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Negative regulation of germination-arrest factor production in Pseudomonas fluorescens WH6 by a putative extracytoplasmic function sigma factor

Negative regulation of germination-arrest factor production in *Pseudomonas fluorescens* WH6 by a putative extracytoplasmic function sigma factor

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Pseudomonas fluorescens WH6 secretes a germination-arrest factor (GAF) that we have identified previously as 4-formylaminooxyvinylglycine. GAF irreversibly inhibits germination of the seeds of numerous grassy weeds and selectively inhibits growth of the bacterial plant pathogen *Erwinia amylovora*. WH6-3, a mutant that has lost the ability to produce GAF, contains a Tn*5* insertion in *prtR,* a gene that has been described previously in some strains of *P. fluorescens* as encoding a transmembrane regulator. As in these other pseudomonads, in WH6, *prtR* occurs immediately downstream of *prtI*, which encodes a protein homologous to extracytoplasmic function (ECF) sigma factors. These two genes have been proposed to function as a dicistronic operon. In this study, we demonstrated that deletion of *prtI* in WT WH6 had no effect on GAF production. However, deletion of *prtI* in the WH6-3 mutant overcame the effects of the Tn*5* insertion in *prtR* and restored GAF production in the resulting double mutant. Complementation of the double *prtIR* mutant with *prtI* suppressed GAF production. This overall pattern of *prtIR* regulation was also observed for the activity of an AprX protease. Furthermore, reverse transcription quantitative real-time PCR analysis demonstrated that alterations in GAF production were mirrored by changes in the transcription of two putative GAF biosynthetic genes. Thus, we concluded that PrtI exerted a negative regulatory effect on GAF production, although the mechanism has not yet been determined. In addition, evidence was obtained that the transcription of *prtI* and *prtR* in WH6 may be more complex than predicted by existing models.

INTRODUCTION

Pseudomonas fluorescens WH6, a pseudomonad isolated from soils of the Willamette Valley in Oregon, USA (Elliott *et al.*, 1998), has been shown in our laboratories to produce a secondary metabolite with selective herbicidal properties (Banowetz *et al.*, 2008, 2009; Armstrong *et al.*, 2009). This compound, which we have termed a germination-arrest factor (GAF), arrests the germination of a large number of grassy weed species without significantly affecting the growth of established grass seedlings or mature plants. The graminaceous weed species affected by GAF include annual

bluegrass (ABG, *Poa annua* L.) and ABG seeds have been used to establish a quantitative bioassay for GAF activity (Banowetz *et al.*, 2008). In addition to its herbicidal properties, GAF also exhibits a selective antimicrobial activity against *Erwinia amylovora*, the bacterial plant pathogen that causes fireblight in orchard crops (Halgren *et al.*, 2011). The compound responsible for GAF activity has been isolated from *P. fluorescens* WH6 culture filtrates and identified as the amino acid analogue 2-amino-4-formylaminooxy-3-butenoic acid (McPhail *et al.*, 2010), a previously undescribed member of a small group of naturally occurring compounds known as oxyvinylglycines.

The genome of *P. fluorescens* WH6 has been sequenced (Kimbrel *et al.*, 2010) and the genetic basis of GAF biosynthesis in WH6 has been investigated by transposon mutagenesis (Halgren *et al.*, 2013). The response of *E. amylovora* to GAF provided a rapid preliminary screen for *P.*

Abbreviations: ABG, annual bluegrass; ECF, extracytoplasmic function; FRT, *flp* recombinase recognition target; GAF, germination-arrest factor; qPCR, quantitative real-time PCR RACE, rapid amplification of cDNA ends; RT, reverse transcription.

Five supplementary figures and two supplementary tables are available with the online version of this paper.

fluorescens WH6 mutants altered in the ability to produce GAF and the ninhydrin reactivity of GAF enabled the presence of this compound in bacterial culture filtrates to be monitored by TLC (Armstrong *et al.*, 2009). Using these screening methods, we identified 11 non-redundant mutations in the WH6 genome that resulted in loss of GAF production (Halgren *et al.*, 2013). Three of these mutations occurred in genes homologous to the regulatory genes *gntR*, *iopB* and *prtR*. The WH6 *gntR* homologue belongs to a large family of transcriptional regulators that control a wide variety of metabolic processes and environmental responses in pseudomonads (Hoskisson & Rigali, 2009). The *iopB* gene has been reported to regulate phenazine production in *Pseudomonas chlororaphis* PCL1391 (van Rij, 2006). The *prtR* homologue and its immediate upstream neighbour (a *prtI* homologue) have high identity (88 %) to the *prtIR* locus that regulates the temperature-sensitive production of an extracellular protease, AprX, in *P. fluorescens* LS107d2 (Burger *et al.*, 2000). The *prtIR* locus has also been reported to regulate the production of a protease in *Pseudomonas entomophila* (Liehl *et al.*, 2006), as well as phase changes in *Pseudomonas* sp. PCL1171 (van den Broek, 2005). Recently, mutations of the *prtR* homologues in *P. fluorescens* strains HCl-07 and SS101 have been reported to suppress the production of both a protease and cyclic lipopeptides (Yang *et al.*, 2014; Song *et al.*, 2014).

PrtI has homology to extracytoplasmic function (ECF) sigma factors (Burger *et al.*, 2000), and it has been proposed that PrtI and PrtR may function as a sigma factor and anti-sigma factor pair (van den Broek, 2005; Liehl *et al.*, 2006), in a manner similar to that described for other ECF sigma factors (Hughes & Mathee, 1998). In bacteria, dissociable sigma factors form part of the multisubunit RNA polymerase, where they direct RNA transcription by binding promoter sequences in DNA. Most bacteria have one housekeeping σ^{70} factor responsible for most transcription and a variable number of alternative sigma factors, including the ECF sigma subgroup, that typically allow the bacterium to respond to environmental conditions (Gruber & Gross, 2003; Paget & Helmann, 2003). Many ECF sigma factors are maintained in an inactive state through direct interactions with a cognate anti-sigma factor, typically a membrane-spanning protein (Helmann, 2002). Upon perception of a signal, the sigma factor is released and engages with the RNA polymerase to initiate transcription.

