PtrA Is Functionally Intertwined with GacS in Regulating the Biocontrol Activity of Pseudomonas chlororaphis PA23

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In vitro inhibition of the fungal pathogen Sclerotinia sclerotiorum by Pseudomonas chlororaphis PA23 is reliant upon a LysR-type transcriptional regulator (LTTR) called PtrA. In the current study, we show that Sclerotinia stem rot and leaf infection are significantly increased in canola plants inoculated with the ptrA-mutant compared to the wild type, establishing PtrA as an essential regulator of PA23 biocontrol. LTTRs typically regulate targets that are upstream of and divergently transcribed from the LTTR locus. We identified a short chain dehydrogenase (scd) gene immediately upstream of ptrA. Characterization of a scd mutant revealed that it is phenotypically identical to the wild type. Moreover, scd transcript abundance was unchanged in the ptrA mutant. These findings indicate that PtrA regulation does not involve scd, rather this LTTR controls genes located elsewhere on the chromosome. Employing a combination of complementation and transcriptional analysis we investigated whether connections exist between PtrA and other regulators of biocontrol. Besides ptrA, gacS was the only gene able to partially rescue the wild-type phenotype, establishing a connection between PtrA and the sensor kinase GacS. Transcriptomic analysis revealed decreased expression of biosynthetic (phzA, prnA) and regulatory genes (phzI, phzR, rpoS, gacA, rsmX, rsmZ, retS) in the ptrA mutant; conversely, rsmE, and rsmY were markedly upregulated. The transcript abundance of ptrA was nine-fold higher in the mutant background indicating that this LTTR negatively autoregulates itself. In summary, PtrA is an essential regulator of genes required for PA23 biocontrol that is functionally intertwined with GacS.

Keywords: antifungal, pyrrolnitrin, phenazine, degradative enzymes, autoinducer

INTRODUCTION

Public concern over the use of chemical pesticides together with the potential for acquiring resistance to these compounds has led to renewed interest in alternative strategies for management of diseases affecting plants. Pseudomonas chlororaphis strain PA23 is a soybean root-tip isolate that demonstrates excellent antifungal (AF) activity against Sclerotinia sclerotiorum (Lib.) de Bary (Savchuk and Fernando, 2004). Strain PA23 produces an arsenal of compounds including the diffusible antibiotics phenazine 1-carboxylic acid (PCA), 2-hydroxyphenazine (2-OH-PHZ), and pyrrolnitrin (PRN) together with degradative enzymes...
upstream of *ptrA* lies a gene encoding a short-chain dehydrogenase, designated *scd*. At present, it is not known what role *scd* plays in PA23 biological control. Moreover, connections between *PtrA* and other members of the regulatory network have not been investigated. The aim of the current study was to conduct greenhouse studies to establish whether *PtrA* is required for PA23-mediated control of Sclerotinia stem rot. We also generated an *scd* mutant and determined its role in PA23 fungal suppression. Finally, a combination of complementation and transcriptional analysis was used to explore interactions between *PtrA* and other regulators of PA23 biocontrol.

**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 Lennoxuria Bertani (LB) agar (Difco Laboratories, Detroit, Michigan). *P. chlororaphis* PA23 and its derivatives were cultured at 28°C on LB agar or M9 minimal media supplemented with 1mM MgSO₄ and 0.2% glucose. For AF assays, bacteria were grown on one-fifth potato dextrose agar (PDA; Difco). As required, media were supplemented with the following antibiotics: tetracycline (Tc; 15 μg/mL), gentamicin (Gm; 15 μg/mL), ampicillin (Amp; 100 μg/mL) for *E. coli*, and rifampicin (Rif; 25 μg/mL), Tc (15 or 100 μg/mL), Gm (20 μg/mL), piperacillin (Pip; 40 or 500 μg/mL) for *P. chlororaphis*. All antibiotics were obtained from Research Products International Corp. (Mt. Prospect, Illinois).

**PCR**

Polymerase Chain Reaction (PCR) was performed as follows. Each reaction contained 2.5 μL of both forward and reverse primers (12 μM), 1 μL of template DNA, 10 μL of 10× Taq Buffer (without Mg added), 1.5 μL of MgSO₄, 1 μL of Taq polymerase (Thermo Fisher Scientific, Carlsbad, USA) and nuclease-free water to a final volume of 100 μL. PCR reaction conditions included an initial denaturation at 98°C for 2 min, followed by 30 cycles of 98°C for 30 s, 55°C for 30 s, and 68°C for 1 min/kb, followed by a final extension of 68°C for 5 min.

**Nucleic Acid Manipulation**

Cloning, purification, electrophoresis, and other manipulations of nucleic acid fragments and constructs were performed using standard techniques (Sambrook et al., 1989).

