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Lipopeptides are essential for *Pseudomonas* sp. DF41 biocontrol of *Sclerotinia sclerotiorum*

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ABSTRACT

Pseudomonas sp. DF41 is a biocontrol agent capable of suppressing *Sclerotinia sclerotiorum*-mediated stem rot of canola. Using transposon mutagenesis, we identified two mutants with greatly reduced antifungal (AF) activity. The first mutant had an insertion in *gacS*, forming part of the GacS/GacA regulatory system. The second mutation was in a gene involved in lipopeptide (LP) synthesis suggesting LPs are essential for biocontrol. Strain DF41 and the *gacS* (DF41-469) and *lp* (DF469-1278) mutants were characterized with respect to their extracellular metabolite production, antifungal (AF) properties and ability to form biofilms. We discovered that DF41 produces a number of compounds that may contribute to biocontrol including hydrogen cyanide (HCN), protease, alginate, and LP molecules. All of these products were found to be under Gac control. In addition, DF41 produces autoinducers, suggesting this bacterium employs quorum sensing as part of its lifestyle. In the greenhouse, the *gacS* and *lp* mutants were unable to protect canola from disease incited by *S. sclerotiorum*. Both mutants were able to sustain themselves in the canola phyllosphere; therefore, the loss of biocontrol activity can be attributed to reduced production of AF compounds and not a declining population size. We conclude that suppression of Sclerotinia stem rot of canola by *Pseudomonas* strain DF41 depends upon LP production and a functional Gac system.

1. Introduction

Pseudomonas sp. DF41 is a biocontrol agent originally isolated from the phyllosphere of canola plants (Savchuk and Fernando, 2004). Interest in this organism derives from its ability to protect canola plants from the devastating effects of stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary (Savchuk and Fernando, 2004; Savchuk, 2002). *S. sclerotiorum* is an economically important soil-borne pathogen that affects more than 400 plant hosts (Purdy, 1979). In commercially grown canola cultivars there is no known resistance against *S. sclerotiorum*. The only methods available to control this disease are crop rotation and/or fungicide application. Both of these methods are of limited use as *S. sclerotiorum* is capable of overwintering in the soil for 3–5 years in the form of sclerotia. Furthermore, fungicide application must occur prior to manifestation of disease symptoms. Given the ever-growing concern over the use of chemical agents in agriculture and the potential for acquired fungal resistance to commercially available fungicides, biological control emerges as an attractive alternative for the containment of fungal diseases.

Biocontrol by certain species of *Pseudomonas* is often mediated via the production of secondary metabolites that antagonize fungal growth. Since the initial report of phenazine production by *Pseudomonas fluorescens* 2–79 in the suppression of take-all disease of wheat (Gurusiddaiah et al., 1986), numerous compounds have been shown to be important for biocontrol activity. Inhibitory compounds include degradative enzymes such as protease, cellulase, chitinase and β-glucanase together with antibiotics such as phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT) and hydrogen cyanide (Dowling and O'Gara, 1994; Haas and Défago, 2005; Thomashow and Weller, 1995). Lipopeptides (LPs) represent another class of molecules associated with biocontrol. Production of LPs by antagonistic Pseudomonads contributes to antimicrobial activity, biofilm formation and motility (Raaijmakers et al., 2006). LP molecules are produced non-ribosomally by multifunctional enzymes called non-ribosomal peptide synthetases (Marahiel et al., 1997). The basic structure of a LP molecule consists of a fatty acid tail joined to a typically cyclic oligopeptide head. Considerable structural diversity exists within this group of molecules due to differences in the length and composition of the fatty acid tail as well the peptide ring structure (Raaijmakers et al., 2006).

In fluorescent Pseudomonads, the GacS–GacA two-component signal transduction system is essential for production of secondary metabolites that mediate biocontrol (Heeb and Haas, 2001). GacS is a membrane-associated sensor kinase that is stimulated by

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a yet-to-be-identified signal resulting in autophosphorylation. The current working model suggests that through phosphotransfer to the response regulator GacA, GacA becomes activated to induce expression of small regulatory RNAs (Heeb and Haas, 2001; Haas and Défago, 2005). These RNAs are believed to function by titrating out repressor proteins, such as RsmA and RsmE that bind to the ribosome binding site of target mRNAs blocking their translation.

In this paper, we describe the characterization of *Pseudomonas* sp. DF41 with respect to extracellular metabolite production. A number of exoproducts were identified, including protease, hydrogen cyanide (HCN), and alginate as well as a LP molecule that is essential for *Sclerotinia* biocontrol. We evaluated how a *gacS* mutation effects expression of these compounds and its impact on disease suppression. Finally, the role of GacS and LPs in DF41 biofilm formation and motility was examined.

2. Materials and methods

2.1. Bacterial strains, growth conditions and plasmids

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* species were routinely cultured on LB agar at 28 °C. As required, antibiotics were added to media at the following concentrations: gentamicin (Gm; 50 μg/ml), ampicillin (Amp; 100 μg/ml), and kanamycin (Km; 50 μg/ml) for *E. coli*, tetracycline (Tc; 15 μg/ml), piperacillin (Pip; 80 μg/ml), rifampicin (Rif; 50 μg/ml), Km (5 μg/ml), Gm (40 μg/ml), for DF41. All antibiotics were obtained from Research Products International Corp. (Mt. Prospect, Illinois).

