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Characterization of the *Pseudomonas* sp. DF41 quorum sensing locus and its role in fungal antagonism

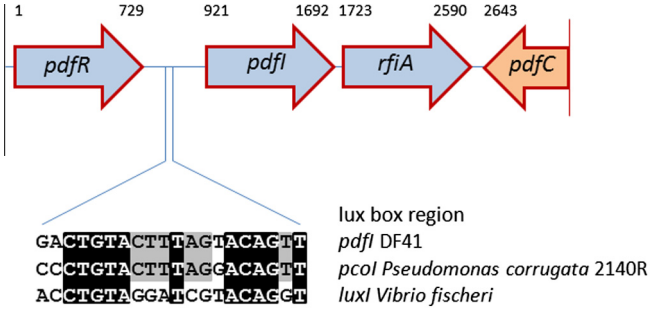
Chrystal L. Berry [a], Munmun Nandi [a], Jerrylynn Manuel [a], Ann Karen C. Brassinga [a], W.G. Dilantha Fernando [b], Peter C. Loewen [a], Teresa R. de Kievit [a,*]

[a] Department of Microbiology, University of Manitoba, Canada
[b] Department of Plant Science, University of Manitoba, Canada

HIGHLIGHTS

- The *Pseudomonas* sp. DF41 quorum-sensing system consists of PdfR and PdfI.
- A gene encoding a novel LuxR-type protein, RfiA, is co-transcribed with *pdfI*.
- Quorum sensing indirectly controls DF41 antifungal activity through RfiA.
- *pdfI* and acyl homoserine lactone production are positively regulated by the Gac–Rsm system.

GRAPHICAL ABSTRACT



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ABSTRACT

Pseudomonas sp. DF41 is able to suppress the fungal pathogen *Sclerotinia sclerotiorum* through production of a lipopeptide called sclerosin. The aim of this study was to isolate the DF41 QS locus and characterize its role in fungal antagonism. Through screening of a fosmid library, one clone was selected that tested positive for AHL production. Sequence analysis revealed the presence of two QS genes: *pdfR* and *pdfI*, encoding a LuxR transcriptional activator and an AHL synthase, respectively. Downstream of *pdfI* lays a gene encoding a transcriptional activator called RfiA followed by *pdfC*, comprising part of an efflux locus. Characterization of an AHL-deficient strain revealed it to be phenotypically identical to the wild type. Conversely *rfiA*, which is co-transcribed with *pdfI*, is essential for both AF activity and sclerosin production. Using a *pdfI-lacZ* fusion analysis, we discovered that *pdfI* is positively autoregulated. Additionally, *pdfI* expression was markedly increased in the *rfiA* mutant and quantification of AHL levels revealed elevated intracellular signal accumulation. We hypothesize that RfiA is a positive activator of the downstream efflux pump which serves to export both sclerosin and AHL signals. In a *gacS* mutant, *pdfI-lacZ* activity was decreased; however, plasmid-borne *rsmZ* was able to restore expression. Collectively, our findings indicate that: (i) QS indirectly controls DF41 suppression of *Sclerotinia* through RfiA; and (ii) *pdfI* expression and AHL signal production are positively regulated by the Gac–Rsm system. Identification of the PdfRI QS system, RfiA and RsmZ add to the increasingly complex network overseeing expression of DF41 biocontrol factors.

1. Introduction

Sclerotinia sclerotiorum is an economically important soil-borne pathogen capable of infecting over 400 plant species (Purdy, 1979). In canola, *S. sclerotiorum* causes a devastating disease called

* Corresponding author. Address: Department of Microbiology, University of Manitoba, 411 Buller Building, Winnipeg, MB R3T 2N2, Canada. Tel.: +1 (204) 474 8987; fax: +1 (204) 474 7603.
E-mail address: Teresa.Dekievit@ad.umanitoba.ca (T.R. de Kievit).

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sclerotinia stem rot that can result in up to 100% yield loss under conditions conducive for fungal infection. Thus far, breeding for Sclerotinia resistance in canola has been unsuccessful, resulting in a heavy reliance on fungicides for disease control. However, increasing public concern regarding agrochemicals coupled with the potential for fungicide resistance has led to interest in alternative strategies for disease management such as biological control. Our lab has been investigating a biocontrol bacterium called *Pseudomonas* sp. DF41 that demonstrates excellent antifungal activity against *S. sclerotiorum* in both greenhouse and field assays (Berry et al., 2010; Savchuk and Fernando, 2004). DF41 produces a number of compounds that are believed to contribute to fungal antagonism, including hydrogen cyanide, a protease, and a novel lipopeptide (LP) called sclerosin (Berry et al., 2010, 2012). The latter has been confirmed to be essential for biocontrol as a sclerosin-deficient mutant, DF41-1278, showed markedly reduced fungal inhibition (Berry et al., 2010).