**Generation of a scd Mutant**

To generate PA23*scd*, a 542-bp internal fragment of the *scd* gene was PCR amplified from PA23 genomic DNA using primers *scd*-pKNOCK FWD and *scd*-pKNOCK REV. The amplicon was gel purified and digested with *Bam*HI and *Xho*I and cloned into the same sites of pKNOCK-Tc. The pKNOCK-*scd* plasmid was then mobilized into PA23 through triparental mating with the donor strain *E. coli* DH5α*p* containing pKNOCK-*scd* and the helper strain DH5α (pRK600). *Pseudomonas Isolation Agar* (PIA, Difco) supplemented with Tc (150 μg/mL) was used to screen for transconjugants. Insertion of the plasmid into *scd* was

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(Poritsanos et al., 2006; Zhang et al., 2006; Selin et al., 2010). PRN is the primary antibiotic responsible for biocontrol; conversely, phenazines (PHZ) are not essential for fungal suppression, but do play a role in biofilm formation (Selin et al., 2010). Despite the fact that many biocontrol agents perform well in the greenhouse, they often exhibit reduced efficacy under field conditions (Cook, 1993; Walsh et al., 2001; Haas and Keel, 2003). Variable expression of genes and gene products required for biocontrol likely contributes to poor performance in the field. It is essential, therefore, that molecular mechanisms underlying biocontrol are well understood so that production of the pathogen-suppressive factors can be optimized in the environment.

In both pathogenic and biocontrol pseudomonads, expression of secondary metabolites is controlled by a multi-tiered network of regulation. Situated at the top of this hierarchy is the GacS/GacA two-component signal transduction system, comprised of the sensor kinase GacS and its cognate response regulator GacA (Heeb and Haas, 2001). For many biocontrol strains, including PA23, a mutation in *gacS* or *gacA* leads to a loss of fungal antagonism (Heeb and Haas, 2001; Poritsanos et al., 2006). A second system, called Rsm, functions in concert with Gac and consists of a combination of RsmA-like repressor proteins and untranslated regulatory RNAs. The repressor proteins act at the post-transcriptional level by binding to and blocking the ribosome-binding site (RBS) of target mRNA (Lapouge et al., 2008). The regulatory RNAs antagonize repression by titrating out the RsmA-like proteins, rendering RBSs accessible to the translational machinery (Lapouge et al., 2008). Several additional regulatory components govern expression of PA23 AF metabolites including the PhzR/PhzI quorum-sensing (QS) system (Selin et al., 2012), the stationary phase sigma factor RpoS (Manuel et al., 2012), a regulator of RpoS called PsrA (Selin et al., 2014), and the stringent response (Manuel et al., 2012). Cross-regulation between the regulators themselves adds to the increasingly complex nature of this regulatory hierarchy (Manuel et al., 2012; Selin et al., 2012, 2014).

We have recently identified a novel regulator in PA23 called *PtrA* (Pseudomonas transcriptional regulator) (Klaponski et al., 2014). The phenotype of a *ptrA* mutant is very similar to that of a *gac*-deficient strain exhibiting a complete loss of AF activity (Poritsanos et al., 2006; Selin et al., 2014). *PtrA* belongs to the LysR-type transcriptional regulator (LTTR) family, which is the most abundant class of transcriptional regulators found among prokaryotes (Schell, 1993). LTTRs act as either activators or repressors and are known to control a diverse range of metabolic functions including cell invasion and virulence, QS, oxidative stress, and amino acid metabolism (Cao et al., 2001; Sperandio et al., 2002; Kim et al., 2004; Heroven and Dersch, 2006; Byrne et al., 2007; Kovaleva and Gelfand, 2007; Hernández-Lucas et al., 2008; Maddocks and Oyston, 2008). Preliminary proteomic and phenotypic analysis of a *ptrA* mutant revealed 59 differentially expressed proteins together with decreased PHZ and PRN production, consistent with the loss of AF activity (Klaponski et al., 2014).

