reduction in HCN expression (Figure 19), and ultimately induction of cyanogenesis. In antifungal plate assays, PA23-63 exhibited equal or more antifungal activity compared to the wild type (Table 2). Therefore, it appeared that additional compounds exhibiting antifungal activity were being produced. PCR analysis (Zhang Y. 2004) and Southern analysis (Figure 18) revealed that PA23 has genes for pyrrolnitrin production. The increased antifungal activity observed in the PA23-63 mutant suggested that if pyrrolnitrin is

indeed produced by PA23, then phenazine may be a negative regulator of pyrrolnitrin production. Consequently, phenazine production would result in decreased levels of pyrrolnitrin. This, however, is not the case. HPLC analysis of cell free supernatants indicated that pyrrolnitrin levels were equivalent between PA23-63 and the wild type (Tim Paulitz, personal communication), and so most likely, phenazine is not a negative regulator of pyrrolnitrin production. Instead the biocontrol activity of *P. chlororaphis* PA23 against *S. sclerotiorum* appears to be primarily dependent on the effects of pyrrolnitrin. Studies have shown pyrrolnitrin to have broad-spectrum activity against plant pathogenic fungi, which include Ascomycetes like *S. sclerotiorum* (Ligon *et al.*, 2000). For PA23-63, efficient biocontrol in suppressing *S. sclerotiorum* on canola plants was proven under green house conditions (Figure 24 and 25). Results showed that both the wild type and mutant PA23-63 had equally suppressive disease ability (Figure 25). These results were surprising since most studies performed in *P. chlororaphis* plant-associated bacteria have shown that the primary antifungal antibiotic is phenazine and not pyrrolnitrin (Chin-A-Woeng *et al.*, 2000; 2001; van Rij *et al.*, 2004; 2005). The absence of phenazine production in PA23-63 (Figure 17) did not affect its ability to colonize the canola petals (Figure 26). This is in agreement with other *P. chlororaphis* mutants deficient in phenazine production which are capable of efficiently colonizing the rhizosphere (Chin-A-Woeng 1998; Schmidt-Eisenlohr *et al.*, 2003). These findings demonstrate that the biocontrol activity of *S. sclerotiorum* is mainly, but not exclusively, due to pyrrolnitrin. Phenazine antibiosis may be a result of a synergistic action with other antibiotics,

such as pyrrolnitrin. To prove that pyrrolnitrin is the primary antifungal metabolite in PA23, biocontrol studies should be performed with a PA23 *prnABCD* knockout mutant, deficient in pyrrolnitrin production.

In class III mutant PA23-443, the gene containing the Tn*5* insertion encodes a novel transcriptional regulator of the LysR family. LysR-type transcriptional regulators are found in *P. aeruginosa*, *P. putida*, *P. syringae,* and *P. fluorescens*, where they function as positive regulators of divergently transcribed genes, as well as positive or negative autoregulators of their own expression (Schell, 1993). In plant-associated bacteria, the production of many secondary metabolites is subject to LysR-mediated regulation. An example is the PltR transcriptional activator of *P. fluorescens*, that binds just upstream of the *pltLABCDEFG* pyoluteorin biosynthetic locus (Nowak-Thompson *et al*., 1999). A mutation in *pltR* resulted in decreased expression of pyoluteorin biosynthetic genes (Nowak-Thompson *et al*., 1999). In another plant-associated bacterium, *P. aeruginosa* PA14, a mutation in the LysR-type MvfR transcriptional regulator resulted in deficiency in pyocyanin expression, as well as a defect in the production of autoinducer molecules and the secretion of a variety of proteins (Cao *et al.*, 2001; Pearson *et al.*, 1994; Pesci *et al.*, 1999). These studies indicate the importance of LysR transcriptional regulators in secondary metabolite production.

Secondary metabolite analysis showed that mutant PA23-443 produces AHL, protease, lipase and an orange growth phenotype, indicative of phenazine production, after 24 h of growth on plates. In *P. chlororaphis* PA23, secondary

metabolite production may be under the regulation of multiple LysR transcriptional regulators acting in a hierarchical way. For example, if PA23-443 is mutated in the region encoding the primary transcriptional regulator for production of secondary metabolites, such as lipase, protease and phenazine, then the delayed production of these products may result from an alternative, less efficient transcriptional regulator. It is interesting that even with a delayed (~40 h) orange growth phenotype in PA23-443, there was no biocontrol activity towards *S. sclerotiorum* in plant assays (Figures 34A, 34B and 35) and plate assays (Table 2). The absence of *S. sclerotiorum* biocontrol, both in plate and plant assays, suggests that even though some secondary metabolites are eventually produced by PA23-443, this is not sufficient for effective biocontrol activity, indicating that this LysR transcriptional regulator may control the production of an additional antifungal metabolite(s). Disease incidence observed on stems and leaves of PA23-443 inoculated-plants was significantly higher, ($P < 0.005$), compared to the plants inoculated with the wild-type PA23 strain (Figures 34A and 34B). Disease severity in PA23-443 was 77% compared to the pathogen only control (4.37 versus 5.7), indicating that there is no disease protection afforded by PA23-443 (Figure 33). The population dynamics on the leaves of canola observed by mutant PA23-443 (Figure 35) were similar the efficient biocontrol strain PA23-63 (Figure 26). Indicating that the deficiency in PA23-443 disease suppression is most likely caused by delayed secondary metabolite production and possibly the absence of an essential antifungal

molecule(s), since PA23-443 was able to establish a population density sufficient for biocontrol (Figure 34).

