An Investigation of the Mechanisms Underlying Biological Control Activity of a Novel Canola-Associated Bacterial Isolate, *Pseudomonas* species DF41

By

Chrystal Louise Berry

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Microbiology

University of Manitoba

Winnipeg, Manitoba, Canada

Abstract

The ability of several plant-associated bacteria to inhibit the proliferation of root-pathogens has been well established whereas considerably less has been reported about bacterial species inhibiting pathogens on the phylloplane. *Sclerotinia sclerotiorum* is the fungal causative agent of stem rot and is capable of infecting over 400 plant species, including flowering canola plants. For this reason, there is a need for disease management strategies targeted at preventing sclerotinia infection.

Pseudomonas species DF41 was isolated from the canola rhizosphere and found to be an excellent antagonist of sclerotinia stem rot. Therefore, research efforts turned towards elucidating the mechanisms underlying DF41 antifungal (AF) activity. A random transposon mutagenesis approach facilitated the identification of genes essential for DF41 fungal antagonism. One gene that was identified, *gacS*, encodes the sensor kinase of the Gac two-component signal transduction system. Characterization of the DF41 *gacS* mutant revealed that this regulator is essential for secondary metabolite production. In other bacteria, the Gac system activates target gene expression by upregulating the transcription of small, untranslated RNA molecules (sRNA). A sRNA molecule called RsmZ was found to act as a downstream regulatory element in the DF41 Gac regulatory cascade.

Furthermore, we discovered that DF41 is producing acyl homoserine lactone (AHL) signalling molecules. This prompted us to investigate the effect of quorum sensing (QS) on phenotypes contributing to AF activity. In DF41, AHL- signalling is not important for secondary metabolite production but does influence motility and may indirectly govern gene expression by controlling other regulatory elements

Screening of our transposon library led to the identification of a non-ribosomal peptide synthetase gene involved in synthesis of a cyclic lipopeptide (CLP) molecule. High-performance liquid chromatography (HPLC) and mass spectrometry (MS) enabled the identification of an unusual CLP and we propose a preliminary structure containing some unique features. The role of this molecule in *Pseudomonas* sp. DF41 AF activity was also elucidated.

Altogether, this investigation has revealed a number of important findings regarding how DF41 functions as a biocontrol agent. This information will allow us to use DF41 more effectively in the future in managing sclerotinia stem rot on canola plants.

Acknowledgements

First, I would like to acknowledge and extend my utmost respect and gratitude to my thesis supervisor Teri, for taking me on as her graduate student. I have gained invaluable lessons from you both professionally and personally that will enable me to carry on to and pursue the next level. Your encouragement, support, patience and mentoring have been deeply appreciated.

I wish to extend my sincere appreciation to my committee members, Dr. Ivan Oresnik, Dr. Betty Worobec and Dr. Dilantha Fernando for all of their time that was dedicated to meetings and for their very helpful suggestions contributed along the way.

The chemical analysis would not have been possible without the assistance of Dr. Lynda Donald who spent countless hours conducting MS experiments and analyzing data. I have enjoyed our brain-storming sessions and cannot thank you enough for all the work you have contributed to this thesis.

My labmates- Carrie and Jer, we have shared so much over these years and you two have helped brighten the long lab days. The at times, much-needed laughs and coffee breaks helped relieve the stressful moments of research and your friendship means the world to me.

Basil- you have been there with me throughout so much of this and I know that I could always turn to you for comfort and solace. Thank you for your companionship and for always being there to lift my spirits.

Finally, to my Mom, Dad and late Nan, for instilling at a very early age, the importance of an education and for your never-ending generosity and support as this degree wouldn't have been possible without your love and encouragment.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND DEFINITIONS

AF = antifungal

AHL = acyl homoserine lactone

AI = autoinducer

BLAST = basic local alignment search tool

Bp = base pair

CLP = cyclic lipopeptide

2,4-DAPG = 2,4-diacetylphloroglucinol

EDTA = ethylenediaminetetraacetic acid

Gac = global activator of cyanide

HPLC = high performance liquid chromatography

IPTG = isopropyl β-D-1-thiogalactopyranoside

ISR = induced systemic response

KB = King's B

LB = Luria-Bertani

LuxR = family of transcriptional regulators

MS = mass spectrometry

MALDI-TOF MS = Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

NRPS = non ribosomal peptide synthetase

PCR = polymerase chain reaction

PGPB = plant growth-promoting bacteria

PGPR = plant growth- promoting rhizobacteria

QS = quorum sensing

Rsm = regulator secondary metabolism

SAR = systemic acquired resistance

X-gal = 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Chapter 1

Literature review

1.1 The role of *Pseudomonas* species in Biological Control

Fungal pathogens destroy millions of crop acres each year, contributing to decreased food production and lost revenue worldwide. Conventional methods of controlling fungal pathogens have included tillage, crop rotation and pesticide application. However, with the ever-increasing awareness of the negative health-implications and environmental concerns regarding prolonged chemical usage there is a burgeoning demand for the development of alternative and safer methods of disease management. Furthermore, not only do pesticide resistant microbes naturally exist, but repeated pesticide treatment has led to the emergence of resistant variants.

Biological control presents a more "environmentally friendly" alternative to chemical pesticides. A concise definition of biological control is "the control of one organism by another" (Beirne 1967; Mazzola et al. 1992). The concept of biological control of pathogenic species arose in part, from the discovery of naturally suppressive soils (Schroth and Hancock 1982; Hornby 1983; Weller et al. 2002). These soils are intrinsically protected from fungal diseases due to the presence of rhizosphere-associated bacteria (Schneider 1982; Schippers et al. 1987). Historically, reports of naturally suppressive soils were published as far back as 1898 and 1933 describing both the varying conductivity of Wisconsin soil types to the pea wilt pathogen and the observation of induced disease suppression, a phenomenon now referred to as "take-all" decline (Roediger 1898; Walker and Snyder 1933). Although naturally suppressive soils are rare, other examples include soils in the Salinas Valley (California, USA), in France's renown Chateaurenard region and in the Broye Valley in Switzerland (Haas and Defago 2005).

The defining characteristics of naturally suppressive soils are as follows: i) the suppressive nature is lost upon harsh antibiotic treatment or other antimicrobial treatments (Shipton 1973; Cook 1976; Scher 1980; Stutz 1986); and ii) the suppressive nature is transferable to other soils that exhibit <10% suppression (Haas et al. 2002). These two characteristics of suppressive soils are attributable to the presence of antagonistic soil-associated microorganisms.

1.1.2 Plant growth- promoting bacteria

Plant growth promoting bacteria (PGPB) are a large group of plant-associated bacteria that stimulate plant growth and soil health. Although they comprise bacteria isolated from the rhizosphere, phyllosphere, spermosphere, and anthosphere (Hallman 1997), the rhizosphere-associated bacteria (PGPR) are the best characterized (Kloepper 1987). PGPR are predominantly comprised of species from the genera *Bacillus, Streptomyces, Burkholderia,* and *Pseudomonas* and are considered harmless to the plant host although their population densities are often quite high (10^8 bacteria/ g root) (Weller 1988; Lugtenberg et al. 2001). PGPR confer protection to their plant hosts through three main modes of action which include: i) achieving sufficient cell numbers on the plant surface to compete with fungal pathogens for available nutrients and growth substrates; ii) induction of plant immune responses such as ISR or systemic acquired resistance (SAR); and iii) antagonism of pathogens by neutralizing virulence factors or antibiosis (Kloepper 1987; Haas and Defago 2005). This review will focus on mechanisms underlying biocontrol in pseudomonads, with emphasis on fluorescent *Pseudomonas*

species. The plant immune responses ISR/ SAR will not be discussed here but have been the subject of several reviews (Thomashow 1996; Compant et al. 2005; Van Loon 2006).

1.1.3 The genus *Pseudomonas*

Pseudomonas is a genus within the gamma proteobacteria. These bacteria are rod-shaped, Gram-negative aerobes, motile by means of one or more polar flagella (Ryan and Ray. 2004). They are a well-studied group due to their broad range of functions and complex interactions with their host. This metabolically diverse genus is capable of using over two hundred substrates for growth, a trait which likely contributes to their ubiquitous nature (Madigan and Martinko. 2005). *Pseudomonas* species have been isolated from various sources, including soil, water and plants. They form a functionally diverse group of bacteria whose members range from xenobiotic degraders to plant pathogens, animal and human opportunistic pathogens, PGPB, and strains that are antagonists of oomycete and fungal pathogens (Madigan and Martinko. 2005).

1.1.4 *Pseudomonas fluorescens*

Pseudomonas species are ubiquitous in the environment and are prevalent in the rhizosphere and phyllosphere. One particular subgroup, the fluorescent pseudomonads, has been the subject of numerous biocontrol studies. These bacteria are distinguished by the formation of a diffusible, yellow-green pigment which manifests due to the production of a siderophore, pyoverdine (pseudobactin) (Meyer 1978). The fluorescent pseudomonads consist of representatives from *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Pseudomonas putida*. Although this group

has members with diverse functions including human and plant pathogens, this review will focus primarily on biocontrol isolates and the factors governing fungal antagonism.

1.1.5 Biological control activity of *Pseudomonas* species

Interest in *Pseudomonas* biocontrol was initiated in the 1970's with research at the University of California, Berkeley where the application of *Pseudomonas* strains on potato, sugar beet and radish seeds or seed pieces improved plant growth (Schroth and Hancock 1981; Schroth and Hancock 1982). Subsequent studies focused on the role of iron sequestering compounds or siderophores on pathogen suppression (Kloepper 1980; Schroth and Hancock 1982). Although *Pseudomonas* antibiotic production had been demonstrated *in vitro*, the first experimental evidence that a *Pseudomonas* antibiotic was responsible for disease suppression on a plant host was provided in 1988 by Thomashow and Weller. Their experiments, which included the examination of null mutants, clearly demonstrated that the antibiotic phenazine 1-carboxylic acid (PCA), produced by *P. fluorescens* 2-79 was capable of suppressing take-all disease caused by *Gaeumannomyces graminis* var. *tritici* on wheat (Thomashow and Weller 1988). A second research article was published shortly thereafter describing the biocontrol activity of another prominent biocontrol strain, *P. fluorescens* CHA0. *P. fluorescens* 2-79 was isolated from the wheat rhizosphere and *P. fluorescens* CHA0 was isolated from soil that was naturally suppressive to tobacco root rot (Voisard et al. 1989). Both of these species have emerged as model biocontrol organisms and a plethora of antibiotics produced by these and other isolates have been identified that contribute to the biological control of various fungal pathogens.

1.1.6 *Pseudomonas* species DF41

Pseudomonas species DF41 was isolated from canola (cv Cresor) root tips in Manitoba, Canada during a screening for novel biocontrol species (Savchuk and Fernando 2004). Biolog^{TM} analysis, 16S rDNA sequencing, and the production of a yellow-green pigment on iron-limiting media classified this isolate as a member of the fluorescent *Pseudomonas* species; however, a species designation was not elucidated (Zhang 2006). This bacterium provoked further interest due to preliminary *in vitro* and field assays in which *Pseudomonas* sp. DF41 demonstrated significant inhibition of the fungal pathogen *Sclerotinia sclerotiorum* (Savchuk and Fernando 2004). Subsequent microscopy studies demonstrated that *Pseudomonas* DF41 strongly inhibited *S. sclerotiorum* ascospore germination and germtube elongation and branching (Savchuk and Fernando 2004). Another interesting finding from the microscopic analysis was the observation that *Pseudomonas* sp. DF41 is able to degrade *S. sclerotiorum* ascospores 24h after co-inoculation onto canola petals (Savchuk and Fernando 2004).

1.1.7 *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary is an economically relevant ascomycete pathogen that is capable of infecting greater than 400 plant species such as peas, potato, dry beans and lentils (Purdy 1979; Hallman 1997). It is the causal agent of sunflower and canola stem rot (Nelson, 1998) and is the most serious disease affecting canola in the Canadian prairies. Furthermore, in 2000, previously resistant varieties of flax became susceptible to *S. sclerotiorum* infection in Manitoba and Saskatchewan (Rashid 2000). Canola yield losses due to *S. sclerotiorum* infection range from 5-100% in years that are

favourable for disease development such as those in which cooler temperatures and high precipitation conditions prevail (Manitoba Agriculture 2002).

The main *S. sclerotiorum* virulence factor is oxalic acid, which creates an acidic environment and causes tissue damage by sequestering calcium from the middle lamellae (Bateman and Beer 1965; Godoy 1990). Oxalic acid disarms the plant immune response by neutralizing the plant's oxidative burst (Cessna et al. 2000). However, cell-wall degrading enzymes such as cellulases, hemi-cellulases, pectinases (Riou et al. 1991), proteases (Poussereau et al. 2001; Girard et al. 2004) and endopolygalacturonases (Cotton et al. 2003) contribute to virulence by facilitating fungal access to internal plant tissues. A complicated and unique life cycle makes management of this fungal pathogen challenging. *S. sclerotiorum* produces hard, irregular- shaped, over-wintering bodies called sclerotia, that may persist in infected soils up to four years (Manitoba Agriculture 2002). Sclerotia germinate under favourable conditions into mushroom-shaped structures called apothecia that release spores which can then become wind-borne and travel up to one kilometre, exposing neighbouring crops to the risk of infection (Purdy 1979; Venette 1998). Canola plants are most susceptible to *S. sclerotiorum* at the flowering stage as the ascospores infect the plant upon landing on senescing flowers and fallen petals which provide the nutrients required for fungal growth (Turkington 1993). Because fungicide must be applied before symptoms appear and all canola varieties are susceptible to sclerotinia infection, chemical treatment becomes costly. This together with the fact that sclerotia are able to persist in the soil for long periods underscores the need for the development of alternative, more effective methods of fungal disease management.

1.1.8 Canola

Canola is one of two cultivars of *Brassica (*rapeseed), specifically, *Brassica napus L*. and *Brassica campetris* L. Rapeseed varieties low in erucic acid were initially developed in the 1960's at the University of Manitoba by two scientists, Dr. Baldur Stefansson and Dr. Keith Downey, and since, canola varieties have been refined to produce seeds that contain less than 2% euric acid and are low in potentially toxic glucosinolates. Approximately 42% of the canola seed is crushed into an oil for food production and the remainder is used a high quality animal feed. According to the Canadian Canola Council, canola production is a multibillion-dollar industry in Canada alone although canola is cultivated world-wide. Canola is susceptible to a number of invasive species including weeds and fungal pathogens. In 1995, a genetically-modified (GM) line of herbicide- resistant canola seeds was introduced into the market and currently over 80% of the canola varieties grown in Western Canada are GM lines. Although disease-resistant varieties of canola and fungicides are available, all canola cultivars are suseptible to *S. sclerotiorum* infection.

1.1.9 Biocontrol compounds produced by *Pseudomonas fluorescens*

The six major classes of biocontrol compounds produced by *Pseudomonas* biocontrol species are shown in Fig. 1.1, encompass phenazines, pyoluteorin, pyrrolnitrin, 2, 4-diacetylphloroglucinol (2,4-DAPG), cyclic lipopeptides (CLPs) and hydrogen cyanide (HCN) (Raaijmakers 2002; Haas and Keel 2003). Although these products are not required for primary bacterial metabolism, they have been experimentally demonstrated to contribute to the biocontrol activity of soil-borne plant pathogens. Many

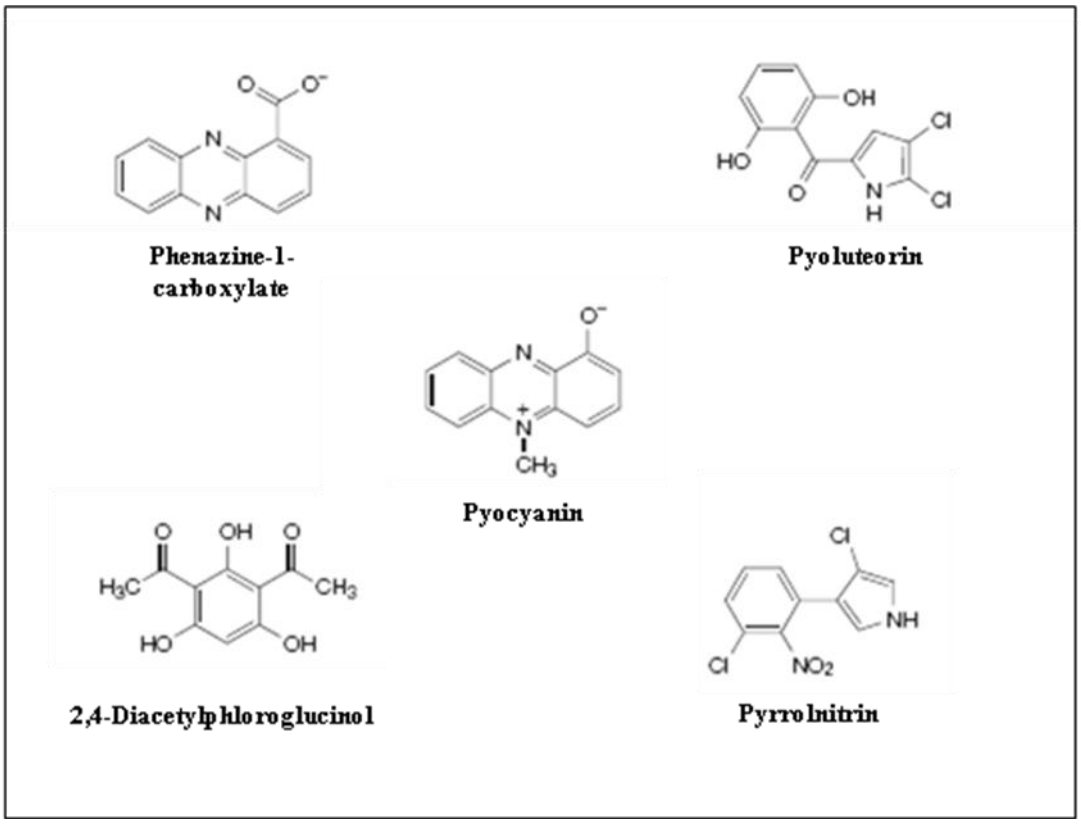


Figure 1.1 The major classes of antibiotics produced by fluoresent *Pseudomonas* species. The figure is modified from Haas and Défago (2005).

of the antibiotics produced have broad-spectrum activity or may act synergistically with other antibiotics reviewed by (Raaijmakers 2002). Due to their lack of pathogen specificity and/or toxic effects at high concentrations, most of these antibiotics are limited to environmental application only, with the exception of pyrrolnitrin which was briefly used as a topical antibiotic against human fungal infections (Ligon 2000; Barrett 2002; Haas and Keel 2003). The antibiotics and other inhibitory compounds depicted in Figureproduced by fluorescent *Pseudomonas* species will be briefly discussed in the following sections, with the exception of CLPs which will be reviewed in section 1.2.

1.1.9.1 Phenazine antibiotics

Phenazines are an extensive group of pigmented, heterocyclic, nitrogen-containing secondary metabolites with broad-spectrum activity and encompass over 50 derivatives produced by fluorescent *Pseudomonas species* and other bacteria (Turner and Messenger 1986; Mavrodi et al. 2006). Although there have been numerous phenazine derivatives reported, phenazine compounds have been implicated in biocontrol activity by several rhizobacteria such as *P. (aureofaciens) chlororaphis* 30-84, *P. fluorescens* 2-79 and *P. chlororaphis* PCL1391 (Thomashow and Weller 1988; Pierson and Thomashow 1992; Chin-A-Woeng 1998; (Mavrodi *et al.* 2010). Phenazines are analogues of flavin coenzymes that inhibit electron transport, and fungal antagonism has been demonstrated against a range of fungal pathogens including *G. graminis* var. *tritici*, *Fusarium oxysporum*, *Pythium* species, *Rhizoctonia solani*, and *Alternaria* species (Gurusiddaiah et al. 1986; Georgakopoulos et al. 1994; Cook 1995; Anjaiah et al. 1998;

Chin-A-Woeng 1998; Morello et al. 2004). Another putative mechanism of phenazine-mediated biocontrol is through lipid and macromolecule damage caused by hydroxyl radical formation in the presence of ferripyochelin (Britigan et al. 1992). Phenazines may have a role in iron mobilization in soils as reduced phenazines can solubilise iron from insoluble sources under neutral soil pH (Hernandez et al. 2004). Experiments with *P. fluorescens* 2-79 demonstrated that phenazine-producing strains may have an advantage in colonizing the roots of wheat plants and exhibit enhanced survival in the rhizosphere compared to phenazine-null mutants (Hernandez et al. 2004). Such beneficial traits may result from phenazine-mediated iron solubilisation. These factors point to the possible contribution of phenazines in ecological competence and long-term survival of bacteria in the soil environment, although this role for phenazines has been disputed (Mazzola et al. 1992; Price-Whelan et al. 2006).

Phenazine biosynthesis occurs via a pathway derived from the shikimic acid pathway, a pathway which is also responsible for the synthesis of siderophores and the aromatic amino acids tyrosine and phenylalanine (McDonald et al. 2001; Vandenende et al. 2004). Chorismic acid has been identified as the branch point for the synthesis of phenazine derivates with most *Pseudomonas* compounds being hydroxyl and carboxy derivatives with antibiotic activity differences arising due to differences in the nature and position of substituents on the heterocyclic ring (Longley 1972; Mavrodi 2006). In the majority of phenazine-producing pseudomonads, the biosynthetic genes are arranged into a core operon, *phzABCDEFG* (Mavrodi 1998; Delaney et al. 2001); however, strains often carry additional genes required for the biosynthesis of phenazine derivatives such as

phzM, phzS, phzO and phzH which can be located close to the core operons or elsewhere in the genome (Chin-A-Woeng 2001).

1.1.9.2 Pyoluteorin

Pyoluteorin is one of two polyketide antibiotics within the arsenal of *P. fluorescens* AF compounds. It is composed of a bichlorinated pyrrole linked to a resorcinol moiety and is produced by *P. fluorescens* Pf-5, *P. fluorescens* CHA0, and *Pseudomonas* species M18 in addition to other bacteria. Although a number of other antibiotics are produced by Pf-5 and CHA0, pyoluteorin is thought to be the most toxic to *Pythium ultimum* damping-off disease on cress, though not on cucumber (Kraus 1992; Maurhofer 1992; Maurhofer 1994). Pyoluteorin is toxic to seed and root-rotting pathogens and to certain Gram-negative and Gram-positive bacteria (Bailey 1973; Howell 1980; Maurhofer 1992). The pyoluteorin biosynthetic gene cluster, *pltABCDEFG* encodes nine products whose functions are sufficient to synthesize pyoluteorin from acetate and proline precursors (Nowak-Thompson 1997; Nowak-Thompson 1999). Two genes encoding LysR-type transcriptional activators are transcribed divergently from the pyoluteorin cluster and have been shown to positively influence expression of the pyoluteorin biosynthetic genes (Nowak-Thompson 1999). Interestingly, pyoluteorin serves as an autoinducer, enhancing its own production and acting as an intercellular signal between bacterial populations in the rhizosphere (Brodhagen 2004). Pyoluteorin is also influenced by the production of other antibiotics. For example, in *Pseudomonas* sp. M18, the production of pyoluteorin is negatively influenced by the accumulation of phenazine 1-carboxamide; however, the reverse is not

true (Ge 2007). In *P. fluorescens* strain Pf-5, pyoluteorin and 2,4,-DAPG are mutually inhibitory to one another and pyrrolnitrin also represses pyoluteorin production. (Schnider-Keel 2000; Brodhagen 2003; Brodhagen 2004; Baehler et al. 2005). Although, the biosynthesis of pyoluteorin has been well-characterized, the mechanism(s) by which pyoluteorin inhibits fungal pathogens remains unknown.

1.1.9.3 2,4-diacetylphloroglucinol (2,4-DAPG)

2,4-DAPG is a phenolic broad-range antibiotic with antiviral, phytotoxic, antibacterial and AF activities. It suppresses plant pathogenic fungi, helminths such as the nematode *Globodera rostochiensis* and the oomycete *Pythium* in which it causes membrane damage, especially to zoospores (Duffy and Defago 1997; de Souza 2003). In terms of biological control, this antibiotic is a broad-range antibiotic which is an important determinant of *P. fluorescens* CHA0-mediated control of *Thielaviopsis basicola*, the causal agent of black root of tobacco and *G. graminis* var. *tritici* which is the causal agent of take-all of wheat (Keel 1990; Keel 1992; Thomashow 1996). At high concentrations, 2,4-DAPG has phytotoxic activity and was found to be herbicidal to tobacco plants at concentrations above 40 mg/g soil (Keel 1992). Similar to the antibiotics described above, 2,4-DAPG synthesis is dependent upon a conserved biosynthetic operon, *phlACBD*. Two genes flanking the operon (*phlE* and *phlF*) encode for efflux and repression of 2,4,-DAPG respectively (Bangera and Thomashow 1999). 2,4-DAPG is subject to autoregulation as exogenous 2,4-DAPG was found to induce expression of *phlA* in *P. fluorescens* CHA0 (Schnider-Keel 2000). In addition to pyoluteorin, negative-regulation of the *phlACBD* operons is exerted by the bacterial

metabolite salicylate as well as the fungal compound fusaric acid (Schnider-Keel 2000). This result is similar to the 2,4-DAPG regulation in *P. fluorescens* Q2-87 (Bangera and Thomashow 1999).

1.1.9.4 Pyrrolnitrin

Pyrrolnitrin (3-chloro-4-(2′nitro-3′chlorophenyl)-pyrrole) is a broad spectrum antibiotic first isolated from *Pseudomonas (Burkholderia) pyrrocinia* that was briefly developed as a topical antibiotic against human pathogenic bacteria and fungal infections (Ligon 2000). Synthetic analogues have since been developed as agricultural fungicides (Ligon 2000). This antibiotic is active against numerous economically important pathogens such as *Rhizoctonia solani*, *Botrytis cinerea*, *Verticillium dahlia* and *S. sclerotiorum* (Howell 1979; Hill 1994). Pyrrolnitrin exerts biological control of fungal pathogens by inhibiting fungal respiratory chains (Tripathi 1969). Using tryptophan as a precursor, pyrrolnitrin is the product of a biosynthetic gene cluster consisting of four genes, *prnABCD*; thus far, pathway-specific regulators have not been identified. However, in another closely-related organism, *Burkholderia ambifaria*, pyrrolnitrin synthesis was found to be strongly dependent on a quorum-sensing system, although the role of this system in pseudomonad pyrrolnitrin biosynthesis remains to be elucidated (de Souza 2003; Schmidt et al. 2009).

1.1.9.5 Hydrogen cyanide

Hydrogen cyanide (HCN) is a volatile antibiotic which was first determined as a biocontrol factor in *P. fluorescens* CHA0 suppression of *T. basicola* black root disease on

tobacco (Voisard et al. 1989). Although long-known as a poisonous substance contributing to *Pseudomonas aeruginosa* virulence, HCN production is also an important determinant in combating fungal diseases. HCN mutants of *P. fluorescens* CHA0 were partially defective in inhibiting the tobacco root rot pathogen (Voisard et al. 1989). HCN exerts inhibitory action by the production of cyanide which is formed when HCN is ionized with water. Cyanide is a potent inhibitor of metalloproteases, especially copper-containing cytochrome c oxidase (Blumer and Haas 2000). HCN is produced from glycine under microaerophilic condition by an HCN synthase which is the product of three structural genes organized into an operon, *hcnABC* (Voisard et al. 1989; Laville et al. 1998).

1.1.9.6 Organic volatile antibiotics

Although their role in biocontrol of fungal pathogens has not been as extensively studied as the AF compounds discussed so far, organic volatiles of bacterial origin may also contribute to biocontrol activity. A study conducted in 2004 examined the *in vitro* effect of volatiles produced by a series of *Pseudomonas* species of canola and soybean origin on the inhibition of *S. sclerotiorum* sclerotia (Fernando et al. 2005). This study identified six compounds which completely inhibited sclerotia germination and mycelial formation and included: nonanol; benzothiazole; cyclohexanol; n-decanal; dimethyl trisulfide; and 2-ethyl 1-hexanol.

1.1.9.7 Siderophores

Fluorescent pseudomonads produce a number of iron-chelating molecules termed siderophores. For example, *P. fluorescens* CHA0 produces at least three siderophores; pyoverdine, salicyclic acid and enantio-pyochelin (Ahl et al. 1986; Meyer et al. 1992; Youard et al. 2007). Pyoverdine is a well-characterized siderophore; its mechanism of suppression is considered due to the sequestration of available iron from pathogenic fungi though not all pyoverdine-producing *Pseudomonas* isolates exhibit biocontrol activity (Kloepper et al. 1980; Schroth and Hancock 1982). Thus, pyoverdine is not considered to be a major determinant of biocontrol activity. Another siderophore, (enantio)-pyochelin, may function in a similar manner to pyoverdine. Pyochelin effectively chelates copper and zinc ions, yet weakly chelates iron (Cuppels et al. 1987; Visca et al. 1992). Despite the fact that siderophores are not single-handedly effective at suppressing fungal pathogens, they may indirectly contribute to biocontrol activity by competing with pathogens for available trace nutrients.

1.1.9.8 Cell-wall degrading enzymes

In addition to antibiotics, *Pseudomonas* species secrete extracellular enzymes which may contribute to pathogen suppression. *P. fluorescens* DR54 produces cellulase, hemi-cellulase and endochitinase in addition to other secondary metabolites that collectively promote antagonism against *P. ultimum*, which contains cellulose in the cell wall, and *R. solani*, which has chitin in the cell wall (Nielsen et al. 1998; Thrane et al. 2001). *P. fluorescens* CHA0 produces four extracellular enzymes; protease, phospholipase C, lipase (Saito et al. 1992; Youard et al. 2007), and alkaline protease

(Siddiqui et al. 2005). Suppression of the root-knot nematode *Meloidogyne incognita* on tomato and soybean plants is highly dependent on production of alkaline protease (Siddiqui et al. 2005). Mutants unable to produce alkaline protease production demonstrated a significant reduction in inhibition of egg hatching and induction of mortality of *M. incognita* juveniles compared to the wild-type strain (Siddiqui et al. 2005).

1.1.10 Additional bacterial traits contributing to biocontrol activity

Biological control activity is determined not only by the production of secondary metabolites but is also known to be dependent on the host environment as well as abiotic factors. Therefore, in addition to antibiosis, other characteristics may confer a selective advantage to the biocontrol agent, thereby indirectly contributing to biocontrol.

The phylloplane can be a harsh environment where bacteria are exposed to temperature gradients, moisture or desiccation, UV light, free radicals and nutrient depletion. In order to persist under these rapidly changing conditions, bacteria have evolved mechanisms to facilitate survival. Motility is one such mechanism that allows the bacteria to migrate from areas that are non-favourable to those that are more favourable for growth. Bacterial migration is achieved via flagellar (swimming) motility and swarming motility. Swarming is an alternative mode of bacterial movement across a semi-solid surface that relies upon both pili and biosurfactant production . Although motility is believed to affect bacterial colonization, there are conflicting reports on the colonization abilities of non-motile mutants of *P. fluorescens* (Lugtenberg et al. 2001).

Therefore it is unclear whether motility is essential for colonization of plant surfaces by all bacteria.

A second trait that may contribute to persistence in the plant environment is biofilm formation. There is strong evidence that in most environments, bacteria exist as attached communities known as biofilms and not as planktonic cells (Costerton et al. 1995). Soil-associated bacteria are typically found attached to soil particles or root surfaces (Ude et al. 2006) and plant-associated bacteria are known to form microcolonies shortly after initial attachment to both root surfaces and the phylloplane (Ude et al. 2006). Biofilms may confer protection to the bacteria from environmental stresses and dessication thus contributing to the long-term survival of bacteria on the plant host. Biofilm formation by plant-associated bacteria is the subject of an extensive review by (Danhorn and Fuqua 2007).

1.2 Cyclic Lipopeptide Production by *Pseudomonas* species

1.2.1 Cyclic lipopeptides

Cyclic lipopeptides (CLPs) are a novel class of small, bioactive molecules composed of a short oligopeptide attached at the N-terminus to a fatty acid. The peptide portion is typically cyclized by a lactone ring between two amino acids. CLPs can be of bacterial or fungal origin and are produced by several plant-associated *Pseudomonas* spp. including pathogenic and saprophytic species. A diverse number of functions are attributed to CLPs ranging from inhibitory effects against human pathogens, such as enveloped viruses, mycoplasmas and gram-positive bacteria to acting as plant pathogenic toxins and biosurfactants. The application of both purified CLP and CLP-producing

Pseudomonas species to seeds, plant tissues and fruits has demonstrated a role in the biological control of fungal pathogens. While the previous section discussed antibiotics that were synthesized ribosomally from biosynthetic operons, CLPs are unique in that synthesis occurs non-ribosomally on large, multimodular enzymes termed non-ribosomal peptide synthetases (NRPS) (Marahiel et al. 1997; Bergendahl et al. 2002). NRPSs function as large, multicarriers via a thiotemplate mechanism that is generally considered less specific than ribosomal protein synthesis thus allowing for variability in the final product with corresponding differences in activities depending on the final structure produced (Doekel and Marahiel 2001). Complete sequences have been determined for a few plant-associated *Pseudomonas* CLP biosynthetic NRPS templates: the phytotoxins syringomycin and syringopeptin; the biosurfactants arthrofactin and putisolvin I and II (Dubern et al. 2008); and the antibiotic massetolide A. Furthermore, genomic mining has led to the identification of several putative CLP biosynthetic clusters (Gross and Loper. 2009).

1.2.2 CLP classification

An array of CLPs produced by pseudomonad species have been classified by Raaijmakers et al. (2006) into four major groups based on structural features. As depicted in Table 1.1, the CLP classification criteria included the length and type of fatty acid tail, the number, configuration and nature of the amino acids present in the peptide moiety, and the placement of the lactone ring. Other noteworthy differences amongst pseudomonad CLPs are the presence of unusual amino acids such as cyclic, branched and non-proteinogenic variants. The four major CLP groups include amphisin, viscosin, tolaasin and syringomycin, which are discussed in more detail below.

Table 1.1 CLPs produced by plant-associated *Pseudomonas* spp.

CLP Group	Fatty acid	AminoAcids in Peptide Moiety*																							
Viscosin		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**															
Viscosin	C10HO	L-Leu	D-Glu	D –*a*Thr	D –Val	L -Leu	D -Ser	L -Leu	D -Ser	L –Ile															
Viscosinamide	C10HO	L-Leu	D-Gln	D –*a*Thr	D –Val	L -Leu	D -Ser	L -Leu	D -Ser	L –Ile															
WLIP	C10HO	L-Leu	D-Glu	D –*a*Thr	D –Val	D –Leu	D -Ser	L -Leu	D -Ser	L –Ile															
Massetolide A	C10HO	L-Leu	D-Glu	D –*a*Thr	D –*a*lle	D –Leu	D -Ser	L -Leu	D -Ser	L –Ile															
Massetolide D	C10HO	L-Leu	D-Glu	D –*a*Thr	D –*a*lle	D –Leu	D -Ser	L -Leu	D -Ser	L –Leu															
Pseudophomin A	C10HO	L-Leu	D-Glu	D –*a*Thr	D -Ile	D –Leu	D -Ser	L -Leu	D -Ser	L –Ile															
Pseudophomin B	C12HO	L-Leu	D-Glu	D –*a*Thr	D –Ile	D –Leu	D -Ser	L -Leu	D -Ser	L –Ile															
Syringomycin		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**															
Syringomycin A	C10HO	L-Ser	D-Ser	D –Dab	L –Dab	L-Arg	L -Phe	Z -Dhb	L –Asp (3-OH)	4-Cl-L –Thr															
Syringomycin E	C12HO	L-Ser	D-Ser	D –Dab	L –Dab	L-Arg	L -Phe	Z -Dhb	L –Asp (3-OH)	4-Cl-L –Thr															
Syringotoxin B	C14HO	L-Ser	D –Dab	Gly	D –Hse	L –Orn	L –*a*Thr	Z -Dhb	L –Asp (3-OH)	4-Cl-L –Thr															
Syringostatin A	C14HO	L-Ser	D –Dab	L –Dab	D –Hse	L -Orn	L –*a*Thr	Z -Dhb	L –Asp (3-OH)	4-Cl-L –Thr															
Pseudomycin A	C14diHO	L-Ser	D –Dab	L –Asp	L –Lys	L –Dab	L –*a*Thr	Z -Dhb	L –Asp (3-OH)	4-Cl-L –Thr															
Cormycin A	C14diHO	L-Ser	D –Orn	L –Asn	D –Hse	L –His	L –*a*Thr	Z -Dhb	L –Asp (3-OH)	4-Cl-L –Thr															
Amphisin		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**													
Amphisin	C10HO	D –Leu	D –Asp	D –*a*Thr	D –Leu	D –Leu	D –Ser	L -Leu	D-Gln	L -Leu	L -Ile	L –Asp													
Tensin	C10HO	D –Leu	D -Asp	D –*a*Thr	D –Leu	D –Leu	D –Ser	L -Leu	D-Gln	L -Leu	L -Ile	L –Glu													
Pholipeptin	C10HO	D –Leu	L –Asp	L –Thr	D –Leu	D –Leu	D -Ser	D-Leu	D-Ser	D-Leu	L -Ile	D –Asp													
Lokisin	C10HO	Leu	Asp	D –*a*Thr	Leu	Leu	D -Ser	Leu	D-Ser	Leu	L -Ile	Asp													
Arthrofactin	C10HO	D –Leu	D -Asp	D -Thr	D –Leu	D –Leu	D -Ser	L -Leu	D-Ser	L –Ile	L –Ile	L –Asp													
Putisolvin		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**												
Putisolvin I	C6	Leu	Glu	Leu	Ile	Gln	Ser	Val	Ile	Ser	Leu	Val	Ser												
Putisolvin II	C6	Leu	Glu	Leu	Ile	Gln	Ser	Val	Ile	Ser	Leu	Leu/Ile	Ser												
Tolaasin		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
Tolaasin	C8HO	Dhb	Pro	Ser	Leu	Val	Ser	Leu	Val	Val	Gln	Leu	Val	Dhb	*a*Thr	Ile	Hse	Dab	Lys							
Fuscopeptin B	C8HO	Dhb	Pro	Leu	Ala	Ala	Ala	Ala	Val	Gly	Ala	Val	Ala	Val	Dhb	*a*Thr	Ala	Dab	Dab	Phe						
Corpeptin A	C10HO	Dhb	Pro	Ala	Ala	Ala	Val	Val	Dhb	Hse	Val	*a*Ile	Dhb	Ala	Ala	Ala	Val	Dhb	*a*Thr	Ala	Dab	Ser	Ile			
Syringopeptin 22	C10HO	Dhb	Pro	Val	Val	Ala	Ala	Val	Val	Dhb	Ala	Val	Ala	Ala	Dhb	*a*Thr	Ser	Ala	Dhb	Ala	Dab	Dab	Tyr			
Syringopeptin 25	C10HO	Dhb	Pro	Val	Ala	Ala	Val	Leu	Ala	Ala	Dhb	Val	Dhb	Ala	Val	Ala	Ala	Dhb	*a*Thr	Ser	Ala	Dhb	Ala	Dab	Dab	Tyr

*Hse: homoserine, *a* Thr , *allo* –threonine, Dab: 2,4-diaminobutyric acid, Dhb: 2,3-dehydroaminobutyric acid

1.2.2.1 Viscosin group

The viscosin group is composed of nine, predominantly hydrophobic amino acids in the D-configuration, with a lactone ring formed between the C-terminal amino acid and the OH group of the *allo*-threonine at position three of the peptide moiety. The N-terminal amino acid is coupled to 3-hydroxydecanoic acid. This group includes the White-Line Inducing Principle (WLIP) CLP, massetolide A D, viscosinamide, viscosin, pseudophomin B and a novel, uncharacterized CLP from *Pseudomonas extremorientalis.* These CLPs are mostly water-soluble, extracellular products with the exception of viscosinamide, which is strongly associated with the cell membrane. The CLPs within this group are typically produced by members of the fluorescent pseudomonads isolated from a variety of environmental sources and have diverse activities attributed to them. For example, viscosin is an important determinant of head rot of broccoli and its potent biosurfactant activity (Laycock et al. 1991) increases membrane permeability to facilitate subsequent invasion by pectolytic enzymes (Laycock et al. 1991).

1.2.2.2 Amphisin group

Amphisin-like CLPs are structurally similar to the viscosin group except that the peptide moiety consists of eleven amino acids linked to 3-hydroxy decanoic acid. Similar to the viscosin group, the lactone ring is formed between the C-terminal amino acid and the D-*allo*-threonine or L-threonine at the third amino acid position in the peptide chain. Additionally, the sequence motif of this group is composed of hydrophilic and hydrophobic amino acids. Amphisin is produced by the sugar beet rhizosphere isolate *Pseudomonas* sp. DSS73 and is antagonistic towards the oomycete pathogen *P. ultimum*

(Nielsen et al. 2005). An interesting trait associated with amphisin-producing fluorescent *Pseudomonas* species is the co-production of HCN and chitinases, a trait which has not been linked with the production of other CLPs (Nielsen et al. 2002).

