Cloning and characterization of the pyrrolnitrin gene cluster of *Pseudomonas chlororaphis* PA23

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Abstract

Pseudomonas chlororaphis strain PA23 is a biocontrol agent that was first isolated from soybean root-tips. Our interest in this strain derives from its ability to protect canola (*Brassica napus* L.) from disease caused by the fungal pathogen *Sclerotinia sclerotiorum* in both greenhouse and field studies. Strain PA23 produces the antibiotics pyrrolnitrin, phenazine 1-carboxylic acid, and 2-hydroxyphenazine, together with a number of other secreted products believed to contribute to biocontrol. Using transposon mutagenesis, we isolated a *phzE* mutant (PA23-63) that no longer produced phenazines but expressed wild-type levels of pyrrolnitrin. In greenhouse studies, both the wild type and PA23-63 exhibited a similar ability to reduce the incidence of leaf infection, stem rot and disease severity in *S. sclerotiorum*-challenged canola. These findings suggest that pyrrolnitrin, rather than phenazines, is the main antibiotic involved in PA23 biocontrol. A PA23 genomic library was created and screened using pyrrolnitrin-specific gene probes. Two clones were found to react with *prnA*- and *prnD*- specific probes, suggesting they contained the entire pyrrolnitrin locus. Through primer walking, we sequenced the 5.8 kb pyrrolnitrin gene cluster. Sequence analysis revealed the PA23 *prn* operon is >93% identical, at the nucleotide level, to that of *Pseudomonas fluorescens* Pf-5. We also discovered that the PA23 *prn* locus contains four open reading frames, *prnA,B,C* and *D* organized in a similar manner to that reported for other bacterial species.

Key words: *Pseudomonas chlororaphis*, canola, *Sclerotinia sclerotiorum*, pyrrolnitrin, phenazine, biocontrol

Introduction

Biological control of plant pathogens by certain antagonistic bacteria is a well-known phenomenon. In *Pseudomonas* spp., biocontrol activity is mediated by production of different antibiotics (Haas and Keel, 2003). *Pseudomonas chlororaphis* strain PA23, first isolated from soybean root tips, is an excellent biocontrol agent and can protect canola from stem rot disease caused by the *Sclerotinia scletiorum* (Lib.) de Bary (Fernando et al. 2007). PA23 produces several non-volatile antibiotics, including phenazine 1-carboxylic acid, 2-hydroxyphenazine and pyrrolnitrin (Zhang et al., 2006) together with numerous volatile compounds (Fernando et al., 2005). In addition to antibiotics, *P. chlororaphis* PA23 produces other compounds thought to be important for biocontrol activity, including lipase, protease and siderophores (Poritsanos et al., 2006). Phenazines play an important role in suppressing a range of fungal infections (Chin-A-Woeng et al., 1998). In PA23, the contribution of individual antibiotics to the biocontrol capacity of this strain is just beginning to be revealed. A phenazine deficient mutant, strain PA23-63, demonstrated increased antifungal activity against *S. sclerotiorum* on PDA plates compared to the PA23 wild type (Selin et al., 2006; Poritsanos, N., 2005). With this finding, we postulated that the production of phenazines is not essential for PA23 to protect canola from stem rot disease (Selin et al., 2006). Instead, the production of pyrrolnitrin seems to have a major role in PA23 biocontrol of stem rot disease of canola. Pyrrolnitrin is an antibiotic with a broad-spectrum antifungal effect (Burkhead et al. 1994) that was first isolated from *Pseudomonas pyrrocinia* (Arima et al., 1964). Pyrrolnitrin is synthesized from tryptophan through a biochemical pathway identified initially by Elander and coworkers (1998).The production of pyrrolnitrin has also been reported for several isolates of *Pseudomonas, Burkholderia* (Homma et al., 1989; Janisiewicz et al., 1988), *Enterobacter agglomerans* (Chernin et al., 1996), *Myxococcus fulvus* (Gerth et al., 1982), *Cytobacter ferrugineus* (Gerth et al., 1982), and *Serratia* sp. (Kalbe et al., 1996). The purpose of the present study is to describe the isolation and genetic analysis of the pyrrolnitrin gene cluster from *P. chlororaphis* strain PA23, probably the best known biocontrol agent against *Sclerotinia sclerotiorum* on the canola phyllosphere (Fernando et. al., 2007; Savchuk and Fernando 2004).

