The FloR master regulator controls flotation, virulence and antibiotic production in Serratia sp. ATCC 39006

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Introduction
Many aquatic bacteria use flagella to move rapidly and explore new environments. Flagellar rotation is an advantageous tool for horizontal and vertical movement. Directionality in flagellar motility is achieved by means of chemical, magnetic and light-dependent attraction (Wadhams and Armitage, 2004; Lefèvre and Bazylinski, 2013; Dieckmann and Mittelmeier, 2016). For instance, some bacteria swim towards carbon sources, downwards to the Earth’s magnetic pole, and towards, or away from, light-exposed environments (Kirschvink, 1980; Schimz and Hildebrand, 1985; Nutsch et al., 2003; Schweinitzer and Josenhans, 2010). Another mechanism for sustainable upward migration in prokaryotes is gas vesicle mediated flotation (Walsby, 1994; Pfeifer, 2012).

Gas vesicles are hollow intracellular protein structures permeable to gases, but impermeable to liquid. These structures are filled with any gases in the environment, lowering the density of cells and, thus, facilitating buoyancy. Gas vesicle synthesis in some bacteria may be sensitive to light, nitrogen, oxygen, phosphorus and potassium availability (Walsby, 1976; Allison and Walsby, 1981; Brookes and Gaf, 2001; Ramsay et al., 2011; Quintero-Yanes et al., 2019).

In contrast to our more detailed knowledge about flagella, the morphogenetic mechanisms and precise arrangement of proteins in gas vesicles remain ill-defined. Various studies have shown that a small hydrophobic protein, GvpA, is the main constituent of the macrostructure (Pfeifer, 2012). Other proteins, such as GvpF, GvpG, GvpJ, GvpK, GvpL and GvpM can be found as minor constituents in gas vesicles of the archaeon Halobacterium salinarum (DasSarma et al., 1994; Shukla and DasSarma, 2004). GvpF was also detected in gas vesicles of the cyanobacterium, Microcystis aeruginosa PCC 7806 (Xu et al., 2014). Most gas vesicle-producing bacteria also contain the hydrophilic protein GvpC (Walsby, 1994; Tashiro et al., 2016). This protein binds to the outer surface of the gas vesicle structure and is important for...
maintaining the shape and strength of the vesicles, preventing their collapse after pressure imbalances (Offner et al., 1996; Tashiro et al., 2016).

*Serratia* sp. ATCC 39006 (S39006) is the only member of the Enterobacteriaceae, to our knowledge, to produce gas vesicles naturally (Ramsay et al., 2011). In addition, S39006 displays diverse mechanisms of motility. It swims using flagellar rotation and swarms over semi-solid surfaces, aided by biosurfactant secretion (Williamson et al., 2008). This strain also produces potentially useful secondary metabolites, such as the carbapenem antibiotic, 1-carbapen-2-em-3-carboxylic acid (carbapenem carboxylate) and the red pigment, prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin) – a member of the prodigine class of bioactive molecules with antibacterial, antifungal, antiprotozoan, immunosuppressant and anti-cancer functionality (Coulthurst et al., 2005; Williamson et al., 2006). Furthermore, S39006 is virulent in plant and animal model systems; it rots potato tubers by producing plant cell wall degrading enzymes (PCWDEs), such as cellulase and pectinases, and kills the microscopic nematode, *Caenorhabditis elegans* (Coulthurst et al., 2004; Fineran et al., 2007; Wilf et al., 2011).

In S39006, gas vesicles are synthesized from a 16.7 Kb gene cluster comprising two contiguous operons (*gvpA1-gvpY* and *gvrA-gvcC*; Fig. 1A). The *gvpA1-gvpY* operon contains genes coding for gas vesicle structural proteins, including three *gvpA* homologues (*gvpA1*, *gvpA2* and *gvpA3*), one *gvpF* homologue (*gvpF1*), plus *gvpG*, *gvpK* and *gvcP*. This operon also contains genes affecting gas vesicle shape (*gvpN* and *gvpV*) and others that are apparently non-essential for buoyancy (*gvpX*, *gvpW* and *gvpY*; Tashiro et al., 2016). The *gvrA-gvcC* operon also contains other structural genes (*gvpF2* and *gvpF3*) and non-essential genes (*gvpH* and *gvpZ*). Furthermore, this operon contains three regulatory genes (*gvrA*, *gvrB* and *gvrC*) required for transcription of the *gvpA1-gvpY* operon (Ramsay et al., 2011; Tashiro et al., 2016). Bioinformatic analyses revealed that GvrA, GvrB and GvrC are two-component system proteins; GvrA and GvrC resemble response regulators, whilst GvrB is a cytoplasmic sensor kinase (Ramsay et al., 2011; Monson et al., 2016). Interestingly, overexpression of these cognate regulators reduced expression of the *gvpA1-gvpY* operon (Monson et al., 2016). Overexpression of GvrA and GvrB also led to elevated prodigiosin elaboration, indicating that the stoichiometry of these regulators is important for cell buoyancy and antibiotic production (Monson et al., 2016).

The diverse phenotypic traits of flotation, gas vesicle biogenesis, motility, virulence, and carbapenem and prodigiosin production in S39006 are modulated by various environmental inputs and are co-regulated by transcriptional and post-transcriptional regulators. Oxygen limitation stimulates expression of the *gvpA1-gvpY* operon, facilitating gas vesicle assembly and bacterial migration to, and persistence at, the air-liquid interface, thereby enabling access to oxygen (Ramsay et al., 2011). In contrast, potassium uptake via the low-affinity transporter, TrkH, downregulates the *gvpA1-gvpY* operon (Quintero-Yanes et al., 2019). TrkH controls cell buoyancy, turgor pressure, motility and prodigiosin production in S39006. Ribose availability also affects flotation. The sugar binds to the LacI-family transcription factor, RbsR (the ribose operon repressor) shown to be a highly pleiotropic regulator in S39006 (Lee et al., 2017). RbsR stimulates gas vesicle biogenesis, and prodigiosin, carbapenem, and cellulase production, whilst inhibiting flagellar motility. The mRNA binding protein RsmA promotes expression of both gas vesicle operons, while repressing prodigiosin production and motility (Ramsay et al., 2011; Hampton et al., 2016). In addition, the quorum sensing system (SmaI/SmaR) controls gas vesicle assembly, motility and secondary metabolite elaboration (Thomson et al., 2000; Fineran et al., 2005b; Williamson et al., 2008; Ramsay et al., 2011; Tashiro et al., 2016). In contrast to most LuxR-like quorum sensing receptors, SmaR acts as a repressor that inhibits transcription of target genes at low cell densities. After growth to high cell density, N-butanoyl-L-homoserine lactone (BHL, synthesized by SmaI) accumulates and binds to SmaR, leading to derepression of multiple biosynthetic and regulatory target genes (Thomson et al., 2000; Ramsay et al., 2011; Tashiro et al., 2016).

