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Using Molecular Techniques to Understand and Enhance Biological Control by *Pseudomonas* spp.

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ABSTRACT

The health and environmental impact of agrochemicals make alternative methods of disease control more attractive for sustainable crop production. Biocontrol strains of bacteria that are able to inhibit plant pathogenic fungi and suppress plant diseases represent one of the most promising alternatives. Various mechanisms of biological control have been recognized including effective root colonization, production of AF metabolites, interference with fungal pathogenic factors, and elicitation of induced systemic resistance in the plant. These mechanisms were initially demonstrated through microbiological and/or biochemical techniques. The advent of molecular techniques to study the genetic basis of biological control has shed light on genes responsible for production of antibiotics and degradative enzymes. Through inactivation, overexpression, and analysis of gene expression patterns, their role in biocontrol has been more clearly defined. In this review we describe the work done in Canada, using molecular techniques, to understand biological control in *Pseudomonas chlororaphis* PA23. The end goal of this research is to develop more reliable products that demonstrate enhanced performance in the field.

Keywords: antibiotics, antifungal, degradative enzymes, regulation, *Sclerotinia sclerotiorum*
Abbreviations: AF, antifungal; **CLPs**, cyclic lipopeptides; **DAPG**, diacetyl phloroglucinol; **LTTR**, LysR-type transcriptional regulator; **NRPS**, non-ribosomal peptide synthetase; **PHZ**, phenazine; **PLT**, pyoluteorin; **PRN**, pyrrolnitrin; **QS**, quorum sensing

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INTRODUCTION

Studies involving biocontrol bacteria have been reported for over 75 years (Massart and Jijakli 2007) and this approach is finally becoming a realistic alternative to chemical treatments (Punja and Utkhede 2003). Of the reported biocontrol bacteria, selected strains of fluorescent *Pseudomonas* and *Bacillus* spp. have received the most attention (Emmert and Handelsman 1999; Ashnaei *et al*. 2009). For many years studies focusing on disease suppression have primarily involved microscopic and/or biochemical techniques (Elad 1996; 2000; Spadaro and Gullino 2004). It is only recently that some of the molecular mechanisms underlying these processes have been discovered (Haas *et al*. 2000). The first complete genome sequence of *Pseudomonas fluorescens* strain Pf-5 that was reported by Paulsen *et al.* (2005) added information on molecular mechanisms of biocontrol, which should ultimately enhance the practical application of these organisms under soil conditions. As more genomes are sequenced, novel biocontrol compounds together with the regulators controlling their expression will be exposed. Information on the molecular mechanisms of beneficial

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Invited Review

plant-microbe interactions enables optimization, enhancement and identification of potential synergistic effects in the process of plant protection. In *Pseudomonas* spp. biocontrol activity is mediated through the action of antibiotics, siderophores and lytic enzymes as well as through competitive exclusion (Lawongsa *et al*. 2008; Couillerot *et al*. 2009). Production of disease-suppressive metabolites is controlled by a multi-tiered network of regulation. Some of the more well-defined global regulators include the GacS/GacA two-component signal transduction system (Chancey *et al*. 1999), sigma factor directed control of RNA polymerase (RNAP), and QS. In addition, a number of transcriptional activator proteins that function to control one or more antibiotics have been identified. In this review, we will discuss antibiotics produced by *Pseudomonads*, regulatory factors governing their expression and the work ongoing in Canada using molecular techniques to elucidate mechanisms of biological control.

ANTIBIOTICS PRODUCED BY BIOCONTROL *PSEUDOMONAS* SPP.

Pseudomonads produce hundreds of secondary metabolites, which include antibiotic compounds such as 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT), phenazines (PHZ), pyrrolnitrin (PRN), and cyclic lipopeptides (CLPs) that contribute to disease suppression in various pathogen–plant systems (Dwivedi and Johri 2003; Raaijmakers *et al*. 2006). Bacteria prefer to multiply during the trophophase and synthesize secondary metabolites during idiophase when nutrients are low and a high cell density has been reached.

