INTRODUCTION

*Serratia* spp. are Gram-negative enterobacteria, and can be opportunistic human, animal, insect and plant pathogens. *Serratia* sp. ATCC 39006 (S39006) produces two secondary metabolites, prodigiosin (Pig) and a β-lactam antibiotic, a carbapenem (1-carbapen-2-em-3-carboxylic acid; Car) (Coulthurst et al., 2005). Pig is a linear tripyrrole that confers the characteristic red pigment of colonies of this strain. It is a member of the prodigines, which are of clinical interest for their anticancer and immunosuppressant properties (Williamson et al., 2007). S39006 also produces the plant cell wall-degrading exo-enzymes pectate lyase and cellulase, which contribute to potato rot (Crow, 2001; Slater et al., 2003). It demonstrates a broad-host-range capacity for pathogenesis and is virulent in the animal *Caenorhabditis elegans* (Coulthurst et al., 2004).

The enzymes for Pig and Car production are encoded, respectively, by the *pigA*–*O* and *carA–H* biosynthetic operons (Slater et al., 2003; Thomson et al., 2000). A complex hierarchical regulatory network involving at least 20 genes regulates the biosynthesis of Pig and Car (Fineran et al., 2005b; Williamson et al., 2005). Environmental inputs, including quorum sensing (QS) (Slater et al., 2003) and phosphate and gluconate levels (Fineran et al., 2005a), are integrated through different genetic circuits to regulate secondary metabolism. QS is a mechanism by which bacteria regulate gene expression in response to population cell density via detection of a diffusible signalling molecule. The S39006 LuxIR-type QS system encoded by the *smalR* locus produces and detects N-acylhomoserine lactones (AHLs) (Poulter et al., 2011; Thomson et al., 2000).

We showed previously that deletion of the RNA chaperone Hfq abolished antibiotic production and attenuated virulence in animal and plant hosts, namely *C. elegans* and potato (Wilf et al., 2011). Hfq, a hexameric protein that forms a doughnut-like structure, is an RNA chaperone

The stationary phase sigma factor, RpoS, regulates the production of a carbapenem antibiotic, a bioactive prodigiosin and virulence in the enterobacterial pathogen *Serratia* sp. ATCC 39006

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*Serratia* sp. ATCC 39006 (S39006) is a Gram-negative bacterium that is virulent in plant (potato) and invertebrate animal (*Caenorhabditis elegans*) models. It produces two secondary metabolite antibiotics, a prodigiosin and a carbapenem, and the exoenzymes pectate lyase and cellulase. We showed previously that deletion of the RNA chaperone Hfq abolished antibiotic production and attenuated virulence in both animal and plant hosts. Hfq and dependent small RNAs (sRNAs) are known to regulate the post-transcriptional expression of rpoS, which encodes σ^S_, the stationary phase sigma factor subunit of RNA polymerase. An S39006 hfq deletion mutant showed decreased transcript levels of rpoS. Therefore, in this study we investigated whether the phenotypes regulated by Hfq were mediated through its control of rpoS. Whereas loss of Hfq abolished prodigiosin and carbapenem production and attenuated virulence in both *C. elegans* and potato, characterization of an S39006 rpoS mutant showed unexpectedly elevated prodigiosin and carbapenem production. Furthermore, the rpoS mutant exhibited attenuated animal pathogenesis, but not plant pathogenesis. Additionally, a homologue of the Hfq-dependent sRNA, RprA, was identified and shown to regulate prodigiosin production in a manner consistent with its role in positively regulating translation of rpoS mRNA. Combined, these results demonstrate that Hfq regulation of secondary metabolism and plant pathogenesis is independent of RpoS and establishes RpoS and RprA as regulators of antibiotic production.
that facilitates the complementary base-pairing between small RNAs (sRNAs) and target mRNAs. A well-studied example is the Hfq-dependent positive regulation of rpoS mRNA translation by the sRNAs DsrA, RprA and ArcZ in *Escherichia coli* and *Salmonella typhimurium* (Majdalani et al., 2002, 2005; Papenfort et al., 2009). Loss of hfq was shown to result in decreased rpoS transcript levels in *E. coli* (Muffler et al., 1996) and *S. typhimurium* (Brown & Elliott, 1996). The absence of Hfq and sRNA binding to the rpoS mRNA, which protects it from degradation by endonucleases (McCullen et al., 2010), results in enhanced degradation of the transcript. Likewise, an S39006 hfq deletion mutant resulted in decreased transcript levels of rpoS, which encodes rpoS, the stationary phase sigma factor subunit of RNA polymerase. RpoS is known to play an important role in the adaptation of the cell to general stresses such as carbon starvation, high temperature and oxidative stress (reviewed by Battesti et al., 2011). Expression of RpoS is highly controlled and increases during stationary phase growth. RpoS directly or indirectly regulates approximately 10% of the *E. coli* genome (481 genes) (Weber et al., 2005).