Additional regulators have been reported to control secondary metabolism, motility and virulence in S39006 – including transcription factors, transport proteins, two-component systems and post-transcriptional regulators (Thomson et al., 1997; Slater et al., 2003; Fineran et al., 2005a; Williamson et al., 2008; Wilf et al., 2011; Wilf and Salmond, 2012). However, fewer regulators have been reported thus far for cell buoyancy control. Here we describe a new regulator identified after random transposon mutagenesis and screening for gas vesicle-defective mutants. Two of the mutants contained transposon insertions in a gene (*floR*; flotation regulator) predicted to encode a previously uncharacterized DeoR-like transcriptional regulator. These *floR* mutants also showed modulated carbapenem and prodigiosin production, changes in swimming and swarming motility, and altered virulence. Proteomic interrogation experiments suggested that FloR had widespread physiological impacts, by affecting the production of a large number of proteins, including other pleiotropic regulators.
Results

Identification of a DeoR-family gas vesicle regulator, FloR

Gas vesicles accumulate and aggregate in the cytoplasm in late stages of growth and appear as phase-bright intracellular structures in individual cells observed by phase contrast microscopy (PCM; Walsby, 1994). In addition, due to light scattering, colonies of some bacteria containing gas vesicles can appear white opaque (Walsby, 1994). Because S39006 colonies are red, due to the production of prodigiosin, we used the prodigiosin mutant NWA19 (pigC) that exhibited the white opaque phenotype to facilitate facile mutant screening (Ramsay et al., 2011; Lee et al., 2017; Quintero-Yanes et al., 2019).

Random transposon mutagenesis was performed on NWA19 and over 18,000 transposon-containing colonies were screened for a translucent phenotype (those likely to be defective in gas vesicle formation). We identified 16 mutants with translucent colony phenotype containing transposon insertions in different loci. Most of the strains had insertions in genes previously defined as affecting gas vesicle production, including gvrA, gvpA1, gvpA2, gvpF1, gvpN, gvpV, gvpW, gvpZ and rbsK (Table S1). Our earlier work showed that in-frame mutants defective in gvrA, gvpA1, gvpA2, gvpF1, gvpN or gvpV did not produce full size gas vesicles, whilst transposon insertions in gvpW, gvpZ and rbsK had polar effects on downstream genes essential for gas vesicle biogenesis (Ramsay et al., 2011; Tashiro et al., 2016; Lee et al., 2017). Specifically, (see Fig. 1A) insertions in gvpW affected structural genes gvpA2, gvpA3 and gvpK from the gvpA1-gvpY operon, and insertions in gvpZ had polar impacts on the structural and regulatory genes, gvpF2, gvpF3, gvrB and gvrC from the gvrA-gvrC operon. Insertions in rbsK were polar on the pleiotropic regulatory gene, rbsR, from the ribose operon (Lee et al., 2017).

Two transparent colony mutants (AQY121 and AQY131, Table S1) contained insertions in an ORF annotated initially as Ser39006_3835 and predicted to encode an uncharacterized transcriptional regulator. This putative regulator contained an N terminal DeoR-like helix-turn-helix domain (Pfam PF08220) and a C terminal DeoR-like sensor domain (Pfam PF00455) (Fig. 1B and C). BLAST analyses showed that the predicted amino acid sequence encoded by Ser39006_3835 had low homology with other characterized DeoR-like regulators (Figs S1 and S2). Here we will refer to Ser39006_3835 as floR (floRation Regulator). This gene is the last in an operon composed of ORFs predicted to encode enzymes related to central metabolism, such as a 4-hydroxythreonine-4-phosphate
dehydrogenase (PdxA)-like protein (Pfam CL0270) and a four carbon acid sugar kinase family protein containing an N-terminal sugar binding domain (Pfam PF07005) and a C-terminal nucleotide binding domain (Pfam PF17042). Further bioinformatic analysis of the floR operon revealed that it is conserved among other enterobacteria, including D. solani and P. carotovorum (Figs S1B and S2). Also, it showed that genes in upstream and downstream operons are divergently transcribed, and are predicted to code for proteins related to central metabolism and transcription regulation (Fig. S1B and Table S2).

The floR mutant shows reduced gas vesicle production and cell buoyancy

Using phage øOT8, we transduced the floR mutation from AQY121 into both the NWA19 parent and wild type (WT) strains, and assessed colony and patch opacity to confirm the transposon phenotype. Colonies on Lysogeny-Broth Lennox Agar (LBA) plates showed that the pigC, floR::TnKRCPN1 transductant (AQY121A, Table 1) was translucent compared to NWA19 (Fig. 2A and B). Samples from patches analysed by PCM were scored for production of phase-bright intracellular organelles (mature gas vesicles) (Fig. 2C) and this showed that the AQY121A transductant lost phase brightness. FloR controlled formation and cell buoyancy because the floR mutation reduced gas vesicle production (Fig. 2D).

Colony translucency, loss of phase brightness and cell buoyancy are also found in mutants producing spindle-like gas vesicles, rather than cylindrical structures (Tashiro et al., 2016). Therefore, we analysed cells carrying the floR mutation by transmission electron microscopy (TEM) to further assess gas vesicle production. TEM analysis of WT and floR::TnKRCPN1 (AQY121B) cells revealed that only a few cylindrical gas vesicles were produced in the mutant (Fig. 2E). This confirmed that the floR mutant was severely down-regulated for production of morphologically-normal gas vesicles, with no evidence for spindle-like vesicle structures. However, unlike some other regulatory mutants (e.g. gvrA, gvrB, gvrC or rbsR) that lose gas vesicle production completely, as seen by TEM (Tashiro et al., 2016; Lee et al., 2017) the floR mutation, although important, was slightly less impactful on gas vesicle morphogenesis than the previously identified regulators.