Diacetyl phloroglucinol

Diacetyl phloroglucinol (DAPG) is a phenolic compound with broad spectrum AF, antibacterial and antihelmenthic properties (Thomashow and Weller 1996). A number of genes involved in DAPG synthesis have been identified including the structural genes *phlACBD* which are arranged as a single transcriptional unit (Bangera and Thomashow 1999). Furthermore, the *phlE* gene is located immediately downstream of *phlD* and encodes a putative transmembrane permease (Bangera and Thomashow 1999; Dwivedi and Johri 2003). Two pathway-specific regulators of PHL biosynthesis have been identified called PhlF and PhlH. PhlF is a TetR-like transcriptional repressor encoded by *phlF* that is upstream of and divergently transcribed from *phlABCD* (Bangera and Thomashaw 1999; Delany *et al.* 2000; Schinder Keel *et al.* 2000). The PhlF recognition sequence, known as *phO*, is a 26 nucleotide palindromic operator sequence located downstream of *phlA*. Interaction of PhlF with this sequence is believed to prevent promoter clearance by RNAP (Bangera and Thomashaw 1996; Abbas *et al*. 2002). Addition of DAPG prevents binding of PhlF to the *phlA* promoter region; consequently DAPG synthesis is subject to positive autoregulation (Delany *et al*. 2000; Abbas *et al*. 2002). Other compounds such as salicylates, PLT and fusaric acid interact with PhlF to antagonise derepression by DAPG (Schnider-Keel *et al*. 2000). The second TetR-like pathway-specific regulator is encoded by the *phlH* gene which is located downstream of *phlF* (Schnider-Keel *et al*. 2000). Inactivation of *phlH* dramatically reduces *phl* operon expression, suggesting that PhlH works as an activator or antirepressor; however, many of the details regarding PhlH regulation have yet to be elucidated (Haas and Keel 2003). In between *phlF* and *phlH* is a gene called *phlG*, the product of which catalyzes the conversion of DAPG to the less-toxic derivative monoacetyl phloroglucinol (Bottiglieri and Keel 2006). Expression of *phlG* is negatively controlled by the pathway-specific regulators PhlF and PhlH (Bottiglieri and Keel 2006).

Pyoluteorin

Pyoluteorin (PLT) is an aromatic polyketide produced by several *Pseudomonas* species that suppresses phytopathogenic fungi (Howell and Stipanovic 1980). Genes required for PLT biosynthesis *(pltLABCDEG* and *pltM*) as well as transport and resistance (*pltHIJKNO*) have been cloned and characterized in strains such as *P. fluorescens* Pf-5 and *Pseudomonas* sp. M18 (Nowak-Thompson *et al*. 1999; Huang *et al*. 2004; Brodhagen *et al*. 2004, 2005; Huang *et al*. 2006). Two pathway-specific regulators of PLT expression (PltR and PltZ) have been identified. PltR is a LysR-type transcriptional regulator (LTTR) encoded by *pltR* which lies upstream of *pltLABCDEFG* and transcribed in the opposite direction (Nowak-Thompson *et al*. 1999). PltR positively regulates expression of *pltLABCDEFG* (Nowak-Thompson *et al*. 1999; Yan *et al*. 2007). The second regulatory gene, *pltZ*, is found upstream of and transcribed divergently from *pltHIJKNO* (Huang *et al*. 2004). PltZ is a TetR-type regulator that functions to inhibit PLT expression in a pathway-specific manner.

Phenazines

Phenazines (PHZ) are a large group of nitrogen-containing heterocyclic pigments that function as redox reactive molecules (Turner and Messenger 1986; Chin-A-Woeng *et al*. 2003). These molecules exhibit broad-spectrum activity against bacteria and fungi and they also play an important role in the rhizosphere colonization of microbes (Raaijmakers *et al*. 1997).

A conserved seven gene operon, *phzABCDEFG,* has been identified in various *Pseudomonas* species that directs the synthesis of phenazine-1-carboxylic acid (PCA) (Chin-A-Woeng *et al*. 2003). Five of the proteins, PhzA, PhzD, PhzE, PhzF and PhzG are required for phenazine biosynthesis (Mavrodi *et al*. 2006). The *phzB* gene is a duplicate of *phzA*, and *phzC*, which is found in most PHZ clusters, encodes a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase. PhzC catalyzes the first step of the shikimic acid pathway and serves to direct intermediates from primary metabolism into the PHZ biosynthetic pathway (Mavrodi *et al*. 2006). Beyond the core cluster, accessory genes are involved modification of PCA to the different PHZ derivatives. For example, in *Pseudomonas chlororaphis* PCL1391, *phzH* lies downstream of *phzG.* PhzH converts PCA to phenazine-1-carboxamide (PCN) (Chin-A-Woeng *et al.* 2001). In *P. chlororaphis* (*aureofaciens*) 30-84, *phzO* is located downstream of the core cluster; PhzO directs the synthesis of 2-hydroxyphenazine carboxylic acid (Delaney *et al*. 2001). *P. aeruginosa* strain PAO1, a bacterium known more for its pathogenic potential than its biocontrol capabilities, has two copies of the *phz* operon in its genome (Stover *et al*. 2000). The *phzM* gene is located upstream of and transcribed divergently from the *phzA1-G1* cluster. The *phzM* product is required for pyocyanin synthesis (Mavrodi *et al*. 2001). A second accessory gene, called *phzS*, is located downstream from *phzG1*; PhzS is involved in biosynthesis of both 1-hydroxyphenazine and pyocyanin (Mavrodi *et al*. 2001). Although global regulators controlling expression of the core *phz* genes have been characterized (discussed below), virtually nothing is known about regulation of the PHZ modifying genes.

