Analysis of the small RNA spf in the plant pathogen Pseudomonas syringae pv. tomato strain DC3000

So Hae Park, Zhongmeng Bao, Bronwyn G. Butcher, Katherine D’Amico, Yun Xu, Paul Stodghill, David J. Schneider, and M. J. Filiatrault

Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, NY 14853, USA

INTRODUCTION

Bacteria contain small non-coding RNAs (ncRNAs) that are typically responsible for altering transcription, translation or mRNA stability. ncRNAs are important because they often regulate virulence factors and susceptibility to various stresses. Here, the regulation of a recently described ncRNA of Pseudomonas syringae DC3000, spot 42 (now referred to as spf), was investigated. A putative RpoE binding site was identified upstream of spf in strain DC3000. RpoE is shown to regulate the expression of spf. Also, deletion of spf results in increased sensitivity to hydrogen peroxide compared with the wild-type strain, suggesting that spf plays a role in susceptibility to oxidative stress. Furthermore, expression of alg8 is shown to be influenced by spf, suggesting that this ncRNA plays a role in alginate biosynthesis. Structural and comparative genomic analyses show this ncRNA is well conserved among the pseudomonads. The findings provide new information on the regulation and role of this ncRNA in P. syringae.

INTRODUCTION

Bacteria contain small RNAs that are often referred to as non-coding RNAs (ncRNAs) (Storz, 2002). ncRNAs are transcripts that are not translated into protein but function as RNA molecules, regulating diverse activities such as responding to environmental changes and regulating expression of virulence factors (Massé et al., 2003; Wassarman, 2002). Most ncRNAs function by interacting directly with proteins or mRNAs to alter transcription, translation or mRNA stability (Storz, 2002). Base-pairing with a target mRNA either activates or represses translation of the transcript, or targets the mRNA for degradation and frequently requires the RNA chaperone Hfq (Storz, 2002) to facilitate binding. In bacteria, the ncRNA is often not a perfect complement to its target mRNA (Waters & Storz, 2009). Because of this, an ncRNA can potentially regulate multiple genes (Waters & Storz, 2009). For example, the ncRNA gcvB in Salmonella enterica regulates mRNA targets oppA, dppA and gll (Sharma et al., 2007). Multiple ncRNAs are also capable of regulating one target gene; ncRNAs such as dsrA, rprA and oxyS regulate the sigma factor RpoS in Escherichia coli (Gottesman, 2004).

Pseudomonas syringae pv. tomato strain DC3000 (DC3000) is responsible for causing bacterial speck on tomato and also infects Arabidopsis thaliana, a commonly used model plant (Cuppels, 1986; Whalen et al., 1991). The bacteria enter the plant via the stomata and grow in the intercellular space, ultimately causing necrotic lesions surrounded by chlorotic haloes (Hirano & Upper, 2000). A major virulence factor of DC3000 is the type III secretion system. This system is responsible for the delivery of effector proteins into plant cells, which act to subvert and suppress plant immunity (Lindgren, 1997). In resistant plants, some effectors induce the hypersensitive reaction, a rapid programmed cell death (apoptosis) (Alfano & Collmer, 1997; Lindgren, 1997). Several other virulence-related proteins have been described in DC3000. For example, a dsbA mutant exhibits reduced virulence on A. thaliana and tomato and partial impairment of type III secretion (Knolle et al., 2000). A novel virulence factor, named TvrR (PSP93_3576) contributes to virulence in A. thaliana (Preiter et al., 2005). Disruption of the malE: quinone oxidoreductase gene (mqo), which encodes an enzyme of the tricarboxylic acid cycle (Mellgren et al., 2003; Wassarman, 2002), also results in reduced virulence. Comparative approaches suggest that other DC3000 proteins may be involved in virulence as well (Lindeberg et al., 2008).
contrast, ncRNAs have not yet been demonstrated to be virulence factors in *P. syringae* DC3000, probably because they are now only being catalogued and characterized.

Few ncRNAs have been confirmed in DC3000, but molecular and computational methods have identified various candidates (Filiatrault et al., 2010; Moll et al., 2010). Among these, *spot 42* (PSPTO_0342) is located between PSPTO_0342 and PSPTO_0345 (polII) (Gottesman et al., 2006) and is expressed in minimal medium under iron-limited conditions (Filiatrault et al., 2010). The *spot 42* genomic context is highly conserved in many bacteria, including *Escherichia coli* where the gene is also known as *spf* (Gottesman et al., 2006). In *E. coli*, this ncRNA influences expression of DNA polymerase I (Polayes et al., 1988a) and mediates discordant expression of the galactose operon.

