CRISPR-Cas gene-editing reveals RsmA and RsmC act through FlhDC to repress the SdhE flavinylation factor and control motility and prodigiosin production in Serratia

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SdhE is required for the flavinylation and activation of succinate dehydrogenase and fumarate reductase (FRD). In addition, SdhE is conserved in proteobacteria (α, β and γ) and eukaryotes. Although the function of this recently characterized family of proteins has been determined, almost nothing is known about how their genes are regulated. Here, the RsmA (CsrA) and RsmC (HexY) post-transcriptional and post-translational regulators have been identified and shown to repress sdhEygfX expression in Serratia sp. ATCC 39006. Conversely, the flagella master regulator complex, FlhDC, activated sdhEygfX transcription. To investigate the hierarchy of control, we developed a novel approach that utilized endogenous CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated) genome-editing by a type I-F system to generate a chromosomal point mutation in flhC. Mutation of flhC alleviated the ability of RsmC to repress sdhEygfX expression, whereas RsmA acted in both an FlhDC-dependent and -independent manner to inhibit sdhEygfX. Mutation of rsmA or rsmC, or overexpression of FlhDC, led to increased prodigiosin, biosurfactant, swimming and swarming. Consistent with the modulation of sdhE by motility regulators, we have demonstrated that SdhE and FRD are required for maximal flagella-dependent swimming. Together, these results demonstrate that regulators of both metabolism and motility (RsmA, RsmC and FlhDC) control the transcription of the sdhEygfX operon.

INTRODUCTION

Serratia sp. ATCC 39006 is a member of the Enterobacteriaceae that was isolated from a salt marsh (Bycroft et al., 1987), and is a model bacterium for the study of the biosynthesis and regulation of antibiotics, particularly prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin) (Williamson et al., 2006). There is pharmaceutical interest in the red tripyrrole prodigiones due to their anticancer, immunosuppressant, antimicrobial and antimalarial properties (Williamson et al., 2006, 2007). We have demonstrated that an interconnected regulatory network controls the biosynthesis of prodigiosin, which responds to various cues, including bacterial cell density through quorum sensing (Fineran et al., 2005b; Slater et al.,...
metabolism\textsuperscript{A}), activation of SDH and FRD enzymes, there is a paucity of widespread SdhE/Sdh5 proteins in the flavinylation and activation of SDH and FRD, respectively. We demonstrated that SdhE is required for the flavinylation and activation of the complex II enzymes succinate dehydrogenase (SDH) and fumarate reductase (FRD) – key enzymes in oxidative phosphorylation and the TCA cycle (McNeil et al., 2012, 2014). Under aerobic conditions, SDH donates electrons to the electron transport chain during the oxidation of succinate to fumarate (Maklashina et al., 2013). For catalysis, the SdhA subunit of SDH requires a covalently bound FAD co-factor (Blaut et al., 1989; Cecchini et al., 2002; Yankovskaya et al., 2003). FRD catalyses the reverse reaction to SDH – the anaerobic reduction of fumarate to succinate. FRD also requires an FAD co-factor within the FrdA subunit (Blaut et al., 1989; Iverson et al., 1999). It was previously thought that FAD attachment was autocatalytic. However, the discovery and characterisation of SdhE demonstrated that SdhE directly interacts with SdhA and FrdA, and is required for the covalent attachment of FAD and the subsequent activation of SDH and FRD (McNeil et al., 2012, 2014). Interestingly, SdhE is conserved in α, β and γ-proteobacteria in addition to eukaryotes, where it is a nuclear-encoded mitochondrial protein termed Sdh5/SdhAF2 (Hao et al., 2009; Huang et al., 2013; Kim & Winge, 2013; McNeil & Fineran, 2013).