Various strains of *P. fluorescens* have been found to have between 19 and 26 ECF-encoding genes in their genomes (Kimbrel *et al.*, 2010). Homologues of the *prtI* and *prtR* pair are present in a number of bacterial strains and species. In addition to the referenced strains of *P. fluorescens* and *P. entomophila*, a BLAST search (Altschul *et al.*, 1997) identified *prtIR* homologues in *Pseudomonas brassicacearum*, *Pseudomonas protegens*, *Pseudomonas putida*, and various species of *Burkholderia* and *Xanthomonas*. Based on a slight overlap of the *prtI* and *prtR* sequences, it has been assumed that the *prtIR* locus functions as a dicistronic operon, with the synthesis of PrtI and PrtR being translationally coupled (Burger *et al.*, 2000).

The role of the *prtIR* locus in the regulation of GAF production in *P. fluorescens* WH6 has been investigated here. We found that PrtR acted to suppress a potentially negative regulatory effect of PrtI on GAF production in WH6. A similar pattern was observed in the regulation of a protease controlled by *prtIR* in WH6. Although the mechanism of these effects remains uncertain, our results suggested that existing models of the interaction and function of *prtI* and *prtR* may need to be revised.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. All strains used in this study were maintained at -80 °C in LB medium (Sambrook & Russell, 2001) with a final concentration of 15 % (v/v) glycerol unless otherwise indicated.

The origin and characterization of *P. fluorescens* strain WH6 were described previously (Elliott *et al.*, 1998; Banowetz *et al.*, 2008). The mutant WH6-3 strain, carrying a Tn*5* insertion in the *prtR* gene, was generated in an earlier study (Armstrong *et al.*, 2009), and characterized by Kimbrel *et al.* (2010) and Halgren *et al.* (2013). *E. amylovora* 153 was obtained from Dr Joyce Loper (USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR, USA).

Preparation of culture filtrates. *Pseudomonas* strains were inoculated into modified *Pseudomonas* Minimal Salts (PMS) medium, cultured and harvested as described previously (Banowetz *et al.*, 2008). Cultures were grown at 28 °C except as indicated and duplicate clones of each deletion mutant or complemented mutant were cultured. To prepare culture filtrates, the *Pseudomonas* culture fluid recovered from 7-day cultures was centrifuged (3000 **g**, 15 min) and the supernatant was passed through a bacteriological filter (Millipore GP Express Steritop, 0.22 μm pore size). The resulting sterile culture filtrate was stored at 4 °C.

TLC analysis. TLC analyses of bacterial culture filtrates were performed on 90 % ethanol extracts of the solids from dried culture filtrates as described previously (Armstrong *et al.*, 2009; Halgren *et al.*, 2011). Silica GHL and microcrystalline cellulose TLC plates (250 μm thick) were purchased from Analtech. Ninhydrin staining was performed as described previously (Armstrong *et al.*, 2009).

Bioassays for GAF activity. Bioassays for GAF activity in bacterial culture filtrates were performed with ABG seeds using the standard *Poa* germination bioassay protocol and scoring system described by Banowetz *et al.* (2008). In this system, a score of 4 represents normal germination and plumule development; a score of 1 represents complete germination arrest immediately after emergence of the radicle and coleoptile. For this assay, the *Poa* seeds were placed in small fluid-filled wells (three seeds per well) and scored after 7 days incubation. Three replicate wells (nine seeds total) were prepared for each concentration of each sample tested.

Tests of culture filtrates for antimicrobial activity against *E. amylovora* were performed as described by Halgren *et al.* (2011).

Assays for protease activity. The WT, mutant and complemented mutant strains of WH6 were assayed for protease activity using a plate-based skim-milk-clearing assay. Bacterial stocks tested in the protease assay were maintained at -60 °C in PMS medium supplemented with 50 % glycerol. Cultures were seeded with 10 μl glycerol stock into liquid PMS medium (6 ml) and grown at 28 °C for 24 h on a rotary shaker (200 r.p.m.). After incubation, cultures were diluted to OD_{600} 1.0 with sterile deionized water. A 5 μl aliquot

Table 1. Bacterial stains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source
Strains		
Pseudomonas fluorescens WH6	WT; from *Triticum* roots; Ap^r	Elliott *et al.* (1998)
Pseudomonas fluorescens WH6-3	GAF mutant, *prtR*::miniTn5*gfp*; Ap^r, Tc^r	Armstrong *et al.* (2009)
Pseudomonas fluorescens WH6-17G	WH6 with 138 nt of *prtI* (PFWH6_3686) deleted (lacking nt 4–141) and containing a premature stop codon at position 148; Ap^r	This study
Pseudomonas fluorescens WH6-3T17G	WH6-3 mutant with the same deletion of *prtI* as in WH6-17G; Ap^r, Tc^r	This study
Pseudomonas fluorescens WH6-40XK	WH6 with 1034 nt of *aprX* (PFWH6_2895) replaced with FRT–KanR–FRT (lacking nt 4–1037); Ap^r, Km^r	This study
Escherichia coli DH5 α	F⁻ *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argF–lacZYA*)1169 (φ80*lacZ* ΔM15)	Invitrogen
Erwinia amylovora 153	WT; isolated originally from a fire blight canker on Gala apple in eastern Oregon (Obtained from Dr Joyce Loper, USDA-ARS, Corvallis, OR, USA)	Halgren *et al.* (2011)
Plasmids		
pEX-18Tc	Mob⁺ *sacB* gene replacement vector; Tc^r	Hoang *et al.* (1998)
pRK2013	Mob⁺ RK2 *tra* ColE1 plasmid; Km^r	Figurski & Helinski (1979)
pKD13	oriR6Kγ bla+ FRT–KanR–FRT; Km^r	Datsenko & Wanner (2000)
pBH474	Suc^s derivative of pTH474 with *flp* expressed constitutively; Gm^r	House *et al.* (2004)
pOSUPrtId-1	pEX-18Tc with deletion in *prtI* generated by overlap extension PCR; contains FRT–KanR–FRT; used to delete 138 nt of *prtI* in WH6; Tc^r, Km^r	This study
pOSUPrtId-2	pEX-18Tc with deletion in *prtI* generated by overlap extension PCR; contains FRT–KanR–FRT; used to delete 138 nt of *prtI* in WH6-3; Tc^r, Km^r	This study
pJet1.2/blunt	Cloning vector for PCR products; Ap^r	Fermentas
pJET-KanR-FRT	Cloning vector with FRT–KanR–FRT sequence from pKD13 flanked by *Bam*HI/*Xho*I sites	This study
pOSUAprXd	pEX-18Tc with deletion in *aprX* generated by overlap extension PCR; contains FRT–KanR–FRT; used to replace 1034 nt of *aprX* with FRT–KanR–FRT in WH6; Tc^r, Km^r	This study
pCR4-TOPO	Cloning vector for PCR products; Ap^r, Km^r	Invitrogen
pBBR1MCS-5	Broad-host-range cloning vector; Gm^r	Kovach *et al.* (1995)
pOSU2000	pBBR1MCS-5 containing a 1055 nt PCR product from WH6, encompassing the 3′ 151 nt of *prtI*, all of *prtR* and 92 nt of downstream ORF PFWH6_3688	This study
pOSU1900	pBBR1MCS-5 containing a 941 nt PCR product from WH6, encompassing the 3′ 33 nt of *prtI*, all of *prtR* and 92 nt of downstream ORF PFWH6_3688; Gm^r	This study
pBBR1EVM	Modified pBBR1MCS-5 in which 654 nt were replaced with 549 nt of new sequence resulting in a constitutive expression vector; Gm^r	This study
pEVW3686	pBBR1EVM containing the coding sequence of the *prtI* gene with altered stop codon (TGA to TAA) to eliminate the overlapping *prtR* start codon; flanked by *Xho*I/*Sac*I sites; Gm^r	This study
pEVW2895	pBBR1EVM containing the coding sequence of the *aprX* gene flanked by *Xho*I/*Bam*HI sites; Gm^r	This study

