Analysis of the small RNA P16/RgsA in the plant pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000

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Bacteria contain small non-coding RNAs (ncRNAs) that are responsible for altering transcription, translation or mRNA stability. ncRNAs are important because they regulate virulence factors and susceptibility to various stresses. Here, the regulation of a recently described ncRNA of *Pseudomonas syringae* pv. *tomato* DC3000, P16, was investigated. We determined that RpoS regulates the expression of P16. We found that deletion of P16 results in increased sensitivity to hydrogen peroxide compared to the wild-type strain, suggesting that P16 plays a role in the bacteria’s susceptibility to oxidative stress. Additionally the P16 mutant displayed enhanced resistance to heat stress. Our findings provide new information on the regulation and role of this ncRNA in *P. syringae*.

INTRODUCTION

Small non-coding RNAs (ncRNAs) are transcripts that function in a bacterial cell structurally as RNA molecules rather than as templates for translation into polypeptides (Majdalani et al., 2005). ncRNAs play important regulatory roles in bacterial stress responses to diverse environmental signals, such as changes in temperature, osmolarity, iron and oxidative stress (Gottesman, 2005; Gottesman et al., 2006; Massé et al., 2007; Romby et al., 2006; Silvaggi et al., 2006), and have key roles in the regulation of virulence factors in a variety of pathogens (Altier et al., 2000; Caswell et al., 2012; De Lay & Gottesman, 2012; Hebrard et al., 2012; Kreikemeyer et al., 2001; Le Rhun & Charpentier, 2012; Lenz et al., 2005, 2004; Mangold et al., 2004; Sonnleitner et al., 2012), including *Pseudomonas aeruginosa* (Burrowes et al., 2005; Heurlier et al., 2004). A single ncRNA species can directly regulate multiple genes, leading to pleiotropic effects (Gottesman et al., 2006).

Many ncRNAs require the bacterial chaperone Hfq to perform their regulatory functions. Hfq facilitates the interaction of ncRNAs with mRNA targets (Majdalani et al., 2005; Vogel & Luisi, 2011). Binding of the ncRNA to the mRNA results in an increase or decrease in the stability and/or translation of the mRNA (Vogel & Luisi, 2011). Several genome-wide approaches, including RNomeics and deep sequencing, have discovered many of the ncRNA and mRNA targets of Hfq and have shown that this chaperone might affect the expression of up to 20% of all genes in some bacteria (Chao & Vogel, 2010). Thus it is not surprising that Hfq mutants often display defects in a wide range of cellular processes, including quorum sensing, biofilm formation, stress tolerance and virulence (Chao & Vogel, 2010).

Several ncRNAs were identified using sRNAPredict2 and found to be conserved in the pseudomonads (Livny et al., 2006). One, termed P16 or RgsA, has been further studied in *Pseudomonas aeruginosa* and expression was found to be dependent upon RpoS, the sigma factor primarily responsible for the regulation of genes during stationary phase (González et al., 2008), and indirectly influenced by the global response regulator GacA. In the wild-type strains of *Pseudomonas fluorescens* and *P. aeruginosa*, expression of this ncRNA is almost absent during mid-exponential growth but abundant during the stationary phase. Also, expression was reduced twofold in a GacA mutant compared to the wild-type strain. However, no GacA binding site was identified, so the observed effect was reported to be most likely indirect.

The function of P16/RgsA is not known; however, it was reported that deletion of this ncRNA resulted in enhanced

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Abbreviations: ChiP, chromatin immunoprecipitation; ncRNA, non-coding RNA.

A supplementary table of primers and two supplementary figures are available with the online version of this paper.
sensitivity to hydrogen peroxide in *P. fluorescens* CHA0 (González et al., 2008), suggesting that this ncRNA targets genes involved in resistance to oxidative stress. In this study we investigated the regulation and function of P16/RgsA in the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000.

**METHODS**

**Bacterial strains/growth conditions.** *Pseudomonas syringae* pv. *tomato* DC3000 (hereafter referred to as DC3000) was routinely cultured on King’s B (KB) agar (King et al., 1954) or on modified Luria medium (LM) (10 g Bacto tryptone, 6 g yeast extract, 0.6 g NaCl, 0.4 g MgSO$_4$$\cdot$7H$_2$O, and 1.5 g K$_2$HPO$_4$ per litre) (Hanahan, 1983) at 28 °C or at room temperature.

**Creation of rpoS and P16 mutants in *P. syringae* pv. *tomato* DC3000.** Construction of the DC3000 rpoS mutant was carried out by PCR amplification of an internal 800 bp sequence of the DC3000 genome and cloning into pKnockout-Ω (Windgassen et al., 2000). The resulting plasmid was introduced into DC3000 via electroporation. Since pKnockout cannot replicate in DC3000, single-crossover integrants were selected for resistance to spectinomycin. Orientation of integration was determined by PCR.

A P16 (PSPTO_5560) deletion mutant (∆P16) was created using a pK18mobsacB plasmid (Schäfer et al., 1994). pK18mobsacB/∆P16 was created by PCR amplification of DNA fragments of approximately 1.0 kb that flank P16. Gel-purified PCR fragments were joined by a second PCR amplification with primers containing BamHI and HindIII restriction sites. The product was gel purified, digested with BamHI and HindIII, and cloned into pK18mobsacB digested with the same restriction enzymes. The pK18mobsacB deletion construct was confirmed by sequencing (Cornell University Life Sciences Core Laboratories Center) before introduction into DC3000 via electroporation. Integration events were selected on KB medium containing 50 μg kanamycin ml$^{-1}$ and then transferred to 10% sucrose medium to select for crossover events that resulted in the loss of the sacB gene. Sucrose-resistant colonies were screened by PCR and positive clones (those containing the deletion) were confirmed by sequencing.