LTTRs frequently control expression of genes that are upstream of and divergently transcribed from the *lttr* locus (Schell, 1993; Maddocks and Oyston, 2008). Immediately
TABLE 1 | Bacterial strains, plasmids, and oligonucleotide sequences.

| Strains | Relevant genotypes | Source or references |
|---------|--------------------|----------------------|
| **PSEUDOMONAS CHLORORAPHIS** | | |
| PA23 | Phz+RifR wild type (soy bean plant isolate) | Savchuk and Fernando, 2004 |
| PA23-443 | Phz−RifR ptrA::Tn5-GT182 genomic fusion | Klaponski et al., 2014 |
| PA23scd | TcR marker inserted into scd gene | This study |
| **ESCHERICHIA COLI** | | |
| DH5α | supE44 ΔU169 (φ80lacZ ΔM15) hadR17 recA1 endA1 gyrA96 thi-1 relA1 | Gibco |
| DH5α-λpir | DH5α-λpir lysogen of DH5α | House et al., 2004 |
| **CHROMOBACTERIUM VIOLACEUM** | | |
| CVO26 | Autoinducer synthase (cviI) mutant from C. violaceum ATCC 31532, autoinducer biosensor | Latifi et al., 1995 |
| **PLASMIDS** | | |
| pUCP23 | Broad-host range vector; AmpR, GmR | West et al., 1994 |
| pUCP22-gacA | 1.65-kb fragment containing gacA and uvrC from P. protegens CHA0 | This study |
| pUCP23-gacS | 3.1-kb fragment containing gacS in pUCP23 | Porsitonas et al., 2006 |
| pUCP22-ptrA | 2.2-kb fragment containing ptrA in pUCP22 | Klaponski et al., 2014 |
| pUCP22-rpoS | 1.3-kb fragment containing rpoS in pUCP22 | Porsitonas et al., 2006 |
| pUCP22-psrA | 950-bp fragment containing psrA in pUCP22 | This study |
| pUCP22-rsmA | 190-bp fragment containing rsmA in pUCP22 | This study |
| pUCP22-rsmE | 600-bp fragment containing rsmE in pUCP22 | This study |
| pUCP23-rsmZ | 400-bp fragment containing rsmZ in pUCP23 | This study |
| pUCP23-phzR | 1.68-kb fragment containing phzR in pUCP23 | Selin et al., 2012 |
| pUCP22-retS | 2.9-kb fragment containing retS in pUCP22 | This study |
| pUCP22-ladS | 2.8-kb fragment containing ladS in pUCP22 | This study |
| pRK600 | Mobilization plasmid containing tra genes, ChlR | Finan et al., 1986 |
| pKNOCK-Tc | Suicide vector designed for insertional mutagenesis; R6K ori; RP4 oriT; TcR | Alexeyev, 1999 |
| pKNOCK-scd | 542-bp internal scd fragment cloned into pKNOCK-Tc | This study |
| **OLIGONUCLEOTIDE SEQUENCES** | | |
| tet FWD | 5′- ACCGCTCTCGTGGATTTCTCTA-3′ | This study |
| new ptrA TL start FWD | 5′- GCAAGCAAGCTTGGCAGCCGATAACCTGCGC-3′ | This study |
| scd-pKNOCK FWD | 5′- TATTGAGATTTCCACGCTTCTTGCGTA-3′ | This study |
| scd-pKNOCK REV | 5′- TATTCTCGAGCCAACGGCACCATAGGTTCA-3′ | This study |
| retS-F2 | 5′- GACGAGATCCAGCGCCGAGCCGATAGTTAT-3′ | This study |
| retS-R2 | 5′- ATGAAGCTTGCGCAAACTCACAGCG-3′ | This study |
| ladS-F-BamHI | 5′- GAGTGGATCCAAACCAATAACAGG-3′ | This study |
| ladS-R-HindIII | 5′- CCAGAAGCTTGGATTTTAAGCACC-3′ | This study |
| gacS RT-PCR FWD | 5′- TGGTCAGCCTGGTGATATC-3′ | This study |
| gacS RT-PCR REV | 5′- TGTCTTCGTGTTCTTCTTG-3′ | This study |
| rpoS RT-PCR FWD | 5′- TGGCTTTCCGAATTGACC-3′ | This study |
| rpoS RT-PCR REV | 5′- CAGACGCTTGGACACC-3′ | This study |
| prnA RT-PCR FWD | 5′- CGTCTCGGCGCTTGAAATGCGC-3′ | This study |
| prnA RT-PCR REV | 5′- GATCTCGGCGTTGAATGCGC-3′ | This study |
| phzI RT-PCR FWD | 5′- GCGATGCCGTTGTTCTGG-3′ | This study |
| phzI RT-PCR REV | 5′- AGCCGCTGCTGGCAGCTC-3′ | This study |
| phzR RT-PCR FWD | 5′- GATTCCGCTTCGCAATGC-3′ | This study |
| phzR RT-PCR REV | 5′- ATCGCGCGCCGATACCC-3′ | This study |
| psrA RT-PCR FWD | 5′- CCATGCTCGGCTTTCTCG-3′ | This study |
| psrA RT-PCR REV | 5′- ATGATCGCGCGAAATTCC-3′ | This study |
| rsmZ RT-PCR FWD | 5′- TGGCGTATGAAAGTGTCTTTTG-3′ | This study |

(Continued)
confirmed by PCR using primers tet FWD and new ptrA TL start FWD followed by sequencing of the amplicon.

