

# Phenazines are not essential for *Pseudomonas chlororaphis* PA23 biocontrol of *Sclerotinia sclerotiorum*, but do play a role in biofilm formation

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## Abstract

*Pseudomonas chlororaphis* strain PA23 is a biocontrol agent capable of suppressing disease caused by the fungal pathogen *Sclerotinia sclerotiorum*. This bacterium produces the diffusible antibiotics phenazine-1-carboxylic acid, 2-hydroxyphenazine and pyrrolnitrin (PRN). Because the individual contribution of these antibiotics to PA23 biocontrol has not been defined, mutants deficient in the production of phenazine (PHZ), PRN or both antibiotics were created. Analysis of the PHZ mutant revealed enhanced antifungal activity *in vitro* and wild-type levels of *Sclerotinia* disease suppression. Conversely, the PRN- and the PRN/PHZ-deficient strains exhibited decreased antifungal activity *in vitro* and markedly reduced the ability to control *Sclerotinia* infection of canola in the greenhouse. These findings suggest that PRN is the primary antibiotic mediating biocontrol of this pathogen. Analysis of *prnA-lacZ* and *phzA-lacZ* transcriptional fusions revealed that PRN and PHZ are not subject to autoregulation; moreover, they do not cross-regulate each other. However, HPLC showed a twofold increase in PRN levels in the PHZ<sup>-</sup> background. Finally, PHZ, but not PRN production, is involved in biofilm development in *P. chlororaphis* PA23.

## Introduction

Compared with chemical pesticides, biological control represents a safer, more environment-friendly approach to managing plant pathogens. Fluorescent pseudomonads are present in high numbers in natural soils and various strains have been found to produce metabolites deleterious to fungal pathogens. Inhibitory compounds include antibiotics, for example 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT), cyclic lipopeptides and hydrogen cyanide (HCN), together with hydrolytic enzymes such as proteases, cellulase,  $\beta$ -glucanase and chitinase (Dowling & O'Gara, 1994; Thomashow & Weller, 1995; Haas & D efago, 2005). This potent arsenal of antimicrobial metabolites enables pseudomonads to inhibit a myriad of plant pathogens.

*Pseudomonas chlororaphis* strain PA23 is a biocontrol agent initially isolated from soybean root tips. In both

greenhouse and field studies, this bacterium is able to protect canola from the devastating effects of stem rot caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Savchuk & Fernando, 2004; Zhang, 2004; Fernando *et al.*, 2007). *Sclerotinia sclerotiorum* is a ubiquitous, soilborne fungus that infects > 400 plant hosts, causing significant loss of economically important crops (Purdy, 1979). In commercially grown canola cultivars, there is no known resistance against *S. sclerotiorum*, making management of this pathogen essential. A well-defined zone of inhibition surrounding PA23 on antifungal plates suggests that antibiotic production is the primary mechanism of pathogen inhibition (Savchuk & Fernando, 2004; Poritsanos *et al.*, 2006). Strain PA23 produces the diffusible antibiotics phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine (2-OH-PHZ) and PRN (Zhang *et al.*, 2006); however, the individual contribution of these antibiotics to PA23 biocontrol is yet to be defined.

Many biocontrol agents display excellent disease control in the greenhouse, but show inconsistent performance in the field, due in part to the variable expression of disease-suppressive factors (Cook, 1993; Walsh *et al.*, 2001; Haas & Keel, 2003). Environmental conditions can have a dramatic impact on antibiotic production and conditions that favor expression of one antibiotic may not be optimal for others. For instance, maximal PHZ production by *P. chlororaphis* strain PCL1391 was found to occur when glucose, L-pyroglutamic acid or glycerol was used as the carbon source (van Rij *et al.*, 2004), whereas fructose and mannitol favored PRN production by *Pseudomonas fluorescens* strain CHA0 (Duffy & D efago, 1999). Other factors that influence the production of PHZ and PRN include pH, temperature, oxygen availability and mineral amendment (Slininger & Jackson, 1992; Slininger & Shea-Wilbur, 1995; Duffy & D efago, 1999; Hwang *et al.*, 2002; van Rij *et al.*, 2004). Identifying metabolites that are essential for PA23 biocontrol is an important first step in the development of a successful biocontrol agent. Once this has been established,

factors affecting the production of key compounds can be determined.

The aim of the present study was to assess the contribution of PHZ and PRN to PA23 inhibition of the fungal pathogen *S. sclerotiorum*. We also examined whether these antibiotics are subject to autoregulatory or coregulatory control. Finally, the impact of PHZ and PRN on biofilm formation was examined.

## Materials and methods

### Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured at 37 °C on Lennox Luria–Bertani (LB) agar (Difco Laboratories, Detroit, MI). *Pseudomonas chlororaphis* PA23 and its derivatives were routinely cultured at 28 °C on LB agar plates and broth, Minimal M9 casamino acid media+0.2% glucose (M9CA; Difco Laboratories), Terrific broth