Secondary metabolite production in pseudomonads is governed by a complex network involving multiple regulatory elements. One such element is the Gac two-component system comprised of the sensor kinase GacS and its cognate response regulator GacA. In several bacteria, including strain DF41, a mutation in either *gacS* or *gacA* results in a loss of biocontrol activity (Berry et al., 2010; Heeb and Haas, 2001). Working in concert with Gac, is a second regulatory network called Rsm (Regulator of secondary metabolism). The Rsm system is comprised of RsmA-type proteins that function as posttranscriptional repressors by binding to the ribosome-binding site (RBS) in target mRNA blocking translation (Lapouge et al., 2008). Repression is antagonized by the action of small RNAs that bind to and titrate out the repressors (Lapouge et al., 2008). Thus far, components of the Rsm system have not been identified in DF41. Another means by which bacteria control expression of exoproducts is through quorum sensing (QS). Quorum sensing enables bacteria to regulate gene transcription according to population density via the production of small, self-generated signals. In Gram negative bacteria, the most common signal molecules are *N*-acyl homoserine lactones (AHL), generated by a LuxI-type AHL synthase (Waters and Bassler, 2005). Once a threshold level of AHL is reached, it binds to and activates a cognate LuxR-type protein, which in turn controls expression of target genes (Waters and Bassler, 2005). In biocontrol bacteria that employ QS as part of their lifestyle, production of key compounds is often governed by these density-dependent networks. For example in *Pseudomonas chlororaphis* strains PA23, 30–84 and PCL1391, as well as *Pseudomonas fluorescens* strain 2–79, phenazine (PHZ) expression is positively regulated by the PHZ QS system (Chin-A-Woeng et al., 2001; Khan et al., 2005; Mavrodi et al., 1998; Selin et al., 2012; Wood and Pierson, 1996). We have previously reported that strain DF41 produces AHL signals (Berry et al., 2010); however the role that QS plays in DF41 biocontrol remains unknown.

The aim of the current study was to investigate how QS affects DF41 antifungal compound production. Genes encoding a QS system, designated PdfI/R, were isolated together with a downstream regulatory gene called *rfiA*. An AHL-deficient strain and an *rfiA* mutant were characterized for fungal antagonism and production of exoproducts including sclerosin. We also isolated an *rsmZ* homolog and determined how this regulatory RNA together with the Gac system affects the QS circuitry.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* were cultured at 37 °C on Lennox Luria

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

Strain/plasmid/primer	Relevant genotype or phenotype	Source or reference
Strain Pseudomonas		
DF41	Rif^R wild type (canola root tip isolate)	Savchuk and Fernando (2004)
DF41-1278	Rif^R *lp*::Tn*5*–1063 genomic insertion	Berry et al. (2010)
DF41–469	Rif^R *gacS*::Tn*5*–1063 genomic insertion	Berry et al. (2010)
DF41*rfiA*	DF41 with Gm^R cassette inserted into the *rfiA* gene	
P. aeruginosa		
QSC105	Strain carries pEAL01 plasmid (*lasB-lacZ* transcriptional fusion)	Ling et al. (2009)
E. coli DH5α	supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Gibco
C. violaceum CVO26	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31532, autoinducer biosensor	Latifi et al. (1995)
Plasmids		
pBSAI	pBluescript containing *pdfI* and *rfiA*	This study
pME6863	pME6000 carrying the *aiiA* gene from *Bacillus* sp. A24 under the constitutive Plac promoter	Reimmann et al. (2002)
pUCGm	Source of Gm^R cassette	Schweizer (1993)
pBSAI-Gm	pBSAI with a Gm^R cassette inserted into *rfiA*	This study
pEX18Tc	Suicide plasmid, Tc^R	Hoang et al. (1998)
pEXrfiA	pEX18Tc containing *rfiA*-Gm^R	This study
pRK600	Contains tra genes for mobilization; Ch^R	Finan et al. (1986)
pUCP22	Broad host-range vector Amp^R Gm^R	West et al. (1994)
pAI-22	pUCP22 containing *rfiA* and *pdfI* on a 3.0-kb *Bam*HI/*Eco*RI fragment	This study
pdfI-lacZ	*pdfI* promoter region cloned as an 852-bp *Xho*I-*Hin*dIII fragment into pLP170	This study
pLP170	Promoterless lacZ transcriptional fusion vector	Preston et al. (1997)
pME3219	pME6010 containing an *hcnA-lacZ* translational fusion	Laville et al. (1992)
pME6032	$lacI^q$-P_{tac} expression vector, Tc^R	Heeb et al. (2002)
pME6359	*rsmZ* gene from *P. fluorescens* CHA0 under control of the P_{tac} promoter in pME6032	Heeb et al. (2002)
Primers		
pdfIjcnF	5′-GAAGCCACTAGATTCGCACTT-3′	
rfiAjcnR	5′-ATGGCGTCGATGCATGTATT-3′	
p170FI*Xho*	5′-CCGCTCGAGTCATCCATACTTGAATAATCC-3′	
p170RI*Hind*	5′-CCCAAGCTTCTAAGGACCTCCTCATAAGT-3′	
RpoSF	5′-TACGTCAGTGCTTACGGCCA-3′	
RsmZR	5′-TATGACCCGCCCACATTTTT-3′	

Bertani (LB) agar (Difco Laboratories, Detroit, MI). *Pseudomonas* species were cultured on King's B (KB; King et al., 1954) medium at 28 °C or in M9CAgly [M9 minimal salts medium amended with 1% casamino acids (Difco) and 240 mM glycerol]. Antibiotics were added to the 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, IL).