To confirm that FloR regulated gas vesicle formation, we cloned the WT allele into an arabinose inducible expression vector (pBAD30) and assessed complementation of the mutation after ectopic expression. After induction with arabinose, AQY121B (pAQY2; pBAD30 + floR) cells were more opaque, contained phase bright structures and fully colonized a static water column, compared with AQY121B carrying the empty vector (Fig. 3A). This suggested that ectopic expression of floR complemented a floR mutation.

FloR controls gas vesicle production via transcriptional regulation of the gvpA1-gvpY and gvrA-gvrC operons

We transduced the transposon insertion in floR into strains GPA1 (gvpA1::uidA) and GRA (gvrA::uidA) reporter strains to assess the impact of the mutation on the expression of the gvpA1-gvpY and gvrA-gvrC operons. The β-glucuronidase activity in AQY121C (gvpA1::uidA, floR:: TnKRCPN1) and AQY121D (gvrA::uidA, floR::TnKRCPN1) showed that the floR mutation significantly down-regulated the expression of both gas vesicle operons (Fig. S3). Complementation experiments with AQY121C and AQY121D with pAQY2 under arabinose induction showed that ectopic expression of floR increased expression of the gvpA1-gvpY and gvrA-gvrC operons (Fig. 3B and C), confirming that FloR controlled expression of both operons at the transcriptional level. Moreover, it suggested that FloR activated gas vesicle production and cell buoyancy, in part, via modulating expression levels of the gvrA, gvrB and gvrC regulatory genes.

The floR mutant displays pleiotropic phenotypic changes

We assessed whether FloR was a pleiotropic regulator by measuring flagellar motility and production of the carbapenem and prodigiosin antibiotics in the mutant. Swimming and swarming assays in media with low agar concentrations showed that the floR mutation increased motility (Fig. 4A and B). Moreover, ectopic expression of floR (using pAQY2) reduced swimming motility of the mutant to WT levels (Fig. 4A) suggesting that FloR negatively regulates motility in S39006. Whilst conducting swimming assays, we noted pigmentation differences in swimming halos of the WT strain, the floR mutant with an empty vector and with pAQY2, indicating that the floR mutation may also affect prodigiosin production (Fig. 4A). This was confirmed by quantitation of pigment produced throughout growth of a complemented mutant (Fig. 4C). Expression of floR in AQY121C (pAQY2) restored prodigiosin production to that of GPA1. In addition, antibiotic sensitivity assays suggested that the production of the carbapenem antibiotic was significantly down-regulated in floR mutants (Fig. 4D) and this was confirmed by assessing carbapenem production using sterile culture supernatants. No significant antibiotic activity was observed in the floR mutant (Data not shown). Taken together, these results confirmed that the floR gene, FloR, was a pleiotropic regulator that affected secondary metabolism, in addition to gas vesicle morphogenesis.
### Table 1. Bacterial strains, phage, plasmids and oligonucleotides.

| Name | Bacteria | Genetic information | References |
|------|----------|---------------------|------------|
| WT | Serratia sp. | Lac^- (LacA), laboratory strain referred to as wild type | Thomson and colleagues (2000) |
| NWA19 | ATCC 39006 (S39006) | LacA, ΔpigC | Ramsay and colleagues (2011) |
| GPA1 | | LacA, gvpA::TnDS1028-uidA, Cm^R | Ramsay and colleagues (2011) |
| GRA | | LacA, gvpA::miniTn5Sm/Msp, Sm^R, Sp^R | Thomson and colleagues (2000) |
| LIS | | LacA, smal::miniTn5Sm/Msp, pgIX::Tn-DS1028, pigZ::miniTn5lacZ1, Sp^R, Cm^R, Km^R | Poulter and colleagues (2010) |
| SP19 | | LacA, rpoS::miniTn5Sm/Msp, pgIX::Tn-DS1028, pigZ::miniTn5lacZ1, Sp^R, Cm^R, Km^R | Poulter and colleagues (2010) |
| NW64 | | LacA, rsmA::TnDS1028-uidA, Cm^R | Hampton and colleagues (2016) |
| pigU::uidA | | LacA, pigU::TnDS1028-uidA, Cm^R | Lab stock |
| rsmB::uidA | | LacA, rsmB::TnDS1028-uidA, Cm^R | Lab stock |
| NMW25 | | LacA, rpoS::TnDS1028-uidA, Cm^R | Wilf and colleagues (2005) |
| MAS1 | | LacA, rap::miniTn5Sm/Msp, Sp^R | Slater and colleagues (2000) |
| AQY121A | | LacA, ΔpigC, floR::TnKRP1, derivative of NWA19 after transduction with floR grown in AQY121, Km^R | This study |
| AQY121B | | LacA, floR::TnKRP1, derivative of WT after transduction with floR grown in AQY121, Km^R | This study |
| AQY121C | | LacA, gvpA::TnDS1028-uidA, floR::TnKRP1, Cm^R, Km^R | This study |
| AQY121D | | LacA, gvpA::TnDS1028-uidA, floR::TnKRP1, Cm^R, Km^R | This study |
| AQY121E | | LacA, rsmA::TnDS1028-uidA, floR::TnKRP1, Cm^R, Km^R | This study |
| AQY121F | | LacA, rsmB::TnDS1028-uidA, floR::TnKRP1, Cm^R, Km^R | This study |
| AQY121G | | LacA, pigU::TnDS1028-uidA, floR::TnKRP1, Cm^R, Km^R | This study |
| AQY121H | | LacA, rpoS::TnDS1028-uidA, floR::TnKRP1, Cm^R, Km^R | This study |
| AQY121I | | LacA, rpoS::TnDS1028-uidA, floR::TnKRP1, Cm^R, Km^R | This study |
| AQY121J | | LacA, rpoS::TnDS1028-uidA, floR::TnKRP1, Cm^R, Km^R | This study |
| Escherichia coli | | (F^−) RP4-2-Tc::Mu dlapA::erm-pir Em^R | Demarre and colleagues (2005) |
| j2163 | | F− (80lacZM15 Δ[lacZYA − argF] U169 recA1 endA1 hsdR17 (rK^−, mK^−) phoA sup E44 λ − thi-1 | Life technology |
| Dh5α | | β− | Bainton and colleagues (1992) |
| ESS | | β-lactam supersensitive indicator strain | |
| floR | | Transducing phage for S39006 | Evans and colleagues (2010) |
| pKRP1 | | Derivative of pDS1028. This plasmid contains a composite transposon, Tn-KRP1, with aaph and a promoterless lacZ, Km^R, Tc^R | Monson and colleagues (2015) |
| pBAD30 | | Expression vector with araBAD promoter, Ap^R | Guzman and colleagues (1995) |
| pAQV2 | | pBAD30 carrying floR from WT | This study |
| pKRP1 | | Derivative of pDS1028. This plasmid contains a composite transposon, Tn-KRP1, with aaph and a promoterless lacZ, Km^R, Tc^R | Monson and colleagues (2015) |
| oMAMV1 | | GGAATTGACCGGTGGAAGTGGAC – Transposon specific oligonucleotide. | Matilla and colleagues (2012) |
| oMAMV2 | | GCATAAAGCTGCTCAATCAGAC – Transposon specific oligonucleotide | Matilla and colleagues (2012) |
| PF106 | | GACCCACGTCGACTGTCAGC – Random primed PCR oligonucleotide 1 | Fineran and colleagues (2005) |
| PF107 | | GACCCACGTCGACTGTCAGC – Random primed PCR oligonucleotide 2 | Fineran and colleagues (2005) |
| PF108 | | GACCCACGTCGACTGTCAGC – Random primed PCR oligonucleotide 3 | Fineran and colleagues (2005) |
| PF109 | | GACCCACGTCGACTGTCAGC – Random primed PCR oligonucleotide 4 | Fineran and colleagues (2005) |
| oAQ46 | | GATGAGCTCAGGATAACTGGAACG – floR F with SaeI restriction sequence | This study |
| oAQ47 | | CTATCTAGAACCAGGAAATTACATCCAC – floR R with PstI restriction sequence | This study |