of diluted culture was spotted onto the centre surface of a Petri dish containing 25 ml PMS agar medium (1.5 % agar) amended with 1 % skim-milk (BD Difco). After briefly drying under sterile air, the plates were incubated inverted at 28 °C (unless otherwise indicated) for 48 h. The Petri plates were scanned and the images cropped to show the centre of each plate. The area cleared around each bacterial colony was measured (in cm^2) using Able Image Analyser software (MU Labs). Each bacterial treatment was tested in triplicate.

DNA manipulation. DNA was isolated from bacteria using a ZR Fungal/Bacterial DNA kit (Zymo Research) or an Ultraclean Microbial DNA Isolation kit (MO BIO). Purity and concentration were determined using a NanoDrop ND1000 (Thermo Scientific). PCR was performed using Platinum *Taq* polymerase (Invitrogen)

unless otherwise specified. Products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced with an ABI 3730 capillary DNA sequence system (Applied Biosystems). Restriction enzymes, T4 DNA ligase, Antarctic Phosphatase and a Quick Blunting kit were purchased from NEB. Sequencing was performed by the Center for Genome Research and Biocomputing Core Laboratories (Oregon State University). All primers used in this study were designed using Primer3Plus software (Untergasser *et al.*, 2007) from Sigma-Aldrich or CLC Main Workbench software from CLC bio.

Construction of in-frame deletion mutants. Plasmids used to construct *prtI* and *aprX* deletion mutants in strain WH6 were pEX-18Tc (Hoang *et al.*, 1998), pKD13 (Datsenko & Wanner, 2000), pRK2013 (Figurski & Helinski, 1979) and pBH474 (House *et al.*,

2004). For each deletion, a genomic fragment containing the region to be deleted and ~1 kb of flanking sequence was amplified in multiple reactions using overlap extension PCR with AccuPrime Pfx (Invitrogen) (Fig. S1, available in the online Supplementary Material). Primers used in PCR amplification are listed in Table S1. Addition of *Xho*I and *Bam*HI sites in primers allowed for insertion of a kanamycin resistance (*kanR*) gene flanked by *flp* recombinase recognition target (FRT) sites derived from pKD13. The fragments were cloned into the pEX-18Tc suicide vector using either recombinant and sticky-end PCR (Thomas *et al.*, 2009) for deletions in *prtI* or standard restriction digests for the deletion in *aprX*. The FRT–KanR–FRT cassette with flanking restriction sites was maintained in the pJET1.2/blunt PCR cloning vector (Fermentas) as pJET-KanR-FRT for use in standard restriction digests. Due to an internal *Bam*HI site in *aprX*, the FRT–KanR–FRT fragment was blunted prior to cloning into the *Xho*I site of the pEX-18Tc vector containing the modified *aprX* and flanking region.

Plasmids were mobilized into recipients through triparental mating using *Escherichia coli* HB101/pRK2013 as the mobilizing helper strain. Plasmid pOSUPrtId-1 was transferred into WT WH6 to create WH6-17G and plasmid pOSUPrtId-2 was transferred into WH6-3 to create WH6-3T17G. Plasmid pOSUAprXd was transferred into WH6 to create WH6-40XK. Mutants that had undergone a double recombination event were selected directly on King's B (KB) agar with chloramphenicol (30 µg ml^{-1}) and kanamycin (100 µg ml^{-1}) or on 925 minimal medium agar (Halgren *et al.*, 2011) with kanamycin (50 µg ml^{-1}). Transformants were transferred to plates containing 10 % sucrose to eliminate transformants with non-integrated constructs. For the mutations in *prtI*, eviction of *kanR* was mediated by pBH474, which encodes the FLP site-specific recombinase, and confirmed by replica plating on agar plates with and without kanamycin (House *et al.*, 2004). In the *aprX* mutant strain WH6-40XK, 1033 nt of *aprX* are replaced by the FRT–KanR–FRT cassette. Mutants were confirmed by colony PCR and sequenced.

Genetic complementation. Complementation of the *prtR* mutation in WH6-3 was attempted with a construct designed to contain sufficient sequence upstream of *prtR* to include any potential native promoter. The *prtR* gene and the associated upstream sequence were amplified from WH6 genomic DNA using primers listed in Table S1 and subcloned into plasmid pJET1.2/blunt (Fermentas) for maintenance. Subsequently, the insert was digested with *Xho*I and *Xba*I, and the resulting fragment was cloned into pBBR1MCS-5 (Kovach *et al.*, 1995) in the opposite orientation to the *lacZ* promoter. The resulting construct, pOSU2000, contained *prtR* and 147 nt of upstream flanking sequence (representing 151 nt of *prtI*) (Fig. 1). This construct was sequenced and introduced into WH6-3 by electroporation (Choi *et al.*, 2006). Transformants expressing resistance to gentamicin from pOSU2000 were selected and tested for GAF activity. A second construct, pOSU1900, containing only 33 nt of the sequence upstream of *prtR*, was made the same way as pOSU2000, but with PCR amplification using a different forward primer (Table S1).