In this study we investigated whether the phenotypes regulated by Hfq were mediated through its control of rpoS. Loss of Hfq abolishes Pig and Car production and attenuates virulence in *C. elegans* and potato. However, surprisingly, characterization of an S39006 rpoS mutant revealed an increase in Pig and Car production and attenuation for animal, but not plant, pathogenesis. Additionally, a homologue of the Hfq-dependent sRNA, RprA, was identified and shown to regulate Pig production in a manner consistent with a role in positively regulating translation of rpoS mRNA. Combined, these results demonstrate that Hfq regulation of secondary metabolism and plant pathogenesis is independent of rpoS, and establishes rpoS as a regulator of antibiotic production.

## METHODS

### Bacterial strains, plasmids, phage and culture conditions.

Bacterial strains and plasmids are listed in Table 1. *Serratia* sp. ATCC 39006 strains were grown at 30 °C and *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) (per litre: 5 g yeast extract, 10 g Bacto tryptone, 5 g NaCl) or minimal medium [0.1% (w/v) (NH₄)₂SO₄, 0.41 mM MgSO₄, 0.2% (w/v) glucose, 40 mM K₂HPO₄, 14.7 mM KH₂PO₄, pH 6.9–7.1] at 200 r.p.m., or on LB medium supplemented with 1.5% (w/v) agar (LBA) (Miller, 1972). For certain experiments, minimal media were supplemented with 0.1% Casamino acids or Casamino acids depleted for vitamins (Sigma). *E. coli* β2163 cultures were supplemented with 300 μM 2,6-diaminopimelic acid. Plasmids were used to transform β2163 and then transferred by conjugation into S39006 (Demarre et al., 2005). Bacterial growth (OD₆₀₀) was measured in a Unicam Helios spectrophotometer. When necessary, media were supplemented with the antibiotics kanamycin (Km; 50 mg ml⁻¹), spectinomycin (Sp; 50 mg ml⁻¹), ampicillin (Ap; 50 mg ml⁻¹) and chloramphenicol (Cm; 25 mg ml⁻¹). The generalized transducing phage φOT8 was used for transduction of chromosomal mutations, as previously described (Thomson et al., 2000).

### DNA manipulations and bioinformatics analyses.

All DNA manipulations were performed as described by Sambrook et al. (1989). DNA was purified using DNA purification kits (Anachem) following the manufacturer’s instructions. Oligonucleotide primers used in cloning and genetic constructions are listed in Supplementary Table S1. DNA sequencing was performed at the DNA Sequencing Facility, Department of Biochemistry, University of Cambridge. Nucleotide sequence data were analysed using BLAST and CLUSTAL W (Altschul et al., 1997; Larkin et al., 2007).

### Plasmid constructs.

To complement the rpoS mutant and express S39006 RprA and *E. coli* DsrA from a plasmid vector, the native S39006 rpoS gene (or the *E. coli* orthologue) and the rprA and dsrA genes were cloned under the control of the IPTG-inducible T5 promoter (which has leaky expression) in pQE8000iT. The coding sequences of the genes were amplified by PCR from genomic DNA of S39006 or *E. coli* and cloned into pQE8000iT. PCR products amplified using primers for S39006 rpoS (oNMW82-R, oNMW83), *E. coli* rpoS (oNMW84-F/R), S39006 rprA (oNMW94-F/R) and *E. coli* dsrA (oNMW95-F/R) generated plasmids pNB43, pNB44, pNB49 and pNB50, respectively.

### Stress tolerance assays.

Susceptibility of S39006 strains to salt, ethanol and SDS was assayed as previously described (Wilf et al., 2011). Overnight cultures of S39006 strains were adjusted to an OD₆₀₀ of 1.0 and serially diluted to 10⁻⁶. Then, 10 μl of each dilution was spotted onto the surface of LB agar supplemented with either 2.5% NaCl, 2.5% ethanol or 0.1% SDS or unsupplemented (as a control). The number of surviving colonies (c.f.u. ml⁻¹) was determined after incubation for 48–56 h.