**Proteomic analysis reveals that FloR is a master regulator**

Previous transcriptomic and proteomic experiments with pleiotropic regulator mutants, such as RsmA and Hfq, provided a holistic appreciation of their roles in some genetic networks of S39006 (Wilf et al., 2013). Given the wide range of phenotypic changes in a floR mutant, we chose to use a quantitative proteomic approach to further understand its effect in *Serratia*. We investigated the floR mutant proteome using TMT peptide labelling and LC–MS/MS analysis in samples taken in stationary phase of growth. A total of 2410 proteins were mapped,

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The mutation in floR results in loss of gas vesicles. Cell number normalized cultures of NWA19 and AQY121A (Table 1) were struck and spotted on LBA plates to produce colonies (A) and patches (B), respectively, and assess their opacity. C. PCM images of cells in patches in panel B were taken to assess gas vesicle formation and shape. Scale bars in PCM and TEM images correspond to 1 μm and 500 nm, respectively. The arrows indicate translucency of the culture associated with loss of cell buoyancy (D) or highlight gas vesicles in cells (E). [Color figure can be viewed at wileyonlinelibrary.com]

FloR controls rsmB and rpoS transcription

Considering the RsmA levels in the floR mutant proteomic profile, and that rsmA and floR mutants lose gas vesicle production capacity (Table 2, Figs 2 and 3; Ramsay et al., 2011), we considered the possibility that FloR positively controlled rsmA expression to regulate cell buoyancy. Therefore, we measured rsmA transcription in a reporter fusion strain carrying the floR mutation (Fig. 6A). Interestingly, the β-glucuronidase activity in NWA64 (rsmA::uidA) and AQY121E (rsmA::uidA, floR::TnKRCPN1) did not differ significantly. This result showed that FloR does not control rsmA transcription but suggested that FloR might regulate rsmA indirectly and post-transcriptionally (through other regulators). RsmA activity is antagonized by the small RNA, rsmB (the homologue of E. coli csrB) by occluding its 5′UTR binding site (Romeo., 1998; Williamson et al., 2008; Ramsay et al., 2011). Therefore, we measured the effect of the floR mutation on rsmB transcription. The transcription activity in double mutant strain AQY121F (rsmB::uidA, floR::TnKRCPN1) was significantly higher than in the rsmB::uidA single mutant (Fig. 6A).

We also used PCM to assess the formation of gas vesicles in floR, rsmA and floR, rsmB double mutants (Fig. 6B). As expected, strain AQY121E mimicked the single floR and rsmA gas vesicle phenotypes. Interestingly, gas vesicle production was lost in the double mutant AQY121F showing that the floR mutation suppressed the rsmB mutant phenotype. This implied that FloR promotes gas vesicle synthesis through regulators other than rsmB.

PigU and RpoS were identified in the proteomic data as up- and down-regulated, respectively, by the floR mutation (Table 2). Previous studies in enterobacteria indicate that RpoS is a positive regulator of RsmA, whilst PigU homologues are negative regulators of RpoS and rsmB (Mukherjee et al., 1998; Mukherjee et al., 2000; Peterson et al., 2006; Dong and Schellhorn, 2009; Wilf and Salmond, 2012). To investigate FloR-mediated regulation of the rsmA circuitry, we measured β-glucuronidase activity in pigU::uidA and rpoS::uidA reporter strains (Fig. 6A). Interestingly, activity of the pigU transcriptional fusion was unaltered whilst activity of the rpoS fusion decreased significantly in a floR mutant background. In addition, we observed gas vesicle formation by PCM in pigU::uidA (pigU), rpoS::uidA (NMW25), AQY121G and the proteomic data confirmed previous genetic and phenotypic observations; proteins involved in flagellar assembly and rotation, and biosurfactant production, were increased, whilst proteins encoded by gas vesicle, carbapenem and prodigiosin operons were reduced (Fig. 5 and Table S3).
AQY121H strains and noted that, compared to WT, the pigU mutant produced phase bright structures but not when combined with a mutation in floR. In contrast, rpoS and rpoS, floR mutants did not (Fig. 6B). Taken altogether, these results indicated that FloR also regulates PigU (apparently indirectly through post-transcriptional regulators) and RpoS to control gas vesicle production.