2.2. Nucleic acid manipulation

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

2.3. Isolation and cloning of the DF41 quorum-sensing genes

A DF41 fosmid library was generated using the CopyControl™ Fosmid Library Production Kit (Epicenter, Madison, WI) according to the manufacturer's instructions. Fosmid clones were screened for AHL production by spotting onto plates seeded with the AHL biosensor strain *Chromobacterium violaceum* CVO26. This bacterial strain is only able to produce the purple pigment violacein in the presence of exogenous autoinducer due to a mutation in the autoinducer synthase gene (Latifi et al., 1995). Four fosmid clones that restored pigment production in CVO26 were identified; one of which (clone 564) was selected for further analysis. Fosmid 564 DNA was partially digested with *Sau*3A1 and fragments between 2 and 4 kb were ligated with *Bam*H1-digested pBluescript SK+. The library of subcloned fragments was mobilized into *E. coli* and transformants were screened for AHL production as above. One AHL-positive clone containing a 3.2-kb insert (pBSAI) was identified.

2.4. Sequence analysis and nucleotide accession numbers

DNA sequencing of fosmid 564 and pBSAI was performed by the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Ontario) and sequences were analyzed with blastn and blastx databases. The GenBank accession numbers for the sequences of the DF41 *pdfI*, *pdfR*, *rfiA* and *rsmZ* genes are HM590003.1, KF703445, KF703444 and KF703443, respectively.

2.5. Generation of autoinducer-deficient strain DF41

Plasmid pME6863 harbouring the AHL lactonase gene *aiiA* from *Bacillus subtilis* was introduced into DF41 by electroporation. Bacteria were plated on LB-Tc15 μg/mL and colonies were patched onto CVO26-seeded plates to identify those unable to restore violacein production due to the presence of the *aiiA* gene.

2.6. Generation of an rfiA null mutant and plasmid pAI-22

To generate an *rfiA*-null mutant, the *rfiA* gene on pBSAI was interrupted through insertion of a Gm-resistance cassette. Plasmid pUCGm was digested with *Sma*I and an 855-bp fragment was inserted into the *Nru*I/*Sph*I sites of *rfiA* made blunt with Klenow. The resulting plasmid, pBSAI-Gm, was digested with *Hin*dIII/*Xba*I to liberate the 3.6-kb insert which was cloned into the same sites of suicide vector pEX18Tc (pEXrfiA). Triparental matings were performed using *E. coli* DH5α (pEXrfiA), *E. coli* DH5α (pRK600) and the DF41 recipient. Transconjugants were screened on LB agar supplemented with Gm. Sucrose plates containing Gm were used to identify bacteria that had undergone a double cross-over event. To confirm the insertion of the Gm marker into the *rfiA* gene, PCR analysis was performed. For complementation of the DF41 *rfiA* mutant, plasmid pAI-22 was created as follows. Plasmid pBSAI was digested with *Bam*H1 and *Eco*R1 and the 3.0-kb fragment containing *rfiA* was cloned into the same sites of pUCP22.

2.7. RT-PCR analysis

To determine if *pdfI* and *rfiA* are co-transcribed, RT-PCR analysis was performed. DF41 was grown in King's B medium for 14 h. Total RNA was purified from 1 ml of the overnight culture using the Qiagen RNeasy Mini Kit (Qiagen, Toronto, ON) according to the manufacturer's protocol. Following a DNase I purification step using the Qiagen DNase I kit (Qiagen), the RNA samples were quantified spectrophotometrically by measuring the OD 260/280 nm. cDNA was then synthesized using 2 μl of the purified RNA and the VILO cDNA synthesis Kit (Invitrogen, Burlington, ON) employing the following conditions: 25 °C for 10 min, 42 °C for 60 min, and 85 °C for 5 min. A 1-μl aliquot of the newly synthesized cDNA was used for PCR analysis employing forward primer (pdfIjcnF) and reverse primer (rfiAjcnR) which were designed to amplify a 750-bp product containing sequences overlapping the *pdfI* and *rfiA* region. PCR conditions were as follows: an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 10 min, followed by a final extension at 72 °C for 10 min.

2.8. Generation of a pdfI-lacZ transcriptional fusion

PCR primers p170FI*Xho* and p170RI*Hin*d were designed to amplify an 852-bp fragment encompassing the promoter region and part of the *pdfI* gene. The primers contained *Xho*I and *Hin*dIII restriction sites which enabled cloning into the same sites of the *lacZ* transcriptional fusion vector pLP170 (Preston et al., 1997).