### Bioassays of Pig, Car, AHLs and motility.

*Bioassays for Pig and Car.* Assays for Pig and Car were performed as described previously (Slater et al., 2003). AHL assays were performed as described previously (Thomson et al., 2000), but using strain SP19 as the sensor (Poulter et al., 2011). Swimming and swarming motility assays were performed as previously described (Williamson et al., 2008). Liquid exoenzyme assays to measure the activity of secreted pectate lyase and cellulase were performed as described previously (Coulthurst et al., 2006).

### Transcriptional fusion assays.

Expression of the lacZ reporter gene was detected based on the fluorescent signal resulting from cleavage by β-galactosidase of the fluorogenic substrate 4-methylumbelliferone (MUG). Ten microlitres of a cell culture was transferred to a 96-well plate and frozen at −80 °C. Samples were thawed at room temperature, 100 μl of reaction mix (400 μg lysozyme ml⁻¹ and 1 × MUG in PBS) was added to each well and samples were mixed by pipetting. The final concentration of MUG was 0.125 mg ml⁻¹. Fluorescent signal (relative light units per second; RLU s⁻¹) was detected in a SpectraMax Gemini XPS fluorescence microplate reader (Molecular Devices) using the following settings: excitation 360 nm, emission 450 nm, read intervals 30 s min⁻¹ for 20 min, temperature 37 °C. β-Galactosidase activity was expressed as RLU min⁻¹ per OD₆₀₀ unit.

### Virulence assays.

*Virulence assays.* Potato-rotting assays were performed as described previously (Fineran et al., 2007). *C. elegans* virulence assays with strain DH26 [fer-15(b26)] were performed as described by Kurz et al. (2003). Survival curves were evaluated using Prism (version 5.0) and were considered statistically significantly different from each other at *P*<0.05 using the log-rank test (Mantel–Cox test).
Table 1. Strains, phage and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain, phage or plasmid</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, φ80AdlacZM15, Δ-lacZYA-argF)U169, endA1, recA1, hsdR17 (r&lt;sup&gt;K&lt;/sup&gt;, m&lt;sup&gt;K&lt;/sup&gt;), deoR, thi-1, supE44, Δ polar gyA96, relA1</td>
<td>Gibco-BRL</td>
</tr>
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<td>ESS</td>
<td>β-Lactam-super-sensitive indicator strain</td>
<td>Bainton et al. (1992)</td>
</tr>
<tr>
<td>β2163</td>
<td>(F&lt;sup&gt;−&lt;/sup&gt;) RP4-2-Tc::Mu ΔapA::(erm-pir) [Km&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt;]</td>
<td>Demarre et al. (2005)</td>
</tr>
<tr>
<td><strong>Serratia strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>ATCC 39006</td>
<td>WT (Car+, Pig+)</td>
<td>Bycroft et al. (1987)</td>
</tr>
<tr>
<td>LacA (WT)</td>
<td>Lac&lt;sup&gt;−&lt;/sup&gt; derivative of ATCC 39006, made by EMS mutagenesis</td>
<td>Thomson et al. (2000)</td>
</tr>
<tr>
<td><strong>Mutants derived from LacA</strong></td>
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<td></td>
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<tr>
<td>HSPIG67</td>
<td>pigP::mini-Tn5lacZI, Km&lt;sup&gt;R&lt;/sup&gt;; random transposon mutant</td>
<td>Fineran et al. (2005b)</td>
</tr>
<tr>
<td>MCP2L</td>
<td>pigA::mini-Tn5lacZI, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Slater et al. (2003)</td>
</tr>
<tr>
<td>MCA54</td>
<td>carA::mini-Tn5lacZI, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Thomson et al. (2000)</td>
</tr>
<tr>
<td>NMW7</td>
<td>rsmA::Tn-KRCPN1lacZI, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(N. Williamson, unpublished results)</td>
</tr>
<tr>
<td>NMW9</td>
<td>bfp::cat, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Will et al. (2011)</td>
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<td>NMW25</td>
<td>rpoS::Tn-uidA, cat, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>NMW26</td>
<td>rpoS::Tn-uidA, cat, Δbfp, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>NMW27</td>
<td>rpoS::Tn-uidA, cat, pigQ::mini-Tn5Sm/Sp, Cm&lt;sup&gt;R&lt;/sup&gt;, Sp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>NMW28</td>
<td>rpoS::Tn-uidA, cat, pigW::mini-Tn5Sm/Sp, Cm&lt;sup&gt;R&lt;/sup&gt;, Sp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>NMW29</td>
<td>rpoS::Tn-uidA, cat, pigP::mini-Tn5lacZI, Cm&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>NMW30</td>
<td>rpoS::Tn-uidA, cat, pigG::mini-Tn5lacZI, Cm&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>NMW32</td>
<td>rpoS::Tn-uidA, cat, carA::mini-Tn5lacZI, Cm&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;; red phenotype</td>
<td>This study</td>
</tr>
<tr>
<td>NMW33</td>
<td>rpoS::Tn-uidA, cat, rsmA::mini-Tn5lacZI, Cm&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>PIG17S</td>
<td>pigQ::mini-Tn5Sm/Sp, Sp&lt;sup&gt;R&lt;/sup&gt;; derivative of HSPIG17, constructed by transposon exchange</td>
<td>Fineran et al. (2005b)</td>
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<td>PIG62S</td>
<td>pigW::mini-Tn5Sm/Sp, Sp&lt;sup&gt;R&lt;/sup&gt;; derivative of HSPIG62, constructed by transposon exchange</td>
<td>Fineran et al. (2005b)</td>
</tr>
<tr>
<td>RAPL</td>
<td>rap::mini-Tn5lacZI, Km&lt;sup&gt;R&lt;/sup&gt;; derivative of RAPS, constructed by transposon exchange</td>
<td>Fineran et al. (2005b)</td>
</tr>
<tr>
<td>SP19</td>
<td>smaI::mini-Tn5Sm/Sp, pigX::Tn-DS1028, pigZ::mini-Tn5lacZI, Sp&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Poulter et al. (2010)</td>
</tr>
<tr>
<td><strong>Phage</strong></td>
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<tr>
<td>φOT8</td>
<td>Serratia generalized transducing phage</td>
<td>Evans et al. (2010)</td>
</tr>
<tr>
<td><strong>Vectors</strong></td>
<td></td>
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<tr>
<td>pQE80oriT</td>
<td>pQE80L (Qiagen) modified to contain the oriT site, medium-copy expression vector, IPTG-inducible T5 promoter, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Ramsay et al. (2011)</td>
</tr>
<tr>
<td>pNB43</td>
<td>S39006 rpoS gene cloned under the T5 promoter in pQE80oriT</td>
<td>This study</td>
</tr>
<tr>
<td>pNB44</td>
<td>E. coli rpoS gene cloned under the T5 promoter in pQE80oriT</td>
<td>This study</td>
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<tr>
<td>pNB49</td>
<td>S39006 rprA cloned into pQE80oriT</td>
<td>This study</td>
</tr>
<tr>
<td>pNB50</td>
<td>E. coli dstA cloned into pQE80oriT</td>
<td>This study</td>
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</table>