The mutation in floR attenuates virulence in S39006

Virulence of the rpoS mutant was attenuated in C. elegans assays (Wilf and Salmond, 2012). Because the proteomic data, transcription, and phenotypic assays suggested that FloR controlled RpoS abundance, we investigated the virulence of floR mutants in C. elegans (Fig. 7). The median survival of worms infected with the E. coli OP50 control strain was 9 days. In contrast, the WT S39006 strain killed 50% of the worms within 3 days and exhibited total lethality within 7 days. The floR mutant had an extended survival median of 2 days compared to that of the WT; half of the worm population was killed in 5 days and the entire population was eliminated in 9 days. This experiment confirmed that the floR mutant was less virulent than the WT. Moreover, as the floR

(A)

|       | WT (pBAD30) | AQY121B (pBAD30) | AQY121B (pAQY2) |
|-------|-------------|------------------|------------------|
| Ara % |             |                  |                  |
| 0     |             |                  |                  |
| 0.2   |             |                  |                  |

Fig 3. Ectopic expression of floR in the mutant restores gas vesicle formation and gene expression. A. PCM analysis of gas vesicle in cultures with and without arabinose induction. β-glucuronidase reporter activity throughout growth in GPA1 (B) and GRA (C) reporter strains carrying the floR mutation. B and C. The data represent the average ± standard deviation (error bars) of three biological replicates. ANOVA two-factor analysis of the β-glucuronidase reporter activity from 6 to 14 h of growth in GPA1 (pBAD30) and AQY121C (pBAD30) found F = 45.75 > Fcrit = 4.35, P = 1.40 × 10⁻⁶; in AQY121C (pBAD30) and AQY121C (pAQY2) F = 55.15 > Fcrit = 4.35, P = 3.60 × 10⁻⁷; in GPA1 (pBAD30) and AQY121C (pAQY2) F = 4.60 > Fcrit = 4.35, P = 0.044; in GRA (pBAD30) and AQY121D (pBAD30) found F = 92.74 > Fcrit = 4.35, P = 5.94 × 10⁻⁹; in AQY121D (pBAD30) and AQY121D (pAQY2) F = 24.35 > Fcrit = 4.35, P = 7.97 × 10⁻³; and in GRA (pBAD30) and AQY121D (pAQY2) F = 0.41 < Fcrit = 4.35, P = 0.53. [Color figure can be viewed at wileyonlinelibrary.com]
mutant was a phenocopy of the rpoS mutant, this suggested that the FloR regulator may control virulence via rpoS.

**Rap dominates antibiotic production in the FloR pathway**

All six regulators impacted by the floR mutation (Table 2) control prodigiosin biosynthesis. Mutations in pstS, rsmA and rpoS increase prodigiosin production, whilst mutations in pigT, pigU and rap result in loss of pigmentation compared to the WT (Thomson et al., 1997; Fineran et al., 2005a,b; Slater et al., 2003; Wilf and Salmond, 2012; Wilf et al., 2013). Increase of PigU and PigT, and reduction of RsmA, RpoS and PstS levels (as seen in the floR mutant proteomic profile) might be expected to lead to hyperproduction of prodigiosin (more details on each of these regulators are given in subsequent sections). Nonetheless, the mutation in floR actually resulted in pigment loss (Fig. 4C). We hypothesized that the reduction in prodigiosin production may be caused by inhibition of Rap, an activator of prodigiosin and carbapenem production that showed the lowest protein levels of the regulators identified (Table 2). Quantitation of prodigiosin production in WT, single mutants (floR, rap, rsmA and rpoS) and double mutants, floR (rap, rsmA and rpoS) showed the impact of mutations (single or in combination) on secondary metabolite production (Fig. 8). First, we noticed that floR, rsmA and floR, rpoS double mutants bypassed the prodigiosin phenotypes seen in the cognate single mutants. In contrast, the floR, rap double mutant and the single rap mutation showed similar low prodigiosin levels; significantly lower than the single floR mutant. These results confirmed that Rap is the dominant regulator in the FloR regulon and that the reduced prodigiosin production seen in floR mutants (compared to WT, and single rsmA and rpoS mutants) is caused by low levels of Rap.

The mutation in floR affects QS

The proteomic analysis showed that the floR mutant had reduced production of the inorganic phosphate (Pi) binding protein (PstS) of the PstSCAB-PhoU system, whilst other proteins (such as PstA, PstB and PhoB) appeared unaltered (Table 2). In high extracellular Pi concentrations, the Pi transport and response complex (PstSCAB-PhoU) inhibits activation of the PhoBR two-component system, whilst at low Pi concentrations PhoR autophosphorylates and drives phosphoryl transfer to PhoB to control expression of multiple genes (Hsieh and Wanner, 2010). In S39006, low Pi concentrations lead to activated PhoB which enhances expression of the QS autoinducer (BHL) synthase gene, smal, and the carbapenem and prodigiosin biosynthetic operons (Slater et al., 2003 and Gristwood et al., 2009). Consequently, mutations in pstSCAB-phoU pleiotropically mimic Pi-depleted conditions leading to elevated BHL, pigment and antibiotic production. Since the floR mutation down-regulated prodigiosin biosynthesis, we considered the possibility that the diminished PstS concentration in the floR mutant may not be a significant player in regulation of the pigment. Nevertheless, to corroborate the negative impact on PstS levels in the floR mutant, we assessed the production of the QS autoinducer throughout growth (Fig. 9). The floR mutant significantly overproduced BHL, as expected, and this QS signal production was higher at late exponential and stationary growth phase compared to WT.
In a floR mutant, production of the upstream gene products is elevated

Bioinformatic analysis of the peptides identified as those most up- and down-regulated in the proteomic profiling revealed that FloR is a global regulator controlling proteins with conserved domains related to central metabolic functions (Tables S3 and S4). First, we noticed that the proteins encoded by genes upstream of floR (Ser39006_3836 and Ser39006_3837) were upregulated and among those most highly expressed (Fig. 10A). In contrast, the levels of proteins encoded by genes downstream of floR were not altered. This suggested that FloR may repress its own operon by a negative feedback loop mechanism while regulating upstream genes, possibly by divergent transcriptional repression (Fig. 10B).