A repressor of PHZ production, called RpeA (repressor of phenazine expression) has been identified in *P. chlororaphis* (*aureofaciens*) 30-84 (Whistler and Pierson 2003). The deduced protein sequence of RpeA revealed that it is similar to a group of two-component sensor kinases of unknown function. Although many of the details regarding how RpeA functions have yet to be resolved, it was found that an *rpeA* mutation leads to increased expression of the *phz* genes and elevated levels of PHZ production in minimal media through a quorum-sensing independent mechanism (Whistler and Pierson 2003).

Pyrrolnitrin

Pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chloro-phenyl) pyrrole; PRN] is a broad spectrum antibiotic effective against a range of fungal pathogens. It's mechanism of action is through inhibition of endogenous and exogenous respiration and glucose metabolism (Tripathi and Gottlieb 1969). The PRN biosynthetic operon consists of four ORFs, *prnABCD* (Hammer *et al*. 1997). Thus far, no pathway-specific regulators of PRN have been identified.

Cyclic lipopeptides

Cyclic lipopeptides (CLPs) are a versatile group of molecules that exhibit antimicrobial, cytotoxic and surfactant properties. In antagonistic Pseudomonads, production of CLPs contributes to antimicrobial activity, biofilm formation and motility (Raaijmakers *et al*. 2006). The basic structure of a CLP molecule consists of a fatty acid tail joined to a cyclic oligopeptide head. However, considerable structural diversity exists within these compounds due to differences in the length and composition of the fatty acid tail, and the peptide ring structure (Raaijmakers *et al*. 2006). CLP molecules are produced non-ribosomally by multifunctional enzymes called non-ribosomal peptide synthetases (NRPSs) (Marahiel *et al*. 1997). NRPSs are arranged as modules with each module acting as building block for the incorporation of one amino acid into the peptide head. The modules are subdivided such that each contains: i) an adenylation (A) domain, responsible for amino acid activation; ii) a thiolation (T) domain for thioesterification of the activated amino acid; and iii) a condensation (C) domain, which results in peptide bond formation between amino acids. The A, T and C catalytic domains create a linear peptide that is cleaved and typically cyclised through the action of a thioesterase domain (Raaijmakers *et al*. 2006). Because CLPs represent such a large and diverse group, a discussion of the biosynthetic genes and regulatory elements controlling their expression will not be covered here. For more information on this topic the reader is referred to an excellent review by Raaijmakers and coworkers (2006).

REGULATORY MECHANISMS CONTROLLING ANTIBIOTIC PRODUCTION IN BIOCONTROL PSEUDOMONADS

Transcriptional control mechanisms

1. Sigma factor-mediated transcription

In the environment bacteria must endure fluctuations in nutrient availability and exposure to stresses including oxidative, osmotic, and temperature shock. In response, they alter their gene expression allowing them to adapt to the prevailing conditions. One means by which bacteria are able to undergo a major shift in gene expression is through the use of sigma factors that direct the RNAP core to an alternative set of genes. In biocontrol pseudomonads, RpoD (σ^{70}), RpoS (σ^{38}) and RpoN (σ^{58}) have all been found to influence the expression of secondary metabolites. For example, in *P. fluorescens* CHA0, production of PLT and DAPG is enhanced by overexpression of *rpoD*, which encodes the housekeeping σ^{70} (Maurhofer *et al*. 1992; Schnider *et al*. 1995). It is speculated that genes for PLT and DAPG biosynthesis are under control of σ^{70} rather than σ^{32}; consequently at higher concentrations σ^{70} can more effectively compete for the core RNAP. Similarly, an *rpoS* mutant of the related strain Pf-5 exhibited increased PLT and DAPG production (Sarniguet *et al*. 1995). In terms of the antibiotic PRN, both *prnA* transcriptional activity and PRN levels were negligible in *rpoS* mutants of Pf-5 and CHA0; therefore, RpoS has a positive effect on expression of this antibiotic (Sarniguet *et al*. 1995; Whistler *et al*. 1998). In *Pseudomonas* sp. M18, RpoS positively and negatively regulates biosynthesis of phenazine-1-carboxylic acid and PLT, respectively (Ge *et al*. 2006). Collectively these findings suggest that the σ^{70}:σ^{38} balance is important for governing antibiotic production under certain conditions. The rationale for why one sigma factor preferentially directs transcription of a given set of antibiotic genes, however, remains an enigma. A third sigma, RpoN (σ^{54}), has been found to control expression of DAPG and PLT in *P. fluorescens* CHA0 (Péchy-Tarr *et al*. 2005). Mutants deficient in *rpoN* exhibited increased *phlA* gene transcription and DAPG production; whereas, PLT production and *plt* gene expression were markedly reduced in this background. The RpoN mutant showed greatly reduced motility, an impaired ability to use several carbon and nitrogen sources and increased sensitivity to salt stress (Péchy-Tarr *et al*. 2005). Thus, RpoN is not only important for controlling the DAPG:PLT balance, it also affects diverse aspects of bacterial physiology.