RESULTS

Pig and Car production is increased in an rpoS mutant

A mutant with a transposon insertion 533 bp after the start codon of rpoS (960 bp) was identified from a random transposon mutagenesis screen. The downstream gene encoding prolyl-tRNA synthetase is arranged convergently to rpoS. The rpoS::Tn mutation was transduced, via phage φOT8, into the lacA wild-type (WT) genetic background, generating mutant strain NMW25. An assessment throughout growth showed that the production of Pig and Car was elevated in the rpoS mutant, with Pig levels increased over twofold (Fig. 1a, b). This was consistent with an observed increased transcription of the pigA and carA genes of the Pig and Car biosynthetic clusters (although transcription of carA was only significantly elevated in stationary phase) (Fig. 1c, d). However, no change was observed for signalling molecule (AHL) production, or the rate of growth, although the rpoS mutant entered stationary phase 1 h earlier and reached a lower optical density (OD<sub>600</sub> 2.09) compared with the WT (OD<sub>600</sub> 2.36). Viable counts throughout growth showed no detectable differences, and there was no difference in the colony morphology of the rpoS mutant.

Expression of RpoS in trans downregulated Pig production to WT levels. Interestingly, expression of E. coli RpoS (90% identical to S39006 RpoS) resulted in enhanced down-regulation (Supplementary Fig. S1).
RpoS is a repressor of swimming and swarming motility

The impact of the loss of RpoS on swimming and swarming motility was examined. Compared with the parent strain, there was no change in the swimming motility of the rpoS mutant, but reduced motility was observed for the complemented strain expressing RpoS from a plasmid vector (Fig. 2a). S39006 WT does not swarm on 0.75% LBA containing Bacto agar, but the rpoS mutant displayed swarming motility (Fig. 2b). Attempts to complement the strain gave inconsistent results, with some replicates showing no swarming motility and others reduced swarming motility. This is probably due to some intrinsic variability in the assay coupled with the sensitivity of the assay conditions. However, taken together, these results show that RpoS downregulates swimming and swarming motility.