2.9. pdfI and hcnA expression in DF41 and derivative strains

Expression of *pdfI* and *hcnA* was measured using *pdfI-lacZ* and pME3219 (Laville et al., 1992), respectively. Bacterial cultures harboring these fusions were grown for 8, 24 and 48 h and then measured for β-galactosidase activity according to Miller (1972). Samples were analyzed in triplicate and the experiment was repeated three times.

2.10. Antifungal and protease activity

Radial diffusion assays to assess fungal inhibition in vitro were performed according to previously described methods (Poritsanos et al., 2006). Protease activity was measured according to Poritsanos et al. (2006). The data represent the average of at least six replicates and the assays were repeated three times.

2.11. AHL signal analysis

External and internal AHL accumulation was monitored by growing bacterial cultures for 18 h at 28 °C in 30 ml of M9 minimal media supplemented with 0.4% glucose and 1 mM $MgSO_4$. Cells were pelleted by centrifuging at 8000 rpm for 15 min. Cell-free supernatants were extracted twice with an equal volume (30 ml) of acidified ethyl acetate. The ethyl acetate fractions were pooled and concentrated to a final volume of 1 ml. Cell pellets were resuspended in 3 ml of phosphate buffered saline and then subject to two 45-s rounds of sonication, separated by 1-min incubation on ice. Cell lysates were centrifuged at 8000 rpm for 10 min to remove

cellular debris and the clarified supernatant was extracted twice with a 3-ml volume of ethyl acetate. The organic layers were pooled and concentrated to a final volume of 1 ml. 100 µl volumes of each extract were subject to AHL quantification using the biosensor strain *P. aeruginosa* QSC105 following the method of Selin et al. (2012). Samples were analyzed in triplicate and the experiment was repeated twice.

2.12. HPLC analysis

To assess sclerosin production, DF41 and derivative strains were grown in 300 mL volumes of M9CAgly medium for 4 days at 28 °C with shaking. Sclerosin was extracted from cultures and analyzed by high-performance liquid chromatography (HPLC) as described by Manuel et al. (2011).

2.13. Motility analysis

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

2.14. Biofilm formation

To examine the ability of DF41 and derivative strains to form biofilms, a 96-well plate assay was employed following the method of Berry et al. (2010).

2.15. Statistical analysis

An unpaired Student's *t* test was used for statistical analysis of antifungal activity, protease, autoinducer and sclerosin production.

3. Results and discussion

3.1. Identification of the PdfI/R quorum sensing system and RfiA

By screening a DF41 fosmid library using the biosensor strain *C. violaceum* CVO26, one clone was selected that tested positive for AHL production. Further subcloning enabled the isolation of the DF41 AHL synthase gene, designated *pdfI*, on plasmid pBSAI. *pdfI*, is 771-bp in length and is predicted to encode a LuxI-type protein. The DF41 *pdfI* gene exhibits the highest degree of nucleotide sequence identity (88%) with the *P. corrugata pcoI* gene (accession no. AF199370.1). Sequencing of fosmid 564 revealed a 729-bp ORF, designated *pdfR*, upstream of *pdfI* that is predicted to encode a LuxR-type protein. This gene exhibits the highest degree of sequence identity (83%) to *P. corrugata pcoR* (accession no. EF189721.2). Immediately downstream of *pdfI*, is an ORF spanning 867 bp that is 81% identical at the nucleotide level to *rfiA* of *P. corrugata* (accession no. AF199370.1). RfiA has been proposed to belong to a novel subfamily of LuxR transcriptional activators that contains a helix-turn-helix DNA-binding domain in the C-terminus but lacks the conserved N-terminal AHL-binding motif (Licciardello et al., 2009). Downstream of the DF41 *rfiA* gene lies a partial ORF

