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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 Tn5 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 Tn5 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 *prtIR* 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 (Banowitz 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 includes annual bluegrass (ABG, *Poa annua* L.) and ABG seeds have been used to establish a quantitative bioassay for GAF activity (Banowitz 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 (Kimbel 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.
fluorescens WH6 mutants altered in the ability to produce GAF and the ninyhdrin 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 σ70 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 prtR 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 dicstronic 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 Tns 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 7–7 days cultures was centrifuged (3000 g, 15 min) and the supernatant was passed through a bacteriophage 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). 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 7–7 days cultures was centrifuged (3000 g, 15 min) and the supernatant was passed through a bacteriophage filter (Millipore GP Express Steritop, 0.22 μm pore size). The resulting sterile culture filtrate was stored at 4 °C.

Bioassays for GAF activity. Bioassays for GAF activity in bacterial culture filtrates were performed with ABG seeds using the standard Psa 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 Psa 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 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 OD600 1.0 with sterile deionized water. A 5 μl aliquot
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 cm²) 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 manipulation 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 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 CLC bio.

Table 1. Bacterial stains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> WH6</td>
<td>WT; from <em>Triticum</em> roots; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Elliott et al. (1998)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> WH6-3</td>
<td>GAF mutant, <em>prtR</em>: miniTn5gfp; Ap&lt;sup&gt;+&lt;/sup&gt;, Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Armstrong et al. (2009)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> WH6-17G</td>
<td>WH6 with 138 nt of <em>prtI</em> (PFWH6-3686) deleted (lacking nt 4–141) and containing a premature stop codon at position 148; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> WH6-3T17G</td>
<td>WH6-3 mutant with the same deletion of <em>prtI</em> as in WH6-17G; Ap&lt;sup&gt;+&lt;/sup&gt;, Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> WH6-4OXK</td>
<td>WH6 with 1034 nt of <em>aprX</em> (PFWH6_2895) replaced with FRT–KanR–FRT (lacking nt 4–1037); Ap&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(argF–lacZYA)1169 (Δ80lacZ ΔM15)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>Erwinia amylovora</em> 153</td>
<td>WT; isolated originally from a fire blight canker on Gala apple in eastern Oregon (Obtained from Dr Joyce Loper, USDA-ARS, Corvallis, OR, USA)</td>
<td>Halgren et al. (2011)</td>
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<tr>
<th><strong>Plasmids</strong></th>
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<tr>
<td>pEX-18Tc</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; sacB gene replacement vector; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Hoang et al. (1998)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; RK2 tra ColE1 plasmid; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pKD13</td>
<td>oriR6K/ bla + FRT–KanR–FRT; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pBH474</td>
<td>Suc&lt;sup&gt;+&lt;/sup&gt; derivative of pTH474 with <em>flp</em> expressed constitutively; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>House et al. (2004)</td>
</tr>
<tr>
<td>pOSUAprPltId-1</td>
<td>pEX-18Tc with deletion in <em>prtI</em> generated by overlap extension PCR; contains FRT–KanR–FRT; used to delete 138 nt of <em>prtI</em> in WH6; Tc&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pOSUPltId-2</td>
<td>pEX-18Tc with deletion in <em>prtI</em> generated by overlap extension PCR; contains FRT–KanR–FRT; used to delete 138 nt of <em>prtI</em> in WH6-3; Tc&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJET-kanR-FRT</td>
<td>Cloning vector for PCR products; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Fermentas</td>
</tr>
<tr>
<td>pOSUAprxD</td>
<td>pEX-18Tc with deletion in <em>aprX</em> generated by overlap extension PCR; contains FRT–KanR–FRT; used to replace 1034 nt of <em>aprX</em> with FRT–KanR–FRT in WH6; Tc&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCR4-TOPO</td>
<td>Cloning vector for PCR products; Ap&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBBR1MCS-5</td>
<td>Broad-host-range cloning vector; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Kovach et al. (1995)</td>
</tr>
<tr>
<td>pOSU2000</td>
<td>pBBR1MCS-5 containing a 1055 nt PCR product from WH6, encompassing the 3’ 151 nt of <em>prtI</em>, all of <em>prtR</em> and 92 nt of downstream ORF PFWH6-3688</td>
<td>This study</td>
</tr>
<tr>
<td>pOSU1900</td>
<td>pBBR1MCS-5 containing a 941 nt PCR product from WH6, encompassing the 3’ 33 nt of <em>prtI</em>, all of <em>prtR</em> and 92 nt of downstream ORF PFWH6-3688; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1evM</td>
<td>Modified pBBR1MCS-5 in which 654 nt were replaced with 549 nt of new sequence resulting in a constitutive expression vector; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pEVW3686</td>
<td>pBBR1evM containing the coding sequence of the <em>prtI</em> gene with altered stop codon (TGA to TAA) to eliminate the overlapping <em>prtR</em> start codon; flanked by Xhol/Saci sites; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pEVW2895</td>
<td>pBBR1evM containing the coding sequence of the <em>aprX</em> gene flanked by Xhol/BamHI sites; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>
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 Xhol and BanHI 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 vector (Fermentas) as pJET-KanR-FRT for use in standard restriction digests. Due to an internal BanHI site in aprX, the FRT–KanR–FRT fragment was blunted prior to cloning into the Xhol 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 pSUPrldt-1 was transferred into WT WH6 to create WH6-17G and plasmid pSUPrldt-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 vectors, the vector pBBR1MCS-5 was modified and somewhat simplified, for complementation with genes controlled by constitutive promoters, the vector pOSU2000, but with PCR amplification using a different forward primer (Table S1).