Overexpression of *spot 42* reduces the expression of GalK, one of the four galactose operon-encoded proteins, by binding to the Shine–Dalgarno sequence and inhibiting ribosome binding (Moller et al., 2002). In *E. coli*, transcription of *spot 42* is repressed by cyclic AMP receptor protein (Crp) and many of the recently identified targets of Spot 42 are involved in the catabolism of diverse carbon sources (Beisel & Storz, 2011). However, in the pseudomonads, the *galK* operon is not present and catabolite repression is mediated by a different factor, catabolite repression control protein (Crc) (Rojo, 2010). Crc may be an RNA-binding protein, although there is contrary evidence (Milojevic et al., 2013). Thus, Spot 42 may have another role not yet described for *E. coli* or, alternatively, a function specific for *P. syringae*.

In this study, we report the first analysis of ncRNA *spot 42* (now referred to as *spf*) in *P. syringae* DC3000. We show that *spf* is regulated by the sigma factor RpoE and that the ncRNA plays a role in resistance to oxidative stress. Several mRNA targets of Spf were also identified.

### METHODS

**Bacterial strains/growth conditions.** The strains and plasmids used for this study are summarized in Table 1. *P. syringae* pv. *tomato* DC3000 was routinely cultured on King’s B (KB) agar (King et al., 1954) at 28°C or at room temperature.

**Creation of *rpoE* and *spf* mutants in DC3000.** A PSPTO_4224 (*rpoE*; alternatively, AlgT and AlgU in some organisms) deletion was created using a pK18mobsacB plasmid (Schäfer et al., 1994). Note that a second DC3000 gene, PSPTO_1043, is erroneously described as yielding ‘RNA polymerase sigma factor RpoE’ as its product in the DC3000 genome annotation. This gene is not the subject of our study. DNA fragments of approximately 1.0 kb upstream and downstream of PSPTO_4224 and *mucB* were PCR amplified, gel purified and then joined by a second PCR amplification with primers containing *Xmal* restriction sites. The product was gel purified using a Gel DNA Recovery kit (Zymo Research), digested with *Xmal* and cloned into pK18mobsacB cut with the same restriction enzyme. The pK18mobsacB deletion construct was confirmed by sequencing (Cornell University Life Sciences Core Laboratories Center) and introduced 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 loss of the *sacB* gene. Sucrose-resistant colonies were screened by PCR and positive clones (those containing the deletion) were confirmed by sequencing. The deletion spans from 4756 056 to 4758 184 and removes *rpoE, mucA* and *mucB* and is referred throughout the text as Δ*rpoE*. Similarly, pK18mobsacB/Δ*spf* was created by PCR amplification of DNA fragments of approximately 1.0 kb that flank *spf*. Gel-purified PCR fragments were joined by a second PCR amplification with primers containing the *SpfI* restriction site. The resulting deletion spanned coordinates 373 016–373 171 and removed the entire *spf* gene.

**Evaluating susceptibility to oxidative stress.** WT DC3000 and the Δ*spf* mutant were grown on KB plates for 2 days (King et al., 1954). Overnight cultures were prepared in liquid KB, and incubated at 28.0°C with shaking. The next morning, each culture was sampled (1 ml) and cells were harvested by centrifugation. Pellets were resuspended in 3 ml of liquid mannitol–glutamate (MG) medium.

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DC3000 strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>Wild-type strain DC3000</td>
<td></td>
</tr>
<tr>
<td>ΔrpoE</td>
<td>rpoE deletion DC3000 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>Δspf</td>
<td><em>spf</em> deletion DC3000 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>WT pBS58/spf promoter</td>
<td>Wild-type strain with the pBS58/spf promoter construct</td>
<td>This study</td>
</tr>
<tr>
<td>WT pBS59/spf promoter</td>
<td>Wild-type strain with the pBS59/spf promoter construct</td>
<td>This study</td>
</tr>
<tr>
<td>ΔrpoE pBS58/spf promoter</td>
<td>ΔrpoE mutant with the pBS58/spf promoter construct</td>
<td>This study</td>
</tr>
<tr>
<td>ΔrpoE pBS59/spf promoter</td>
<td>ΔrpoE mutant with the pBS59/spf promoter construct</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBS58</td>
<td><em>lux</em> reporter construct; KanR, TetR</td>
<td>Markel et al. (2011), Swingle et al. (2008)</td>
</tr>
<tr>
<td>pBS59</td>
<td><em>lux</em> reporter construct (control); KanR, TetR</td>
<td>Markel et al. (2011), Swingle et al. (2008)</td>
</tr>
<tr>
<td>pBS58/spf promoter</td>
<td><em>lux</em> reporter construct containing <em>spf</em> promoter inserted in the correct orientation; KanR, TetR</td>
<td>This study</td>
</tr>
<tr>
<td>pBS59/spf promoter</td>
<td><em>lux</em> reporter construct containing <em>spf</em> promoter inserted in the opposite orientation; KanR, TetR</td>
<td>This study</td>
</tr>
</tbody>
</table>