**Table 1.** Bacterial strains, plasmids and primers used in this study

Strain/plasmid/primer	Relevant genotype or phenotype	Source or reference
<b>Strain</b>		
<i>P. chlororaphis</i>		
PA23	PRN <sup>+</sup> PHZ <sup>+</sup> Rif <sup>R</sup> wild type (soybean plant isolate)	Savchuk & Fernando (2004)
PA23-63	PHZ <sup>-</sup> Rif <sup>R</sup> <i>phzE</i> ::Tn5-OT182 genomic fusion	This study
PA23-8	PRN <sup>-</sup> Rif <sup>R</sup> <i>prnBC</i> deletion mutant	This study
PA23-63-1	PHZ <sup>-</sup> PRN <sup>-</sup> Rif <sup>R</sup> <i>phzE</i> ::Tn5-OT182 genomic fusion; <i>prnBC</i> deletion mutant	This study
<i>E. coli</i>		
DH5�	<i>supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Gibco
SM10	Mobilizing strain; RP4 <i>tra</i> genes integrated in chromosome; Km <sup>R</sup> Tc <sup>R</sup>	Simon <i>et al.</i> (1983)
<i>C. violaceum</i> CVO26	Autoinducer synthase ( <i>cvil</i> ) mutant from <i>C. violaceum</i> ATCC 31532, autoinducer biosensor	Latifi <i>et al.</i> (1995)
<b>Plasmids</b>		
pOT182	pSUP102(GM)::Tn5-OT182 Cm <sup>R</sup> Gm <sup>R</sup> Amp <sup>R</sup> Tc <sup>R</sup>	Merriman & Lamont (1993)
pOT182-63 (EcoRI)	pOT182 containing <i>phzE</i> ::Tn5-OT182 genomic fusion	This study
pCR <sup>R</sup> 2.1-TOPO	Cloning vector for PCR products	Invitrogen
pTOPO-prn	pCR <sup>R</sup> 2.1-TOPO containing <i>prnABCD</i> genes	This study
pEX18Ap	Suicide vector	Hoang <i>et al.</i> (1998)
pUCGm	Source of Gm <sup>R</sup> cassette	Schweizer (1993)
pEX18Ap-prn	5.7-kb prn cluster containing a Gm <sup>R</sup> cassette insertion within <i>prnB</i> and <i>prnC</i>	This study
pPHZA- <i>lacZ</i>	pLP170 containing the <i>phzA</i> promoter region from <i>P. chlororaphis</i> PA23	This study
pPRNA- <i>lacZ</i>	pLP170 containing the <i>prnA</i> promoter region from <i>P. chlororaphis</i> PA23	This study
pLP170	Promoterless <i>lacZ</i> transcriptional fusion vector	Preston <i>et al.</i> (1997)
pME3219	pME6010 containing an <i>hcnA-lacZ</i> translational fusion	Laville <i>et al.</i> (1998)
<b>Primers</b>		
prnF	5'-ggtcggtgttatgaacaagccgatcaa-3'	This study
prnR	5'-tctagatgaactgacgcccgtcg-3'	This study
phzAR-F	5'-aatcctgcatccaactc-3'	This study
phzAR-R	5'-aagttgttcgaaggggttca-3'	This study
prnA-F	5'-ccaatgcccttgcatcttgag-3'	This study
prnA-R	5'-tgccggttcgagccaga-3'	This study
Tn5-ON82	5'-gatcctgaaaaagggaagg-3'	Poritsanos <i>et al.</i> (2006)
Tn5-OT182 right	5'-atgtaggaggtcacatg-3'	Poritsanos <i>et al.</i> (2006)

(Sambrook *et al.*, 1989) or peptone tryptic soy broth (PTSB; Ohman *et al.*, 1980). For PRN and PHZ analysis, cells were grown in 523 (Kato & Heskett, 1970) and PPM media (Levitch & Stadtman, 1964), respectively. As required, media were supplemented with the following antibiotics: tetracycline (Tc; 15 µg mL<sup>-1</sup>), gentamicin (Gm; 15 µg mL<sup>-1</sup>) and ampicillin (100 µg mL<sup>-1</sup>) for *E. coli*, and rifampicin (25 µg mL<sup>-1</sup>), tetracycline (15 or 100 µg mL<sup>-1</sup>), gentamicin (25 µg mL<sup>-1</sup>) and piperacillin (13 or 100 µg mL<sup>-1</sup>) for *P. chlororaphis*. All antibiotics were obtained from Research Products International Corp. (Mt. Prospect, IL).

### Nucleic acid manipulation

Cloning, purification, electrophoresis and other manipulations of nucleic acid fragments and constructs were performed using standard techniques (Sambrook *et al.*, 1989). PCR was performed under standard conditions as suggested by Invitrogen Life Technologies data sheets supplied with their *Taq* polymerase.

### Tn5-OT182 transposon mutagenesis

Bacterial conjugations were performed to introduce Tn5-OT182 into *P. chlororaphis* PA23 by biparental mating following the method of Lewenza *et al.* (1999). For each mating, 5–10 Tc<sup>R</sup> colonies were screened by PCR to ensure that transconjugants contained a Tn5 insertion using TNP5-Forward and TNP5-Reverse primers (Table 1). To determine the site of Tn5-OT182 insertion, rescue cloning was performed following previously described methods (Lewenza *et al.*, 1999).

### Generation of PA23 PRN-deficient strains

The PRN biosynthetic gene cluster was PCR amplified from PA23 genomic DNA using primers prnF and prnR (Table 1). The 5.6-kb PCR product was cloned into pCR2.1-TOPO and the resulting plasmid, pTOPO-prn, was digested with ClaI and BspE1 to remove a 785-bp fragment containing a portion of *prnB* and *prnC*. The ends were polished with Klenow and ligated to an 840-bp SmaI-cut Gm<sup>R</sup> cassette isolated from pUCGm. The interrupted *prn* operon was released from pTOPO-prn with XbaI and BamHI and cloned into the same sites of suicide vector pEX18Ap, yielding pEX18Ap-prn (Table 1). Plasmid pEX18Ap-prn was mobilized into PA23 and PA23-63 by triparental mating. Putative PRN-deficient mutants PA23-8 and PA23-63-1 were screened by PCR analysis to verify that a double-crossover event had occurred.

### Sequence analysis

The sequence of the *prnABCD* operon on plasmid pTOPO-prn was determined using a combination of universal

primers and primer walking. The PA23 *prnABCD* sequence was submitted to GenBank (accession number EU188755). Sequence analysis of the Tn5 XhoI rescue clones was performed using oligonucleotide primer Tn5-ON82 (Table 1), which anneals to the 5' end of Tn5-OT182. Sequencing was performed at the University of Calgary Core DNA Services facility, and sequences were analyzed with BLASTN and BLASTX databases.