Materials and Methods

Bacterial strains, plasmids and growth conditions. All 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, Michigan). *Pseudomonas chlororaphis* PA23 and its derivatives were cultured at 28°C on LB agar. Transformed bacterial cultures were maintained in presence of appropriate antibiotics: gentamicin (Gm; 15 μg/mL), ampicillin (100 μg/mL), chloramphenicol (Chl; 12.5 μg/mL) for *E. coli,* and Gm (25 μg/mL), pipericillin (13 or 100 μg/mL) for *P. chlororaphis.* All antibiotics were obtained from Research Products International Corp. (Mt. Prospect, Illinois).

DNA isolation. Plasmid and genomic DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA

fragment purification, cloning, and DNA ligation were all performed by standard techniques (Ausubel, 1989).

PCR and DNA sequencing. PCR primers used in this study are listed in the Table 1. Polymerase Chain Reaction (PCR) was performed under standard conditions as suggested by Invitrogen Life Technology data sheets supplied with their taq polymerase.

All sequencing was carried out at the university of Calgary Core DNA Services facility. Sequences were analyzed using blastn and blastx databases.

Generating a Fosmid library of *Pseudomonas chlororaphis* PA23. To clone the PA23 pyrrolnitrin gene cluster, a Fosmid library containing PA23 genomic DNA was generated according to manufacturer's instructions (EPICENTRE®).

Southern Blotting. Detection of nucleic acid by DNA Dot Blotting was carried out using the non-radioactive digoxigenin (DIG) DNA labeling. A *prnD* PCR product was labeled with DIG-11-dUTP provided with the DIG labeling kit (Boehringer Mannheim). Fosmid DNA isolated from 1300 clones was spotted directly onto a nitrocellulose membrane (DNA Dot Blotting). For all procedures: DIG labeling of *prnD*, hybridization and subsequently chemiluminescence detection of the prn gene cluster followed manufacturer's instruction (The DIG System User's Guide, BOEHRINGER MANNHEIM).

Results and Discussion

A Fosmid library of genomic DNA of strain PA23 was created and screened using prn-specific gene probes (Fig. 1). From approximately 1300 fosmid clones, two clones were identified to react with *prnA*- and *prnD*-specific probes indicating they contain the entire prn locus (Fig. 2). Through primer walking, the 5.8 kb prn gene cluster was sequenced. Sequence analysis revealed the *prn* operon of PA23 is >93% identical, at the nucleotide level, to that of *Pseudomonas fluorescence* Pf-5. The *prn* operon of strain PA23 contains four ORFs, arranged as a single transcriptional unit (Fig.3). In addition, the organization of genes is identical to the order in which the encoded products function in the pyrrolnitrin biosynthetic pathway (Fig.4). The same *prnABCD* genetic arrangement was reported for other bacteria including, *Pseudomonas fluorescence, Pseudomonas pyrrocinia* and *Burkheldria cepacia* (Hammer et al. 1999). This finding indicates that the biochemical pathway for pyrrolnitrin production is highly conserved. By definition of the FRAMS analysis, the three prn ORFs, *prnA, prnC* and *prnD* begin with ATG as start codon. In contrast, the *prnB* coding sequence begins with the unusual start codon GTG and is translationally coupled by overlapping one base of the *prnA* stop codon to the *prnB* ORF. These results confirm and extend data obtained by other researchers (Hammer et al. 1999 and Kirner et al. 1998). Gene overlaps is a common phenomenon and has been observed in chromosomal DNA of different bacteria and even higher organisms (Wellington et al. 1992 and Fukuda et al. 1999). Overlapping genes have reported to be potentially important in transcriptional and translational regulation of gene expression (Keese and Gibbs 1992; Krakauer 2000). Therefore, the translational coupling of *prnA* with *prnB* by the strain PA23 could be considered as a mechanism to regulate the production of PrnB protein and consequently the accumulation of the 7-chlorotryptophan in the cell (Fig.1). We are in the process subcloning of the entire *prn* gene cluster and creating a *prn* null mutant to uncover the role of pyrrolnitrin production in PA23 biocontrol.