Other proteins identified among those most up-regulated in the mutant supported the idea that FloR is a global regulator controlling carbon metabolism and other functions, including cell buoyancy, motility and secondary metabolism. For instance, a glucose 1-phosphatase
homologue (NCBI ID: WP_021016594.1), the ribose dehydrogenase RbsD, the aerobic C4-dicarboxylate transporter DctA and various uncharacterised proteins with conserved domains related to carbohydrate hydrolysis, recognition and transport were abundant in the mutant (Table S4). In addition, proteins with miscellaneous predicted functions were down-regulated, including a 3-dehydro-L-gulonate-6-phosphate decarboxylase homologue, an uncharacterized amidase (NCBI ID: WP_021015566.1), and a protein chaperone of the small heat shock (HSP20) family (NCBI ID: WP_021015453.1) (Table S5).

Discussion

Screening gas vesicle mutants of S39006 led to the identification of a novel pleiotropic regulator (FloR). FloR is a DeoR-family protein and an important regulator of cell buoyancy, motility, virulence, and aspects of central and secondary metabolism. Members of the DeoR family are

Fig 6. Transcription activity of \( rsmA \), \( rsmB \), \( pigU \) and \( rpoS \) in \( floR \) mutants and gas vesicle formation in single and double mutants. A. The \( floR \) mutation alters \( rsmB \) and \( rpoS \), but not \( rsmA \) and \( pigU \) transcription. Black bars correspond to reporter fusions strains NWA64 (\( rsmA::uidA \)), \( rsmB::uidA \), \( pigU::uidA \) and NMW25 (\( rpoS::uidA \)), whilst green bars to reporter fusions carrying the \( floR \) mutation; AQY121E, AQY121F, AQY121G and AQY121H (Table 1). These data represent the average ± standard deviation (error bars), *\( P < 0.05 \) and **\( P < 0.01 \). B. PCM analysis of gas vesicle production in single and double mutants. Samples were taken from cells OD600 1.0 normalized liquid cultures. Scale bars correspond to 1 \( \mu \)m.
transcriptional repressors known for regulating many phenotypes in bacteria including infection, motility, antibiotic production and resistance, LPS synthesis, cell differentiation, osmotic and temperature stress response, in addition to aspects of carbon, fatty-acid, nucleotide, deoxynucleotide and peptide metabolism (Saxild et al., 1996; Hava and Camilli, 2002; Gaurivaux et al., 2000a,b; Hava et al., 2003; Ramos-Aires et al., 2004; Haine et al., 2005; Elgrably-Weiss et al., 2006; Ulanova et al., 2013; Wang et al., 2014; Turner et al., 2020). To our knowledge, this is the first report of a DeoR family regulator involved in biogenesis of gas vesicles and production of β-lactam and prodigiosin antibiotics.

FloR regulates gas vesicle production via transcriptional control of cognate regulatory genes gvrA, gvrB and gvrC. As with rsmA, rbsR and QS smal mutants, the floR mutant showed reduced transcriptional activity of the gvrA-gvrC operon (Ramsay et al., 2011; Lee et al., 2017). Proteomic analysis showed that, in addition to the gas vesicle proteins, RsmA abundance was negatively affected, whilst RbsR and Smal/SmaR were not detected. RsmA also inhibits flagellar motility and prodigiosin production in S39006 via negative control of the gvrA-gvrC operon (Ramsay et al., 2008; Will et al., 2013; Hampton et al., 2016). In a floR mutant, we observed increased RsmA levels and the high levels of motility related proteins. Additionally, we found that mutations in both floR or rsmA reduced gas vesicle production and up-regulated motility. Thus, we believe it is possible that FloR controls cell buoyancy via, RsmA-GvrABC, and motility via RsmA-FlhDC (Fig. 11A). We are now beginning to investigate the mechanism of FloR-dependent regulation of RsmA.

Proteins reported in other enterobacteria as part of the RsmA regulatory network, such as PigU and RpoS, were also altered in a floR mutant. In P. carotovorum, which contains a floR orthologue, RpoS positively controls rsmA transcription and the PigU-homologue (HexA) represses rsmB expression and RpoS protein levels (Mukherjee et al., 1998; Mukherjee et al., 2000). Our results suggested that the PigU-RpoS-RsmA/B circuitry functions differently in S39006. We showed that, despite high levels of PigU in the floR mutant, transcription of rsmB was higher than in the WT. Moreover, despite the altered PigU and RpoS levels, rsmA transcription was not
affected in the floR mutant. Considering that pigU mutants hyper-produced GVs and given that floR (rsmB, pigU and rpoS) double mutants lost GVs, FloR might regulate GVs independently via PigU-RpoS-GvrABC and rsmB-RsmA-GvrABC in S39006 (Fig. 11A).

RpoS regulates production of secondary metabolites oppositely to FloR (Fig. 8; Wilf and Salmond, 2012; Wilf et al., 2013). Our data indicated that the SlyA-like transcriptional regulator Rap, instead of RsmA or RpoS, governed antibiotic production in the FloR regulatory network (Fig. 11A and B). Rap is also a negative regulator of swarming motility (Williamson et al., 2008). Therefore, we suggest that, although Rap is a hierarchical regulator for antibiotic production, it also acts synergistically with RpoS and RsmA to downregulate swarming dependent of the FloR regulon (Fig. 11A and B).

In S39006, Rap is also under direct control of the inorganic Pᵢ sensing, transport and response system PstSCAB-PhoU, PhoB/PhoR and indirectly, via QS regulation (Gristwood et al., 2009). Because prodigiosin production and Rap abundance was reduced, despite low PstS levels, in the floR mutant, our data suggest that FloR controls Rap production independent of the PstSCAB-PhoU, PhoB/R and QS regulatory pathways (Fig. 11B). In contrast to Rap, but consistent with diminution of PstS, BHL production was up-regulated in the floR mutant (Fig. 9). BHL binding to the LuxR-family regulator, SmaR, derepresses transcription to promote motility, buoyancy and secondary metabolite production in S39006. Given the floR mutant phenotypes, it is likely that enhanced BHL production contributes to the observed hyper-motility. Also, since production of gas vesicles and prodigiosin were not completely abolished in the mutant (Figs 2E and 4C) BHL-dependent regulation in the FloR pathway may be also important for cell buoyancy and pigment production (Fig. 11B). This is a curious observation as initial work in S39006 postulated that gas vesicle production and swimming motility were inversely regulated (Ramsay et al., 2011). However, these data suggest that a more nuanced regulatory hierarchy exists where different regulators respond to a diversity of signals (Ramsay and Salmond, 2012).