### Antifungal assays

Radial diffusion assays to assess fungal inhibition *in vitro* were performed according to previously described methods (Poritsanos *et al.*, 2006). Six replicates were analyzed for each strain and assays were repeated four times.

### PHZ and PRN gene expression

To monitor PHZ gene expression, a *phzA-lacZ* transcriptional fusion was created using PCR primers phzAR-F and phzAR-R (Table 1). The *phzA* promoter region was PCR amplified and cloned into pCR2.1-TOPO. A 1.1-kb HindIII and EcoRV fragment was removed and cloned into HindIII-SmaI-digested pLP170, yielding pPHZA-*lacZ*. To generate a *prn* (PRN) transcriptional fusion, PCR primers prnA-F and prnA-R (Table 1) were used to amplify a 1.5-kb fragment corresponding to the *prnA* promoter region of PA23. The PCR product was first cloned into pCR2.1-TOPO, and then excised using HindIII and XbaI and ligated into the same sites of pLP170, yielding pPRNA-*lacZ*. The *phzA-lacZ* and *prnA-lacZ* transcriptional fusions were mobilized into PA23, PA23-63 (*phzE*), PA23-8 (*prnBC*) and PA23-63-1 (*phzE;prnBC*). Cultures harboring these plasmids were grown in PTSB and β-galactosidase assays were performed at various points throughout growth (Miller, 1972). Samples were analyzed in triplicate and the experiment was repeated three times.

### Quantification of PRN

The amount of PRN produced by PA23, PA23-8, PA23-63 and PA23-63-1 was quantified by HPLC. Each strain was grown in 10 mL of 523 media on a rotary shaker for 4 days. The 10 mL volume was extracted with 50 mL ethyl acetate according to the method of Hwang *et al.* (2002). Samples were dissolved in 500 µL of methanol and 500-µL aliquots of each sample were injected into a Gemini C<sub>18</sub> column (100 × 4.6 mm; 5-µm particle diameter) (Phenomenex Inc, Torrance, CA) and analyzed in an isocratic solution of 45% acetonitrile/35% water/20% methanol. The eluent flow rate was 1.0 mL min<sup>-1</sup>. Peaks were detected by UV absorption at 225 nm using a Dionex AD20 Detector (Dionex, Sunnyvale, CA). The concentration of PRN in each sample was based on standard curves prepared from purified PRN (Sigma,

St. Louis, MO). HPLC-grade solvents were obtained from Fisher Scientific. Cultures were analyzed in triplicate and the PRN analysis was repeated twice.

### Quantification of PHZ

Overnight cultures of *P. chlororaphis* strain PA23, PA23-63, PA23-8 and PA23-63-1 grown in PPM were subjected to PHZ extraction following the method of Chancey *et al.* (1999). Extractions were quantified with UV-visible spectroscopy according to Maddula *et al.* (2008); the absorption maxima for PCA and 2-OH-PHZ were measured at 367 and 490 nm, respectively. The relative amounts of PCA and 2-OH-PHZ were calculated by dividing the absorption maxima by their standard extinction coefficients (PCA: 3019; 2-OH-PHZ: 7943; Olson & Richards, 1967). The PHZ quantification was repeated three times.

### HCN analysis

Production of HCN was determined qualitatively using Cyantesmo paper (Machery-Nagel GmbH & Co., Düren, Germany). To monitor expression of the genes encoding HCN, plasmid pME3219 containing an *hcnA-lacZ* translational fusion was transformed into PA23, PA23-63, PA23-8 and PA23-63-1. Cultures were grown in PTSB until they reached the stationary phase ( $OD_{600\text{ nm}} = 2.5\text{--}3.0$ ), at which point *hcnA* expression was assessed using  $\beta$ -galactosidase assays (Miller, 1972). Samples were analyzed in triplicate and experiments were repeated three times.

### Exoproduct analyses

Protease activity, lipase activity and the production of homoserine lactone autoinducer molecules were assessed according to previously described methods (Poritsanos *et al.*, 2006). Data represent the average of six replicates and assays were repeated three times.

### Motility analysis

Flagellar (swimming) and swarming motility were monitored according to Poritsanos *et al.* (2006). For motility assays, five replicates were analyzed and the experiment was repeated three times.

### Growth analysis

Growth rate analysis of PA23, PA23-63 (*phzE*), PA23-8 (*prnBC*) and PA23-63-1 (*phzE;prnBC*) was performed in rich (LB, PTSB, TB) and minimal (M9CA+0.2% glucose) media using a Bioscreen<sup>R</sup> C automated turbidometer. Overnight cultures were adjusted to an  $OD_{600\text{ nm}}$  of 0.1 by diluting with the same media and 100  $\mu\text{L}$  of culture was inoculated into each well of a Bioscreen<sup>R</sup> C microtiter plate. Control wells

contained an equal volume of sterile media. Growth of the cultures at 28 °C was monitored every 15 min over a 45-h period. Samples were analyzed in triplicate and the growth rate analysis was repeated three times.

### Biofilm development

A static, 96-well plate assay was used to assess the ability of PA23, PA23-63, PA23-8 and PA23-63-1 to form biofilms (O'Toole & Kolter, 1998). Briefly, overnight cultures grown in M9 minimal casamino acid media+0.2% glucose were adjusted to an OD of 1.0 and then diluted 1 in 100 in fresh media. One hundred-microliter aliquots of the diluted culture were inoculated into 96-well plates (Becton-Dickenson, Oakville, ON) and allowed to form biofilms. After 24 and 48 h, the adherent cell population was quantified by crystal violet (CV) staining and measuring the  $OD_{600\text{ nm}}$ . Results are averages of eight replicates ( $\pm$  SD) and representative of five independent experiments. For direct enumeration of the adherent cell population, cultures were prepared as described above, except that 1.5-mL aliquots were added to each well of a six-well plate (Becton-Dickenson). Biofilms were allowed to form for 24 and 48 h, after which wells were washed four times with phosphate-buffered saline (PBS) to remove planktonic cells. A 1-mL aliquot of PBS was added to each well and the surface-attached cells were removed by a combination of scraping and sonication. Cell suspensions were diluted and viable plate counting was performed. Results are averages of four replicates ( $\pm$  SD) and representative of three independent experiments.