Despite our increasing understanding of the function of the widespread SdhE/Sdh5 proteins in the flavinylation and activation of SDH and FRD enzymes, there is a paucity of information about how SdhE genes are regulated. Previously, we showed that sdhE and ygX were co-transcribed and expressed at similar levels during aerobic or anaerobic growth, which is consistent with both SDH and FRD requiring flavinylation by SdhE (McNeil et al., 2012, 2014). In this study, we show that the DNA-binding master transcriptional activator of flagella biosynthesis, FlhDC, promoted sdhEygfX expression. Furthermore, a post-translational anti-FlhDC factor, RsmC (regulator of secondary metabolism C), strongly reduced sdhEygfX expression by acting through FlhDC. A post-transcriptional mRNA-binding protein, RsmA (regulator of secondary metabolism A), also reduced sdhEygfX levels, but through both FlhDC-dependent and -independent routes. In addition to their role in sdhEygfX regulation, RsmA, RsmC and FlhDC exhibited co-ordinate control of motility and prodigiosin production. Consistent with the regulation of sdhEygfX by proteins that control metabolism and motility, SdhE controlled metabolism through SDH and FRD (McNeil et al., 2012, 2014), and with FRD was required for maximal flagella-dependent swimming. Finally, to assist our genetic analyses we developed, and describe here, a novel method for genome-editing in bacteria that uses an endogenous type I-F CRISPR (clustered regularly interspaced short palindromic repeats)-(Cas (CRISPR associated) system to generate chromosomal point mutations.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids used in this study are listed in Tables S1 and S2 (available in the online Supplementary Material), respectively. Serratia sp. ATCC 39006 (Fineran et al., 2013) and Escherichia coli strains were grown at 30 and 37 °C, respectively. Bacteria were grown in lysogeny broth (LB) (5 g yeast extract l\(^{-1}\), 10 g bacto tryptone l\(^{-1}\) and 5 g NaCl l\(^{-1}\)), minimal medium (0.1 %, w/v, (NH\(_4\))\(_2\)SO\(_4\); 0.41 mM MgSO\(_4\); 0.2 %, w/v, glucose; 40 mM KH\(_2\)PO\(_4\); pH 6.9–7.1) at 180 r.p.m., or on LB agar (LBA) (LB supplemented with 1.5 %, w/v, agar) (Miller, 1972). Growth (OD\(_{600}\)) and absorbance were measured in a Jenway 6300 spectrophotometer. When required, media were supplemented with antibiotics at final concentrations as follow: kanamycin, 50 µg ml\(^{-1}\); ampicillin, 100 µg ml\(^{-1}\); streptomycin, 50 µg ml\(^{-1}\); and chloramphenicol (Cm), 25 µg ml\(^{-1}\). Unless noted otherwise, experiments were carried out at least in biological triplicates. For statistical analysis, either one-way ANOVA with a Dunnet post-test, or unpaired \(t\)-tests, were used. A \(P\) value less than 0.0001 is indicated by ****, less than 0.001 by *** and less than 0.05 by *.

**Movement of mutations via generalized transduction.** When required, mutations were moved by generalized transduction between strains to generate single, double and triple mutants. For these transductions, phage \(\Phi\)O18 was used, as described previously (Evans et al., 2010). The genetic nature of transductants was confirmed by antibiotic-resistance profile and PCR.

**Transposon mutagenesis.** Random transposon mutagenesis of Serratia 39006 strain HSPIG46 (sdhEygfX::mini-Tn5lacZI) (Fineran et al., 2005b) was performed by conjugation with E. coli BW20767 harbouring the Tn-DS1028uidA delivery plasmid pDS1028uidA (Ramsay et al., 2011). Cultures of the sdhEygfX::mini-Tn5lacZI mutant and E. coli BW20767 donor were grown overnight in LB and 20 µl of each was mixed, pelleted by centrifugation, resuspended in 40 µl LB, spotted onto LBA containing kanamycin, Cm and X-Gal (30 µg ml\(^{-1}\)). By using this X-Gal screen on plates, transposon mutants were identified that caused altered sdhEygfX expression. Transposon insertion mutations were moved into a clean sdhEygfX::lacZ background via generalised transduction as described above.
RsmA, RsmC and FlhDC regulate sdhEygF\textit{X} in \textit{Serratia}

quantitative assessment of \textit{sdhEygF}\textit{X}::\textit{lacZ} expression throughout growth in different mutant backgrounds, standard \(\beta\)-galactosidase assays were used as described previously and expressed as Miller units (MU) (Przybilski \textit{et al}, 2011).