For complementation with genes controlled by constitutive promoters, the vector pBBR1MCS-5 was modified and somewhat simplified, as illustrated and described in Fig. S2, resulting in the vector pBBR1EVM. The ORFs of the *prtI* and *aprX* genes were amplified from WH6 genomic DNA using Phusion High-Fidelity DNA Polymerase (NEB). The PCR products were cloned separately into the *Xho*I/*Sac*I sites (*prtI*) or *Xho*I/*Bam*HI sites (*aprX*) of pBBR1EVM in the same orientation as the *lacZ* promoter. The resulting plasmids, pEVW3686 (*prtI*) and pEVW2895 (*aprX*), were sequenced and mobilized into the appropriate mutant strain of WH6 via triparental mating as above. Plasmid pEVW3686 was transferred to WH6-3T17G and plasmid pEVW2895 was transferred to WH6-40XK.

Transformants expressing resistance to gentamicin from the plasmid and ampicillin from WH6 were tested for GAF activity.

Reverse transcription quantitative real-time PCR (RT-qPCR). RNA for RT-qPCR analyses was extracted from WT WH6, the mutant strain WH6-3, the double mutant WH6-3T17G and the complemented double mutant WH6-3T17G/pEVW3686. Bacteria were cultured in 60 ml PMS medium in Wheaton bottles and 3 ml aliquots of mid-exponential-phase cultures were added directly to 6 ml RNA Protect Bacterial Reagent (Qiagen). Total RNA was extracted using an RNeasy Mini kit (Qiagen) and contaminating genomic DNA removed by Turbo DNase treatment (Ambion). The cDNA was synthesized using a SuperScript III First-Strand Synthesis System (Invitrogen) following the standard protocol with random hexamer primers.

The expression of two putative GAF biosynthetic genes, PFWH6_5256 (putative aminotransferase) and PFWH6_5257 (putative formyltransferase), was examined by RT-qPCR in each of the mutant strains and compared with WT WH6. Plate design, SYBR Green reaction mixtures, cycling conditions, primer design, optimization and validation were performed as described previously (Halgren *et al.*, 2013). Data were normalized to the previously validated reference genes *tufB* and *rpsL* (Halgren *et al.*, 2013). Post-run data analyses were performed with REST2009 (Qiagen) (Pfaffl *et al.*, 2002). Using REST2009, the C_q values of the genes were converted to relative quantities and normalized using the mean of the two reference genes (Halgren *et al.*, 2013).

Transcriptional analysis of *prtI* and *prtR*. *P. fluorescens* WH6 was grown to mid-exponential phase in PMS medium, total RNA was extracted and cDNA synthesized as described above for qRT-PCR. To demonstrate that the mutated *prtI* transcript from the deletion mutant was shortened compared with the transcript in WT WH6, PCR primers promoter-upper-F and Right-201 were used to span the region of deletion mutagenesis. The presence of a single transcript containing *prtI* and *prtR* was determined by amplifying WH6 cDNA using Phusion Polymerase and OneTaq (both NEB) with PCR primers prtI-RT-F and prtR-RT-R. All RT-PCR products were sequenced for confirmation.

To determine the transcriptional start sites for all genes of interest, rapid amplification of cDNA ends (RACE) was carried out with a GeneRacer kit (Invitrogen). GeneRacer RNA Oligo was ligated to non-dephosphorylated mRNA 5′ ends and the WH6 mRNA was reverse transcribed according to the manufacturer's instructions with random hexamers. Reverse primers (Table S1) were designed to anneal at two separate sites within each of the two genes examined. PCR was carried out using the GeneRacer 5′ primer and individual reverse primers, with thermocycling tailored to primer annealing temperatures and run using a Touchdown PCR protocol and a DNA Engine Thermal Cycler (Bio-Rad). RACE cDNA products were cloned into the pCR4-TOPO cloning vector for sequencing and maintenance. Cloned RACE products were sequenced twice in both directions, for confirmation of an exact transcriptional start site.

RESULTS

Structure of *prtIR* in *P. fluorescens* WH6 and WH6-3

The *prtIR* genes in *P. fluorescens* WH6 correspond to ORF PFWH6_3686 (*prtI*) and ORF PFWH6_3687 (*prtR*) (Kimbrel *et al.*, 2010). As shown in Fig. 1, the two genes share a 4 nt overlap that includes the stop codon of *prtI* and the start codon of *prtR*. The configuration of the overlap
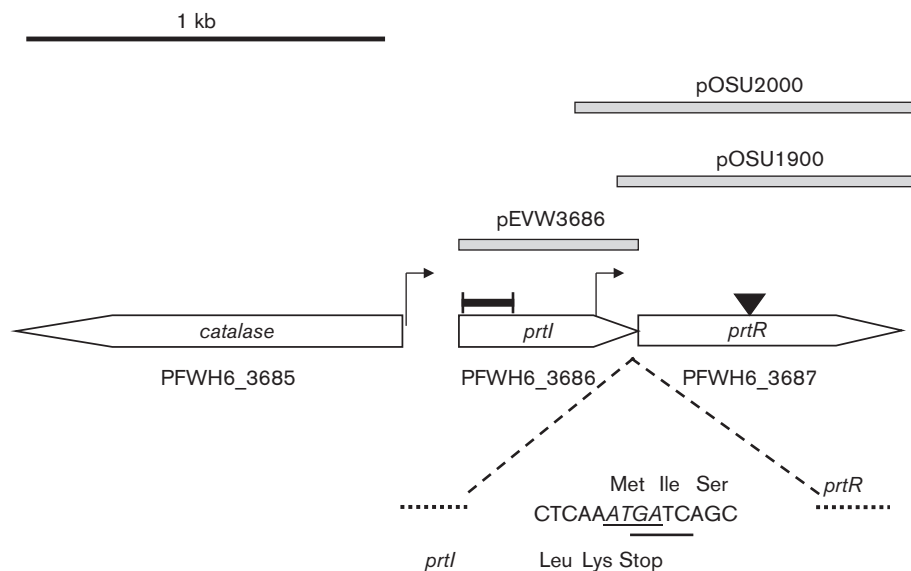
Fig. 1. Structure of the WH6 *prtI–prtR* region. The site of Tn*5* insertion in the chromosome of the WH6-3 mutant is shown by an inverted triangle. The site of the deletion in *prtI* WH6-17G and WH6-3T17G is shown by a black bar over the gene diagram. The direction of transcription is indicated by ORF arrows. Cloned regions for complement construction, putative gene products (based on sequence homology) and ORF numbers are indicated. Arrows above ORFs represent transcription start sites determined through 5′-RACE experiments (163 nt upstream of *prtI* and 108 nt upstream of *prtR*).

results in the two genes being in different reading frames. A similar arrangement occurs in *P. fluorescens* LS107d2 (Burger *et al.*, 2000), in which this locus was identified originally, as well as in other bacterial strains containing homologues of *prtIR* (van den Broek, 2005; data not shown). As indicated in Fig. 1, the mutant WH6-3, which had lost the ability to produce GAF, had a Tn*5* insertion in *prtR* at nt 417, within codon Asp139 (Halgren *et al.*, 2013).