**Evaluating susceptibility to oxidative stress.** Wild-type (WT) DC3000 and ∆P16 were grown on KB plates for 2 days (King et al., 1954). Overnight cultures were prepared in liquid KB, and incubated at 28 °C with shaking. The next morning, 1 ml of overnight culture was pelleted and resuspended in 3 ml liquid MG (mannitol-glutamate medium; Bronstein et al., 2008). A 30% hydrogen peroxide solution was added to the culture to a final concentration of 30 mM (Péchy-Tarr et al., 2005). No hydrogen peroxide was added to the control cultures. Cultures were incubated at 28 °C with shaking for 30 min then serially diluted. Dilutions were plated on KB plates and incubated at room temperature until colonies were visible and the number of colonies could be enumerated. Three biological replicates were evaluated. The number of colonies for both the control and the experimental tests was averaged for the three biological replicates. The statistical significance was analysed by using a one-tailed t-test for two independent samples with unequal variances.

**Sensitivity to heat stress.** Sensitivity to heat shock was based on the protocol described by Schurr et al. (1995). WT DC3000 and the ∆P16 and ∆rpoS mutants were grown on KB plates for 2 days. Cultures were prepared in liquid KB, and incubated at 28 °C with shaking. The next morning, 1 ml of each overnight culture was centrifuged for 5 min at 15,000 r.p.m., the supernatant was removed, and the cell pellet was resuspended in 3 ml liquid MG. Next, the cultures were serially diluted in fresh liquid MG for the first time point (t=0). Aliquots (100 μl) of the dilutions were spread on KB plates and were incubated until colonies were visible and the number of colonies could be enumerated. The cultures were then incubated at 42 °C. Every 15 min, the cultures were serially diluted in fresh medium and 100 μl aliquots of the dilutions were spread on KB plates. The plates were incubated at room temperature until colonies were visible and the number of colonies could be enumerated.

**NaCl sensitivity assay.** WT DC3000 and the ∆rpoS and ∆P16 mutants were grown overnight in KB medium at 28 °C with aeration. The next day cells were pelleted, and washed and resuspended in MG supplemented with 1.5 M sodium chloride. Resuspended cells were incubated at 28 °C with aeration, aliquots were taken periodically, and serial dilutions of the samples were plated on KB plates to determine the c.f.u.

**Creation of reporter constructs.** The putative promoter region for P16 was amplified via PCR using chromosomal DNA isolated from DC3000. Primers were designed to amplify a region spanning 150 nt upstream of the transcriptional start site and including the first 14 nt of the P16 gene. The sense primer was designed to have a CACC overhang on the 5′ end to ensure directional cloning into pENTR/D-TOPO vector (Invitrogen). The amplicons were separated by agarose gel electrophoresis. The DNA fragment was extracted from the gel using the Qiagen Gel Extraction kit and cloned into the pENTR vector (pENTR Directional TOPO Cloning kit; Invitrogen). To ensure that the putative P16 promoter region was successfully cloned into the pENTR vector, the insert was sequenced (Life Sciences Core Laboratories Center at Cornell University).

The promoter region was moved into destination vectors pBS58 and pBS59 (Markel et al., 2011) using Gateways LR Clonase II Enzyme mix (Invitrogen). The Gateway cassette in these plasmids is located upstream from a promoterless lux gene. The destination vector pBS58 was designed so that the cloned promoter is in the same orientation as the lux gene, while pBS59 was designed as a control with the cloning site reversed so that the promoter is cloned in the opposite orientation to the lux gene. Plasmids were transformed into One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen).

**Promoter fusion assay.** pBS58/P16 promoter and pBS59/P16 promoter plasmids were introduced into WT DC3000 and the ∆rpoS mutant via electroporation. Each strain was grown in KB or LM (Pessler et al., 1988), with appropriate antibiotics for approximately 22 h at 28 °C with shaking. Optical density (OD$_{600}$) of the overnight cultures was measured, and each strain was diluted to an OD$_{600}$ of 0.1 in fresh KB, LM or MG. Aliquots (600 μl) of the culture were dispensed into three 200 μl wells in a 96-well plate for three technical replicates. OD$_{600}$ and relative luminescence were measured immediately for an initial measurement (t=0) with a Tecan GENios microplate reader, using Magellan Data Analysis software. Cultures were shaken at room temperature. Both OD$_{600}$ and relative luminescence were measured at 1 h intervals. Relative luminescence values for each technical replicate were normalized by dividing the raw luminescence value by the OD$_{600}$ (Schagat et al., 2007). Three biological replicates were obtained. Technical replicates were averaged for each biological replicate. Means and standard deviations for each of the biological replicates were calculated. Statistical significance was assessed using a one-way ANOVA test.