2.2. Nucleic acid manipulation

Standard techniques were used in the purification, cloning and other manipulations of DNA (Sambrook et al., 1989). Polymerase chain reaction (PCR) was performed following standard conditions suggested by Invitrogen Life Technologies data sheets supplied with their *Taq* polymerase.

2.3. Tn*5*-1063 transposon mutagenesis

Random mutagenesis was performed using transposon Tn*5*-1063 which contains a Km^R marker as well as a p15a *oriV* origin of replication to allow rescue cloning of Tn*5*-flanking DNA (Wolk et al., 1991). Bacterial conjugations were carried out using pRL1063a (Wolk et al., 1991), helper plasmid pRK2013 (Ditta et al., 1980) and DF41. Km^R transconjugants were screened in plate assays to identify mutants that exhibited altered antagonistic activity towards *S. sclerotiorum* compared to the wild type. For each mating, 3–5 Km^R colonies were screened by PCR to confirm that the transconjugants contained a Tn*5*-1063 insertion using primers Kan-FWD (5′-ttga acaagatggattgcacg-3′) and Kan-REV (5′-tcgtcaagaaggcgatagaa-3′). To determine the site of Tn*5*-1063 insertion, genomic DNA from the mutant strains was digested with *Eco*R1 which does not cut within Tn*5*-1063. DNA was ligated and transformed into *E. coli* DH5α. Selection on Km-containing media resulted in isolation of plasmids p1063-1278 and p1063-469. To verify that the mutants harbored a single chromosomal Tn*5*-1063 insertion, Southern hybridization was performed using a digoxygenin-labelled probe. The probe was generated by PCR amplification of the 929-bp Km^R cassette harbored on Tn*5*-1063 using primers KmF (5′-ttgaacaagatggattgcacg-3′) and KmR (5′-tcgtcaagaaggcgatagaa-3′). Southern hybridizations were carried out at 42 °C following previously described methods (de Kievit et al., 1995).

2.4. Sequence analysis

Plasmids p1063-1278 and p1063-469 were sent for sequencing using oligonucleotide primer TnOT182-LT (5-gatcctggaaaacg ggaaagg-3) which anneals to the 3 end of Tn*5*-1063. All sequencing was performed at the University of Calgary Core DNA Services Facility. Sequences were analyzed using the blastx and blastn databases. The GenBank accession number for the sequence of the DF41 *gacS* gene is GQ328782.

2.5. Cloning of the DF41 *gacS* gene

The DF41 *gacS* gene was cloned using oligonucleotide primers GacS–F (5′-gatcgcagattggaaagcaa-3′) and GacS–R (5′-acggtgtccaggt accaag-3′) which were designed from the DF41 *gacS* gene sequence obtained from sequencing Tn*5*-1063 chromosomal insertions. A TOPO-pCR2.1 cloning kit (Invitrogen Life Technologies, Burlington, ON) was used to clone the 3.2-kb *gacS* PCR product into the pCR2.1-TOPO vector according to the manufacturer's instructions. The 3.2-kb *gacS* PCR product was excised from the pCR2.1 vector with *Bam*H1 and *Xba*1 and cloned into the same sites of pUCP23 to generate pUCP23-*gacS*.

2.6. Antifungal assays

Radial diffusion assays to assess fungal inhibition *in vitro* were performed according to previously described methods

Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant genotype or phenotype	Source or References
Pseudomonas		
DF41	Rif^R wild type (canola root tip isolate)	Savchuk and Fernando (2004)
DF41-1278	Rif^R lp::Tn*5*-1063 genomic insertion	This study
DF41-469	Rif^R gacS::Tn*5*-1063 genomic insertion	This study
DF41-469 (pUCP23-gacS)	Rif^R gacS::Tn*5*-1063 genomic insertion, gacS complemented strain	This study
E. coli DH5α	supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Gibco
C. violaceum CVO26	Autoinducer synthase (cvil) mutant from *C. violaceum* ATCC 31532, autoinducer biosensor	Latifi et al. (1995)
Plasmids		
pRL1063a	Delivery plasmid for Tn*5*-1063; Km^R	Wolk et al. (1991)
pRK2013	Transposon delivery vector; Km^R	Ditta et al. (1980)
p1063-1278	Rescue plasmid containing lp::Tn*5*-1063 genomic insertion	This study
p1063-469	Rescue plasmid containing gacS::Tn*5*-1063 genomic insertion	This study
pCR2.1TOPO	Cloning vector for PCR products	Invitrogen
pUCP23	Broad-host-range vector; IncP OriT, Amp^R Gm^R	West et al. (1994)
pUCP23-gacS	pUCP23 containing gacS from *Pseudomonas* DF41	This study
pME3219	pME6010 containing an hcnA-lacZ translational fusion	Laville et al. (1998)

(Poritsanos et al., 2006). Eight replicates were analyzed for each strain and assays were repeated four times.