**Plasmid Construction**

For complementation analysis, pUCP22-ptrA were generated as follows. ptrA was PCR amplified from PA23 genomic DNA using primers tet FWD and new ptrA TL start. The 2.9-kb amplicon was digested with BamHI & HindIII before cloning into the same sites of pUCP22. The ladS gene was amplified using primers ladS-F-BamHI and ladS-R-HindIII. The 2.8-kb ladS-containing fragment was subject to digestion with BamHI & HindIII and cloned into pUCP22 digested with the same enzymes. pUCP22-ptrA and pUCP22-ladS were verified through sequence analysis.

**Antifungal Assays**

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

**Greenhouse Assays**

Strains PA23 (pUCP22), PA23-443 (pUCP22), and PA23-443 (ptrA-pUCP22) were assessed for their ability to control stem rot of canola [Brassica napus (cv. Westar)] under greenhouse conditions. Canola plants were grown in pots (21 × 20 cm) at 24/16°C with a 16-h photoperiod. The plants were sprayed at 30% flowering with bacterial strains (2.0 × 10^8 CFU mL^{-1}) suspended in sterile distilled water with 0.02% Tween 20 and kept in a growth chamber (24/16°C, 16-h photoperiod). Twenty-four hours after bacterial inoculation, plants were sprayed with ascospores of S. sclerotiorum (8 × 10^4 spores mL^{-1}) suspended in water containing 0.02% Tween 20. Pathogen control plants were inoculated with ascospores only, while healthy control plants were sprayed with water (0.02% Tween 20). After pathogen inoculation, plants were incubated in a humidity chamber for 72 h, after which they were placed back in the growth chamber. Fourteen days after ascospore inoculation, symptom development on the stem and leaves was scored according to Poritsanos et al. (2006). Ten plants were used for each treatment and the plant studies were repeated two times.

**Phenazine Analysis**

Production of PCA and 2-OH-PHZ was quantified according to the methods outlined by Chancey et al. (1999). Overnight cultures (5 mL) were grown in M9 minimal media (1 mM MgSO_4; 0.2% glucose) and subjected to PHZ extraction. Spectrophotometric quantification was performed at 367 nm and 490 nm for PCA and 2-OH-PHZ, respectively (Maddula et al., 2008). PHZ analysis was performed in triplicate.

**Pyrrolnitrin Analysis**

Production of the antibiotic PRN was quantified according to the methods outlined by Selin et al. (2010). Briefly, 20 mL cultures of PA23 and its derivatives were grown for 5 days in M9 minimal media (1 mM MgSO_4; 0.2% glucose) and PRN was extracted with
an equal volume of ethyl acetate. Before extraction, toluene was added to each sample as an internal control. Toluene and PRN UV absorption maxima were recorded at 225 nm with a Varian 335 diode array detector. PRN peaks were detected at 4.7 min. Samples were analyzed in duplicate.

**Autoinducer Analysis**
The production of AHL was analyzed by spotting 10 µL of an overnight culture onto LB agar plates seeded with C. violaceum CV026. This strain is able to detect exogenous AHLs with carbon chain length structures ranging from C4 to C8, resulting in a purple halo surrounding the colonies. The diameter of the purple zones was measured at 24 h.

**Semi-Quantitative Reverse Transcriptase PCR**
To monitor expression of genes involved in biocontrol, semi-quantitative reverse transcriptase (RT) PCR was used. PA23 and its derivatives were grown to early stationary phase and total RNA was extracted using a RNeasy Mini Kit (QIAGEN, Valencia, USA). Residual genomic DNA was removed by treatment with TURBO RNase-free DNase 1 (Ambion, Carlsbad, USA) during the RNA isolation procedure. RNA concentrations were measured at 260 and 280 nm and only RNA samples with A260/A280 between 1.8 and 2.0 were used in subsequent steps. cDNA was generated by reverse transcription using the Maxima First Strand cDNA Synthesis Kit (ThermoScientific, Rockford, USA) and random hexamer primers in a 20 µL total reaction volume. The following conditions were employed: initial heating at 25°C for 10 min, 30°C for 15 min for reverse transcription and 85°C for 5 min for enzyme denaturation. Sequences for the genes of interest from PA23 were obtained from GenBank. The primer sequences are listed in **Table 1**. PCR was performed using a CFX96 Connect™ Real-Time PCR Detection System (Bio-rad, Hercules, USA) and SsoFast™ EvaGreen® Supermix (Bio-rad). The final 10-µL volume mixture in each well contained 0.4 µL of both forward and reverse primers (12 µM), 1 µL of 1:20 diluted cDNA, 5 µL of SsoFast™ EvaGreen® Supermix and 3.4 µL of nuclease-free water. PCR reaction conditions included an initial denaturation at 98°C for 2 min, followed by 39 cycles of 98°C for 5 s, 60°C for 30 s, and 60°C for 5 s. Melt-curve analysis was performed to evaluate the formation of primer dimers and other artifacts to validate results. Each reaction was performed in triplicate and experiments were repeated three times with three biological replicates. Relative gene expression was calculated using the ΔΔCt method as described by Livak and Schmittgen (2001) using *rpoB* as the reference gene and the CFX Manager™ software (Bio-rad).