**RNA isolation.** Total RNA was prepared with the RNeasy kit (Qiagen) following the manufacturer’s instructions, using the optional on-column DNase I digestion and with the exception that lysozyme was used at a concentration of 5 mg ml$^{-1}$. RNA was treated twice with DNase I (Ambion) to remove residual DNA and then cleaned and concentrated using the MinElute kit (Qiagen).

http://mic.sgmjournals.org
Quantitative real-time PCR (qRT-PCR). Total RNA (100 ng) extracted from DC3000 was reverse transcribed in a thermocycler using the qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer’s instructions. qRT-PCR was performed using iQ SYBR green SuperMix (Bio-Rad) on an iQ5 multicolor real-time detection system (Bio-Rad). The PCR assay was carried out with one cycle at 95 °C for 2.5 min followed by 35 cycles of 95 °C for 15 s and 60 °C for 30 s. The resulting cycle threshold (Ct) values were calculated by the software and analysed using the relative standard curve method (Vencato et al., 2006). Ct values of each gene tested were normalized to the Ct values of the housekeeping gene gap1 (PSPTO_1287) to obtain relative expression data for each gene.

Creation of a strain expressing a FLAG-tagged RpoS. The rpos coding region was amplified with primers oSWC01564 and oSWC01565 (which contains the FLAG sequence followed by a stop codon; see Table S1, available with the online version of this paper, for sequences of all primers) using the Expand High Fidelity PCR System (Roche). The 1.04 kb PCR product was gel purified using the Zymoclean Gel DNA Recovery kit (Zymo Research) and cloned into pENTR/SD/D (Invitrogen) by directional TOPO cloning (Invitrogen) to create pZA01. The pZA01 expression construct (where pZA01.FLAG is expressed under the control of the constitutive nptII promoter) was created by an LR reaction with pBS46 (Swingle et al., 2011) with the following modifications. After seeds were vernalized for 4 days at 4 °C to break the dormancy and then incubated at 26 °C for 12 h light/12 h dark photoperiod and 75 % humidity, they were germinated on half-strength MS medium distilled water containing 0.025 % Silwet L-77 (OSI Specialties) to allow a 12 h light/12 h dark photoperiod and 75 % humidity. Seedlings that were 14 days post-germination were infected with bacteria using a 12 h light/12 h dark photoperiod and 75 % humidity. Seedlings that were 14 days post-germination were used for the virulence assay.

Co-immunoprecipitation of RNAs bound to Hfq. Co-immunoprecipitation of RNAs bound to FLAG-tagged Hfq was performed as described by Berghoff et al. (2011) with the following modifications. WT DC3000 and hfq-FLAG strains were inoculated in KB medium to a starting OD600 of 0.02 and grown with shaking at 28 °C for 24 h (OD600 of approximately 6–7). The cells were harvested by centrifugation at 5000 g for 5 min at 4 °C and washed twice with cold 50 ml Tris-buffered saline (TBS). Pellets were resuspended in 2 ml cold Celllytic B Lysis reagent (Sigma) with 20 μl Longlife Lysozyme (G-Biosciences), 1 mM PMSF and 5 μl RNaseOUT (Invitrogen) added. The cells were lysed by sonication (twice for 15 s at 15 % power using a Fisher Scientific 550 Sonic Dismembrator with a microtip). Insoluble material was removed by centrifugation at 16000 g for 10 min at 4 °C, then 180 μl supernatant was removed for preparation of total RNA (lysat control). The remaining cleared lysate was mixed with 40 μl anti-FLAG M2 Affinity Gel (Sigma), which had been washed twice with TBS as described by the manufacturer, and incubated for 2 h at 4 °C under rotation. The resin was collected by centrifugation at 8000 g for 30 s at 4 °C and the supernatant carefully removed and discarded. The resin was resuspended in 500 μl cold Celllytic B Lysis reagent, transferred to a Spin-X centrifuge tube filter (Sigma), and centrifuged for 30 s at 5000 g. The resin was washed another four times with 500 μl cold Celllytic B Lysis reagent and finally resuspended in 200 μl Celllytic B Lysis reagent and transferred to a new tube for RNA isolation (IP sample). Total RNA was isolated from the lysate control or IP samples using TRI reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA samples were treated twice with DNase I (Ambion) to remove residual DNA. RNA was extracted using acid phenol/chloroform and then precipitated with sodium acetate and ethanol using standard protocols. RNA was stored at −80 °C.

Chromatin immunoprecipitation (ChIP) of RpoS. Strains ZAPS01 and ZAPS03 were grown in LB overnight and ChIP performed as described by Butcher et al. (2011). Enrichment of P16 was determined by qRT-PCR as described above, but using 10 ng purified DNA from the lysate or immunoprecipitated (IP) samples as the template. Enrichment was determined relative to regions within the gap1 gene (primers oSWC00381/oSWC00382).

Construction of a strain expressing FLAG-tagged Hfq. Integration of the FLAG epitope-encoding tag at the 3’ end of hfq was achieved using a pK18mobbsacB (Schäfer et al., 1994) based construct as follows. A region containing hfq and upstream sequence was amplified using oligomers oSWC05086 and oSWC05087. Primer oSWC05087 inserts the FLAG sequence in front of the hfq stop codon. A downstream region was amplified using primers oSWC05088 and oSWC05089. oSWC05089 contains a sequence complementary to oSWC05087. These fragments were amplified using the Expand High Fidelity PCR System (Roche). The PCR products were gel purified using the Zymoclean Gel DNA Recovery kit (Zymo Research) and were joined using the Expand High Fidelity PCR System (Roche) with primers oSWC05086 and oSWC05089 and the up and down fragments as the PCR template. The product (including the hfq gene with a FLAG epitope-encoding sequence at the 3’ end, up and downstream sequences, and flanking XbaI sites) was cloned into pK18mobbsacB. This suicide plasmid was introduced into DC3000 by electroporation and integration events were selected on modified LB with 50 μg kanamycin ml−1. A selected colony was then subjected to counter-selection on 10 % sucrose to select for the loss of the sacB gene. Kanamycin-sensitive colonies were then screened for the presence of the FLAG-tag by PCR with primers oSWC05086 and oSWC02103 (a primer specific to the FLAG sequence) using Premix Taq (Ex Taq version 2.0; Takara) and a correct colony selected for further experiments. The hfq−FLAG and flanking areas in this strain was sequenced to confirm that no other mutations had been introduced in these regions during strain construction. The presence of the FLAG-tagged Hfq was confirmed by Western analysis (data not shown).