2.7. HCN analysis

Hydrogen cyanide production was determined qualitatively using Cyantesmo paper (Machery-Nagel GmbH & Co., Düren, Germany). Expression of genes encoding hydrogen cyanide was monitored using plasmid pME3219 which contains an *hcnA–lacZ* translational fusion. Plasmid pME3219 was transformed into DF41, DF41-469 and DF41-1278. Cultures were grown in Peptone Tryptic Soy broth [PTSB (Ohman et al., 1980)] until they reached stationary phase (OD_{600nm} = 2.5–3.0), at which point *hcnA* expression was measured using β-galactosidase assays (Miller, 1972). Samples were analyzed in triplicate and experiments were repeated twice.

2.8. HPLC

For preliminary characterization of AF compound(s) produced by DF41 and its derivatives, high-performance liquid chromatography (HPLC) analysis was performed on culture extracts. Bacteria were grown in M9 minimal salts medium (Difco) for 3 days at 28 °C. Cells were removed by centrifugation and the supernatants extracted with ethyl acetate containing 1% formic acid, followed by an extraction with pure ethyl acetate. The ethyl acetate was then removed using rotary evaporation and the remaining material was dissolved in methanol. HPLC analysis of compounds was performed using a Gemini C_{18} column (100 × 4.6 mm; 5-μm particle diameter) (Phenomenex Inc., Torrance, CA) and UV detection (200–220 nm) by a Varian 335 diode array detector. Samples were analyzed in a linear gradient of 85% eluent A (0.1% *o*-phosphoric acid) and 15% eluent B (acetonitrile) at 0 min, increasing eluent B to 100% over 40 min. The eluent flow rate was 1.0 ml per min. HPLC-grade solvents were obtained from Fisher Scientific.

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

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

2.10. Exoproduct analysis

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

2.11. Alginate analysis

Bacterial strains DF41 (pUCP23), DF41-1278 (pUCP23), DF41-469 (pUCP23) and DF41-469 (pUCP23-*gacS*) were streaked onto King's Medium B agar [KMB (King et al., 1954)] and incubated at 28 °C for 5 days. Cells were washed from the plates with 0.9% NaCl. Cellular material was removed by centrifugation and the cell pellet washed with 0.9% NaCl as described by May and Chakrabarty (1994). Alginate production was quantified using a carbazole assay for assaying uronic acids (Knutson and Jeanes, 1968). Protein concentrations were determined using the Bradford assay (Bradford, 1972).

2.12. 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 repeated three times.

2.13. Biosurfactant assays

To test for biosurfactant activity, drop-collapse assays were performed on HPLC-purified extracts of DF41, DF41-1278, and DF41-469 following the protocol of Kuiper et al. (2004). A 50-μl aliquot of the fraction exhibiting bioactivity was applied to a sheet of parafilm "M" (Pechiney, Menasha, WI). A 2-μl aliquot of 0.1% crystal violet was added to each of the samples and the droplets were visually examined for the ability to reduce surface tension by observing the contact angle of the droplets on the parafilm. Sterile distilled water was used as a negative control.

2.14. Biofilm formation

We employed a 96-well plate assay described by O'Toole and Kolter (1998) to examine the ability of DF41 (pUCP23), DF41-1278 (pUCP23), DF41-469 (pUCP23) and DF41-469 (pUCP23-*gacS*) to form biofilms. Briefly, overnight cultures grown in M9 casamino acid media + 0.2% glucose were adjusted to an OD_{600} of 1.0 and then diluted 1 in 100 in fresh media. Hundred micro liters aliquots of the diluted culture were inoculated into 96-well plates (Becton-Dickenson, Oakville, ON) and allowed to form biofilms. After 24 h, the adherent population was quantified by crystal violet staining and measuring the optical density at a wavelength of 600 nm.

2.15. Biocontrol under greenhouse conditions

DF41 (pUCP23), DF41-469 (pUCP23), DF41-1278 (pUCP23) and DF41-469 (pUCP23-*gacS)* were assessed for their ability to suppress Sclerotinia stem rot on canola plants following previously described methods (Poritsanos et al., 2006). Briefly, an overnight culture of bacteria grown in 300 mL of LB broth was washed and resuspended in 1× PBS (pH 7.0) amended with 0.02% Tween 20 to an OD_{600nm} = 2.0. For each treatment ten *Brassica napus* (cv. Westar) canola plants were sprayed with bacteria at the 30–50% flowering stage, then incubated overnight in a humidity chamber (24 °C:16 °C; 16 h:8 h light:dark regime). The following day, the plants were removed from the humidity chamber sprayed with a *S. sclerotiorum* ascospore suspension of 2×10^4 ascospores/mL. The pathogen control plants were sprayed with 2×10^4 ascospores/mL and the healthy control plants were sprayed with 1 X PBS buffer amended with 0.02% Tween 20. The plants were incubated in the greenhouse for fourteen days and scored for the percent leaf incidence (PLI) and stem rot disease severity (DS). The plant studies were repeated twice.