* KanR, kanamycin resistance marker; TetR, tetracycline resistance marker.
(Bronstein et al., 2008) and 30% hydrogen peroxide was added to the culture to a final concentration of 30 mM (Pečý-Tarr et al., 2005). Two control cultures (WT and Aaspf) without hydrogen peroxide were also included. All cultures were incubated at 28.0 °C with shaking for 30 min before dilutions were plated onto solid KB medium. Plates were incubated at room temperature until colonies were visible and the number of colonies was determined for both the control and the experimental samples. Viable counts were averaged (mean) for three biological replicates. Statistical significance was evaluated using a one-tailed t-test for two independent samples with unequal variances.

**Sensitivity to heat stress.** Sensitivity to heat shock was assessed using the protocol described by Schurr et al. (1995). WT DC3000, Δaspf and ΔrpoE cells were grown on KB plates for 2 days. Cultures were prepared in liquid KB, and incubated at 28.0 °C with shaking overnight. Then, 1 ml from each culture was centrifuged for 5 min at 15,000 rpm, the supernatant was removed and the cell pellet was resuspended in 3 ml of liquid MG medium. The cultures were incubated at 42 °C. Every 15 min, the cultures were serially diluted in MG medium, spread onto solid KB medium and incubated at room temperature to determine viable cell counts.

**Creation of reporter constructs.** The putative promoter for spf, identified in earlier work as a region containing an RpoE-dependent promoter motif (Filitratrault et al., 2011), was amplified via PCR using chromosomal DNA isolated from DC3000. Primers were designed to amplify a region containing 28 bp of spf in addition to 110 bp upstream of the transcriptional start site of spf for a total product length of 138 bp. The sense primer was designed with a CACC overhang on the 5’ end to ensure directional cloning into pENTR/D-TOPo vector (Invitrogen). The PCR products were separated by agarose gel electrophoresis. The DNA fragment was extracted from the gel using a Gel Extraction kit (Qiagen) and cloned into the pENTR vector (pENTR Directional TOPO Cloning kit; Invitrogen). To confirm that the putative spf 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; Swingle et al., 2008) using Gateway LR Clonase II Enzyme mix (Invitrogen). The Gateway cassette in these plasmids is located upstream from a promoterless lux operon (luxCDABE). The destination vector pBS58 was designed so that the cloned promoter is in the same orientation as the lux operon, 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 operon. Plasmids were transformed into One Shot TOP10 Chemically Competent E. coli (Invitrogen).

**Promoter fusion assays.** The pBS58/spf and pBS59/spf promoter fusion constructs were introduced into WT DC3000 and the ΔrpoE mutant via electroporation. Each strain was grown in KB or LM (Pressler et al., 1988), with appropriate antibiotics, for approximately 22 h at 28.0 °C with shaking. Optical density (OD₆₀₀) of the overnight cultures was measured, and each strain was diluted to an OD₆₀₀ of 0.1 in fresh KB, LM or MG. Then, 600 μl of the culture was dispensed into three 200 μl wells in a 96-well plate for three technical replicates. OD₆₀₀ 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 OD₆₀₀ 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 OD₆₀₀ values (Schagat et al., 2007). Three biological replicates were obtained. Technical replicates were averaged for each biological replicate. Means and SD for each of the three biological replicates were calculated and statistical significance was assessed using a one-way ANOVA test.

DC3000 containing the pBS58/spf and pBS59/spf constructs were maintained on KB + kanamycin plates. Overnight cultures were prepared in KB medium + kanamycin, and incubated at 28.0 °C with shaking. OD₆₀₀ of the overnight cultures was measured, and each strain was diluted to an OD₆₀₀ of 0.1 in LM. Subsequently, 200 μl of the subculture was dispensed into six separate wells of a 96-well plate; 10 μl of 0.2 mM paraquat was added to three of the wells (final concentration of 0.01 mM) and the remaining three were left as controls. The plates were incubated at 28.0 °C with shaking in a Biotek Synergy 2 microplate reader. OD₆₀₀ and relative luminescence were measured every 10 min for 5 h and relative luminescence calculated as luminescence/OD₆₀₀. The experiment was performed three times and a representative graph is shown in Fig. 2 below.

**RNA isolation.** Total RNA was prepared with an RNaseasy kit (Qiagen) using the optional on-column DNase I digestion following the manufacturer’s instructions, except that lysozyme was used at a final concentration of 5 μg ml⁻¹. RNA was treated twice with DNase I (Ambion) to remove residual DNA and then cleaned and concentrated using a MinElute kit (Qiagen).