### Biocontrol under greenhouse conditions

Strains PA23, PA23-63, PA23-8 and PA23-63-1 were assessed for their efficiency in suppressing stem rot of canola [*Brassica napus* (cv. Westar)] under greenhouse conditions. *Brassica napus* (cv. Westar) plants were grown in pots (21 cm  $\times$  20 cm) at 24/16 °C with a 16-h photoperiod. The plants were sprayed at 30% flowering with bacterial strains ( $2.0 \times 10^8$  CFU mL<sup>-1</sup>) suspended in 100 mM phosphate buffer, pH 7.0, with 0.02% Tween 20 and kept in a humidity chamber (24/16 °C, 16-h photoperiod). Twenty-four hours after bacterial inoculation, canola petals were sprayed with ascospores of *S. sclerotiorum* ( $8 \times 10^4$  spores mL<sup>-1</sup>) suspended in 100 mM phosphate buffer, pH 7.0, containing 0.02% Tween 20. The pathogen control plants were inoculated with ascospores, while the healthy control plants were sprayed with phosphate buffer. All plants were incubated in a humidity chamber. Fourteen days after inoculation of *Sclerotinia* ascospores, symptom development on stem and leaves was observed and recorded using a 0–7 scale (0, no lesions on the stem; 1, leaf lesion with no stem symptom; 2, 1–20-mm stem lesion; 3, 21–40-mm stem lesion; 4, 41–60-mm stem lesion; 5, 61–80-mm stem lesion; 6,

81–100-mm stem lesion; and 7, > 100-mm stem lesion or plant death). Based on symptom development, percent leaf incidence by *Sclerotinia* (PLI) and stem rot disease severity (DS) were calculated. Ten plants were used for each treatment. For assessing infection on leaves, the first 10 leaves, from top to bottom, were scored for the presence or the absence of the symptom per plant.

$$\text{PLI} = \frac{\text{Number of leaves infected with } Sclerotinia}{\text{Number of leaves observed}} \times 100$$

$$\text{DS} = \frac{\text{Total points for all plants using a 0–7 scale}}{\text{Number of plants observed}}$$

The plant studies were repeated two times.

### Statistical analysis

An unpaired Student's *t*-test was used for statistical analysis of antifungal, protease and autoinducer activity, PRN and PHZ levels, CV staining and viability counts.

## Results

### Isolation of a *P. chlororaphis* PHZ-deficient mutant

To identify genes involved in PA23 biocontrol, Tn mutagenesis was performed. Out of approximately 5000 mutants, one mutant was identified (PA23-63) that was white in color, indicative of no PHZ production. Surprisingly, this strain exhibited increased antifungal activity compared with the wild type (Table 2). Sequence analysis of DNA flanking the Tn insertion in this mutant showed 92% identity to the *phzE* gene of *P. chlororaphis* PCL1391 (GenBank accession #AF195615). PhzE is an enzyme involved in PHZ biosynthesis (Chin-A-Woeng *et al.*, 2003). Sequencing of the PHZ operons from a number of *Pseudomonas* spp. has revealed a conserved seven-gene operon comprised of *phzABCDEFG*

**Table 2.** Phenotypic characterization of *Pseudomonas chlororaphis* PA23 and mutants PA23-8 (PRN<sup>-</sup>), PA23-63 (PHZ<sup>-</sup>) and PA23-63-1 (PHZ<sup>-</sup> PRN<sup>-</sup>)

Strain	Extracellular metabolite activity				
	Antifungal*	Protease*	Autoinducer*	HCN <sup>†</sup>	Lipase
PA23	5.8 (1.3)	19.3 (1.3)	12.4 (0.5)	+	+
PA23-8 (PRN <sup>-</sup> )	3.8 (0.7) <sup>‡</sup>	20.3 (1.5) <sup>  </sup>	12.2 (0.3) <sup>  </sup>	+	+
PA23-63 (PHZ <sup>-</sup> )	9.9 (0.5) <sup>§</sup>	20.1 (1.4) <sup>  </sup>	13.3 (1.3) <sup>  </sup>	+	+
PA23-63-1 (PHZ <sup>-</sup> PRN <sup>-</sup> )	0 (0) <sup>§</sup>	20.1 (1.1) <sup>  </sup>	12.2 (1.0) <sup>  </sup>	+	+

\*Mean (SD) of the zones of activity (mm) obtained from six replicates.

<sup>†</sup>Determined using Cyantesmo paper.

<sup>‡</sup>Significantly different from the wild type ( $P < 0.01$ ).

<sup>§</sup>Significantly different from the wild type ( $P < 0.0001$ ).

<sup>||</sup>Not significantly different from the wild type.

(Mavrodi *et al.*, 2006). As such, the Tn insertion in *phzE* is expected to also affect the expression of *phzF* and *phzG*.

### Isolation of the *prn* gene cluster and generation of PRN-deficient mutants

Because no PRN biosynthetic mutants were identified in our Tn screen, they were generated using allelic exchange. To accomplish this, the PA23 *prn* biosynthetic operon was isolated through PCR amplification. Sequence analysis revealed that the *prn* cluster is comprised of four genes, *prnABCD*, exhibiting 93% identity at the nucleotide level to the *prn* operon of *P. fluorescens* Pf-5 (GenBank accession #NC004129). The same *prnABCD* genetic arrangement has been reported for other bacteria including *P. fluorescens*, *Pseudomonas pyrrocinia* and *Burkholderia cepacia* (Hammer *et al.*, 1997). PRN-deficient derivatives of PA23 and PA23-63, called PA23-8 (PHZ<sup>+</sup>, PRN<sup>-</sup>) and PA23-63-1 (PHZ<sup>-</sup>, PRN<sup>-</sup>), respectively, were created as described in Materials and methods.