2.16. Bacterial persistence

The ability of DF41 and its derivatives to effectively colonize canola petals was analyzed. Cultures were grown in 10 mL of LB broth at 28 °C. The next day, the bacteria were subcultured to a

starting OD_{600nm} = 0.1 in a total volume of 300 mL of LB and allowed to grow overnight at 28 °C. Bacteria were washed and resuspended in an equal volume of PBS before spraying onto canola (8 plants/strain). Random sampling of canola petals was performed on days 1 though 5 from plants treated with DF41, DF41-469, DF41-469 (*gacS*) or DF41-1278. For each test strain, 10 petals were selected from the plants and placed in 1 mL of 1× PBS. Cells were removed from the petals by vigorous vortexing for 30 s. The bacterial suspensions were serially diluted and plated in triplicate on LB or LB-km 5 μg/mL using an Autoplate® 4000 Automated Spiral Plater (Spiral Biotech, Inc., Bethesda, MD). After overnight incubation at 28 °C, the CFU/mL was determined using an aCOLyte colony counter (Don Whitley Scientific, Frederick, MD).

2.17. Statistical analysis

An unpaired Student's *t* test was used for statistical analysis of AF, autoinducer and protease activity, alginate production, flagellar motility, and bacterial persistence on canola.

3. Results

3.1. Isolation of Pseudomonas DF41 mutants deficient in antifungal activity

Transposon mutagenesis was performed to identify molecular mechanisms underlying the biocontrol activity of *Pseudomonas* sp. DF41. Approximately five thousand mutants were screened in radial diffusion assays for altered AF activity. Two mutants, DF41-469 and DF41-1278, exhibiting significantly decreased inhibition of the fungal pathogen *S. sclerotiorum* were selected for further analysis (Fig. 1).

In the case of mutant DF41-469, sequence analysis of the chromosomal DNA flanking the Tn insertion showed >90% identity to the *gacS* genes of annotated *Pseudomonas* species, including *Pseudomonas fluorescens* strains 2P24 [Accession # AY623898] and PfO-1 [Accession # NC007492]. Because *gacS* is found as a single gene and not part of a large operon, we were able to complement this mutant. Transfer of the wild-type *gacS* gene into DF41-469 restored antifungal activity (Table 2), confirming that the DF41-469 phenotype results from inactivation of *gacS*.

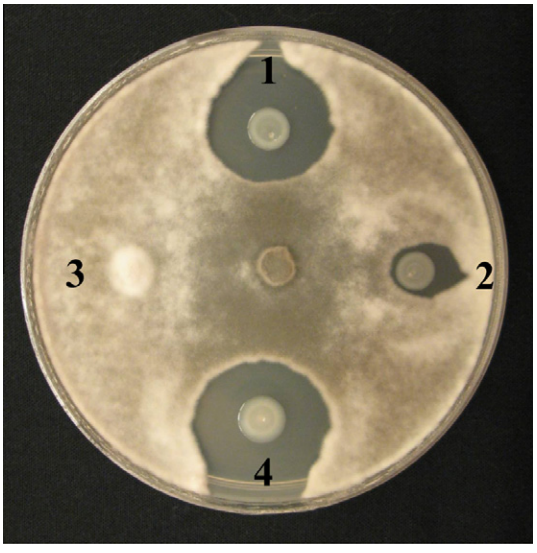

Fig. 1. Antifungal activity of *Pseudomonas sp.* DF41 and derivative strains against *Sclerotinia sclerotiorum*. A 5-μl aliquot of an overnight bacterial culture was spotted onto a potato dextrose agar plate. After 24 h, a fungal plug was placed in the centre of the plate, which was incubated at 24 °C until the fungal mycelia reached the periphery of the plate. Sample 1: DF41 (wild type); sample 2: DF41-1278 (*lp*); sample 3: DF41-469 (*gacS*); sample 4: DF41-469 (pUCP23-*gacS*).

For mutant DF41-1278, the Tn interrupted a gene with highest homology at the amino acid level (82%; 226/275 amino acids) to the *sypC* gene of *Pseudomonas syringae* pv. *syringae* B301D (Accession #AA072425). *sypC* encodes a peptide synthetase involved in production of the LP syringopeptin (Scholz-Schroeder et al., 2001). The sequence flanking the Tn insertion in DF41-1278 is homologous to a SypC condensation domain (data not shown). LP biosynthetic operons are extremely large, for example the *sypC* ORF spans 40.6 kb and the entire *syp* gene cluster is estimated to be 80 kb (Scholz-Schroeder et al., 2001); as a result, we did not attempt to genetically complement this mutant. Southern blot analysis revealed that both DF41-1278 and DF41-469 harbored a single Tn insertion (data not shown).

3.2. Analysis of culture supernatants from wild type and mutant strains of Pseudomonas DF41

We employed C_{18} reverse-phase HPLC to isolate DF41 compounds exhibiting AF activity. When extracts were prepared from DF41 culture supernatants and fractionated by HPLC, several large peaks were observed and AF bioactivity was found in one peak, eluting at 25 min (Fig. 2). Analysis of DF41-1278 (*lp*) and DF41-469 (*gacS*) extracts revealed no peak at 25 min (Fig. 2) and there was no AF activity found in any of the fractions (data not shown). Thus it appears that DF41-1278 (*lp*) and DF41-469 (*gacS*) are deficient in the production of the active compound(s). Preliminary chemical analysis employing electrospray ionization and MALDI-MS have revealed a 2123 Da molecule present in the DF41 extract (fraction 25) that is absent in DF41-469 and DF41-1278 (data not shown).