Role of rpoS in stress tolerance

E. coli RpoS has been shown to play a critical role in coordinating the expression of genes involved in withstanding various stresses, including oxidative stress and starvation (Dong & Schellhorn, 2010). Therefore, the rpoS mutant was investigated for its ability to withstand different stresses. Unlike the hsfq mutant, the rpoS mutant was unable to grow in minimal medium (MM) with glucose as the sole carbon source. However, colonies grew when cells were diluted in LB media and plated onto the MM plates, suggesting that the mutant was auxotrophic for...
essential amino acids. This was confirmed by supplementing the media with Casamino acids, which allowed normal growth of the rpoS mutant. It was also observed that addition of Casamino acids depleted for vitamins resulted in increased pigment production in both the WT and the rpoS mutants, suggesting that particular vitamins may suppress pigment production (Fig. 3). Additionally, when the rpoS mutant was plated out at high density on MM plates, spontaneous revertants arose that could grow on MM (data not shown).

Additional assays of stress-inducing factors showed no difference between the WT and the rpoS mutant for growth and survival on media supplemented with SDS (0.1 %) or ethanol (2.5 %) to test for the effect of membrane-perturbing agents. However, the rpoS mutant exhibited a five- to 10-fold growth reduction compared with WT on media supplemented with NaCl (2.5 %) to test for the effect of high osmolarity, a phenotype which was genetically complemented (Supplementary Fig. S2).

Finally, the ability of the rpoS mutant to withstand oxidative stress was tested using an H2O2 disc diffusion assay on a lawn of bacteria. Compared with the WT, the rpoS mutant showed a marginally larger halo of inhibition of growth from the H2O2, which could be complemented by the expression of rpoS in trans (Supplementary Fig. S3). This was in contrast to the much larger halo observed for the hfq mutant, and indicates that rpoS plays a small role in resistance to oxidative stress when compared with hfq.

The results of the different assays indicate that RpoS plays a role in withstanding nutrient-limiting conditions and osmotic stress.

The rpoS gene is epistatic to other regulators of Pig production

The interaction of rpoS with other factors involved in the wider regulatory network for secondary metabolism was explored. Double mutants of rpoS::Tn and various Pig regulations.
regulator transposon insertion mutants were generated by transduction. Pig production was measured in the double mutants and compared with that in the isogenic single mutants to determine whether the rpoS phenotype was suppressed. 

rpoS::Tn was found to bypass mutations in pigQ and pigW, which are part of a two-component system, and partially restore pigment production (Fig. 4). However, it was not able to bypass mutations in pigP, rap, rsmA and hfq (data not shown).

The sRNAs DsrA and RprA regulate Pig production

Previous research has shown that the E. coli rpoS mRNA contains an inhibitory stem–loop structure in the 5' untranslated region (UTR), occluding the ribosome-binding site from translation initiation (Brown & Elliott, 1997). Hfq facilitates the complementary base-pairing of three small regulatory RNAs, namely DsrA, RprA and ArcZ, to the 5' UTR, weakening the structure of the inhibitory stem–loop and resulting in increased rpoS translation (Majdalani et al., 1998, 2002; Soper et al., 2010). The E. coli rpoS mRNA has been shown to have an unusually long 567 nt 5' UTR, with transcription initiating midway within the upstream nlpD gene (Takayanagi et al., 1994). Likewise, nlpD precedes rpoS in S39006. A comparison of the hypothetical S39006 rpoS 5' UTR with the homologous region in E. coli showed a high degree of conservation, with complete identity for the overlapping DsrA/RprA/ArcZ interaction site in the mRNA, indicating that sRNA homologues in S39006 may positively regulate the translation of rpoS. sRNAs are located in intergenic regions, and a comparison of the genomic context of E. coli rpoS (105 bp) with the homologous region in S39006 identified a highly conserved region (111 bp), here referred to as S39006 rprA, with a similar predicted secondary structure (Fig. 5a, b). Interestingly, the 5' half of the sequence of S39006 rprA showed a high degree of variation, whereas the 3' half was nearly identical to its E. coli homologue. The RNAhybrid algorithm was used to predict potential RNA duplexes formed between S39006 RprA and rpoS mRNA, and identified two short pairing regions in RprA which overlapped with but differed from the pairing of E. coli RprA (Fig. 5c) (Rehmsmeier et al., 2004). A homologue for arcZ was also identified, but no homologue of dsrA was found after comparing the genomic context of E. coli dsrA and after scanning the S39006 genome for conserved sRNAs using Rfam (Gardner et al., 2009).