**Quantitative real-time PCR (qRT-PCR).** Total RNA (100 ng) extracted from DC3000 was reverse transcribed in a thermocycler using a qScript cDNA Supermix (Quanta Biosciences) according to the manufacturer’s instructions. qPCR was performed using IQ SYBR green Supermix (Bio-Rad) on an IQ5 multi-colour real-time detection system (Bio-Rad). The production of non-specific products was determined by the dissociation protocol included with the software provided with the machine. The PCR assay was carried out with one cycle at 95 °C for 2.5 min followed by 35 cycles at 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 those of the housekeeping gene gap1 (PSPTO_1287) to obtain relative expression data for each gene.

**Co-immunoprecipitation of RNAs bound to Hfq.** Co-immunoprecipitation of RNAs bound to FLAG-tagged Hfq was performed as described by Park et al. (2013). One hundred nanograms of total RNA (from the LYSATE controls) or enriched RNA (from the IP samples) were reverse transcribed to cDNA using qScript cDNA SuperMix (Quanta) as described by the manufacturer. A total of 15 ng of cDNA was used to perform real-time PCR as described above. Under each condition the Ct values were normalized to those for the housekeeping gene, gap1. Enrichment was calculated relative to the values in the WT (untagged) control as follows: Enrichment = 1-2^(-([IPFLAG-IPWT]/(LYSATEFLAG-LYSATEWT))).

**Virulence assays.** Plant assays were performed as described by Bronstein et al. (2005) and Park et al. (2013), except that Silvet L-77 (Lehle Seeds) was used at a concentration of 0.04% for the tomato dip assay.

**Identification of mRNA targets.** TargetRNA (http://snowwhite.wellesley.edu/targetRNA; Tjaden et al., 2006), RNApredator (http://rna.tbi.univie.ac.at/RNApredator; Eggelhofer et al., 2011) and IntaRNA (http://www.bioinf.uni-freiburg.de/Software; Busch et al., 2008) were used to identify potential Spf mRNA targets. The region used to search for Spf targets bracketed the translation start codon for each mRNA (± 100 bp). The hybridization seed was set as 7 or 9, and all other parameters were left as default settings. The first 100 mRNA targets generated by each server were compared with one another and those that were commonly shared candidates among these servers were selected for further investigation by qRT-PCR. One exception was PSPTO_4459 (soDA), which was selected because of its association with oxidative stress. Beacon Designer (http://www.premierbiosoft.com/molecular_beacons/) was used to design each pair of primers for...
each mRNA target. Parameters for primer searching were set as follows: $T_m$ at $60 \pm 5^\circ C$, length of primers within the range 15–25 bp and amplicon length within the range 70–150 bp. qRT-PCR was performed as described above.

Computational analyses. A list of all genomes used in this study and their GenBank and Refseq accession numbers is included in Tables S1 and S2. Prediction of secondary structure, consensus model building, calibration and searching were performed as described by Filiatrault et al. (2013). The ‘mlocarna’ tool from the LOCARNA v1.7.1 package (Will et al., 2007) was used to align the sequences and predict a secondary structure. ‘RNAalifold’ from the ViennaRNA v2.1.3 package (Gruber et al., 2008) was used to generate figures of the secondary structures. The resulting alignment was converted to Stockholm format using a custom script for use by the Infernal toolset (Nawrocki et al., 2009).

The method used to search the genomes using the Spf model was similar to that reported in (Filiatrault et al., 2013). The following programs from the Infernal v1.1rc4 package (Nawrocki et al., 2009) were used following the package’s documentation and with the default parameters (except as noted):

- cmbuild
- cmcalibrate – seed 1
- cmsearch

The infernal matches were found to fall into two sets, matches with E-values greater than 0.23 and matches with E-values less than 2.9 x 10^-14. We discarded the former and converted the latter into GFF-formatted files using a custom script.

Syntenic regions were identified based on homology of neighbouring genes using the method described by Filiatrault et al. (2013).

To evaluate regulatory features upstream of the ncRNA candidates, the sequences between 40 and two bases upstream of the ncRNA candidates were extracted and aligned using MUSCLE v3.8.31 (Edgar, 2004) with the default parameters. The program WEBLOGO 3.3 (Crooks et al., 2004) was used to generate a logo from the alignment.

**RESULTS**

The ncRNA spf is regulated by RpoE

In previous work, a MEME (Bailey et al., 2009) analysis of genomic regions immediately upstream from the 5’ ends of DC3000 transcripts revealed a motif that was similar to the promoter sequence recognized by RpoE (also known as AlgT or AlgU in some species) adjacent to spf (Fig. 1, underlined italics) (Filiatrault et al., 2011). To investigate if RpoE regulates expression of spf, lux promoter fusions were constructed and introduced into WT DC3000 and ΔrpoE backgrounds (see Methods). Using reporter expression as the proxy, the results show that the spf promoter is active in three different media (KB, LM and MG) but activity is reduced in the ΔrpoE mutant compared with the WT strain (approx. a 100-fold difference in all cases) (Fig. 2). The relative luminescence generated by the ΔrpoE mutant was not significantly different from the controls in which the cloned promoter had been reversed relative to the lux operon (strains with pBS59/spf promoter).