Evaluating virulence in Arabidopsis thaliana seedlings. To assess virulence, the Arabidopsis seedling flood-inoculation assay was used (Ishiga et al., 2011) with the following modifications. After seeds were sterilized, they were germinated on half-strength MS medium that was solidified with 0.7 % Phytagel (instead of 0.3 %). The seeds were vernalized for 4 days at 4 °C to break the dormancy and then plated. Plates were incubated at 26 °C with a 12 h light/12 h dark photoperiod and 75 % humidity. Seedlings were 14 days post-germination were used for the virulence assay.

To perform the inoculation, 40 ml bacterial suspension (sterile distilled water containing 0.025 % Silwet L-77 (OSI Specialties)) was dispensed into the Petri dish containing 14-day-old Arabidopsis seedlings, and the plates were incubated for 3 min at room temperature to allow bacteria to adhere. The bacterial culture was poured off and then plates containing inoculated plants were sealed with 3M MicroPore 2.5 cm surgical tape and incubated at 26 °C with a 12 h light/12 h dark photoperiod and 75 % humidity. To determine the initial number of c.f.u., the inoculum was serially diluted and plated on KB containing 50 μg rifampicin ml−1. Rifampicin was used throughout the experiment to ensure that all c.f.u. were DC3000 since the strain is resistant to this antibiotic (Dong et al., 1991). To determine the bacterial growth in Arabidopsis leaves, internal bacterial c.f.u. were determined at 72 h post-inoculation. The
bacterial population inside the plants was evaluated from two independent seedlings grown in a single Petri dish. Inoculated seedlings were collected by cutting the hypocotyls in order to separate the plants above the agar from the roots in the Phytagel plate, and the total weight of inoculated seedlings was determined. Next, seedlings were surface-sterilized with 30% hydrogen peroxide for 5 min. After washing three times with sterile distilled water, each seedling was placed into a single well of a 96-deep-well plate, and the total weight of inoculated seedlings was determined. Next, seedlings were surface-sterilized with 30% hydrogen peroxide for 5 min. After washing three times with sterile distilled water, each seedling was placed into a single well of a 96-deep-well plate, and 200 μl sterile distilled water and two steel beads were added to the well. The plate was covered using two sealing films to prevent the steel beads from breaking through the film during the grinding process. The seedlings were ground using a 5G Mixer (Fluid Management) for 2.5 min. The solution containing the ground seedlings was serially diluted and plated onto KB plates containing 50 μg rifampicin ml⁻¹. C.f.u. were counted after 48 h and normalized to c.f.u. per mg plant material.

**Tomato dip-inoculation.** Tomato cv. Moneymaker was grown in 16 h light/8 h dark cycles at 25 °C in a greenhouse for 4 weeks. Plants were acclimatized on a bench top in open air for 24 h and were then bagged to create a sealed, high-humidity chamber for 24 h prior to dip inoculation. Prior to infection, bacterial cultures were spread onto KB agar plates and incubated at 27 °C for 24 h. The resulting bacterial lawns were then suspended in 10 mM MgCl₂ to an OD₆₀₀ of 0.2 (~10⁷ c.f.u. ml⁻¹). Tomato plants were infected by gentle agitation for 30 s in 10-fold dilutions of the initial bacterial suspensions made in 1 M MgCl₂ + 0.01–0.2% Silwet L-77. Following bacterial inoculation, the tomato plants were allowed to air dry on the bench top for 1 h, then incubated in a growth chamber at 80% relative humidity, 25 °C and 16 h light/8 h dark. Tomato leaf tissue samples were harvested using a 4 mm cork borer. Bacteria were extracted from plant tissue samples by shaking them in 0.2 ml fresh 10 mM MgCl₂ at room temperature with steel beads for 2.5 min. Extracted bacteria were then serially diluted in 96-well plates and spotted on KB containing 50 μg rifampicin ml⁻¹. Spots containing >5 and <30 colonies were used to quantify the number of c.f.u. per g leaf tissue. Plants were observed daily for the development of disease symptoms.

**RESULTS**

The ncRNA P16 is directly regulated by RpoS

In DC3000, an Rfam prediction for P16 (PSPTO_5660) is located between PSPTO_3823 and PSPTO_3824 (Filiatrault et al., 2010) [Rfam: http://rfam.sanger.ac.uk]. Our group reported transcriptional activity for this region when cells were grown in MG medium under low-iron conditions (Filiatrault et al., 2010). However, we have since noticed a discrepancy in length between the P16 family reported in Rfam and the reported P16/RgsA (González et al., 2008) (Fig. 1). The predicted coordinates reported by Rfam appear to contain an additional 87 nt upstream of the transcriptional start site and the sequence contains the reported RpoS promoter region (González et al., 2008). Since González et al. (2008) reported an RpoS binding site located upstream of P16 in DC3000 (Fig. 1) we investigated if RpoS regulates expression of P16 in DC3000. Promoter fusion constructs