Due to their amphipathic nature, LP molecules frequently exhibit biosurfactant properties. Therefore, we tested the biosurfactant properties of DF41, DF41-1278 (*lp*), DF41-469 (*gacS*) in a drop-collapse assay. The HPLC fraction that exhibited AF activity (fraction 25) was analyzed from each of these strains. While the DF41 droplet showed some degree of collapse, the DF41-1278 (*lp*) and DF41-469 (*gacS*) HPLC fractions more closely resembled the negative control (Fig. 3).

3.3. Hydrogen cyanide expression

Using cyantesmo paper, which is specific for the detection of HCN, strains DF41 and DF41-1278 (*lp*) were found to produce this volatile antibiotic; whereas DF41-469 (*gacS*) does not (Table 2). Complementation with pUCP23-*gacS* restored HCN production in the *gacS* mutant (Table 2). Expression analysis using a *hcnA–lacZ* translational fusion further substantiated these findings. A fourfold decrease in *hcnA* expression was observed in the *gacS* mutant (486 ± 76 Miller units) compared to DF41 (2194 ± 145 Miller units). Expression of the *hcnA–lacZ* fusion in the LP-deficient strain was similar to wild type (2357 ± 350 Miller units). Although a definitive role for HCN in the biocontrol activity of DF41 has not yet been established, it is evident that expression of this compound is dependent on GacS.

3.4. Phenotypic analysis of Pseudomonas DF41 and mutants deficient in AF properties

Many biocontrol bacteria produce compounds that act together with antibiotics to synergistically increase their efficacy. Production of these molecules can be under control of a population density-dependent signaling mechanism known as quorum sensing (QS). We tested wild-type DF41 as well as DF41-1278 (*lp*), DF41-469 (*gacS*), and DF41-469 (pUCP23-*gacS*) for production of lipase,

Table 2
Phenotypic characteristics of *Pseudomonas* sp. DF41, Tn5 mutants 8 DF41-1278 (*clp*) and DF41-469 (*gacS*), and *gacS* complemented strain DF41-469 9 (pUCP23-*gacS*

Strain	AF[a]	Autoinducer[a]	Protease[a]	Alginate[b] (μg/mg protein)	HCN[c]
DF41 (pUCP23)	7.1 (0.9)	10.7 (0.5)	5.8 (0.7)	568 (52)	+
DF41-1278 (pUCP23)	2.6 (1.4)[d]	9.9 (0.6)[g]	6.3 (1.2)[g]	543 (95)[g]	+
DF41-469 (pUCP23)	0 (0.0)[d]	8.0 (0.5)[e]	0.0 (0.0)[d]	118 (16)[e]	–
DF41-469 (pUCP23-*gacS*)	8.4 (0.7)[f]	9.3 (0.5)[f]	7.3 (0.7)[g]	582 (13)[g]	+

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

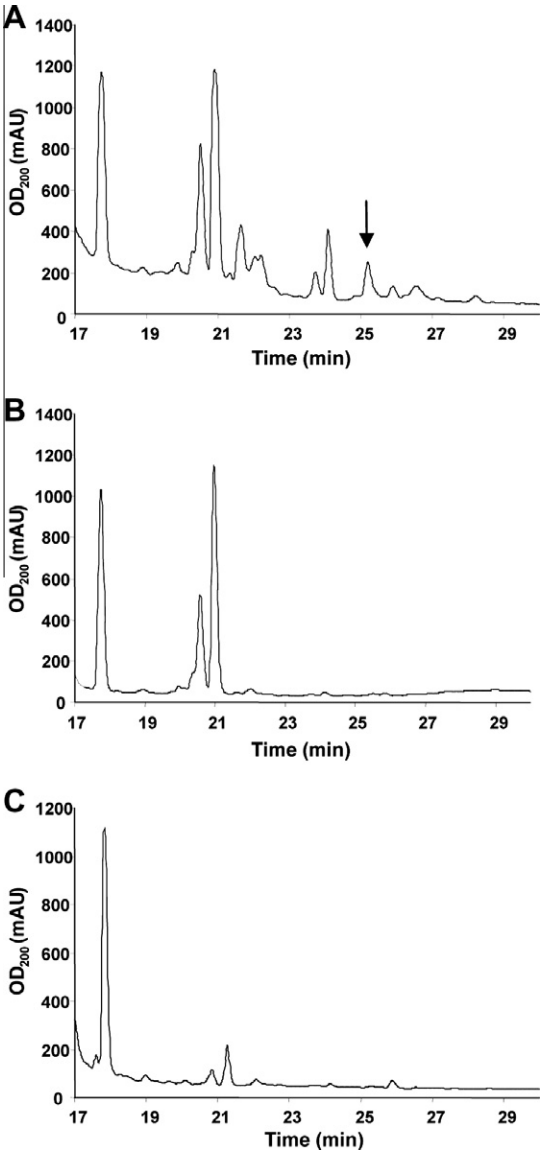

Fig. 2. C_{18}-reverse-phase HPLC analysis of bioactive compounds of *Pseudomonas* sp. DF41. Ethyl acetate fractions derived from supernatants of DF41 (A); DF41-1278 (*lp*) (B) and DF41-469 (*gacS*) (C) were separated by HPLC and 1 ml fractions were collected and tested for AF activity. Peaks representing fractions with AF activity are indicated with an arrow.