Biological activity of WH6-3 culture filtrates

The loss of GAF production in WH6-3 as a result of Tn*5* insertion in *prtR* has been repeatedly confirmed in our laboratories (Kimbrel *et al.*, 2010; Halgren *et al.*, 2013). However, Burger *et al.* (2000) observed that the loss of protease activity associated with mutation of *prtR* in *P. fluorescens* LS107d2 was temperature dependent. Protease production was restored when the temperature at which LS107d2 was cultured was reduced from 29 to 23 °C. Our cultures were routinely grown at 28 °C. Therefore, it was of interest to determine whether *prtR* regulation of GAF production might also be temperature sensitive. At a reduced culture temperature of 20 °C, WH6-3 continued to grow vigorously, but the ninhydrin-reactive band characteristic of GAF was not present when extracts of the culture filtrates were analysed by TLC, indicating that a reduction in growth temperature did not restore GAF production (Fig. S3). Moreover, GAF activity was not detected in these culture filtrates when they were tested in the *E. amylovora* or *Poa* bioassay (Table S2).

Restoration of GAF activity in WH6-3 by genetic complementation of *prtR*::Tn*5*

To confirm that insertion of Tn*5* into the *prtR* gene was alone responsible for the loss of GAF activity in WH6-3, complementation of this mutation was attempted with a construct, pOSU2000, which contained *prtR* and 151 nt of *prtI* (which might be expected to contain any native promoter of *prtR*). GAF activity was restored completely in culture filtrate from the WH6-3/pOSU2000 transformant, as evidenced by the return of both antimicrobial activity against *E. amylovora* and germination-arrest activity in the *Poa* bioassay (Table 2), as well as the reappearance of the ninhydrin-reactive TLC band corresponding to GAF (Fig. 2).

The ability to complement the *prtR*::Tn*5* mutation with pOSU2000 supported the presence of a *prtR* promoter located within 151 nt of the 3′ end of *prtI*. To eliminate the possibility that transcription of *prtR* in pOSU2000 was occurring fortuitously from other promoters within the plasmid, WH6-3 was transformed with a second construct, pOSU1900, which contained *prtR* with only 33 nt of upstream sequence. This construct contained a putative ribosome-binding site, but would be unlikely to contain any native promoter sequence. As pOSU1900 failed to complement the *prtR* mutation (data not shown), it was unlikely that pOSU2000 complemented the *prtR*::Tn*5* mutation as an artefact of the transcription of *prtR* from a promoter elsewhere in the plasmid.

Table 2. GAF activity in culture filtrates from *P. fluorescens* WH6 mutants and complements

Culture filtrates from 7-day cultures were tested for GAF activity in the *Poa* and *E. amylovora* bioassays as described in Methods.

Bacterial strain	GAF activity in the *Poa* bioassay (germination score) at different culture filtrate concentrations*					Anti-*Erwinia* activity: area of zone of inhibition (cm²)†
	0	0.03×	0.1×	0.3×	1.0×	
WH6 (WT)	4.0±0.00	2.2±0.05	1.3±0.10	1.0±0.00	1.0±0.00	17.5±0.15
WH6-3 (*prtR*::Tn*5* mutant)	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	3.5±0.20	0.0±0.00
WH6-3/pOSU2000 (*prtR* complement of *prtR* mutant WH6-3)	4.0±0.00	1.8±0.04	1.0±0.00	1.0±0.00	1.0±0.00	20.5±0.47
WH6-17G (partial deletion mutant of *prtI*)	4.0±0.00	2.2±0.08	1.1±0.06	1.0±0.00	1.0±0.00	14.3±0.27
WH6-3T17G (double mutant of *prtIR*)	4.0±0.00	2.1±0.02	1.6±0.03	1.0±0.00	1.0±0.00	18.4±0.24
WH6-3T17G/pEVW3686 (constitutive *prtI* complement of double mutant of *prtIR*)	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	0.0±0.00

*The scoring system for the *Poa* bioassay has been described in detail by Banowetz *et al.* (2008). A score of 4 indicates germination arrest immediately after emergence of the radicle and coleoptile; a score of 1 indicates normal seedling development; a score of 1 indicates germination arrest. In the *Poa* assay, nine seeds were evaluated for each strain tested. Results are expressed as mean values ± SEM.

†For evaluation of anti-*Erwinia* activity, three replicate plates were measured for each strain tested. Results are expressed as mean values ± SEM.

GAF phenotype of a *prtI* deletion mutant

The role of *prtI* in the regulation of GAF production in *P. fluorescens* WH6 was examined by construction of an in-frame, deletion mutation in *prtI*. This mutation, designated Δ*prtI*-138, lacked nt 4–141 (corresponding to a putative DNA-binding domain; Paget & Helmann, 2003) and was constructed to contain a premature stop codon at position 148. It was predicted to produce a polypeptide of 34 aa, of which 31 were derived from *Xho*I, *Bam*HI and the scar from the FRT sites. These changes were expected to result in a loss of function of *prtI* without affecting any putative promoter region for *prtR*. The presence of a shortened transcript reflecting this alteration in the *prtI* gene was confirmed by RT-PCR in the mutant strain (Fig. S4). The level of GAF activity present in culture filtrate from this mutant WH6 strain, designated WH6-17G, was indistinguishable from that of WT WH6, as judged by either antimicrobial activity against *E. amylovora* or by the *Poa* bioassay (Table 2). Moreover, when extracts of the mutant culture filtrate were analysed by TLC, the characteristic ninhydrin-reactive GAF band was still evident on the chromatograms (Fig. 2).

GAF phenotypes of a *prtIR* double mutant and its *prtI* complement

The continued production of GAF by the WH6 mutant containing a deletion mutation in *prtI* was surprising. Given that this gene codes for a putative ECF sigma factor, we had expected that mutation of *prtI* would eliminate GAF production. To further test for a possible role of *prtI* in the regulation of GAF production, a double mutant with an identical Δ*prtI*-138 deletion mutation of *prtI* was created in the WH6-3 mutant that carried the Tn*5* insertion in *prtR*. Culture filtrates of this double mutant, designated WH6-3T17G, exhibited GAF activity equivalent to that of WT WH6 in both the *E. amylovora* and *Poa* bioassays (Table 2), and the ninhydrin-reactive GAF band was once again present when extracts of the culture filtrate were analysed by TLC (Fig. 2). Thus, mutation of *prtI* overcame the effects of the *prtR* mutation and fully restored GAF production. From this result, PrtI appeared to exert a negative regulatory effect on GAF production in the absence of PrtR.