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TTGGAAAACTCACCCTGCAAACTCCGGGCAAACGCCTGAAAACCCCTTG
←-----PSPTO_3823-----
GCACACTGACCCCTCGCTGCGCCTAGAGACGCCAAGTGCTTTT

TCGAGGACTCAAGAAGCGCTTTTGATATTCCAAAAACTGTATTTAAAAAC

GACAAAAAGACAAGACGGCGGCCCCCCGCTCTGAGCATTACCTTTAA

+1
GGGACGCGCCTGATATCCAAAAAGACCTCGCAAGCGCGGCGGCGAGGTGCCA

ATGGATACGGCGCACCATTGTTTTAATCAAATGCTCTGAACCACCGGCTT

CACCCCCCAAGATCCGGGGTTTTTTGTGCGATTGAGGCGTCTCT

TTTTACGGGGCAATCGCGCTGAAGGTCGGCAAC

←-----PSPTO_3824-----
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**Fig. 1.** Genomic sequence of P16 and the putative RpoS promoter sequence. The transcriptional start sites (reported by Filiatrault et al., 2011) are highlighted in grey. The putative RpoS promoter sequence predicted by González et al. (2008) is underlined. A predicted terminator sequence is indicated by the solid arrow and the beginning and end of the neighbouring coding sequences are indicated by dashed arrows. The sequence of P16 predicted in Rfam and annotated as PSPTO_5660 extends upstream of the reported P16 transcriptional start site and is shown in italics. Primers used to clone the promoter region are shown in bold.
were introduced into WT DC3000 and the ΔrpoS mutant strain. Fig. 2 shows that there was a significant reduction in the relative luminescence of the lux reporter in the ΔrpoS mutant compared to the WT strain in two media (KB and MG). However, when cells were grown in MG, the expression was not completely abolished and some expression for P16 was detected in the ΔrpoS mutant. The results also indicate that P16 is expressed in both media, with expression increasing throughout growth and highest expression occurring during stationary phase.

The data from the lux reporter assay indicate the activity of the P16 promoter. To determine the abundance of P16 transcript, qRT-PCR was performed between the WT strain and the ΔrpoS mutant. The ΔrpoS mutant showed an eightfold reduction in the level of P16 transcript compared to the WT in KB medium and an 18-fold reduction in the level of P16 transcript compared to the WT in MG medium (Fig. S1). Taken together, these data indicate that RpoS regulates expression of P16. The regulation of P16 by RpoS could be direct or indirect but the presence of a putative RpoS binding site suggests direct regulation. ChIP was used to detect the binding of RpoS to the upstream region of P16. A 1.8–5-fold enrichment of P16 over the gap1 gene was observed (Fig. S2). These results show that RpoS directly regulates the expression of P16.

**P16 influences the response of *P. syringae* DC3000 to diverse environmental conditions**

RpoS has also been shown to play a role in protecting bacterial cells from oxidative stress (González et al., 2008), heat stress (Jørgensen et al., 1999; Suh et al., 1999) and osmotic stress (Kidambi et al., 1995). Therefore, we evaluated the role of P16 in the response of DC3000 to these environmental factors. Susceptibility to oxidative stress was analysed by an assay in which the bacteria were exposed to hydrogen peroxide. Fig. 3(a) shows that the addition of 30 mM hydrogen peroxide to ΔP16 cultures resulted in a significant reduction (>50%) in the number of colonies ($P<0.01$). Based on these data, it is likely that P16 plays a role in protecting the bacteria from oxidative stress.

As shown in Fig. 3(b), the proportion of surviving cells for the ΔP16 mutant remained unchanged after 15 min exposure to heat stress, whereas the WT and ΔrpoS mutant demonstrated a significant reduction in the proportion of surviving cells when exposed to heat shock at any of the time points tested. In addition, the ΔrpoS mutant showed a significant reduction in the proportion of surviving cells at $t=15$ min and $t=30$ min compared to the WT. Therefore, it is likely that P16 participates in the tolerance to heat stress.

No difference in growth in 1.5 M sodium chloride was observed between WT and ΔP16 mutant, while the ΔrpoS mutant displayed a significant reduction in the number of colonies (Fig. 3c). This suggests that it is unlikely P16 plays a role in protecting the bacteria from osmotic stress.

**P16 does not influence virulence**

Since exposure of bacteria to oxidative stress is an important part of the plant defence response, we investigated whether P16 contributes to the growth and virulence of DC3000. Tomato plants were dipped in suspensions of the WT, the ΔP16 mutant and the ΔrpoS mutant. Although the ΔP16 mutant and the ΔrpoS mutant appeared to be slightly less abundant than the WT at 5 days post-inoculation, by day 7 all the strains produced approximately the same number of colonies (Fig. 4a). All of the inoculated plants developed lesions and no differences were observed among the lesions produced by the three strains at day 5 (Fig. 4b). These data suggest that P16 and rpoS may be critical for growth in early stages in infection in tomato plants.

We additionally examined growth and virulence of the WT, the ΔP16 mutant and the ΔrpoS mutant strains in
Arabidopsis seedlings. At 3 days post-inoculation, the ΔP16 mutant and the ΔrpoS mutant were as efficient at growing in planta as the WT strain (Fig. 4c). In addition, Arabidopsis seedlings infected with the WT, the ΔP16 mutant and the ΔrpoS mutant displayed the same necrotic symptoms (Fig. 4d). Based on these data, it is unlikely that P16 plays a role in virulence in DC3000.