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

Strain/plasmid/primer	Relevant genotype or phenotype	source or reference
P. chlororaphis	wild type (soybean plant isolate)	(Savchuk and Fernando, 2004)
PA23	Phz^+, Rif^R	
PA23-63	Phz^-, Rif^R	(Selin,de Kievit et al, unpublished)
E. coli	supE44 ΔlacU169 (φ80 lacZM15)	Gibco
DH5-α	hsdR17, recA1, endA1, gyrA96 thi-1, relA1	
Primers		
prnAF	5-gtgttcttcgacttcctcgg-3	(Zhang et al. 2006)
prnAR	5-tgccggttcgcgagccaga-3	(Zhang et al. 2006)
prnDF	5-ggggcgcgtggtgatgga-3	(Zhang et al. 2006)
prnDR	5-ycccgcsgcctgyctggtctg-3	(Zhang et al 2006)

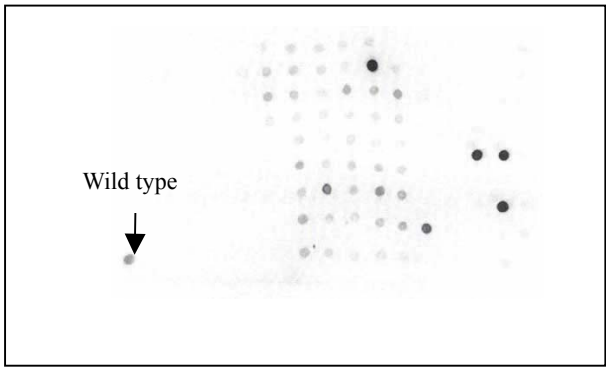

Fig.1: Probing of PA23 Fosmid library using a Dig-labeled *prnD* probe

1-wild type, *prnD;* 2-clone 1, *prnD;* 3-clone 2, *prnD;* 4-clone 3, *prnD;* 5-clone1, *prnA;* 6-clone 2, *prnA;* 7-clone 3, *prnA* 8-wild type, *prnA*

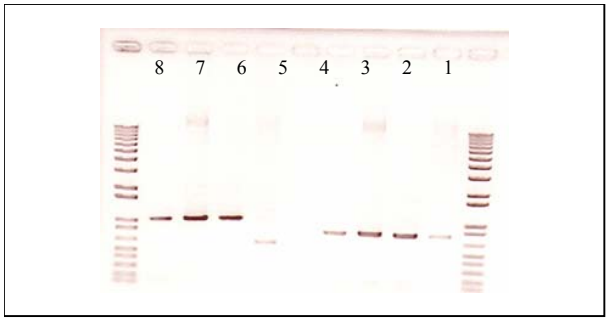

Fig.2: PCR analysis of Fosmid library

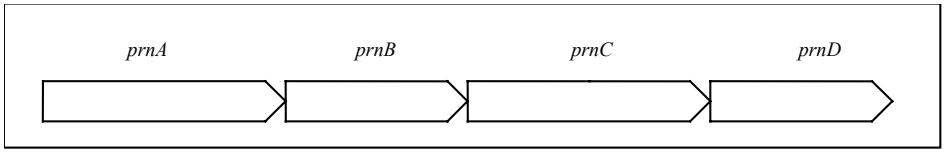

Fig.3: Organization of the pyrrolnitrin gene cluster in *Pseudomonas chlororaphis* PA23

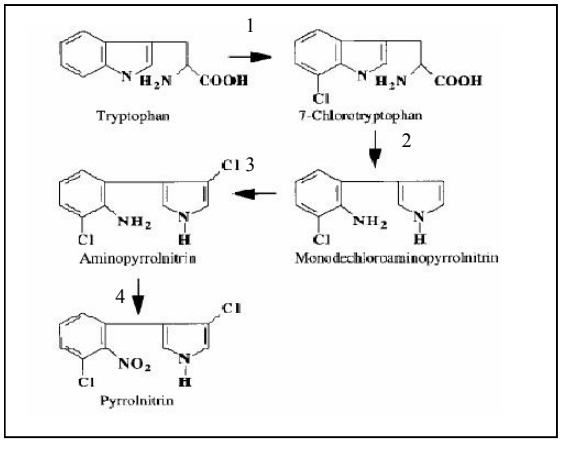

Fig.4: Proposed biosynthetic pathway for the synthesis of pyrrolnitrin according to van Pee et al. 1980