Growth analysis in minimal (Figure 29A) and rich media (Figures 29B, 30A and 30B) showed that the LysR mutant PA23-443 enters a dormant growth phase (20 h) at an earlier time point than the wild type. Surprisingly, after a prolonged growth period, the LysR mutant increases in population density that reaches the levels of the wild type. These findings suggest that under the conditions tested, the LysR mutant may not be as metabolically active as the wild type, resulting in an earlier entrance into stationary phase where the majority of population dies. The latent PA23-443 population increase may be due to a small portion of cells that were initially dormant and were later rapidly dividing, exhibiting a growth advantage compared to their counterparts. A growth advantage in stationary phase (GASP) has been previously described in *E. coli* (Zambrano and Kolter. 1996). Under prolonged periods of nutrient limitation a small portion of cells acquired *rpoS* mutations that rendered them viable at a time when the majority of the cell population died. Another explanation for the PA23-443 growth phenotype is that it is possible for a mutation in this LysR transcriptional regulator, over a long-term period, to provide a greater fitness in stressful environmental conditions found in the phyllosphere or rhizosphere of crop plants. Considering that PA23-443 LysR mutant exhibits the ability to scavenge higher levels of iron (Table 2), as well as the latent secondary metabolite production, at a time point when the wild-type population may not be producing any more antifungal products, then it may be advantageous to have

PA23-443 as part of the total PA23 biocontrol population in plant studies. Additional factors contributing to the biocontrol defect in PA23-443 background may be its lower rate of flagellar motility (Figure 28) and altered swarming phenotype, as well as, reduced biofilm formation ($P < 0.005$) compared to the wild type (Figure 29). This is supported by previous studies emphasizing the importance of swarming and flagellar motility contributing to the biocontrol activity both in the phyllosphere and rhizosphere (de Weger *et al.*, 1987; Simons *et al.*, 1996; Chin-A-Woeng *et al.*, 2000; Haefele and Lindow, 1987). Addition of a functional *gacS* allele in PA23-443 restored the production of extracellular products and motility to that of the wild type. In addition, the antifungal activity of PA23-443 (pUCP23-*gacS*) was equal to the wild-type PA23 strain (Table 2). It is possible that GacS activates expression of the putative transcriptional activator(s) that substitutes for the mutated regulator. Therefore, providing multiple copies of *gacS* increases production of that second activator(s) thereby restoring the wild-type phenotype.

CONCLUSION

P. chlororaphis PA23 is a bacterial antagonist of *S. sclerotiorum* mediating disease suppression through the production of secondary metabolites including PCA, 2-OH-PHZ, and pyrrolnitrin (Paulitz, de Kievit and Fernando, unpublished data). To better understand the molecular mechanisms underlying biocontrol activity in *P. chlororaphis* PA23, three different types of mutants were isolated and characterized: (i) GacS sensor kinase of a two-component global regulatory system, (ii) phenazine biosynthetic genes and (iii) a novel LysR-type transcriptional regulator.

Phenazine biosynthetic mutants of PA23 exhibited antifungal activity equivalent to the wild type when tested in plate and green house assays. A mutation in the *phzE*-coding region did not alter the levels of pyrrolnitrin production, indicating that phenazine is not a negative regulator of pyrrolnitrin biosynthesis in this genetic background. The findings presented here suggest that PA23 biocontrol activity against *S. sclerotiorum* is mainly, but not exclusively, due to pyrrolnitrin.

Regulatory mutants in the coding regions for GacS and a novel LysR-type transcriptional regulator were deficient in production of a number of extracellular products. Not surprising, they were impaired in their biocontrol activity when tested *in vitro* in plate assays and *in situ* under green house conditions. These mutants efficiently colonized canola petals, regardless of a significant reduction in flagellar motility and biofilm formation. Addition of a functional *gacS* allele *in trans* to either the *gacS* mutant (PA23-314) or the LysR mutant (PA23-443),

restored secondary metabolite production, as well as motility and biofilm formation to wild-type levels. These findings suggest that GacS is at the top of the regulatory hierarchy controlling biocontrol in *P. chlororaphis* PA23.

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Appendix 1.