P16 interacts with Hfq

Many bacterial ncRNAs use the chaperone Hfq to facilitate the interaction of ncRNAs with target mRNAs (Majdalani et al., 2005; Vogel & Luisi, 2011). Hfq is an RNA-binding protein that controls a number of different cellular processes and is highly conserved in a wide variety of bacteria, including all completely sequenced pseudomonads. Mutations in hfq attenuate virulence in several pathogenic bacteria (Berghoff et al., 2011; Christiansen et al., 2004; Ding et al., 2004; Geng et al., 2009; Liu et al., 2010; Sonnleitner et al., 2003; Torres-Quesada et al., 2010). In DC3000, we are unable to investigate phenotypes associated with the loss of hfq because we are not able to construct an hfq mutant (the loss of hfq in DC3000 is probably lethal). To further characterize P16 we expressed an epitope-tagged version of Hfq and determined if P16 was able to interact with Hfq. We found that P16 is enriched following RNA immunoprecipitation of strains containing a FLAG-tagged Hfq (Fig. 5).

DISCUSSION

The stationary-phase sigma factor RpoS (σ5) has been shown to be important for optimal stress response in Pseudomonas. rpoS mutants of Pseudomonas frequently display reduced survival in stationary phase when exposed to environmental stresses, such as UV radiation, desiccation, heat and osmotic stress. For example, RpoS is important for survival of P. aeruginosa under osmotic shock, heat shock and oxidative stress conditions (Jørgensen et al., 1999; Suh et al., 1999). RpoS has been shown to contribute to tolerance to stresses such as oxidative stress in P. fluorescens (Heeb et al., 2005; Stockwell et al., 2009). Although differences in the response to various stresses and fitness has been observed between various strains (Hagen et al., 2009; Stockwell & Loper, 2005; Stockwell et al., 2009), few studies have evaluated the role of RpoS in the plant pathogen P. syringae. The rpoS gene in P. syringae pv. syringae B728a is important in surviving exposure to the near-UV in sunlight (Miller et al., 2001), but no studies have reported its role in response to other environmental stresses. Here we report that an RpoS mutant of P. syringae DC3000 is sensitive to heat stress, but is not altered in virulence. As noted above, specific

![Fig. 3. P16 is involved in response to various stresses. (a) P16 is involved in the response to hydrogen peroxide. The numbers of c.f.u. were compared between the WT and the ΔP16 mutant without (grey bars) and with (black bars) the addition of hydrogen peroxide. There was a significant reduction in c.f.u. (more than 50%) when hydrogen peroxide was added to the ΔP16 mutant cells (P<0.01, as denoted by **). (b) P16 influences susceptibility to heat shock. The proportion of surviving cells after incubation at 42 °C was compared between WT (black bars), ΔP16 mutant (grey bars) and ΔrpoS mutant (white bars) cells. Significant difference was observed compared to WT (P<0.01, as denoted by *). (c) P16 does not play a role in the response to salt stress. The proportion of surviving cells after addition of 1.5 M NaCl was compared between WT (black bars), ΔP16 mutant (grey bars) and ΔrpoS mutant (white bars) cells. Significant difference was observed compared to WT (P<0.01, as denoted by *).](http://mic.sgmjournals.org/301)
environmental conditions influence the outcomes that have been observed with RpoS mutants. Therefore, it is possible that RpoS could play a significant role in P. syringae when other environmental situations are encountered.

The data presented here show that the transcription of P16/RgsA is regulated by RpoS; this is consistent with reports in other pseudomonads. GacA also controls expression of rpoS in DC3000 (Chatterjee et al., 2003). Therefore it is likely that P16/RgsA in DC3000 is also indirectly controlled by this two-component system.

Oxidative stress plays an important role in the plant environment. Oxidative stress can be caused by a number of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H2O2) and hydroxyl radical. ROS are involved in many biological processes. ROS can be produced as a result of normal aerobic metabolism, but also serve as a mechanism to reduce the viability of invading pathogens (Bolwell, 1999). Generation of ROS occurs as part of the defence mechanism in plants against invading pathogens (Bolwell, 1999). In fact, it is recognized that upon infection of plants with DC3000, there is activation of the plant defence responses and as a result there is increased production of ROS (Alvarez et al., 1998; Levine et al., 1994). We observed that the ΔP16 mutant was more sensitive to hydrogen peroxide than the WT strain. This suggests that P16 may play a role in resistance to plant defence mechanisms during infection. However, deletion of
P16 did not affect the ability of the pathogen to infect *Arabidopsis* or tomato. It is possible that the amount of hydrogen peroxide produced during this particular infection model is not sufficient to detect a difference in viability or that the number of cells used in our experiments is too overwhelming to observe an effect. Interestingly, Miguel et al. (2000) reported that an oxyR mutant in *Erwinia chrysanthemi* is more sensitive to hydrogen peroxide in *vitro*, but this mutant retains full virulence. The data reported by Miguel et al. (2000) suggest that there is no direct antimicrobial effect of hydrogen peroxide in plant defence against *E. chrysanthemi* and raise the question as to whether this phenomenon occurs in other plant pathogens. Our data show that this phenomenon occurs in *P. syringae* DC3000 as well. As noted by Miguel et al. (2000), more experiments are needed to determine the mechanism involved. It is however still possible that P16 plays a role in resistance to other ROS present during infection.