### Production of antifungal metabolites in the wild-type and mutant strains

Strain PA23 produces the compounds PCA and 2-OH-PHZ, resulting in an orange phenotype (Zhang *et al.*, 2006). The loss of orange color, together with the presence of a *phzE*-Tn insertion in PA23-63, suggested that this strain was not producing PHZ. Spectral analysis confirmed that PHZ compounds were not present in culture extracts of this mutant (Table 3). The increased antifungal activity led us to hypothesize that some other antifungal metabolite(s) was upregulated in PA23-63. Through HPLC analysis, we found that PRN production is increased over twofold in the PHZ<sup>-</sup> background (Table 3). However, the reverse was not true; wild-type levels of PHZ were produced by the PRN-deficient mutant PA23-8 (Table 3). As expected, no PRN was detected in the culture supernatants of the *prnBC* mutants PA23-8 and PA23-63-1 (Table 3).

**Table 3.** PRN and PHZ production by *Pseudomonas chlororaphis* PA23 and mutants PA23-8 (PRN<sup>-</sup>), PA23-63 (PHZ<sup>-</sup>) and PA23-63-1 (PHZ<sup>-</sup> PRN<sup>-</sup>)

Strain	Antibiotic (μg mL <sup>-1</sup> )			
	PRN*	PCA*	2-OH-PCA*	Total PHZ*
PA23	1.37 (0.31)	28.5 (0.3)	4.9 (0.2)	33.7 (0.6)
PA23-8 (PRN <sup>-</sup> )	ND	29.2 (0.4) <sup>‡</sup>	4.7 (0.7) <sup>‡</sup>	33.4 (1.0) <sup>‡</sup>
PA23-63 (PHZ <sup>-</sup> )	3.05 (0.14) <sup>†</sup>	ND	ND	ND
PA23-63-1 (PHZ <sup>-</sup> PRN <sup>-</sup> )	ND	ND	ND	ND

\*Mean (SD) from three replicates.

<sup>†</sup>Significantly different from the wild type ( $P < 0.01$ ).

<sup>‡</sup>Not significantly different from the wild type.

ND, not detectable.

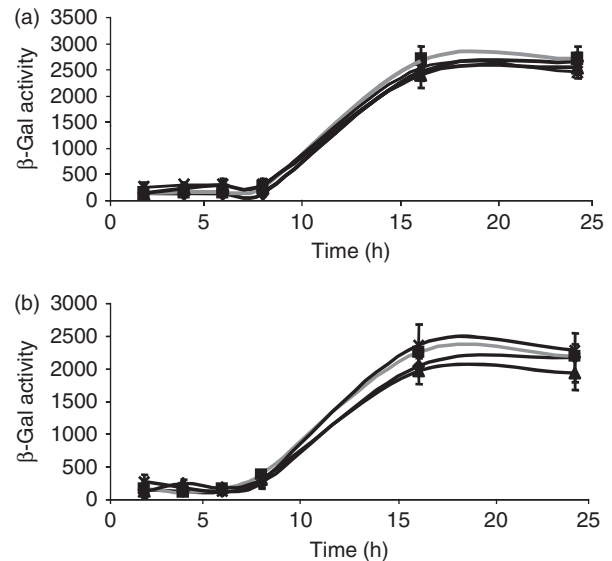
In addition to PHZ and PRN, PA23 produces a number of compounds believed to contribute to biocontrol, including HCN, protease, lipase and autoinducer molecules (Poritsanos *et al.*, 2006). Accordingly, PA23, PA23-63 (*phzE*), PA23-8 (*prnBC*) and PA23-63-1 (*phzE;prnBC*) were analyzed for the production of these molecules. Using Cyantesmo paper, all three strains demonstrated the production of the volatile antibiotic HCN (Table 2). Analysis of an *hcnA-lacZ* translational fusion revealed no significant differences in expression between PA23-63 ( $10\,200 \pm 230$  Miller units), PA23-63-1 ( $10\,481 \pm 318$  Miller units), PA23-8 ( $10\,889 \pm 311$  Miller units) and the PA23 wild-type strain ( $10\,545 \pm 655$  Miller units). Similarly, no differences were detected in protease activity, lipase activity and autoinducer production among the strains (Table 2).

### Expression of *phzA* and *prnA* in the wild type and antibiotic-deficient mutants

The increased production of PRN in PA23-63 led us to speculate that PHZ might be acting as a repressor of the *prn* operon. Therefore, we generated a *prnA-lacZ* transcriptional fusion (*pPRNA-lacZ*) to monitor expression of the PRN biosynthetic genes. We were particularly interested in two parameters: (1) the onset of *prnA* gene expression and (2) the level of transcriptional activity. As illustrated in Fig. 1a, no difference in either the initiation of *prnA* transcription or the level of expression was observed between PA23 and PA23-63. Similarly, *prnA* expression in PA23-8 and PA23-63-1 was identical to that of the wild type (Fig. 1a). These findings indicate that PHZ does not have a repressive effect on *prn* transcription. Moreover, the *prn* operon is not subject to autoregulation. When we monitored *phz* gene activity, the expression kinetics of the *phzA-lacZ* fusion were nearly identical in all four strains (Fig. 1b). Therefore, we conclude that (1) PRN has no impact on *phz* gene expression and (2) PHZ neither stimulates nor represses the PA23 *phz* operon.