protease, and autoinducer molecules. The results of our analyses are summarized in Table 2. We were unable to detect lipase production by any of the four strains (data not shown). On skim milk agar, a clear zone of proteolysis was observed surrounding the

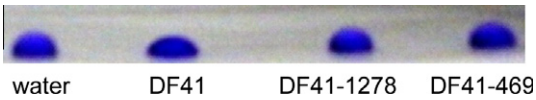

Fig. 3. Biosurfactant activity associated with culture extracts of *Pseudomonas* sp. DF41 (wild type), DF41-1278 (*lp*), and DF41-469 (*gacS*). HPLC fractions corresponding to the retention time of the LP peak were collected, dried and resuspended in water. The samples were stained with crystal violet and deposited onto a hydrophobic surface (parafilm). Note that the DF41 sample shows a modest degree of spreading; whereas the DF41-1278 and DF41-469 extracts, which are devoid of LP, more closely resemble the water control.

DF41, DF41-1278 (*lp*) and *gacS* complemented mutant DF41-469 (pUCP23-*gacS*) colonies, indicating these strains are positive for protease production (Table 2). DF41-469 (*gacS*) on the other hand, was protease deficient. The biosensor strain CV026 is unable to produce acyl homoserine lactone (AHL) molecules due to a mutation in the AHL synthase gene. Exogenous AHLs in the C_4- to C_8-size range complement this mutation resulting in production of the QS-controlled purple pigment violacein. Agar plates seeded with CV026 revealed that DF41 and DF41-1278 (*lp*) secreted similar levels of AHL (Table 2). For DF41-469 (*gacS*), a noticeable decrease in AHL production was consistently observed; whereas production was restored to near wild-type levels when *gacS* was added *in trans* (Table 2). Collectively, our findings indicate that DF41 secretes protease and AHL molecules, with the former being tightly regulated by the Gac system.

3.5. The role of GacS in alginate production

We have observed that DF41 develops a mucoidy phenotype when grown for an extended period (120 h) on King's B agar; whereas the *gacS* mutant does not. These findings suggest that DF41 is producing alginate in a GacS-dependent manner. We discovered that DF41 does secrete alginate and production of this exopolysaccharide is markedly decreased in the *gacS*-minus strain (Table 2). As expected, parental levels of alginate were produced by the DF41-1278 (*lp*) mutant and complemented *gacS* mutant.

3.6. Biofilm formation by Pseudomonas DF41 and its derivatives

Using a highly reproducible 96-well PVC microtitre plate assay, the ability of DF41 and its derivatives to form biofilms was examined under minimal nutrient conditions. We discovered equivalent biofilm formation by the wild-type strain ($OD_{600} = 2.17 \pm 0.25$), DF41-1278 (2.07 ± 0.15), the *gacS* mutant ($OD_{600} = 2.24 \pm 0.17$), and the *gacS* complemented mutant ($OD_{600} = 2.22 \pm 0.32$).

3.7. Effect of LP and GacS on DF41 motility

Because motility can impact biocontrol swimming and swarming motility was examined. There was little difference in

the swimming ability of the four strains, with the exception of the *gacS* mutant, which showed increased swimming at both 24 and 72 h (Table 3). Because of the irregular pattern of swarming, quantitative analyses were not performed. We did observe that the wild-type and LP-deficient strain swarmed in a similar manner with fork-like tendrils extending out from the colony that began to form at approximately 24 h (data not shown). In contrast, the *gacS* mutant did not swarm (data not shown).

3.8. GacS and LP production are essential for biocontrol of S. sclerotiorum

DF41, the *lp*- and *gacS* mutants and the complemented *gacS* mutant were evaluated for their ability to inhibit disease on canola plants caused by *S. sclerotiorum* infection. The incidence of leaf infection and disease severity were evaluated. In greenhouse studies, DF41 provided significant protection against fungal infection on the canola leaves and considerably reduced disease severity (Fig. 4). In contrast, the *lp* and *gacS* mutants showed little antagonism toward *S. sclerotiorum* (Fig. 4). Addition of *gacS* in trans restored DF41-469 disease suppression to wild-type levels. Our findings indicate that LPs are an essential component of DF41 biocontrol of *S. sclerotiorum* infection in canola. Because Gac controls production of LPs as well as other AF compounds, it is not surprising that a functional Gac system is required for disease suppression.

3.9. Bacterial persistence on canola petals

To determine whether poor persistence contributed to the lack of biocontrol by the *gacS* and *lp* mutants, bacteria isolated from the canola petals were enumerated over 5 days. Random sampling of canola petals was performed on days 1 though 5 from plants treated with DF41, DF41-469, DF41-469 (*gacS*) or DF41-1278. Although there was some fluctuation in the population sizes over the 5 day period, there was no significant difference in the ability of the four strains to persist in the canola phyllosphere (data not shown).

4. Discussion

Pseudomonas sp. DF41, originally isolated from the phyllosphere of canola plants, has consistently demonstrated strong antagonism of Sclerotinia stem rot of canola (Savchuk and Fernando, 2004; Savchuk, 2002). Neither Biolog™ analysis nor 16s rDNA sequencing have enabled species designation for DF41 (Zhang et al., 2006). The DF41 16s rDNA exhibited the highest degree of similarity with *Pseudomonas migulae* (95%); therefore, DF41 may represent a novel species of *Pseudomonas*. In a previous screen for DF41 antibiotic biosynthetic genes, those involved in phenazine, pyoluteorin, 2,4-diacetylphloroglucinol and pyrrolnitrin synthesis could not be detected, suggesting other product(s) must be responsible for the AF activity associated with DF41 (Zhang et al., 2006). This study represents the first detailed report of metabolites that contribute to DF41 biocontrol, as well as regulatory factors influencing their expression.