Another possible explanation for the lack of a role for P16 during infection is that stationary phase may not be reached in the plant infection or that RpoS is not needed for *P. syringae* under the conditions tested and other sigma factors known to play a role in the stress response of *P. syringae*, such as AlgU and RpoN, are used in the plant infection when exposed to various environmental stresses. One way to determine if stationary phase is reached in the plant is to determine the expression of genes known to be controlled by RpoS. Currently we do not have any evidence that P16 is expressed during infection or in planta. Studies are under way in our lab to examine the global transcript profile of *P. syringae* during infection.

Temperature is recognized as an important environmental signal and is known to influence production of virulence factors in a number of pathogens. Little is known about how temperature affects the virulence of plant-pathogenic bacteria, though temperature has been shown to influence the pathogenicity of *P. syringae pv. glycinea* (Palmer & Bender, 1993), coronatine production (Palmer & Bender, 1993), and the expression of antibiotic compounds and hydrogen cyanide in *P. fluorescens* CHA0 (Humair et al., 2009). Our results are consistent with those reported for RpoS in other pseudomonads in that deletion of *rpoS* results in increased sensitivity to elevated temperatures. Surprisingly, deletion of P16 resulted in a slight increase in resistance to exposure to higher temperatures. A possible explanation for this discrepancy is that P16 may target an mRNA that decreases the stability of RpoS. If this were the case then it might be expected that deletion of P16 would result in an increase in resistance to heat stress. It is known that RpoS is regulated at the transcriptional, translational and post-translational level, with different stresses acting at different levels (Battesti et al., 2011), and this would be consistent with our findings. Even so, further experiments will be required to dissect these pathways.

It is possible that P16 is transcribed at low levels in the Δ*rpoS* mutant and this residual expression results in the observed phenotype. In fact, our data show that P16 expression is not completely abolished in a Δ*rpoS* mutant background (Fig. 2). This is not uncommon for genes regulated by RpoS. Some genes regulated by RpoS are only induced specifically by RpoS under particularly stressful conditions, whereas others are expressed constitutively by the housekeeping sigma factor RpoD and then expression becomes boosted by RpoS (Battesti et al., 2011). Also, some RpoS-regulated genes require additional transcriptional activators for their expression (Battesti et al., 2011). González et al. (2008) noted the presence of a regulatory sequence upstream of P16 that appears to be conserved in the pseudomonads. The role of this sequence in the regulation of P16 has yet to be investigated, but it could explain the various responses to different stresses we observed.

Because different responses were observed for P16 when exposed to oxidative stress and heat stress, it is possible that P16 may be regulated by several different mechanisms (as noted above). Alternatively, different mRNA targets may be involved in the response to these particular stresses and P16 may regulate these targets in different ways. mRNA targets have not been reported for the *Pseudomonas* ncRNA P16. To identify possible targets for this ncRNA, the DC3000 genome was scanned for target of *P16* using the program IntaRNA (http://rna.informatik.uni-freiburg.de:8080/v1/IntaRNA.jsp). Interestingly, a candidate from this analysis was PSPTO_5535. The interaction between P16 and PSPTO_5535 is predicted to occur from positions −17 to 22 on PSPTO_5535 and 30−75 on P16. PSPTO_5535 is annotated as a hypothetical protein, with an SPFH domain (http://www.pseudomonas.com/). The SPFH superfamily of proteins contain ‘SPFH’ domains named after the proteins stomatin, prohibitin, flotillin and HflK/C (Browman et al., 2007; López & Kolter, 2010).
While these proteins are commonly distributed in bacteria, their functions in these organisms are unclear. However, there are reports that they may be involved in stress responses such as those to high salt and antibiotic treatment (Butcher & Helmann, 2006).

We believe this is the first study of P16 in the plant pathogen P. syringae DC3000. Studies are under way to confirm the direct binding of P16 to PSPTO_5535 and perform a more detailed investigation of the role of P16 in the tolerance to heat shock.

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REFERENCES

Altier, C., Suyemoto, M. & Lawhon, S. D. (2000). Regulation of Salmonella enterica serovar Typhimurium invasion genes by csrA. Infect Immun 68, 6790–6797.

Alvarez, M. E., Pennell, R. I., Meijer, P. J., Ishikawa, A., Dixon, R. A. & Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92, 773–784.

Battesti, A., Majdalani, N. & Gottesman, S. (2011). The RpoS-mediated general stress response in Escherichia coli. Annu Rev Microbiol 65, 189–213.

Berghoff, B. A., Glaeser, J., Sharma, C. M., Zobawa, M., Lottspeich, F., Vogel, J. & Klug, G. (2011). Contribution of Hfq to photooxidative stress resistance and global regulation in Rhodobacter sphaeroides. Mol Microbiol 80, 1479–1495.

Bolwell, G. P. (1999). Role of active oxygen species and NO in plant defence responses. Curr Opin Plant Biol 2, 287–294.

Bronstein, P. A., Filiatrault, M. J., Myers, C. R., Rutzke, M., Schneider, D. J. & Cartinhour, S. W. (2008). Global transcriptional responses of Pseudomonas syringae DC3000 to changes in iron bioavailability in vitro. BMC Microbiol 8, 209.

Browman, D. T., Hoegg, M. B. & Robbins, S. M. (2007). The SPFH domain-containing proteins: more than lipid raft markers. Trends Cell Biol 17, 394–402.

Burrows, E., Abbas, A., O’Neill, A., Adams, C. & O’Gara, F. (2005). Characterisation of the regulatory RNA RsmB from Pseudomonas aeruginosa PA01. Res Microbiol 156, 7–16.

Butcher, B. G. & Helmann, J. D. (2006). Identification of Bacillus subtilis sigma-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by bacilli. Mol Microbiol 60, 765–782.