Nevertheless, the complete conservation of the sRNA interaction site in the S39006 rpoS 5' UTR indicated that E. coli DsrA could pair with S39006 rpoS mRNA and regulate its translation. To test this, the S39006 rprA and the E. coli dsrA sequences were cloned into plasmid vectors and expressed in trans in S39006 WT. Expression of RpoS in trans in WT repressed Pig production (Fig. 5d). Therefore, positive regulation of rpoS mRNA translation by DsrA/RprA should result in a suppression of pigment production. This was confirmed by the expression of DsrA/RprA in trans in the WT, which decreased pigment levels (Fig. 5d). Moreover, pigment production was nearly abolished by inducing the expression of the sRNAs from the plasmid vectors. However, growth of the WT strain at stationary phase was only marginally decreased by overexpression of the sRNAs, whereas overexpression of the rpoS CDS from the same plasmid vector caused a 10-fold reduction in growth (data not shown). This is explained by the current model in which the increased translation of rpoS mRNA by the sRNAs is limited by the fixed amount of the rpoS message naturally expressed in the cell, whereas expression of rpoS directly from the plasmid vector results in a much greater translation of the protein, thereby affecting the growth of the cell.

The rpoS mutant is attenuated for virulence in animal, but not plant, hosts

RpoS has been shown to regulate virulence factors in E. coli, S. typhimurium and several other species involved in both animal and plant pathogenesis (Dong & Schellhorn, 2010). The pathogenesis of the S39006 rpoS mutant was investigated using potato and C. elegans as model hosts. The rpoS mutant was significantly altered for production of the plant cell wall-degrading exoenzymes: decreased for cellulase but increased for pectate lyase activity (Fig. 6). Nevertheless, the altered production of exoenzymes for the rpoS mutant did not lead to any clear effect on plant virulence, as no obvious difference was detected in the level of potato rot compared with WT (data not shown).

However, the rpoS mutant was attenuated for virulence in C. elegans. The rate of killing by the rpoS mutant was
significantly reduced compared with that of the WT (Fig. 6). The survival median (representing the time required for 50% of nematodes to die) was 4 days for WT, 8 days for the rpoS::Tn strain, 7 days for the partially complemented rpoS::Tn strain, 9 days for the hfg::cat strain and 9 days for the conventional control strain, E. coli OP50 (OP50) (Kurz et al., 2003). Additionally, worms fed on WT were impaired for growth compared with rpoS mutant-fed worms, which reached the full adult size at the same rate as worms fed on OP50 (data not shown). Moreover, Pujol et al. (2001) have shown that C. elegans avoid lawns of the pathogenic bacterium Serratia marcescens. A similar

**Fig. 5.** sRNA pairing results in downregulation of pigment production. (a) The most energetically favoured structure of S39006 RprA predicted by mfold (Zuker, 2003). (b) An alignment of the E. coli and S39006 RprA DNA sequences was produced using CLUSTAL W. (c) A comparison of the pairing of the E. coli and S39006 RprA sRNAs with the section of the rpoS mRNA 5' UTR conserved in both E. coli and S39006. The region of the rpoS mRNA involved in sRNA pairing is from position −111 to −85 relative to the rpoS translation start. E. coli RprA–rpoS mRNA pairing is shown as described previously (Majdalani et al., 2002), and the S39006 RprA–rpoS mRNA pairing is based on the prediction by the RNAhybrid algorithm. E. coli RprA forms a duplex of 19 bp, while S39006 RprA is predicted to form a duplex of 20 bp differing from the E. coli pairing by 7 bp. S39006 and E. coli RprA nucleotides involved in pairing with rpoS are shown in red and blue, respectively. (d) Pig production was measured after 12 h of growth of the WT carrying different plasmid constructs expressing RpoS (pNB43), RprA (pNB49) or E. coli DsrA (pNB50). IPTG (+1) was added at the start of growth to induce overexpression of DsrA and RprA.
behaviour toward lawns of S39006 WT and the rpoS mutant, but not the hfq mutant, was observed while tracking worm survival.

**DISCUSSION**

The RpoS sigma factor plays an important role in regulating the stress response and pathogenesis in a variety of bacterial species (Dong & Schellhorn, 2010). In this study, the role of RpoS in the Gram-negative bacterium *Serratia* 39006 was investigated. In addition to affecting stress tolerance and virulence in animal but not plant hosts, RpoS was found to be a repressor of both swimming and swarming motility and to play a major role in regulating the production of two bioactive secondary metabolites, Pig and Car.