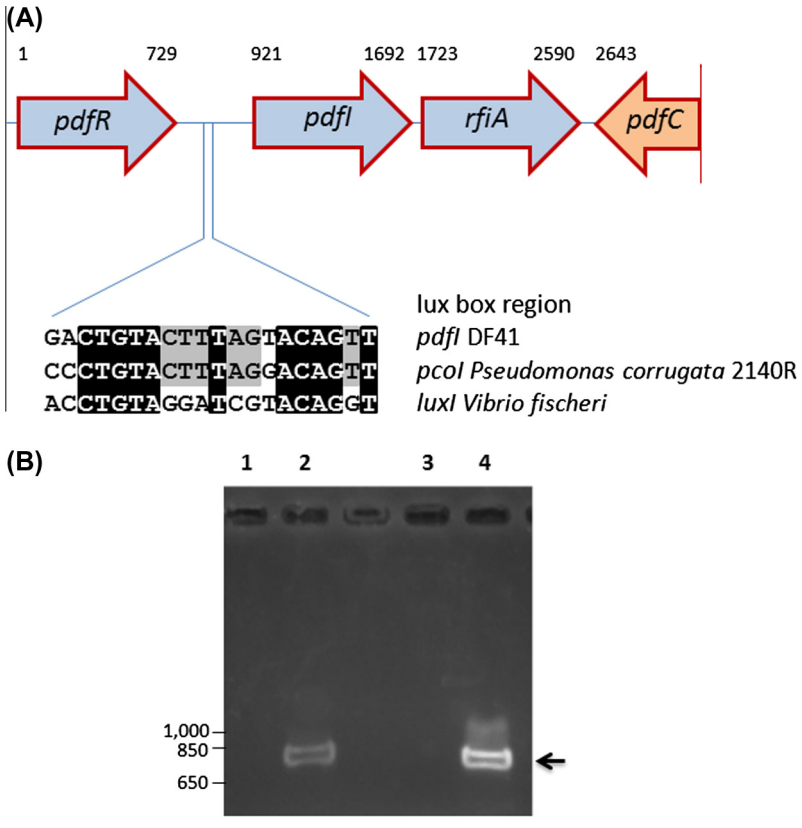

Fig. 1. The *Pseudomonas* sp. DF41 quorum sensing locus. (A) Genetic map of the quorum sensing genes including *pdfR*, *pdfI*, and *rfiA*, with the position of each gene located above. Downstream of and divergently transcribed from *rfiA* lays *pdfC*, which is believed to encode part of an efflux pump. A putative 20-bp lux-box sequence is located in the *pdfI* promoter. Light and dark shading highlights nucleotides identical to those found in the lux boxes of *Pseudomonas corrugata* 2140R and *Vibrio fischeri*. (B) Reverse transcription PCR indicates that *pdfI* and *rfiA* are co-transcribed. Using a forward primer that anneals with *pdfI* and a reverse primer annealing to *rfiA* resulted in a single 750-bp amplicon, indicated with an arrow, from DF41 cDNA (lane 2) and pAI-22 (lane 4). When total RNA (lane 1) and pUCP22 vector (lane 3) were used as template, no amplicon was observed.

exhibiting 85% identity to the *pcoC* gene of *P. corrugata* (accession no. EF189721.2). The *P. corrugata pcoABC* genes encode a tripartite RND transport system involved in the export of multiple compounds (Licciardello et al., 2009). Overall, the genetic arrangement of the DF41 QS locus and flanking region (Fig. 1) most closely resembles that of phytopathogenic *P. corrugata* strain CFBP 5454 (Licciardello et al., 2007; Licciardello et al., 2009).

3.2. RfiA but not AHL signals are essential for DF41 antifungal activity and LP production

In many pseudomonads, QS plays an important role in regulating exoproduct expression. When we analyzed what effect if any the ability to produce AHL signals has on fungal antagonism, we discovered no difference between the AHL-deficient strain DF41 (pME6863) and the wild type (Table 2). Not surprisingly, HPLC analysis revealed wild-type levels of sclerosin produced by DF41 (pME6863), the key metabolite involved *S. sclerotinia* suppression (Table 2). Strain DF41-1278 was included as a negative control because it contains a Tn insertion in the LP biosynthetic locus and does not produce sclerosin (Berry et al., 2010). When we analyzed a DF41 *rfiA* mutant, a very different phenotypic pattern was observed. This strain was devoid of AF activity and showed markedly reduced sclerosin production. Similarly in the phytopathogen *P. corrugata*, mutants no longer producing RfiA were attenuated for virulence (Licciardello et al., 2009). RfiA belongs to a novel LuxR subfamily of regulatory proteins that includes SalA, SyrF and ToxJ; these regulators control expression of genes for toxin biosynthesis and export in phytopathogenic bacteria (Kim et al., 2004; Licciardello et al., 2009; Wang et al., 2006). Detailed characterization of SalA and SyrF in *Pseudomonas syringae* pv. *syringae* revealed that unlike LuxR, AHL is not required for dimerization and binding to their target DNA (Wang et al., 2006). Because DF41-*rfiA* showed dramatically reduced sclerosin production, we hypothesize that RfiA is an essential regulator of sclerosin biosynthesis and or secretion. No differences in protease activity were observed for either the AHL-deficient strain or the *rfiA* mutant (Table 2).

3.3. pdfI and rfiA are co-transcribed

pdfI and *rfiA* are separated by only 31 bp; therefore, we explored the possibility that the two genes are co-transcribed. Reverse transcription employing a forward primer that annealed within the *pdfI* gene and a reverse primer annealing to the *rfiA* coding sequence was performed. A single 750-bp amplicon was observed when both DF41 cDNA and pAI-22 were used as template DNA (Fig. 1B), indicating that *pdfI* and *rfiA* are cotranscribed. A similar situation occurs in *P. corrugata*, where *rfiA* is co-transcribed with the upstream AHL synthase gene *pcoI* (Licciardello et al., 2009).