PigT, a GntR-family transcription regulator was upregulated in the floR mutant proteomic profile (Table 2). A mutation in pigT results in pigment loss and reduced motility (Fineran et al., 2005a). FloR control of PigT may be important in augmenting negative regulation of motility, whilst limiting prodigiosin regulation (Fig. 11A). PigT-dependent transcription of the prodigiosin biosynthetic operon in S39006 is inhibited by gluconate, whilst the role of this carbon source in motility has not been investigated in detail (Fineran et al., 2005b). In E. coli,
gluconate induces expression of cognate metabolic enzymes by binding to GntR (Tong et al., 1996; Izu et al., 1997). Since our data indicate that PigT is negatively controlled by FloR, it is likely that FloR modulates the genetic response to gluconate in S39006 (Table S3). The altered production of proteins involved in central metabolism in the floR mutant (Table S3) is consistent with the idea that FloR is a global regulator connecting carbon metabolism with gas vesicle morphogenesis, and the production of flagella and prodigiosin and carbapenem antibiotics. The ribose operon regulator, RbsR, also connects carbon metabolism with cell buoyancy, motility, antibiotic production and virulence in S39006 (Lee et al., 2017). Moreover, the proteomic profile revealed that the ribose mutarotase, RbsD, which is transcriptionally regulated by RbsR, was detected among the most abundant proteins in the proteomic profile of the floR mutant (Table S4). This suggests that FloR may also control ribose metabolism, and possibly RbsR, linking the two regulatory networks together.

Bioinformatical analyses showed that the S39006 floR operon is conserved in other species of the Enterobacteriaceae family, such as D. solani, and P. carotovorum (Fig. S1). Furthermore, we identified FloR homologues in several bacterial species that are not plant pathogens and demonstrated that they form a discrete phylogenetic group, separate from DeoR in E. coli. Unlike S39006, these other bacterial species do not produce gas vesicles or prodigiosin. This suggests that FloR may have been coopted into regulating gas vesicles in S39006 yet can act as a regulator of other phenotypes in different bacteria.

DeoR-like transcription factors such as GlpR, SugR and UlaR, among others, are susceptible to allosteric modulation by phosphorylated metabolites, such as deoxyribose-5-phosphate, glycerol-3-phosphate, fructose-1-6 bisphosphate, fructose-6-phosphate, glucose-6-phosphate and L-ascorbate-6-phosphate (Mortensen et al., 1989; Gaigalat et al., 2007; Garces et al., 2008). These effectors control the DNA binding affinity of the regulators and, hence, their transcriptional activities. Moreover, these ligands are metabolic intermediates physiologically related to the operons and pathways controlled by the cognate DeoR-like regulators. Therefore, future experiments will try to define candidate FloR effectors and corresponding DNA binding motifs to further dissect the complex regulatory networks operating in S39006.

Methods

Bacterial strains, media and growth conditions

Bacterial strains are listed in Table1 and Table S1. S39006 strains were grown at 30 °C in either LB (10gl−1 tryptone, 5gl−1 yeast extract and 5gl−1 NaCl) for 16h or on LBA (LB+1.5% agar) solid medium for 48h. E. coli strains were grown in LB at 37 °C. E. coli p2163 cultures were supplemented with 200μgml−1 erythromycin (Em) and 0.3mM diaminopimelic acid (DAPA). Seed cultures were grown from single colonies inoculated into 30ml sealed universal tubes containing five ml of LB and

Fig 11. Model for the impacts of the FloR regulator in S39006. A. Model of FloR-dependent regulation via PigU-RpoS, rsmB-RsmA, and PigT. B. Model of FloR-dependent regulation via PstSCAB-PhoU-PhoB/R, QS (SmaI/R) and Rap under high inorganic phosphate concentrations (P) as these mutations were studied in complex media (LB). In both models, positive interactions are indicated by an arrowhead, whilst a perpendicular line indicates negative regulation and a line ending with roundhead, control by phosphorylation. Black lines indicate pathways that dominate the FloR regulatory network and determine the formation of gas vesicles, motility, carbapenem and prodigiosin production. In contrast, grey lines represent pathways that, although present in the network, have minor impacts on determining the phenotypes studied.
incubated on tube rollers overnight (14–16h). Growth assays were done in 250ml flasks containing 25ml of LB. Cultures were inoculated to an initial density of OD_{600} 0.05 and aerated by shaking at 215rpm. Flotation assays were performed with cell cultures inoculated to an initial density of OD_{600} 0.05 and grown for 24h on a tube roller. Then, the tubes were set upright and static for five days. Complementation experiments were performed by inducing gene expression with 0.2% arabinose. Plasmid maintenance was achieved by selection with 50μg/ml ampicillin (Amp).

Transposon mutagenesis and screen for GV mutants

Fresh overnight cultures of β2163 (pKRCPN1) and NWA19 were mixed at different volume ratios (1:1, 2:1, 3:1, 1:2) for transfer of pKRCPN1 via conjugation. This plasmid contains a promoterless lacZ gene, thus if insertions are in the correct orientation, a transcriptional fusion will be formed (Table S1). Selection of gas vesicle mutants was performed as indicated previously (Ramsay et al., 2011; Monson et al., 2015). The transposon insertion sites in transparent mutants were identified using random primed PCR (RP-PCR; Jacobs et al., 2003) with oligos oMAMV1, oMAMV2, PF106, PF107, PF108, PF109 as indicated by Fineran and colleagues (2005a) and Matilla and colleagues (2012).

Bioinformatic analysis

Artemis 16.0 (Carver et al., 2011) was used for identification of the transposon insertion sites. This information was obtained through nucleotide alignment with the genome sequence of S39006 (Fineran et al., 2013). The homologous proteins of FloR and the predicted protein products from upstream and downstream genes were analysed using the PSI-BLAST algorithm from NCBI (Waterhouse et al., 2019). Analysis of floR homologous, and upstream and downstream genes in different bacteria, was performed using the gene sequence viewer from NCBI. The identity and similarity of proteins was determined using the EMBOSS needle protein alignment tool (Li et al., 2015). To identify FloR homologues in other organisms, the amino acid sequence was examined by PSI-BLAST and the amino acid sequences chosen from the top 500 hits. These were aligned using ClustalOmega and a phylogenetic tree generated using JalView (Waterhouse et al., 2009; Sievers et al., 2011).