1.2.2.3 Tolaasin group

Contrary to the amphisin- and viscosin-like CLPs, the tolaasin molecules are significantly larger in size and display higher variability in structure between individual molecules. The peptide moiety contains 19-25 amino acids and harbours some "unusual" amino acids such as homoserine, 2,4-diaminobutyric acid, and 2,3-dihydro-2-aminobutyric acid. The fatty acid moiety displays some variability as well and is typically either 3-hydroxy octanoic acid or 3-hydroxy decanoic acid. Tolaasin-like CLPs are cyclized between the C-terminal amino acid the *allo*-threonine residue and are composed of five to eight amino acids. CLPs within this group are commonly associated with plant pathogenic strains such as *P. syringae*; syringopeptin (Ballio et al. 1991; Iacobellis et al. 1992), *P. tolaasi*; tolaasin (Nutkins et al. 1991) and *P. fuscovaginae*; fuscopeptin (Ballio et al. 1996).

1.2.2.4 Syringomycin group

Although the number of amino acids in the peptide portion is similar to the viscosin-like CLPs, the syringomycin class differs in that they harbour a number of atypical and modified amino acids such as ornithine, 2,4-diaminobutyric acid and 4-chloro-threonine. Another significant characteristic is that the entire peptide moiety is cyclized, with a lactone ring formed between the N-terminal serine and the C-terminal 4-chloro-threonine.

Whereas the afore-mentioned CLPs have lipid tails consisting of C_8-C_{10}, the syringomycin-like CLPs have C_{10} to C_{14} 3-hydroxy or 3,4-dihydroxy fatty acid tails. This group consists mainly of broad-spectrum molecules with phytotoxic and AF activities and are produced by *P. syringae* (Harrison et al. 1991; Quigley and Gross 1994) and *Pseudomonas corrugata* (Scaloni et al. 2004)

1.2.2.5 Additional CLPs

A small number of unique CLPs have been isolated and characterized from *Pseudomonas* species which lie outside of the classification scheme employed by Raaijmakers et al, (2006). *P. putida* produces two CLPs designated putisolvin I and II which have twelve amino acids in the peptide head and a hexanoic lipid tail (Kuiper et al. 2004). As the genome sequences of *Pseudomonas* species become available, *in silico* analysis is yielding exciting findings with the identification of previously undetected CLPs. In the biocontrol strain *P. fluorescens* Pf-5, for example, a gene cluster encoding a putative lipodecapeptide has been identified (Paulsen et al. 2005). Genome mining of *P. syringae* pv. tomato DC3000 and subsequent chemical analysis led to the identification of syringafactins A-F, which are a set of closely related linear peptides consisting of eight amino acids and ten to twelve carbon atoms in the lipid tail (Berti et al. 2007).

1.2.3 Non-ribosomal peptide synthesis of CLP molecules

CLPs are synthesized non-ribosomally via a thiotemplate mechanism on NRPS modules. An NRPS module typically consists of three domains which are collectively required for the stepwise incorporation of one amino acid into the peptide chain. For

example a CLP with nine amino acids in the peptide moiety would require nine modules for its biosynthesis. A module harbours an adenylation (A) domain which is responsible for amino acid recognition and activation, a condensation (C) domain which catalyzes peptide bond formation between adjacent amino acids, and a thiolation (T) domain, alternatively referred to as peptide carrier protein (PCP) as it is responsible for thioesterification of the adenlyated amino acid. The modules responsible for amino acid incorporation are typically organized on the chromosome in the same order in which the amino acids appear in the final CLP peptide moiety, and this is considered adherent to the "colinearity rule" (Marahiel et al. 1997). However, there are a few exceptions to this rule, such as in the case of the syringomycin NRPS modules. Here the *syrB1* gene, encoding the domains responsible for the incorporation of the ninth amino acid, is located upstream of the *syrE* gene, which incorporates the first eight amino acids into the peptide head (Guenzi et al. 1998).

1.2.3.1 Arrangement of NRPS modules

Modules can be further subdivided into initiation modules and elongation modules (Keating and Walsh 1999). An initiation module is the first enzymatic unit of the NRPS template and typically consists of two domains, an A domain and a T domain (Keating and Walsh 1999). The A domain is responsible for recognition and selection of the first amino acid of the peptide chain (Dieckmann et al. 1995; Stachelhaus and Marahiel 1995; Mootz and Marahiel 1997; May et al. 2002). The amino acid is activated as its corresponding aminoacyl adenylate derivative in an ATP-dependent reaction (Dieckmann et al. 1995). The activated amino acid is then transferred as a thiol ester to the adjacent T domain, releasing AMP (Stachelhaus et al. 1996; Ehmann et al. 2000).

However, one distinguishing feature of CLP-producing NRPS modules is the presence of a C domain in the first module (Guenzi et al. 1998; Konz et al. 1999; Scholz-Schroeder et al. 2003; Roongsawang et al. 2005; Gross et al. 2007). Based on studies of *Bacillus* and *Streptomyces* CLP-encoding NRPS templates, the C-domain in this atypical initiation domain is responsible for linking the first amino acid tethered to the adjacent T domain to a fatty acid, thus forming a lipidiated CLP (Vollenbroich al. 1994; Hojati et al. 2002; Miao et al. 2006).

An elongation domain consists of a C, A, and T domain and is responsible for elongation of the nascent peptide chain. C domains are situated at the carboxyl end of T domains which harbour a prosthetic phosphopantetheine group protruding off the T domain which acts in a "swinging arm" fashion to accept the activated amino acid from the A domain and transport it towards the next catalytic module (Linne and Marahiel 2000; Weber et al. 2000; Linne et al. 2001). The C domain subsequently catalyzes peptide bond formation between the N-terminal thiol-esterified amino acid and the elongation intermediate tethered to the adjacent C-terminal module (Stachelhaus et al. 1998). In this assembly-line manner, peptide biosynthesis proceeds from module to module until it reaches the PCP domain in the final module in the NRPS machinery where it is subsequently transferred to and cleaved from the NRPS by a thioesterase (TE) domain also called a peptide cyclase (Schneider and Marahiel 1998; Sieber and Marahiel 2003). This step results in the release of either a linear peptide or a circularized derivative resulting from an intramolecular cyclization reaction. TE domains are highly specific as they select a particular residue of the peptide chain as a substrate for

cyclization (Schneider and Marahiel 1998). Fig. 1.2 illustrates a schematic of a NRPS gene cluster encoding a modular template for the biosynthesis of a CLP molecule.

It should be noted that unusual amino acids such as non-proteinogenic D-amino acids, carboxy acids or fatty acids can be incorporated into the NRPS assembly line as well. NRPS templates may possess additional domains such as epimerization (E) domains that convert L- or D- amino acids to the opposite configuration. In many pseudomonad NRPS templates for CLP biosynthesis, certain modules contain C-domains that have additional amino acids at the N-terminus (Balibar et al. 2005; Berti et al. 2007). These larger C-domains are associated with dual function, as they not only catalyze peptide bond formation but also epimerize the amino acid incorporated by that specific module (Balibar et al. 2005).

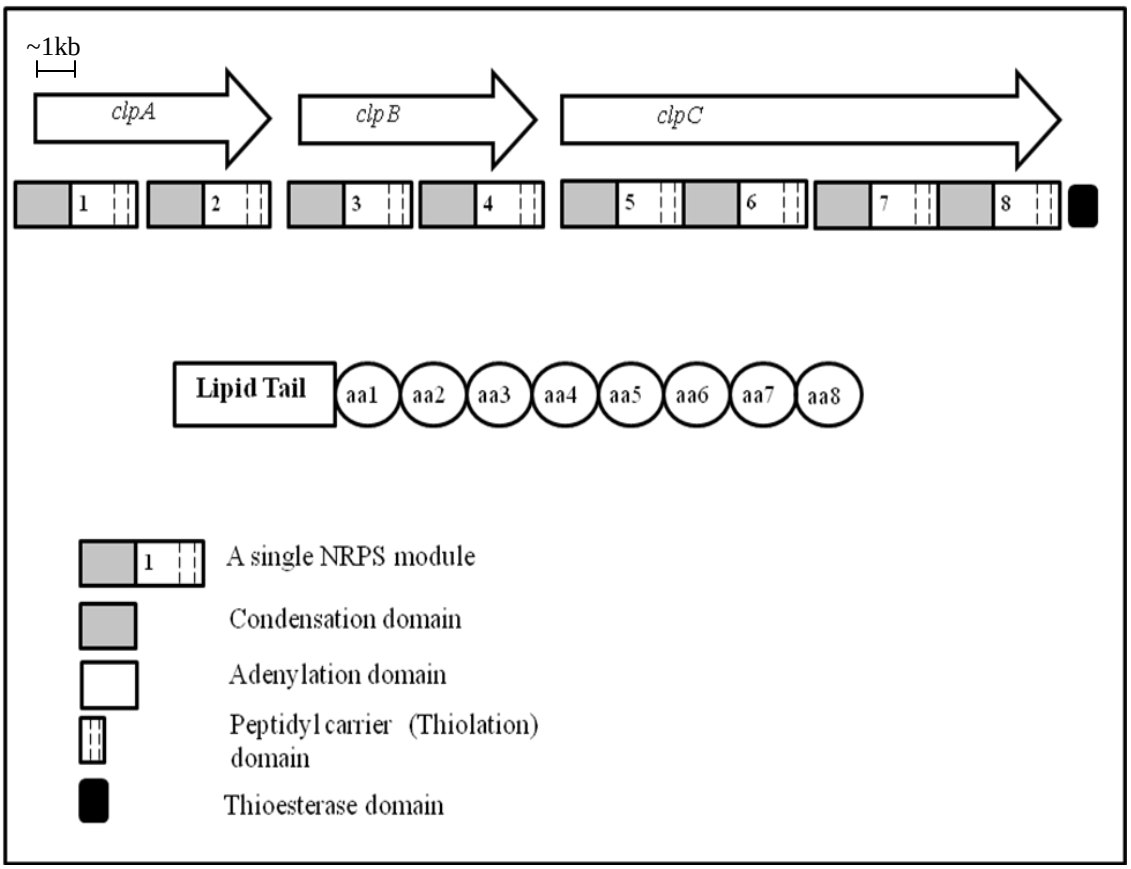


Figure 1.2 Schematic of the NRPS modular structure showing three large genes, *clpA, clpB* and *clpC* with *clpA* and *clpB* encoding two modules each and *clpC* encoding four modules for the biosynthesis of a CLP structure containing eight amino acids in the peptide moiety. The lipid tail is indicated however the genes responsible for FA synthesis are not encoded by NRPS gene clusters.

NRPS biosynthesis is not limited to CLP molecules but also contributes to the production of an array of important metabolite such as the antibiotics penicillin (Byford et al. 1997) and vancomycin (van Wageningen et al. 1998), the immunosuppressant; cyclosporin A (Weber et al. 1994), and pyoverdine and pyochelin, iron-chelating siderophores produced by *Pseudomonas* spp. (Quadri et al. 1999; Quadri 2000).

1.2.3.2 NRPS genes involved in CLP synthesis

To date, a total of ten NRPS templates encoding pseudomonad CLPs have been entirely sequenced, in addition to a significant amount of annotated partial genetic information. Of the sequenced templates, two very well characterized templates are those of arthrofactin (Roongsawang et al. 2003) and the syringopeptin-syringomycin pathogenicity island (Guenzi et al. 1998; Bender et al. 1999). NRPS gene clusters are substantial in size with the most impressive amongst them being the syringomycin and syringopeptin gene clusters. Together, these clusters span 132 kb, accounting for approximately two percent of the *P. syringae* pv. s*yringae* genome (Spiers et al. 2000; Stover et al. 2000; Scholz-Schroeder et al. 2001) with the syringopeptin synthetase encoding twenty-two modules, the largest NRPS system to be described in prokaryotes (Scholz-Schroeder et al. 2003).

Arthrofactin biosynthesis requires three large genes, *arfA*, *arfB* and *arfC,* the products of which form eleven modules consisting of the standard A, C and T domains. Biosynthesis proceeds according to the colinearity rule. Although four of the eleven amino acids in the peptide moiety are in the D-configuration, none of the modules harbour an internal racemase or epimerase domain (Roongsawang et al. 2003). This is

also true for the other six completely described NRPS biosynthetic clusters; syringomycin (Scholz-Schroeder et al. 2001), syringopeptin (Scholz-Schroeder et al. 2003), massetolide A (de Bruijn et al. 2008), a viscosin-like CLP produced by *P. fluorescens* SBW25 (de Bruijn et al. 2007), syringafactins (Berti et al. 2007), and orfamide A produced by *P. fluorescens* Pf-5 (Gross et al. 2007). A recent analysis of the arthrofactin condensation domains has revealed that certain condensation domains also have epimerization (E) activity and that these dual C/E domains are involved in the epimerization of the amino acid loaded onto the preceding module (Balibar et al. 2005). These dual C/E domains have since been identified in syringomycin, syringopeptin and massetolide A (de Bruijn et al. 2008).

A domains have also been particularly well studied as they are considered the "gate-keepers" of the NRPS template. A comparison of the *Bacillus* phenylalanine-binding pocket of gramicidin S (Conti et al. 1997) with other NRPS sequences revealed that the ten residues lining the substrate-binding pocket were highly conserved and contained "signature sequences" for the incorporation of a specific amino acid (Stachelhaus et al. 1999). Thus, the sequence of a peptide could be predicted based on the translated sequence of adenylation domains. This strategy has proven useful when mining new genomes for NRPS templates and it was instrumental in the discovery of orfamide A (Gross et al. 2007) and syringactins A-F (Berti et al. 2007; de Bruijn et al. 2007).

1.2.4 CLP secretion

The regions flanking NRPS genes often harbour genes for CLP transport out of the cell. Sequence analysis of the regions flanking the syringomycin-syringopeptin (*syr*-

syp) gene cluster has resulted in the identification and subsequent characterization of a few interesting products. One of these is *syrD,* a gene encoding a product with homology to membrane proteins of the ABC transporter family (Quigley et al. 1993). Strains with an inactivated *syrD* exhibit significantly decreased virulence compared to the wild type (Quigley et al. 1993). A second secretion system with homology to the resistance-nodulation-cell division efflux genes was located upstream of the *syr-syp* gene cluster and has been demonstrated to function in toxin secretion (Kang and Gross 2005).

1.2.5 Biological activities of *Pseudomonas*-associated CLPs

CLP activity is determined by the number, type and configuration of amino acids in the peptide moiety and by the length and composition of the fatty acid tail. CLPs are functionally diverse and have roles ranging from biosurfactant activity, regulation of surface attachment and detachment, enhancing motility, antimicrobial activity and pathogenicity, all of which will be discussed in more detail below.

1.2.5.1 CLP biosurfactant activity and inhibition of biofilm formation

Biosurfactants are surface active, structurally amphipathic molecules that reduce surface tension. Given their hydrophobic and hydrophilic moieties, it is not surprising that *Pseudomonas* CLPs exhibit biosurfactant activity. Putisolvin I and II are two biosurfactants produced by *P. putida* PCL1445, a bacterium isolated from plant roots growing on a site polluted with polycyclic aromatic hydrocarbons (Kuiper et al. 2001). Putisolvin I and II differ from each other only in the nature of the amino acid in position eleven of the peptide moiety which is valine in putisolvin I and leucine/isoleucine in

putisolvin II (Kuiper et al. 2004). Putisolvins demonstrate strong biosurfactant activity and are able to reduce the surface tension of growth medium by forty percent, increase toluene emulsions formation, and promote swarming motility on agar plates (Kuiper et al. 2001). However, an intriguing aspect of putisolvins is the observation that they are capable of inhibiting biofilm formation (Kuiper et al. 2004). Not only do mutants impaired in putisolvin production produce thicker biofilms with a higher number of cells and cell aggregates as compared to the wild type, but purified putisolvins have been demonstrated to break down exisiting biofilms produced by *P. aeruginosa* UCBPP-PA14 and *P. fluorescens* WCS365 (Kuiper et al. 2004). Although biofilm inhibition has been described for non-CLP surfactants produced by *P. aeruginosa* PAO1 (Davey et al. 2003) and Gram-positive bacteria lipopeptides (Busscher et al. 1997; Velraeds et al. 2000; Mireles et al. 2001), this was the first report of biofilm degradation by a *Pseudomonas* CLP molecule (Kuiper et al., 2004).

1.2.5.2 Involvement of CLPs in bacterial motility

The biosurfactant properties of CLPs promote bacterial movement over surfaces such as the phylloplane and roots (Hildebrand et al. 1998; Lindow and Brandl 2003). Studies have shown that CLP production is an importantl trait for bacterial swarming, a form of motility involving type IV pili and biosurfactant production (Lindum et al. 1998; Nielsen et al. 2002; Andersen et al. 2003; Roongsawang et al. 2003; Kuiper et al. 2004). For example, a *Pseudomonas* DSS73 mutant impaired in amphisin production was deficient in the colonization of sugar beet seed and barley straw residues, compared to the wild type. (Nielsen et al. 2005). Furthermore, amphisin-mediated motility was

determined to be a requirement for efficient suppression of the root pathogens, *P. ultimum* and *R. solani* (Andersen et al. 2003).

1.2.5.3 CLPs as AF compounds

Certain *Pseudomonas* species are antagonistic towards phytopathogenic and economically important fungi. CLP-mediated antagonism has been partially attributed to membrane disruption via pore formation and membrane solubilization. Membrane disruption has been postulated to promote accessibility of extracellular enzymes which further antagonize target pathogens (Hutchison et al. 1995; Hildebrand et al. 1998; Lindow and Brandl 2003). This activity has been demonstrated for oomycete zoospores as well as fungal mycelia (Thrane et al. 1999; Thrane et al. 2000; Nybroe and Sorensen 2004). Recently, a role has been proposed for CLPs as elicitors of ISR, a plant immune response (Tran et al. 2007).

An example of a CLP that demonstrates AF activity is the cell-associated CLP viscosinamide which is produced by antagonistic *Pseudomonas* sp. DR54 (Nielsen et al. 1999). *In situ* and *in vitro* production of viscosinamide has been recorded with observations of reduced sclerotia formation by *R. solani*, as well as reduced mycelium growth, zoospore formation, and intracellular activity, and that purified viscosinamide induced zoospore encystment by *P. ultimum* (Thrane et al. 1999; Hansen et al. 2000; Thrane et al. 2000).

One of the beneficial aspects of CLPs is their broad-spectrum AF activity; however, the degree of AF activity may vary depending on the CLP-pathogen interaction. To illustrate this point, putisolvins produced by *P. putida* 267 are effective at lysing

zoospores of the oomycete pathogen *Phytophthora capsici* (Tran et al. 2008), yet putisolvin-deficient mutants are not impaired in biocontrol of damping-off of cucumber caused by the same pathogen (Kruijt et al. 2009). Although putisolvins significantly inhibit *B. cinerea* mycelial growth (Kruijt et al. 2009), they are not as effective at suppressing growth of *R. solani* mycelia or a number of other oomycete and fungal pathogens (Kruijt et al. 2009). The variability in pathogen susceptibility was attributed to differences in membrane compositions or the ability of pathogens to resist or neutralize CLPs (Kruijt et al. 2009). Similar observations of pathogen-host CLP dependent suppression have been observed with massetolide A (Mazzola et al. 2007; Tran et al. 2007), suggesting there may be a CLP structure - pathogen determinant of CLP-mediated biocontrol.

1.2.5.4 CLPs and antiprotozoan activity

Raaijmakers et al. conducted a series of experiments examining the effect of massetolide A and viscosin on *Naegleria americana*, a protozoan which preys on bacteria (Mazzola et al. 2009). They found that massetolide A and viscosin enhanced bacterial survival by promoting bacterial evasion from the predator (Mazzola et al. 2009). Purified viscosin was found to lyse *N. americana* more effectively than massetolide A; however, it remains unclear whether or not the CLP achieves high enough concentration *in situ* to lyse the predator. Nevertheless, the experiments demonstrated that strains defective in CLP production were present in the soil and wheat rhizosphere in lower numbers than the CLP-producing strains, and this difference was only observed in the presence of *N. americana* (Mazzola et al. 2009). Another interesting finding from these studies was the

observation that interaction with *N. americana* triggered CLP production. Based on these experiments, the authors speculate that the natural role for CLPs may be enabling bacteria to evade protozoan predators, rather than competition through their antibacterial and AF activity. Interestingly, viscosin has also been shown to lyse trypomastigotes of the human pathogen *Trypanosoma cruzi*, the causal agent of Chagas's disease (Mercado and Colon-Whitt 1982; Burke et al. 1999).

1.2.5.5 CLP production by plant pathogens

There are a number of plant pathogenic species that produce CLPs as virulence factors. *P. syringae* mutants deficient in syringomycin or syringopeptin production exhibit decreased virulence on cherry leaves compared to the wild-type strain (Scholz-Schroeder et al. 2001). Other phytotoxic CLPs including tolaasin and viscosin have also been implicated in plant pathogenesis and similar to syringomycin and syringopeptin, their toxic effect is through formation of pores in the membrane, thus disturbing the ion potential which ultimately leads to cell death (Hildebrand). The biosurfactant properties of CLPs have also been postulated to contribute to virulence indirectly as they facilitate colonization of plant tissues.

1.2.6 Regulation of CLP production

The majority of *Pseudomonas* CLP research has focused on their biosynthesis and mode of action, whereas relatively little has been elucidated regarding regulation of the NRPS genes. A few studies which have examined abiotic, nutrient, and plant signal

molecules and sequence analysis of the regions flanking NRPS operons have yielded some interesting findings, which are discussed in the following sections.

1.2.6.1 Influence of abiotic factors on CLP expression

Given the complex interactions between *Pseudomonas* species and their environment, one would speculate that the host plant environment would markedly influence the production of secondary metabolites. Studies with putisolvin production by *P. putida*, a root isolate, demonstrates that salt stress and low oxygen concentration positively influenced transcription of *psoA*, the first gene in the putisolvin biosynthetic cluster. Carbon and nitrogen sources have also been found to influence CLP accumulation (Dubern and Bloemberg 2006). In the case of syringomycin, production of this CLP is dependent upon a threshold level of iron but is repressed by inorganic phosphate (Gross 1985).

1.2.6.2 Additional regulators of CLP production

Genome mining of regions flanking the NRPS biosynthetic gene clusters of certain CLPs has revealed regulatory elements controlling expression of these molecules. For example, analysis of regions flanking the syringomycin and syringopeptin genes identified SalA, a protein with homology to the LuxR family of transcriptional regulators that is part of the Gac regulon (Kitten et al. 1998). SalA is an important contributor to phytotoxin production by *P. syringae* pv. *syringae* (Wang et al. 2006). *salA* mutants were completely impaired in syringomycin production and displayed markedly reduced virulence on cherry fruit (Lu et al. 2002). SalA was shown to affect syringomycin

production through the positive regulation of *syrB1* and *syrG, a* syringomycin biosynthetic gene and a Lux-R type regulator, respectively (Lu et al. 2002).

A second regulator of CLP biosynthesis is *gidA* (glucose inhibited division), which is involved in the regulation of syringomycin and syringopeptin synthesis in *P. syringae* (Kinscherf and Willis 2002). *gidA* mutants were unable to produce CLPs and displayed altered pyoverdine synthesis and surface motility (Kinscherf and Willis 2002). Interestingly, *salA* and *syrB1* are positively regulated by GidA, however, this induction is not mediated via the Gac two-component system (Kinscherf and Willis 2002) discussed below.

Studies investigating the production of putisolvin biosynthesis found that members of the heat-shock protein family played a role in CLP biosynthesis (Dubern et al. 2005). DnaK is positively regulated by GacS/GacA and has homology with the Hsp70 heat shock protein family (Cowing et al. 1985; Hughes and Mathee 1998). At low temperatures, putisolvin production was upregulated and required a functional DnaK protein (Dubern et al. 2005). Further sequencing of regions flanking *dnaK*, revealed two additional genes, *dnaJ* and *grpE*, that inhibited putisolvin production (Dubern et al. 2005). The authors hypothesized that the three gene products, DnaK, DnaJ and GrpE, collectively may be required for proper folding or activity of either an unidentified regulator of the putisolvin biosynthetic genes or the NRPS protein assembly complex (Dubern et al. 2005).

1.3 Regulatory elements underlying biological control activity

1.3.1 Regulatory elements involved in biocontrol activity

Regulation of secondary metabolites occurs at the transcriptional and posttranscriptional level and involves small untranslated RNA molecules, cell-density dependent and two-component signal transduction systems as well as sigma factors. Collectively, a number of elements form a complex hierarchy of regulation affecting biological control in *Pseudomonas* species.

1.3.2 The GacS-GacA two-component regulatory system

The Gac two-component regulatory system has been identified in over twenty diverse bacteria including *Pseudomonas, Erwinia, Vibrio, Escherichia, Legionella, and Salmonella* (Heeb and Haas 2001); however, most of our knowledge regarding the Gac system comes from studies focused on fluorescent *Pseudomonas* spp and enteric bacteria. In human, animal and plant pathogens, the Gac system mediates expression of virulence factors. However, Gac regulation is not limited to virulence but encompasses an array of traits such as plant growth-promoting ability, biofilm formation, production of secondary metabolites and secreted enzymes. For example, in fluorescent *Pseudomonas* spp., the Gac system is a global regulator of extracellular enzyme and secondary metabolite production required for biocontrol activity (Thomashow 1996; Haas et al. 2000).

1.3.2.1 GacS and GacA proteins

The Gac two-component signal transduction pathway is composed of a membrane bound sensor kinase, GacS and its cognate response regulator, GacA. GacS was initially described in the plant pathogen *P. syringae* pv. *syringae* B728a as a factor required for lesion manifestation on bean leaves (Willis et al. 1990) and was subsequently identified as a sensor kinase belonging to a bacterial family of two-component regulators (Hrabak and Willis 1992). The response regulator GacA was first characterized in *P. fluorescens* CHA0 as a global activator of antibiotic and cyanide production (Laville et al. 1992) and was described as essential for the AF activity and ecological fitness of this important biocontrol strain (Laville et al. 1992; Natsch et al. 1994). Together, GacS and GacA function at a post-transcriptional level in response to an unknown environmental cue or bacterial signal (de Souza et al. 2003). Perhaps the best-characterized Gac System is that of *P. fluorescens* strain CHA0 (Zuber et al. 2003). The *P. fluorescens* CHA0 GacS protein has two transmembrane segments separated by a periplasmic loop and three conserved phosphoryl transfer sites each with a conserved residue essential for phosphorelay. The phosphorelay mechanism depicted in Fig. 1.3 is as follows; in response to detection of an unknown signal, GacS undergoes a conformational change accompanied by autophosphorylation of a conserved histidine residue (Robinson et al. 2000; Zuber et al. 2003). The phosphoryl group is then relayed from histidine-294 in the GacS primary transmitter domain to aspartate-717 in the receiver domain and finally to a conserved histidine-863 in the carboxy-terminal secondary transmitter domain (Zuber et al. 2003). Transfer then proceeds to a conserved aspartate in the receiver domain of the response regulator GacA (Zuber et al. 2003). Once activated, GacA, induces the

expression of small, untranslated RNA molecules which are part of a downstream regulatory system, known as Rsm (regulator of secondary metabolism). Aside from the conserved residues essential for the phosphorelay mechanism, other relevant domains have been identified such as the GacS linker domain. Normally, GacS/GacA regulation takes place during the switch from primary metabolism to secondary metabolism, which occurs at the end of the exponential growth phase. However, deletions in the GacS linker domain result in signal-independent and elevated exoproduct formation at low-cell densities (Zuber et al. 2003). Moreover, the Gac system is constitutively expressed if the linker domain is deleted, suggesting that this domain may have a role in signal recognition.

1.3.2.2 Contribution of GacS and GacA to biological control

In the plant-beneficial, root-colonizing strains *P. fluorescens* CHA0 and Pf-5, as well as *P. chlororaphis* PCL1391, 30-84 and PA23, GacS and GacA are responsible for the regulation of an array of antimicrobial products. These products include pyoluteorin, 2,4-DAPG, hydrogen cyanide, 2-hexyl-5-propyl-resorcinol, phenazine compounds and secreted enzymes (protease, phospholipase C and chitinase) which contribute to protecting colonized plants from fungal diseases (Corbell and Loper 1995; Haas et al. 2000; Poritsanos et al. 2006)

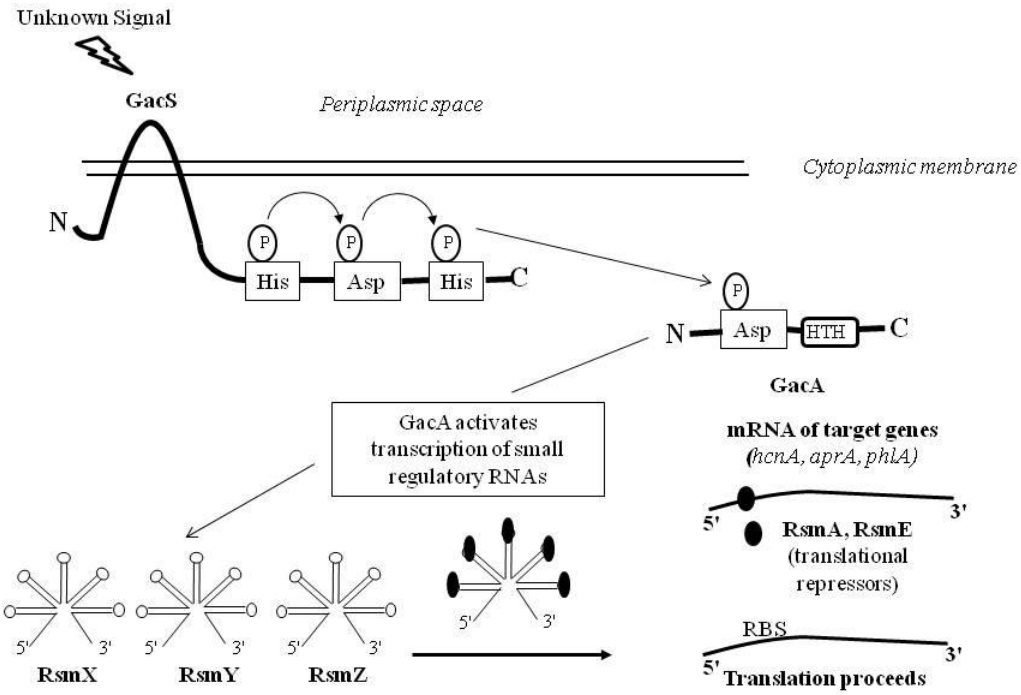


Figure 1.3 ***P. fluorescens*** **CHA0 GacS/GacA signal transduction model**. As outlined in the text, GacS undergoes a conformational change in response to an unknown signal followed by autophosphorylation at a conserved histidine residue. The phosphate is then transferred via a phosphophorelay mechanism to GacA which upon activation proceeds to induce target gene expression. Figure was modified from Heeb and Haas (2005).

The significance of GacS and GacA in biocontrol activity has been highlighted by the drastic effect of null mutations on the AF activity of these biocontrol bacteria. In both the *P. fluorescens* and *P. chlororaphis* strains mentioned above, Gac mutants exhibit a complete loss of exoproduct and antibiotic production which translates into a loss of biocontrol activity (Heeb and Haas 2001).

Although generally considered a positive post-transcriptional regulator, examples of GacS/GacA negative regulation do exist. In *P. fluorescens* CHA0, *gacS* and *gacA* mutants produce higher amounts of pyochelin and of the siderophore pyoverdine, suggesting that the biosynthetic genes encoding these metabolites are repressed by the GacS/GacA system (Schmidli-Sacherer et al. 1997; Duffy and Defago 2000).

1.3.2.3 Gac regulation of CLPs

The GacS/GacA system has been identified as the key regulator controlling the expression of a number of CLPs. For example, syringomycin (Hrabak and Willis 1992; Kitten et al. 1998), amphisin (Koch et al. 2002), massetolide A (de Bruijn and Raaijmakers 2009), viscosin (de Bruijn et al. 2007) and putisolvin (Dubern et al. 2005) expression are all dependent on functional GacS/GacA proteins. Furthermore, although the nature of the GacS-inducing signal remains unknown, a study of factors affecting amphisin production in *P. fluorescens* DSS73 found that sugar beet exudates contain a small, solvent-extractable, and heat-stable signal that enhanced amphisin production, and the positive effects of this exudate were effected through the GacS/GacA system (Koch et al. 2002).

1.3.2.4 Additional traits regulated by GacS and GacA

In addition to secondary metabolite production, colony morphology and surface motility are affected by *gacS* and *gacA* mutations. In *P. fluorescens* CHA0, Gac mutants are larger and flatter compared to the wild type and display altered motility (Duffy and Defago 2000; Goodier and Ahmer 2001). A second and more unique role of GacS/ GacA-controlled traits is the indirect induction of plant immune responses in rice. The production of a 12-membered peptide, syringolin, is under GacS control in *P. syringae* pv. *syringae* B301D-R. Syringolin elicits acquired resistance in rice against the rice blast fungus *Pyricularia oryzae*, although syringolin itself is not toxic to the fungal pathogen (Wäspi et al. 1998).

1.3.2.5 GacA as a master switch in a complex regulatory cascade

Numerous studies investigating the genetic regulation of secondary metabolite production have shown that GacS/GacA is a master regulatory system overseeing a complex regulatory cascade that includes alternative sigma factors, small, untranslated RNA molecules and cell-density dependent regulatory systems. The tomato pathogen, *P. syringae* DC3000 provides a clear example of the complex intricacies underlying secondary metabolite production. In this strain, GacA affects at least three alternate sigma factors, the quorum-sensing (QS) genes, and the expression of the small, untranslated RNA molecules, *rsmB* and *rsmZ.* The interaction of GacS/GacA with a few of these systems and their relevance to exoproduct production and biocontrol in fluorescent pseudomonads will be discussed in the following sections.

1.3.3 The Rsm system in *P. fluorescens*

As was briefly introduced in section 1.3.2.1 and depicted in Fig. 1.2, GacS/GacA exerts its regulatory effects via interaction with downstream regulatory components, which collectively form the Rsm system. Specifically at late exponential phase, phosphorylated GacA is proposed to upregulate the transcription of small, untranslated RNA molecules, *rsmX*, *rsmY*, and *rsmZ* which exert a positive effect by titrating out a repressive protein complex (Kay et al. 2005). The repressive complex is composed of small, well-conserved RNA-binding proteins, RsmA (Blumer et al. 1999; Heeb et al. 2002) and RsmE (Reimmann et al. 2005) that block translation by binding to repeated ANGGA motifs in the leader region of target mRNA. Similar to their homologs in *E. coli* and *E. carotovora*, in *P. fluorescens* CHA0, RsmZ, RsmY, and RsmX are small at 127, 118, and 119 nucleotides respectively, and contain several unpaired GGA motifs within stem-loop structures that bind multiple copies of RsmA and RsmE. Through RsmA/E binding, these repressor proteins are effectively titrated out enabling translation to proceed (Heeb, Blumer et al. 2002; Valverde et al. 2003; Kay et al. 2005). Given that expression of *rsmZ* and *rsmY* relies on a functional GacS/GacA (Heeb et al. 2002; Valverde et al. 2003) and *rsmZ* overexpression suppresses *gacS* and *gacA* mutations, RsmZ and RsmY are considered downstream regulatory elements in the GacS/GacA cascade (Heeb et al. 2002). Further support for *rsmZ* and *rsmY* dependence on GacS/GacA was provided by the findings that *rsmZ* expression was enhanced by a solvent extractable signal produced by *P. fluorescens* CHA0 at high cell densities (Heeb et al. 2002). Interestingly, a single mutation of either *rsmX*, *rsmY* or *rsmZ* alone was insufficient to cause a significant reduction in HCN, exoprotease or 2,4-DAPG synthesis,

however double mutants exhibited a complete loss of these products and a concomitant loss of biocontrol activity against *P. ultimum* infection of cucumber (Kay et al. 2005). Furthermore, deleting *rsmA* and *rsmE* in a *gacS* mutant fully suppressed the *gacS* mutation (Reimmann et al. 2005). As illustrated in Fig 1.3, these studies culminated in the proposal of a model in which GacS/GacA stimulates the expression of *rsmX*, *rsmY* and *rsmZ* during the transition from exponential to stationary phase. RsmX, RsmY and RsmZ then bind multiple copies of the repressor RNA-binding proteins RsmA and RsmE and target mRNA is translated.

Adding to the layers of complexity underlying the Gac/Rsm posttranscriptional control mechanisms, in *P. fluorescens* CHA0, *rsmE* is subject to positive regulation by GacA and negative regulation by RsmA and RsmE (Reimmann et al. 2005). Furthermore, RsmA and RsmE have been shown to stabilize RsmZ and RsmY potentially protecting these small, untranslated RNA species from degradation by cellular RNases (Reimmann et al. 2005). The expression levels of the two regulatory RNA proteins differ from one another during cell growth, with RsmA levels remaining fairly constant throughout growth while RsmE attains higher levels during stationary phase (Reimmann et al. 2005). Similarly, positive and negative regulatory roles for RsmA have been described in *P. aeruginosa* suggesting that aspects of Rsm-mediated control of secondary production in *P. fluorescens* are yet to be revealed. In contrast to *E. coli* and *E. carotovora* which harbour only a single *rsmA* gene (Heeb et al. 2004), *in silico* analysis of *Pseudomonas* genomes reveals a certain degree of redundancy. For example, the number of putative *rsmA* and *rsmE* homologs ranges from two in *P. fluorescens* Pf-5 to at least five in *P. syringae* pv. *phaseolica* (Reimmann et al. 2005). This may indicate that

RNA species play a greater role in the timing and regulation of secondary metabolite production in *Pseudomona*s species.

1.3.4 Quorum sensing

QS is a population density dependent system, which relies on cell-cell communication via small diffusible signal molecules termed *N*-acylhomoserine lactones (AHL) or autoinducers (AI). This system was first described in the marine symbiont *Vibrio fischeri* where it is essential for bioluminescence (Hanzelka and Greenberg 1995). Based on the *V. fischeri* model, a QS system constitutes two proteins. The first protein is an AHL synthase encoded by the *luxI* gene that is responsible for the biosynthesis of AHL molecules (Fuqua et al. 1994). The second protein is a cognate transcriptional regulator (R-protein) called LuxR (Fuqua et al. 1994). AHLs are composed of a conserved lactone ring attached to a fatty acid chain where the length of the acyl side chain can range in number of carbons from C4-C16 and can vary in the type of side chain modifications (Fuqua et al. 1996). Most AHLs freely diffuse across the cell envelope (Kaplan and Greenberg 1985); consequently the AHL concentration increases as the population grows. After a threshold level is reached, the AHL binds to its cognate R-protein, enabling it to bind as a dimer to a twenty-nucleotide region of dyad symmetry in the promoter of target genes called the "lux box" (Fuqua et al. 1994). LuxR binding promotes target gene transcription. QS systems are an example of a positive autoinduction loop as LuxR activates *luxI* transcription (Fuqua et al. 1994).

1.3.4.1 The role of QS in Pseudomonas biological control

Numerous plant-associated bacteria utilize small, diffusible AHLs to regulate a range of biological traits. In *P. chlororaphis* strains 30-84 and PCL1391, phenazine production which contributes to the biocontrol activity against the take-all of wheat pathogen, is subject to QS regulation (Pierson et al. 1994; Wood et al. 1997). However, not all secondary metabolites in these two biocontrol isolates are subject to QS control as HCN and exoprotease production are regulated independent of QS (Chin-A-Woeng et al. 2001; Zhang and Pierson 2001). Moreover, *Pseudomonas* species may harbour more than one QS system as in *P. aeruginosa* (Gambello and Iglewski 1991; Passador et al. 1993; Ochsner et al. 1994; Latifi et al. 1995) and *P. chlororaphis* 30-84 (Pierson et al. 1994; Wood et al. 1997; Zhang and Pierson 2001). In *P. chlororaphis* 30-84, the PhzI/PhzR QS system regulates expression of the phenazine antibiotic genes, *phzABCDEFG* (Wood et al. 1997), while a second QS system, CsaR/CsaI, regulates cell surface properties as well as survival in the rhizosphere (Zhang and Pierson 2001). In contrast to the well-known interactions between the *P. aeruginosa* LasI/LasR and RhlI/RhlR QS systems (Pesci and Iglewski 1997), in *P. chlororaphis* 30-84, a hierarchy has not been established between the two QS systems; rather, they appear to function independent of one another (Zhang and Pierson 2001). A recently isolated take-all biocontrol strain, *P. fluorescens* 2P24 produces several AF compounds including 2,4-DAPG, HCN and siderophores (Lei and Zhang, 2006). Although a PcoR/PcoI QS system has been identified and found to be involved in biofilm formation, wheat rhizosphere colonization and biocontrol of take-all disease of wheat, the production of secondary metabolites was not affected in QS mutants of this strain (Wei and Zhang 2006). In *P.*

fluorescens 2P24 and *P. chlororaphis* 30-84, the GacS/GacA system upregulates AHL transcription (Chancey et al. 1999; Yan et al. 2009).

1.3.4.2 QS regulation of CLP production

Although, population-density dependent regulation of CLP expression is not nearly as wide spread as the GacS/GacA system, QS systems have been identified in a number of CLP-producing *Pseudomonas* species. The presence of a QS system, however, does not dictate its involvement in CLP production; in fact only a few CLPs are QS regulated. The first CLP found to be under QS control was viscosin, produced by the plant pathogenic isolate *P. fluorescens* 5064 (Cui et al. 2005). Mutants defective in the main signalling molecule, *N*-3-acyl-hydroxyoctanoyl-HSL failed to produce viscosin; however, CLP production was restored by the addition of purified acyl homoserine lactones (Cui et al. 2005). Similarly, the production of two biosurfactants which inhibit biofilm formation and degrade existing biofilms, putisolvin I and II, is positively regulated by the *P. putida* PCL1445 PpuR/PpuI QS system. Also, a *lux* box, typical for activation of QS controlled genes, was detected in the promoter region of the *psoA* gene, the first gene of the putisolvin biosynthetic gene cluster (Dubern et al. 2006).