2. LysR-type transcriptional regulators (LTTRs)

The ability of fluorescent *Pseudomonas* spp. to survive environmental stress conditions in the plant environment is in part attributed to the diverse regulatory mechanisms that control the production of secondary metabolites. The LTTR family comprises the most abundant group of transcriptional regulators in prokaryotes (Maddocks and Oyston 2008). One LTTR regulating secondary metabolite production by *P. aeruginosa*, *P. putida*, and *P. syringae* is Trpl. Trpl transcriptionally activates the expression of divergently transcribed *trpBA* genes, an enzyme that catalyzes the last step in tryptophan biosynthesis (Crawford 1989). Trpl affects antibiotic production because tryptophan is a precursor of PRN (Kirner *et al*. 1998). Another LTTR has been identified, called PqsR, which binds to the quorum-sensing signal molecule PQS (Pseudomonas quinolone signal) leading to increased expression of the PHZ biosynthetic genes (Deziel *et al*. 2005). The *pqsR* gene was recently isolated and characterized from *Pseudomonas* sp. M18 (Lu *et al*. 2009). PqsR was found to act as a repressor of PLT biosynthesis and an activator of PHZ biosynthesis. In mutants lacking *pqsR*, there was increased transcription of the *plt* biosynthetic operon; however *pltR* expression was unaffected suggesting that regulation is independent of the pathway-specific regulator PltR (Lu *et al*. 2009). Conversely, expression of the *phz* operon was greatly diminished in the *pqsR*-mutant background (Lu *et al*. 2009). The details regarding how PqsR acts as a transcriptional repressor of the PLT genes while at the same time activating PHZ gene expression has yet to be determined. Because LTTRs are such a large family of transcriptional regulators, it is only a matter of time before additional members that regulate secondary metabolite production are identified.

3. The TetR-family transcriptional regulator PsrA

In various *Pseudomonas* species including *P. aeruginosa*, *P. putida*, *P. chlororaphis* and the pathogenic *P. syringae* pv. tomato strain DC3000, a TetR family regulator called PsrA (*Pseudomonas* sigma regulator) has been identified that controls expression of *rpoS* (Kojic and Venturi 2001; Kojic *et al*. 2002; Chin-A-Woeng *et al*. 2005; Chatterjee *et al*. 2007). PsrA acts as a positive regulator of *rpoS* and it also negatively regulates its own transcription through binding to a palindromic C/GAAACN$_{2\text{-}4}$ GTTTG/C sequence located in the promoter regions of these genes (Kojic *et al*. 2002). Because RpoS controls production of secondary metabolites, PsrA also influences the biocontrol capabilities of these bacteria. A computer search of the *P. aeruginosa* genome identified the PsrA binding motif upstream of fourteen genes (Kojic *et al*. 2005). It was confirmed that PsrA directly binds to and regulates four of the fourteen promoter regions; therefore, PsrA, appears to function as a global regulator involved in adaptation to the stationary phase.

4. Quorum sensing

Quorum-sensing (QS) systems rely on self-generated signal molecules to coordinate gene expression in response to population density. Gram negative bacteria typically use N-acyl-homoserine lactones (AHLs) as signalling molecules, which are synthesized by LuxI-type proteins also known as AHL synthases. Most AHLs freely diffuse across the cell envelope; consequently, the AHL concentration increases as the population grows. After a threshold level is achieved, the AHL binds to a cognate LuxR-type protein, which can then activate or repress target genes.

In *Pseudomonas* spp., production of PHZ antibiotics is controlled by QS. In *P. fluorescens* 2-79, *P. chlororaphis* (*aureofaciens*) 30-84 and *P. chlororaphis* PCL1391, genes encoding the AHL synthase (*phzI*) and the transcriptional activator (*phzR*) are located upstream of the *phz* biosynthetic cluster (see Mavrodi *et al*. 2006 for a review). Transcription of *phzR* and *phzI* is under PhzR control; hence the system is autoinducible (Mavrodi *et al*. 2006). The LuxR-type regulator binds to a palindromic sequence known as the "phz box" located between *phzA* and the divergently transcribed *phzR,* which is essential for *phz* gene expression (Khan *et al*. 2005). A second QS system has been identified in *P. chlororaphis* (*aureofaciens*) 30-84 called CsaI/R (Zhang and Pierson 2001). The Csa system has no impact on PHZ rather it controls protease production as well as cell surface properties of this bacterium (Zhang and Pierson 2001).