Transformation of the double mutant WH6-3T17G with *prtI* was attempted to confirm that PrtI acted to negatively regulate GAF production in the absence of a functional PrtR. If this hypothesis were correct, expression of *prtI* in the double mutant was expected to suppress GAF production, mimicking the phenotype of WH6-3. For this purpose, *prtI* was placed under control of a constitutive promoter in the construct pEVW3686. Transformation of WH6-3T17G with this plasmid resulted in complete suppression of GAF production as the culture filtrate lacked activity in the *E. amylovora* and *Poa* bioassays (Table 2), and no GAF band was present in TLC chromatograms of extracts of the culture filtrate (Fig. 2). Thus, a negative
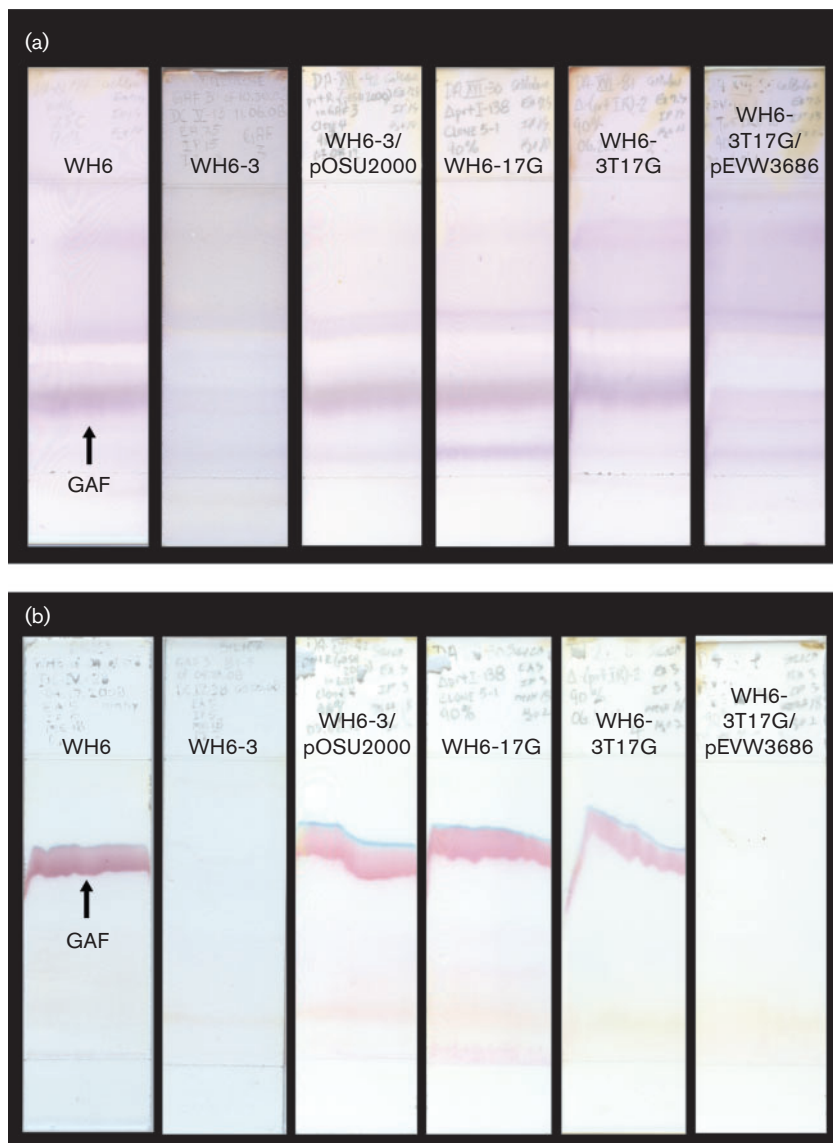
Fig. 2. TLC analyses of culture filtrates from *P. fluorescens* WH6 and mutant bacterial strains and complements. Culture filtrates from the indicated bacterial strains were taken to dryness *in vacuo.* As described in Methods, the recovered solids were extracted with 90 % ethanol and the ethanol extracts were fractionated by TLC. Strains were WH6 (WT WH6), WH6-3 (*prtR*::Tn*5*), WH6-3/pOSU2000 (*prtR*::Tn*5* complemented with *prtR* under control of a native promoter), WH6-17G (partial deletion in *prtI*), WH6-3T17G (double mutant of *prtIR*) and WH6-3T17G/pEVW3686 (double mutant of *prtIR* complemented with *prtI* under control of a constitutive promoter). (a) Ninhydrin-stained cellulose TLC plates. (b) Ninhydrin-stained silica TLC plates.

regulatory role for *prtI* in the control of GAF production was confirmed.

Regulation of two putative GAF biosynthetic genes by *prtIR*

We have shown previously that Tn*5* insertion in *prtR*, in addition to eliminating GAF production in WH6, also sharply reduced transcription of two genes that code for enzymes that appeared to be involved in the GAF biosynthetic pathway (Halgren *et al.*, 2013). The two presumptive biosynthetic genes, PFWH6_5256 and PFWH6_5257, encoded putative aminotransferase and formyltransferase enzymes, respectively, and likely contributed to the formation of the formylamino group of the GAF molecule (McPhail *et al.*, 2010). In the present study, qRT-PCR analyses were performed to examine the effects of *prtI* mutation on the

transcription of these two genes. As shown in Fig. 3, although the *prtR* mutation in WH6-3 almost completely eliminated the transcription of both the PFWH6_5256 and PFWH6_5257 genes, mutation of *prtI* in WH6-17G did not affect their transcription. Transcription was near to WT levels in the WH6-3T17G double mutant, but was again suppressed when this genotype was complemented with *prtI* under the control of a constitutive promoter. These results are qualitatively consistent with the proposed negative regulatory role of PrtI in the control of GAF biosynthesis.