The data from the lux reporter assay suggest that spf ncRNA is abundant in WT cells under the conditions we tested. To determine the level of spf transcript more directly, qRT-PCR was performed in both the WT and the ΔrpoE strains. The ΔrpoE mutant showed a more than eightfold reduction in the level of spf transcript compared with the WT (Fig. S1). Taken together, these data indicate that RpoE regulates the expression of spf.

Spf and the response to stress

RpoE is essential for the production of the exopolysaccharide alginate and is involved in the tolerance to desiccation, heat, osmotic stress and oxidative stress in the genus Pseudomonas (Keith & Bender, 1999; Schnider-Keel et al., 2001; Yu et al., 1995). RpoE is also known to play a role in protecting P. syringae pv. syringae from the toxic effects of copper sulfate (Kidambi et al., 1995). Therefore, we investigated whether spf has comparable functionality in DC3000. Susceptibility to oxidative stress was analysed by exposing bacteria to hydrogen peroxide. We first compared growth of WT, Δspf and ΔrpoE cells in media containing increasing concentrations of hydrogen peroxide (Fig. S2). All three strains showed similar growth of WT DC3000, Δspf and ΔrpoE mutant strains were also tested for susceptibility to paraquat. Paraquat can induce the generation of reactive oxygen species, usually superoxide, by transferring electrons to molecular oxygen (Bus & Gibson, 1982). We compared growth of WT, Δspf and ΔrpoE cells in media containing increasing concentrations of paraquat (Fig. S3). All three strains showed similar responses when challenged with paraquat (Fig. S3).

The response of the ΔrpoE mutant contrasts with data described...
by Keith & Bender (1999) using P. syringae pv. syringae FF5, in which an algU/rpoE transposon insertion mutant displayed increased sensitivity to paraquat. These differences in results could be due to the use of different strains (P. syringae pv. syringae FF5 vs. P. syringae pv. tomato DC3000) or possibly differences in how susceptibility was evaluated. For example, Keith & Bender (1999) used a disc diffusion assay to evaluate susceptibility of strains that were grown overnight in MG medium supplemented with yeast extract whereas we compared growth of the strains over a time course in KB or LM medium in the presence of various concentrations of paraquat. To probe this result, we used lux reporter fusions to examine spf promoter activity in the presence of sublethal concentrations of paraquat. Fig. 4 shows that luminescence increases in WT cells under these conditions. Based on this, and on the fact that spf confers resistance to hydrogen peroxide (Fig. 3), we conclude that spf plays a role in the DC3000 response to oxidative stress.

As shown in Fig. 5, survival was not significantly different between WT and Δspf cells after heat shock at any of the time points tested. However, the ΔrpoE mutant showed a reduction in the proportion of surviving cells at T=15 min compared with the WT (P<0.05), as expected. Therefore, it
is unlikely that spf plays a major role in the heat shock response. No differences were observed in growth in various concentrations of copper sulfate (Fig. S4).

**Spf does not influence virulence.** In *P. syringae* pv. *glycinea*, RpoE strongly promotes *in planta* growth, survival and disease symptom development (Schenk *et al.*, 2008). We therefore investigated whether spf plays a role in the virulence of DC3000 in both arabioidpsis and tomato. Fig. 6 shows that at 3 and 5 days post-infection, the disease symptoms in arabioidpsis caused by WT DC3000 and the Δspf mutant are not significantly different (Fig. 6). We additionally examined growth and virulence of WT, Δspf and ΔrpoE in tomato plants. Tomato plants were dipped in suspensions of WT, Δspf and ΔrpoE. As expected, plants exposed to WT DC3000 exhibited disease symptoms at 5 days post-infection (Fig. 7). Tomato plants dipped in a suspension of Δspf displayed similar symptoms and grew as well as the WT. Based on these experiments, we find no evidence that spf influences virulence in DC3000. In contrast, the ΔrpoE strain was attenuated in symptoms by day 5 and also displayed reduced growth by days 5 and 7 (Fig. 7). These data suggest rpoE maybe critical for growth in tomato plants.

**Spf interacts with the RNA chaperone Hfq *in vivo***

The RNA-binding protein Hfq is known to be important in regulating stress-related responses in several bacterial species (Vogel & Luisi, 2011). Unfortunately we have not been able to construct an *hfq* mutant and therefore we are unable to evaluate if disruption of *hfq* influences expression of spf. To investigate whether Spf interacts with this important global regulator, we expressed an epitope-tagged version of Hfq and used it to enrich bound RNAs.