Bioinformatic analysis of proteomics data was performed by the Cambridge Proteomics Centre as follows: Proteome Discoverer v2.1 (Thermo Fisher Scientific) and Mascot (Matrix Science) v2.6 were used to process raw data files. Data were aligned with the UniProt S39006 database, in addition to using the common repository of adventitious proteins (cRAP) v1.0. Protein identification allowed an MS tolerance of ±10 ppm and an MS/MS tolerance of ±0.8 Da ppm along with permission of up to 2 missed tryptic cleavages. Quantitation was achieved by calculating the sum of centroided reporter ions within a ±2 millimass unit (mmu) window around the expected m/z for each of the four TMT reporter ions.

All comparative analyses were performed with the R statistical language (R Core Team, 2013). The R package MSnbase (Gatto and Lilley, 2011) was used for processing proteomics data. Briefly, this entailed missing value removal (instances where a protein was identified but not quantified in all channels were rejected from further analysis), log_{2}-transformation of the raw data, followed by sample normalization; utilizing the ‘diff. median’ method in MSnbase (this translates all sample columns so that they all match the grand median). Protein differential abundance was evaluated using the Limma package (Smyth, 2005). Differences in protein abundances were statistically determined using the Student’s t-test with variances moderated by Limma’s empirical Bayes method. P-values were adjusted for multiple testing by the Benjamini Hochberg method (Benjamini and Hochberg, 1995).

Phage transduction

Transduction of mutations into different S39006 strains was performed using phage OT8 following the protocol previously reported (Evans et al., 2010). Mutants were selected on LBA plates supplemented with either 25 μg ml⁻¹ kanamycin (Km) or 35 μg ml⁻¹ chloramphenicol (Cm) depending on the nature of the transposon used for mutagenesis and selection.

Microscopy

Phase contrast microscopy (PCM) images were obtained from cells grown overnight as patches on LBA. Samples were analysed under oil immersion using an Olympus BX-51 microscope with a 100X lens. Images were taken with a QICAM monochrome camera adapted to QCapture Pro-6 software. Transmission electron microscopy (TEM) images were obtained from cells in static liquid cultures prepared as reported previously (Quintero-Yanes et al., 2019). Cell images were obtained using a FEI Tecnai G2 TEM in the Cambridge Advance Imaging Centre, University of Cambridge.
β-Glucuronidase (UidA) reporter assays

Samples (100 μl) from growth assays of S39006 strains containing uidA gene fusions (Table 1) were taken at different time points and kept in 96 well microtiter plates at −80 °C until assays were performed. Samples were thawed at 37 °C and 10 μl aliquots were diluted into 90 μl LB and frozen again for no less than 30 min. Thereafter, diluted samples were thawed at room temperature and 10 μl were mixed with 100 μl of PBS (8 g l⁻¹ NaCl, 0.2 g l⁻¹ KCl, 1.15 g l⁻¹ Na₂HPO₄, 0.2 g l⁻¹ KH₂PO₄), 20 mg ml⁻¹ lysozyme (Sigma) and 250 μg ml⁻¹ 4'-Methylumbelliferyl-D-glucuronide (MUG, Melford laboratories) solution. The β-glucuronidase activity was quantified using a Gemini XPS plate reader following the parameters described in Ramsay et al., (2011). The activity at each time point was normalized to culture OD₆₀₀, and expressed as RFU min⁻¹ OD₆₀₀⁻¹.

pAQY2 plasmid construction and transformation

PCR amplification of floR from WT samples was performed with oligonucleotides oAQ46 (and oAQ47 for cloning into the arabinose inducible expression plasmid, pBAD30 (Guzman et al., 1995). PCR and plasmid DNA were double-digested using SacI and XbaI restriction enzymes, according to the manufacturer’s protocol (NEB®). Ligation reactions were performed with T4 DNA ligase (NEB®) following manufacturer’s recommendations to obtain pAQY2 (pBAD30 + floR). Reaction mixtures were used for transformations by electroporation and selected on LBA plates with Amp. pAQY2 construction was confirmed using Sanger DNA sequencing (Eurofins).

Phenotypic assays

S39006 strains were spotted on plates using 10 μl of cultures normalized to OD₆₀₀ 1.0 for assessment of carbapenem production, swimming and swarming motility as indicated previously (Slater et al., 2003; Fineran et al., 2005a; Williamson et al., 2008). Prodigiosin and QS signal (BHL) production was assessed using samples (1 ml) from growth assays taken at different time points, as reported before (Poultier et al., 2010). Prodigiosin was also assessed in WT, single floR, rsmA, rpoS, rap and double floR + rsmA, rpoS or rap mutants using 1 ml of fresh overnight cultures normalized to cell number OD₆₀₀ 1.0. C. elegans virulence assays were performed based on the methodologies developed by Kurz et al., (2003) and adapted to S39006 by Coulthurst et al., (2004).

TMT labelling and LC–MS/MS

Cultures for protein analysis were inoculated in 500 ml flasks with 50 ml LB for TMT-labelling. Samples were collected at 16 h, normalized to 2.0 at OD₆₀₀, pelleted at 8000 g and 4 °C, and resuspended in 1.25 ml with CHAPS lysis buffer containing 1X protease inhibitor cocktail set I (Calbiochem; Coulthurst et al., 2006). The lysis solution was kept on ice and sonicated for 10s × 6 cycles (60s off per cycle). Cell debris and insoluble material were pelleted at 13,000 g and 4 °C. Supernatants containing soluble protein extracts were transferred to pre-chilled tubes kept at 4 °C.

The amount of proteins in the samples was quantified with the DCTM (detergent compatible) Protein Assay from Bio-RAD following the manufacturer’s instructions. Thereafter, 100 μg of proteins was diluted with six times (volume) of ice-cold acetone and kept at −20 °C overnight for precipitation. Samples were sent to the Cambridge Centre for Proteomics (CCP) for analysis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Appendix S1. Supporting Information.