Unlike the hfq mutant, which could grow in nutrient-limiting conditions, the rpoS mutant could not be cultured in the same minimal medium, consistent with the observation that rpoS mutants in related bacteria have more stringent nutritional requirements (Lange & Hengge-Aronis, 1994; Mukherjee et al., 1998). It appears that the rpoS mutant is auxotrophic, as the addition of Casamino acids to the medium allowed growth of the strain. Interestingly, Casamino acids depleted for certain vitamins resulted in hyperpigmentation, indicating that vitamin metabolism influences Pig production. The rpoS mutant was also affected by osmotic stress.

The rpoS mutant was unchanged for swimming motility, but expression of rpoS in trans in the WT reduced swimming. The mutant showed increased swarming and this phenotype could be complemented. This is opposite to the phenotype of the hfq mutant, which showed reduced swarming and swimming motility. Similarly, the *Pseudomonas aeruginosa* rpoS mutant is a hyperswarmer, whereas the hfq mutant shows reduced swarming (Sonnleitner et al., 2003). Although swimming and swimming motility are both flagella-dependent, it is unclear why the S39006 rpoS mutant shows increased swarming but not swimming motility. *E. coli* RpoS was shown to negatively regulate FliA, the flagella sigma factor, explaining the increase in swimming motility in the *E. coli* rpoS mutant (Dong et al., 2011). S39006 RpoS is 91 % identical and 97 % similar to *E. coli* K-12 RpoS. Therefore, the negative regulation of FliA by *E. coli* RpoS may explain the decreased swimming motility in the S39006 WT expressing rpoS from a multicopy plasmid.

The key phenotype of the S39006 rpoS mutant was the clear increase of Pig and Car production, independent of an effect on AHL levels. Additionally, transcription of the pigA and carA biosynthetic genes was increased, reflecting pigA–O and carA–G operon expression. Studies from this laboratory have shown that multiple environmental signals sensed by various genetic factors are involved in regulating secondary metabolism, including QS, and gluconate and phosphate levels (Fineran et al., 2005a; Gristwood et al., 2009; Thomson et al., 2000). One of these genetic systems involved in regulating Pig production includes the PigQW two-component signal transduction system homologous to the BarA/UvrY system in *E. coli*. PigQW activity expression

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*Fig. 6.* The *rpoS* mutant is altered for exoenzyme production and attenuated for virulence in *C. elegans*. (a, b) Cellulase and pectate lyase activity in the culture supernatant of the WT and the *rpoS* mutant (NMW25) was measured after 17 h growth at 25 °C in Pel minimal broth PMB medium (Coulthurst et al., 2006). Bars represent the mean ± SEM from three independent experiments. (c) Kinetics of killing of *C. elegans* infected by S39006 WT, *rpoS*::Tn, complemented *rpoS*::Tn and *hfq*::cat. *pRpoS* refers to *pNB43*. Included for comparison is the survival of worms fed on the normal feeder strain *E. coli* OP50. Fifty worms of the temperature-sensitive strain DH26 were used for each bacterial strain and were grown at 25 °C using nematode growth medium plates.
of the sRNA RsmB, which interacts with the RNA-binding protein RsmA and prevents it from binding to and repressing target mRNAs (Williamson et al., 2008). Mutations in pigQ or pigW cause a significant decrease in pigment levels due to the inactivation of RsmB expression, leading to continued repression, by RsmA, of its mRNA targets, including those involved in Pig production. In E. coli, mutants deficient in barA or uvrY displayed reduced rpoS transcription (Hengge, 2008). If this function were conserved for pigQW in S39006 then it would be expected that decreased levels of rpoS would result in increased pigment levels, contrary to the observed result. Analysis of double mutants of rpoS with pigQ and pigW showed that an rpoS mutation could bypass mutations in pigQ and pigW and partially restore pigment production, indicating that rpoS may be regulated by pigQ and pigW. Therefore, it would be of interest to investigate in a future study the expression level of rpoS in the pigQ and pigW mutants and the interaction between these factors in regulating secondary metabolite production.