**Phylogenetic Analysis**
Genetic sequences for *ptrA* homologs were obtained through BLAST (Altschul et al., 1990). The accession number for *ptrA* (EF054873.1) was used as a query against Pseudomonadales (taxid:72274) sequences in the non-redundant nucleotide collection (nr/nt) employing the blastn algorithm. For amino acid sequences, this query was used against the non-redundant protein database using the blastp algorithm. Forty nucleotide and 41 amino acid sequences were selected with *E*-values of <2 × 10^{-100}. Both sets of sequences were aligned using Mafft servers (Katoh et al., 2005). Phylogenetic trees were constructed with the MEGA6 program package (Tamura et al., 2013). For nucleotide sequences, the Maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) was applied, with genetic distances estimated using the Maximum composite likelihood approach. Bootstrap analysis with a thousand substitutions was utilized to test the trees. The nucleotide sequence for a LTR from *Serratia marcescens* strain RSC-14 (gi: 926475601) was included as an outgroup for phylogenetic tree construction. For amino acid sequences, the Maximum likelihood method based on the Le-Gascuel 2008 model (Le and Gascuel, 1993) was employed with genetic distances estimated using the JTT model. Bootstrap analysis with a thousand substitutions was used to test the trees. The amino acid sequence for a LTR from *S. marcescens* (gi: 759524346) was included as an outgroup for phylogenetic tree construction.
**Statistical Analysis**

All statistical analysis was performed using unpaired Student’s t-test.

**RESULTS**

**PtrA is Essential for PA23 Biocontrol of S. sclerotiorum in the Greenhouse**

The wild-type PA23, PA23-443 and the complemented PA23-443 (ptrA-pUCP22) were tested for their ability to protect canola from stem rot disease caused by S. sclerotiorum. Two parameters were evaluated, namely incidence of leaf infection and stem rot disease severity. As illustrated in Figure 1, the ptrA mutant showed a significant reduction in its ability to control fungal infection of leaves and stems and reduce overall disease severity. Compared to the disease control, the ptrA mutant mediated a modest decrease in leaf infection (Figure 1A) and no difference in disease severity (Figure 1B). Addition of ptrA in trans restored the ability of PA23-443 to prevent both leaf infection and stem rot to wild-type levels (Figure 1).
scd, Which Lies Upstream of PtrA, Does Not Appear to Be Involved in PA23 AF Activity

A 115-bp intergenic region separates ptrA and an upstream gene encoding a short-chain dehydrogenase, designated scd. To determine whether this allele is involved in PtrA regulation, an scd insertional mutant was generated. Unlike the ptrA mutant that is devoid of AF activity, the scd mutant showed near wild-type fungal suppression (Figure 2). Due to the plasmid insertion, only the first 100 nt (of 600) of the scd open reading frame remain intact (data not shown). It seems highly unlikely that a functional truncated Scd is being produced, but we cannot rule out this possibility entirely.

gacS is Able to Partially Complement the PtrA Mutant

To reveal interactions between PtrA and other members of the regulatory hierarchy overseeing PA23 biocontrol, plasmid-borne copies of regulatory genes constitutively expressed from the lac promoter were transformed into the ptrA mutant. Genes that are able to fully or partially complement the mutant are predicted to lie downstream of PtrA in the regulatory cascade. We began our characterization of the PA23-443 transformants by analyzing AF activity. As expected, providing ptrA in trans restored AF activity close to wild-type levels (Figure 2). The only other gene that resulted in partial complementation of fungal suppression was gacS (Figure 2).

PA23 produces the diffusible antibiotics PHZ and PRN. Antibiotic analysis revealed that strain PA23-443 synthesized markedly lower levels of both compounds, whereas no difference in antibiotic production was observed for the scd mutant (Table 2). Addition of ptrA in trans led to partial and full restoration of PHZ and PRN production, respectively. Consistent with the AF analysis, plasmid-borne gacS resulted in partial complementation of antibiotic synthesis in the ptrA mutant (Table 2). Our protease activity profiles closely mirrored what was observed for the antibiotics. The ptrA mutant was devoid of protease production, whereas the scd mutant showed wild-type activity (Figure 3). Addition of ptrA and gacS in trans lead to full and partial rescue of protease activity in the ptrA mutant background, respectively (Figure 3).