1.3.4.3 Regulation of QS systems

Population density is not the only factor affecting QS systems. GacA positive regulation of AHL signals has been demonstrated in *P. aeruginosa* (Reimmann et al. 1997), *P. syringae* (Chatterjee et al. 2003), *P. fluorescens* (Wei and Zhang 2006) and *P. putida* (Bertani and Venturi 2004). Microarray analyses of the *P. aeruginosa* QS system

systems have identified over 600 genes constituting the QS regulon (Wagner et al. 2003). Thus it is not surprising that QS regulation overlaps that of numerous other global regulators in addition to RpoS and Gac/Rsm reviewed in (Schuster and Greenberg 2006; Venturi 2006). In *P. putida* and *P. aeruginosa*, negative regulators of QS have been identified, such as RsaL, which acts as a strong repressor of *ppuI* (Dubern et al. 2006) and *lasI* transcription (de Kievit et al. 1999). A recent search for factors influencing QS in *P. putida* WCS358 identified Lon protease as a negative regulator of AHL production (Bertani et al. 2007). The stationary phase sigma factor, RpoS negatively regulates AHL synthesis in *P. fluorescens* 2P24 (Yan et al. 2009) and *P. aeruginosa.* However, in 2P24 this negative effect was dependent upon a functional Gac system, leading the authors to speculate that the negative effect of RpoS may be due to RpoS repression of the Gac system either directly or indirectly (Yan et al. 2009). Adding to the conflicting reports, another study found that the RhlR/RhlI system was required for *rpoS* expression (Latifi et al., 1996), whereas Whiteley et al. (Whiteley et al. 2000) concluded that *rpoS* expression was not dependent on the Rhl QS system, rather RpoS repressed *rhlI* transcription. Transcriptional profiling of the *P. aeruginosa* RpoS regulon has determined that there is significant overlap between the RpoS and QS- regulated genes. The results indicated that QS slightly increases *rpoS* expression and conversely, RpoS slightly upregulates the QS system at stationary phase (Schuster et al. 2004).

Analysis of *P. aeruginosa* has determined that the RNA-binding protein, RsmA has a negative impact on the QS signal molecules as *rsmA* mutants overproduced both AHL-signalling molecules and RsmA overexpression resulted in significantly decreased AHL production (Pessi et al. 2001). Additionally, in the biocontrol strain *P. fluorescens*

CHA0, the non-coding regulatory RNAs, *rsmX*, *rsmY*, and *rsmZ* were experimentally demonstrated to enhance the expression of a small, non-AHL, signal which induced the Gac/Rsm system in a positive feedback loop (Kay et al. 2005).

1.4 Thesis objectives

As discussed in the sections above, biological control strains present an intriguing alternative to chemical pesticides to control diseases caused by fungal pathogens. Several *Pseudomonas* species produce secondary metabolites which are antagonistic to other microorganisms. One promising isolate, *Pseudomonas sp.* DF41, was isolated from the canola rhizosphere and tested for inhibition of the fungal pathogen *S. sclerotiorum*. DF41 demonstrated excellent biocontrol activity against this pathogen, reducing mycelial growth and germtube elongation (Savchuk and Fernando 2004). Furthermore, it was able to successfully colonize and persist on the canola phylloplane for several days (Savchuk and Fernando 2004). These preliminary experiments demonstrated the biocontrol potential of this strain; however, the traits required for AF activity had yet to be elucidated. The overall goal of this study is to understand the mechanisms underlying the biocontrol activity of *Pseudomonas* sp. DF41. The main objectives are as follows:

1. To identify genes and gene products essential for DF41 biocontrol of *S. sclerotiorum*.

2. To identify traits which may affect the ecological fitness and antagonism exhibited by DF41, including motility, biofilm formation and alginate production.

3. To elucidate the regulatory hierarchy governing secondary metabolite production.

4. To characterize the biological activity and chemical structure of a novel DF41 compound that is essential for biocontrol.

The tools used to accomplish these goals include transposon mutagenesis, transcriptional fusion analysis, HPLC, MS and MALDI-TOF MS analysis, together with various phenotypic assays.

Chapter 2

Materials and Methods

2.0 Materials and Methods

2.1 Bacterial strains and plasmids:

The bacterial strains and plasmids used in this study are outlined in Table 2.1

2.2 Media and growth conditions.

Escherichia coli strains were routinely cultured at 37^{o}C in LB Broth (Difco Laboratories, Detroit, MI, USA) or on LB Miller agar (VWR International Ltd, Mississauga, ON). *Pseudomonas* species DF41 was routinely cultured at 28^{o}C in King's medium B (KB) (King, Ward et al. 1954) and in M9 minimal media (Difco Laboratories, Detroit, MI, USA). KB Media was solidified by the addition of 1.5% agar (Bacto Agar, VWR). Antibiotics were added when appropriate at the following concentrations: ampicillin (Amp; 100 μg/mL), chloramphenicol (Chl; 30 μg/mL), gentamicin (Gm; 50 μg/mL), kanamycin (Km; 50 μg/mL), tetracycline (Tc; 15 μg/mL) for *E. coli,* piperacillin (Pip; 80 μg/mL), rifampicin (Rif; 100 μg/mL), Gm (40 μg/mL), Tc (15 μg/mL) for *Pseudomonas* sp. DF41. All antibiotics were purchased from Research Products International Corp. (Mt. Prospect, Illinois), with the exception of Tc and Pip, which were purchased from Sigma-Aldrich (St. Louis, MO).

2.3 DNA manipulations

Small-scale plasmid preparations were performed using one of three methods: i) an alkaline lysis protocol (Sambrook *et al.* 1989); ii) a rapid alkaline extraction protocol (Birrboim and Doly 1979); and iii) a Qiagen mini plasmid preparation kit (Qiagen Inc.).

Table 2.1 Bacterial Strains and plasmids

Strain/plasmid	Relevant genotype or phenotype[a]	Source or reference
Pseudomonas		
DF41	Rif^R wild type (canola root tip isolate)	(Savchuk and Fernando 2004)
DF41-469	Rif^R *gacS*::Tn*5*-1063 genomic fusion	This study
DF41-469 (pUCP23-*gacS*)	Rif^R *gacS*::Tn*5*-1063 genomic fusion, *gacS* complemented strain	This study
P. aeruginosa PAO1	Wild type	(Holloway et al., 1979)
E. coli DH5α	*supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco
C. violaceum CVO26	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31532, autoinducer biosensor	(Latifi et al., 1995)
Plasmids		
pBluescript II SK	Cloning vector, ColE1, *oriV*, Ap^R	Stratagene
pRL1063a	Delivery plasmid for Tn*5*-1063; Km^R	(Merriman and Lamont 1993)
pRK2013	Transposon delivery vector; Km^R	(Ditta et al., 1980)
p1063-469	Rescue plasmid containing *gacS*::Tn*5*-1063 genomic fusion	This study
pCR2.1GacS	3.2-kb *gacS* PCR product cloned into the *Eco*R1 site in pCR2.1	This study
pCR2.1RpoS	1.7-kb *rpoS-rmsZ* PCR-fragment cloned into the *Eco*R1 site in pCR2.1	This study
pCR2.1TOPO	Cloning vector for PCR products	Invitrogen
pLP170	Promoterless *lacZ* transcriptional fusion vector, Amp^R	(Preston et al., 1997)
pPDFI-*lacZ*	pLP170 containing the *pdfI* promoter region from DF41	This study
pPROS-*lacZ*	pLP170 containing the *rpoS* promoter region from DF41	This study

pRSMZ-*lacZ*	pLP170 containing the *rsmZ* promoter region from *P. chlororaphis* PA23	(Selin et al., unpublished)
pUCP23	Broad-host-range vector; IncP OriT, Amp^R Gm^R	(West, Schweizer et al. 1994)
pUCP23-*gacS*	pUCP23 containing *gacS* from *Pseudomonas* DF41	This study
pME3219	pME6010 containing an *hcnA-lacZ* translational fusion	(Laville, Blumer et al. 1998)
pME6032	*Nru*I-*Eco*R1 $lacI^q$-P_{tac} fragment of pJF118EH subcloned in [*Bam*H1]-*Eco*R1-digested pME6031; $lacI^q$-P_{tac} expression vector	(Heeb et al.,2002)
pME6359	*rsmZ* gene from *P. fluorescens* CHAO under control of the P_{tac} promoter in pME6032	(Heeb et al., 2002)
pME6863	pME6000, Tc^R cloning vector with the *aiiA* gene from *Bacillus* A24 under control of the P_{tac} promoter	(Reimmann et al.2002)

[a] Antibiotic abbreviations: Amp^R, ampicillin; Tc^R, tetracycline; Gm^R, gentamycin; Km^R, kanamycin; Rif^R, rifampicin

Table 2.2 Primers used in this study

No.	Primer Name	Sequence (5'→ 3')*
1	Tn5-OT182	GATCCTGGAAAACGGGAAAGG
2	KmF	TTGAACAAGATGGATTGCACG
3	KmR	TCGTCAAGAAGGCGATAGAA
4	GacSF	GATCGCAGATTGGAAAGCAA
5	GacSR	ACGGTGTCCAGGTACCAAG
6	M13 Forward	CTGGCCGTCGTTTTAC
7	M13 Reverse	CAGTATCGACAAAGGAC
8	p170FI*Xho*	CCGCTCGAGTCATCCATACTTGAATAATCC
9	p170RI*Hind*	CCCAAGCTTCTAAGGACCTCCTCATAAGTT
10	M13Fwd	CTGGCCGTCGTTTTAC
11	M13Rev	CAGTATCGACAAAGGAC
12	RpoSF	TACGTCAGTGCTTACGGCCA
13	RsmZR	TATGACCCGCCCACATTTTT
14	p170FecorpoS	TGTGAATTCGGGAGGGACA
15	p170Rxbarpos	AGTCTAGAATCACCACTTCCCATTGCTT

* Restriction endonuclease sites introduced into the primer are underlined

Large-scale plasmid purification was performed using the Qiagen midi plasmid purification kit.

2.4 Standard DNA procedures

DNA was digested with appropriate restriction endonucleases (Invitrogen) according to the manufacturer's instructions.

2.5 Tn*5*-1063 mutagenesis

Transposon mutagenesis of *Pseudomonas* DF41 was achieved by tri-parental mating between the following three bacterial strains: i) *E. coli* strain DH5α harbouring the Tn*5luxAB* Tn on pRL1063a (Wolk *et al.*, 1991); ii) *E. coli* strain MT607 carrying the helper plasmid pRK600 (Finan et al. 1986); and iii) the DF41 recipient. The three strains were propagated separately in LB medium containing appropriate antibiotics until stationary phase (~OD_{600nm} = 2.0) at which point the cells were sedimented by centrifugation (5,000 x g) and resuspended in an equal volume of LB medium. Donor-to-helper-to-recipient ratios of 1:1:2, 1:1:3, 1:1:5 were added to a total volume of 1.5 mL and pelleted by centrifugation (5,000 x g). The cells were resuspended in 0.1 mL of LB broth and spotted onto sterile 0.45 μM-pore sized nitrocellulose filters on LB agar plates amended with 10 mM $MgSO_4$. After overnight incubation at 28^{o}C, the cells were washed from the filters using 1 mL of LB broth and plated as 100 μL aliquots onto pre-warmed LB agar plates containing Km 5 μg/mL and Rif 100 μg/mL. Transposants were isolated after 36-48 h at 28^{o}C. For each mating, 5-10 Km^R colonies were screened by PCR to ensure that transconjugants contained a Tn*5* insertion. For the Tn*5*-1063 PCR, primers

KmF (5′-ttgaacaagatggattgcacg-3′) and KmR (5′-tcgtcaagaaggcgatagaa-3′) were designed to the kanamycin cassette found Tn. Tn*5*-1063 contains an origin of replication which allows rescue cloning of the Tn and flanking DNA. To accomplish this, genomic DNA from the transposants was cleaved with *Eco*R1 and ligation reactions containing 2 μg of DNA in a total volume of 20 μL were allowed to incubate at 15^{o}C overnight. The ligations were precipitated by the addition of two volumes of cold 95% ethanol and incubated at -70^{o}C for 30 min. After 30 min. centrifugation, the DNA pellet was washed with 70% ethanol and centrifuged for an additional 30 min. The air-dried pellet was resuspended in 10 μL sd$H_2$0 and a 5-μl aliquot of plasmid DNA was electroporated into *E. coli* DH5α cells. After electroporation, cells were resuspended in 1 mL of prewarmed LB broth and incubated overnight at 37^{o}C with no aeration. Cells were pelleted by centrifugation (5,000 x g) and plated on LB agar supplemented with Km 50 μg/mL, to select for *E. coli* harbouring rescue clones.

2.6 Sequence analysis of Tn*5*-1063 mutants

Plasmids isolated from Km^{R} *Eco*R1 clones were sequenced using oligonucleotide primer Tn*5*-OT182 (5′-gatcctggaaaacgggaaagg-3′) which anneals to the 3′ end of the Tn*5*-1063. The *Eco*R1 rescue clones were sequenced at the University of Calgary Core DNA Facility. Sequence analysis was done using BLASTN and BLASTX databases.

2.7 Southern hybridization and generation of DIG-labelled probe

To verify single chromosomal Tn*5*-1063 insertions in DF41, Southern hybridization was performed using a *kan*-DIG labelled probe. The probe was generated

by PCR amplification of the 929-bp kanamycin cassette harboured on pRL1063a using PCR primers KmF (5′-ttgaacaagatggattgcacg-3′) and KmR (5′-tcgtcaagaaggcgatagaa-3′). For Southern blot analysis, genomic DNA was isolated from the Tn mutants, digested with *Eco*R1 or *Eco*RV restriction enzymes and separated on an agarose gel. The DNA was depurinated by treating the gel with 0.25M HCl for 10 min., followed by 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 agarose gel to Hybond-N^{TM} nitrocellulose membrane (Amersham Biosciences) by alkaline capillary transfer overnight in 10X SSC. After a 10 min. rinse in 5X SSC with gentle agitation, the nucleic acids were cross linked to the membrane by exposure to UV light for 5 min. A 40 mL aliquot of prehybridization solution was added to the membrane, which was allowed to equilibrate overnight at 65^{o}C in a ProBlot hybridization oven (Mandel, Canada). The blot was hybridized at 65^{o}C for 24 h with a *kan*-DIG DNA labelled probe. Unbound probe was removed by two 15 min. washes with a solution of 2X SSC and 0.1% SDS at room temperature, followed by two 30 min. washes with 0.5X SSC and 0.1% SDS at 65^{o}C. The membrane was incubated with washing buffer for 5 min. followed by blocking solution for 30 min. A solution containing anti-DIG polyclonal antibody conjugated to alkaline phosphatase (diluted 1:10, 000 in 1X blocking solution) was added to the blot for 60 min. The membrane was rinsed three times with water, incubated with washing buffer for 15 min., and then rinsed three more times with water before equilibration in 10 mL of detection buffer for 5 min. The colourimetric detection substrate nitroblue tetrazolium chloride/ 5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP) (40 μL in 2 mL of detection buffer) was evenly spread over the entire

surface of the blot which was sealed in an acetate sheet and stored in the dark to enable colour development (0.5 h - 16 h).

2.8 Isolation and cloning of the DF41 *gacS* gene.

The DF41 *gacS* gene was PCR amplified using oligonucleotide primers GacS-F (5′-gatcgcagattggaaagcaa-3′) and GacS-R (5′-acggtgtccaggtaccaag-3′). These primers were designed from the Tn*5*-1063 rescue clone sequence as well as the *Pseudomonas fluorescens* 2P24 *gacS* sequence (92.3% sequence identity). DF41 DNA template (1 μL; 20 ng) was amplified with 1.25 μL (2 pmol) of each primer, 2.5 μL of 10 X High Fidelity PCR buffer minus Mg [60 mM Tris-SO_4 (pH 8.9), 18 mM Ammonium Sulfate], 1.5 μL $MgS0_4$ (1.5 mM) and 0.2 mM of each deoxyribonucleotide (dATP, dCTP, dTTP, dGTP). One unit of Platimum^R *Taq* DNA Polymerase High Fidelity (Invitrogen) was added (0.2 μL) to a total volume of 25 μL. The PCR conditions were as follows: an initial denaturation for 3 min. at 95^{o}C, followed by denaturation at 95^{o}C for 1 min., annealing at 58^{o}C for 1 min. and extension at 72^{o}C for 1 min. for 30 cycles. The final extension was set at 72^{o}C for 10 min.

The 3.2-kb *gacS* PCR product was gel purified using the Ultra TM Kit and resuspended in 20 μl of 1X Tris-EDTA buffer (pH 7.5). Addition of 3′ A-overhangs to a 3 μL aliquot of the purified product was achieved by adding 2 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), 0.5 μL of 10 mM dATP and 0.5 μL of *Taq* Polymerase (2.5 units) in a total reaction volume of 20 μL. The reaction was incubated at 72^{o}C for 15 min. The 3.2-kb *gacS* product was gel purified again and cloned into the pCR2.1-TOPO vector according to the TOPO-pCR2.1 cloning

kit instructions (Invitrogen Life Technologies, Burlington, ON). In brief, the cloning reaction was performed using 4 μL of the *gacS* 3′-A overhang product, 1 μL pCR2.1-TOPO vector and 1 μL of TOPO salt. The reaction was mixed and incubated at room temperature for 30 min., which enhances the cloning of larger (>1-kb) products. The TOPO cloned *gacS* PCR product was transformed into *E. coli* TOP10F′ by adding 3 μL of the cloning reaction into a vial of TOP10F′ chemically competent cells, gently mixing the tube and incubating on ice for 5 min. After heat shock at 42^{o}C for 1.5 min., the cells were incubated at 37^{o}C for 1 h with aeration (200 RPM). Blue-white selection of the putative *gacS* clones was performed by plating on LB agar supplemented with IPTG, X-gal and 100 μg/ mL Amp. The orientation of the *gacS* insert was determined by sequencing using M13 Forward Primer (5′-ctggccgtcgttttac-3′) and M13 Reverse Primer (3′-cagtatcgacaaaggac-5′).

2.9 Subcloning of the DF41 *gacS* gene into pUCP23

For expression in a *Pseudomonas* background, the DF41 *gacS* gene was cloned into vector pUCP23. A 3.2-kb fragment containing the *gacS* gene was excised from pCR2.1-TOPO using *Bam*H1 and *Xba*1. The fragment was then gel purified and ligated into the same sites of pUCP23, generating pUCP23-*gacS*.

2.10 *gacS* nucleotide sequence accession number.

The GenBank accession number for the DF41 *gacS* gene is GQ328782.

2.11 Generation of a DF41 fosmid library

A DF41 fosmid library was generated using the CopyControl^{TM} Fosmid Library Production Kit (Epicenter, Madison, WI) according to the manufacturer's instructions. Briefly, high molecular weight (≥ 25 kb) DF41 genomic DNA was ligated into linearized and dephosphorylated pCC1FOS copy control vector, packaged into MaxPlax Lambda Packaging extract and plated on the *E. coli* EPI300^{TM} cells supplied. Fosmid clones were selected on LB agar supplemented with Chl. (12.5 μg/mL). The CopyControl pCC1FOS^{TM} vector is advantageous for fosmid library production as it contains the *E. coli* F-factor single-copy origin of replication and an inducible high copy origin of replication (*oriV*). Clones were selected and maintained as single copy to ensure stability and induced to high copy number (10-50 copies per cell) by the addition of 0.2% L-arabinose.

2.12 Isolation and cloning of the DF41 AHL synthase gene, *pdfI*

The DF41 genomic fosmid library was screened for autoinducer-positive clones by patching onto CV026-seeded plates. One fosmid clone, F564, which restored violacein production was selected for further analysis. Fosmid DNA was partially digested using *Sau*3A and ligated into *Bam*H1-digested pBluescript. Ligation reactions were incubated overnight at 16^{o}C and transformed into chemically competent *E. coli* DH5α cells by heat shock at 42^{o}C for 1.5 min, followed by incubation for 1 h at 37^{o}C. Blue-white selection of the putative *pdfI* clones was performed by isolation on LB agar supplemented with IPTG, X-gal and 100 μg/ mL Amp. White clones were patched onto

CV026-seeded LB-agar plates and incubated at 28^{o}C overnight. Clones which had a purple pigment surrounding the bacteria were selected for sequence analysis.

2.13 *pdfI* nucleotide sequence accession number.

The GenBank accession number for the DF41 *pdfI* gene is HM590003.

2.14 Overexpression of QS genes

Sequence analysis of putative *pdfI*-positive clones described in section 2.12, revealed the presence of an autoinducer synthase gene, *pdfI* as well as a transcriptional activator gene, *rfiA.* For expression in *Pseudomonas* DF41, a 3.2-kb fragment containing the *pdfI* and *rfiA* genes was excised from pBluescipt using *Eco*R1 and *Bam*H1 and ligated into the same sites of pUCP23. The ligation reactions were transformed into *E. coli* DH5α and plated on LB agar containing Amp100, Xgal and IPTG. White colonies were patched onto CV026-seeded plates and observed for violacein restoration. Plasmids were isolated from colonies that formed a purple zone on CV026-seeded plates. Plasmids were then digested with *Bam*H1 and *Eco*R1 to confirm the insertion of a 3.2-kb PCR product and positive clones were electroporated into DF41.

2.15 Generation of autoinducer-deficient strains

To obtatin an AHL-minus phenotype, plasmid pME6863 harbouring the AHL lactonase gene, *aiiA* from *Bacillus subtilis* (obtained from D. Haas) was introduced into DF41, DF41-469 and DF41-1278 by electroporation. AiiA cleaves the AHL ring rendering cells AHL negative. Bacteria were plated on LB-Tc15 μg/mL and colonies that

appeared were patched onto CV026-seeded plates to identify those unable to restore violacein production due to the presence of the *aiiA* gene. Plasmid preps were performed to confirm the presence of pME6863.

2.16 Generation of *pdfI-lacZ* transcriptional fusions

PCR primers (p170FI*Xho*; 5′-ccgctcgagtcatccatacttgaataatcc-3′) and (p170RI*Hind*; 5′-cccaagcttctaaggacctcctcataagtt-3′) were designed to amplify an 852-bp fragment encompassing the promoter region and part of the *pdfI* gene. The primers contained *Xho*I and *Hin*dIII restriction sites to enable cloning into the promoterless *lacZ* transcriptional fusion vector, pLP170 (Preston, Seed et al. 1997).

2.17 Isolation and cloning of the DF41 *rpoS* gene

The PCR primers that enabled isolation of the *P. chlororaphis* PA23 *rpoS* gene were used to amplify a 1.7-kb PCR product from DF41. PCR primers (rpoSF; 5'-tacgtcagtgcttacggcca-3') and (rsmZR 5'-tatgacccgcccacattttt'-3'). The PCR product was TOPO-cloned into pCR2.1, as described for the *gacS* gene, creating pCR2.1-*gacS*. Sequencing of pCR2.1-*gacS* was performed at Toronto Sick Kids TCAG facility and sequences were analyzed using BLASTN and BLASTX databases.

2.18 *rpoS* nucleotide sequence accession number.

The GenBank accession number for the sequence of the DF41 *rpoS* gene is EU595545.1

2.19 Generation of an *rpoS-lacZ* transcriptional fusion

An 1125-bp segment encompassing the DF41 *rpoS* promoter region and 3′-truncated coding region was cloned into the promoterless *lacZ* transcriptional fusion vector pLP170 (Preston, Seed et al. 1997). To accomplish this, PCR primers containing restriction enzyme sites *Eco*R1 (P170fecorpoS; 5'-tgtgaattcgggagggaca-3') and *Xba*1 (P170rxbarpoS; 5'-agtctagaatcaccacttcccattgctt-3') were used to amplify an 1125-bp product from the pCR2.1-*rpoS* template. The resulting 1125-bp PCR product was digested with *Eco*R1 and *Xba*I and cloned into the same sites of pLP170. The ligation mix was transformed into *E.* coli DH5α and blue-white selection was performed on LB agar supplemented with IPTG, X-gal and 100 μg/mL Amp. Blue colonies were selected for further analysis. Plasmids preps were used to confirm the 1125-bp insertion into pLP170 and the resulting plasmid, p170*rpoS*, was transformed into DF41 and DF41-469 (*gacS*).

2.20 RsmZ overexpression

Plasmid pME6359 carrying the *P. fluorescens* CHA0 *rsmZ* gene (provided by D. Haas) was introduced into DF41, DF41-469 and DF41-1278 by electroporation. Cultures were incubated overnight in 2 mL of LB at 28^{o}C, after which 100 μl aliquots were plated onto LB-Tet15 μg/mL. Introduction of pME6359 into the bacterial strains was confirmed by plasmid extraction and analysis.

2.21 AF assays

Fungal inhibition was detected by spotting 5 μL of an overnight culture onto Potato Dextrose Agar (VWR) 0.5 cm from the edge of the plate. After 24 h incubation at 30^{o}C, a 0.6-cm agar plug containing fungal mycelia was placed into the center of the plate and incubated at room temperature. After 3 days of growth, fungal activity was assessed by measuring the distance between the edges of the bacterial colony and *S. sclerotiorum* mycelium.

2.22 Autoinducer assays

N-acyl-homoserine lactone production was assessed using bioreporter strain *Chromobacterium violaceum* CV026 which produces the pigment violacein in the presence of exogenous HSL. Briefly, indicator plates were made by adding 3 mL of an overnight culture of CV026 to 100 mL of LB agar. The CV026-seeded plates were dried and then spotted with 5 μl of 16-h test cultures and incubated at 30^{o}C overnight. A purple zone surrounding the bacterial colonies indicates violacein production, which results from the presence of exogenous autoinducer.

2.23 Protease activity

Extracellular protease production was determined semi-quantitatively by inoculating 5 μL of an overnight culture onto a 1.5% agar plate supplemented with 1.5% skim milk. Proteolysis was indicated by a clear zone surrounding the bacterial colony after 96 h at 28^{o}C. Protease production was determined quantitatively using the Sigma Universal Protease Assay. Briefly, strains to be tested were cultured for 96 h in either

PTSB or skim-milk media. The cells were removed by centrifugation and the supernatant was assayed for protease activity according to the protocol described on the Sigma website (www.sigmaaldrich.com/life-science/learning-center/life-science-video.htmL). This assay uses casein as a substrate for protease activity. Protease-digestion of casein liberates the amino acid tyrosine which reacts with Folin & Ciocalteu's (F-C) reagent to produce a blue-coloured chromophore (Folin 1927). Chromophore production is measured as an absorbance value using a spectrophotometer and test absorbance values are compared to a standard curve generated by the reaction of free tyrosine with the F-C reagent (Folin 1927; Anson 1938). All reagents were purchased from Sigma-Aldrich, St. Louis, MO.

2.24 Alginate assay

Alginate production was quantified using a modified carbazole assay for assaying uronic acid polymers. DF41 (pUCP23), DF41-1278 (pUCP23), DF41-469 (pUCP23) and DF41-469 (pUCP23-*gacS*) were streaked onto KB agar and incubated at 28^{o}C for 5 days. Alginate was separated from cells by washing each plate with 0.9 % NaCl. Cellular material was removed by centrifugation and the cell pellet washed with 0.9 % NaCl as described by May and Chakrabarty (May 1994). The cell pellet is kept and used to determine the total cell protein by the Bradford method (Bradford 1976). The supernatants were combined and alginate production was quantified using a microassay modification of the Knutson and Jeanes carbazole assay (Knutson and Jeanes 1968). Briefly, a borate stock solution is prepared by dissolving 24.74 mg of H_3BO_3 in 45 mL of 4 M KOH and diluting to 100 mL with distilled water. The borate stock solution is

diluted 1:40 (v/v) with concentrated H_2SO_4 to prepare a working solution which is then equilibrated in an ice-water bath. A 0.1% (w/v) carbazole solution is prepared in absolute ethanol. Keeping all reagents on ice, 70 μL of supernatant containing uronic acids is layered on top of 600 μL of ice-cold borate working solution, vortexed for 4 sec. and 20 μL of carbazole solution is layered on top. Again, the mixture is vortexed for 4 sec. and heated for 30 min. at 55°C to allow for colour development. The absorbance is measured at a wavelength of 530 nm and compared to a standard curve generated by the reaction of alginic acid with the carbazole solution. The quantity of uronic acid present is described as mg per mg cell protein. Boric acid was purchased from Fisher Scientific and carbazole and alginic acid were purchased from Sigma-Aldrich, St. Louis, MO.

2.25 Siderophore production

Siderophore production was qualitatively assayed using the protocol described by Schwyn and Neilands (1987). Briefly, overnight cultures were spotted onto Chrome azurol S (CAS) agar plates and incubated for 16 h at 28^{o}C. Siderophore-mediated iron-chelation is indicated by the presence of an orange zone around the bacterial colony.

2.26 Hydrogen cyanide (HCN) detection and expression of *hcn* genes.

HCN production was detected using cyantesmo paper (Machery-Nagel GnbH & Co., Düren, Germany). Briefly, strains were streaked onto a LB agar plate and incubated for 24 h at 28^{o}C. A 2-inch strip of cyantesmo paper was taped to the inside lid of the petri dish, which was sealed with parafilm and allowed to incubated at 28^{o}C overnight. The presence of a blue stripe on the cyantesmo paper is indicative of HCN production.

To monitor HCN gene expression, β-galactosidase assays were performed using pME3219 (kindly provided by D. Haas) which contains an *hcnA'-'lacZ* translational fusion. DF41, DF41-469 (*gacS*) and DF41-1278 (*clp)* containing pME3219 were grown overnight in Peptone Tryptic Soy broth [PTSB (Ohman, Cryz et al. 1980)]. Cultures were adjusted to an OD_{600nm} = 0.1 and incubated until stationary phase was reached (OD_{600nm}= 2.5-3.0). Cells were solubilized using chloroform and SDS and β-galactosidase activity 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.

2.27 Swimming motility

Swimming (flagellar) motility was assayed on LB plates solidified with 0.3% agar. Overnight cultures were adjusted to an OD_{600nm}= 1.0. A 3-μL aliquot of culture was inoculated below the surface of the agar and the plates were incubated at 28^{o}C. At least ten replicates of each culture were assessed by measuring the diameter of the swim zones at 24 h and 48 h.

2.28 Swarming motility

Swarming motility was monitored on 0.5% peptone, 0.3% yeast extract and 0.5% agar or on KB plates solidified with 0.5 % agar. Swarm media was cooled to approximately 60^{o}C and 25-mL volumes were poured into petri plates and dried for 2 h prior to use. An applicator stick was used to transfer a small amount of bacteria from a 48 h plate to the centre of the swarm plates, which were then incubated at 28^{o}C. A

minimum of 10 plates per strain were inoculated and observed at 48 h and 72 h for swarming motility.

2.29 Biofilm formation

Biofilm formation was assessed by the method described by O'Toole (O'Toole and Kolter 1998). Sstrains were tested for their ability to adhere to the walls of a 96-well polyvinylchloride plastic plate (Becton Dickinson Labware). Strains were grown overnight in 1X M9-minimal salts medium amended with 240 mM glycerol, 1% casamino acids and 1 mM $MgSO_4$. Overnight cultures were diluted 1:100 in fresh medium and a 200-µL aliquot was added to each well of the microtitre plate. After 24 h incubation at 28^{o}C, 25 µL of a 1% Crystal Violet (CV) solution was added to each well and incubated for 15 min. Unbound cells were removed by extensive washing with distilled H_20. A 200-µL aliquot of 95% ethanol was added to each well to solubilize the CV contained in the adherent cell population. The optical density was then measured at a wavelength of 600 nm to quantify biofilm formation.

2.30 Greenhouse assays

DF41 (pUCP23), DF41-469 (pUCP23), DF41-1278 (pUCP23) and DF41-469 (pUCP23-*gacS)* were assessed for their ability to suppress sclerotinia stem rot on canola plants. An overnight culture of bacteria grown in 300 mL of LB broth was washed and resuspended in 1X PBS (pH 7.0) amended with 0.02% Tween 20 to an OD_{600nm}= 2.0. Ten *Brassica napus* (cv. Westar) canola plants per treatment were sprayed with bacteria at the 30% and 50% flowering stages, then incubated overnight in a humidity chamber

(24°C:16°C; 16 h:8 h light:dark regime). The following day, the plants were removed from the humidity chamber sprayed with a *S. sclerotiorum* ascospore suspension of 2 x 10^4 ascospores/mL. The plants were incubated in the greenhouse for fourteen days and scored for the percent leaf incidence and stem rot disease severity. The pathogen control plants were sprayed with 2 x 10^4 ascospores/mL and the healthy control plants were sprayed with 1X PBS buffer amended with 0.02% Tween 20. Disease development was scored on a scale of 0-7 where 0 = no lesions; 1 = lesions present on leaves but not the stem; 2= 1-20 mm stem lesions; 3 = 21-40 mm stem lesions; 4= 41-60 mm stem lesions; 5 = 61-80 mm stem lesions; 6 = 81-100 mm stem lesions; 7, > 100 mm stem lesion or plant death. The percent leaf disease incidence and stem rot disease severity were calculated based on disease. The plant assays were conducted as two separate trials.

2.31 Petal colonization

The ability of DF41 and its derivatives to effectively colonize canola petals was analyzed. Starter cultures were grown in 10 mL of LB broth at 28°C. The next day, the bacteria were subcultured to a starting $OD_{600\ nm}$ = 0.1 in a total volume of 300 mL of LB and allowed to grow overnight at 28°C. Bacteria were washed and resuspended in an equal volume of PBS before spraying onto canola (8 plants/strain). For each test strain, 10 petals were randomly selected from the plants and placed in 1 mL of 1X PBS. Cells were removed from the petals by vigorous vortexing for 30 sec. The bacterial suspensions were serially diluted and plated in triplicate on LB or LB-kan 5 μg/mL using an Autoplate® 4000 Automated Spiral Plater (Spiral Biotech, Inc., Bethesda, MD). After

overnight incubation at 28^{o}C, the CFU/mL was determined using an aCOLyte colony counter (Don Whitley Scientific (Frederick, MD).

2.32 Acyl homoserine lactone extraction

DF41 was grown overnight at 28^{o}C in M9-minimal medium amended with glucose and 0.1 mM $MgSO_4$. The following day, bacteria were subcultured at a starting OD_{600nm}= 0.1 and grown for 30 h at 28^{o}C with aeration (200 RPM). Cells were removed by centrifugation and the cell-free supernatant was extracted once with an equal volume of ethyl acetate acidified with 0.1% glacial acetic acid. The suspension was vigorously shaken for 5 min. and centrifuged at 8000 x g to fully separate the phases. The organic layer was removed and extracted a second time with acidified ethyl acetate. The ethyl acetate layers were pooled and any aqueous material was removed with a spoonful of anhydrous sodium sulfate. The extract was dried to completion under a steady stream of nitrogen, after which the dried material was resuspended in a 1-mL volume of ethyl acetate containing 0.1% acetic acid. The extract was tested for autoinducer activity by spotting 100 µL of extract that had been diluted 1/20, 1/50 and 1/100 times into an empty petri plate and drying the extract to completion in a laminar flow hood before the addition of 15 mL of CV026-seeded LB agar. The plates were incubated at 28^{o}C overnight and observed for the presence of a purple zone in the agar plates which is indicative of autoinducer. The resuspended extracts were stored at -20^{o}C prior to use.

2.33 Addition of exogenous autoinducer

AHL that had been purified as described in section 2.32, was added to DF41 and its derivatives carrying *lacZ* transcriptional fusions to examine the effect of exogenous autoinducer on gene expression. A 1/1000 dilution of purified extract was added to each test tube and the ethyl acetate was allowed to evaporate in the laminar flow hood before addition of the bacterial cultures.

2.34 Cyclic lipopeptide extraction

To purify the bioactive lipopeptide from DF41 and its derivatives, strains were grown for 72 h in 300 mL of M9 minimal media amended with 240 mM glycerol, 200 mM $MgS0_4$ and 1 % Casamino acids. Cells were removed by centrifugation (7000 x g) for 20 min., culture supernatants were acidified to pH 3.0 and extracted twice with an equal volume of ethyl acetate (Fisher Scientific). Ethyl acetate extracts were evaporated to dryness under a steady stream of nitrogen. The flasks containing dried ethyl acetate extracts were then rinsed in succession with a methanol gradient increasing from 20% methanol to 100% methanol. The compound of interest eluted in the 80% methanol wash.

2.35 HPLC separations

100 µL of each culture extract was separated by high-performance liquid chromatography (HPLC) using a C_{18} reverse-phase column (100 x 4.6 mm; 5-µm particle diameter) (Phenomenex Inc, Torrance, CA) and monitored by UV detection at 200 nm and 220 nm using a Varian 335 diode array detector. The HPLC separation consisted of

a linear gradient of 85% eluent A (0.1% phosphoric acid) and 15% eluent B (acetonitrile) at 0 min. increasing to 85% eluent B and 15% eluent A over 40 min. The flow rate was 1.0 mL/min. HPLC-grade solvents were obtained from Fisher Scientific.

2.36 Detection of a biologically active compound from DF41 cell-free supernatants.

Strains DF41, DF41-469 and DF41-1278 were cultured as described in the above sections. 100 µL of each extract was separated by HPLC and collected as 1 min. fractions. Fractions from a minimum of six HPLC elutions were pooled together, dried under nitrogen and tested for inhibitory activity of *S. sclerotiorum.* Briefly, dried fractions were resuspended in 100 µl of 80% methanol and spotted onto glass fibre filters (Whatmann) on a PDA plate. The methanol was evaporated in a laminar flow hood for 30 min. prior to placement of an agar plug containing *S. sclerotiorum* mycelia onto the centre of the plate. The plates were incubated at room temperature for 3 days and observed for fungal inhibition which was defined as the inability of the fungus to grow over the filter. To ensure that methanol was not having an inhibitory effect on fungal inhibition, 100 µL of 80% methanol was also spotted onto a glass filter and tested for *S. sclerotiorum* inhibition.

For detection of biosurfactant activity the dried fractions were resuspended in 100 µL of distilled water and 50 µL droplets were deposited onto a hydrophobic surface (parafilm M). The droplets were visually examined for the ability to reduce surface tension by observing the contact angle of the droplets on the Parafilm M. Sterile distilled water was used as a negative control.

Chapter 3

The role of the GacS/GacA Two-Component

Signal Transduction System in DF41 Biocontrol Activity

3.1 Introduction

Pseudomonas strain DF41 is a canola rhizosphere isolate, discovered during a screening for bacteria capable of inhibiting the growth of the pathogenic fungus *S. sclerotiorum* (Savchuk and Dilantha Fernando 2004). This fungus infects a number of economically important crops such as canola and sunflower. In canola, it is the causal agent of stem rot diseases leading to large lesions on the leaves and stem, eventually culminating in plant death. Sclerotinia-mediated infections are difficult to contain and prevent due to a complex fungal life cycle and the production of small, overwintering bodies called sclerotia which may persist in the soil for 4-6 years post-infection (Manitoba Agriculture 2002). Currently, all of the commercially grown canola cultivars are susceptible to *S. sclerotiorum* and the only methods of disease control, crop rotation and fungicide application, are of limited success due to the persistence of sclerotia in the soil (Nelson 1998). Furthermore, fungicides must be applied prior to the manifestation of disease symptoms which can be costly. These facts together with the public concern over the application of chemical pesticides, has lead to an increasing demand for alternative methods of pathogen control. The discovery that certain soils are naturally suppressive to fungal pathogens has sparked research pursuits into biological control (Schroth and Hancock. 1982; Hornby. 1983; Weller *et al.* 2002).

There are a number of bacterial species exhibiting AF activity due to the production of volatile and non-volatile antibiotics, extracellular enzymes and siderophores which act in concert to inhibit fungal growth and propagation on host plants. The four major antibiotics associated with *Pseudomonas*-mediated biocontrol activity are pyrrolnitrin (Hill 1994), 2,4 -DAPG (de Souza 2003), pyoluteorin (Howell 1980; Laville

et al. 1992) and phenazines (Anjaiah et al. 1998; Haas et al. 2000). When *Pseudomonas* strain DF41 was screened by PCR for genes encoding these antibiotics, PCR products were not obtained for any of these four antibiotics, suggesting that *Pseudomonas* strain DF41 produces alternative AF compounds. Therefore, to identify genes and/ or gene products involved in DF41 biological control activity, random transposon mutagenesis experiments were undertaken. Transposon insertion mutants were phenotypically screened for altered protease, siderophore, acyl homoserine lactone production and AF activity. These methods resulted in the identification of a few interesting mutants, one of which lacked AF and protease activity. Sequence analysis of the regions flanking the Tn*5* insertion revealed that the insertion had occurred in a global regulatory gene, *gacS.*

In fluorescent Pseudomonads, the GacS/GacA two-component signal transduction system is essential for production of secondary metabolites that mediate biocontrol (Heeb and Haas, 2001). GacS is a membrane-associated sensor kinase that is stimulated by a yet-to-be-identified signal resulting in autophosphorylation. The current working model suggests that through phosphotransfer to the response regulator GacA, GacA dimerizes and induces the expression of small regulatory RNAs (Heeb and Haas, 2001; Haas and Défago, 2005). These RNAs are believed to function by alleviating translational repression of RNA-binding proteins (Heeb et al. 2002).