Control of the *aprX* protease by *prtIR* in WH6

P. fluorescens WH6 contains a protease gene (*aprX*) homologous to that shown previously to be under the control of *prtIR* in *P. fluorescens* LS107d2 (Burger *et al.*, 2000) and *P. entomophila* (Liehl *et al.*, 2006). In the latter
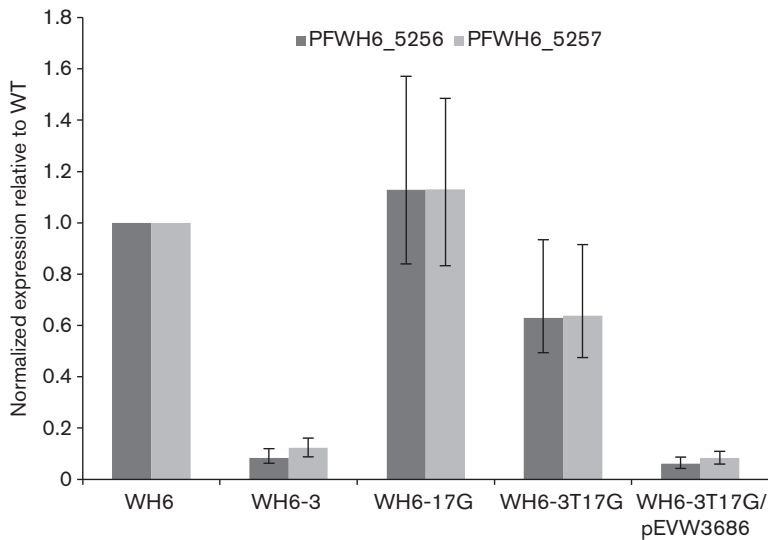
Fig. 3. Effects of Tn5 and deletion mutagenesis on the transcription of the putative aminotransferase and formyltransferase genes (PFWH6_5256 and PFWH6_5257) predicted to be involved in the synthesis of GAF. Transcription levels, as evaluated by qRT-PCR, were compared with those observed in WT *P. fluorescens* WH6. The mutant strains evaluated were WH6 (WT WH6), WH6-3 (*prtR*::Tn5), WH6-17G (partial deletion in *prtI*), WH6-3T17G (double mutant of *prtIR*) and WH6-3T17G/pEVW3686 (double mutant of *prtIR* complemented with *prtI*). Bars, SEM. Expression levels of PFWH6_5256 and PFWH6_5257 were significantly different from WT in WH6-3 and WH6-3T17G/pEVW3686 with $P<0.05$.

strains, the enzymes encoded by their *aprX* loci were active in digesting skim-milk. Although protease activity could not be detected in WH6 suspension cultures grown in PMS medium, it was present in culture filtrates from WH6 grown in PMS medium supplemented with skim-milk (data not shown) and was evident as zones of clearing around WH6 colonies spotted on agar plates containing skim-milk (Fig. 4). The WH6 *aprX* gene was mutated by replacing nt 4–1037 of *aprX* with a FRT–KanR–FRT cassette, resulting in strain WH6-40XK. Mutation of *aprX* in this strain resulted in loss of the skim-milk-clearing activity of WH6 and this activity could be restored by complementation with the *aprX* gene under the control of a constitutive promoter in plasmid pEVW2895 (Fig. 4). The effects of the various *prtIR* mutations and their complements on skim-milk clearing are also shown in Fig. 4. The skim-milk-clearing activity of WH6 was lost in the WH6-3 mutant (containing a Tn5 insertion in *prtR*).

Lowering the temperature from 28 to 20 °C did not restore protease activity in this mutant (data not shown). Mutation of *prtI* alone (mutant strain WH6-17G) had no detectable effect on skim-milk-clearing ability. Mutation of *prtI* in WH6-3, however, restored skim-milk-clearing activity in the resulting double mutant (WH6-3T17G). Thus, *prtIR* regulation of skim-milk-clearing activity followed a pattern identical to that observed with respect to GAF production.

Transcriptional organization of *prtIR* in WH6

The ability of pOSU2000 to complement WH6-3 suggested that a native promoter for *prtR* was present within the 151 nt of the *prtI* coding sequence included in the construct. To further test this possibility, the transcriptional organization of *prtIR* was examined using 5′-RACE experiments. RACE reactions carried out from two
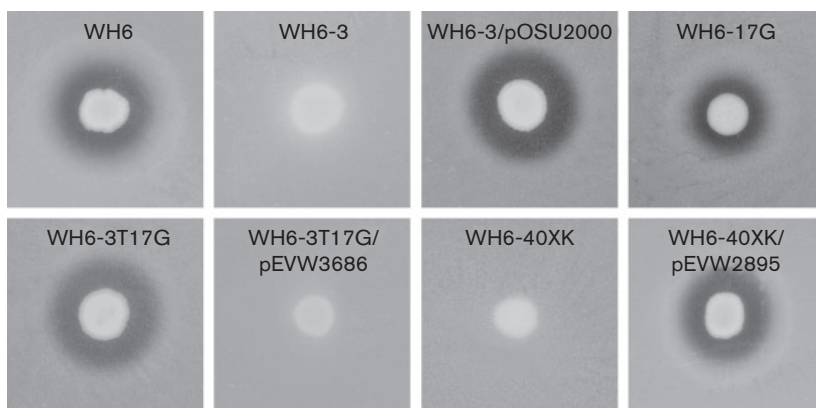

Fig. 4. Protease activity of *P. fluorescens* WT, mutant and complemented strains. A skim-milk agar assay was used to characterize the protease activity of various strains. Cleared zones around bacterial colonies on the centre of an agar plate indicated the presence of protease activity. Strains were WH6 (WT WH6), WH6-3 (*prtR*::Tn5), WH6-3/pOSU2000 (*prtR*::Tn5 complemented with *prtR*), WH6-17G (partial deletion in *prtI*), WH6-3T17G (double mutant of *prtIR*), WH6-3T17G/pEVW3686 (double mutant of *prtIR* complemented with *prtI*), WH6-40XK (Δ*aprX*::KanR) and WH6-40XK/pEVW2895 (Δ*aprX*::KanR complemented with *aprX*).

separate primer-binding positions within each of the two genes confirmed that *prtI* and *prtR* could be transcribed independently. Transcription of *prtI* was initiated at an intergenic adenine, located 163 nt upstream of its putative ATG translational start codon. The initiation of *prtR* transcription was mapped to an adenine 108 nt upstream of its putative ATG translational start codon and within the coding sequence of the *prtI* gene. The positions of these transcriptional start sites are indicated in Fig. 1. As expected, the transcriptional start site of *prtR* lay well within the 151 nt of *prtI* included in plasmid pOSU2000. Thus, the 5′-RACE results were consistent with the *prtR* complementation results. In addition, a potential promoter with the conserved GAA motif of some ECF sigma −35 recognition sites (Staroń *et al.*, 2009) lies immediately upstream of the predicted transcriptional start site of *prtR*.