Butcher, B. G., Bronstein, P. A., Myers, C. R., Stodghill, P. V., Bolton, J. J., Markel, E. J., Filiatrault, M. J., Swingle, B., Gaballa, A. & other authors (2011). Characterization of the Fur regulon in Pseudomonas syringae pv. tomato DC3000. J Bacteriol 193, 4598–4611.

Caswell, C. C., Gaines, J. M., Ciborowski, P., Smith, D., Borchers, C. H., Roux, C. M., Sayood, K., Dunman, P. M. & Roop, R. M., II (2012). Identification of two small regulatory RNAs linked to virulence in Brucella abortus 2308. Mol Microbiol 85, 345–360.

Chao, Y. & Vogel, J. (2010). The role of Hfq in bacterial pathogens. Curr Opin Microbiol 13, 24–33.

Chatterjee, A., Cui, Y., Yang, H., Collmer, A., Alfano, J. R. & Chatterjee, A. K. (2003). GacA, the response regulator of a two-component system, acts as a master regulator in Pseudomonas syringae pv. tomato DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. Mol Plant Microbe Interact 16, 1106–1117.

Christiansen, J. K., Larsen, M. H., Ingmer, H., Segaard-Andersen, L. & Kallipolitis, B. H. (2004). The RNA-binding protein Hfq of Listeria monocytogenes: role in stress tolerance and virulence. J Bacteriol 186, 3355–3362.

De Lay, N. & Gottesman, S. (2012). A complex network of small non-coding RNAs regulate motility in Escherichia coli. Mol Microbiol 86, 524–538.

Ding, Y., Davis, B. M. & Waldor, M. K. (2004). Hfq is essential for Vibrio cholerae virulence and downregulates sigma expression. Mol Microbiol 53, 345–354.

Dong, X., Mindrinos, M., Davis, K. R. & Ausubel, F. M. (1991). Induction of Arabidopsis defense genes by virulent and avirulent Pseudomonas syringae strains and by a cloned avirulence gene. Plant Cell 3, 61–72.

Filiatrault, M. J., Stodghill, P. V., Bronstein, P. A., Moll, S., Lindeberg, M., Grills, G., Schweitzer, P., Wang, W., Schroth, G. P. & other authors (2010). Transcriptome analysis of Pseudomonas syringae identifies new genes, noncoding RNAs, and antisense activity. J Bacteriol 192, 2399–2372.

Filiatrault, M. J., Stodghill, P. V., Myers, C. R., Bronstein, P. A., Butcher, B. G., Lam, H., Grills, G., Schweitzer, P., Wang, W. & other authors (2011). Genome-wide identification of transcriptional start sites in the plant pathogen Pseudomonas syringae pv. tomato strain DC3000. PLoS ONE 6, e29335.

Geng, J., Song, Y., Yang, L., Feng, Y., Qiu, Y., Li, G., Guo, J., Bi, Y., Qu, Y. & other authors (2009). Involvement of the post-transcriptional regulator Hfq in Yersinia pestis virulence. PLoS ONE 4, e26123.

González, N., Heeb, S., Valverde, C., Kay, E., Reimmann, C., Junier, T. & Haas, D. (2008). Genome-wide search reveals a novel GacA-regulated small RNA in Pseudomonas species. BMC Genomics 9, 167.

Gottesman, S. (2005). Micros for microbes: non-coding regulatory RNAs in bacteria. Trends Genet 21, 399–404.

Gottesman, S., McCullen, C. A., Guillem, M., Vanderpool, C. K., Majdalani, N., Benhammou, J., Thompson, K. M., FitzGerald, P. C., Sowa, N. A. & FitzGerald, D. J. (2006). Small RNA regulators and the bacterial response to stress. Cold Spring Harb Symp Quant Biol 71, 1–11.

Hagen, M. J., Stockwell, V. O., Whistler, C. A., Johnson, K. B. & Loper, J. E. (2009). Stress tolerance and environmental fitness of Pseudomonas fluorescens A506, which has a mutation in RpoS. Phytopathology 99, 679–688.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. J Mol Biol 166, 557–580.

Hébrard, M., Kröger, C., Srikumar, S., Colgan, A., Händler, K. & Hinton, J. C. (2012). sRNAs and the virulence of Salmonella enterica serovar Typhimurium. RNA Biol 9, 437–445.

Heeb, S., Valerde, C., Gigot-Bonnefoy, C. & Haas, D. (2005). Role of the stress sigma factor RpoS in GacA/RsmA-controlled secondary metabolism and resistance to oxidative stress in Pseudomonas fluorescens CHA0. FEMS Microbiol Lett 243, 251–258.
*meliloti* RNA chaperone Hfq influences central carbon metabolism and the symbiotic interaction with alfalfa. *BMC Microbiol* **10**, 71.

Vencato, M., Tian, F., Alfano, J. R., Buell, C. R., Cartinhour, S., DeClerck, G. A., Gutman, D. S., Stavrinides, J., Joardar, V. & other authors (2006). Bioinformatics-enabled identification of the HrpL regulon and type III secretion system effector proteins of *Pseudomonas syringae* pv. *phaseolicola* 1448A. *Mol Plant Microbe Interact* **19**, 1193–1206.

Vogel, J. & Luisi, B. F. (2011). Hfq and its constellation of RNA. *Nat Rev Microbiol* **9**, 578–589.

Windgassen, M., Urban, A. & Jaeger, K. E. (2000). Rapid gene inactivation in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **193**, 201–205.

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