Growth Media

Minimal medium
10 g M9 minimal salts (Gibco)
(6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl)
dH_2O to 1 L

autoclave, and then add 1 mL 20% $MgSO_4$
10 mL 20% D-glucose

PTSB
5% Peptone
0.25% Tryptic Soy Broth
adjust to pH 7.0

Terrific broth (TB)
(i) Solution 1
12g Tryptone
24g Yeast Extract
4 mL glycerol
900 mL dH_2O

autoclave, cool to 60 C

(ii) Solution 2
2.31g KH_2PO_4
12.54g K_2HPO_4
100 mL dH_2O

autoclave, and then add to solution 1 (60 C)

Electrophoresis buffer

TAE
40 mM Tris base
20 mM Acetic acid
2 mM EDTA
pH 8.1

Southern blot analysis-hybridization and washing buffers

Prehybridization/hybridization buffer
5X SSC
2% blocking reagent
0.1% M-lauroyl sarcosine
0.02% SDS
50% formamide
autoclaved and stored at -20°C

20X SSC
0.3 M sodium citrate
3.0 M NaCl
adjust to pH 7 with NaOH
autoclaved and stored at room temperature

Washing solutions
Low stringency wash: 2X SSC/0.1% SDS
High stringency wash: 0.1X SSC/0.1% SDS

Buffer 1
0.1 M maleic acid
0.15 M NaCl
autoclaved and stored at room temperature

Blocking solution
10% blocking reagent (Boehringer Mannheim) in buffer 1
autoclaved and stored at -20°C

Buffer 2
1% blocking solution in buffer 1

Buffer 3
0.1 M Tris base
0.1 M NaCl
50 mM $MgCl_2$
adjust to pH 9.5 with HCl
autoclaved and stored at room temperature

Washing buffer
buffer 1 with 0.3% (v/v) Tween-20

Solutions for DNA analysis

Phosphate-buffered Saline (PBS)
137 mM NaCl
2 mM KH_2PO_4
10 mM Na_2HPO_4 $12H_2O$
2.7 mM KCL
dH_2O to 1L

DNase-free RNase
10 mg/mL RNase A
10 mM Tris base (pH 7.5)
15 mM NaCl
boil for 15 min.

Sarcosyl Protease solution
5 mg/mL Protease
10% Sarcosyl in $T_{20}E_1$
incubate at 37°C for 2 h prior to use

Alkaline lysis solution
0.2N NaOH
1% (w/v) SDS

10X TE
100 mM Tris-Cl (pH 7.6)
10 mM EDTA (pH 8.0)

autoclave and store at room temperature

TES
0.5 mL 1 M Tris
3 mL 0.5 M EDTA
1.5 mL 5 M NaCl
dd H_2O 50 mL

$T_{10}E_{25}$
0.5 mL 1 M Tris
2.5 mL 0.5 M EDTA
dd H_2O 50 mL

Appendix 2

Tn*5*-OT182 insertions in PA23 mutants

PA23-314 *Xho*I-clone

Harboring 11 kb of cloned PA23 sequence. Tn*5*-OT182 insertion site in the *gacS* ORF: (*gacS* transcriptional start)1-544 bp**--Tn*5*-OT182 transcriptional orientation-->**555-3121 bp (*gacS*) in *Pseudomonas chlororaphis* strain PCL1391.

PA23-2490 *BamH*I- and *Cla*I-clone

Harboring ~2.2 kb of cloned PA23 sequence. Tn*5*-OT182 insertion site in the *gacS* ORF: (*gacS* transcriptional start)1-544 bp**<-- transcriptional orientation Tn*5*-OT182-** 555-3009 bp (*gacS*) in *Pseudomonas chlororaphis* strain PCL1391.

PA23-63 *Xho*I-clone

Harboring 7 kb of cloned PA23 sequence. Tn*5*-OT182 insertion site in the *phzE* ORF: (*phzE* transcription start) 4873-6218 bp**--Tn*5*-OT182 transcriptional orientation-->**6219-6786 bp (*phzE*) in *Pseudomonas chlororaphis* strain PCL1391.

PA23-754 *EcoR*I- and *Cla*I-clone

Harboring ~18 kb of cloned PA23 sequence. Tn*5*-OT182 insertion site in the *phzC* ORF: (*phzC* transcriptional start) 3035-3568bp**--Tn*5*-OT182 transcriptional orientation-->**3569-4237 bp in *Pseudomonas chlororaphis* PCL1391 *phz* biosynthetic operon.

PA23-443 *Xho*I-, *BamH*I- and *Cla*I-clone

Harboring ~8 kb of cloned PA23 sequence. Tn*5*-OT182 insertion site in the ORF: (transcriptional start of the mutated gene) 1461933 bp**--transcriptional orientation Tn*5*-OT182-->**1462961-1463352 bp in *Pseudomonas fluorescens* Pf-5.