The role of the GacS/GacA system in DF41 biocontrol activity was analyzed by observing the effect of the *gacS* mutation on a number of factors such as; extracellular enzyme production, motility, biofilm and alginate production and *in planta* inhition of *Sclerotinia* stem rot on canola plants.

3.2 Results

3.2.1 Isolation of *Pseudomonas* DF41 mutants deficient in AF activity

To identify molecular mechanisms underlying the biocontrol activity of *Pseudomonas* sp. DF41, Tn*5* mutagenesis was performed. Approximately five thousand mutants were screened in radial diffusion assays for altered AF activity. One mutant, DF41-469 exhibited significantly decreased inhibition of the fungal pathogen *S. sclerotiorum* (Fig. 3.1).

Sequence analysis of the chromosomal DNA flanking the Tn*5* insertion showed >90% identity to the *gacS* genes of annotated *Pseudomonas* species, including *P. fluorescens* strains 2P24 [accession # AY623898] and PfO-1 [accession # NC007492]. Transfer of the wild-type *gacS* gene into DF41-469 restored AF activity (Table 3.1), confirming that the DF41-469 phenotype results from inactivation of *gacS.* The increased level of AF activity in DF41-469 (pUCP23-*gacS*) is likely due to a gene dosage effect.

3.2.2 Verification of a single transposon insertion using Southern analysis

Southern analysis was performed to confirm that a single transposon insertion event had occurred in DF41-469. DF41 and DF41-469 (*gacS*) genomic DNA was digested with *Eco*R1. Hybridization using a DIG-labelled kanamycin cassette from Tn*5*-1063 showed a single probe-reactive band for DF41-469. As expected, DF41 genomic DNA did not react with the probe (Fig. 3.2). The uncut plasmid harbouring Tn*5*-1063 was used as a positive control to test the efficiency of the Southern hybridization.

Figure 3.1 ***In vitro*** **inhibition of** ***Sclerotinia sclerotiorum*****.** *Pseudomonas* strain DF41 and Tn*5* derivatives were spotted onto PDA plates and incubated at 30°C for 24 h prior to the addition of an agar plug containing fungal mycelia to the centre of the plate. A and C, *Pseudomonas* DF41; B, DF41-469 (*gacS*); D, DF41-469 (pUCP23-*gacS*).

Table 3.1 Phenotypic characteristics of *Pseudomonas* sp. DF41, Tn*5* mutant DF41-469, and *gacS* complemented strain DF41-469 (pUCP23-*gacS*).

Strain	AF[a]	Autoinducer[a]	Protease[a]	Alginate[b] (μg/mg protein)	HCN[c]
DF41 (pUCP23)	7.1 (0.9)	10.7 (0.5)	5.8 (0.7)	568 (52)	+
DF41-469 (pUCP23)	0 (0.0) [d]	8.0 (0.5) [e]	0.0 (0.0[d]	118 (16) [e]	-
DF41-469 (pUCP23-*gacS*)	8.4 (0.7) [f]	9.3 (0.5) [f]	7.3 (0.7)[g]	582 (13)[g]	+

[a] Mean (SD) of the zones of activity (mm) from at least six replicates.
[b] Alginate concentration determined from cells grown on KB agar for 120 h; mean (SD) from three replicates.
[c] Determined using Cyantesmo paper.
[d] Significantly different from the wild type ($p < 0.0001$).
[e] Significantly different from the wild type ($p < 0.001$).
[f] Significantly different from the wild type ($p < 0.05$).
[g] Not significantly different from the wild type.

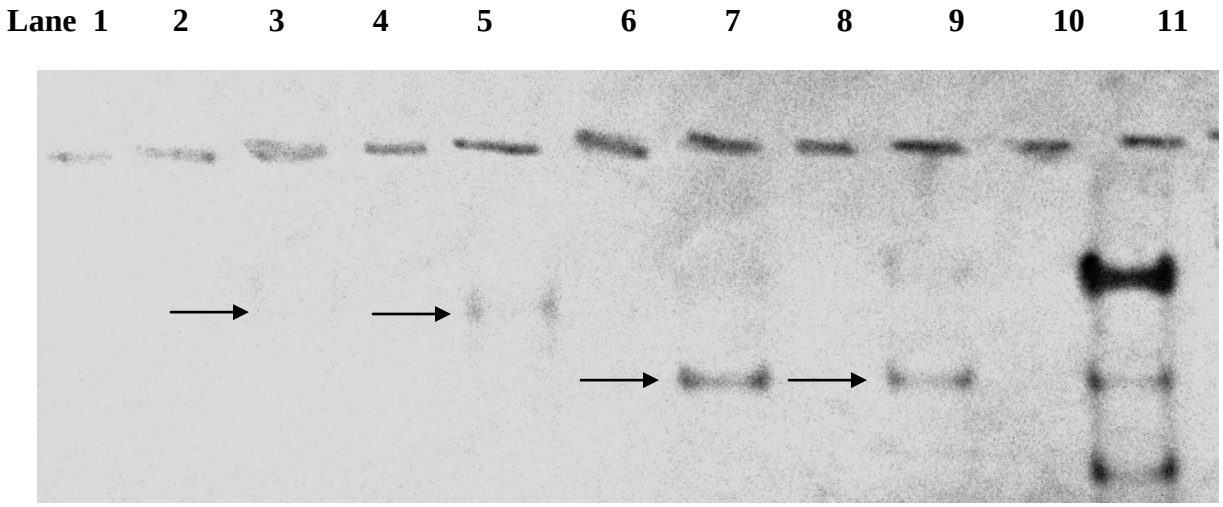


Figure 3.2 Southern blot analysis of DF41 and Tn*5*-insertion mutants confirming a single Tn*5* insertion. Genomic DNA was digested with *Eco*R1 and *Eco*RV and probed with a DIG-labelled kanamycin cassette from pRL1063a. Lane 1, DF41 genomic DNA cut with *Eco*R1; Lanes 3 and 5, DF41-469 genomic DNA cut with *Eco*R1 and *Eco*RV respectively; Lane and 7 and 9, DF41-1278 (an additional Tn*5* mutant with an insertion in a gene producing an AF compound) genomic DNA cut with *Eco*R1 and *Eco*RV; Lane 11, uncut pRL1063a (positive control). Lanes 1,2,4,6,8 and 10 were left empty.

3.2.3 Phenotypic analysis of *Pseudomonas* DF41 and DF41-469

Many biocontrol bacteria produce compounds that act together with antibiotics to synergistically increase their efficacy. Production of these molecules can be under control of a population density-dependent signaling mechanism known as QS. We tested wild-type DF41 as well as DF41-469 (*gacS*), and DF41-469 (pUCP23-*gacS*) for production of siderophore, lipase, protease, and autoinducer molecules. The results of our analyses are summarized in Table 3.1. We were unable to detect lipase production by any of the four strains. Iron-chelating activity was observed on CAS plates by all strains tested, however, the *gacS* mutant formed a larger zone of iron chelation compared to the wild type, suggesting elevated siderophore production by this mutant (Fig 3.3). The introduction of the wild-type allele into DF41-469 (*gacS*) decreased siderophore production to wild-type levels. On skim milk agar, a clear zone of proteolysis was observed surrounding the DF41 and *gacS* complemented mutant DF41-469 (pUCP23-*gacS*) colonies, indicating these strains are positive for protease production. DF41-469 (*gacS*) on the other hand, was protease deficient (Fig. 3.4). The biosensor strain CV026 is unable to produce acyl homoserine lactone (AHL) molecules due to a mutation in the AHL synthase gene (Fig. 3.5). Exogenous AHLs in the C4- to C8- size range complement this mutation resulting in production of the QS-controlled purple pigment violacein. Agar plates seeded with CV026 revealed that DF41 produced an AHL capable of restoring violacein production (Table 3.1). For DF41-469 (*gacS*), a noticeable decrease in AHL production was consistently observed; whereas production was restored to near wild-type levels when *gacS* was added *in trans* (Table 3.1). Collectively, our

Figure 3.3 CAS media depicting siderophore production by *Pseudomonas* DF41. An orange halo surrounding the bacterial colony indicates siderophore production. A and C, *Pseudomonas* DF41; B, DF41-469 (*gacS*); D, DF41-469 (pUCP23-*gacS*).

Figure 3.4 Protease production by *Pseudomonas* DF41 and derivatives. Bacteria were spotted onto 1.5% skim milk agar plates and examined for hydrolysis (clearing) as a result of extracellular protease production. A and C, *Pseudomonas* DF41; B, DF41-469 (*gacS*); D, DF41-469 (pUCP23-*gacS*).

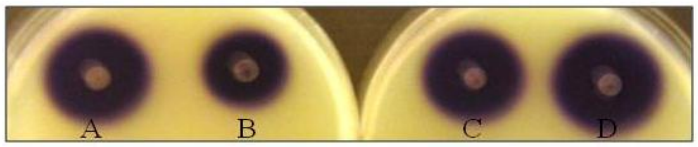


Figure 3.5 Acyl-homoserine lactone production assayed using *Chromobacterium violaceum* seeded plates. Violacein (as indicated by the formation of a purple zone) is restored due to AHL production by *Pseudomonas* strain DF41. A and C, *Pseudomonas* DF41; B, DF41-469 (*gacS*); D, DF41-469 (pUCP23-*gacS*).

findings indicate that DF41 secretes protease and AHL molecules, with the former being tightly regulated by the Gac system.

3.2.4 Hydrogen cyanide expression

Using cyantesmo paper, which is specific for the detection of HCN, strain DF41 was found to produce this volatile antibiotic; whereas DF41-469 (*gacS*) does not (Tables 3.1 and 3.2). Complementation with pUCP23-*gacS* restored HCN production in the *gacS* mutant (Table 3.1). Expression analysis using a *hcnA-lacZ* translational fusion further substantiated these findings. A four-fold decrease in *hcnA* expression was observed in the *gacS* mutant (486 ± 76 Miller units) compared to DF41 (2,194 ± 145 Miller units). Although a definitive role for HCN in the biocontrol activity of DF41 has not yet been established, it is evident that expression of this compound is dependent on GacS.

3.2.5 Effect of a *gacS* mutation on DF41 motility

Because motility can impact biocontrol, swimming and swarming motility were examined in *Pseudomonas* DF41, DF41-469 (*gacS*) and the *gacS*-complemented mutant. There was little difference in the swimming ability of these strains, with the exception of the *gacS* mutant, which showed increased swimming at both 24 and 72 h (Table 3.3). Because of the irregular pattern of swarming, quantitative analyses were not performed. While DF41 swarming could be characterized as fork-like tendrils extending out from the colony that began to form at approximately 24 h, the *gacS* mutant consistently failed to swarm yet did form a colony at the inoculation point (Fig. 3.6).

Table 3.2 Expression of an *hcnA-lacZ* translational fusion in DF41 and DF41-469.

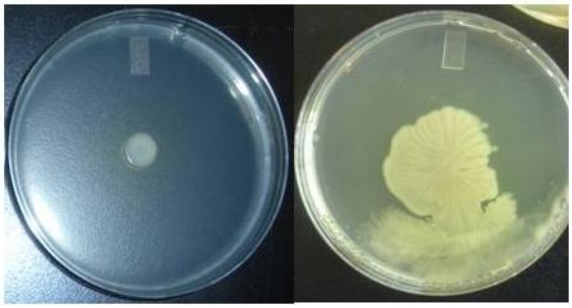
Strain[a]	Genotype	β-Galactosidase activity (Miller Units)[b]
P. fluorescens Pf-5 (pME3219)	Wild-type	10361 (1023)
DF41 (pME3219)	Wild-type	2711 (294)
DF41-469 (pME3219)	*gacS*:: Tn5-1063	486 (76)

[a] Strains harbouring pME3219 were grown overnight to an OD_{600} of 1.5-2.0 and tested for β-galactosidase activity.
[b] Values are the mean and standard deviation from three replicates.

Table 3.3 Flagellar (swimming) motility of *Pseudomonas* sp. DF41, Tn*5* mutants DF41-1278 and DF41-469, and *gacS* complemented strain DF41-469 (pUCP23-*gacS*).

Strain	Swim Zone Diameter (mm) [a]	
	24 h	72 h
DF41 (pUCP23)	20.5 (1.0)	47.0 (2.6)
DF41-469 (pUCP23)	25.5 (1.1) [c]	56.4 (2.2) [c]
DF41-469 (pUCP23-*gacS*)	19.2 (4.8) [b]	49.4 (7.2) [b]

[a] Mean (SD) from five replicates.
[b] Not significantly different from the wild type.
[c] Significantly different from the wild type ($p < 0.001$).



A B

Figure 3.6 Swarming motility on King's B amended with 0.5% agar by *Pseudomonas* DF41 and DF41-469 (*gacS*). A, DF41-469 (*gacS*); B, DF41

3.2.6 The role of GacS in alginate production

We have observed that DF41 develops a mucoidy phenotype when grown on King's B agar; whereas the *gacS* mutant does not. These findings suggest that DF41 is producing alginate in a GacS-dependent manner. We discovered that DF41 does secrete alginate and production of this exopolysaccharide is markedly decreased in the *gacS*-minus strain (Table 3.1). As expected, parental levels of alginate were observed for the complemented *gacS* mutant.

3.2.7 Biofilm formation by *Pseudomonas* DF41 and DF41 derivatives

Using a highly reproducible 96-well PVC microtitre plate assay (O'Toole and Kolter. 1998), the ability of DF41 and its derivatives to form biofilms was examined under minimal nutrient conditions. We discovered equivalent biofilm formation by the wild type strain (OD_{600} = 2.17 ± 0.25), the *gacS* mutant (OD_{600} = 2.24 ± 0.17), and the *gacS* complemented mutant (OD_{600} = 2.22 ± 0.32). Therefore, it appears that the Gac system does not influence DF41 biofilm formation (Table 3.4).

3.2.8 GacS is essential for biocontrol of *S. sclerotiorum*

DF41, DF41-469 (*gacS*) and the complemented *gacS* mutant were evaluated for their ability to inhibit *S. sclerotiorum* mediated stem rot disease symptoms on canola plants. The incidence of leaf infection and disease severity were evaluated. In greenhouse studies, DF41 provided significant protection against fungal infection of the canola leaves and considerably reduced disease severity (Fig. 3.7). In contrast, the *gacS* mutant showed little antagonism toward *S. sclerotiorum* (Fig. 3.7). Addition of *gacS* in

Table 3.4 Biofilm Formation by DF41 (pUCP23), DF41-469 (pUCP23) and DF41-469 (pUCP23-*gacS*).

Strain [a]	Genotype	OD_{600} [b]
DF41 (pUCP23)	Wild-type	2.17 (0.25)
DF41-469 (pUCP23)	*gacS*:: Tn5-1063	2.24 (0.17)
DF41-469 (pUCP23-*gacS*)	*gacS*:: Tn5-1063, *gacS* complemented strain	2.22 (0.32)

[a] Strains were grown overnight in 96-well plates to OD_{600} of 1.5-2.0 and assayed for biofilm formation with crystal violet staining.
[b] Values are the mean and standard deviation from three replicates.

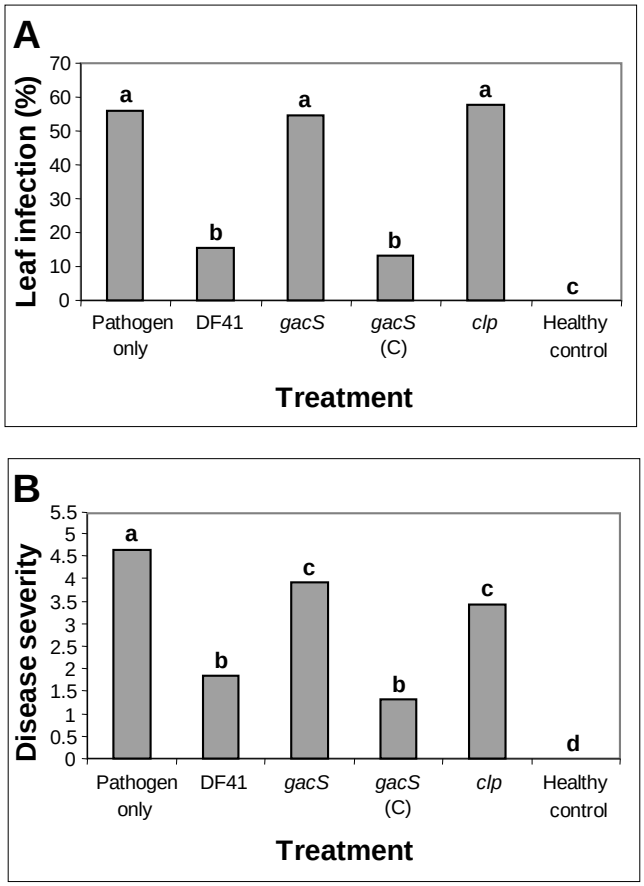


Figure 3.7 Efficiency of *Pseudomonas* sp. DF41 (wild type), *gacS*$^-$ (DF41-469), *gacS* complemented (C) [DF41-469 (pUCP23-*gacS*)], and *clp*$^-$ (DF41-1278) strains in managing *Sclerotinia sclerotiorum* ascospore infection on canola plants. Panel A: percent incidence of leaf infection. Panel B: disease severity on stem. In all treatments except the healthy control, plants were sprayed with *S. sclerotiorum* ascospores. The healthy control plants were sprayed with phosphate buffer. Column means labelled with the same letter do not differ significantly by Duncan's Multiple Range Test (DMRT; P>0.05).

trans restored DF41-469 disease suppression to wild-type levels. Because Gac controls production of secondary metabolites, it is not surprising that a functional Gac system is required for disease suppression.

3.2.9 The colonization of canola petals by DF41 and its derivatives

DF41, DF-41-469 (*gacS*) and the *gacS*–complemented strain were tested for their ability to colonize canola petals over a period of 5 days. All of the strains tested displayed similar rates of colonization and persistence during the course of the sampling period. Based on this observation, we concluded that the loss of biocontrol exhibited by the *gacS*-minus strain was not due to an inability to establish and maintain sufficient population size on the canola phyllosphere (Fig. 3.8).

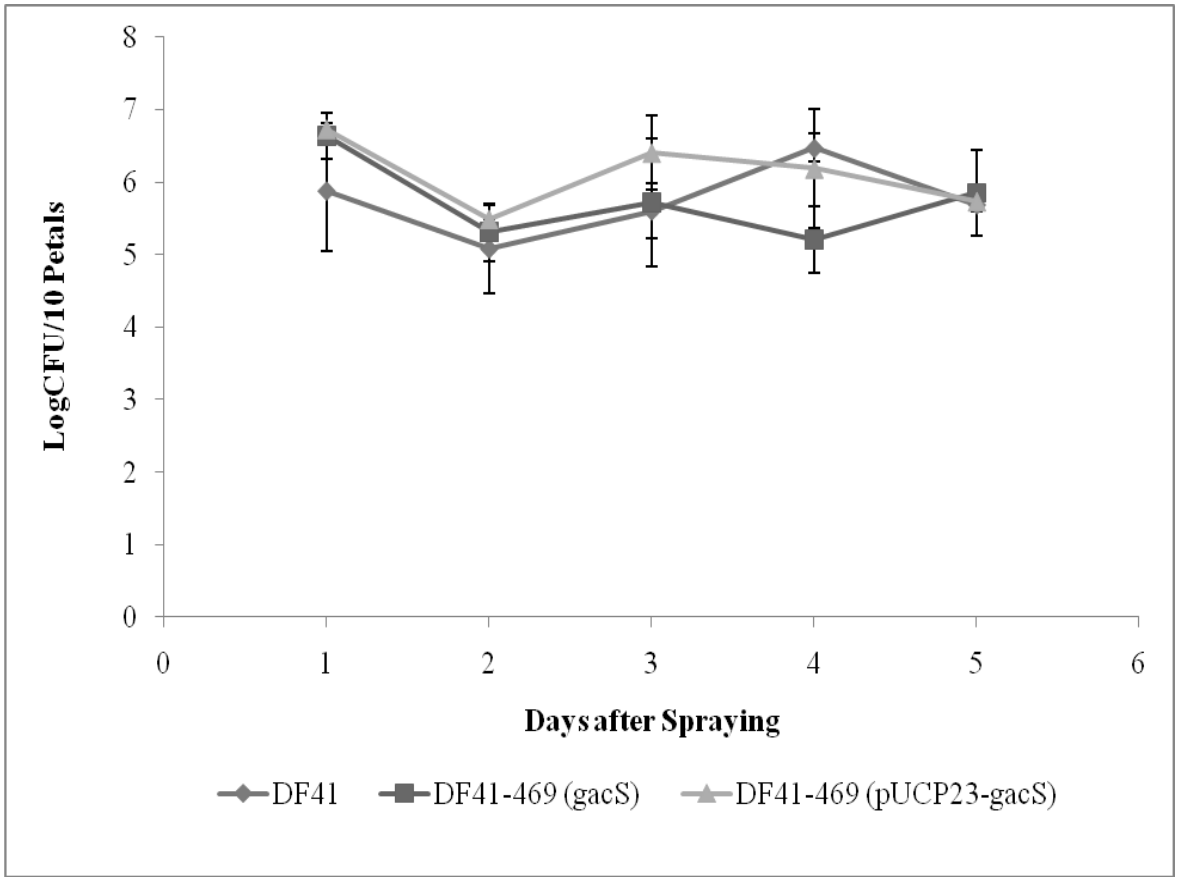


Figure 3.8 Sustainability of *Pseudomonas* DF41, DF41-469 (*gacS*) and DF41-469 (pUCP23-*gacS*) on canola petals. Overnight cultures of each strain were sprayed onto canola petals at a starting OD_{600}=1.5. 10 petals were randomly selected from plants, the bacteria were removed from the petals by vigorous vortexing in 1X PBS and plated onto agar plates supplemented with selective antibiotics for determination of bacterial titres.

3.3 Discussion

Pseudomonas sp. DF41, originally isolated from the phyllosphere of canola plants, has consistently demonstrated strong antagonism of Sclerotinia stem rot of canola (Savchuk and Fernando, 2004, Savchuk 2002). Neither Biolog^{TM} analysis nor 16S rDNA sequencing have enabled a species designation for DF41 (Zhang et al., 2006). Thus, we believe DF41 may represent a novel species of *Pseudomonas*. Through screening of a Tn*5* library, a mutant was isolated that exhibited a complete loss of AF activity against *S. sclerotiorum*. This mutant, designated DF41-469, had a Tn*5* insertion in a gene with >90% homology to the *gacS* gene of *P. fluorescens* 2P24 and PfO-1.

A comparison between the wild type and a *gacS*$^{-}$ strain provided insight into secondary metabolites and bacterial characteristics contributing to DF41 biocontrol activity. We tested DF41 for biocontrol-associated traits and discovered that this bacterium is producing alginate, HCN, protease, and an AHL signalling molecule. The *gacS*-mutant displayed significant decreases in production of the first three aforementioned products. A modest decrease in AHL signal production was observed in the GacS-minus background, suggesting the two regulatory systems are linked in some manner.

An effective biocontrol bacterium must not only be capable of *in vitro* inhibition and AF compound production but must also be able to overcome the harsh environment of the phylloplane. For example, bacterial motility enables bacterial migration to and subsequent colonization of nutrient-rich niches. Not only can motility assist in sustaining bacterial survival on the plant, but it may also contribute to efficient containment of fungal pathogens. Two modes of bacterial motility were tested, swimming or flagellar

motility and swarming motility. DF41 was found to swim on low-percentage agar plates as was DF41-469. The rate of swimming motility by the *gacS* mutant, however, was increased compared to the wild type. Previously, a *gacA* mutant of *P. fluorescens* F113 demonstrated an increase in both *fliC*-encoded flagellin expression and swimming motility (Sanchez-Contreras et al., 2002). In a later proteomics study, a *P. aeruginosa gacA* mutant showed elevated levels of flagellin (FliC) and flagellum capping protein (FliD) (Kay et al., 2006). Therefore, increased swimming and upregulated flagellar protein expression may be common phenotypes associated with *Pseudomonas gac* mutations. In terms of swarming motility, DF41 and the complemented *gacS* mutant exhibited similar swarming on King's B plates solidified with 0.5% agar. The *gacS* mutant in contrast was unable to swarm. Swarming motility is believed to involve flagellar motility and surfactant production. Since DF41-469 (*gacS*) swims at a higher rate than the wild type, yet is deficient in swarming motility, it is unlikely that the swarming defect results from impaired swimming or flagellar motility. Altered expression of other factors which can facilitate motility such as biosurfactant production, likely accounts for the loss of swarming motility observed in the *gacS* mutant.

In nature, the bulk of bacterial biomass exists as an adherent multicellular community encased in an extracellular matrix collectively called a biofilm (Geesey *et al.* 1977). There are many advantages of adopting the biofilm mode of growth in the plant environment. Biofilm bacteria are sheltered from environmental stresses including desiccation, ultraviolet radiation, changes in humidity, and they are afforded protection from grazing predators (Ramey et al., 2004). Studies have shown that the Gac system is important for biofilm formation in various *Pseudomonas* species (Anderson et al., 2005;

Parkins et al., 2001 Poritsanos et al., 2006) prompting us to investigate whether the same would hold true for DF41. Interestingly, it was discovered that the wild type and the *gacS*-minus DF41-469 are both able to form thick biofilms in a static plate assay. In *P. syringae* B728a, GacS is required for alginate production (Willis et al., 2001). A similar finding was observed in this study; the DF41 *gacS* mutant produced over four-fold less alginate than the parent (Table 3.1). If alginate forms part of the DF41 biofilm matrix, the residual level of alginate secreted by DF41-469 appears to be sufficient to form biofilms comparable to wild type.

In greenhouse assays, DF41-469 (*gacS*) showed decreased biocontrol of Sclerotinia stem rot of canola compared to the wild type. Both the incidence of leaf infection (Fig. 3.7a) and disease severity (Fig. 3.7b) were markedly increased, leading to the conclusion that the GacS/GacA system is essential for DF41 control of *Sclerotinia*. Survival of DF41 and DF41-469 (*gacS*) and the *gacS*-complemented strain were tested by determination of bacterial titres on the canola petals over a period of five days. No significant differences in bacterial numbers were observed, indicating that at least under the conditions tested, there were no differences in phylloplane persistence between the three strains. The lack of biocontrol activity by the *gacS* mutant must be due to a lack of AF compound production rather than reduced viability on the plant surface.

In summary, strain DF41 produces an array of extracellular metabolites that may contribute to plant disease suppression. The Gac system is required for the production of a number of secondary metabolites which are required for efficient biocontrol activity of *S. sclerotiorum*.

Chapter 4

The elucidation of regulatory mechanisms and their role in controlling the production of secondary metabolites contributing to DF41 AF activity

4.1 Introduction

Gene expression in *Pseudomonas* is multi-faceted and a plethora of regulatory elements have been identified that control gene expression at transcriptional, translational or post-translational levels. A prominent global regulator in several *Pseudomonas* species is the GacS/GacA two-component signal transduction system which post-transcriptionally regulates gene expression. GacA controls the activation of small, untranslated RNA by interaction with promoter elements. The Gac/Rsm mechanistic link was first recognized in *P. fluorescens* CHA0 (Blumer et al. 1999; Aarons et al. 2000) where it is essential for the expression of several secondary metabolites required for suppression of soil-borne fungal pathogens. In this bacterium, GacA activates the synthesis of three RNA molecules called RsmX, RsmZ and RsmY (Kay et al. 2005). These functionally redundant RNAs relieve translational repression of target genes by capturing multiple copies of the mRNA binding proteins RsmA and RsmE. RsmA and RsmE contain a RNA-binding motif that enables them to bind to the Shine-Dalgarno site as well as additional sites in the 5'-untranslated leader region of target mRNA. RsmA/E binding blocks the translational machinery from accessing the RBS, which in turn prevents protein synthesis. Rsm homologues have been described in numerous other Gram-negative bacteria including the plant pathogens *E. carotovora* subsp. *carotovora* (Chatterjee et al. 1995; Cui et al. 1995), and *P. syringae* pv. tomato (Chatterjee et al. 2003), and the human opportunistic pseudomonad, *P. aeruginosa* (Pessi et al. 2001; Heurlier et al. 2004).

RsmA can also act as a positive regulator by promoting translation and increasing mRNA stability. For example, RsmA acts as both a negative regulatory element and a

positive regulator in *P. aeruginosa* PAO1 (Heurlier et al. 2004). Although the production of several PAO1 virulence factors are positively regulated by the Gac/Rsm cascade, swarming motility, together with rhamnolipid and lipase production are abolished in a *rsmA* mutant, indicating that these traits are positively regulated by RsmA (Heurlier et al. 2004).

Additional complexity of the Gac/Rsm system occurs at the level of interaction with downstream regulatory elements, such as QS. QS is a mechanism that enables bacteria to coordinate gene expression with increasing population density. QS systems typically consist of a *luxR* gene and a *luxI* gene. *luxI* encodes an AHL synthase enzyme responsible for synthesis of an AHL-signalling molecule. At high cell densities, the AHLs complex with a cognate transcriptional activator, encoded by the *luxR* gene. AHL-mediated signalling pathways have been identified in many gram-negative bacteria including a number of *Pseudomonas* species. The most extensively studied *Pseudomonas* QS-system is that of *P. aeruginosa* where a hierarchy of two AHL-based QS systems control virulence factor production and biofilm formation (Winson et al. 1995; de Kievit and Iglewski 1999). A number of plant-associated pseudomonads including strains of *P. fluorescens, P. putida* and *P. chlororaphis* have been found to employ QS (Bertani and Venturi 2004; Yan et al. 2009). In these strains, QS controls a multitude of traits. For example, compounds underlying AF activity, such as phenazine production by *P. chlororaphis* (Pierson et al. 1994) or plant pathogenic activity, such as viscosin production by *P. fluorescens* 5064 (Cui et al. 2005) are QS regulated. Conversely in other strains, QS does not govern expression of extracellular enzymes or secondary metabolites but contributes to bacterial motility and epiphytic fitness (Wei and

Zhang 2006). Links between QS and the Gac/Rsm cascade have been observed in various pseudomonads. In *P. aeruginosa,* GacA activation of RsmZ is essential to alleviating RsmA repression of AHL signals. Furthermore, the biological activity of the plant-associated strains *P. fluorescens* 2P24 and *P. chlororaphis* is also partially dependent on GacA activation of AHL molecules (Wei and Zhang 2006; Yan et al. 2009).

Another downstream global regulator in the Gac/Rsm cascade is the stress or stationary- phase sigma factor, RpoS. In *E. coli* RpoS promotes the expression of genes in response to starvation and oxidative stress, whereas in *Pseudomonas,* RpoS additionally directs the transcription of genes required for antibiotic biosynthesis and extracellular enzyme production. In *P. fluorescens* Pf-5, an *rpoS* mutant demonstrated reduced survival under oxidative stress and altered secondary metabolite production (Whistler et al. 1998). In *P. aeruginosa* and *P. fluorescens* CHA0, expression of RpoS is subject to Gac/Rsm control although the extent of Gac/Rsm regulation varies (Heeb et al. 2005). For example, in *P. fluorescens* Pf-5 *gacA*/*gacS* mutants, RpoS levels ranged from 20% in exponentially growing cells to a maximum of 50% of wild-type levels at the entry into stationary phase (Whistler et al. 1998). Further complicating the role of RpoS in global regulation is its corresponding control by other regulators. For example, QS and RpoS have been found to mutually regulate one another's expression and have overlapping regulons (Latifi et al. 1996; Whiteley et al. 2000; Wagner et al. 2003; Schuster and Greenberg 2007).

We have established that a GacS/GacA system controls secondary metabolism in DF41, which led us to postulate that its activity is being mediated via an RsmZ homolog.

The aim of Chapter 4 is to investigate the roles of RsmZ, RpoS, and QS in DF41 biocontrol. In addition, the interaction between these three regulators is examined.

4.2 Results

4.2.1 Identification of *rsmZ* in *Pseudomonas* sp. DF41

Since the Gac/Rsm regulatory cascade is present in a number of Gram-negative bacteria, including plant-associated *Pseudomonas* strains, it is reasonable to assume that a similar regulatory hierarchy may be overseeing secondary metabolite production in *Pseudomonas* sp. DF41. In a number of *Pseudomonas* strains, the *rsmZ* gene lies immediately downstream of the gene encoding the stationary phase sigma factor, *rpoS*. Therefore, degenerate PCR primers were designed using annotated *rpoS* and *rsmZ* gene sequences to isolate the *rsmZ* gene from DF41. These primers successfully amplified a 1.0-kb product encompassing the 3'-end of *rpoS*, the *rpoS-rsmZ* intergenic region and the entire *rsmZ* gene. BLASTN analysis of the sequenced product identified a 122-nt sequence downstream of *rpoS* with significant homology to *rsmZ* genes from a number of other *Pseudomonas* species (Fig. 4.1). The DF41 *rsmZ* gene shares the highest level of identity with that of *P. fluorescens* 2P24 (96%) and has 83% sequence identity with the *P. fluorescens* CHA0 *rsmZ.* Analysis of the 122-bp sequence using the RNA-fold program for predicting secondary structure revealed several stem-loop structures containing unpaired AGG(G)A motifs that are believed to be essential for RsmZ titration of RsmA. The +1 transcriptional start site was also identified along with a putative *rho*-independent termination region (Fig. 4.1).

4.2.2 Identification of the DF41 QS system

AHL production can be readily detected using bioreporter strains which respond to exogenous AHL. In this study, the biosensor strain *C. violaceum* CV026 was used. Strain CVO26 carries a mutation in the gene encoding the AHL synthetase so the cells

A.

5'-**t**gtcgac**gga**cagacacacccgtcac**gga**cgatgggaaggaaggacatcgca**gga**cgcgattcatca**gga**cgatgaat

aggattaaag**gga**ttag**gga**aaatggggcggtcaaaaaaggcga-3'

B.

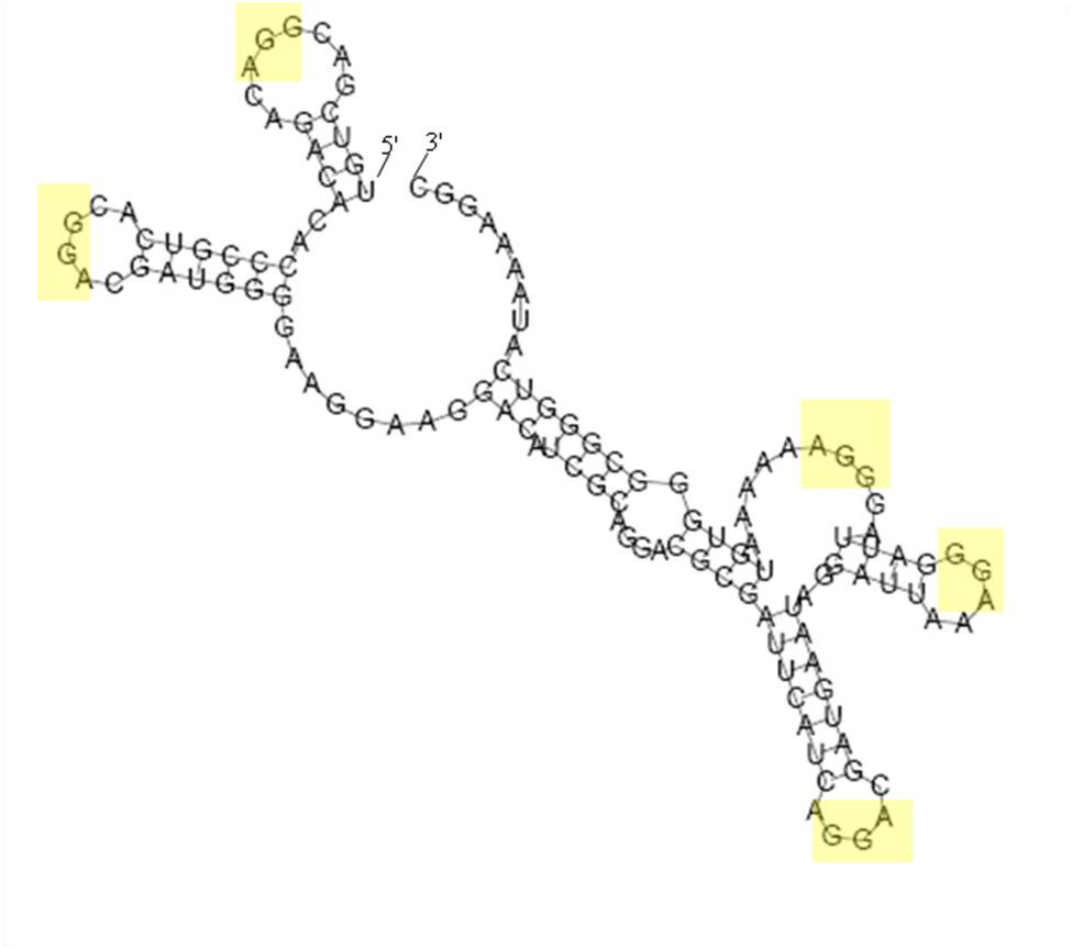


Figure 4.1 DF41 *rsmZ* sequence and RNA-fold prediction of RsmZ secondary structure depicting unpaired AGG motifs and stem-loop structures formed at 30°C with a minimum free energy of -90.90 kcal/mol. A; DF41 RsmZ promoter region and gene sequence cloned into pCR2.1. The putative transcriptional start site is underlined and shown in bold-face. B; Predicted RsmZ secondary structure using the free RNAfold software available at http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi

are unable to produce the QS-controlled pigment violacein and retain a whitish colour in the absence of exogenous AHL. However, in the presence of AHL molecules containing acyl chain lengths between 4 and 8 carbons, violacein production is restored and the cells become purple (McClean *et al.* 1997). DF41 was streaked in close proximity to CV026 on L-agar plates and the plates visualized for violacein production after overnight incubation at 28^{o}C. DF41 restored violacein production indicating that AHLs are being produced. To isolate the DF41 *luxI* gene, a genomic fosmid library was patched onto CV026-seeded L-agar plates and incubated overnight. A single fosmid clone able to restore violacein production was identified. The fosmid DNA was partially digested with *Sau*3A and cloned into *Bam*H1-digested pBluescript SK^{-}. One clone harbouring a 3.2-kb insert was capable of restoring violacein production. BLASTN analysis of the 3.2-kb sequence revealed an ORF with 88% sequence identity to the *P. corrugata pdfI* quorum-sensing gene (accession no. AF199370.1). The DF41 QS system consists of a 772-bp AHL synthetase gene designated *pdfI* (accession no. HM590003).

4.2.3 Identification of *rpoS* in *Pseudomonas* sp. DF41

To ascertain the role of RpoS in the regulatory cascade controlling DF41 biocontrol, *rpoS* was isolated through PCR amplification. Using PCR primers specific to the *P. chlororaphis* PA23 *rpoS* gene, a 1.3 kb product was amplified from DF41. The 1.3 kb PCR product was cloned into TOPO vector pCR2.1 and sequenced using the oligonucleotide universal primers, M13 forward (5'-gtaaaacgacggccag-3') and M13 reverse (5'-caggaaacagctatgac-3'). Sequence analysis confirmed the presence of an ORF encoding a protein of 335 amino acids that shares 98% identity with the RpoS of *P.*

fluorescens 2P24 (accession no. ABQ65863), 97% identity with the RpoS of *P. fluorescens* PfO1 (accession no. YP_346864) and 96% identity with the RpoS of *P. chlororaphis* PfO-1 (accession no. CP000094.2). The DF41 *rpoS* sequence has been entered into the NCBI database (accession no. EU595545.1).

4.2.4 Effect of RsmZ overexpression on biocontrol-associated phenotypes.

Plasmid pME6359 harbouring the *P. fluorescens* CHA0 *rsmZ* gene under the control of the inducible *tac* promoter was introduced into DF41 and DF41-469 (*gacS*). We subsequently examined the effect of multiple copies of RsmZ on secondary metabolites contributing to the AF activity of DF41. We were foremost interested in determining whether RsmZ overexpression could suppress a *gacS* mutation as has been reported for *P. fluorescens* CHA0 Gac-mutants (Heeb et al. 2002). In the *in vitro* AF assays, we observed that surplus copies of RsmZ partially restored AF activity to the GacS-mutant, DF41-469. However, the zones of inhibition were only one third those of the wild type (Table 4.1).

In *P. fluorescens* CHA0, protease production is controlled by the Gac/Rsm regulatory pathway (Blumer et al. 1999). Therefore, we tested DF41 (pME6359) and DF41-469 (pME6359) for altered protease levels. In DF41 (pME6359), protease levels were increased 2-fold compared to the wild type. Similar to the results reported for *P. fluorescens* CHA0 *gac*-mutants, *rsmZ* overexpression complemented protease production by the DF41 *gacS*-mutant to wild-type levels (Table 4.1).

To determine if RsmZ mediates AHL synthesis in DF41, AHL production in DF41 and DF41-469 (*gacS*) was assayed using CV026-indicator plates. AHL production

Table 4.1 Phenotypic characteristics of *Pseudomonas* sp. DF41 and derivatives harbouring the *rsmZ*-overexpression plasmid pME6359.