PtrA Regulates AHL Signal Production

The AHL signal generated by the PhzRI QS system activates the CVO26 biosensor resulting in a white to purple color change due to the production of the QS-controlled pigment violacein. While CVO26 cells form a purple halo around colonies of PA23 and the scd mutant, this is not observed for PA23-443 indicating that the latter is AHL deficient. Addition of ptrA, gacA, and gacS in trans rescued AHL production in the ptrA mutant to varying degrees (Figure 4).

Multiple Genes Show Altered Expression in the PtrA Mutant

To better understand how PtrA functions as a regulator of PA23 biocontrol, expression analysis of biosynthetic and regulatory genes associated with fungal suppression was undertaken (Figure 5). The two biosynthetic genes analyzed, phzA (PHZ) and prnA (PRN), showed a dramatic reduction in expression levels in the ptrA mutant background. In terms of regulatory

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**TABLE 2 | Quantification of phenazines and pyronitrin present in cultures of Pseudomonas chlororaphis PA23, PA23scd and PA23-443 harboring empty vector or various overexpression plasmids.**

| Strain          | PCAa | 2-OH-PHZa | Total PHZa | PRNa  |
|-----------------|------|-----------|------------|-------|
| PA23 (pUCP22)   | 65.46| 11.04     | 76.49      | 3.48  |
| PA23scd (pUCP22)| 77.47| 13.89     | 91.35      | 3.74  |
| PA23-443 (pUCP22)| 11.04| 0.98      | 12.02      | ND    |
| PA23-443 (ptrA) | 38.24| 5.11      | 43.36      | 3.90  |
| PA23-443 (gacA) | 3.42 | 0.45      | 3.86       | ND    |
| PA23-443 (gacS) | 25.85| 3.92      | 29.71      | 2.56  |
| PA23-443 (psaA) | 6.81 | 0.70      | 7.52       | ND    |
| PA23-443 (pspS) | 4.98 | 0.50      | 5.48       | ND    |
| PA23-443 (ptrR) | 10.42| 1.03      | 11.46      | ND    |
| PA23-443 (ramE) | 0.94 | 0.02      | 0.96       | ND    |
| PA23-443 (ramZ) | 11.42| 1.33      | 12.75      | ND    |
| PA23-443 (retS) | 5.22 | 0.67      | 5.89       | ND    |
| PA23-443 (ladS) | 4.76 | 0.44      | 5.16       | ND    |

Note: Activity close to wild-type levels (Figure 2).

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*Not significantly different from wild type.*

<sup>a</sup>Mean (standard deviation) of concentrations of PCA, 2-OH-PHZ and total PHZ (µg/mL) from a triplicate set.

<sup>b</sup>Mean (standard deviation) of amounts of PRN (µg) extracted from 20 ml culture volumes from a duplicate set.

<sup>c</sup>Significantly different from wild type (P < 0.01).

<sup>d</sup>Significantly different from wild type (P < 0.05).

<sup>e</sup>Significantly different from wild type (P < 0.01).

ND, not detectable.
genes, gacA and gacS exhibited a 50% reduction and no change in expression, respectively. Expression of both phzI, encoding the AHL synthase and phzR, encoding the LysR-type transcriptional regulator PhzR, were significantly downregulated in the ptrA mutant (Figure 5). Interestingly, psrA transcription was modestly increased, while the PsrA-regulated target gene rpoS was downregulated in the mutant background. Analysis of genes encoding Rsm repressor proteins (rsmA, rsmE) and regulatory RNAs (rsmX, rsmY, rsmZ) revealed elevated rsmE and rsmY expression in the ptrA mutant. Conversely rsmA, rsmX and rsmZ were
FIGURE 4 | Autoinducer production by PA23 and derivative strains. (A) Autoinducer production by PA23, PA23-443 (ptrA-mutant) and derivative strains determined using Chromobacterium violaceum CVO26-seeded agar. (B) Zone of purple pigmentation (mm) indicative of bacterial autoinducer production after 48 h on CVO26-indicator plates. Samples are as follows: 1, PA23 (pUCP22); 2, PA23scd (pUCP22); 3, PA23-443 (pUCP22); 4, PA23-443 (pUCP22 ptrA); 5, PA23-443 (pUCP22 rsmA); 6, PA23-443 (pUCP22 rsmE); 7, PA23-443 (pUCP23 rsmZ); 8, PA23-443 (pUCP22 rpoS); 9, PA23-443 (pUCP22 psrA); 10, PA23-443 (pUCP23 phzR); 11, PA23-443 (pUCP22 gacA); 12, PA23-443 (pUCP23 gacS); 13, PA23-443 (pUCP22 retS), PA23-443 (pUCP22 ladS). For strains that differ significantly from PA23-443, columns have been marked with an asterisk (*p < 0.0001).