**Identification of mRNA putative targets of Spf**

To identify RNAs that potentially interact with Spf, computational methods were used to identify sequences that could base pair with this ncRNA in DC3000 (see Methods: Identification of mRNA targets). Targets were predicted using multiple programs and then compared to generate a list of high-value candidates. A subset of putative mRNA targets was selected (Table 2) for experiments in which expression of the predicted target was evaluated in Δspf and WT strains. As shown in Fig. 9, PSPT0_1242 (alginate biosynthesis protein Alg8) and PSPT0_2927 (outer membrane porin, OprE) were more highly expressed in the Δspf mutant compared with the WT. Deletion of rpoE resulted in decreased expression for both of these genes.

**Structure and conservation of Spf in pseudomonads**

To further investigate spf, we modelled its secondary structure based on sequences from DC3000 and several
other pseudomonads. Using the genomic coordinates reported by Gottesman et al. (2006), and the putative transcription start site reported by Filiatrault et al. (2011) (see Fig. 1), spf coordinates were identified in *P. syringae* B728A (Feil et al., 2005), *P. syringae* 1448A (Joardar et al., 2005), *Pseudomonas putida* KT2440 (Nelson et al., 2002) and *Pseudomonas aeruginosa* PAO1 (Stover et al., 2000). The RNA in the pseudomonads is slightly larger (~115 nt versus 105 nt) than that reported for *spot 42* (Gottesman et al., 2006). The proposed secondary structure is shown in Fig. 10(a). For comparison, a secondary structure for the Rfam (the RNA family database) Spot 42 model (Griffiths-Jones et al., 2005) was also generated (Fig. 10b). Note that our predicted secondary structure for the Rfam Spot 42 is different from the structure reported on the Rfam website (http://rfam.sanger.ac.uk/family/RF00021). Nevertheless, the structure of the *Pseudomonas* ncRNA is different from that of the Rfam model for ‘Spot 42’ (RF00021).

To determine the taxonomic distribution of the *Pseudomonas* ncRNA, a co-variance model (CM) was constructed using the Infernal toolset. The CM was used to scan all the closed *Pseudomonas* genomes and the 19 genomes used for the Spf model generated in Rfam (RF00021). A single match was detected in all 44 closed *Pseudomonas* genomes, while no matches were observed in any of the 19 genomes used to build the Spot 42 Rfam model. The results indicate that the ncRNA present in DC3000 is highly conserved among the pseudomonads and appears to be distinct from the ncRNA represented by Rfam model RF00021.

Next we investigated if the ncRNAs detected in the pseudomonads by the CM are syntenic (reside in a conserved genomic location). We found that orthologues of PSPTO_0343 (*engB*) are located downstream and orthologues of PSPTO_0344 (*polA/polI*) are located upstream of the ncRNA in all of the pseudomonads except *Pseudomonas stutzeri*. For *P. stutzeri* A1501 and *P. stutzeri* DSM 4166, the ncRNA is located between a gene homologous to PSPTO_5469 (a hypothetical protein) and PSPTO_0344. For *P. stutzeri* CCUG 29243, *P. stutzeri* DSM 10701 and *P. stutzeri* RCH2 the ncRNA is located between a homologue to PSPTO_5435 (*hcp-2*) and a homologue to PSPTO_0344. In summary, the spf candidates are always closely linked to *polA/polI* and almost always located between *polA/polI* and *engB*.  

---

**Fig. 7.** Evaluation of virulence in tomato. (a) Four-week-old tomato cv. Moneymaker plants were dipped in suspensions containing $\sim 1 \times 10^7$ c.f.u. ml$^{-1}$ of either WT, Δspf or ΔrhoE. At the time points indicated below the graph, bacteria were extracted from leaves and plated on KB containing rifampicin for enumeration. The values on the graph are the means obtained from technical replicates, and error bars represent SD. Similar results were obtained in two repetitions of the experiment. (b) Infected tomato leaves were photographed at 5 and 7 days post-inoculation (DPI). Similar results were obtained in a repeated experiment.
Conserved regulation of DC3000 *spf*

The upstream regions of the candidate *Pseudomonas* ncRNAs were aligned to identify potential conserved regulatory features. The analysis revealed the existence of a highly conserved motif that is similar to the RpoE-dependent promoter motif (Fig. 11), suggesting that all of the candidate ncRNAs in the pseudomonads are regulated by RpoE.

**DISCUSSION**

Although *spf* has been previously investigated in *E. coli*, its role and regulation have not been investigated in the pseudomonads. The data presented in this study suggest that the transcription of *spf* is regulated by RpoE, and that *spf* plays a role in the response to oxidative stress but not to other environmental stresses such as heat shock or exposure to copper sulfate. Additionally, the data suggest that *spf* may play a role in alginate biosynthesis in DC3000.