The observation that *prtI* and *prtR* overlap suggested that the two genes could also be co-regulated by the same promoter and transcribed as a dicistronic message. RT-PCR experiments were performed using primers that annealed to the 5′ end of *prtI* and near the 3′ end of *prtR* (Table S1). Indeed, we successfully amplified a transcript with a size that was consistent with a dicistronic message carrying *prtI* and *prtR* (Fig. S5). Based on the combined results from RT-PCR, complementation of WH6-3 with pOSU2000 and the 5′-RACE experiments, the transcription of *prtIR* may take place by more than one route and be more complex than would be expected from a simple dicistronic operon.

DISCUSSION

The *prtIR* locus in *P. fluorescens* WH6 is necessary for regulating GAF production. As reported previously (Kimbrel *et al.*, 2010), mutation of *prtR* by Tn*5* insertion leads to a loss of GAF production in the resulting mutant strain WH6-3. In the present study, the role of *prtR* in the regulation of GAF production was confirmed by demonstrating that complementation of the Tn*5* mutation with the *prtR* gene resulted in restoration of GAF production in WH6-3. Thus, PrtR appears to act to promote GAF production. In contrast to these results, a deletion of *prtI* in WT WH6 had no apparent effect on GAF production. However, similar mutagenesis of *prtI* in WH6-3 restored GAF production that had been lost as a result of the mutation of *prtR*. Moreover, transformation of this double *prtIR* mutant with a plasmid bearing the *prtI* gene under the control of a constitutive promoter resulted in the suppression of GAF production. Based on these results, we conclude that PrtI acts to negatively regulate GAF production in WH6, although the mechanism of this regulation remains to be determined.

The *prtIR* genes were identified originally in *P. fluorescens* strains LS107d2 and B52 (Burger *et al.*, 2000). In LS107d2, *prtIR* was shown by Burger *et al.* (2000) to regulate the temperature-sensitive production of an extracellular protease encoded by *aprX*. Mutation of either *prtR* or *prtI* in LS107d2 was reported to result in the loss of protease production at 29 °C and this loss of function was restored by complementation with the respective gene. Protease production at 23 °C was unaffected by either mutation. Based on these results and the respective sequence homologies of *prtI* and *prtR*, Burger *et al.* (2000) proposed that *prtI* codes for a σ^{70}-like ECF sigma factor, and *prtR* codes for a novel member of a group of anti-sigma factors and transmembrane sensors and activators that interact with ECF sigma factors (Hughes & Mathee, 1998; Brooks & Buchanan, 2008; Staroń *et al.*, 2009). Our WH6 strain possesses a homologous protease gene, *aprX*. As with the LS107d2 protease, the WH6 enzyme can be detected by its ability to clear skim-milk and is regulated by *prtIR*. However, the protease phenotypes of *prtI* and *prtR* mutants in WH6 followed the pattern observed for GAF production rather than that reported for the protease observed by Burger *et al.* (2000). Thus, PrtI in WH6 appears to exert a negative regulatory effect on both GAF production and expression of the *aprX* protease.

Our results also suggest that the transcription of *prtIR* in WH6 may be more complex than represented by the simple dicistronic operon model proposed by Burger *et al.* (2000) in which *prtI* and *prtR* are transcribed together as a single transcript. Their proposal appears to have been based largely on functional analogies to sigma and anti-sigma factor pairs in other systems (Hughes & Mathee, 1998; Gruber & Gross, 2003), as well as on the fact that the overlap between the stop codon of *prtI* and the start codon of *prtR* suggested that their respective proteins might be transcriptionally and translationally coupled. Whilst our RT-PCR results indicate that these genes can be transcribed in a single transcript, our complementation studies and 5′-RACE analyses suggest that *prtR* has its own promoter, and is also transcribed independently of *prtI*. It should be noted that there is precedence for an anti-sigma factor gene downstream of a sigma factor to have an additional independent promoter, as in *rseA* from *E. coli* (Missiakas *et al.*, 1997) and *Salmonella enterica* (Homerova *et al.*, 2010). However, further work, beyond the scope of what has been attempted here, will be needed to resolve the transcriptional organization of *prtIR*.

One additional case in which the interactions of *prtI* and *prtR* have been investigated previously is in the control of phase transitions in *Pseudomonas* sp. PCL1171 (van den Broek, 2005). In this case, mutation of *prtR* in WT PCL1171, which showed a dense colony morphology (Phase I), resulted in a transition to a thin, translucent colony morphology (Phase II), as well as changes in extracellular polysaccharide production. Mutation of *prtI* in PCL1171 did not affect phase change or extracellular polysaccharide production. The lack of effect of *prtI* mutation in this system resembles what we observed in WH6. Unfortunately, a double *prtI* and *prtR* mutant was not constructed in that study, so further comparison with our results concerning the regulation of GAF production in WH6 is not possible.

The genetic evidence presented here indicates that the putative sigma factor PrtI exerts a negative regulatory influence on GAF production and AprX protease activity. Although our data do not permit any conclusion concerning the mechanism by which PrtI exerts this influence, models have been proposed that describe negative regulation by an ECF sigma factor in other systems. Negative regulation of flagellar development by ECF sigma factors has been reported in studies of RpoE1 in *Brucella melitensis* (Ferooz *et al.*, 2011) and AlgT in *Pseudomonas aeruginosa* (Garrett *et al.*, 1999; Tart *et al.*, 2006). In the latter case, AlgT regulates transcription of a gene encoding the repressor AmrZ, which controls transcription of *fleQ* – the master switch for flagellar gene regulation. A similar mechanism may be involved in the negative regulatory effects of PrtI on GAF production and protease activity in WH6. Alternatively, the observed repression may be an indirect effect of the manipulation of relative levels of sigma factors. When the relative abundance of one ECF sigma factor is elevated, competition between sigma factors for binding to core RNA polymerase may result in reduced transcription of genes controlled by other ECF sigma factors (Farewell *et al.*, 1998, Österberg *et al.*, 2011). Given that *prtIR* occurs in a number of *P. fluorescens* strains as well as in other pseudomonads, elucidation of the mechanism by which *prtIR* controls the production of GAF and regulates the synthesis of other secondary metabolites will be of considerable interest.

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