In *P. aeruginosa* PHZ expression is under QS control; however, many of the details regarding regulation have yet to be defined. This is in part due to the fact that *P. aeruginosa* QS is extremely complex (see Mavrodi *et al*. 2006 for a review). There are two AHL-based QS systems called Las (comprised of the *lasR* and *rhlI* genes) and Rhl (comprised of *rhlR* and *rhlI*). This bacterium also uses a quinolone molecule known as PQS (Pseudomonas quinolone signal) to regulate gene expression. The systems are all interconnected with the Las circuitry positively regulating *rhlI* and *rhlR*. What's more, PQS, which is produced during late stationary phase, modulates expression of both the Rhl and Las QS systems. A number of additional regulators (QscR, RsaL, MvaT) feed into this hierarchy, adding even more complexity to this intricate system of regulation (Mavrodi *et al*. 2006). Therefore QS control of PHZ production is not straightforward in *P. aeruginosa*, and more research is required to clarify this multi-tiered system of regulation.

Post transcriptional regulation

1. The Gac/Rsm pathway

The sensor kinase GacS and the response regulator GacA are members of a two-component regulatory system that is present in a wide variety of Gram-negative bacteria (Heeb and Haas 2001). It has been shown in numerous studies that a mutation in either *gacS* or *gacA* results in two main characteristics: a partial or complete loss of biocontrol ability in plant-beneficial pseudomonads and markedly reduced virulence in plant- or animal-pathogenic bacteria. The *gacS/gacA* mutant phenotype is attributed to the loss of secondary metabolite and extracellular enzyme production (Barta *et al*. 1992; Reimman *et al*. 1997; Whistler *et al.* 1998; Duffy and Defago 2000). In *P. fluorescens* CHA0, *gacS* and *gacA* mutants exhibit altered colony morphology and motility in addition to the loss of secondary metabolite production (Schmidli-Sacherer *et al*. 1997; Duffy and Defago 2000), suggesting that the GacS/GacA system impacts a variety of cellular activities.

Activation of the Gac system occurs when an unknown signal stimulates autophosphorylation of the GacS sensor (Heeb *et al*. 2004). The phosphate group is then transferred by a phosphorelay mechanism to GacA. Regulation of secondary metabolites by Gac involves a second system called Rsm (regulation of secondary metabolites). In *P. fluorescens* CHA0, one of the best-studied organisms with respect to this circuitry, the Rsm system is comprised of two RNA-binding proteins, RsmA and RsmE, and three small regulatory RNAs, *RsmZ*, *RsmX*, *RsmY* (Lapouge *et al*. 2008). The RsmA/E proteins function as post-transcriptional repressors by binding to a specific ribosome-binding sequence (RBS) in the target mRNA and blocking translation. Translational repression can be alleviated by the action of *RsmY*, *RsmZ* and *RsmX* which bind multiple copies of RsmA/E, rendering the ribosome-binding site of target genes accessible for the translational machinery (Heeb *et al.* 2002; Kay *et al.* 2005; Reimmann *et al*. 2005). GacA positively regulates the transcription of *rsmZ*, *rsmX* and *rsmY* and this is thought to occur through GacA binding to a conserved upstream element termed the GacA box (TGAAGN6-CTTACA) (Lapouge *et al*. 2008). There are homologues of the Gac/Rsm system found in other bacteria including *P. aeruginosa*, *E. coli* and *Erwinia caratovora* (Hyytiäinen *et al.* 2001; Dubey *et al.* 2003; Mulcahy *et al.* 2008). In all cases, the Gac/Rsm system serves to modulate expression of secondary metabolites and extracellular enzymes during the transition from low to high cell density states (Bejerano-Sagie and Xavier 2007).

As discussed above, for many Pseudomonads regulatory elements like RpoS and QS systems control production of secondary metabolites (Heeb *et al*. 2004; Ge *et al*. 2006; Girard *et al.* 2006). Interestingly, the expression of both RpoS and the PhzI/PhzR QS genes are subject to positive regulation by the GacS/GacA system (Heeb *et al.* 2005; Girard *et al.* 2006). These findings suggest that secondary metabolite and extracellular enzyme production is governed by a complex regulatory cascade, with the GacS/GacA pair at the top of the hierarchy. The picture becomes even more complicated by the fact that the activity of the GacS/GacA system can be modulated by accessory regulators called RetS (regulator of exopolysaccharide and type III secretion) and LadS (lost adherence). Studies in *P. aeruginosa* and *P. fluorescens* CHA0 have shown that RetS inhibits the Gac system, whereas LadS activates it (Goodman *et al*. 2004; Humair *et al.* 2009). It is postulated that LadS interacts with GacS, facilitating autophosphorylation, while RetS functions as a phosphatase removing phosphates from GacS (Humair *et al.* 2009). A recent study in *P. aeruginosa* demonstrated that both RetS and LadS physically interact with GacS (Goodman *et al.* 2009; Workentine *et al*. 2009), but the actual mechanism whereby the sensors communicate with one another has yet to be determined. Of interest, some chemoreceptor proteins of enteric bacteria are known to assemble in the cytoplasmic membrane, which allows bacteria to integrate several signals in the chemotactic response (Hazelbauer *et al.* 2008). It is possible that LadS and RetS behave like chemoreceptors, in that they are able to sense additional signals and modulate the activity of GacS accordingly.