Unfortunately, for *Pseudomonas syringae* pv. *tomato*, there are no reports of the total number of genes thought to be regulated by RpoE. There are reports of RpoE (AlgU, AlgT)-regulated genes in the other pseudomonads (Firoved et al., 2002; Martin et al., 1994; Wood & Ohman, 2012; Wozniak & Ohman, 1994). Firoved et al. (2002) reported that in all cases (10) the transcript (of predicted AlgU promoters) was completely absent in the *algU* mutant strain, with one exception. Our results are consistent with other reports in that *spf* expression appears to be absent in an RpoE mutant.

In *P. syringae* pv. *glycinea*, RpoE strongly promotes *in planta* growth, survival and symptom development (Schenk et al., 2008). One possible interpretation is that the primary function of RpoE is to mobilize bacterial defences against plant-induced oxidative stress. However, deletion of *spf* did not impair the ability of DC3000 to infect *Arabidopsis* or

**Table 2. Candidate mRNA targets of Spf**

| Target         | Predicted binding sites* | Description|| |
|----------------|--------------------------|-------------|
| PSPTO_1255     | -29 to -1, -43 to -1, -42 to +2 | Amino acid ABC transporter periplasmic amino acid-binding protein |
| PSPTO_3210     | -40 to -13, -41 to -14 | Filamentous haemagglutinin family protein |
| PSPTO_4380     | +522 to +534, -32 to +5 | Short chain dehydrogenase/reductase family oxidoreductase |
| PSPTO_2029     | +356 to +364, +15 to +45 | Oxidoreductase zinc-binding protein |
| PSPTO_1242     | -14 to -9, -16 to -10, -40 to +50 | Alginate biosynthesis protein Alg8 |
| PSPTO_2470     | -170 to -161, -30 to +12, -30 to +12 | Senescence marker protein-30 family protein |
| PSPTO_1221     | +275 to +299, -32 to +13, -18 to +19 | Transporter, LysE family |
| PSPTO_4459     | +13 to +23, -58 to -8 | Superoxide dismutase |
| PSPTO_2927     | -32 to -16, +13 to +40, -32 to +5 | Outer membrane porin OprE |

*The binding sites are positions relative to the translational start codon of the mRNAs. A negative number corresponds to nucleotides upstream from nucleotide 1 of the translational start codon, whereas a positive number corresponds to nucleotides downstream of the translational codon.
†Eggenhofer et al. (2011).
‡Busch et al. (2008).
§Tjaden et al. (2006).
‖Functions listed for each gene were obtained from http://www.pseudomonas.com.
tomato plants. Similarly, in *P. syringae*, we have shown that deletion of the ncRNA P16 (regulated by RpoS) renders bacteria more sensitive to hydrogen peroxide, but does not influence virulence (Park *et al.*, 2013). An *oxyR* deletion in *Erwinia chrysanthemi* also confers sensitivity to hydrogen peroxide but no apparent virulence phenotype (Miguel *et al.*, 2000; Park *et al.*, 2013). These results demonstrate the importance of performing more detailed studies to understand how oxidative stress impacts the outcome of the infection process.

Hfq has been carefully investigated in *E. coli* to identify the diverse RNAs with which it associates. However, *spf* from *P. syringae* appears to be different in structure from *spot 42* in *E. coli* and may have distinct properties. We therefore investigated the interaction between Hfq and Spf in *P. syringae* rather than rely on inferences from *E. coli*. The results are consistent with a model in which *P. syringae* Spf functions by interacting with mRNA targets in trans with the assistance of Hfq. To further investigate the function of Spf in DC3000, we identified putative mRNA targets using publicly available software. This approach has been successful elsewhere (Vogel & Wagner, 2007) but generates large numbers of potential candidates. We attempted to obtain more refined results by combining the outputs from three different prediction algorithms before testing the predictions using qRT-PCR. Many putative targets on the final list are predicted to encode putative membrane proteins, such as outer-membrane proteins PSPTO_3210 and PSPTO_2927 (*oprE*) and inner-membrane proteins PSPTO_1242 (*alg8*) and PSPTO_1221. Differential expression of membrane proteins during conditions of oxidative stress has been reported for DC3000 (Cao *et al.*, 2012). Therefore, it is possible that the sensitivity of the Δ*spf* mutant to hydrogen peroxide is a result of differences in the membrane protein profile.