### Effect of PHZ and PRN production on growth rate and motility

One of the determining factors for the success of a biocontrol agent in managing plant disease is how quickly it can achieve a sufficient population size to begin the production of pathogen-inhibiting compounds. To explore whether antibiotic production represents a significant metabolic burden for the bacteria, growth rate analysis was undertaken. No differences were detected in the growth rate between PA23, PA23-63, PA23-8 and PA23-63-1 in either rich or minimal media (data not shown). Motility is a trait that can also impact biocontrol (Lugtenberg *et al.*, 2001); accordingly, the influence of PHZ and/or PRN deficiency on bacterial translocation was assessed. We previously reported



**Fig. 1.** Time course of *prnA* and *phzA* expression in *Pseudomonas chlororaphis* PA23 (◆, wild type), PA23-63 (■, PHZ<sup>-</sup>), PA23-8 (▲, PRN<sup>-</sup>) and PA23-63-1 (×, PRN<sup>-</sup> PHZ<sup>-</sup>). Cultures containing a *prnA-lacZ* fusion (a) or a *phzA-lacZ* fusion (b) were analyzed for β-galactosidase activity throughout growth as described in Materials and methods. Note: for some data points, error bars representing SD are too small to be shown on the graph.

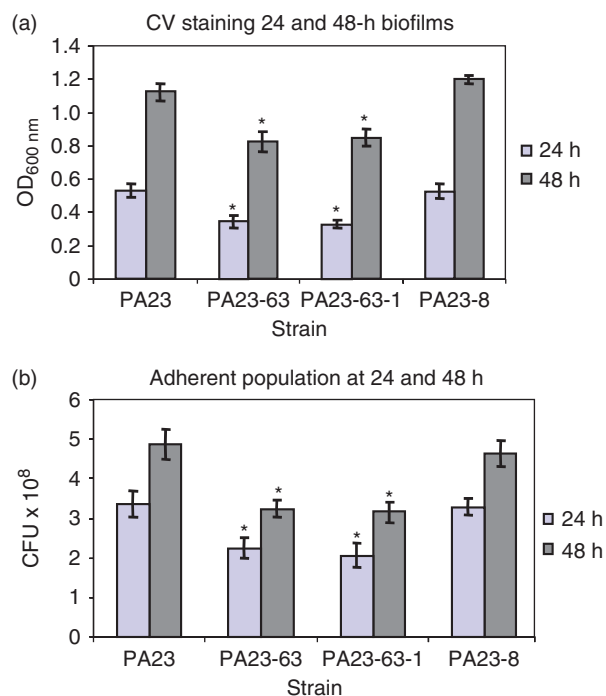
that strain PA23 is capable of swimming and swarming motility (Poritsanos *et al.*, 2006). Over the course of 72 h, PA23-63 (*phzE*), PA23-8 (*prnBC*) and PA23-63-1 (*phzE;prnBC*) were able to swim and swarm at the same rate as the wild type (data not shown), indicating that the production of these two antibiotics does not affect motility *in vitro*.

### The effect of PHZ and PRN production on PA23 biofilm formation

We used a highly reproducible 96-well plate assay to assess the ability of PA23, PA23-63, PA23-8 and PA23-63-1 to form biofilms. As illustrated in Fig. 2a, a statistically significant decrease in biofilm production was observed for the PHZ strains, compared with the wild-type and mutant PA23-8. Conversely, PRN was shown to have no impact on biofilm formation (Fig. 2a). Because CV stains biofilm matrix components as well as bacterial cells, we performed direct enumeration of the adherent cell population. The results of these analyses revealed the same outcome: PHZ, but not PRN, production leads to increased attached biomass (Fig. 2b).

### The contribution of PHZ and PRN to PA23 biocontrol of *S. sclerotiorum* in the greenhouse

The wild-type PA23, PA23-63 (*phzE*), PA23-8 (*prnBC*) and PA23-63-1 (*phzE;prnBC*) were evaluated for their ability to protect canola from stem rot disease caused by *S.*

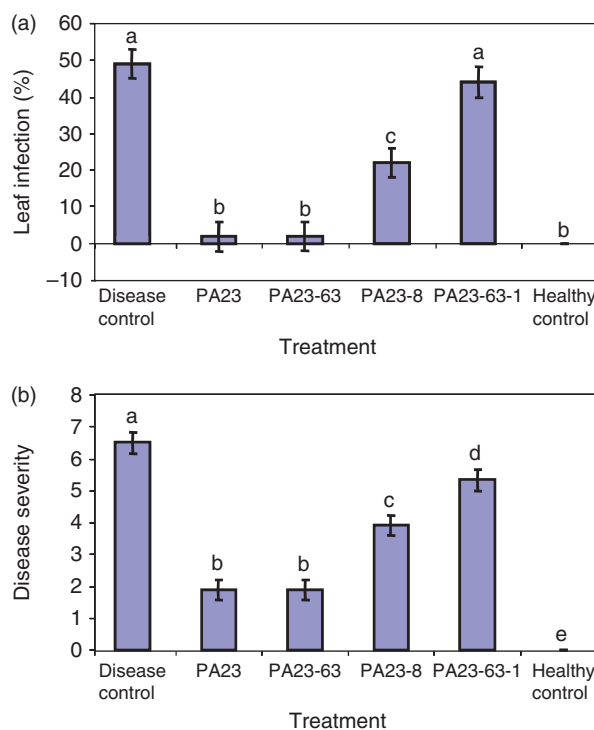


**Fig. 2.** Biofilm formation by *Pseudomonas chlororaphis* PA23 (wild type), PA23-63 (PHZ<sup>-</sup>), PA23-8 (PRN<sup>-</sup>) and PA23-63-1 (PRN<sup>-</sup> PHZ<sup>-</sup>). (a) Cultures were grown in 96-well microtiter plates containing M9CA (0.2% glucose) for 24 and 48 h at 28 °C. Biofilm formation, indicated by CV staining, was measured at A<sub>600 nm</sub>. (b) Cultures were grown in M9CA (0.2% glucose) in six-well tissue culture plates. After 24 and 48 h, the adherent cell population was enumerated by viable plate counting. Column means labeled with (\*) are significantly different from the wild type ( $P < 0.01$ ). Error bars indicate SD.