Table 3
Flagellar (swimming) motility of *Pseudomonas* sp. DF41, Tn5 mutants DF41-1278 (*lp*) and DF41-469 (*gacS*), and *gacS* complemented strain DF41-469 (pUCP23-*gacS*).

Strain	Swim zone diameter (mm)[a]	
	24 h	72 h
DF41 (pUCP23)	20.5 (1.0)	47.0 (2.6)
DF41-1278 (pUCP23)	17.6 (2.6)[b]	45.2 (0.5)[b]
DF41-469 (pUCP23)	25.5 (1.1)[c]	56.4 (2.2)[c]
DF41-469 (pUCP23-*gacS*)	19.2 (4.8)[b]	49.4 (7.2)[b]

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

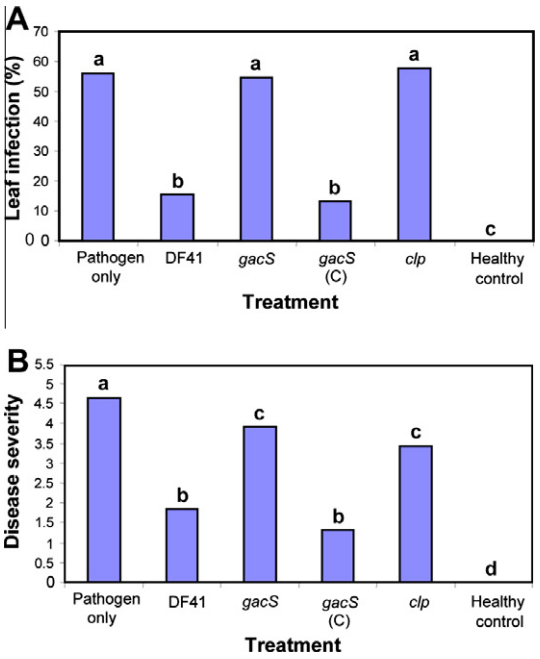

Fig. 4. Efficiency of *Pseudomonas* sp. DF41 (wild type), *gacS*⁻ (DF41-469), *gacS* complemented [DF41-469 (pUCP23-*gacS*)], and *lp*⁻ (DF41-1278) strains in managing *Sclerotinia sclerotiorum* ascospore infection on canola plants. (A) Percent incidence of leaf infection. (B) Disease severity on stem. 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 (DMRT; $P > 0.05$).

Through screening of a Tn mutant library, we were able to isolate mutants exhibiting greatly reduced AF activity against *S. sclerotiorum*. One mutant, designated DF41-1278, had a Tn insertion in a gene with high homology to the *sypC* gene of *P. syringae* pv. *syringae* B301D. This gene encodes a peptide synthetase involved in production of the LP syringopeptin (Scholz-Schroeder et al., 2003). These findings led us to postulate that DF41 is producing a LP involved in biocontrol.

The main antagonistic mechanism of LPs is believed to be pore formation which disrupts the electrical potential across the fungal cell membrane (Brodney et al., 1991; Raaijmakers et al., 2006). Several LPs have been implicated in the control of plant pathogens; however, in many instances comparative data from LP-deficient and wild-type strains is missing. In studies where LP mutants have been analyzed, the role of LPs in antagonism is variable. For example in *Bacillus subtilis* strain 6051, surfactin production is essential for control of *P. syringae* infection of Arabidopsis (Bais et al., 2004). Furthermore, a massetolide-deficient derivative of *P. fluorescens* strain SS101 was less effective in suppressing late blight of tomato (Tran et al., 2007), but exhibited wild-type biocontrol of *Pythium*-mediated root rot of apple and wheat (Mazzola et al., 2007). In *P. putida* strain 267, LPs are not required for control of Phytophthora damping-off of cucumber (Kruijt et al., 2009). From these examples it is clear that biocontrol agents may produce LPs but in many instances these molecules are not required for disease suppression. In our greenhouse assays, DF41-1278 (*lp*) showed decreased biocontrol of Sclerotinia stem rot of canola compared to the wild-type. Both the incidence of leaf infection (Fig. 4a) and disease severity (Fig. 4b) were markedly increased, leading us to conclude that this metabolite is essential for DF41 control of *S. sclerotiorum*. In addition to LPs, DF41 produces HCN and protease (Table 2). The small

zone of inhibition surrounding DF41-1278 on AF plates (Fig. 1) may be due to protease activity; however, DF41-1278 and the protease-deficient DF41-469 demonstrated equivalent protection in the greenhouse indicating this low-level AF activity is insufficient for disease suppression.