MOLECULAR CHARACTERIZATION OF THE BIOCONTROL BACTERIUM *PSEUDOMONAS CHLORORAPHIS* PA23 (WORK DONE IN CANADA)

Pseudomonas chlororaphis PA23 is a biocontrol agent first isolated from the root tips of soybean plants. This strain inhibits mycelial growth and sclerotial germination, and reduces the disease incidence and severity of *Sclerotinia sclerotiorum*-mediated stem rot of canola (Savchuk and Fernando 2004; Zhang 2004). PA23 also inhibits several root pathogens in both greenhouse and field studies (Kavitha *et al*. 2005). Historically, biological approaches to disease management have shown inconsistent performance in the field which is due in part to the variable expression of disease suppressive factors. Therefore, identifying metabolites that are essential for PA23 biocontrol is a critical step in the development of a successful biocontrol agent. A number of different approaches have been taken to identify biocontrol compounds produced by PA23, as well as regulatory factors governing their expression. The results of these analyses are

discussed in the following sections.

Identification of antibiotics and other AF compounds produced by PA23

Antibiotic related genes from PA23 were identified using standard PCR techniques. Thirty different primer combinations based on conserved sequences were used for PCR amplification (Zhang *et al.* 2006). PA23 was found to harbour genes required for the biosynthesis of PHZ and PRN. Through HPLC analysis of culture supernatant extracts, it was discovered that PA23 generates phenazine-1-carboxylic acid and 2-hydroxyphenazine with higher amounts of the former (Zhang *et al*. 2006). PRN was also confirmed to be present in PA23 extracts (Selin *et al*. 2010). Besides antibiotics, PA23 produces an array of compounds that likely contribute to biocontrol including proteases, lipases, and siderophores (Poritsanos *et al*. 2006). PA23 also generates autoinducer signal molecules, suggesting that at least some of these biocontrol metabolites are regulated through QS (Poritsanos *et al*. 2006).

The role of PHZ and PRN in PA23 biocontrol

Most biocontrol Pseudomonads produce more than one antibiotic; however, not necessarily all of these compounds are required for the control of a particular pathogen. To understand the individual contribution of PHZ and PRN to inhibition of *S. sclerotiorum*, antibiotic-deficient mutants were generated and analyzed for their AF capabilities.

Isolation and characterization of a PA23 PHZ-deficient mutant

A PHZ-minus mutant, called PA23-63, was isolated from a transposon (Tn) mutant library (Selin *et al*. 2010). Mutant PA23-63 was identified because it exhibited increased *S. sclerotiorum* AF activity compared to the wild-type but it was white in color, suggesting no PHZ production. Sequencing revealed a Tn insertion in the *phzE* gene, which forms part of the PHZ biosynthetic cluster. The AF activity exhibited by PA23-63 was supported by our greenhouse assays, where PA23-63 demonstrated wild-type suppression of canola stem rot (Selin *et al*. 2010). These findings led us to postulate that another metabolite might be upregulated in the PHZ-minus background. Analysis of PA23-63 culture supernatants revealed PRN levels were elevated 2-fold over wild type. Using a *prnA-lacZ* transcriptional fusion we discovered no change in transcription of the PRN biosynthetic genes; consequently, another mechanism must account for the increased PRN production in PA23-63. Both PHZ and PRN are produced by the shikimic acid pathway and we believe that it is through this shared pathway that PRN levels are elevated. Looking at this more closely, PhzE is responsible for converting chorismic acid into 2-amino-2-deoxy-isochorismic acid. The *phzC* gene, which lies upstream of *phzE*, encodes a deoxy-arabino-heptulosonate-7-phosphate (DAHP) synthase which shunts C3 and C4 organic phosphates into the shikimic acid pathway. PhzC is regulated at both the transcriptional level and through feedback inhibition. Therefore in a *phzE* mutant background, there is no endproduct formation and no feedback inhibition of PhzC. As a result, PhzC continuously pumps carbon into the shikimic acid pathway resulting in elevated PRN production. Collectively our findings revealed that PHZ production is not essential for PA23 biocontrol of *S. Sclerotiorum* rather other metabolite(s), like PRN, play a more important role.