downregulated, indicating positive regulation by PtrA. Next, we examined the orphan sensor kinase-encoding retS and ladS genes. A decrease in retS activity was observed; whereas ladS transcription remained at wild-type levels. The gene showing the most dramatic change in transcript abundance was ptrA. In the PA23-443 background, ptrA expression increased nine fold indicating that this LTTR is subject to negative autoregulation. No change in scd transcription was observed further supporting that PtrA regulation is not mediated through this divergently transcribed gene. Quantitative RT-PCR was used to monitor
expression of three genes immediately upstream of *scd*, encoding a membrane protein (AIC18438.1; primers Up1), a hypothetical protein (AIC18437.1; primers Up2) as well as *nhaA* (AIC18435.1; primers Up3). No differences in transcript abundance were detected in the *ptrA* mutant compared to the wild type (data not shown).

**Phylogenetic Analysis of *PtrA* Homologs**

Phylogenetic analysis revealed that *PtrA* was conserved amongst pseudomonads, including both biocontrol and pathogenic strains. Homologs from well-known biocontrol strains *P. chlororaphis* O6 and *P. protegens* Pf-5 were found to cluster with *PtrA* (Figure 6). A similar pattern was observed when a phylogenetic tree was constructed using genomic sequences (Supplementary Figure 1). As closely related homologs of *PtrA* are found in several biocontrol strains, they are expected to play a similar role in regulating genes responsible for the production of antifungal compounds. A list of the *PtrA* homologs used for the phylogenetic analysis can be found in Supplementary Tables 1, 2.

**DISCUSSION**

LTTRs, which represent the largest family of prokaryotic transcriptional regulators, frequently regulate divergently transcribed genes; however, targets can be located elsewhere on the chromosome (Schell, 1993; Maddocks and Oyston, 2008). Several pieces of evidence suggest that *PtrA* fits into this second paradigm, functioning as a global transcriptional regulator. First, 59 differentially regulated proteins distributed across 16 different COG categories were identified in the *ptrA* mutant (Klaponski et al., 2014) and genes encoding these proteins are scattered about the chromosome (Loewen et al., 2014). Second, insertional inactivation of the divergently transcribed *scd* gene upstream of *ptrA* resulted in no observable phenotype. AF activity, antibiotic and AHL production, and protease activity were similar to wild type (Figures 2–4; Table 2). Moreover, expression of *scd* was unchanged in the *ptrA* mutant (Figure 5). These findings indicate that *scd* is not involved in PA23 biocontrol and does not appear to be linked to *PtrA*. Finally, our qRT-PCR results showed altered expression of several biosynthetic and regulatory genes involved in PA23 biocontrol (Figure 5). For the most part, gene expression profiles corresponded well with the *ptrA* mutant phenotype. For example, *phzA* and *prnA* were both significantly downregulated (Figure 5). Negligible expression of both genes is consistent with the loss of orange pigmentation, fungal suppression (Figure 2) and antibiotic production (Table 2) exhibited by the *ptrA* mutant. In strain PA23, the Phz QS system regulates expression of antibiotics and degradative enzymes and so it is necessary for biocontrol (Selin et al., 2012). Transcriptional profiling revealed that *phzl* and *phzR* were significantly decreased in the *ptrA* mutant compared to the wild type. Reduced *phzl* expression coincides with the loss of AHL signal production in this background (Figure 4). While *rpoS* expression was markedly down in the *ptrA*-deficient strain, *psrA* transcription was elevated, albeit modestly (Figure 5). Because RpoS is a negative regulator of PA23 biocontrol (Manuel et al., 2012) the decrease in *rpoS* transcription was unexpected as the *ptrA* mutant is no longer capable of fungal suppression. It is important to note, however, that cross regulation occurs between the PhzRI QS system and RpoS (Selin et al., 2012), which may obscure interpretation of findings. We also explored genes
Figure 6 | Molecular phylogenetic analysis of PtrA homologs by the Maximum Likelihood method. The evolutionary history was inferred using the Maximum Likelihood method based on the Le Gascuel Method (Le and Gascuel, 1993). This analysis involved 41 amino acid sequences including PtrA. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is indicated next to the branches.
belonging to the Gac-Rsm regulatory network. In *P. protegens* strain CHAO, this regulatory network functions as follows. Upon binding to an unknown signal, the sensor kinase GacS undergoes autophosphorylation and phosphotransfer to the response regulator GacA (Lapouge et al., 2008). Phosphorylated GacA can then activate expression of sRNAs, including RsmXYZ, that titrate out the translational repressors RsmA and RsmE allowing expression of target genes (Lapouge et al., 2008). While all of these components have been identified in strain PA23, the way in which they function has not yet been explored. In the closely related *P. chlororaphis* 30–84, RsmE was found to repress production of PHZ and AHL signaling molecules; however, RsmA exerted no regulatory effect over these compounds (Wang et al., 2013). Moreover, of the three small RNA molecules, only constitutively expressed *rsmZ* was able to rescue a *gacA* mutant for PHZ and AHL production; *rsmX* exhibited no effect and elevated levels of *rsmY* were reportedly lethal (Wang et al., 2013). Collectively, these findings suggest that in terms of PHZ and AHL production in *P. chlororaphis* 30–84, RsmE is the primary repressor protein and RsmZ is the small RNA responsible for lifting repression. If we assume that this circuitry functions in a similar manner in strain PA23, the elevated *rsmE* expression and reduced *rsmZ* transcription are consistent with the loss of AF activity exhibited by the *ptrA* mutant.