Table 2
Phenotypic characteristics of *Pseudomonas* sp. DF41 and derivative strains.

Bacterial strain	AF[a]	Protease[a]	Sclerosin production[b] Absorbance (mAU at 210 nm)
DF41 (pUCP22)	9.7 (0.7)	7.0 (1.5)	812.2 (212.7)
DF41*rfiA* (pUCP22)	0.0 (0.0)[c]	7.8 (1.4)[d]	37.2 (12.7)[e]
DF41*rfiA* (pAI-22)	9.0 (0.8)[d]	6.5 (0.4)[d]	688.4 (14.5)[d]
DF41 (pME6863, pUCP22)	10.0 (0.8)[d]	6.6 (0.8)[d]	751.6 (212.9)[d]
DF41-1278	n.d.	n.d.	3.3 (4.6)[e]

[a] Mean (SD) of the zones of activity (mm) from at least six replicates.
[b] Mean (SD) of the LP antibiotic extracted from 300-ml culture volume. The experiment was performed in duplicate and the repeated twice.
[c] Significantly different from the wild type ($p < 0.001$).
[d] Not significantly different from the wild type.
[e] Significantly different from the wild type.

3.4. pdfI is autoregulated

Sixty-five bp upstream of the predicted *pdfI* translational start lies a 20-bp imperfect inverted repeat with a high degree of similarity to previously reported lux-box elements (Fig. 1). *luxI*-type genes are typically autoregulated through binding of the LuxR–AHL complex to this *lux*-box region. Analysis of a *pdfI-lacZ* transcriptional fusion in DF41 revealed that expression increased according to population density (Fig. 2). However, *pdfI* expression was extremely low in the presence of the AHL-degrading lactonase enzyme [DF41(pM6863)] (Fig. 2). Collectively, these findings indicate that *pdfI* is subject to positive autoregulation.

3.5. pdfI expression and intracellular AHL accumulation is increased in an rfiA mutant

Next, we were interested to see what effect if any RfiA had on *pdfI* expression. We were surprised to discovered that in the *rfiA* mutant, *pdfI-lacZ* activity was increased fourfold over wild type at 48 h (Fig. 2). To determine whether this phenomenon occurred with other QS-regulated genes, an *hcnA-lacZ* reporter was analyzed. We observed markedly increased expression of this fusion in DF41-*rfiA* compared to DF41 (Fig. 3). One possible explanation for these findings is that AHL accumulation is higher in the *rfiA* mutant. Accordingly, we analyzed cell-free supernatants and cell pellets for AHL levels. We discovered that AHL accumulation was 1.6-fold lower in supernants of the *rfiA* mutant compared to wild type; whereas the cell pellet contained 2.7-fold higher amounts of AHL (Fig. 4). These findings suggest that the signalling molecules are being retained inside of the *rfiA* mutant. As expected, levels of AHL in both the cell-free supernatant and cell pellet of DF41 (pME6863) were extremely low (Fig. 4). In *P. corrugata* 5454, expression of the PcoABC pump operon was markedly reduced in an *rfiA* mutant leading to reduced phytotoxin export (Licciardello et al., 2009). We hypothesize that increased AHL accumulation occurs in the *rfiA* mutant due to decreased expression of the down stream pump through which these signals are exported. A similar finding was observed for *Pseudomonas aeruginosa* where the longer-chain 3O–C_{12}–AHL is actively effuxed by the MexAB-OprM RND system (Pearson et al., 1999). Conversely the shorter chain C_4–AHL

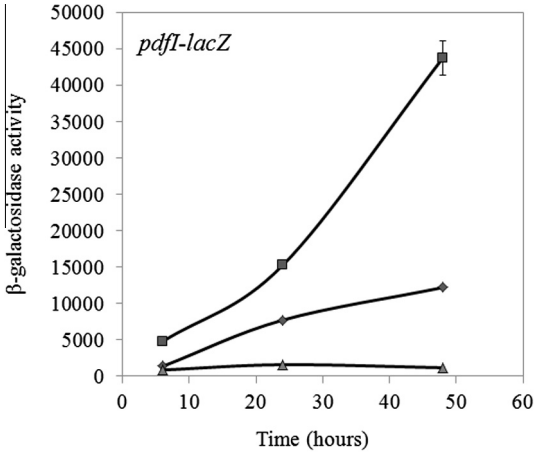

Fig. 2. *pdfI* is subject to autoregulation. The activity of a *pdfI-lacZ* transcriptional fusion was monitored in DF41 (diamonds), DF41*rfiA* (squares), and DF41 (pME6863) (triangles). Strains were grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose. Each value is the mean from three different cultures ± standard error. Experiments were performed three times; one representative data set is shown. Note the reduced *pdfI* expression in the AHL-deficient strain [DF41 (pME6863)] and the significantly higher expression in the *rfiA* mutant, compared to the wild type.