Isolation and characterization of PA23 PRN-deficient mutants

To confirm the involvement of PRN in PA23-mediated control of *S. sclerotiorum*, mutants unable to synthesize this antibiotic were required. We did not isolate any mutants

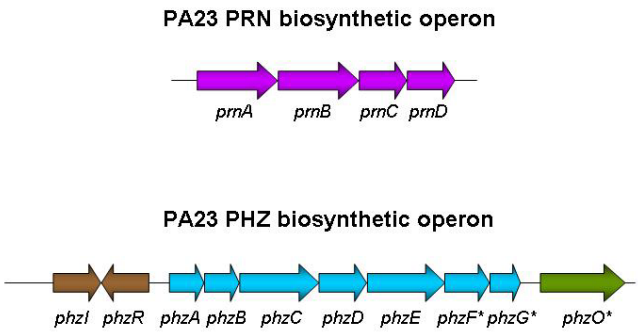

Fig. 1 *Pseudomonas chlororaphis* PA23 pyrrolnitrin and phenazine gene clusters. The pyrrolnitrin gene cluster is arranged as a four-gene operon consisting of *prnABCD*. The phenazine biosynthetic gene cluster consists of *phzABCDEFG*. Two upstream genes, *phzI* and *phzR* encode for a quorum sensing system. The region encompassing *phzI* through *phzE* has been sequenced in PA23; the *phzF-phzO* (*) genetic arrangement is based on that found in the closely related strain *P. chlororaphis* (*aureofaciens*) 30-84. PhzO directs the synthesis of 2-hydroxyphenazine carboxylic acid, which spontaneously converts to 2-hydroxyphenazine.

harbouring a Tn insertion in the PRN biosynthetic cluster in our initial screen; therefore, they were created via allelic exchange (Selin *et al*. 2010). This was accomplished by isolating the *prn* biosynthetic genes through PCR. Sequence analysis revealed that the *prn* cluster is comprised of four genes, *prnABCD* (**Fig. 1**), which are 93% identical at the nucleotide level to the *prn* operon of *P. fluorescens* Pf-5. A plasmid containing the *prnABCD* cluster in which a portion of the *prnBC* genes had been replaced by a gentamicin-resistance cassette was used to generate the PRN mutants. Two PRN-deficient strains were generated: PA23-8 (PRN-; PHZ+) and PA23-63-1 (PRN-; PHZ-). Analysis of these mutants in plate assays and in the greenhouse revealed a dramatic decrease in AF activity compared to the wild type (Selin *et al*. 2010). Our discovery that PRN is the primary antibiotic underlying PA23 biocontrol of *S. sclerotiorum* is pivotal for our understanding of this bacterial-pathogen interaction. We can now turn our attention towards elucidating biotic and abiotic factors influencing PRN expression as a means of enhancing PA23 biocontrol.

Identification and characterization of regulatory genes affecting *P. chlororaphis* biocontrol

1. The Gac/Rsm system

One isolate was identified from our Tn mutant library that no longer exhibited AF activity. This strain was found to harbour a Tn insertion in the *gacS* gene and our analysis revealed that it was no longer producing antibiotics and degradative enzymes (Poritsanos *et al*. 2006). Furthermore, autoinducer signalling molecules were not being generated by the *gacS* mutant, suggesting that in PA23, expression of QS is under Gac control. Not surprisingly, the *gacS* mutant was unable to suppress *Sclerotinia*-mediated stem rot of canola in our greenhouse studies. The loss of biocontrol activity was attributed to a lack of AF compound production rather than phyllosphere persistence because the presence or absence of *gacS* had no impact on the latter (Poritsanos *et al*. 2006). The *gacS* mutation did, however, affect the ability of strain PA23 to form biofilms. The *gacS*-deficient strain was found to produce 9-fold less biofilm compared to the wild type. With this in mind, we were surprised to discover that the mutant and wild-type biofilms were equally resistant to antibiotics (Poritsanos *et al*. 2006). Thus, even though the biofilm formed by the *gacS* mutant was much thinner, it remained fully functional in terms of its ability to protect cells from toxic agents.