Strain	Relevant Genotype	AF[a]	Autoinducer[a]	Protease[a]	HCN[b]
DF41 (pME6032)	Wild type	7.8 (0.8)	8.2 (0.7)	5.2 (0.5)	+
DF41 (pME6359)	Wild type, *rsmZ* overexpression	8.0 (0.6) [c]	9.3 (0.3) [e]	10.2 (0.4) [f]	+
DF41-469 (pME6032)	*gacS*$^-$	Overgrown	4.6 (0.5)	0.0 (0.0)	-
DF41-469 (pME6359)	*gacS*$^-$, *rsmZ* overexpression	2.8 (0.3) [f]	7.8 (1.2) [e]	5.6 (0.9) [f]	+

[a] Mean (SD) of the zones of activity (mm) from at least five replicates.
[b] Determined using Cyantesmo paper.
[c] Not significantly different from the wild type.
[d] Significantly different from the strain carrying the empty vector ($p < 0.05$).
[e] Significantly different from the strain carrying the empty vector ($p < 0.001$).
[f] Significantly different from the strain carrying the empty vector ($p < 0.0001$).

in DF41 is not entirely abolished in a *gacS* mutant, but is significantly decreased. Overexpression of RsmZ in the *gacS* mutant restored AHL production to those of wild type and also elevated AHL levels in the DF41 parent strain (Table 4.1).

4.2.5 RsmZ has pleiotropic effects on DF41 motility

Swarming and swimming motility were determined on low-percentage agar KB plates. As described in chapter 3, a GacS deficiency in DF41 results in increased flagellar motility (swimming) and a complete loss of swarming motility. However, when multiple copies of RsmZ were introduced into DF41-469, swarming motility was restored (Fig. 4.2). In contrast, swimming motility in all of the strains tested was decreased when RsmZ was provided in surplus (Table 4.2). These results indicate that RsmZ positively controls swarming motility and represses flagellar motility in DF41.

4.2.6 RsmZ is part of the GacS/GacA regulatory cascade

To determine if RsmZ is a downstream regulatory element of the GacS/GacA regulon in DF41, a promoterless transcriptional fusion vector carrying the *P. chlororaphis* PA23 *rsmZ* promoter was introduced into DF41 and DF41-469 (*gacS*). Not surprisingly, expression of the *rsmZ'-'lacZ* transcriptional fusion was very low in a GacS-minus background (Fig. 4.3). In the wild type, *rsmZ* expression increased throughout the growth phase and was maximal at later stages of growth (Fig. 4.4). Since *rsmZ* expression depends on GacS/GacA in other *Pseudomonas* strains, this result supports a similar Gac/Rsm regulatory cascade in DF41.

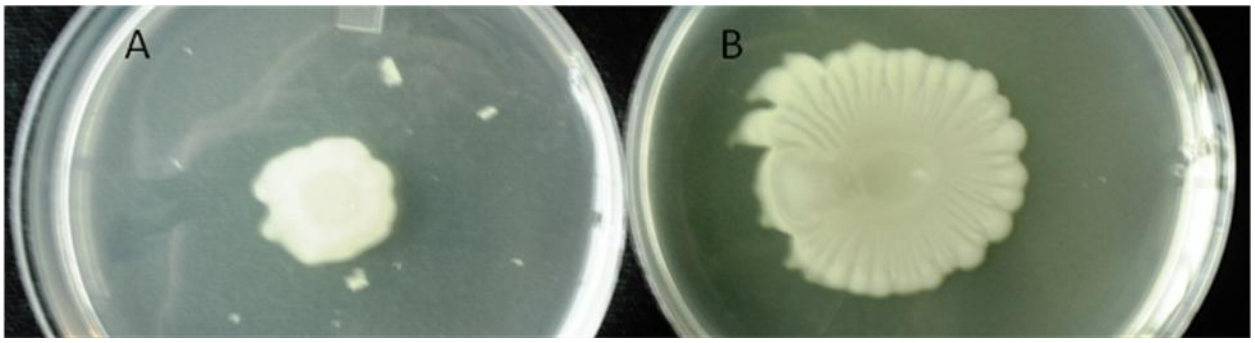


Figure 4.2 The effect of the RsmZ multicopy plasmid, pME6359, on swarming motility in DF41-469 (*gacS*). Panel A: DF41-469 (pME6032), Panel B: DF41-469 (pME6359).

Table 4.2 Flagellar (swimming) motility of *Pseudomonas* sp. DF41 and DF41-469 (*gacS*) harbouring the *rsmZ* overexpression plasmid pME6359.

Strain	Swim Zone Diameter (mm) [a]	
	24 h	72 h
DF41 (pME6032)	11.4 (1.9)	34.0 (0.7)
DF41 (pME6359)	7.2 (0.8) [b]	29.4 (1.1) [c]
DF41-469 (pME6032)	18.6 (1.5)	49.8 (1.6)
DF41-469 (pME6359)	16.8 (0.4) [b]	42.4 (1.1) [c]

[a] Mean (SD) from five replicates.
[b] Significantly different than strain carrying the empty vector ($p < 0.05$).
[c] Significantly different than strain carrying the empty vector ($p < 0.0001$).

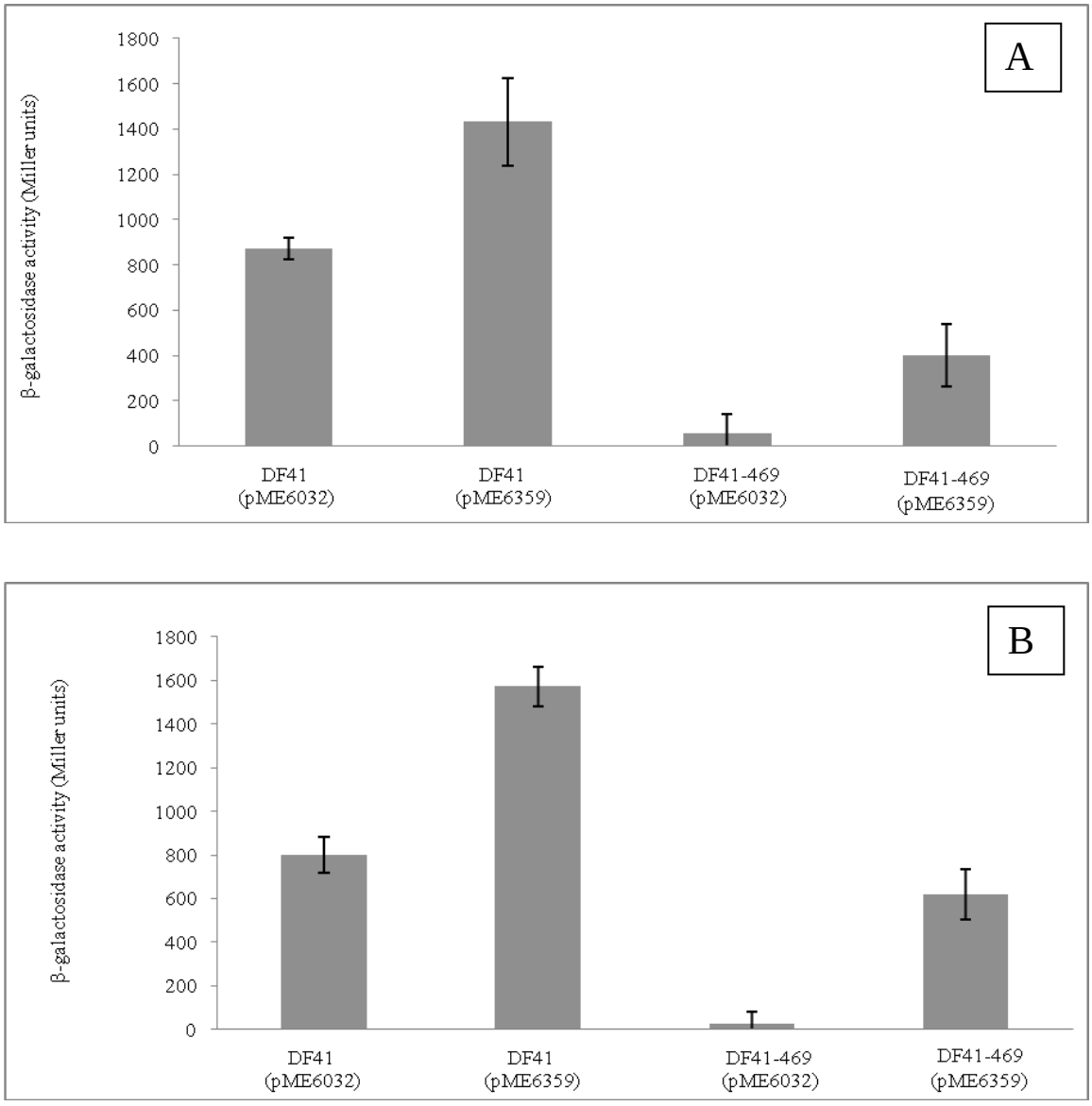


Figure 4.3 The effect of the RsmZ multicopy plasmid (pME6359) on expression of an *rsmZ'-'lacZ* transcriptional fusion in DF41 and DF41-469 (*gacS*). Panel A: *rsmZ* expression in strains cultured for 24 h in PTSB. Panel B: *rsmZ* expression in strains cultured for 48 h in PTSB. Activities (Miller units) are mean values of triplicate samples ± SD. pME6032 is the empty vector.

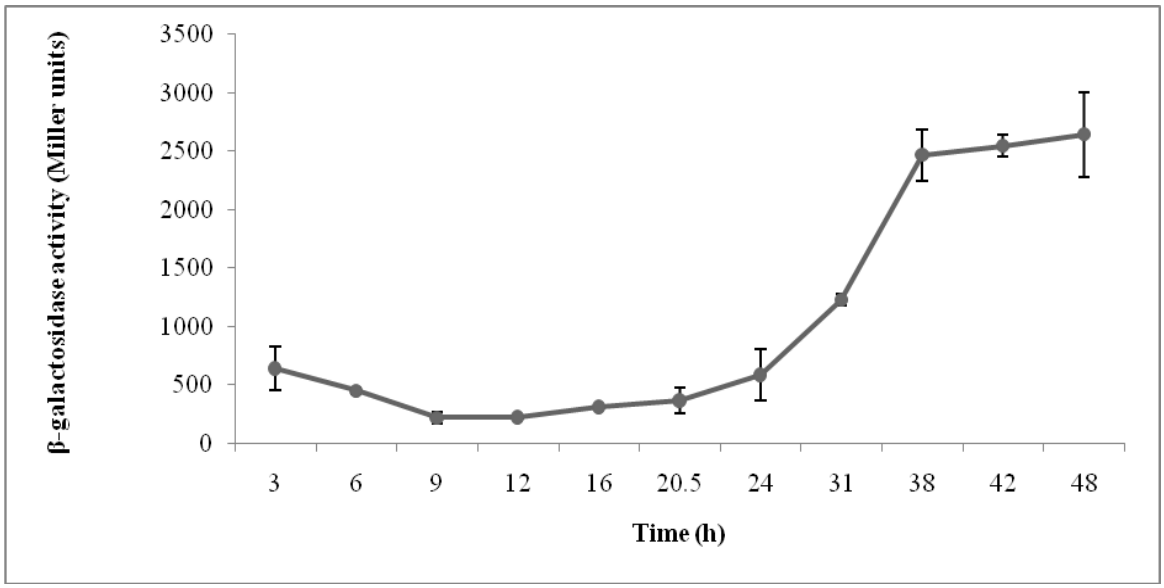


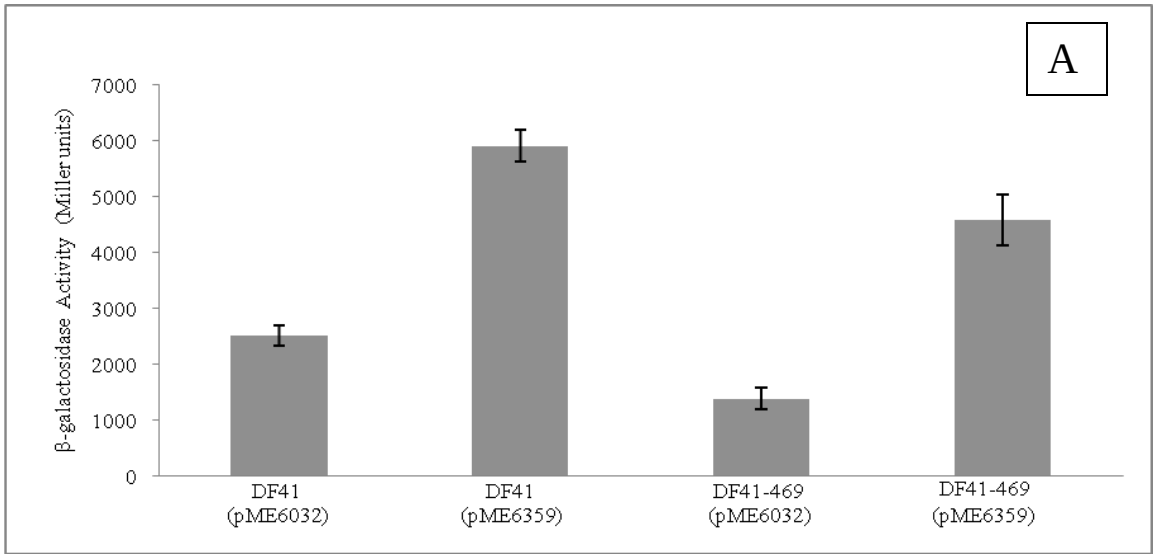
Figure 4.4 Expression of an *rsmZ'-'lacZ* transcriptional fusion in DF41. *rsmZ* expression slowly increases throughout the growth phase with maximal expression at late stages of growth. DF41 was grown in PTSB and assayed for β-galactosidase activity at the times indicated. Activities (Miller units) are mean values of triplicate samples ± SD.

4.2.7 RsmZ affects expression of *rsmZ* and other regulatory genes

To understand whether RsmZ is autoregulated, *rsmZ* transcription was analyzed in strains overexpressing the *rsmZ* gene on plasmid pME6359. In addition, the effect of RsmZ overexpression on transcription of two other regulatory genes, namely *rpoS* and *pdfI* was examined. Plasmid pME6359 was introduced into DF41 and DF41-469 (*gacS*) harbouring *rsmZ, rpoS* and *pdfI-lacZ* transcriptional fusions. Since the expression profile of *rsmZ* in DF41 is similar to that in other *Pseudomonas* strains, with maximal expression occurring at stationary phase (Fig. 4.4), *rsmZ*, *rpoS* and *pdfI* activity were assayed at 24 h and 48 h of growth.

Our results indicate that multiple copies of RsmZ in the *gacS* mutant [DF41-469 (pME6359)] significantly increased *rsmZ* transcription compared to the mutant carrying the empty vector [DF41-469 (pME6032)] (Fig.4.3). In DF41, RsmZ overexpression led to a 2-fold increase in *rsmZ* transcription at 48h (Fig. 4.3). Thus it appears that RsmZ is able to upregulate its own expression.

Similar to the AHL bioassay plates, the transcriptional activity of *pdfI* in DF41-469 (*gacS*) is modestly decreased compared to DF41 (Fig. 4.5). When RsmZ is provided in surplus, at 24 h *pdfI* transcription increased over 2-fold in both DF41 and DF41-469 (Fig. 4.5A) and a further increase in *pdfI* expression was observed at 48 h (Fig. 4.5B). Together, these results indicate that RsmZ is a strong positive regulator of *pdfI* expression in DF41 (Fig. 4.5).



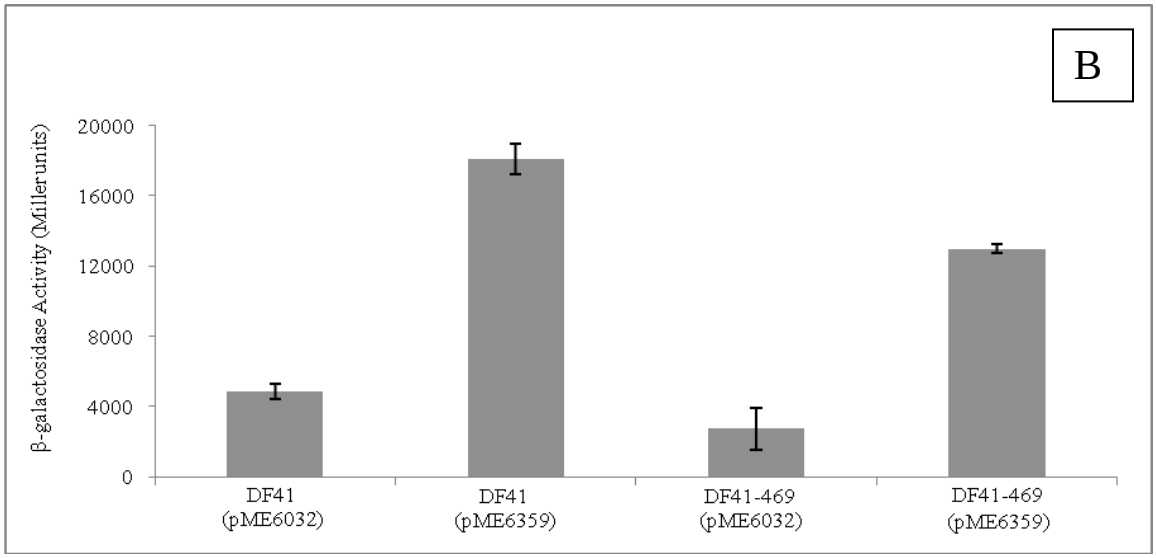
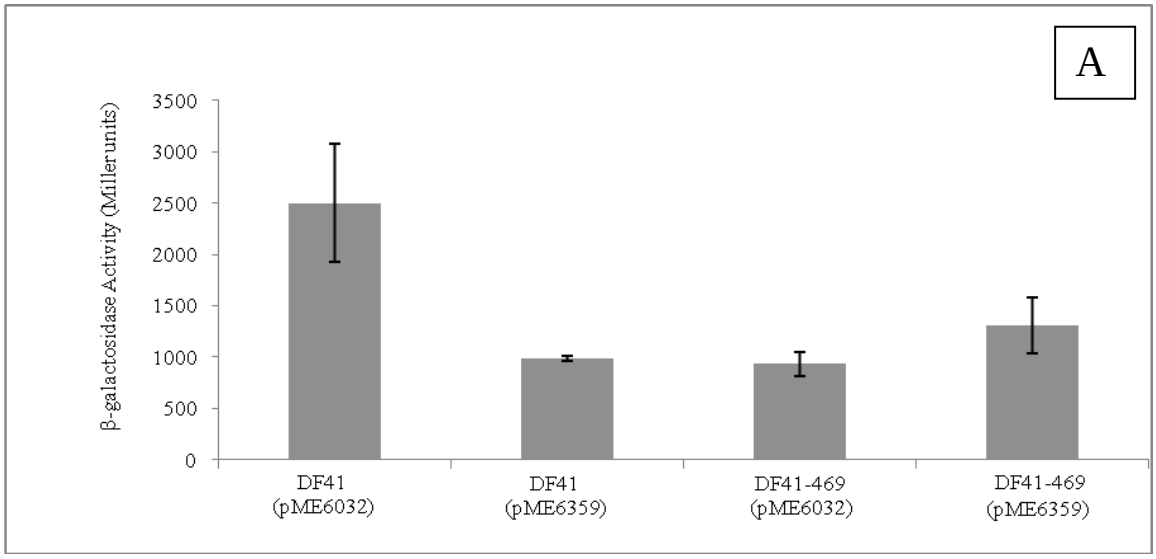


Figure 4.5 The effect of the RsmZ multicopy plasmid (pME6359) on expression of a *pdfI'-'lacZ* transcriptional fusion in DF41 and DF41-469 (*gacS*). Panel A: *pdfI* expression in strains cultured for 24 h in PTSB. Panel B: *pdfI* expression in strains cultured for 48 h in PTSB. Activities (Miller units) are mean values of triplicate samples ± SD.

To gain insight into the regulation of RpoS by the DF41 Gac/Rsm regulatory cascade, *rpoS* expression was examined in the wild-type background and the *gacS* mutant. The effect of RsmZ overexpression on *rpoS* expression was also determined (Fig. 4.6). In the *gacS* mutant, *rpoS* transcription was reduced over two-fold by 48 h compared to the wild-type suggesting that GacS is controlling *rpoS* expression to some extent. GacS exerts its regulatory effects through RsmZ which counters RsmA and RsmE translational repression, therefore, the addition of multiple copies of RsmZ is expected to suppress the negative effects of a *gacS* mutation. However, we observed conflicting results in DF41 and the *gacS*–minus strain. When RsmZ was introduced into DF41 harbouring an *rpoS'-'lacZ* transcriptional fusion, RsmZ overexpression reduced *rpoS* transcription approximately 2-fold approximating levels observed in a *gacS* mutant. In contrast, when RsmZ was provided in surplus to the *gacS* mutant, a significant effect was not observed after 24h of growth (Fig. 4.6A). However, when cells were assayed at 48h, RsmZ overexpression led to a 1.6-fold increase in *rpoS* transcription (Fig. 4.6B). This inverse effect suggests that control of *rpoS* expression is complex and a number of regulatory elements are likely involved.

4.2.8 QS does not influence DF41 biocontrol traits

Previous attempts to generate an AHL-minus phenotype by allelic exchange in DF41 were unsuccessful leading us to speculate that there may be a second, unidentified QS system present in this strain. Therefore, to generate a QS-minus phenotype in DF41, plasmid pME6863 harbouring the *B. subtillus aiiA* gene encoding an acyl homoserine



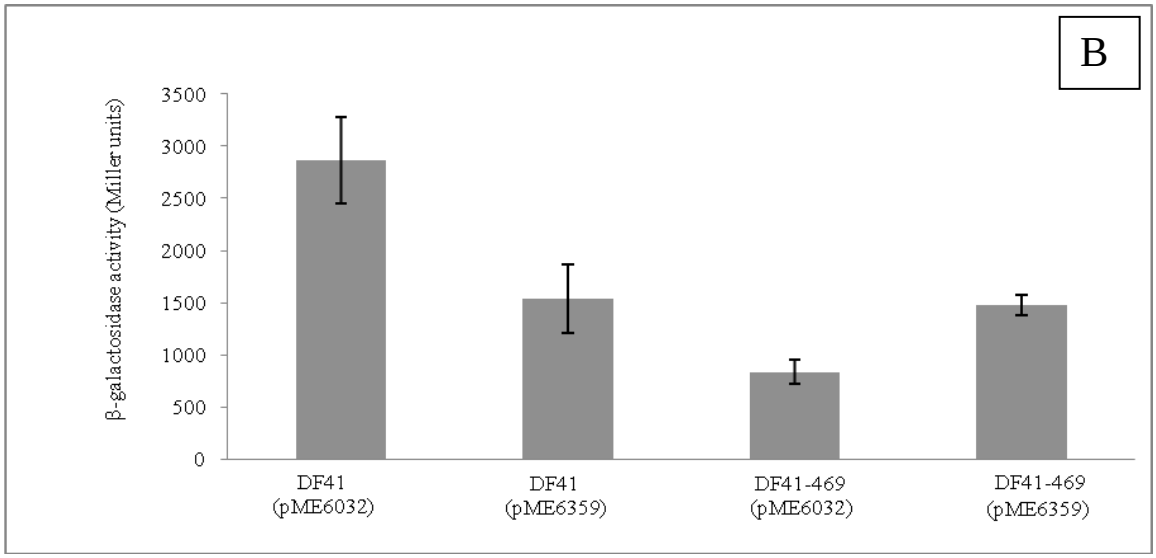


Figure 4.6 The effect of the RsmZ multicopy plasmid (pME6359) on expression of an *rpoS'-'lacZ* transcriptional fusion in DF41 and DF41-469 (*gacS*). Panel A: *rpoS* expression in strains grown for 24 h in PTSB. Panel B: *rpoS* expression in strains grown for 48 h in PTSB. Activities (Miller units) are mean values of triplicate samples ± SD.

lactonase was introduced into DF41 and DF41-469 (*gacS*). An AHL-minus phenotype was verified on CV026-seeded agar plates. HCN, protease and AF activity were assayed; however, none of these factors were found to be influenced by AHL (Table 4.3), indicating that QS may have a more unique regulatory role in DF41.

4.2.9 QS affects motility in DF41

The effect of either exogenous AHL addition or AHL degradation on swimming and swarming motility in DF41 and DF41-469 (*gacS*) was determined on low-percentage agar KB plates. Interestingly, QS appears to negatively regulate both modes of motility. The addition of exogenous AHL to the media completely abolished swarming motility in DF41. As illustrated in Table 4.4, a significant decrease in swimming motility was observed in the presence of exogenous AHL. Conversely, AHL-degradation resulting from the *aiiA* gene product led to an overall increase in swimming motility. Together, these results indicate that in DF41, QS is a negative regulator of motility.

4.2.10 AHL is a negative regulator of *rpoS* and *rsmZ* transcription

The effect of AHL degradation or excess autoinducer on *rpoS* and *rsmZ* transcription was determined. AHL degradation was found to have a strong inducing effect on *rpoS* transcription in DF41 and DF41-469 at both 24 and 48 hours (Fig. 4.7). In strains harbouring the *aiiA* gene, *rpoS'-'lacZ* transcription was increased nearly 3-fold in DF41 and DF41-469. In contrast, when cultures were grown in the presence of exogenous AHL, *rpoS* transcription was reduced to below wild-type levels. AHL had a

Table 4.3 Effect of quorum quenching and addition of exogenous AHL on the phenotypic characteristics of DF41 and Tn*5* mutant DF41-469 (*gacS*).

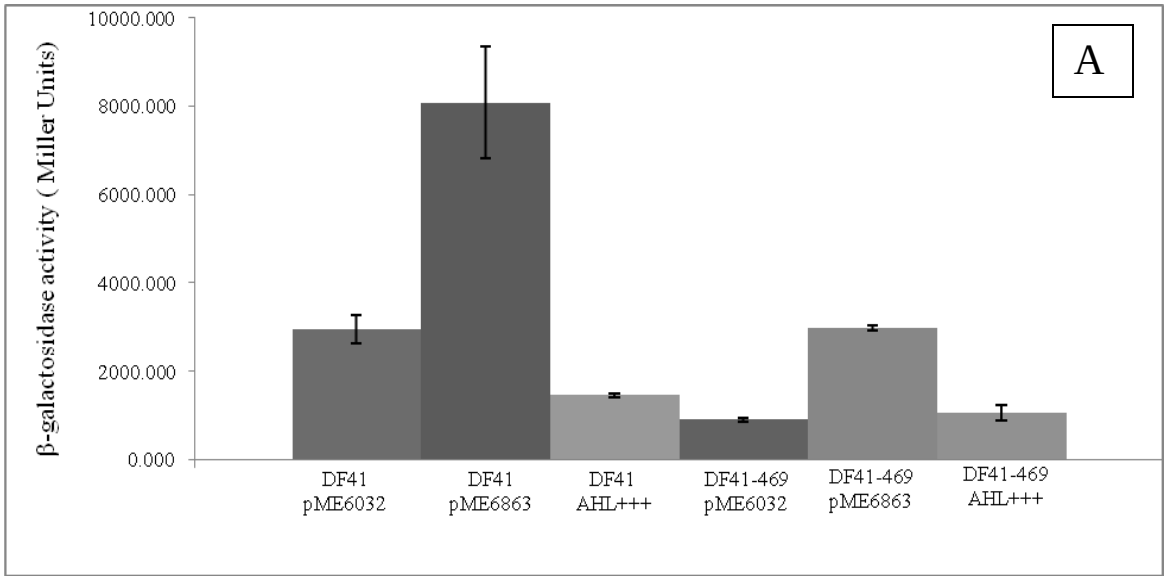
Strain	AF [a]	Autoinducer [a]	Protease [a]	HCN [b]
DF41 (pME6032)	7.8 (0.8)	8.2 (0.7)	5.2 (0.5)	+
DF41 (AHL +++)	8.1 (0.5) [e]	10.3 (0.5) [c]	5.2 (0.4) [e]	+
DF41 (pME6863	7.9 (0.5) [e]	0.0 (0.0) [c]	5.9 (0.6) [d]	+
DF41-469 (pME6032)	Overgrown	4.6 (0.5)	0.0 (0.0)	-
DF41-469 (AHL +++)	Overgrown [e]	6.2 (0.3) [c]	0.0 (0.0) [e]	-
DF41-469 (pME6863	Overgrown [e]	0.0 (0.0) [c]	0.0 (0.0) [e]	-

[a] Mean (SD) of the zones of activity (mm) from a minimum of five replicates.
[b] Determined using Cyantesmo paper.
[c] Significantly different from strain carrying empty vector ($p < 0.0001$).
[d] Significantly different from strain carrying empty vector ($p < 0.001$).
[e] Not significantly different from strain carrying empty vector.

Table 4.4 The effect of AHL addition and quorum quenching on swimming motility of DF41 and *gacS* mutant DF41-469.

Strain	Swim Zone Diameter (mm) [a]	
	24 h	72 h
DF41 (pME6032)	11.4 (1.9)	34.0 (0.7)
DF41 (pME6863)	13.0 (1.0) [d]	37.2 (1.3) [c]
DF41 (AI +++)	9.2 (1.3) [d]	29.0 (1.4) [c]
DF41-469 (pME6032)	18.6 (1.5)	49.8 (1.6)
DF41-469 (pME6863)	18.2 (0.4) [e]	42.8 (1.6) [b]
DF41-469 (AI +++)	14.6 (1.3) [c]	44.0 (1.0) [b]

[a]Mean (SD) from five replicates.
[b] Significantly different from strain carrying empty vector ($p < 0.0001$).
[c] Significantly different from strain carrying empty vector ($p < 0.001$).
[d] Significantly different from strain carrying empty vector ($p < 0.05$).
[e] Not significantly different.



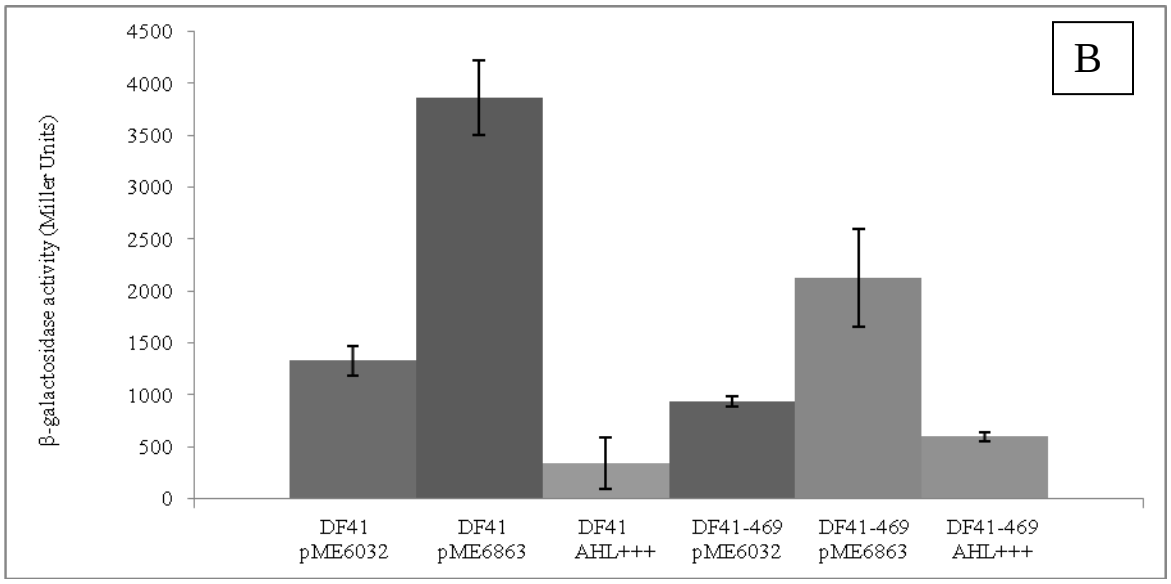


Figure 4.7 The effect of plasmid pME6863 harbouring the AHL lactonase gene, *aiiA* and excess AHL on expression of an *rpoS'-'lacZ* transcriptional fusion in DF41 and DF41-469 (*gacS*). Panel A: *rpoS* expression in strains grown in for 24 h in PTSB. Panel B: *rpoS* expression in strains grown for 48 h in PTSB. Activities (Miller units) are mean values of triplicate samples ± SD. pME6032 is the empty vector.

similar effect on *rsmZ* transcription, although the results were not quite as dramatic (Fig. 4.8). In DF41, *rsmZ* transcription was significantly repressed at both 24 h and 48 h of growth upon the addition of excess AHL. In the *gacS*-minus strain, DF41-469, AHL addition did not significantly impact *rsmZ* expression until 48 h of growth (Fig. 4.8). Likewise, AHL degradation resulted in considerable increases in *rsmZ* transcription in the wild-type background at 24 h and 48 h (Fig. 4.8) with a siginificant increase observed in DF41-469 only at the 48h time point.

4.2.11 *pdfI* expression is controlled by a positive feedback mechanism

Many QS-regulated genes contain a *lux* box in their promoter regions for binding of the cognate QS LuxR transcriptional activator. The presence of a putative *lux* box-like sequence in the *pdfI* promoter region suggests that *pdfI* may be subject to autoregulation. To investigate this, the effect of AHL degradation or excess autoinducer on *pdfI* transcription was determined. AHL degradation was found to significantly decrease *pdfI* expression at both 24 h and 48 h whereas the addition of exogenous AHL led to considerable increases in *pdfI* transcription in both DF41 and the *gacS*-minus strain (Fig. 4.9). *pdfI* expression was also determined to be strongly linked to the growth phase with maximal expression observed at 38 h growth (Fig. 4.10). Together, these results indicate that *pdfI* gene expression is considerably affected by AHL in a positive feedback manner and is growth-phase dependent.

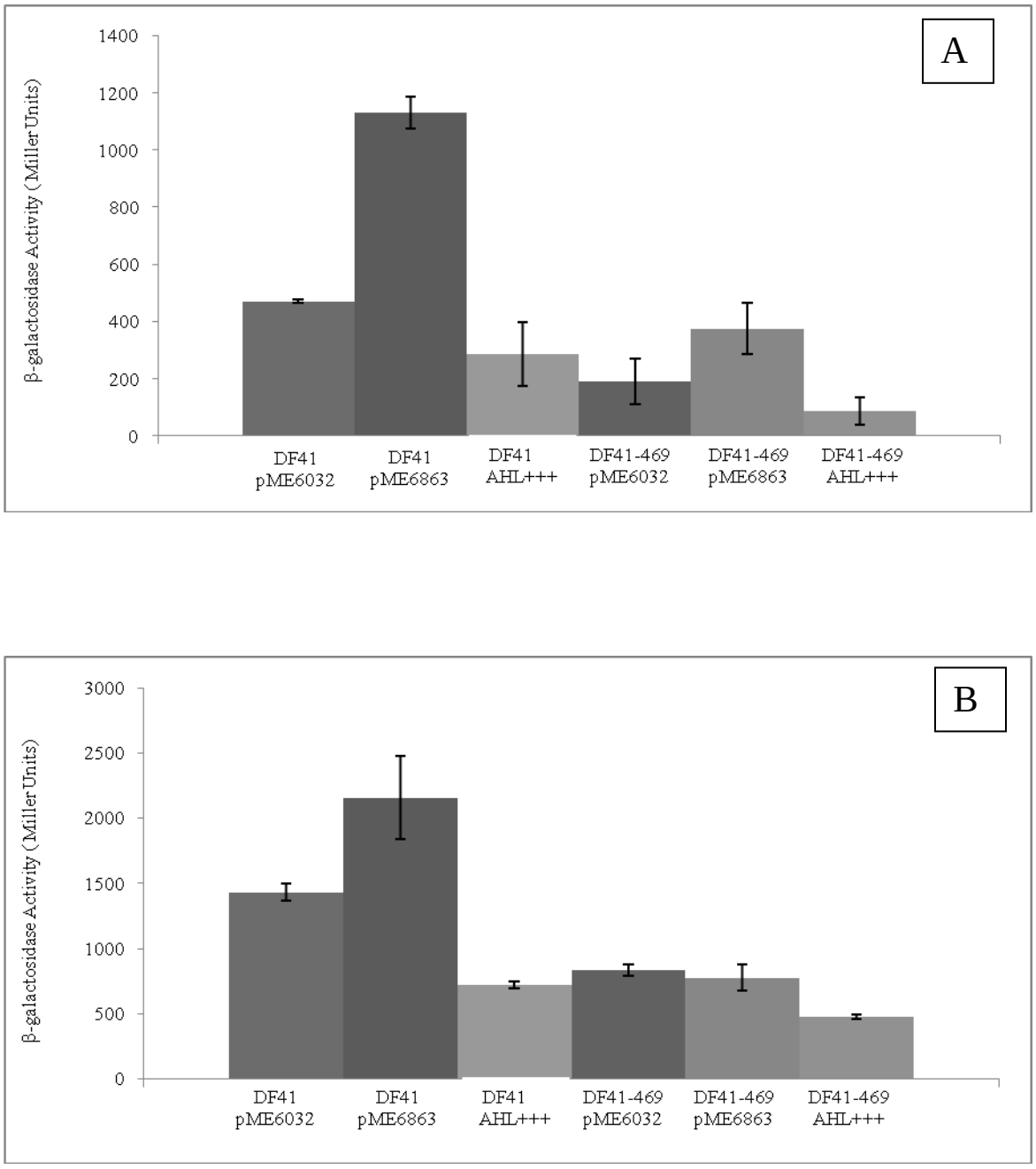


Figure 4.8 The effect of plasmid pME6863 harbouring the AHL lactonase gene, *aiiA* and excess AHL on expression of an *rsmZ'-'lacZ* transcriptional fusion in DF41 and DF41-469 (*gacS*). Panel A: *rsmZ* expression in strains grown for 24 h in PTSB. Panel B: *rsmZ* expression in strains grown for 48 h in PTSB. Activities (Miller units) are mean values of triplicate samples ± SD.

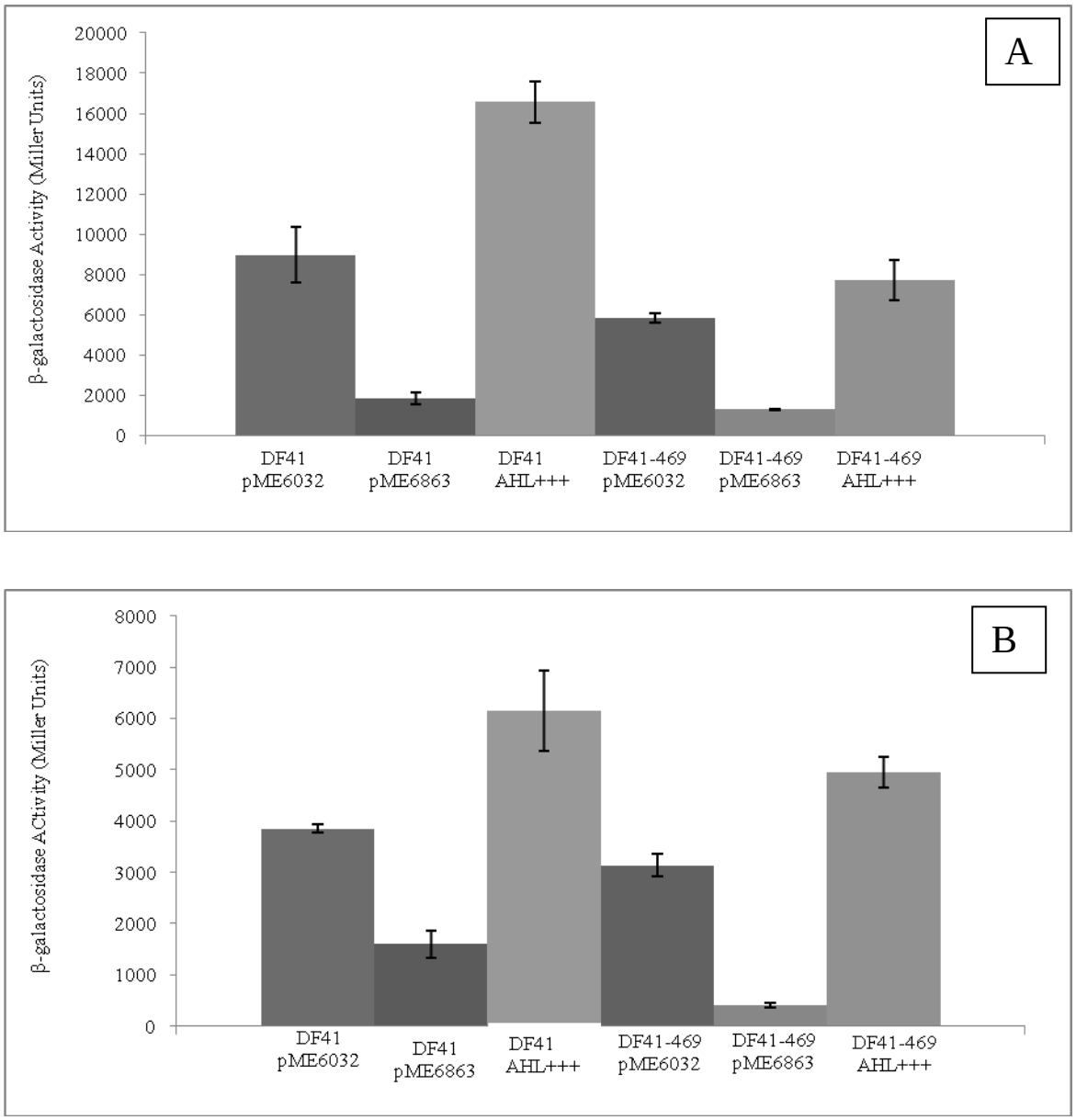


Figure 4.9 The effect of plasmid pME6863 harbouring the AHL lactonase gene, *aiiA* and excess AHL on a *pdfI'-'lacZ* transcriptional fusion in DF41 and DF41-469 (*gacS*). Panel A: *pdfI* expression in strains grown for 24 h in PTSB. Panel B: *pdfI* expression in strains grown for 48 h in PTSB. Activities (Miller units) are mean values of triplicate samples ± SD.