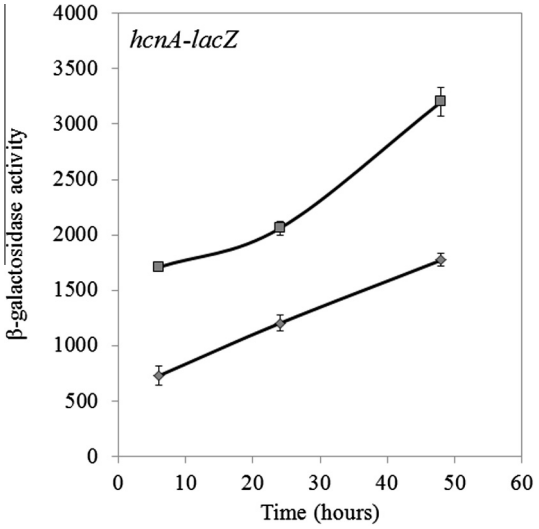

Fig. 3. *hcnA* expression is increased in a DF41 *rfiA* mutant. The activity of an *hcnA-lacZ* reporter fusion was monitored in DF41 (diamonds) and DF41*rfiA* (squares). Strains were grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose. Each value is the mean from three different cultures ± standard error. Experiments were performed three times; one representative data set is shown. Note the significantly higher expression in the *rfiA* mutant, compared to the wild type.

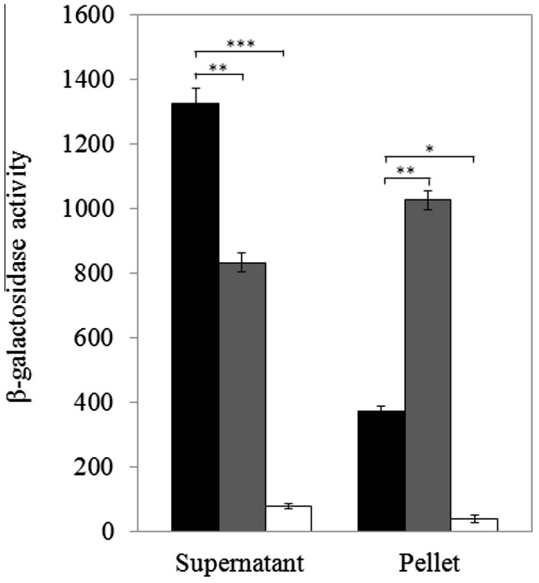

Fig. 4. Intracellular and extracellular acyl homoserine lactone accumulation. Cell-free supernatants and bacterial pellets of DF41 (black bars), DF41-*rfiA* (gray bars) and DF41 (pME6863) (white bars) were subject to AHL extraction using ethyl acetate. A 100-µl aliquot of each AHL preparation was added to cultures of *P. aeruginosa* QSC105 (pEAL01), which were grown for 18 h prior to β-galactosidase analysis. Each value is the mean from three different cultures ± standard error. Experiments were performed twice and one representative data set is shown. For strains that differ significantly from the wild type, columns are labelled with asterisks ($^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0001$).

molecules primarily move by passive diffusion (Pearson et al., 1999). The exact structure of the DF41 AHL has not been deduced; however, the fact that it activates the CVO26 biosensor strain suggests it is in the C4-C8 HSL range. At the moment secretion of sclerosin and AHL signals by the downstream efflux pump is merely speculative; a pump mutant is required to better understand how these molecules are exported.

3.6. Identification of rsmZ in strain DF41

In a number of pseudomonads, *rsmZ* lies immediately downstream of the gene encoding the stationary phase sigma factor, *rpoS*. Degenerate PCR primers (RpoSF/RsmZR) were designed using annotated *rpoS* and *rsmZ* gene sequences to isolate the *rsmZ* gene from DF41. These primers successfully amplified a 1.0-kb product encompassing the 3′-end of *rpoS*, the *rpoS–rsmZ* intergenic region and the entire *rsmZ* gene. Blastn analysis of the sequenced product identified a 129-nt sequence with significant homology to the *rsmZ* genes from a number of other *Pseudomonas* species. The DF41 *rsmZ* gene shares the highest level of identity with that of *P. fluorescens* 2P24 (96%) and has 84% sequence identity with the *P. fluorescens* CHA0 *rsmZ* (accession no. AF245440). Analysis of the *rsmZ* sequence using an RNA-fold program for predicting secondary structure (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) revealed several stem-loop structures containing unpaired AGG(G)A motifs (data not shown). Similar structures have been predicted for RsmX, Y and Z of *P. fluorescens* CHAO (Haas and Défago, 2005). This configuration is believed to be essential for RsmZ titration of RsmA-like repressor proteins (Lapouge et al., 2008).