*sclerotiorum*. Two parameters were evaluated: (1) incidence of leaf infection and (2) stem rot disease severity. The PHZ-deficient strain PA23-63 was equivalent to the wild type in its ability to control fungal infection of stems and leaves and reduce disease severity (Fig. 3). For strain PA23-8, which produces the diffusible antibiotic PHZ, but no PRN, some biocontrol was observed, but it was significantly decreased from that of the wild type (Fig. 3). Compared with the disease control, no difference in the incidence of leaf infection (Fig. 3a) and only a modest decline in disease severity were observed for the PRN<sup>-</sup>/PHZ<sup>-</sup> double mutant PA23-63-1 (Fig 3b). Collectively, these findings indicate that PRN is the primary antibiotic underlying PA23 biocontrol of *S. sclerotiorum* infection in canola. Although PHZ can inhibit the fungus to some degree, it plays a more minor role in disease suppression.

## Discussion

*Sclerotinia sclerotiorum* is the causative agent of *Sclerotinia* stem rot, one of the most important diseases of canola in

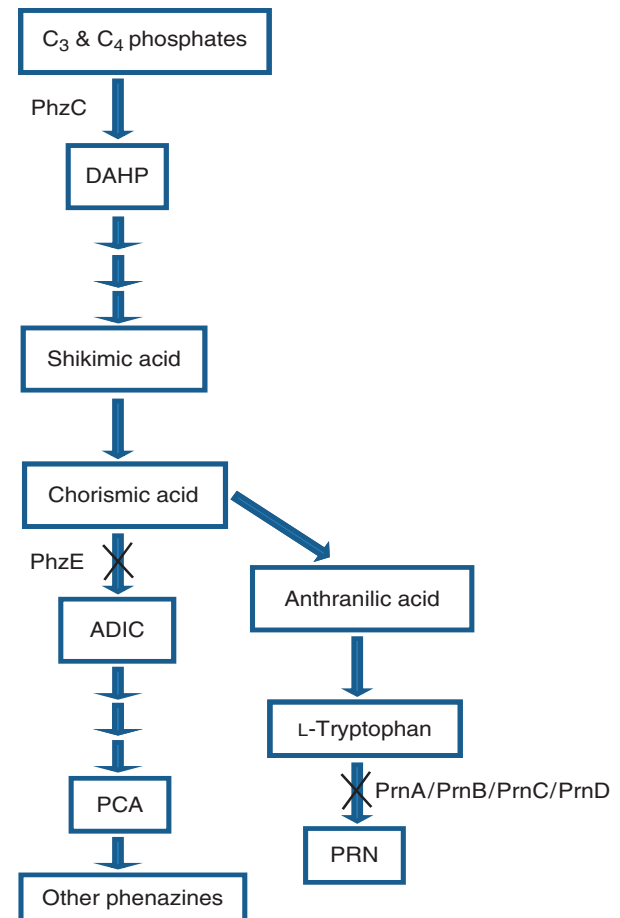


**Fig. 3.** Efficiency of *Pseudomonas chlororaphis* PA23 (wild type), PA23-63 (PHZ<sup>-</sup>), PA23-8 (PRN<sup>-</sup>) and PA23-63-1 (PRN<sup>-</sup> PHZ<sup>-</sup>) in managing *Sclerotinia sclerotiorum* ascospore infection on canola plants. (a) Percent incidence of leaf infection. (b) Disease 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 ( $P > 0.05$ ). Error bars indicate SD.

Western Canada (Martens *et al.*, 1994). Much of the research on *S. sclerotiorum* biocontrol is focused on hyperparasitism by fungal antagonists that degrade sclerotia in the soil (McLaren *et al.*, 1996; Gerlagh *et al.*, 1999). This approach is limited, however, by the fact that reducing the sclerotial soil load does not eliminate the risk of crop infection. Therefore, our group has focused on biocontrol of *S. sclerotiorum* through application of the antagonist directly at the site of pathogen entry, the canola petal. In greenhouse and field trials, *P. chlororaphis* strain PA23 has demonstrated excellent biocontrol of *S. sclerotiorum* (Savchuk & Fernando, 2004; Zhang, 2004; Fernando *et al.*, 2007). This bacterium produces metabolites that are believed to inhibit mycelial growth (Poritsanos *et al.*, 2006) and it also stimulates the production of plant defense enzymes (Fernando *et al.*, 2007); therefore, fungal antagonism is multifactorial.

*Pseudomonads* that function as biocontrol agents frequently produce more than one antibiotic (Haas & Keel, 2003). *Pseudomonas chlororaphis* PA23 produces the PHZ derivatives PCA and 2-OH-PHZ, together with PRN (Zhang *et al.*, 2006). PHZ production plays an essential role in the