**Plasmid construction.** The resulting construct, pOSU2000, contained the prtR gene and the associated upstream sequence as illustrated and described in Fig. 1, 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 in 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 pBBRIEMV. 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 Xhol/SacI sites of pRl1 or Xhol/BanHI sites (aprX) of pBBRIEMV 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/pEWV3686. 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 ProtectBacterial 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 Cq 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 OneTag (both NEB) with PCR primers prtI-RF 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) (Kimbrick 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
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 Tn5 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 Tn5 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*::Tn5**

To confirm that insertion of Tn5 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*::Tn5 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*::Tn5 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.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>GAF activity in the <em>Poa</em> bioassay (germination score) at different culture filtrate concentrations*</th>
<th>Anti-<em>Erwinia</em> activity: area of zone of inhibition (cm²)†</th>
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<tbody>
<tr>
<td>WH6 (WT)</td>
<td>4.0 ± 0.00</td>
<td>17.5 ± 0.15</td>
</tr>
<tr>
<td>WH6-3 (prtR::Tn5 mutant)</td>
<td>4.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>WH6-3/pOSU2000 (prtR complement of <em>prtR</em> mutant WH6-3)</td>
<td>4.0 ± 0.00</td>
<td>20.5 ± 0.47</td>
</tr>
<tr>
<td>WH6-17G (partial deletion mutant of <em>prtI</em>)</td>
<td>4.0 ± 0.00</td>
<td>14.3 ± 0.27</td>
</tr>
<tr>
<td>WH6-3T17G (double mutant of <em>prtR</em>)</td>
<td>4.0 ± 0.00</td>
<td>18.4 ± 0.24</td>
</tr>
<tr>
<td>WH6-3T17G/pEVW3686 (constitutive <em>prtR</em> complement of double mutant of <em>prtR</em>)</td>
<td>4.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
</tbody>
</table>

*The scoring system for the *Poa* bioassay has been described in detail by Banowetz *et al.* (2008). A score of 4 indicates normal seedling development; a score of 1 indicates germination arrest immediately after emergence of the radicle and coleoptile. For each filtrate concentration tested 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.

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

The continued production of GAF by the WH6 mutant containing a deletion mutation in *prtI* was surprising. The presence of a fragment 148 bp in size, which was predicted to contain a premature stop codon at position 432, was confirmed by RT-PCR in the mutant strain (Fig. S4). The fragment was derived from the FRT sites. These changes were expected to result in a loss of function of *prtI* without affecting any putative promoter region for *prtI*. The presence of a shortened promoter reflecting this alteration in the *prtI* gene was confirmed by RT-PCR in the *prtI* mutant and *P. fluorescens* WH6 (Fig. S4). It was predicted to contain a premature stop codon at position 432, which would eliminate DNA-binding domain (Paget & Helmann, 2003) and was constructed by replacing the *prtI* gene with a tet-trap cassette (Fig. S4). The transcript reflecting this alteration in the *prtI* gene was confirmed by RT-PCR in the *prtI* mutant and *P. fluorescens* WH6 (Fig. S4). The presence of a shortened promoter reflecting this alteration in the *prtI* gene was confirmed by RT-PCR in the *prtI* mutant and *P. fluorescens* WH6 (Fig. S4).
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 Tn5 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
strains, the enzymes encoded by their \( \text{aprX} \) 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 \( \text{aprX} \) gene was mutated by replacing nt 4–1037 of \( \text{aprX} \) with a FRT–KanR–FRT cassette, resulting in strain WH6-40XK. Mutation of \( \text{aprX} \) in this strain resulted in loss of the skim-milk-clearing activity of WH6 and this activity could be restored by complementation with the \( \text{aprX} \) gene under the control of a constitutive promoter in plasmid pEVW2895 (Fig. 4). Lowering the temperature from 28 to 20 °C did not restore protease activity in this mutant (data not shown). Mutation of \( \text{prtI} \) alone (mutant strain WH6-17G) had no detectable effect on skim-milk-clearing ability. Mutation of \( \text{prtI} \) in WH6-3, however, restored skim-milk-clearing activity in the resulting double mutant (WH6-3T17G). Thus, \( \text{prtIR} \) regulation of skim-milk-clearing activity followed a pattern identical to that observed with respect to GAF production.

### Transcriptional organization of \( \text{prtIR} \) in WH6

The ability of pOSU2000 to complement WH6-3 suggested that a native promoter for \( \text{prtR} \) was present within the 151 nt of the \( \text{prtI} \) coding sequence included in the construct. To further test this possibility, the transcriptional organization of \( \text{prtIR} \) was examined using 5′-RACE experiments. RACE reactions carried out from two...
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 factors was shown by Burger et al. (2000) proposed that prtI codes for a σ70-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 transcribed 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 rsaA 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 AmiZ, 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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