3.7. The Gac/Rsm system controls AHL production and pdfI expression

Next, we sought to determine how the Gac/Rsm system affects QS in DF41. On CVO26 indicator plates, significantly decreased ($p < 0.01$) purple zones were observed for the *gacS* mutant [4.6 (±0.5) mm] as compared to the wild-type strain [8.2 (±0.7) mm]. Because the *rsmZ* gene of DF41 shares a high degree of nucleotide identity with that of *P. fluorescens* CHA0 we mobilized *rsmZ*-containing pME6359, into DF41 and the DF-469 *gacS* mutant. In the wild-type background, the presence of pME6359 significantly ($p < 0.05$) increased AHL production from 8.2 (±0.7) to 9.3 (±0.3) mm. Furthermore, this plasmid restored AHL production in the *gacS* mutant to near wild type levels (7.8 ± 1.2). Thus, RsmZ has a positive effect on AHL production in DF41. Analysis of the *pdfI-lacZ* fusion in DF41 and DF41-469 (*gacS*) revealed that *pdfI* expression was significantly decreased in the *gacS* mutant compared to the wild type (Fig. 5). Addition of pME6359 resulted in elevated *pdfI-lacZ* activity in both the wild-type and *gacS* mutant backgrounds (Fig. 5). Collectively, our findings indicate that AHL

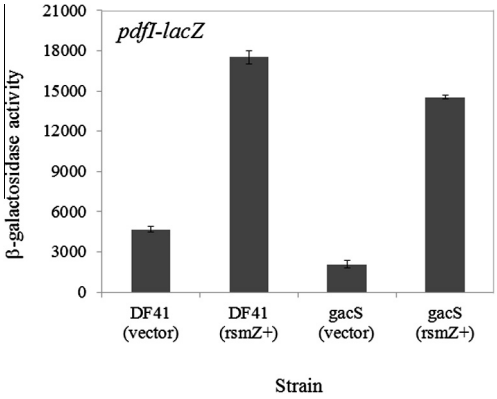

Fig. 5. *pdfI* expression is under control of the Gac–Rsm system. The activity of a *pdfI-lacZ* transcriptional fusion was monitored in DF41 (pME6032; empty vector), DF41 (pME6359; *rsmZ*+), DF41–469 (pME6032; empty vector) and DF41–469 (pME6359; *rsmZ*+) after 24 h growth. Bacteria were grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose. Each value is the mean from three different cultures ± standard error. Experiments were performed three times; one representative data set is shown. Note that in the wild type and *gacS* mutant (DF41–469), overexpression of *rsmZ* leads to increased *pdfI* expression.

production and *pdfI* expression are under positive control of the Gac/Rsm system. At the moment, the mechanism by which GacS and RsmZ control *pdfI* expression remains unclear. In other pseudomonads, one or more small regulatory RNAs, like RsmZ, function to titrate out RsmA translational repressors (Haas and Défago, 2005). The repressor proteins bind to the Shine–Dalgarno sequence of target mRNA blocking ribosome binding and inhibiting translation. It is possible that *pdfI* is a direct target for RsmA-like proteins, and providing RsmZ in trans leads to derepression and increased *pdfI-lacZ* activity. Alternatively, yet-to-be identified regulators of *pdfI* under Gac–Rsm control may be responsible for these effects. Thus far, RsmZ is the only member of the Rsm circuitry that has been identified in DF41. Further studies are required to determine whether this strain harbors additional sRNAs as well as RsmA-like proteins.

3.8. QS and RfiA have no effect on DF41 biofilm formation or flagellar motility

In other pseudomonads, such as *P. chlororaphis* and *P. aeruginosa*, QS has been found to positively control biofilm formation (Poritsanos et al., 2006; Davies et al., 1998). Furthermore, flagellar motility can be important for attachment to and movement across solid surfaces (O'Toole et al., 2000). Therefore, we examined whether DF41 (pME6863) and the *rfiA* mutant showed any differences in their ability to swim (flagellar motility) or to form biofilms on a plastic surface. No difference in swimming motility between the aforementioned strains and the wild type were observed at 24, 48 and 72 h (data not shown). Additionally, all three strains showed equivalent, robust biofilm formation on 96-well plates (data not shown). Thus it appears that neither AHL production nor RfiA affects the ability of DF41 to move by flagellar motility or colonize plastic surfaces under minimal media conditions.

In conclusion, we have identified and characterized components of the DF41 QS network. We have discovered that AHL signalling is not directly involved in either sclerosin production or fungal antagonism. *rfiA*, on the other hand, is co-transcribed with *pdfI* and its product, RfiA, was found to be an essential regulator of DF41 antifungal activity. Thus, QS indirectly controls the ability of DF41 to suppress *Sclerotinia* through RfiA. Furthermore, expression of *pdfI* and by extension, *rfiA*, is positively regulated by the Gac–Rsm network. Identification of PdfI, PdfR, RfiA and RsmZ add to the increasing complex network overseeing expression of biocontrol factors by DF41. Having a multi-tiered system of regulation presumably allows bacteria to respond to a plethora of diverse signals, allowing them to regulate production of antifungal metabolites according to prevailing conditions.

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