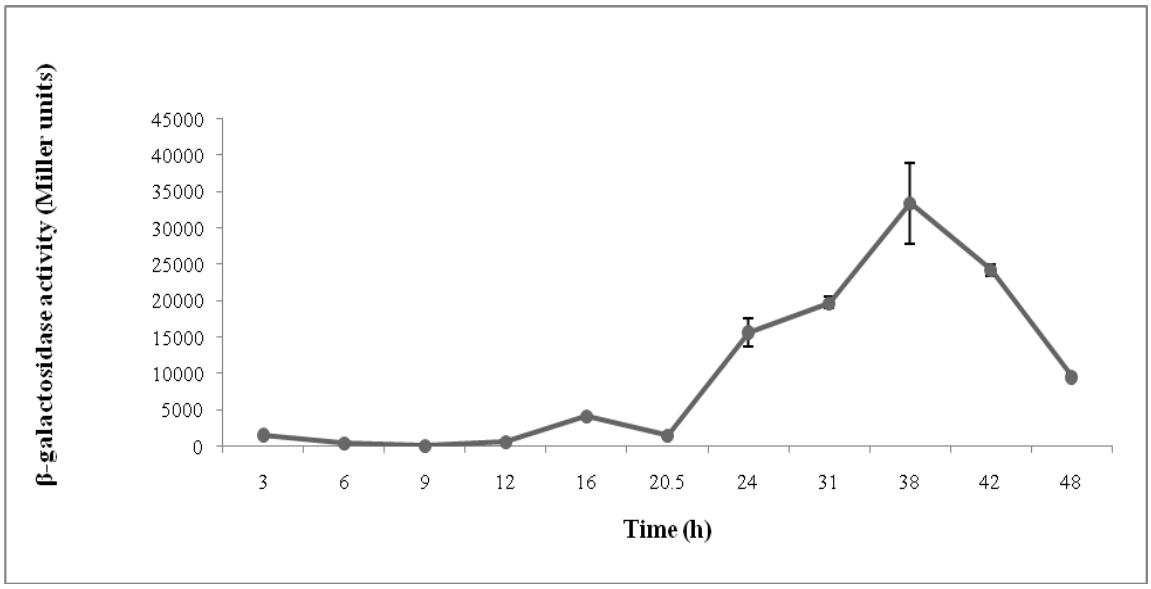


Figure 4.10 Expression of a *pdfI'-'lacZ* transcriptional fusion in DF41 is growth-phase dependent. DF41 was cultured in PTSB and β-galactosidase activity was assayed at times indicated. Activities (Miller units) are mean values of triplicate samples ± SD.

4.3 Discussion

Our initial characterization of *Pseudomonas* sp. DF41 focused on the identification of gene products that directly and indirectly contributed to the biological control activity of this bacterium. To gain further insight into the underlying mechanisms of biocontrol activity, it is necessary to examine the regulatory systems governing *Pseudomonas* sp. DF41 AF activity. We first identified and characterized the two-component signal transduction system GacS/GacA (Chapter 3), which is a conserved system in numerous Gram-negative bacteria. The mechanisms underlying GacS/GacA post-transcriptional control of target gene expression has been examined in a few *Pseudomonas* species, particularly in *P. aeruginosa* PAO1 and *P. fluorescens* CHA0. In each of these strains, GacA posttranscriptional control is coupled to the Rsm system. The Rsm system includes mRNA-binding repressor proteins and Gac-controlled regulatory RNAs.

In PAO1, the Gac/Rsm cascade activates expression of the QS signal, *N*-butanoyl-homoserine lactone as well as extracellular virulence factors such as HCN and pyocyanin. This is achieved via GacA upregulation of two, functionally redundant small RNAs, RsmZ and RsmY. Together RsmZ and RsmY induce translation by titrating the RNA-binding protein RsmA. A homologous system in *P. fluorescens* CHA0 consists of three, small RNA regulators, RsmZ, RsmY and RsmX that antagonize translational repression of target genes by sequestration of the RNA-binding proteins RsmA and RsmE. While we were in the process of PCR-screening for an *rsmZ* gene in *Pseudomonas* sp. DF41, we obtained an RsmZ overexpression plasmid, pME6359, from

the D. Haas lab. This plasmid was introduced into DF41 and the *gacS* mutant to examine the multi-copy effect of RsmZ in a *gacS*-deficient strain. When pME6359 was introduced into DF41-469, *rsmZ* partially suppressed the effects of a *gacS* mutation, restoring protease and HCN production and enabling some inhibition of *S. sclerotiorum* (Table 4.1). RsmZ overexpression in the DF41 wild type resulted in a substantial increase in protease production, which translated into a slightly elevated level of fungal inhibition (Table 4.1). Although a DF41 *gacS* mutant produced approximately 60% AHL compared to the wild type, RsmZ overexpression compensated for the *gacS* deficiency and AHL levels increased to those of the wild type. This result is similar to those observed in *P. fluorescens* CHA0 and in *P. fluorescens* F113, where overexpression of RsmX, RsmY or RsmZ, or PrrB (an RsmZ homologue) respectively, can compensate for *gacS* and *gacA* mutations. Moreover, these findings further support our earlier observations that in *Pseudomonas* sp. DF41, a Gac/Rsm regulatory cascade is essential for production of secondary metabolites contributing to AF activity.

Contrasting results were observed for the effect of RsmZ on bacterial motility. While multiple copies of *rsmZ* restored swarming motility to the *gacS*-minus derivative, an inhibitory effect was observed on swimming motility. The *Pseudomonas* sp. DF41 *gacS* mutant consistently displays markedly increased swimming motility on low-percentage agar plates (Chapter 3). However, upon introduction of the *rsmZ* multicopy plasmid, pME6359, swimming motility was considerably decreased in the wild-type and in the *gacS* mutant. There is some precedent for this provided in the literature as comparable results were observed in *E. coli.* In this strain, the RsmA homolog, CsrA is a positive regulator of flagellar motility (Wei et al. 2001). RsmA binding to the 5'-

segment of *flhDC* mRNA stimulates its translation and increases its half-life (Wei et al. 2001). Although swarming motility in *Pseudomonas* sp. DF41 is positively regulated by Gac/Rsm similar to *P. fluorescens* CHA0, it is notably different from that observed in other *Pseudomonas* strains. For example, in *P. aeruginosa* PAO1, RsmA has a positive effect on rhamnolipids and swarming motility; therefore, GacA mutants display increased swarming compared to the wild type due to the loss of RsmZ antagonism (Heurlier et al. 2004). These results provide evidence that in *Pseudomonas* DF41, RsmZ acts as a positive and a negative regulator of bacterial motility possibly by influencing the level of free RNA-binding proteins homologous to RsmA and RsmE. Furthermore, the Gac/Rsm regulons appear to display a notable amount of variability amongst different bacterial strains.

The DF41 RsmZ secondary structure was analyzed using RNA-fold to confirm the presence of repeated, unpaired GGA motifs in the hairpin loops believed to be essential for RsmZ titration effects. Based on a high level of sequence identity and identification of GGA motifs, we hypothesize that the DF41 and CHA0 RsmZ regulatory RNA will function in a similar manner. Furthermore, the gene organization in *Pseudomonas* sp. DF41 is similar to that of a number other pseudomonads, with the *rsmZ* gene located immediately downstream of the *rpoS* gene.

Several *lux*-based QS systems have been identified in plant-associated pseudomonads that control an array of traits from AF compound production to bacterial motility and epiphytic fitness. We were interested in determining QS-regulated phenotypes in *Pseudomonas* sp. DF41 and investigating their role in biological control. The generation of a genomic library facilitated the identification of a LuxR-LuxI type QS

system. Sequence analysis revealed 77% nucleotide identity shared with the QS genes of the tomato pathogen *P. corrugata*. Interestingly, the Blastn search tool did not indicate homology with other *Pseudomonas luxI* genes other than *P. corrugata*. The *Pseudomonas* sp. DF41 QS system consists of an autoinducer synthase gene located upstream of a cognate transcriptional activator gene. Immediately downstream of the DF41-QS locus are three genes coding for components of a tripartite resistance nodulation- cell-division transporter, analogous to the *P. corrugata* QS gene arrangement. In *P. corrugata*, an AHL-mediated QS system controls the production and secretion of cyclic lipopeptides important for virulence on tomato plants and inhibition of *Rhodotorula pilimanae* and *Bacillus megaterium* (Licciardello et al. 2007).

To generate an AHL-minus phenotype in *Pseudomonas* sp. DF41, plasmid pME6863 was introduced into DF41 and the *gacS* minus strain. The *aiiA* gene on this plasmid encodes a lactonase enzyme from the soil bacterium *Bacillus* sp. A24 (Reimmann et al. 2002), which allowed us to determine the effect of a QS-minus background on the expression of AF factors. Phenotypic analysis indicated that HCN production, protease and AF activity was indistinguishable in strains harbouring the *aiiA* plasmid compared to those strains carrying the empty vector (Table 4.3). These results are analogous to those observed for a *pcoI* mutant of the biocontrol bacterium *P. fluorescens* 2P24 where production of several biocontrol factors including siderophore, HCN, proteinase and 2,4-DAPG remained unchanged from the wild type (Wei and Zhang 2006). Finally, addition of exogenous AHL to growing cultures did not affect AF activity or protease production (Table 4.3), further substantiating that in DF41, QS does not appear to regulate expression of biocontrol factors.

When the phenotypic analysis was extended to include the effects of QS on bacterial motility, differences were observed. AHL addition resulted in slight decreases in swimming motility. The most dramatic effects were observed upon AHL-degradation by the lactonase enzyme. Swimming motility was significantly increased in both the wild type and the *gacS* mutant carrying pME6863, supporting QS-mediated repression of flagellar motility (Table 4.4). Identical trends were observed when swarming motility was assayed. Addition of exogenous AHL to swarm plates completely abolished swarming motility by the wild type strain. Whereas in the presence of the AHL degradation enzyme, increased swarming was observed.

Next, the effects of RsmZ and AHL on the regulatory cascade underlying AF activity were examined. An *rsmZ'-'lacZ* transcriptional fusion was introduced into DF41 and DF41-469 (*gacS*). As expected, in the *gacS* minus background *rsmZ* expression was negligible. However, RsmZ overexpression resulted in markedly increased transcription, attaining maximum expression at late stationary phase in both strains, indicating that the *rsmZ* gene is subject to positive autoregulation. It appears as if *rsmZ* may be subject to alternative regulation compared to other *Pseudomonas* strains. In *P. aeruginosa,* RsmZ represses its own transcription (Kay et al. 2006), whereas in *P. fluorescens* CHA0, an *rsmZ* mutant had only transient effects on an *rsmZ'-'lacZ* transcriptional fusion (Valverde et al. 2003).

Further insight into the Gac/Rsm regulon was provided by determining the influence of GacS, AHL and RsmZ on *rpoS, rsmZ* and *pdfI* expression. β-galactosidase assays indicated that RsmZ positively regulates *pdfI* expression at high cell densities (Fig. 4.5). However, since a *gacS* mutation only slightly decreased *pdfI* expression, it would

appear that there are other regulatory elements responsible for QS expression. In *P. aeruginosa*, C4-HSL production is stimulated by the Gac/Rsm pathway, and similarly, in *Pseudomonas* sp. DF41, overexpression of RsmZ in both the wild type and the *gacS* mutant significantly increased the activity of a *pdfI'-'lacZ* transcriptional fusion. These findings are consistent with the increased levels of AHL produced by RsmZ overexpressing strains that can be observed on agar plates seeded with the AHL-bioreporter strain.

When the effect of RsmZ on an *rpoS'-'lacZ* transcriptional vector was examined, conflicting results were obtained (Fig. 4.6). In the wild-type background, RsmZ decreases *rpoS* expression 2-fold; however at 48 h, the opposite trend was observed in the *gacS* mutant. A possible explanation could be that since RsmZ has a strong positive influence on AHL production which in turn negatively regulates *rpoS* expression, the different expression profiles observed between the wild type and the GacS mutant may be due to a balance in the timing of AHL production and its corresponding negative regulatory effects.

In *P. aeruginosa* PAO1, RpoS and AHL regulate each other and studies have often drawn conflicting conclusions. A recent transcriptome analysis of the QS regulon in this strain concluded that RpoS controls approximately 40% of all genes in the QS regulon and that RpoS positively influences the *rhl* system which in turn, positively regulates RpoS (Schuster and Greenberg 2007). When the influence of QS degradation and exogenous AHL addition on *rpoS* (Fig. 4.7) and *rsmZ* (Fig.4.8) expression was examined in *Pseudomonas* sp. DF41, the results clearly indicated that QS represses both *rsmZ* and *rpoS* as expression was increased when AHL was degraded. This result was

somewhat unexpected given that in other strains with QS systems, such as *P. putida* and *P. aeruginosa* PAO1, AHL has been reported to have a positive effect on *rpoS* transcription (Bertani and Venturi 2004). Whether this negative effect is a matter of timing of expression or mediated through indirect effects remains to be elucidated.

In conclusion, we have established that Gac/Rsm regulates a complex network of regulatory elements underlying AF activity in *Pseudomonas* sp. DF41. The Gac/Rsm cascade partially influences RpoS and AHL expression in this strain, suggesting that additional unknown regulatory elements may be involved. An interesting role for QS is proposed which differs significantly from that reported for other bacteria, because in DF41, QS is a negative regulator of *rpoS* and *rsmZ* expression. Few studies have examined possible *rsmZ* regulatory elements, and our preliminary results suggest a negative AHL- mediated influence on *rsmZ* transcription. Although it is highly probable that *Pseudomonas* sp. DF41 produces RsmA homologs which act as negative regulators of biocontrol, they have yet to be identified.

Chapter 5

Investigation of the biological activity and structure of a compound essential for DF41 AF activity

5.1 Introduction

Cyclic lipopeptides are a novel class of functionally versatile molecules that have been isolated from a number of bacterial species, including various *Pseudomonas* strains reviewed in (Raaijmakers et al. 2006). A defining feature of CLPs is their biosynthesis via a non-ribosomal thiotemplate mechanism on large peptide synthetases (Finking and Marahiel 2004; Grunewald and Marahiel 2006). These non-ribosomal peptide synthetase (NRPS) templates consist of repeating sets of core modules which are each divided into enzymatic domains (Marahiel et al. 1997). Each module is typically responsible for the incorporation of a single amino acid precursor into the non-ribosomal peptide. Although the modules are formed of repeating sets of three standard domains, namely, adenylation (A), peptidyl carrier protein (PCP) and condensation (C), variability in the number and arrangement of domains as well as the incorporation of different and non-standard amino acid precursors leads to a number of structurally diverse peptide products. The inclusion of additional catalytic domains into the core protein template also contributes to the structural diversity associated with CLPs. Despite the structural variations, CLPs all consist of a short oligopeptide moiety linked to a fatty acid tail. The majority of NRPS lipopeptides produced by *Pseudomonas* species are cyclized by lactone ring formation between two amino acids in the peptide moiety; however, recently, two linear NRPS-produced lipopeptides, syringafactin and peptin31 have been isolated from *P. syringae* strains (Berti et al. 2007; Fiore et al. 2008).

In *Pseudomonas* species, a number of metabolites are produced on NRPSs such as siderophores, biosurfactants, phytotoxins and AF compounds. CLPs produced by plant-associated pseudomonads are often antagonistic toward other microorganisms and

demonstrate phytotoxic (Bender et al. 1999), antibacterial (Gerard et al. 1997), AF (Nielsen et al. 2002) or antiprotozoan activity (Mazzola et al. 2009). CLPs also contribute to bacterial motility and colonization and as such, contribute to biofilm formation, bacterial proliferation and survival. The main biological action of CLPs is mediated through formation of small pores in target membranes, thus disrupting the flow of ions across the membrane and leading to membrane collapse. CLPs are believed to exert their effect on cell membranes by working synergistically with cell wall degrading enzymes (CWDEs).

Many regulatory elements of CLP synthesis have been identified. The global regulatory Gac system has been identified as a major determinant controlling putisolvin (Dubern et al. 2005), syringomycin and syringopeptin (Hrabak and Willis 1992; Kitten et al. 1998), tolaasin (Grewal et al. 1995) amphisin (Koch et al. 2002), entolysin (Vallet-Gely et al. 2010) and massetolide A biosynthesis (de Bruijn et al. 2008). This two-component signalling pathway appears to play a global role in CLP production in contrast to a plethora of other strain-dependent regulatory elements such as QS (Cui et al. 2005; Dubern et al. 2006), heat-shock proteins (Dubern et al. 2005), and a serine protease (de Bruijn and Raaijmakers 2009). LuxR-type transcriptional activators of the non-QS family are emerging as a wide-spread regulator of CLP biosynthesis (de Bruijn and Raaijmakers 2009). As regulatory elements are often found in the genomic regions flanking NRPS genes, continued sequencing of NRPS genes and *Pseudomonas* genomes coupled with *in silico* analysis is advancing our understanding of CLP regulation and leading to the identification of NRPS gene clusters for the biosynthesis of new CLP molecules.

5.2 Results

5.2.1 Isolation of a *Pseudomonas* sp. DF41 mutant deficient in AF activity

Transposon mutagenesis of *Pseudomonas* sp. DF41 was performed with the aim of identifying metabolites demonstrating biological control activity against *S. sclerotiorum.* Approximately five thousand mutants were screened in radial diffusion assays for altered AF activity. In addition to DF41-469 (*gacS)* discussed in chapter 3, a second Tn*5* mutant, DF41-1278 also exhibited significantly reduced inhibition of the fungal pathogen *S. sclerotiorum* and was selected for further analysis (Fig. 5.1).

Rescue-cloning experiments and subsequent sequencing of the regions flanking the Tn*5*-1063 insertion, indicated that the Tn interrupted a gene with highest homology at the amino acid level (82%; 226/275 amino acids) to the *sypC* gene of *Pseudomonas syringae* pv. *syringae* B301D (accession #AA072425). *sypC* encodes a peptide synthetase involved in non-ribosomal production of the CLP syringopeptin (Scholz-Schroeder et al., 2001). The sequence flanking the Tn*5* insertion in DF41-1278 is homologous to a SypC condensation domain. NRPS operons are extremely large, for example the *sypC* ORF spans 40.6 kb and the entire *syp* gene cluster is estimated to be nearly 80 kb (Scholz-Schroeder et al., 2001); as a result, genetic complementation of this mutation was not attempted.

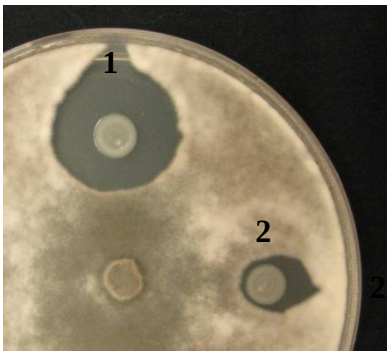


Figure 5.1 AF activity of DF41 and 1278 (*clp*). 5µL of an overnight culture was spotted onto a PDA plate and grown for 16 h at 28°C, at which point an agar plug carrying *S. sclerotiorum* hyphae was placed onto the middle of the plate. 1: DF41. 2: DF41-1278

5.2.2 A putative CLP-like compound is a major determinant of DF41 AF activity

We hypothesized that Tn*5* insertion into a NRPS gene should not alter DF41-1278 expression of other AF factors including protease, HCN and AHL signal molecules. Analysis of this strain confirmed that there was no difference in production of these molecules in DF41-1278 compared to the wild type. Thus it appears that the significantly decreased AF activity associated with DF41-1278 is due to the loss of the NRPS-generated product (Table 5.1 and Fig. 5.1). A single transposon insertion in this mutant was verified by Southern analysis (refer to Fig. 3.2)

5.2.3 DF41-1278 (*clp*) displays a loss of biocontrol activity against *S. sclerotiorum*

DF41 and DF41-1278 (*clp*) were evaluated for their ability to inhibit disease on canola plants caused by *S. sclerotiorum* infection. The incidence of leaf infection and disease severity were evaluated. In greenhouse studies, DF41 provided significant protection against fungal infection on the canola leaves and considerably reduced disease severity (Fig. 5.2 and Fig. 5.3). In contrast, the putative CLP mutant showed little antagonism toward *S. sclerotiorum* (Fig. 5.2 and Fig 5.3). These findings indicate that CLPs are an essential factor for DF41 biocontrol of *Sclerotinia* infection in canola.

5.2.4 DF41-1278 (*clp*) is not affected in motility

To determine if the DF41-1278 (*clp*) mutant was affected in motility, swarming and flagellar motility was compared to that of the wild-type strain. On 0.5% KB agar plates, a change in swarming pattern was not observed as DF41-1278 swarmed in a blebbing pattern comparable to the wild type. Flagellar motility was assayed on 0.3%

Table 5.1 Phenotypic characteristics of *Pseudomonas* sp. DF41 and Tn*5* mutant DF41-1278 (*clp*)

Strain	AF [a]	Autoinducer [a]	Protease [a]	HCN [b]
DF41	7.1 (0.9)	10.7 (0.5)	5.8 (0.7)	+
DF41-1278	2.6 (1.4) [c]	9.9 (0.6) [d]	6.3 (1.2) [d]	+

[a] Mean (SD) of the zones of activity (mm) from at least six replicates.
[b] Determined using Cyantesmo paper.
[c] Significantly different from the wild type ($p < 0.0001$).
[d] Not significantly different from the wild type.

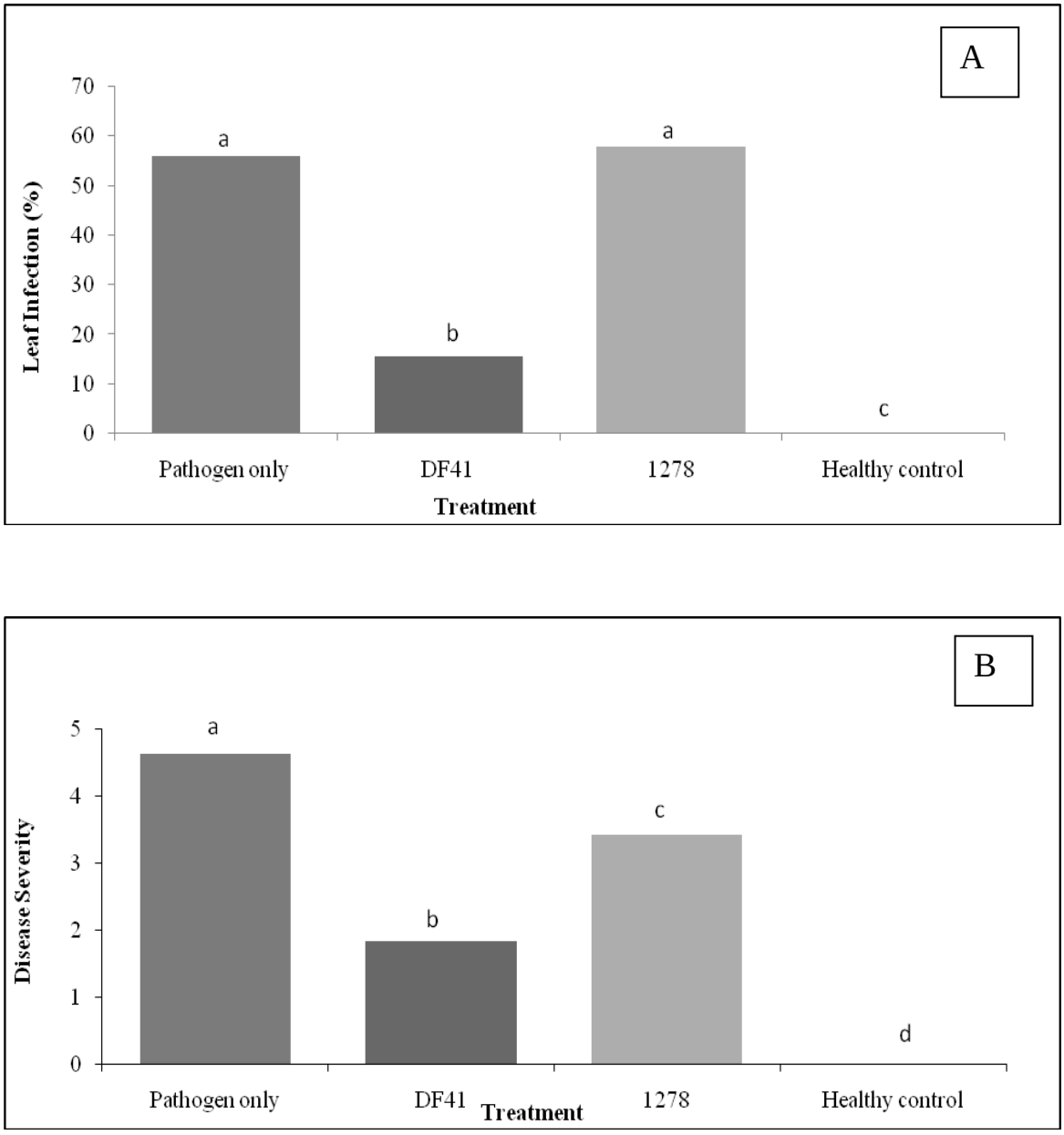


Figure 5.2 Efficiency of *Pseudomonas* sp. DF41 (wild type) and DF41-1278 (*clp*) in managing *Sclerotinia sclerotiorum* ascospore infection on canola plants. Panel A: percent incidence of leaf infection. Panel B: disease severity on stem. In all treatments except the healthy control, plants were sprayed with *S. sclerotiorum* ascospores. The healthy control plants were sprayed with phosphate buffer. Column means labeled with the same letter do not differ significantly by Duncan's Multiple Range Test (DMRT; P>0.05).

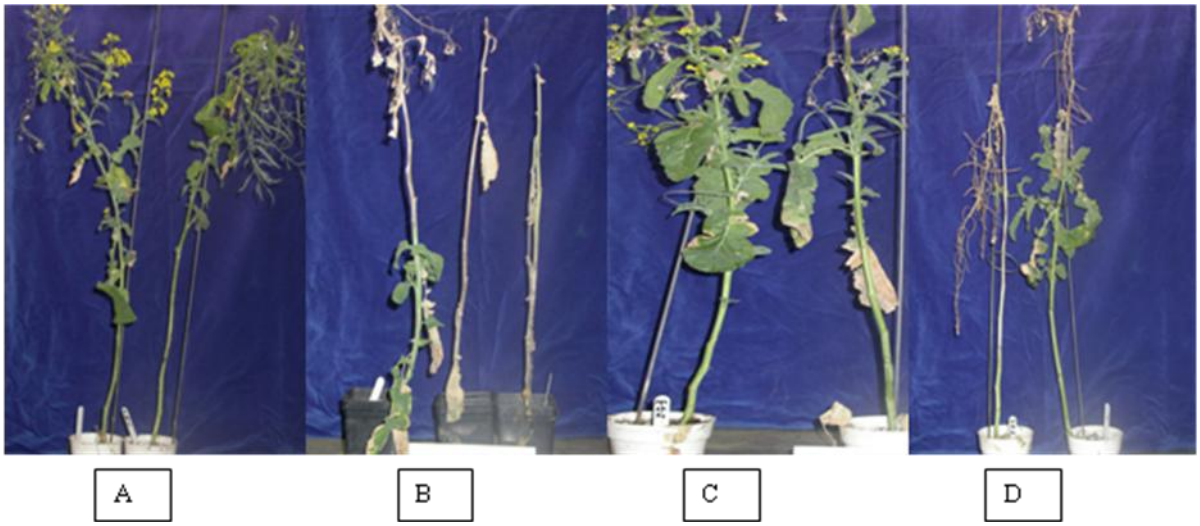


Figure 5.3 Efficiency of *Pseudomonas* sp. DF41 (wild type), DF41-469 (*gacS*) and DF41-1278 (*clp*) in managing *Sclerotinia sclerotiorum* ascospore infection on canola plants in greenhouse assays. Petals were sprayed with bacterial suspensions of 2 x 10^8 cells/ml 24 h prior to spraying with a *S. sclerotiorum* spore suspension of 2 x 10^4 ascospores/ml. Panel A: DF41, Panel B: DF41-469 (gacS), Panel C: DF41-1278 (*clp*), Panel D: disease control only

KB agar plates and similarly, the Tn*5* insertion mutant swam at wild-type levels (Table 5.2).

5.2.5 Analysis of culture supernatants from wild type and mutant strains of *Pseudomonas* sp. DF41.

We employed C_{18} reverse-phase HPLC to isolate DF41 metabolites exhibiting AF activity. When extracts prepared from DF41, DF41-1278 and DF41-469 spent culture supernatants were separated by HPLC, a large peak with a retention time of 27 minutes was observed in the DF41 extract that was absent in DF41-1278 (*clp*) and DF41-469 (*gacS*) (Fig. 5.4). To test whether this peak may represent an AF metabolite, one-minute fractions of DF41, DF41-469 and DF41-1278 were collected and tested for AF activity. The DF41 fractions corresponding to this region demonstrated inhibition of *S. sclerotiorum,* whereas the fungi overgrew the filters spotted with DF41-469 and DF41-1278 fractions (data not shown).

5.2.6 The AF metabolite displays biosurfactant properties

Due to their amphipathic nature, CLP molecules frequently exhibit biosurfactant properties. Therefore, we tested the biosurfactant properties of DF41, DF41-1278 (*clp*) and DF41-469 (*gacS*) in a drop-collapse assay. The HPLC fraction corresponding to the peak with a retention time of 27 minutes was collected and tested for surface-tension reducing activity. While the DF41 droplet showed some degree of collapse, the DF41-1278 (*clp*) and DF41-469 (*gacS*) HPLC fractions more closely resembled the water droplet used as a negative control (Fig. 5.5).

Table 5.2 Flagellar (swimming) motility of *Pseudomonas* sp. DF41 and Tn*5* mutant DF41-1278 (*clp*)

Strain	Swim Zone Diameter (mm) [a]	
	24 h	72 h
DF41 (pUCP23)	20.5 (1.0)	47.0 (2.6)
DF41-1278 (pUCP23)	17.6 (2.6) [b]	45.2 (0.5) [b]

[a] Mean (SD) from five replicates.
[b] Not significantly different from the wild type.

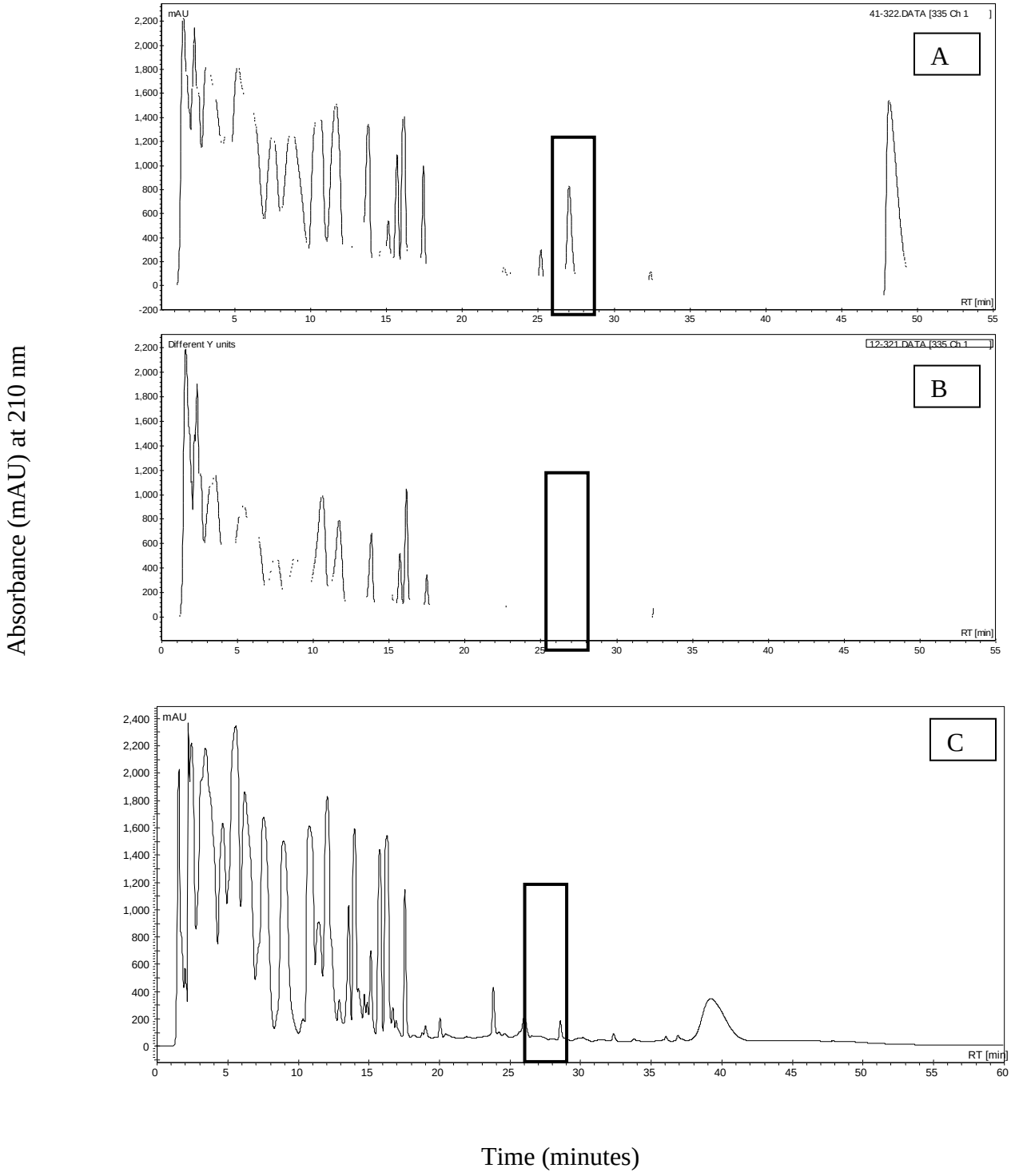


Figure 5.4 HPLC chromatograms of A: DF41; B: DF41-1278 (*clp*) and C: DF41-469 (*gacS*) crude extracts dissolved in 80% methanol. The boxed regions show the region corresponding to the fraction demonstrating AF activity with a retention time of 27 minutes.

Water DF41 DF41-1278 DF41-469

Figure 5.5 Biosurfactant activity associated with culture extracts of *Pseudomonas* sp. DF41 (wild type), DF41-1278 (*clp*), and DF41-469 (*gacS*). HPLC fractions corresponding to the retention time of the AF metabolite were collected, dried and resuspended in water. The samples were stained with crystal violet and deposited onto a hydrophobic surface (parafilm). Note that the DF41 sample shows a modest degree of spreading; whereas the DF41-1278 and DF41-469 extracts, which are devoid of AF activity, more closely resemble the water control.

5.2.7 The DF41 AF metabolite is not regulated by AHLs

To determine if the AF metabolite was subject to AHL-mediated regulation, DF41 harbouring the AHL-degradation plasmid, pME6863, or grown in the presence of exogenous AHL was analyzed. Two approaches were employed to determine the effect of AHL addition or degradation on DF41 AF activity. First, strains were tested for *in vitro* inhibition of *S. sclerotiorum* in AF plate assays and second, culture extracts of DF41 were analyzed by HPLC. As shown in Table 5.3, DF41 AF activity was not altered by AHL addition or degradation. Since the CLP is a major biocontrol determinant, any regulatory elements affecting its production would be expected to have an effect on the overall AF activity of DF41.

Interestingly, for the CLP-deficient DF41-1278 harbouring the AHL-degradation plasmid, increased AF activity was observed. Moreover, addition of exogenous AHL resulted in slightly decreased inhibition (Table 5.3). Protease and HCN production were included in the assay to determine if any observed changes in AF activity could be due to changes in these metabolites; however, neither protease nor HCN appear to be AHL regulated.

To confirm these results, culture extracts of DF41, DF41 (pME6383) and DF41 grown in the presence of exogenous AHL were analyzed by HPLC separation. All strains were prepared as previously described in Section 2.34 and equal volumes of each sample were loaded onto the HPLC column. Similar peaks were observed for DF41 grown in the presence of exogenous AHL or in the absence due to AHL-degradation (Fig. 5.6). These results are consistent with the phenotypic assays, indicating that the AF metabolite is not subject to AHL-regulation.

Table 5.3 Effect of additional AHL and AHL degradation on phenotypic characteristics of DF41 and Tn*5* mutant DF41-1278.

Strain	AF[a]	Autoinducer[a]	Protease[a]	HCN[b]
DF41 (pME6032)	8.5 (0.7)	11.3 (0.9)	2.5 (0.5)	+
DF41 (AHL +++)	9.0 (0.6) [e]	13.5 (0.5) [d]	2.5 (0.5) [e]	+
DF41 (pME6863)	9.0 (0.8) [e]	0.0 (0.0) [c]	2.4 (0.4) [e]	+
DF41-1278 (pME6032)	2.0 (1.6)	11.2 (0.6)	2.7 (0.4)	+
DF41-1278 (AHL+++)	0.0 (0.0) [c]	13.2 (0.8) [d]	2.7 (0.3) [e]	+
DF41-1278 (pME6863)	5.0 (0.0) [c]	0.0 (0.0) [c]	2.8 (0.3) [e]	+

[a] Mean (SD) of the zones of activity (mm) from a minimum of three replicates.
[b] Determined using Cyantesmo paper.
[c] Significantly different ($p < 0.0001$).
[d] Significantly different ($p < 0.001$).
[e] Not significantly different.

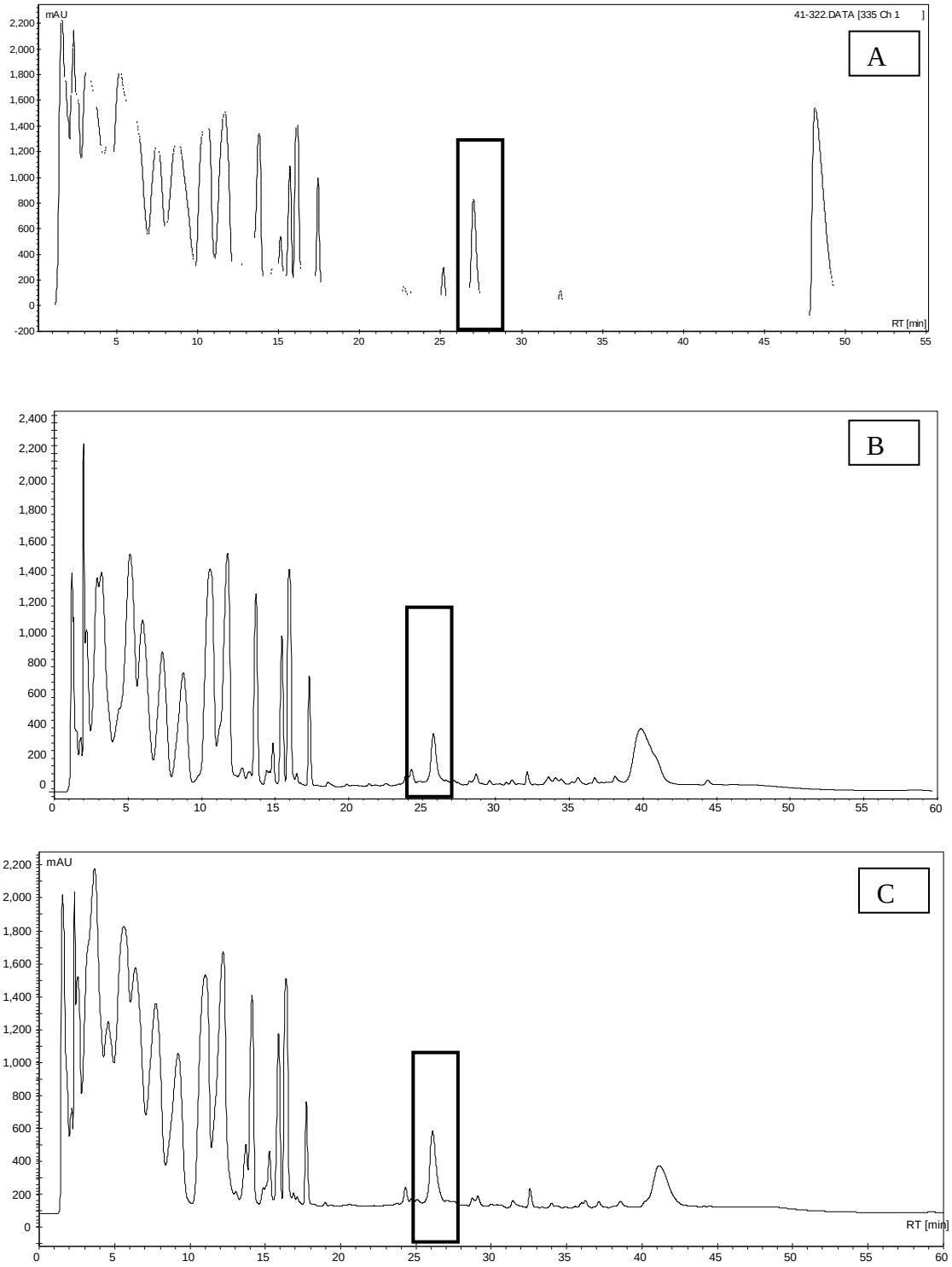


Figure 5.6 The effect of AHL degradation or the presence of additional AHL on production of the DF41 AF metabolite. Panel A: DF41 (pME6032); B: DF41 (AHL+); C: DF41 (pME6863). The rectangular box outlines the peak with AF activity and a retention time of 27 minutes.