Even though *gacS* is not under *ptrA* transcriptional control, a regulatory link clearly exists between the two. Our phenotypic assays showed either full or partial complementation of the *ptrA* mutant by *gacS* when provided in trans. We hypothesized that *PtrA* might be controlling expression of regulatory elements that impact signal transduction through the Gac system and that overexpression of *gacS* is able to overcome this effect. In *P. aeruginosa* PA01, the orphan sensor kinases RetS and LadS modulate the Gac-Rsm circuitry (Ventre et al., 2006; Goodman et al., 2009). RetS blocks GacS autophosphorylation and subsequent activation of GacA, while LadS exerts a positive effect on Gac regulation (Ventre et al., 2006; Goodman et al., 2009). To explore whether RetS and LadS are in some way linked to *PtrA*, we attempted to complement the *ptrA* mutant by providing *retS* and *ladS in trans*. No changes in fungal suppression, protease activity, antibiotic and AHL production were observed in the *ptrA* mutant harboring plasmid-borne copies of these genes (Figures 2–4, Table 2). We did, however, discover a two-fold decrease in retS transcription in PA23-443 (Figure 5). Because RetS functions to antagonize the Gac system, decreased *retS* expression is expected to have a positive impact on biocontrol. Taken together, these findings do not support a role for RetS and LadS in the *PtrA*-GacS interaction.

Phylogenetic analysis illustrates that a wide range of *Pseudomonas* species harbor a *PtrA* homolog (Figure 6). For both symbiotic and pathogenic pseudomonads, secreted products play a significant role in the biocontrol and virulence properties of these organisms. In addition, regulatory factors overseeing their expression are in many cases conserved. For example, the Gac two-component system and QS positively regulate exoproducts that play a key role in symbiotic and pathogenic interactions (Heeb and Haas, 2001; Bassler, 2002). It is not surprising, therefore, to find that *PtrA* is highly conserved amongst *Pseudomonas* species. To the best of our knowledge, this transcriptional regulator has not been characterized in other pseudomonads.

In summary, we have shown that *PtrA* is essential for biocontrol of *S. sclerotiorum* stem rot of canola. *PtrA* appears to function as a global regulator controlling expression of unlinked genes across the chromosome. Future studies will be directed at analyzing the *PtrA* transcriptome on a global scale so we can better comprehend how this LTTR is controlling expression of biocontrol factors in PA23. Due to the conserved nature of this regulator, we hypothesize that *PtrA* governs expression of secondary metabolites in biocontrol strains and pathogenic pseudomonads alike. Elucidating the mechanisms underlying *PtrA* regulation will have far-reaching implications for our understanding of how these bacteria interact with other members of their environment including prokaryotic and eukaryotic organisms.

**AUTHOR CONTRIBUTIONS**

NS, NK, WF, MB, and Td conceived and designed the study. NS and NK drafted the manuscript with input from Td. NS, NK, and RR performed the phenotypic characterization of the *ptrA* mutant; CS was responsible for the greenhouse analysis. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01512

**Supplementary Figure 1 | Molecular phylogenetic analysis of *Pseudomonas chlororaphis* PA23 *ptrA* by the Maximum Likelihood method.** The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura Nei model (Tamura and Nei, 1993). This analysis involved 40 nucleotide sequences including *ptrA*. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is indicated next to the branches.

**Supplementary Table 1 | Protein sequences used in phylogenetic analysis of *PtrA* and homologs.**

**Supplementary Table 2 | Nucleotide sequences used in the construction of the phylogeny tree in Supplementary Figure 1.**
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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