disease-suppressive ability of many biocontrol agents. For example, *phzB* and *phzH* mutants of *P. chlororaphis* strain 1391, deficient in phenazine-1-carboxamide (PCN), were unable to control *Fusarium oxysporum* f. sp. *radicis-lycopersici* tomato foot and root rot (Chin-A-Woeng et al., 1998). *Pseudomonas fluorescens* 2-79 mutants no longer producing PCA exhibited dramatically decreased suppression of take-all disease of wheat (Thomashow & Weller, 1988). Similarly, a PHZ-deficient mutant of *Pseudomonas aeruginosa*, PNA1, exhibited reduced ability to suppress Fusarium wilt of chickpea or Pythium damping off of bean compared with the wild type (Anjaiah et al., 1998). In light of these findings, we were surprised to discover an increased zone of inhibition surrounding the PHZ-deficient mutant PA23-63 (Table 2). The antifungal activity observed *in vitro* was supported by our greenhouse analysis, where PA23-63 was able to suppress stem rot of canola as well as the wild type (Fig. 3). We postulated that a second metabolite, most likely PRN, was upregulated in the *phz*<sup>-</sup> background, accounting for the increased antifungal activity *in vitro*. Balanced metabolite production has been observed for *P. fluorescens* CHA0, which produces the antibiotics 2,4-DAPG and PLT (Schnider-Keel et al., 2000; Baehler et al., 2005). Regulation of these two antibiotics occurs at the transcriptional level; 2,4-DAPG and PLT activate their own biosynthesis while at the same time repressing synthesis of the other (Schnider-Keel et al., 2000; Baehler et al., 2005). Strain CHA0 also produces PRN, but expression of this antibiotic had no impact on the 2,4-DAPG:PLT balance (Baehler et al., 2005). When we analyzed culture supernatants of the PHZ-deficient mutant PA23-63, we observed a twofold increase in PRN production (Table 3). This finding led us to explore whether PHZ is acting as a transcriptional repressor of the *prn* biosynthetic operon. Analysis of a *prnA-lacZ* TS fusion revealed no difference in the expression kinetics between the *phzE* mutant and the wild type (Fig. 1a). Therefore, a mechanism other than TS repression must account for the increased PRN production. As depicted in Fig. 4, both PHZ and PRN are produced via the shikimic acid pathway. PhzE is responsible for converting chorismic acid into 2-amino-2-deoxy-isochorismic acid (Pierson & Thomashow, 1992; Mavrodi et al., 1998; McDonald et al., 2001). In the PHZ locus, *phzC* lies upstream of *phzE*. The *phzC* gene encodes a deoxy-arabino-heptulosonate-7-phosphate (DAHP) synthase, which shunts C3 and C4 organic phosphates into the shikimic acid pathway (Wilson et al., 1998). Bacterial DAHP synthases are regulated at the transcriptional level and through feedback inhibition (Herrmann, 1983); therefore, PhzC is an important control point for regulating carbon flow into the shikimic acid pathway. The *phzE* mutation in PA23-63 prevents end-product formation and consequently feedback inhibition. As a result, PhzC continuously pumps carbon into the shikimic acid pathway, which leads to elevated PRN production by PA23-63.



**Fig. 4.** Biosynthesis of PRN and PHZ from shikimic acid. DAHP, deoxy-arabino-heptulosonate-7-phosphate; ADIC, 2-amino-4 deoxychorismic acid; PCA, phenazine-1-carboxylic acid; PRN, pyrrolnitrin. Strain PA23-63 has a Tn insertion in *phzE*. Strains PA23-8 and PA23-63-1 have a portion of *prnBC* replaced with a Gm<sup>R</sup> cassette.

Through our expression analysis, we discovered that PRN is not subject to autoregulation as there was no change in *prn* transcription in the PRN-deficient mutants (Fig. 1a). Examination of the PA23 *prn* upstream region revealed the presence of genes encoding a putative efflux pump; however, no regulatory genes were detected (data not shown). These findings are consistent with others who have reported an absence of pathway-specific regulators for the *prn* operons of *P. fluorescens* strains BL915 and CHA0 (Hammer et al., 1997; Kirner et al., 1998; van Pée & Ligon, 2000; Baehler et al., 2005). In terms of the *phz* operon, a deficiency in PRN and/or PHZ production had no impact on transcription, suggesting that these genes are neither autoregulated nor controlled by PRN. In *P. chlororaphis* strains 30-84 and PCL1391, PHZ gene expression is under QS control (Pierson et al., 1994; Chin-A-Woeng et al., 2001). We expect the same will hold true for PA23 because this bacterium produces AI

signal molecules (Table 2), and two genes with high homology to the *phzI* and *phzR* QS genes are located immediately upstream of the PA23 PHZ operon (data not shown).

It was previously demonstrated that strain PA23 is capable of forming a biofilm and cells within this adherent community are significantly more resistant to antibiotics (Poritsanos *et al.*, 2006). When growing as a biofilm, the increased antimicrobial resistance as well as protection from environmental stresses, such as desiccation and UV radiation, likely facilitates PA23 survival in the environment. In the current study, the role of PHZ and PRN in PA23 biofilm formation was investigated. A static, microtiter biofilm assay was used to mimic the phyllosphere/rhizosphere, which can be best characterized as a no-flow or a low-flow environment, depending on the prevailing conditions. We discovered that PHZ production enhances biofilm formation in PA23 (Fig. 2). A role for PHZ in biofilm development has been reported previously by Maddula *et al.* (2008). Not only did PHZ production enhance *P. chlororaphis* (*aureofaciens*) 30–84 biofilm production by amounts similar to those reported here, but altering the PCA:2-OH-PCA ratio affected the initial attachment, dispersal and the mature biofilm structure (Maddula *et al.*, 2008). Our discovery that PRN does not affect biofilm development (Fig. 2) suggests that this trait is not linked to antibiotic production in general; rather it may be unique to PHZ. Recently, it has been proposed that antibiotics play multiple, concentration-dependent roles (see Davies *et al.*, 2006; Fajardo & Martínez, 2008 for reviews). At lower concentrations, antibiotics act as signaling molecules capable of modulating gene expression, whereas at higher concentrations, they function as inhibitors. Consistent with this, Dietrich *et al.* (2006) demonstrated that the PHZ derivative pyocyanin can induce expression of a set of *P. aeruginosa* genes called the 'PYO stimulon', indicating that pyocyanin can function both as an antibiotic and a QS signal. In a number of bacterial species, a connection has been established between QS and biofilms (Irie & Parsek, 2008). It is possible that PHZ is functioning as a signaling molecule, and in this manner, affects *P. chlororaphis* biofilm development.

In summary, our findings indicate that PRN is the primary antibiotic responsible for PA23 biocontrol of *S. sclerotiorum*. Our next step is to elucidate the factors influencing PRN production, with the end goal of maximizing disease suppression in the field. Although PHZ production is not essential for *Sclerotinia* biocontrol, it leads to enhanced biofilm formation by PA23. Others have shown that PHZ-producing biocontrol strains are better able to colonize roots and persist in the wheat rhizosphere compared with PHZ<sup>-</sup> mutants (Mazzola *et al.*, 1992). Whether PHZ contributes to PA23 persistence in the environment is yet to be established.

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