5.2.8 Mass spectrometry analysis of the DF41 CLP

To determine the structure of the putative CLP molecule produced by DF41, culture extracts from DF41 and DF41-1278 were purified by HPLC separation and collected as one minute fractions for subsequent MALDI-TOF MS analysis. MS fragmentation and subsequent comparison of the ions present indicated the presence of two ions of 2095 m/z and 2123 m/z present in the DF41 fraction with an HPLC retention time of 27 minutes that were absent in DF41-1278 (Fig 5.4 and 5.7). These two ions were selected for second generation MS fragmentation which gave structural insight into the AF metabolite.

First, a partial amino acid sequence was proposed based on the fragmentation patterns of the parent ions (2095 m/z and 2123 m/z). The putative peptide is composed of a total number of 21 amino acids and contains leucine/isoleucine, threonine/homoserine, valine, alanine, proline and 2,3,-dehyhdroaminobutyric acid (Fig. 5.8). A difference in the MS fragmentation patterns of the two major ions was not observed, suggesting that the two ions are analogs with a difference either in a lipid moiety or in unidentified amino acids.

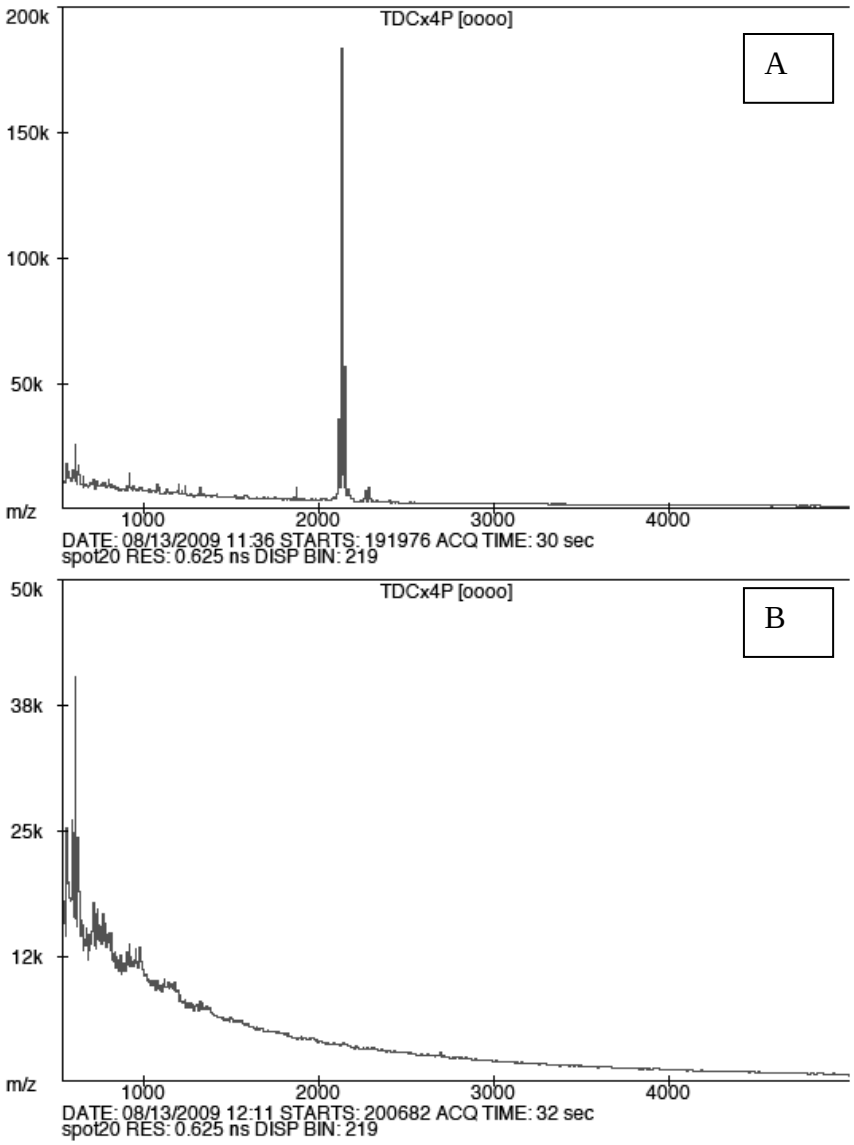


Figure 5.7 MALDI-TOF MS analysis of DF41 and 1278 HPLC peak with a retention time of 27 minutes. Panel A: MS profile of DF41 with a major ion of 2123 m/z. Panel B: MS profile of 1278 showing an absence of dominant ions. (MALDI and MS analysis done by Dr. Lynda Donald, Department of Chemistry, University of Manitoba)

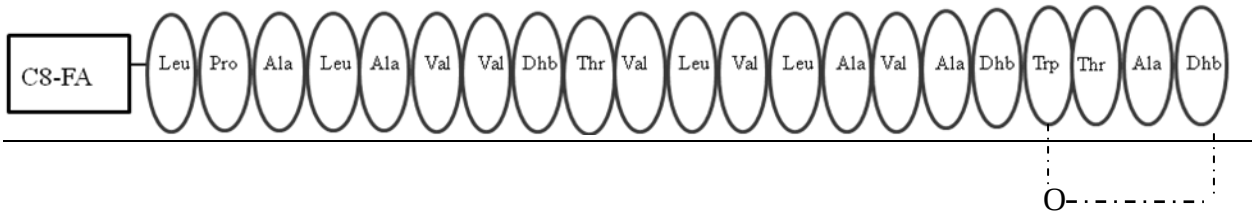


Figure 5.8 Putative sequence of the DF41 AF metabolite eluting at 27 minutes. MALDI-TOF MS analysis of two major ions (2095 m/z and 2123 m/z) yielded similar peptide sequences. Aea: 2,3-dehydroaminobutyric acid. Leu: leucine or Isoleucine; Thr: threonine or homoserine. The putative location of a lactone ring is shown, although experimental evidence suggests that the metabolite is linear, this result remains to be conclusively demonstrated.

5.3 Discussion

Pseudomonas strains produce an array of biologically active metabolites, a feature which contributes to their metabolic diversity and enables this genus to colonize a wide variety of ecological niches. Amongst environmental isolates, the production of biologically active compounds may provide a competitive edge to the producing strain, as many of these secondary metabolites are harmful to other microorganisms. One class of functionally diverse metabolites are the CLPs. CLPs are molecules with an amphipathic structure; thus it is not surprising that several CLP species are strong biosurfactants. In other instances, CLPs demonstrate AF activity and are promising biocontrol compounds.

The canola rhizosphere isolate *Pseudomonas* sp. DF41 was identified in a screen for bacteria displaying inhibition of the fungal pathogen *S. sclerotiorum* (Savchuk and Fernando 2004). Although this strain demonstrated significant inhibition of advancing fungal mycelia, the AF compounds being produced were unknown. To identify DF41 biocontrol factors, a Tn*5* mutagenesis strategy was employed which led to the identification of a mutant, DF41-1278, which was significantly attenuated in AF activity. Sequencing and Blastn analysis of the genomic region surrounding the insertion indicated that Tn*5* had interrupted a gene with homology to the condensation domains of NRPS genes from a number of CLP-producing bacteria. Currently, there are a total of ten entirely sequenced CLP biosynthetic templates. Interestingly, Blastn analysis of this sequence revealed high sequence identity with NRPS condensation domains of the tolaasin CLP group (Raaijmakers et al. 2006). This group of CLPs as classified by Raaijamkers et al. contains a higher number of amino acids in the peptide moiety (18-25) as well as a number of unusual amino acids such as 2,3,-dihydro-2-aminobutyric acid and

homoserine (Raaijmakers et al. 2006). In addition to tolaasin (Grewal et al. 1995), the other CLPs in this class, syringopeptin (Ballio, Barra et al. 1991), corpeptin (Emanuele et al. 1998) and fuscopeptin (Ballio et al. 1996) are all phytotoxins.

DF41-1278 consistently displayed reduced inhibition of *S. sclerotiorum* on AF plates. As protease activity, HCN and AHL production were similar to wild type, the reduced AF activity appears to be caused by the loss of the compound produced by the NRPS cluster. Consistent with this notion, when *S. sclerotiorum* was sprayed onto canola plants and challenged with DF41 and DF41-1278, the mutant was unable to prevent the development of sclerotinia stem rot symptoms on canola leaves and plant stems (Fig. 5.2).

Phenotypic analysis of the DF41 CLP molecule suggests that it may represent a novel class of CLPs. There are several factors supporting this idea. First, although CLPs are typically associated with biosurfactant activity, the metabolite produced by DF41 appears to have weak surface-tension reducing activity compared to other CLPs. The HPLC-purified AF compound as verified on AF plates, demonstrates some surface-tension reduction between a hydrophobic surface (parafilm) and a drop of water (Fig. 5.4), whereas the fractions collected from DF41-469 and DF41-1278 did not alter the shape of the water droplet. The activity observed was not as dramatic as that reported for CLPs from other *Pseudomonas* species such as putisolvins, which when added to an aqueous solution completely flatten the water droplet (Kuiper et al. 2004). CLPs often influence swarming motility on soft-agar plates as they function as wetting agents; however, DF41-1278 swarmed in a comparable fashion to the wild-type strain. A possible explanation for this finding is that in DF41, additional factors contribute to

swarming motility. There is some precedent for this as siderophores and lipopolysaccharide have been implicated in swarming motility of a *Salmonella typhimurium* strain (Wang, Frye et al. 2004). Furthermore, motility is reported to be reduced but not absent in *Pseudomonas* sp. deficient in production of the CLPs orfamide and putisolvin (Kuiper et al. 2004; Gross et al. 2007).

HPLC separation and MALDI-MS analysis were undertaken to gain insight into the structure of the DF41 CLP. To isolate the molecule, spent culture supernatants from DF41 and the mutants DF41-469 (*gacS*) and DF41-1278 (*clp*) were extracted with ethyl acetate and separated by RP-HPLC. This method consistently revealed the presence of a peak present in DF41 with a retention time of 27 minutes that was missing in the two mutants. Analysis of one-minute fractions revealed AF activity associated with the DF41 sample in which this peak eluted. To confirm that this peak represented the CLP molecule, DF41 and DF41-1278 compounds eluting from HPLC were collected at one-minute intervals between 20 and 35 minutes and analyzed by MALDI-MS. A comparison of the ions present in the fractions from the two strains identified two dominant ions of 2123 m/z and 2095 m/z as well as several smaller ions present in the DF41 fractions that were missing in DF41-1278. To determine the structure of the DF41 AF compound, the fractions were further analyzed by MALDI-TOF MS. The two ions that were identified in the 27-minute fraction are of a similar size to larger CLP molecules such as syringopeptin which has 22-25 amino acids in the peptide moiety and have highly similar molecular weights with corpeptin A and corpeptin B (2094 m/z and 2120 m/z respectively). Corpeptin A and corpeptin B have an identical amino acid

sequence in the peptide moiety and differ only in the nature of the attached lipid tail (Emanuele, Scaloni et al. 1998).

A comparison of the amino acid sequence of the DF41 CLP molecule with other CLPs revealed that its peptide sequence shares the most similarities with members of the tolaasin group. As depicted in Table 5.4, the DF41 CLP is hypothesized to contain the unusual amino acids, Dhb and Dab as well as a high number of hydrophobic amino acids which is characteristic of the CLPs in this group. Another defining trait of this group is the presence of Dhb before the threonine residue, a feature that our proposed sequence also includes. In contrast to the other members of the tolaasin group, MS analysis of the CLP molecule strongly suggests that it is a linear molecule as a decreased mass of 18 Da was not observed upon exposure to various ring-opening treatments. There are currently two reported linear *Pseudomonas* sp. lipopeptides produced by NRPS templates, syringafactin and peptin 31, both of which are produced by *P. syringae* strains (Berti et al. 2007; Fiore et al. 2008). Furthermore, linear lipopeptides have been reported to have decreased activity compared to their cyclic counterparts as cyclization has been found to stabilize the molecule by reducing conformational freedom (Sieber and Marahiel 2003). This may help to explain the lower level of biosurfactant activity and non-existent impact on swarming motility associated with the DF41 molecule.

CLPs are known to be regulated by a number of factors, including the global regulatory system, GacS/GacA and AHL-mediated QS. To ascertain if one or both of these systems was underlying production of the CLP molecule, the *gacS* mutant, DF41-469 was analyzed by HPLC. This strain revealed a lack of peaks in the region corresponding to the ions of 2123 and 2095 Da and completely lacks AF activity, thus

Table 5.4 Alignment of the proposed DF41 CLP structure with members of the tolaasin CLP class

Amino Acid no.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
CorpeptinA	C_{10}HO acid	Dhb	Pro	Ala	Ala	Ala	Val	Val	Dhb	Hse	Val	*a*Ile	Dhp	Ala	Ala	Ala	Val	Dhb	*a*Thr	Ala	Dab	Ser	Ile			
Tolaasin	C_8HO acid	Dhb	Pro	Ser	Leu	Val	Ser	Leu	Val	Val	Gln	Leu	Val	Dhb	*a*Thr	Ile	Hse	Dab	Lys							
Syringopeptin 25	FA	Dhb	Pro	Val	Ala	Ala	Val	Leu	Ala	Ala	Dhb	Val	Dhb	Ala	Val	Ala	Ala	Dhb	*a*Thr	Ser	Ala	Val	Ala	Dab	Dab	Tyr
Syringopeptin 22	FA	Pro	Val	Leu	Ala	Ala	Leu	Val	Dhp	Ala	Val	Ala	Ala	Dhb	*a*Thr	Ser	Ala	Dhb	Ala	Dab	Dab	Tyr				
DF41	C8	Leu	Pro	Ala	Leu	Ala	Val	Val	Dhb	Thr	Val	Leu	Val	Leu	Ala	Val	Ala	Dhb	Trp	Thr	Ala	Dhb				
Syringafactin	FA	Leu	Leu	Gln	Leu	Thr	Val	Leu	Leu																	

Table Legend:
Dab = 2,4-diaminobutyric acid
Dhb = 2,3,-dehyhdroaminobutyric acid
Dhp = dehydro-2-aminopropanoic acid
Dhb = 2-aminobutyric-2-enoic-acid= 2,3-dehydro-2-aminobutyric acid

supporting that CLP regulation in DF41 is Gac regulated. This is an expected result as the role of the Gac system in CLP production has been described for a number of molecules such as entolysin (Vallet-Gely et al. 2010), putisolvin (Dubern et al. 2005) and tolaasin (Grewal et al. 1995). AHLs are widespread regulators of secondary metabolites in *Pseudomonas* sp.; however, the role of this system in CLP production is much less common. Since an AHL-based QS system was identified in DF41, we were interested in determining if it was a factor in CLP regulation. Our results indicated that DF41 AF activity was not affected by the addition or degradation of AHL. Furthermore, analysis of culture extracts revealed no significant change in the size of the peak eluting at 27 minutes.

In summary, our results indicate that DF41 produces a NRPS-synthesized lipopeptide which is an essential metabolite for this strain's biocontrol activity. Our preliminary peptide sequencing data indicate that a linear lipopeptide is being produced. This CLP most closely resembles members of the tolaasin group, as deduced by the size of the intact molecule and the nature of the amino acids in the peptide head. Further chemical analysis such as NMR is required to resolve the remaining structural ambiguities, including: i) the identity of the remaining amino acids postulated to be present; ii) the isomers of the amino acids; and iii) the structure of the lipid moiety. In addition, sequencing of the entire NRPS template would be advantageous as it may enable the identification of additional regulatory elements underlying CLP production since regulatory genes are often located in the regions flanking the NRPS biosynthetic genes.

Acknowledgements

Much of the structural analysis described in this chapter was performed with assistance from Lynda Donald in the Department of Chemistry. I am indebted to Lynda for the MS and MALDI-TOF MS analysis together with her help interpreting the data.

Chapter 6

Thesis General Conclusions and Future Perspectives

6.1 Thesis Conclusions

Biological control strains present an intriguing alternative to chemical pesticides currently in use to combat a number of economically relevant fungal pathogens. Several *Pseudomonas* species are associated with the production of secondary metabolites which are antagonistic to other microorganisms and nematodes. One such promising isolate is *Pseudomonas sp.* DF41 which was isolated from the canola rhizosphere and tested for inhibition of the fungal pathogen *S. sclerotiorum*. DF41 demonstrated significant biocontrol activity against this pathogen, significantly reducing mycelial growth and germtube elongation (Savchuk and Fernando 2004). Furthermore, it was able to successfully colonize and persist on the canola phylloplane for several days (Savchuk and Fernando 2004). These preliminary experiments demonstrated the biocontrol potential of this strain; however, the mechanisms underlying its antagonistic activity had yet to be elucidated.

We employed a random transpogenesis approach to identify DF41 genes essential for biocontrol. The first mutant that was identified and characterized had an insertion in a global regulatory gene, *gacS*. Homologs of this gene have been described for a number of other bacterial species, including several Pseudomonads. The GacS/GacA system was determined to play an essential role in DF41 biocontrol activity as a mutation in the sensor kinase gene *gacS* affected a number of important traits. In a DF41 *gacS*-minus derivative, both *in vitro* and *in planta* AF activity was abolished, as were protease production and swarming motility. Now that we have clearly established that GacS is a master regulator of *Pseudomonas sp.* DF41 biocontrol activity, several questions remain

to be addressed. First, the gene encoding the cognate response regulator *gacA* has not been identified in this strain, the elucidation of which may provide further insight into the regulatory cascade. Second, the nature of the cue or signals believed to trigger Gac phosphorylation is not known (Heeb et al. 2002; Zuber et al. 2003; Dubuis and Haas 2007). Moreover, factors involved in regulation of the Gac/Rsm system have yet to be determined. In *P. aeruginosa* and *P. fluorescens* CHA0, two, additional sensors, LadS (lost adherence) and RetS (regulator of exopolysaccharide and type III secretion) activate and inhibit the Gac/Rsm pathway respectively, possibly by direct interaction with GacS (Goodman et al. 2004; Laskowski and Kazmierczak 2006; Ventre et al. 2006; Goodman et al. 2009; Humair et al. 2009; Workentine et al. 2009). One could speculate that similar elements might influence the Gac/Rsm pathway in DF41.

A number of studies have determined that the GacS/GacA cascade exerts its regulatory effects via upregulation of small non-coding RNA molecules which in turn counter translational repression of target genes by RNA-binding proteins. We were able to successfully PCR-amplify and clone an *rsmZ* homolog from DF41 and demonstrate that it is a downstream regulatory element in the Gac regulon. RsmZ was found to function as a positive activator of genes involved in DF41 biocontrol activity. Although we have identified a single regulatory RNA species, RsmZ, homologs of the RNA-binding proteins have not yet been identified. The identification of additional sRNA species and RsmA homologs would enable a study of their interaction and regulation. One possible regulator of the Rsm components is PsrA, a transcriptional activator of *rpoS* expression and an inhibitor of fatty acid degradation (Kojic et al. 2005; Kang et al. 2009). In *P. fluorescens* CHA0, PsrA has been found to induce expression of the *rsmZ* promoter

(Humair et al. 2010). The mechanisms governing Gac regulation of *rsmZ* in DF41 also remain to be determined. Sequences upstream of sRNA genes in *P. aeruginosa* and *P. fluorescens* CHA0 have been identified sequences which are essential for GacA activation (Brencic et al. 2009; Humair et al. 2010). A detailed analysis of DF41 promoter elements may shed further insight into the regulatory elements controlling the Rsm system.

Another regulatory system that is in part controlled by Gac/Rsm is QS. AHL production in DF41 was detected using a bioreporter strain which responds to AHL signals with acyl side chains between four and eight carbons. We identified an AHL synthase gene, designated *pdfI* that displays a high level of sequence identity with the *P. corrugata pcoI* gene. A second ORF located immediately downstream of *pdfI* also displayed high homology to another *P. corrugata* gene, *rfiA*, a non, AHL-binding, transcriptional activator gene which is cotranscribed with *pcoI* and is thus intricately linked to QS (Licciardello et al. 2009). Interestingly, in *P. corrugata*, RfiA regulates secretion of the CLPs corpeptin A and corpeptin B (Licciardello et al. 2009) via a multidrug efflux system located downstream of the *rfiA* gene (Licciardello et al. 2009). We also identified a partial sequence in DF41 located downstream of the *rfiA* homolog which had high sequence identity to this efflux system. Given the highly similar genetic arrangement in DF41, it would be interesting to determine the roles of the DF41 *rfiA* and efflux system homologs in DF41 CLP secretion and biocontrol activity.

We were able to mimic an AHL-minus derivative of DF41 by introducing the *B. subtillus* AHL lactonase-encoding gene into DF41 and Tn*5* derivatives DF41-469 and DF41-1278. This enabled us to study the role of AHLs in DF41 biocontrol activity.

Although QS regulates an array of traits contributing to both virulence and AF activity in many other pseudomonads, we did not detect changes in protease, HCN or CLP production. Not surprisingly, AF activity remained unchanged in the AHL-deficient strain. A phenotype that was affected, swimming and swarming motility, appear to be negatively regulated by AHLs. Notably, a similar effect on swarming motility has been reported for *P. corrugata* (Licciardello et al. 2007). In *P. corrugata*, the cognate QS PcoR activator was found to have a more pronounced regulatory effect on the production of virulence determinants than did AHL signalling (Licciardello et al. 2007; Licciardello et al. 2009). Whether or not the DF41 QS system operates in a similar manner remains to be determined. It would be beneficial to identify and characterize the role of a *pcoR* homolog in DF41 as it may be an important regulator of CLPs and other secondary metabolites.

To gain insight into the interaction between different regulatory elements in DF41, we determined the expression of *rpoS, pdfI,*and *rsmZ* transcriptional fusions in the wild type and the *gacS*-minus strain. β-galactosidase assays revealed that GacS influences *rpoS* and *pdfI* expression, although it doesn't appear to be the sole regulator. In the *gacS* mutant, *rpoS* and *pdfI* expression were significantly decreased but not turned off completely, indicating that other regulatory elements are at play. As expected, *rsmZ* expression was nearly abolished in the *gacS* mutant compared to the wild type.

The effect of RsmZ overexpression yielded some interesting results. In DF41, *rsmZ* expression increased in the presence of multiple copies of RsmZ, which is contrary to the results reported for *P. aeruginosa* in which *rsmZ* expression was substantially increased in an *rsmZ*-deletion mutant (Heurlier et al. 2004). Furthermore, RsmZ

overexpression strongly increased *pdfI* expression, but was found to negatively impact *rpoS* expression. The regulation of *rpoS* appears to involve additional regulators, as *rpoS* expression increased in the *gacS* minus background in the presence of multiple copies of RsmZ. The conflicting results obtained for the *gacS* mutant could be due to the fact that there are decreased levels of other regulators, including AHL, RpoS and RsmZ, present in this strain. We also discovered that AHLs negatively affect *rpoS* and *rsmZ* expression; so it would appear that interactions between these regulators enable fine-tuning of gene expression in DF41. The construction of *rpoS,* QS and *rsmZ* null mutants would help to clarify the interactions occurring between these different regulatory systems. The findings outlined above are summarized in Figure 6.1 which depicts the regulatory cascade underlying DF41 biological control activity.

A main determinant of DF41 biocontrol activity is proposed to be a CLP molecule which is synthesized via an NRPS template. We have deduced a significant portion of the peptide moiety constituting the novel DF41 CLP; the sequence of which is most similar to the phytotoxic tolaasin group. A number of experiments are required to confirm our proposed sequence. NMR should enable a determination of the amino acid isomers in the peptide head and resolve discrepancies between the amino acids leucine and isoleucine which, due to identical masses, are not distinguishable by the MS experiments. NMR will also enable identification of the lipid moiety attached to the peptide. Sequence elucidation of the entire NRPS biosynthetic operon would further validate our proposed CLP structure. NRPS genes contain conserved residues for the incorporation of a single amino acid. *In silico* analysis would enable prediction of the nature and sequence of amino acids present in the peptide moiety. In addition, because

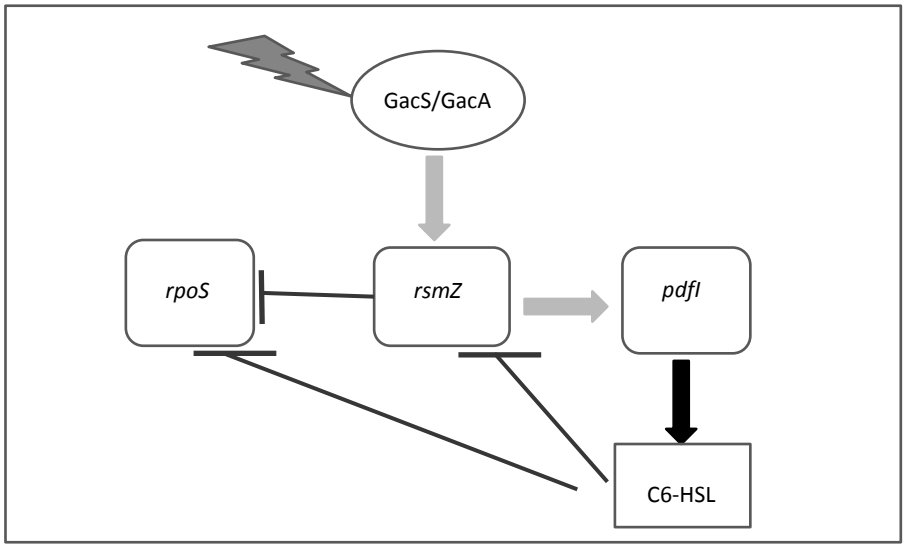


Figure 6.1 Proposed regulatory hierarchy underlying DF41 biological control activity.

genes involved in the regulation of CLPs are often located in the NRPS flanking regions, these sequences may provide important clues regarding how these molecules are regulated.

CLPs often have varying biological activity depending on their concentration and environment. For example, corpeptin A and B are associated with phytotoxicity and virulence on tomato plants, but also exhibit antibacterial activity (Licciardello et al. 2009). Since the CLP produced by DF41 is most closely related to a group of phytotoxins, it is tempting to speculate that it may exhibit toxic effects on a different host. It should be emphasized that DF41 does not display any discernable toxicity towards canola plants. Furthermore, we have only tested the biocontrol activity of DF41 against *S. sclerotiorum* on the canola phylloplane. As a rhizosphere isolate, one might expect that it would exhibit biological activity against additional root-associated pathogens.

In summary, we have identified and examined the roles of different regulatory systems on DF41 biocontrol activity and outlined a number of traits which may contribute to the AF activity of this novel and promising biocontrol agent. Although further studies are required to fully understand the intricacies and timing of secondary metabolite production in DF41, our results clearly demonstrate the important role of the Gac/Rsm cascade as a master regulator of DF41 biocontrol. QS may be found to contribute to secondary metabolism as well, but it is not likely to be AHL-mediated. In *P. corrugata,* the LuxR-type transcriptional activator, PcoR, binds AHL to activate the expression of genes mediating virulence on tomato plants (Licciardello et al. 2009). However, an AHL-minus mutant was not significantly affected in virulence. In contrast,

deletion of the *pcoR* gene did significantly decrease the manifestation of disease symptoms in tomato plants, suggesting that the PcoR protein is more important for gene expression than PcoR-AHL complexes (Licciardello et al. 2009). The authors of this intriguing study have suggested that PcoR may function directly in the presence of AHL or indirectly in its absence to activate target gene expression (Licciardello et al. 2009). Based on these results and the high degree of similarity between the *P. corrugata* and DF41 QS systems, it is possible that DF41 gene expression may be more dependent on LuxR regulation than on AHL molecules.

We have also identified this unique CLP molecule as a substantial inhibitor of *S. sclerotiorum* stem rot of canola. Direct application of the purified CLP onto plants would help to ascertain the full activity of this molecule. Smaller nonapeptide CLPs along with derivatives of the larger, predominant CLP are often secreted by strains producing the larger molecules. Although we have not undertaken a search for other CLPs in DF41, additional MS experiments could be conducted to achieve this goal. Finally, DF41 has never been given a species designation despite 16SrDNA sequencing and Biolog^{TM} analysis; therefore, it may represent a novel *Pseudomonas* biocontrol species.

References

Aarons, S., A. Abbas, et al. (2000). "A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113." J Bacteriol **182**(14): 3913-3919.

Ahl, P., C. Voisard, et al. (1986). "Iron bound-siderophores, cyanic acid, and antibiotics involved in suppression of *Thielaviopsis basicola* by a *Pseudomonas fluorescens* strain." J. Phytopathology **116**: 121-134.

Andersen, J. B., B. Koch, et al. (2003). "Surface motility in *Pseudomonas* sp. DSS73 is required for efficient biological containment of the root-pathogenic microfungi *Rhizoctonia solani* and *Pythium ultimum*." Microbiology **149**(Pt 1): 37-46.

Anjaiah, V., N. Koedam, et al. (1998). "Involvement of Phenazines and Anthranilate in the Antagonism of *Pseudomonas aeruginosa* PNA1 and Tn*5* Derivatives Toward *Fusarium* spp. and *Pythium* spp." Molecular Plant-Microbe Interactions **11**(9): 847-854.

Anson, M. L. (1938). "The Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Hemoglobin." J Gen Physiol **22**(1): 79-89.

Baehler, E., M. Bottiglieri, et al. (2005). "Use of green fluorescent protein-based reporters to monitor balanced production of AF compounds in the biocontrol agent *Pseudomonas fluorescens* CHA0." J Appl Microbiol **99**(1): 24-38.

Bailey, D. M., Johnson, R. E., and Salvador, U. J. (1973). "Pyrrole antibacterial agents. 1. Compounds related to pyoluteorin." J Med Chem **16**(11): 1298-1300.

Balibar, C. J., F. H. Vaillancourt, et al. (2005). "Generation of D amino acid residues in assembly of arthrofactin by dual condensation/epimerization domains." Chem Biol **12**(11): 1189-1200.

Ballio, A., D. Barra, et al. (1991). "Syringopeptins, new phytotoxic lipodepsipeptides of *Pseudomonas syringae* pv. *syringae*." FEBS Lett **291**(1): 109-112.

Ballio, A., F. Bossa, et al. (1996). "Structure of fuscopeptins, phytotoxic metabolites of *Pseudomonas fuscovaginae*." FEBS Lett **381**(3): 213-216.

Bangera, M. G. and L. S. Thomashow (1999). "Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87." J Bacteriol **181**(10): 3155-3163.

Barrett, D. (2002). "From natural products to clinically useful AFs." Biochim Biophys Acta **1587**(2-3): 224-233.

Bateman, D. F. and S. V. Beer (1965). "Simultaneous Production and Synergistic Action of Oxalic Acid and Polygalacturonase during Pathogenesis by *Sclerotium rolfsii*." Phytopathology **55**: 204-211.

Beirne, B. P. (1967). "Biological control and its potential." World Rev. Pest Control **6** 7-20.

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

Bergendahl, V., U. Linne, et al. (2002). "Mutational analysis of the C-domain in nonribosomal peptide synthesis." Eur J Biochem **269**(2): 620-629.

Bertani, I., G. Rampioni, et al. (2007). "The *Pseudomonas putida* Lon protease is involved in *N*-acyl homoserine lactone quorum sensing regulation." BMC Microbiol **7**: 71.

Bertani, I. and V. Venturi (2004). "Regulation of the *N*-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase RpoS sigma factor and the global regulator GacA." Appl Environ Microbiol **70**(9): 5493-5502.

Berti, A. D., N. J. Greve, et al. (2007). "Identification of a biosynthetic gene cluster and the six associated lipopeptides involved in swarming motility of *Pseudomonas syringae* pv. tomato DC3000." J Bacteriol **189**(17): 6312-6323.

Birnboim, H. C. and J. Doly (1979). "A rapid alkaline extraction procedure for screening recombinant plasmid DNA." Nucleic Acids Res **7**(6): 1513-1523.

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

Blumer, C., S. Heeb, et al. (1999). "Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites." Proc Natl Acad Sci U S A **96**(24): 14073-14078.

Bradford, M. M. (1976). "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. " Anal Biochem **72**: 248-254.

Brencic, A., K. A. McFarland, et al. (2009). "The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs." Mol Microbiol **73**(3): 434-445.

Britigan, B. E., T. L. Roeder, et al. (1992). "Interaction of the *Pseudomonas aeruginosa* secretory products pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to endothelial cells. Implications for *Pseudomonas*-associated tissue injury." J Clin Invest **90**(6): 2187-2196.

Brodhagen, M., Henkels, M. D., Loper, J. E. (2003). "Regulation of the antibiotic pyoluteorin in *Pseudomonas fluorescens* Pf-5 includes positive autoregulation and repression by 2,4-diacetylphloroglucinol and pyoluteorin." Phytopathology **93**: S11.

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

Burke, T., Jr., B. Chandrasekkar, et al. (1999). Analogs of viscosin and uses therof. U.S.A. **5,965,524**.

Busscher, H. J., C. G. van Hoogmoed, et al. (1997). "*Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *Candida* spp. on silicone rubber." Appl Environ Microbiol **63**(10): 3810-3817.

Byford, M. F., J. E. Baldwin, et al. (1997). "The Mechanism of ACV Synthetase." Chem Rev **97**(7): 2631-2650.

Cessna, S. G., V. E. Sears, et al. (2000). "Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant." Plant Cell **12**(11): 2191-2200.

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

Chatterjee, A., Y. Cui, et al. (1995). "Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. *carotovora* in the absence of the starvation/cell density-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone." Appl Environ Microbiol **61**(5): 1959-1967.

Chatterjee, A., Y. Cui, et al. (2003). "GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. *tomato* DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors." Mol Plant Microbe Interact **16**(12): 1106-1117.

Chin-A-Woeng, T. F., D. van den Broek, et al. (2001). "Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium." Mol Plant Microbe Interact **14**(8): 969-979.

Chin-A-Woeng, T. F. C., Bloemberg, G.V., van der Bij, A.J., van der Drift, K.M.G.M., Schripsema, J., Kroon, B., Scheffer, R.J., Keel, C., Bakker, P.A.H.M., Tichy, H.V., de Bruijn, F.J., Thomas-Oates, J.E., Lugtenberg, B.J.J. (1998). "Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*." Mol. Plant-Microbe Interact. **11**: 1069-1077.

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

Compant, S., B. Duffy, et al. (2005). "Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects." Appl Environ Microbiol **71**(9): 4951-4959.

Conti, E., T. Stachelhaus, et al. (1997). "Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S." EMBO J **16**(14): 4174-4183.

Cook, R. J., and Rovira, A.D. (1976). "The role of bacteria in the biological control of *Gaeumannomyces graminis* by suppressive soils." Soil. Biol. Biochem. **8**: 269-273.

Cook, R. J., Thomashow, L. S., Weller, D. M., Fujimoto, D., Mazzola, M., Bangera, G., Kim, D. S. (1995). "Molecular mechanisms of defense by rhizobacteria against root disease." Proc Natl Acad Sci U S A **92**(10): 4197-4201.

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

Costerton, J. W., Z. Lewandowski, et al. (1995). "Microbial biofilms." Annu Rev Microbiol **49**: 711-745.

Cotton, P., Z. Kasza, et al. (2003). "Ambient pH controls the expression of endopolygalacturonase genes in the necrotrophic fungus *Sclerotinia sclerotiorum*." FEMS Microbiol Lett **227**(2): 163-169.

Cowing, D. W., J. C. Bardwell, et al. (1985). "Consensus sequence for *Escherichia coli* heat shock gene promoters." Proc Natl Acad Sci U S A **82**(9): 2679-2683.

Cui, X., R. Harling, et al. (2005). "Identification of *N*-3-hydroxyoctanolyl-homoserine lactone production in *Pseudomonas fluorescens* 5064, pathogenic to broccoli, and

controlling biosurfactant production by quorum sensing." Eur. J. Plant. Pathol. **111**: 297-308.
Cui, Y., A. Chatterjee, et al. (1995). "Identification of a global repressor gene, *rsmA*, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, *N*-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp." J Bacteriol **177**(17): 5108-5115.
Cuppels, D. A., R. D. Stipanovic, et al. (1987). "The constitution and properties of a pyochelin-zinc complex." Can. J. Chem. **65**: 2126-2130.
Danhorn, T. and C. Fuqua (2007). "Biofilm formation by plant-associated bacteria." Annu Rev Microbiol **61**: 401-422.
Davey, M. E., N. C. Caiazza, et al. (2003). "Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1." J Bacteriol **185**(3): 1027-1036.
de Bruijn, I., M. J. de Kock, et al. (2008). "Massetolide A biosynthesis in *Pseudomonas fluorescens*." J Bacteriol **190**(8): 2777-2789.
de Bruijn, I., M. J. de Kock, et al. (2007). "Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in *Pseudomonas* species." Mol Microbiol **63**(2): 417-428.
de Bruijn, I. and J. M. Raaijmakers (2009). "Diversity and functional analysis of LuxR-type transcriptional regulators of cyclic lipopeptide biosynthesis in *Pseudomonas fluorescens*." Appl Environ Microbiol **75**(14): 4753-4761.
de Bruijn, I. and J. M. Raaijmakers (2009). "Regulation of cyclic lipopeptide biosynthesis in *Pseudomonas fluorescens* by the ClpP protease." J Bacteriol **191**(6): 1910-1923.
de Kievit, T., P. C. Seed, et al. (1999). "RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*." J Bacteriol **181**(7): 2175-2184.
de Kievit, T. R. and B. H. Iglewski (1999). "Quorum sensing, gene expression, and *Pseudomonas* biofilms." Methods Enzymol **310**: 117-128.
de Souza, J. T., and Raaijmakers, J.M. (2003). "Polymorphisms within the *prnD* and *pltC* genes from pyrrolnitrin and pyoluteorin- producing *Pseudomonas* and *Burkholderia* spp." FEMS Microbiol Ecol **43**: 21-34.
de Souza, J. T., Arnould, C., Deulvot, C., Lemanceau, P., Gianinazzi-Pearson, V., Raaijmakers, J.M. (2003). "Effect of 2,4-Diacetylphloroglucinol on *Pythium*: Cellular Responses and Variation in Sensitivity Among Propagules and Species." Phytopathology **93**(8): 966-975.
de Souza, J. T., M. Mazzola, et al. (2003). "Conservation of the response regulator gene *gacA* in *Pseudomonas* species." Environ Microbiol **5**(12): 1328-1340.
Delaney, S. M., D. V. Mavrodi, et al. (2001). "*phzO*, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30-84." J Bacteriol **183**(1): 318-327.
Dieckmann, R., Y. O. Lee, et al. (1995). "Expression of an active adenylate-forming domain of peptide synthetases corresponding to acyl-CoA-synthetases." FEBS Lett **357**(2): 212-216.
Doekel, S. and M. A. Marahiel (2001). "Biosynthesis of natural products on modular peptide synthetases." Metab Eng **3**(1): 64-77.

Dubern, J. F. and G. V. Bloemberg (2006). "Influence of environmental conditions on putisolvins I and II production in *Pseudomonas putida* strain PCL1445." FEMS Microbiol Lett **263**(2): 169-175.

Dubern, J. F., E. R. Coppoolse, et al. (2008). "Genetic and functional characterization of the gene cluster directing the biosynthesis of putisolvin I and II in *Pseudomonas putida* strain PCL1445." Microbiology **154**(Pt 7): 2070-2083.

Dubern, J. F., E. L. Lagendijk, et al. (2005). "The heat shock genes *dnaK*, *dnaJ*, and *grpE* are involved in regulation of putisolvin biosynthesis in *Pseudomonas putida* PCL1445." J Bacteriol **187**(17): 5967-5976.

Dubern, J. F., B. J. Lugtenberg, et al. (2006). "The *ppuI-rsaL-ppuR* quorum-sensing system regulates biofilm formation of *Pseudomonas putida* PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II." J Bacteriol **188**(8): 2898-2906.

Dubuis, C. and D. Haas (2007). "Cross-species GacA-controlled induction of antibiosis in pseudomonads." Appl Environ Microbiol **73**(2): 650-654.

Duffy, B. K. and G. Defago (1997). "Zinc Improves Biocontrol of *Fusarium* Crown and Root Rot of Tomato by *Pseudomonas fluorescens* and Represses the Production of Pathogen Metabolites Inhibitory to Bacterial Antibiotic Biosynthesis." Phytopathology **87**(12): 1250-1257.

Duffy, B. K. and G. Defago (2000). "Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains." Appl Environ Microbiol **66**(8): 3142-3150.

Ehmann, D. E., C. A. Shaw-Reid, et al. (2000). "The EntF and EntE adenylation domains of *Escherichia coli* enterobactin synthetase: sequestration and selectivity in acyl-AMP transfers to thiolation domain cosubstrates." Proc Natl Acad Sci U S A **97**(6): 2509-2514.

Emanuele, M. C., A. Scaloni, et al. (1998). "Corpeptins, new bioactive lipodepsipeptides from cultures of *Pseudomonas corrugata*." FEBS Lett **433**(3): 317-320.