We found that the levels of several predicted mRNA targets increased in the Δ*spf* mutant. As ncRNAs can influence RNA levels by binding to targets and promoting their degradation, it is possible that Spf may be directly responsible for the degradation of these transcripts in WT cells, or may do so...
indirectly by affecting the levels of other regulatory factors. Of particular interest, mRNA levels for PSPTO_1242, which encodes the alginate biosynthesis protein Alg8, were increased in the Δspf mutant. Alginate in _P. aeruginosa_ provides protection against oxidative stress because it acts as a ‘scavenger’ for superoxide radicals (Simpson et al., 1989). In _P. syringae_ alginate is synthesized at times of oxidative stress, increases the survival rate of the bacteria in an oxidative environment, and contributes to virulence by facilitating colonizing and dissemination of the bacteria in the host plant (Keith & Bender, 1999; Yu et al., 1994). If _spf_ plays a role in alginate production in DC3000, one might predict that a Δspf mutant would exhibit a mucoid phenotype as compared with the WT. However, we saw neither more nor less mucoidity in the mutant, based on visual inspection of colony morphology (data not shown), although it is possible that the changes were too subtle to observe easily.

In contrast to the results for _alg8_, the levels of several predicted mRNA targets were unchanged in the Δspf mutant. For example, PSPTO_4380 and PSPTO_4459 (sodA) mRNA levels were very similar in the WT and Δspf242 mutant strains, suggesting that _spf_ does not affect these mRNAs. SodA is a potentially interesting target because of its likely role in detoxifying peroxide. However, it is possible that Spf impacts translation rather than turnover by base pairing to the ribosome-binding site or to the 5’-untranslated region in these cases. Spf is known to function as a translational inhibitor in _E. coli_ (see below).

Although the genes flanking _spf_ are conserved among _E. coli_, _S. enterica_, _P. aeruginosa_, _P. putida_, and _P. syringae_ DC3000, the intergenic sequence at this locus has diverged between the _Enterobacteriaceae_ and _Pseudomonaceae_ (Gottesman et al., 2006). The Rfam model (RF00021) does not detect similar ncRNAs in the pseudomonads, suggesting that _spf_ does not affect these ncRNAs. SodA is a potentially interesting target because of its likely role in detoxifying peroxide. However, it is possible that Spf impacts translation rather than turnover by base pairing to the ribosome-binding site or to the 5’-untranslated region in these cases. Spf is known to function as a translational inhibitor in _E. coli_ (see below).

In _E. coli_ Spf works with Crp and regulates the uptake and the catabolism of non-preferred carbon sources when glucose levels are low (Beisel & Storz, 2011). Overexpression of _spf_ results in reduced growth of _E. coli_ on minimal media with non-preferred carbon source such as N-acetylneuraminic acid (Beisel & Storz, 2011). Crp also binds to the _spf_ promoter region where it functions as a negative regulator of _spf_ gene expression (Polayes et al., 1988b). Does _spf_ cooperate with a Crp-like protein partner in _P. syringae_ and other pseudomonads to regulate the uptake and catabolism of non-preferred carbon sources? A closer look at catabolite repression in the pseudomonads reveals a complex picture. In _P. aeruginosa_, the virulence factor regulator (Vfr) is homologous to _E. coli_ Crp (West et al., 1994) and is an obvious candidate for interaction with _spf_. However, this protein is not involved in catabolite repression despite its homology to Crp (Suh et al., 2002). Rather, Vfr acts as a transcriptional regulator for various virulence factors (Jones et al., 2010) and is unlikely to directly regulate the expression of _spf_ as the Vfr binding site (Kanack et al., 2006) does not appear in the _spf_ promoter region of DC3000 (data not shown). Moreover, catabolite repression in the pseudomonads is mediated by a different protein, Crc. In _P. putida_ this protein inhibits the expression of the OprB1 porin and other proteins that are responsible for assimilation of non-preferred carbon sources (Rojo, 2010). Unlike Crp in _E. coli_, Crc does not appear to be a DNA-binding protein but regulates expression post-transcriptionally by binding to the 5’-end of transcripts and inhibiting their translation (Moreno et al., 2007). Whether _spf_ co-regulates catabolite repression with Crc is not yet known, although a growth analysis of _spf_ and WT cells show no difference in media containing various carbon sources, such as fructose, glucose or mannitol (data not shown). The role of Spf in carbon source discrimination in the pseudomonads is therefore unclear and will require additional experiments to elucidate.

**ACKNOWLEDGEMENTS**

We thank Bryan Swingle for contributing to the design of the ΔrhoE mutant. We thank Bryan Swingle and Eric Markel for pBS58 and pBS59. Research support for Y. X. was provided by the Fredrick N. Gabler ’93 Memorial Research Endowment. The US Department of Agriculture (USDA) is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purposes of providing specific information and does not imply recommendation or endorsement by the USDA.

**REFERENCES**


control protein Crc is devoid of RNA binding activity. PLoS ONE 8, e6469.


homeostasis in either the planktonic or the sessile mode of growth. *MBio* 3, e00094-12.


Edited by: G. Preston