However, the rpoS mutation was not able to bypass mutations in the other Pig regulators pigP, rap, rsmA and hfq. It was surprising that the rpoS mutation did not bypass an hfq deletion. Hfq positively regulates the translation of rpoS mRNA by the dependent sRNAs DsrA, RprA and ArcZ in E. coli and S. typhimurium (Majdalani et al., 2002, 2005; Papenfort et al., 2009). The Hfq-mediated regulation of rpoS in S39006 was previously shown by the decreased rpoS transcript levels in an hfq mutant, and through the effects seen when E. coli Hfq binds to a section of the S39006 rpoS 5′ UTR containing the conserved sRNA pairing sites (Wilf et al., 2011). Moreover, homologs of RprA and ArcZ, but not DsrA, were identified in S39006. Expression of S39006 RprA and E. coli DsrA in trans in the WT resulted in decreased Pig production in a manner consistent with their role in positively regulating translation of rpoS mRNA, which downregulates Pig production (Fig. 7). Thus, it is surprising that despite a decrease in rpoS transcript levels, the hfq mutant was abolished for Pig and Car production and showed decreased swarming, whereas the rpoS mutant was upregulated for both secondary metabolism and swarming. Additionally, expression of rpoS in trans in the hfq mutant did not restore Pig production (data not shown). This indicates that Hfq-mediated regulation of secondary metabolism is independent of its effect on rpoS expression, and that additional Hfq-dependent factors are required for the activation of secondary metabolism.

It has been shown previously that S39006 kills C. elegans by an infection-like process of the intestine and that C. elegans avoids lawns of S39006 (Wilf et al., 2011). The rpoS mutant was increased for Pig production but attenuated for virulence in C. elegans, although to a lesser degree than the hfq mutant. This provides evidence that Pig does not appear to play a significant role in pathogenesis in this particular strain. An rpoS mutant of S. typhimurium was also attenuated for virulence in C. elegans (Labrousse et al., 2000). Moreover, it was observed that worms continued to avoid lawns of the S39006 rpoS mutant but not the hfq mutant, indicating that the factor inducing lawn avoidance is downregulated in the hfq but not the rpoS mutant. This factor was identified in the related strain S. marcescens Db10 as serrawettin W2, a secreted surfactant encoded by the swrA gene predicted to encode a non-ribosomal peptide synthase (Pradel et al., 2007). A BLAST search identified a homologue of swrA in S39006 preceded by a cyclic peptide transporter, suggesting that the product is synthesized and exported from the cell. Also, expression of the swarming-dependent rhlA gene involved in production of another surfactant (Williamson et al., 2008) has been observed to be elevated in mutants increased for swarming motility, such as the rpoS mutant. It may also be involved in inducing lawn avoidance behaviour in C. elegans. Thus, this study demonstrates that the rpoS mutant is attenuated for virulence in an animal host despite being increased for Pig production and swarming motility. RpoS has also been shown to be required for full virulence of Serratia entomophila in the New Zealand grass grub Costelytra zealandica (Giddens et al., 2000).

Nevertheless, the rpoS mutant was not attenuated for virulence in plants, unlike the hfq mutant, suggesting that Hfq regulates plant pathogenesis independently of its effect on rpoS expression. Interestingly, the activity of the plant cell wall-degrading exoenzymes was differentially regulated in the rpoS mutant: cellulase activity decreased and pectate lyase increased. However, an rpoS mutation in other plant pathogens results in differing impacts on pathogenesis. An rpoS mutant of Pectobacterium carotovorum subsp. carotovorum (formerly

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**Fig. 7.** Proposed model for RpoS regulation in S39006. The rpoS mRNA contains an inhibitory stem–loop region which is altered by Hfq-dependent RprA binding. Exposure of the RBS and initiation codon in rpoS results in increased translation of RpoS (σs). Expression of RpoS results in repression of Pig and Car production and motility. Repression is depicted by flat arrowheads and the dashed line indicates proposed regulation.
Erwinia carotovora subsp. carotovora) is more virulent for infection in celery and tobacco, but not potato, whereas mutants of the rice and tomato pathogens Burkholderia plantariorum andRalstonia solanacearum are attenuated for pathogenesis (reviewed by Dong & Schellhorn, 2010). A Serratia plymuthica rpoS mutant is decreased in production of exoprotease, endochitinase and the antibiotic pyrrolnitrin, and reduced for antifungal activity against Rhizoctonia solani and Pythium aphanidermatum (Ovadis et al., 2004).

In summary, this study demonstrates that RpoS regulates secondary metabolism and animal pathogenesis in S3906. It identifies a homologue of the Hfq-dependent RprA sRNA and demonstrates its ability to downregulate Pig production, consistent with its role in positively regulating rpoS translation. It also demonstrates that the Hfq-mediated regulation of secondary metabolism and plant pathogenesis is independent of Hfq regulation of rpoS translation.

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REFERENCES


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