Our analysis of DF41 revealed that this bacterium is producing QS signal molecules. In *P. putida*, the LPs putisolvin I and II are under QS control (Dubern et al., 2006). Whether there is a connection between DF41 QS and LP expression is currently under investigation. We did identify one essential regulator of biocontrol in this study; analysis of the *gacS* Tn insertion mutant DF41-469 revealed a complete loss of biocontrol activity in both *in vitro* (Fig. 1) and greenhouse assays (Fig. 4). This is not surprising considering that this mutant was protease- and HCN-deficient (Table 2) and, even more importantly, did not produce LPs. Several other LP molecules have been shown to be under control of the GacS/GacA two-component regulatory system, including putisolvins I and II (Dubern et al., 2005), syringomycin (Bender et al., 1999; Willis and Kinscherf, 2004), amphisin (Koch et al., 2002), massetolide A and viscosin (Raaijmakers et al., 2006).

Many of the LPs that have been characterized to date are biosurfactants (Raaijmakers et al., 2006). We were able to demonstrate weak biosurfactant activity associated with culture extracts of DF41, but not the LP- and GacS-deficient strains. For several bacteria, LPs are essential for swarming motility and this is believed to be due to their biosurfactant properties (Andersen et al., 2003; Kuiper et al., 2004; Lindum et al., 1998; Nielsen et al., 2002; Roongsawang et al., 2003). We observed no difference in the swarming ability of the LP-deficient DF41-1278 and the DF41 wild type. Interestingly, the *gacS* mutant was unable to swarm, indicating that the Gac system plays a role in DF41 surface motility. Although swarm-deficient, the *gacS* mutant swam at an increased rate compared to the wild type (Table 3). Previously, a *gacA* mutant of *Pseudomonas fluorescens* F113 demonstrated an increase in both *fliC*-encoded flagellin expression and swimming motility (Sanchez-Contreras et al., 2002). In a later proteomics study, a *P. aeruginosa gacA* mutant showed elevated levels of flagellin (FliC) and flagellum capping protein (FliD) (Kay et al., 2006). Therefore, increased swimming and upregulated flagellar protein expression may be common phenotypes associated with *Pseudomonas gac* mutations.

In nature, the bulk of bacterial biomass exists as an adherent multicellular community encased in an extracellular matrix collectively called a biofilm. There are many advantages of adopting the biofilm mode of growth in the plant environment. Biofilm bacteria are sheltered from environmental stresses including desiccation, ultraviolet radiation, changes in humidity, and they are afforded protection from grazing predators (Ramey et al., 2004). Studies have shown that the Gac system is important for biofilm formation in various *Pseudomonas* species (Anderson et al., 2005; Parkins et al., 2001; Poritsanos et al., 2006) leading us to investigate whether the same would hold true for DF41. We discovered that the wild type and the *gacS*-minus DF41-469 are both able to form thick biofilms in a static plate assay. In *P. syringae* B728a, GacS is required for alginate production (Willis et al., 2001). A similar finding was observed in this study; the DF41 *gacS* mutant produced over fourfold less alginate than the parent (Table 2). If alginate forms part of the DF41 biofilm matrix, the residual level of alginate excreted by DF41-469 appears to be sufficient to form biofilms comparable to wild type.

There are reports of LPs being able to inhibit biofilm development on plastic surfaces (Kuiper et al., 2004; Roongsawang et al., 2003). Accordingly, the LP-minus strain DF41-1278 was analyzed to see if this compound influenced biofilm formation. We discovered no difference in biofilm production between the mutant and the wild type. These findings are consistent with those of the *gacS* mutant, which is also deficient in LP production but still able to form robust biofilms. A newly identified LP from *P. fluorescens* Pf-5, called orfamide A, was found to have no effect on biofilm formation (Gross et al., 2007). Most likely, disruption of biofilms is a trait associated with a subset of LPs. We speculate that for a given LP, there is a correlation between the degree of biosurfactant activity and its impact on biofilms. There are a number of examples of potent biosurfactants that alter biofilm development. For instance rhamnolipids, putisolvins I and II, and arthrobactin exhibit strong biosurfactant activity and all of these LPs have been found to either reduce biofilm formation or alter biofilm architecture (Davey et al., 2003; Kuiper et al., 2004; Roongsawang et al., 2003). At this point, we cannot definitively conclude that DF41 LPs play no role in biofilm formation or swarming motility. When the three strains grown under the corresponding test conditions were subjected to drop-collapse assays, there was no difference in their ability to reduce surface tension (data not shown). As such, the conditions used to test for biofilm formation and swarming motility appear to be suboptimal for LP production.

A great deal of diversity exists in terms of the structure and biological activity of LP molecules and we are currently in the process of elucidating the chemical structure of the compound produced by DF41. Using electrospray ionization and MALDI-MS analysis we have discovered a molecule of 2123 Da present in the DF41 extract (fraction 25) that is absent in DF41-469 and DF41-1278 (data not shown). Preliminary data suggests that DF41 produces a novel, linear molecule with a peptide moiety similar to that of the syringopeptin LP molecules (results to be presented elsewhere). Once the DF41 LP structure has been fully elucidated, comparisons with other known LPs should help clarify why the DF41 molecule has only weak biosurfactant properties.

In summary, strain DF41 produces an array of extracellular metabolites that may play a role in plant disease suppression. Now that we have determined LPs play a pivotal role in DF41 AF activity, our next step is to identify how the biosynthesis of these molecules is regulated to maximize disease suppression in the field.

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