Fernando, W. G. D., R. Ramarathnam, et al. (2005). "Identification and use of potential bacterial organic AF volatiles in biocontrol." Soil. Biol. Biochem. **37**: 955-964.

Finan, T. M., B. Kunkel, et al. (1986). "Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes." J Bacteriol **167**(1): 66-72.

Finking, R. and M. A. Marahiel (2004). "Biosynthesis of nonribosomal peptides1." Annu Rev Microbiol **58**: 453-488.

Fiore, A., J. M. Laparra, et al. (2008). "Lipodepsipeptides from *Pseudomonas syringae* are partially proteolyzed and are not absorbed by humans: an in vitro study." J Food Prot **71**(5): 979-985.

Fiore, A., L. Mannina, et al. (2008). "Bioactive lipopeptides of ice-nucleating snow bacterium *Pseudomonas syringae* strain 31R1." FEMS Microbiol Lett **286**(2): 158-165.

Folin, O. a. C., V. (1927). "On Tyrosine and Tryptophane Determinations in Proteins." J. Biol. Chem **73**: 627-650.

Fuqua, C., S. C. Winans, et al. (1996). "Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators." Annu Rev Microbiol **50**: 727-751.

Fuqua, W. C., S. C. Winans, et al. (1994). "Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators." J Bacteriol **176**(2): 269-275.

Gambello, M. J. and B. H. Iglewski (1991). "Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression." J Bacteriol **173**(9): 3000-3009.

Ge, Y. H., Pei, D. L., Zhao, Y. H., Li, W. W., Wang, S. F., Xu, Y. Q. (2007). "Correlation between AF agent phenazine-1-carboxylic acid and pyoluteorin biosynthesis in *Pseudomonas* sp. M18." Curr Microbiol **54**(4): 277-281.

Geesey, G. G., W. T. Richardson, et al. (1977). "Microscopic examination of natural sessile bacterial populations from an alpine stream." Can J Microbiol **23**(12): 1733-1736.

Georgakopoulos, D. G., M. Hendson, et al. (1994). "Cloning of a phenazine biosynthetic locus of *Pseudomonas aureofaciens* PGS12 and analysis of its expression in vitro with the ice nucleation reporter gene." Appl Environ Microbiol **60**(8): 2931-2938.

Gerard, J., R. Lloyd, et al. (1997). "Massetolides A-H, antimycobacterial cyclic depsipeptides produced by two pseudomonads isolated from marine habitats." J Nat Prod **60**(3): 223-229.

Girard, V., M. Fevre, et al. (2004). "Involvement of cyclic AMP in the production of the acid protease Acp1 by *Sclerotinia sclerotiorum.*" FEMS Microbiol Lett **237**(2): 227-233.

Godoy, G., Steadman, J.R., Dickman, M.B., and Dam, R. (1990). "Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*." Physiol. Mol. Plant Pathol. **37**: 179-191.

Goodier, R. I. and B. M. Ahmer (2001). "SirA orthologs affect both motility and virulence." J Bacteriol **183**(7): 2249-2258.

Goodman, A. L., B. Kulasekara, et al. (2004). "A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*." Dev Cell **7**(5): 745-754.

Goodman, A. L., M. Merighi, et al. (2009). "Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen." Genes Dev **23**(2): 249-259.

Grewal, S. I., B. Han, et al. (1995). "Identification and characterization of a locus which regulates multiple functions in *Pseudomonas tolaasii*, the cause of brown blotch disease of *Agaricus bisporus.*" J Bacteriol **177**(16): 4658-4668.

Gross, D. C. (1985). "Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. syringae and defined conditions for its production." J Appl Bacteriol **58**(2): 167-174.

Gross, H. and J. E. Loper (2009). "Genomics of secondary metabolite production by *Pseudomonas* spp." Nat Prod Rep **26**(11): 1408-1446.

Gross, H., V. O. Stockwell, et al. (2007). "The genomisotopic approach: a systematic method to isolate products of orphan biosynthetic gene clusters." Chem Biol **14**(1): 53-63.
Grunewald, J. and M. A. Marahiel (2006). "Chemoenzymatic and template-directed synthesis of bioactive macrocyclic peptides." Microbiol Mol Biol Rev **70**(1): 121-146.
Guenzi, E., G. Galli, et al. (1998). "Characterization of the syringomycin synthetase gene cluster. A link between prokaryotic and eukaryotic peptide synthetases." J Biol Chem **273**(49): 32857-32863.
Gurusiddaiah, S., D. M. Weller, et al. (1986). "Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and Pythium spp." Antimicrob Agents Chemother **29**(3): 488-495.
Haas, D., C. Blumer, et al. (2000). "Biocontrol ability of fluorescent pseudomonads genetically dissected: importance of positive feedback regulation." Curr Opin Biotechnol **11**(3): 290-297.
-Haas, D. and G. Defago (2005). "Biological control of soil-borne pathogens by fluorescent pseudomonads." Nat Rev Microbiol **3**(4): 307-319.
Haas, D. and C. Keel (2003). "Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease." Annu Rev Phytopathol **41**: 117-153.
Haas, D., C. Keel, et al. (2002). "Signal transduction in plant-beneficial rhizobacteria with biocontrol properties." Antonie Van Leeuwenhoek **81**(1-4): 385-395.
Hallman, J., A. Quadt-Hallman, W. F. Mahafee, and J. W. Kloepper. (1997). "Bacterial endophytes in agricultural crops." Can. J. Microbiol. **43**: 895-914.
Hansen, M., C. Thrane, et al. (2000). "Confocal imaging of living fungal hyphae challenged with the AF antagonist." Mycologia **92**: 216-221.
Hanzelka, B. L. and E. P. Greenberg (1995). "Evidence that the N-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain." J Bacteriol **177**(3): 815-817.
Harrison, L., D. B. Teplow, et al. (1991). "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum AF activity." J Gen Microbiol **137**(12): 2857-2865.
Heeb, S., C. Blumer, et al. (2002). "Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0." J Bacteriol **184**(4): 1046-1056.
Heeb, S. and D. Haas (2001). "Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria." Mol Plant Microbe Interact **14**(12): 1351-1363.
Heeb, S., K. Heurlier, et al. (2004). Post-transcriptional regulation in *Pseudomonas* spp. via the Gac/Rsm regulatory network. *Pseudomonas*. J. L. Ramos. New York, N.Y., Kluwer Academic Publishers. **2:** 239-255.
Heeb, S., C. Valverde, et al. (2005). "Role of the stress sigma factor RpoS in GacA/RsmA-controlled secondary metabolism and resistance to oxidative stress in *Pseudomonas fluorescens* CHA0." FEMS Microbiol Lett **243**(1): 251-258.

Hernandez, M. E., A. Kappler, et al. (2004). "Phenazines and other redox-active antibiotics promote microbial mineral reduction." Appl Environ Microbiol **70**(2): 921-928.
Heurlier, K., F. Williams, et al. (2004). "Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1." J Bacteriol **186**(10): 2936-2945.
Hildebrand, P. D., P. G. Braun, et al. (1998). "Role of the biosurfactant viscosin in broccoli head rot caused by a pectolytic strain of *Pseudomonas fluorescens*." Can. J. Plant Pathol. **20**: 296-303.
Hill, D. S., Stein, J. I., Torkewitz, N. R., Morse, A. M., Howell, C. R., Pachlatko, J. P., Becker, J. O. and Ligon, J. M. (1994). "Cloning of Genes Involved in the Synthesis of Pyrrolnitrin from *Pseudomonas fluorescens* and Role of Pyrrolnitrin Synthesis in Biological Control of Plant Disease." Appl Environ Microbiol **60**(1): 78-85.
Hojati, Z., C. Milne, et al. (2002). "Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*." Chem Biol **9**(11): 1175-1187.
Hornby, D. (1983). "Suppressive soils." Annual Review of Phytopathology **21**: 65-85.
Howell, C. R., and Stipanovic, R.D. (1979). "Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium." Phytopathology **69**: 480-482.
Howell, C. R., and Stipanovic, R.D. (1980). "Suppression of *Pythium ultimum* induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. ." Phytopathology **70**: 712-715.
Hrabak, E. M. and D. K. Willis (1992). "The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators." J Bacteriol **174**(9): 3011-3020.
Hrabak, E. M. and D. K. Willis (1992). "The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators." J. Bacteriol. **174**: 3011-3020.
Hughes, K. T. and K. Mathee (1998). "The anti-sigma factors." Annu Rev Microbiol **52**: 231-286.
Humair, B., N. Gonzalez, et al. (2009). "Temperature-responsive sensing regulates biocontrol factor expression in *Pseudomonas fluorescens* CHA0." ISME J **3**(8): 955-965.
Humair, B., B. Wackwitz, et al. (2010). "GacA-Controlled Activation of Promoters for Small RNA Genes in *Pseudomonas fluorescens*." Appl Environ Microbiol.
Hutchison, M. L., M. A. Tester, et al. (1995). "Role of biosurfactant and ion channel-forming activities of syringomycin in transmembrane ion flux: a model for the mechanism of action in the plant-pathogen interaction." Mol Plant Microbe Interact **8**(4): 610-620.
Iacobellis, N. S., P. LAvermicocca, et al. (1992). "Phytotoxic properties of *Pseudomonas syringae* pv. *syringae* toxins." Physiol. Mol. Plant Pathol. **40**: 107-116.
Kang, H. and D. C. Gross (2005). "Characterization of a resistance-nodulation-cell division transporter system associated with the *syr-syp* genomic island of *Pseudomonas syringae* pv. syringae." Appl Environ Microbiol **71**(9): 5056-5065.

Kang, Y., V. V. Lunin, et al. (2009). "The long-chain fatty acid sensor, PsrA, modulates the expression of *rpoS* and the type III secretion *exsCEBA* operon in *Pseudomonas aeruginosa*." Mol Microbiol **73**(1): 120-136.

Kaplan, H. B. and E. P. Greenberg (1985). "Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system." J Bacteriol **163**(3): 1210-1214.

Kay, E., C. Dubuis, et al. (2005). "Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0." Proc Natl Acad Sci U S A **102**(47): 17136-17141.

Kay, E., B. Humair, et al. (2006). "Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*." J Bacteriol **188**(16): 6026-6033.

Keating, T. A. and C. T. Walsh (1999). "Initiation, elongation, and termination strategies in polyketide and polypeptide antibiotic biosynthesis." Curr Opin Chem Biol **3**(5): 598-606.

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D., Défago, G., (1992). "Suppression of Root Diseases By *Pseudomonas fluorescens* CHA0: Importance of the Bacterial Secondary Metabolite 2,4-Diacetylphloroglucinol
" Mol. Plant-Microbe Interact. **5**(1): 4-13.

Keel, C., Wirthner, P., Oberhansli, T., Voisard, C., Burger, U., Haas D., and Defago, G. (1990). "Pseudomonads as antagonists of plant-pathogens in the rhizosphere - Role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black root-rot of tobacco. ." Symbiosis **9**: 327-341.

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

Kinscherf, T. G. and D. K. Willis (2002). "Global regulation by gidA in *Pseudomonas syringae*." J Bacteriol **184**(8): 2281-2286.

Kitten, T., T. G. Kinscherf, et al. (1998). "A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*." Mol Microbiol **28**(5): 917-929.

Kloepper, J. W., and Schroth, M.N. (1987). Plant growth-promoting rhizobacteria on radishes. *In* Station de pathologie vegetale et phyto-bacteriologie: Proceedings of the 4th International Conference on Plant Pathogenic Bacteria. . 4th International Conference on Plant Pathogenic Bacteria, Tours, France, Gilbert-Clarey.

Kloepper, J. W., LEong, J., Teintze, M. and Schroth, M.N. (1980). "Enhancing plant growth by siderophores produced by plant growth-promoting rhizobacteria." Nature **286**: 885-886.

Knutson, C. A. and A. Jeanes (1968). "A new modification of the carbazole analysis: application to heteropolysaccharides." Anal Biochem **24**(3): 470-481.

Koch, B., T. H. Nielsen, et al. (2002). "Lipopeptide production in *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudate via the Gac two-component regulatory system." Appl Environ Microbiol **68**(9): 4509-4516.

Kojic, M., B. Jovcic, et al. (2005). "Novel target genes of PsrA transcriptional regulator of *Pseudomonas aeruginosa*." FEMS Microbiol Lett **246**(2): 175-181.

Konz, D., S. Doekel, et al. (1999). "Molecular and biochemical characterization of the protein template controlling biosynthesis of the lipopeptide lichenysin." J Bacteriol **181**(1): 133-140.
Kraus, J., and Loper, J.E. (1992). "Lack of evidence for a role of AF metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of Pythium damping-off of cucumber." Phytopathology **82**: 264-271.
Kruijt, M., H. Tran, et al. (2009). "Functional, genetic and chemical characterization of biosurfactants produced by plant growth-promoting *Pseudomonas putida* 267." J Appl Microbiol **107**(2): 546-556.
Kuiper, I., G. V. Bloemberg, et al. (2001). "Selection of a plant-bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria." Mol Plant Microbe Interact **14**(10): 1197-1205.
Kuiper, I., E. L. Lagendijk, et al. (2004). "Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms." Mol Microbiol **51**(1): 97-113.
Laskowski, M. A. and B. I. Kazmierczak (2006). "Mutational analysis of RetS, an unusual sensor kinase-response regulator hybrid required for *Pseudomonas aeruginosa* virulence." Infect Immun **74**(8): 4462-4473.
Latifi, A., M. Foglino, et al. (1996). "A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS." Mol Microbiol **21**(6): 1137-1146.
Latifi, A., M. K. Winson, et al. (1995). "Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1." Mol Microbiol **17**(2): 333-343.
Laville, J., C. Blumer, et al. (1998). "Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0." J Bacteriol **180**(12): 3187-3196.
Laville, J., C. Voisard, et al. (1992). "Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco." Proc Natl Acad Sci U S A **89**(5): 1562-1566.
Laycock, M. V., P. D. Hildebrand, et al. (1991). "Visocin, a potent peptidolipid biosurfactant and phytopathogenic mediator produced by a pectolytic strain of *Pseudomonas fluorescens*." J. Agric. Food Chem. **39**: 483-489.
Licciardello, G., I. Bertani, et al. (2007). "*Pseudomonas corrugata* contains a conserved *N*-acyl homoserine lactone quorum sensing system; its role in tomato pathogenicity and tobacco hypersensitivity response." FEMS Microbiol Ecol **61**(2): 222-234.
Licciardello, G., I. Bertani, et al. (2009). "The transcriptional activator *rfiA* is quorum-sensing regulated by cotranscription with the *luxI* homolog *pcoI* and is essential for plant virulence in *Pseudomonas corrugata*." Mol Plant Microbe Interact **22**(12): 1514-1522.
Ligon, J. M., Hill, D.S., Hammer, P.E., Torkewitz, N.R., Hofmann,D, *et al.* (2000). "Natural products with AF activity from *Pseudomonas* biocontrol bacteria." Pest Manage. Sci. **56**: 688-695.

Lindow, S. E. and M. T. Brandl (2003). "Microbiology of the phyllosphere." Appl Environ Microbiol **69**(4): 1875-1883.
Lindum, P. W., U. Anthoni, et al. (1998). "*N*-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1." J Bacteriol **180**(23): 6384-6388.
Linne, U., S. Doekel, et al. (2001). "Portability of epimerization domain and role of peptidyl carrier protein on epimerization activity in nonribosomal peptide synthetases." Biochemistry **40**(51): 15824-15834.
Linne, U. and M. A. Marahiel (2000). "Control of directionality in nonribosomal peptide synthesis: role of the condensation domain in preventing misinitiation and timing of epimerization." Biochemistry **39**(34): 10439-10447.
Longley, R. P., Halliwell, J. E., Campbell, J. J., Ingledew, W. M. (1972). "The branchpoint of pyocyanine biosynthesis." Can J Microbiol **18**(9): 1357-1363.
Lu, S. E., B. K. Scholz-Schroeder, et al. (2002). "Characterization of the *salA*, *syrF*, and *syrG* regulatory genes located at the right border of the syringomycin gene cluster of *Pseudomonas syringae* pv. *syringae*." Mol Plant Microbe Interact **15**(1): 43-53.
Lugtenberg, B. J., L. Dekkers, et al. (2001). "Molecular determinants of rhizosphere colonization by *Pseudomonas*." Annu Rev Phytopathol **39**: 461-490.
Manitoba Agriculture, F. A. R. I. (2002). "Pest Management Plant Disease. Sclerotinia." from http://www.gov.mb.ca/agriculture/crops/diseases/fac07s00.html.
Marahiel, M. A., T. Stachelhaus, et al. (1997). "Modular Peptide Synthetases Involved in Nonribosomal Peptide Synthesis." Chem Rev **97**(7): 2651-2674.
Marahiel, M. A., T. Stachelhaus, et al. (1997). "Modular peptide sythetases involved in nonribosomal peptide synthesis." Chem. Rev. **97**: 2651-2673.
Maurhofer, M., Keel, C., Schnider, U., Voisard, C., Haas, D., and Defago, G. (1992). "Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. 190–195." Phytopathology **82**: 190-195.
Maurhofer, M., Keel,C., Haas, D., Défago, G. (1994). "Pyoluteorin production by *Pseudomonas fluorescens* strain CHA0 is involved in the suppression of *Pythium* damping-off of cress but not of cucumber." Eur. J. Plant. Pathol. **100**: 221-232.
Mavrodi, D. V., Blankenfeldt, W., Thomashow, L. S. (2006). "Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation." Annu Rev Phytopathol **44**: 417-445.
Mavrodi, D. V., Ksenzenko, V. N., Bonsall, R. F., Cook, R. J., Boronin, A. M., Thomashow, L. S. (1998). "A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79." J Bacteriol **180**(9): 2541-2548.
Mavrodi, D. V., T. L. Peever, et al. (2010). "Diversity and evolution of the phenazine biosynthesis pathway." Appl Environ Microbiol **76**(3): 866-879.
May, J. J., N. Kessler, et al. (2002). "Crystal structure of DhbE, an archetype for aryl acid activating domains of modular nonribosomal peptide synthetases." Proc Natl Acad Sci U S A **99**(19): 12120-12125.
May, T. B., and Chakrabarty, A.M. (1994). " Isolation and assay of *Pseudomonas aeruginosa* alginate. ." Methods Enzymol **235**: 295-304.

Mazzola, M., R. J. Cook, et al. (1992). "Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats." Appl Environ Microbiol **58**(8): 2616-2624.
Mazzola, M., I. de Bruijn, et al. (2009). "Protozoan-induced regulation of cyclic lipopeptide biosynthesis is an effective predation defense mechanism for *Pseudomonas fluorescens*." Appl Environ Microbiol **75**(21): 6804-6811.
Mazzola, M., X. Zhao, et al. (2007). "Cyclic Lipopeptide Surfactant Production by *Pseudomonas fluorescens* SS101 Is Not Required for Suppression of Complex *Pythium* spp. Populations." Phytopathology **97**(10): 1348-1355.
McClean, K. H., M. K. Winson, et al. (1997). "Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones." Microbiology **143 (Pt 12)**: 3703-3711.
McDonald, M., D. V. Mavrodi, et al. (2001). "Phenazine biosynthesis in *Pseudomonas fluorescens*: branchpoint from the primary shikimate biosynthetic pathway and role of phenazine-1,6-dicarboxylic acid." J Am Chem Soc **123**(38): 9459-9460.
Mercado, T. I. and A. Colon-Whitt (1982). "Lysis of *Trypanosoma cruzi* by *Pseudomonas fluorescens*." Antimicrob Agents Chemother **22**(6): 1051-1057.
Merriman, T. R. and I. L. Lamont (1993). "Construction and use of a self-cloning promoter probe vector for gram-negative bacteria." Gene **126**(1): 17-23.
Meyer, J. M., and Abdallah, M.A. (1978). "The Fluorescent Pigment of *Pseudomonas fluorescens*: Biosynthesis, Purification and Physicochemical Properties." J Gen Microbiol **107**(2): 319-328.
Meyer, J. M., P. Azelvandre, et al. (1992). "Iron metabolism in *Pseudomonas:* salicylic acid, a siderophore of *Pseudomonas fluorescens* CHAO." Biofactors **4**(1): 23-27.
Miao, V., R. Brost, et al. (2006). "The lipopeptide antibiotic A54145 biosynthetic gene cluster from *Streptomyces fradiae*." J Ind Microbiol Biotechnol **33**(2): 129-140.
Miller, J. H. (1972). "Experiments in molecular genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mireles, J. R., 2nd, A. Toguchi, et al. (2001). "*Salmonella enterica* serovar *typhimurium* swarming mutants with altered biofilm-forming abilities: surfactin inhibits biofilm formation." J Bacteriol **183**(20): 5848-5854.
Mootz, H. D. and M. A. Marahiel (1997). "The tyrocidine biosynthesis operon of *Bacillus brevis*: complete nucleotide sequence and biochemical characterization of functional internal adenylation domains." J Bacteriol **179**(21): 6843-6850.
Morello, J. E., E. A. Pierson, et al. (2004). "Negative cross-communication among wheat rhizosphere bacteria: effect on antibiotic production by the biological control bacterium *Pseudomonas aureofaciens* 30-84." Appl Environ Microbiol **70**(5): 3103-3109.
Natsch, A., C. Keel, et al. (1994). "Contribution of the Global Regulator Gene *gacA* to Persistence and Dissemination of *Pseudomonas fluorescens* Biocontrol Strain CHA0 Introduced into Soil Microcosms." Appl Environ Microbiol **60**(7): 2553-2560.
Nelson, B. (1998). Biology of Sclerotinia. . 10th International Sclerotinia workshop, Fargo, N.D., U.S.A., North Dakota State University Department of Plant Pathology.

Nielsen, M. N., J. Sorensen, et al. (1998). "Secondary metabolite- and endochitinase-dependent antagonism toward plant-pathogenic microfungi of *Pseudomonas fluorescens* isolates from sugar beet rhizosphere." Appl Environ Microbiol **64**(10): 3563-3569.

Nielsen, T. H., C. Christophersen, et al. (1999). "Viscosinamide, a new cyclic depsipeptide with surfactant and AF properties produced by *Pseudomonas fluorescens* DR54." J Appl Microbiol **87**(1): 80-90.

Nielsen, T. H., O. Nybroe, et al. (2005). "Genes involved in cyclic lipopeptide production are important for seed and straw colonization by *Pseudomonas* sp. strain DSS73." Appl Environ Microbiol **71**(7): 4112-4116.

Nielsen, T. H., D. Sorensen, et al. (2002). "Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizosphere." Appl Environ Microbiol **68**(7): 3416-3423.

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

Nowak-Thompson, B., Gould, S. J., Loper, J. E. (1997). "Identification and sequence analysis of the genes encoding a polyketide synthase required for pyoluteorin biosynthesis in *Pseudomonas fluorescens* Pf-5." Gene **204**(1-2): 17-24.

Nutkins, J. C., R. J. Mortishire-Smith, et al. (1991). "Structure determination of tolaasin, an extracellular lipodepsipeptide produced by the mushroom pathogen *Pseudomonas tolaasii* Paine." J. Am. Chem. Soc. **113**: 2621-2627.

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

O'Toole, G. A. and R. Kolter (1998). "Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis." Mol Microbiol **28**(3): 449-461.

Ochsner, U. A., A. K. Koch, et al. (1994). "Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*." J Bacteriol **176**(7): 2044-2054.

Ohman, D. E., S. J. Cryz, et al. (1980). "Isolation and characterization of *Pseudomonas aeruginosa* PAO mutant that produces altered elastase." J Bacteriol **142**(3): 836-842.

Passador, L., J. M. Cook, et al. (1993). "Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication." Science **260**(5111): 1127-1130.

Paulsen, I. T., C. M. Press, et al. (2005). "Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5." Nat Biotechnol **23**(7): 873-878.

Pesci, E. C. and B. H. Iglewski (1997). "The chain of command in *Pseudomonas* quorum sensing." Trends in Microbiology **5**(4): 132-134.

Pessi, G., F. Williams, et al. (2001). "The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*." J Bacteriol **183**(22): 6676-6683.

Pierson, L. S., 3rd, V. D. Keppenne, et al. (1994). "Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density." J Bacteriol **176**(13): 3966-3974.

Pierson, L. S., 3rd and L. S. Thomashow (1992). "Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84." Mol Plant Microbe Interact **5**(4): 330-339.

Poritsanos, N., C. Selin, et al. (2006). "A GacS deficiency does not affect *Pseudomonas chlororaphis* PA23 fitness when growing on canola, in aged batch culture or as a biofilm." Can J Microbiol **52**(12): 1177-1188.

Poussereau, N., S. Gente, et al. (2001). "*aspS* encoding an unusual aspartyl protease from *Sclerotinia sclerotiorum* is expressed during phytopathogenesis." FEMS Microbiol Lett **194**(1): 27-32.

Preston, M. J., P. C. Seed, et al. (1997). "Contribution of proteases and LasR to the virulence of *Pseudomonas aeruginosa* during corneal infections." Infect Immun **65**(8): 3086-3090.

Price-Whelan, A., L. E. Dietrich, et al. (2006). "Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics." Nat Chem Biol **2**(2): 71-78.

Purdy, L. H. (1979). "*Sclerotinia sclerotiorum*: History, Diseases and Symptomatology, Host Range, Geographic Distribution, and Impact." Phytopathology **69**: 875-880.

Quadri, L. E. (2000). "Assembly of aryl-capped siderophores by modular peptide synthetases and polyketide synthases." Mol Microbiol **37**(1): 1-12.

Quadri, L. E., T. A. Keating, et al. (1999). "Assembly of the *Pseudomonas aeruginosa* nonribosomal peptide siderophore pyochelin: In vitro reconstitution of aryl-4, 2-bisthiazoline synthetase activity from PchD, PchE, and PchF." Biochemistry **38**(45): 14941-14954.

Quigley, N. B. and D. C. Gross (1994). "Syringomycin production among strains of *Pseudomonas syringae* pv. *syringae*: conservation of the *syrB* and *syrD* genes and activation of phytotoxin production by plant signal molecules." Mol Plant Microbe Interact **7**(1): 78-90.

Quigley, N. B., Y. Y. Mo, et al. (1993). "SyrD is required for syringomycin production by *Pseudomonas syringae* pathovar *syringae* and is related to a family of ATP-binding secretion proteins." Mol Microbiol **9**(4): 787-801.

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

Raaijmakers, J. M., Vlami, M., and de Souza, J. T. (2002). "Antibiotic production by bacterial biocontrol agents." Antonie Van Leeuwenhoek **81**(1-4): 537-547.

Rashid, K. Y. (2000). "Sclerotinia on flax in Western canada-warning for a potential disease problem." Can. J. Plant Pathol. **21**: 175.

Reimmann, C., M. Beyeler, et al. (1997). "The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase." Mol Microbiol **24**(2): 309-319.

Reimmann, C., N. Ginet, et al. (2002). "Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1." Microbiology **148**(Pt 4): 923-932.

Reimmann, C., C. Valverde, et al. (2005). "Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHA0." J Bacteriol **187**(1): 276-285.

Riou, C., G. Freyssinet, et al. (1991). "Production of Cell Wall-Degrading Enzymes by the Phytopathogenic Fungus *Sclerotinia sclerotiorum*." Appl Environ Microbiol **57**(5): 1478-1484.

Robinson, V. L., D. R. Buckler, et al. (2000). "A tale of two components: a novel kinase and a regulatory switch." Nat Struct Biol **7**(8): 626-633.

Roediger, H. (1898). "Report of Branches. Gawler River, September 30, Take-all." Journal Department of Agriculture South Australia **2**: 371.

Roongsawang, N., K. Hase, et al. (2003). "Cloning and characterization of the gene cluster encoding arthrofactin synthetase from *Pseudomonas* sp. MIS38." Chem Biol **10**(9): 869-880.

Roongsawang, N., S. P. Lim, et al. (2005). "Phylogenetic analysis of condensation domains in the nonribosomal peptide synthetases." FEMS Microbiol Lett **252**(1): 143-151.

Ryan, K. J. and C. G. Ray (2004). Sherris Medical Microbiology, McGraw Hill.

Saito, H., S. Kurata, et al. (1992). "Purification and characterization of a hemocyte proteinase of Sarcophaga, possibly participating in elimination of foreign substances." Eur J Biochem **209**(3): 939-944.

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

Scaloni, A., M. Dalla Serra, et al. (2004). "Structure, conformation and biological activity of a novel lipodepsipeptide from *Pseudomonas corrugata*: cormycin A." Biochem J **384**(Pt 1): 25-36.

Scher, F. M., and Baker, R. (1980). "Mechanism of biological control in a *Fusarium*-suppressive soil." Phytopathology **70**: 412-417.

Schippers, B., A. W. Bakker, et al. (1987). "Interactions of Deleterious and Beneficial Rhizosphere Microorganisms and the Effect of Cropping Practices." Annual Review of Phytopathology **25**(1): 339-358.

Schmidli-Sacherer, P., C. Keel, et al. (1997). "The global regulator GacA of *Pseudomonas fluorescens* CHA0 is required for suppression of root diseases in dicotyledons but not in graminae." Plant Pathol. **46**: 80-90.

Schmidt, S., J. F. Blom, et al. (2009). "Production of the AF compound pyrrolnitrin is quorum sensing-regulated in members of the *Burkholderia cepacia* complex." Environ Microbiol **11**(6): 1422-1437.

Schneider, A. and M. A. Marahiel (1998). "Genetic evidence for a role of thioesterase domains, integrated in or associated with peptide synthetases, in non-ribosomal peptide biosynthesis in *Bacillus subtilis*." Arch Microbiol **169**(5): 404-410.

Schneider, R. W. (1982). Suppressive soils and plant disease St. Paul, Minnesota, American Phytopathological Society.

Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Defago, G., Haas, D., Keel, C. (2000). "Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent

Pseudomonas fluorescens CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin." J Bacteriol **182**(5): 1215-1225.
Scholz-Schroeder, B. K., M. L. Hutchison, et al. (2001). "The contribution of syringopeptin and syringomycin to virulence of *Pseudomonas syringae* pv. *syringae* strain B301D on the basis of *sypA* and *syrB1* biosynthesis mutant analysis." Mol Plant Microbe Interact **14**(3): 336-348.
Scholz-Schroeder, B. K., J. D. Soule, et al. (2003). "The *sypA*, *sypB*, and *sypC* synthetase genes encode twenty-two modules involved in the nonribosomal peptide synthesis of syringopeptin by *Pseudomonas syringae* pv. *syringae* B301D." Mol Plant Microbe Interact **16**(4): 271-280.
Scholz-Schroeder, B. K., J. D. Soule, et al. (2001). "A physical map of the syringomycin and syringopeptin gene clusters localized to an approximately 145-kb DNA region of *Pseudomonas syringae* pv. *syringae* strain B301D." Mol Plant Microbe Interact **14**(12): 1426-1435.
Schroth, M. N. and J. G. Hancock (1981). "Selected topics in biological control." Annu Rev Microbiol **35**: 453-476.
Schroth, M. N. and J. G. Hancock (1982). "Disease-Suppressive Soil and Root-Colonizing Bacteria." Science **216**(4553): 1376-1381.
Schuster, M. and E. P. Greenberg (2006). "A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*." Int J Med Microbiol **296**(2-3): 73-81.
Schuster, M. and E. P. Greenberg (2007). "Early activation of quorum sensing in *Pseudomonas aeruginosa* reveals the architecture of a complex regulon." BMC Genomics **8**: 287.
Schuster, M., A. C. Hawkins, et al. (2004). "The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing." Mol Microbiol **51**(4): 973-985.
Shipton, P. J., Cook, R.J., and Sitton, J.W. (1973). "Occurrence and Transfer of a Biological Factor in Soil that Suppresses Take-all of Wheat in Eastern Washington." Phytopathology **63**: 511-517.
Siddiqui, I. A., D. Haas, et al. (2005). "Extracellular protease of *Pseudomonas fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode *Meloidogyne incognita*." Appl Environ Microbiol **71**(9): 5646-5649.
Sieber, S. A. and M. A. Marahiel (2003). "Learning from nature's drug factories: nonribosomal synthesis of macrocyclic peptides." J Bacteriol **185**(24): 7036-7043.
Spiers, A. J., A. Buckling, et al. (2000). "The causes of *Pseudomonas* diversity." Microbiology **146 (Pt 10)**: 2345-2350.
Stachelhaus, T., A. Huser, et al. (1996). "Biochemical characterization of peptidyl carrier protein (PCP), the thiolation domain of multifunctional peptide synthetases." Chem Biol **3**(11): 913-921.
Stachelhaus, T. and M. A. Marahiel (1995). "Modular structure of peptide synthetases revealed by dissection of the multifunctional enzyme GrsA." J Biol Chem **270**(11): 6163-6169.
Stachelhaus, T., H. D. Mootz, et al. (1998). "Peptide bond formation in nonribosomal peptide biosynthesis. Catalytic role of the condensation domain." J Biol Chem **273**(35): 22773-22781.

Stachelhaus, T., H. D. Mootz, et al. (1999). "The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases." Chem Biol **6**(8): 493-505.

Stover, C. K., X. Q. Pham, et al. (2000). "Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen." Nature **406**(6799): 959-964.

Stutz, E. W., Defago, G., and Kern, H. (1986). "Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco." Phytopathology **76**: 181-185.

Thomashow, L. S. (1996). "Biological control of plant root pathogens." Curr Opin Biotechnol **7**(3): 343-347.

Thomashow, L. S. and D. M. Weller (1988). "Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*." J Bacteriol **170**(8): 3499-3508.

Thomashow, L. S. a. W., D.M. (1996). Current Concepts in the Use of Introduced Bacteria for Biological Disease Control: Mechanisms and AF Metabolites. Plant-Microbe Interactions. G. a. K. Stacey, N.T. NY, Chapman and Hall**:** 187-235.

Thrane, C., T. Harder Nielsen, et al. (2000). "Viscosinamide-producing *Pseudomonas fluorescens* DR54 exerts a biocontrol effect on *Pythium ultimum* in sugar beet rhizosphere." FEMS Microbiol Ecol **33**(2): 139-146.

Thrane, C., M. N. Nielsen, et al. (2001). "*Pseudomonas fluorescens* DR54 Reduces Sclerotia Formation, Biomass Development, and Disease Incidence of *Rhizoctonia solani* Causing Damping-Off in Sugar Beet." Microb Ecol **42**(3): 438-445.

Thrane, C., S. Olsson, et al. (1999). "Vital fluorescent stains for detection og stress in *Pythium ultimum* and *Rhizoctonia solani* challenged with viscosinamide from *Pseudomonas fluorescens* DR54." FEMS Microbiol Ecol **30**: 11-23.

Tran, H., A. Ficke, et al. (2007). "Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*." New Phytol **175**(4): 731-742.

Tran, H., M. Kruijt, et al. (2008). "Diversity and activity of biosurfactant-producing *Pseudomonas* in the rhizosphere of black pepper in Vietnam." J Appl Microbiol **104**(3): 839-851.

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

Turkington, T. K., and Morrall, R.A.A. (1993). "Use of petal infestation to forecast sclerotinia stem rot of canola: the influence of inoculum variation over the flowering period and canopy density." Phytopathology **83**: 682-689.

Turner, J. M. and A. J. Messenger (1986). "Occurrence, biochemistry and physiology of phenazine pigment production." Adv Microb Physiol **27**: 211-275.

Ude, S., D. L. Arnold, et al. (2006). "Biofilm formation and cellulose expression among diverse environmental *Pseudomonas* isolates." Environ Microbiol **8**(11): 1997-2011.

Vallet-Gely, I., A. Novikov, et al. (2010). "Association of hemolytic activity of *Pseudomonas entomophila*, a versatile soil bacterium, with cyclic lipopeptide production." Appl Environ Microbiol **76**(3): 910-921.

Valverde, C., S. Heeb, et al. (2003). "RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0." Mol Microbiol **50**(4): 1361-1379.

Van Loon, L. C., and Bakker, P.A.H.M. (2006). Induced systemic resistance as a mechanism of disease suppression by rhizobacteria. PGPR: Biocontrol and Biofertilization. Z. A. Siddiqui. Dordrecht, The Netherlands, Springer**:** 39-66.

van Wageningen, A. M., P. N. Kirkpatrick, et al. (1998). "Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic." Chem Biol **5**(3): 155-162.

Vandenende, C. S., M. Vlasschaert, et al. (2004). "Functional characterization of an aminotransferase required for pyoverdine siderophore biosynthesis in *Pseudomonas aeruginosa* PAO1." J Bacteriol **186**(17): 5596-5602.

Velraeds, M. M., B. van de Belt-Gritter, et al. (2000). "Inhibition of uropathogenic biofilm growth on silicone rubber in human urine by lactobacilli--a teleologic approach." World J Urol **18**(6): 422-426.

Venette, J. (1998). Sclerotinia spore formation, transport and infection. . Proceedings of the Sclerotinia Workshop. Fargo, North Dakota, U.S.A.

Ventre, I., A. L. Goodman, et al. (2006). "Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes." Proc Natl Acad Sci U S A **103**(1): 171-176.

Venturi, V. (2006). "Regulation of quorum sensing in *Pseudomonas*." FEMS Microbiol Rev **30**(2): 274-291.

Visca, P., G. Colotti, et al. (1992). "Metal regulation of siderophore synthesis in *Pseudomonas aeruginosa* and functional effects of siderophore-metal complexes." Appl Environ Microbiol **58**(9): 2886-2893.

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

Vollenbroich, D., N. Mehta, et al. (1994). "Analysis of surfactin synthetase subunits in *srfA* mutants of *Bacillus subtilis* OKB105." J Bacteriol **176**(2): 395-400.

Wagner, V. E., D. Bushnell, et al. (2003). "Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment." J Bacteriol **185**(7): 2080-2095.

Walker, J. C. and W. C. Snyder (1933). Pea wilt and root rots. Madison, Agricultural Experiment Station, University of Wisconsin.

Wang, N., S. E. Lu, et al. (2006). "Characterization of the transcriptional activators SalA and SyrF, Which are required for syringomycin and syringopeptin production by *Pseudomonas syringae* pv. *syringae*." J Bacteriol **188**(9): 3290-3298.

Wang, Q., J. G. Frye, et al. (2004). "Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes." Mol Microbiol **52**(1): 169-187.

Wäspi, U., D. Blanc, et al. (1998). "Syringolin, a Novel Peptide Elicitor from *Pseudomonas syringae* pv. *syringae* that Induces Resistance to *Pyricularia oryzae* in Rice." Molecular Plant-Microbe Interactions **11**(8): 727-733.

Weber, G., K. Schorgendorfer, et al. (1994). "The peptide synthetase catalyzing cyclosporine production in *Tolypocladium niveum* is encoded by a giant 45.8-kilobase open reading frame." Curr Genet **26**(2): 120-125.

Weber, T., R. Baumgartner, et al. (2000). "Solution structure of PCP, a prototype for the peptidyl carrier domains of modular peptide synthetases." Structure **8**(4): 407-418.

Wei, B. L., A. M. Brun-Zinkernagel, et al. (2001). "Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*." Mol Microbiol **40**(1): 245-256.

Wei, H. L. and L. Q. Zhang (2006). "Quorum-sensing system influences root colonization and biological control ability in *Pseudomonas fluorescens* 2P24." Antonie Van Leeuwenhoek **89**(2): 267-280.

Weller, D. M. (1988). "Biological control of soilborne plant pathogens in the rhizosphere with bacteria." Ann. Rev. Phytopathol. **26**: 379-407.

Weller, D. M., J. M. Raaijmakers, et al. (2002). "Microbial populations responsible for specific soil suppressiveness to plant pathogens." Annu Rev Phytopathol **40**: 309-348.

West, S. E., H. P. Schweizer, et al. (1994). "Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*." Gene **148**(1): 81-86.

Whistler, C. A., N. A. Corbell, et al. (1998). "The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor sigmaS and the stress response in *Pseudomonas fluorescens* Pf-5." J Bacteriol **180**(24): 6635-6641.

Whiteley, M., M. R. Parsek, et al. (2000). "Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*." J Bacteriol **182**(15): 4356-4360.

Willis, D. K., E. M. Hrabak, et al. (1990). "Isolation and characterization of a *Pseudomonas syringae* pv. *syringae* mutant defecient in lesion forming ability on bean." Mol. Plant-Microbe Interact. **3**: 149-156.

Winson, M. K., M. Camara, et al. (1995). "Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*." Proc Natl Acad Sci U S A **92**(20): 9427-9431.

Wood, D. W., F. Gong, et al. (1997). "*N*-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere." J Bacteriol **179**(24): 7663-7670.

Workentine, M. L., L. Chang, et al. (2009). "The GacS-GacA two-component regulatory system of *Pseudomonas fluorescens*: a bacterial two-hybrid analysis." FEMS Microbiol Lett **292**(1): 50-56.

Yan, Q., X. G. Wu, et al. (2009). "Differential control of the PcoI/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24 by sigma factor RpoS and the GacS/GacA two-component regulatory system." Microbiol Res **164**(1): 18-26.

Youard, Z. A., G. L. Mislin, et al. (2007). "*Pseudomonas fluorescens* CHA0 produces enantio-pyochelin, the optical antipode of the *Pseudomonas aeruginosa* siderophore pyochelin." J Biol Chem **282**(49): 35546-35553.

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

Zhang, Z. and L. S. Pierson, 3rd (2001). "A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*." Appl Environ Microbiol **67**(9): 4305-4315.

Zuber, S., F. Carruthers, et al. (2003). "GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0." Mol Plant Microbe Interact **16**(7): 634-644.