As discussed above, the Gac two-component circuitry affects secondary metabolite production through a second

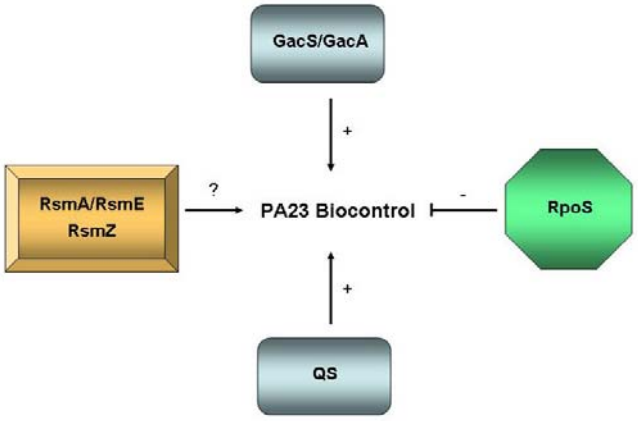

Fig. 2 Regulation of *Pseudomonas chlororaphis* PA23 biocontrol. In PA23, the GacS/GacA two-component signal transduction system positively regulates expression of antibiotics and degradative enzymes. The stationary phase sigma factor, RpoS, has a repressive effect on AF activity, while the PhzI/R QS system positively affects fungal inhibition. Two Rsm protein components have also been identified (RsmA and RsmE) together with a regulatory RNA (*RsmZ*). The way in which the Rsm components influence PA23 biocontrol is currently under investigation.

system called Rsm. In our laboratory, we have begun to characterize the PA23 Rsm system. Through PCR amplification, we have isolated genes encoding putative RsmA and RsmE regulatory proteins as well as an RsmZ regulatory RNA (Selin and de Kievit unpublished data). We are currently generating knock-out mutants as well as overexpression strains for each of these genes.

2. RpoS

Preliminary studies looking at the role of RpoS have demonstrated that this sigma factor negatively impacts PA23 biocontrol (Selin and de Kievit unpublished data). An *rpoS* mutant generated through allelic exchange exhibited increased AF activity compared to the wild type. The mechanism(s) underlying the enhanced fungal inhibition is currently under investigation. We hypothesize that AF compounds like PRN, PHZ or degradative enzymes are being produced at higher levels in the *rpoS* mutant background. If this turns out to be the case, it suggests that other sigma factors like RpoD or RpoN control expression of these key factors.

3. Quorum sensing

We have identified two genes upstream of the PHZ biosynthetic operon, called *phzI* and *phzR* that encode a quorum-sensing system in PA23 (**Fig. 1**). QS mutants have been made using two different strategies. First, a *phzR* mutant was created through allelic exchange. Second, an autoinducer-deficient mutant was generated through introducing a plasmid-borne copy of *aiiA* into PA23. This gene encodes an enzyme that degrades autoinducer signal molecules. Analysis of both mutants revealed a reduction in AF activity suggesting QS positively regulates PA23 biocontrol (Selin and de Kievit, unpublished data).

4. PA23 regulation of biocontrol – concluding remarks

Our analysis of PA23 gene regulation has revealed that, like other biocontrol Pseudomonads, secondary metabolite production is governed by a complex, multi-tiered network of regulation (**Fig. 2**). It is only through creation of PA23 derivatives, either lacking or overexpressing these regulatory factors that we can begin to piece together the puzzle of PA23 biocontrol. Analysis of genes and gene products in the aforementioned strains will clarify how these AF metabolites are regulated. Such studies are essential if we are to create a PA23-based product that performs in a predictable and reliable fashion.

CONCLUSIONS

Biological control agents represent an environmental friendly, promising alternative to chemicals for the management of plant diseases. Several commercial preparations of *Pseudomonas* and *Bacillus* genera are currently in use. One of the primary drawbacks associated with these biocontrol agents is their inconsistent performance in the field. Fluctuations in environmental conditions influence expression of key biocontrol factors, which in turn can result in unreliable field performance. Thus we need to understand, at a molecular level, biotic and abiotic factors influencing expression of AF compounds if these products are to become a viable alternative to chemical pesticides. Our understanding of biocontrol can be improved by sequencing the genomes of a number of bacteria that control plant pathogens. Such studies will undoubtedly reveal novel genes and gene products that contribute to biocontrol. Furthermore, biocontrol studies have primarily focused on bacterial genes related to antibiosis; consequently, only a few genes involved in induction of the plant defence response or competition for nutrients and space have been characterised. Additional research is needed to unravel the molecular mechanisms of antagonistic bacteria with emphasis on rhizosphere competence, competition or parasitism and induced resistance in addition to antibiosis. Through transcriptomics and proteomics, we can identify genes and proteins that are differentially expressed in not only the bacteria, but the fungus and the plant host as well. Collectively these findings will provide us with a global perspective of the complex physiological changes that take place in all three organisms, namely the bacterium, the fungus and the plant, upon interaction in the environment. As essential genes are identified, their expression can be monitored using reporter fusions like green fluorescent protein, or through quantitative PCR. Together, these molecular approaches will enhance our understanding of mechanisms involved in biocontrol, which should ultimately lead to the creation of new tools for improving plant health and soil productivity.

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