\textbf{Arbitrary PCR.} Transposon insertion sites of mutants of interest were mapped using arbitrary PCR, as described elsewhere (Fineran \textit{et al}, 2005a; Jacobs \textit{et al}, 2003). Briefly, PCR was performed on colony DNA using a random primer mix (PF106, PF107 and PF108) and a Taq-DS1028\textit{r6\alpha}\textit{dA} specific primer (PF225 or PF338, hybridizing at either end of the transposon). All oligonucleotides used in this study are shown in Table S3. A second PCR was then performed on a 2 \(\mu\)l aliquot of undeleted purified DNA from the first PCR with an adapter primer (PF109) that binds to the 5’ ends of PF106-PF108, and a nested Taq-DS1028\textit{r6\alpha}\textit{dA} specific primer (either PF226 or PF294). The resulting mix of PCR fragments was purified and sequenced with the nested Taq-DS1028\textit{r6\alpha}\textit{dA} specific primer. The Taq-DS1028\textit{r6\alpha}\textit{dA} insertion site and orientation were determined by aligning with the \textit{Serratia} 39006 genome (Fineran \textit{et al}, 2013).

\textbf{Phenotypic assays.} Prodigiosin production was assessed as previously described (Slater \textit{et al}, 2003). For complementation studies of prodigiosin production, plasmid expression was induced at time zero with 1 mM IPTG and assays were performed after 12 h growth. For swimming assays, bacterial cultures were grown overnight in 5 ml LB. The OD\textsubscript{600} was adjusted to 0.2 and 3 \(\mu\)l was spotted onto tryptic swimming agar (5 g NaCl l\(^{-1}\); 10 g tryptone l\(^{-1}\); 0.3 \%, w/v, agar) plate. Plates were incubated at 30 \(^\circ\)C for 16 h, and swimming measured by the area of the swimming halo. Swarming was assessed as described previously and measured by the swarm area (Williamson \textit{et al}, 2008). To measure surfactant production, overnight cultures of bacteria were adjusted to an OD\textsubscript{600} of 0.2 and 5 \(\mu\)l spotted onto LBA plates solidified with 0.75 \% (w/v) agar. Plates were incubated for 16 h at 30 \(^\circ\)C and surfactant production determined by the diameter (mm) of the clear ring surrounding the bacterial colony (Williamson \textit{et al}, 2008). For complementation assays of swimming, swarming and surfactant production, plasmid expression was induced by the addition of 1 mM IPTG to appropriate plates. For \(\beta\)-galactosidase assays on complemented strains, appropriate cultures were grown overnight in 5 ml LB. The OD\textsubscript{600} was adjusted to give a starting OD\textsubscript{600} of 0.02 in 25 ml LB with 1 mM IPTG. Strains were grown at 30 \(^\circ\)C at 180 r.p.m., and OD\textsubscript{600} and \(\beta\)-galactosidase activity measured at 12 h.

\textbf{Generation of RsmC, RsmA and FlhDC expression plasmids.} Plasmids for expression of RsmC, RsmA and FlhDC were constructed as follows. Firstly, the genes were amplified by PCR using \textit{Serratia} 39006 genomic DNA as a template and primer pairs PF786 and PF787 for \textit{rsmC}, PF788 and PF789 for \textit{rsmA}, and PF796 and PF797 for \textit{flhDC}. The forward primers contained a ribosome-binding site and EcoRI sites. The reverse primers contained \textit{HindIII} sites, except PF797, which had an \textit{XmaI} site. PCR products were digested with the appropriate enzymes and ligated to pQE-80LooriT that had been previously cut with the same endonucleases. \textit{E. coli} DH5\(\alpha\) was transformed with the ligations and plasmids were verified by sequencing. Plasmids were introduced into \textit{Serratia} 39006 by conjugation using either \textit{E. coli} SM10 Ap\(\beta\)ir or S17-1 Apir donors and minimal medium or appropriate antibiotics to counter-select the donors. The nature of the transconjugants was confirmed by antibiotic-resistance testing and PCR.

\textbf{Construction of a \textit{flhDC\textit{::Cm}} mutant.} The \textit{flhDC\textit{::Cm}} deletion plasmid (pPF39S) was made by overlap extension PCR. Using \textit{Serratia} 39006 genomic DNA as a template, the left hand fragment contained a 3’ 20 bp sequence that overlapped the 5’ end of the \textit{Cm}-resistance cassette, whilst the right hand fragment contained a 5’ 20 bp sequence that overlapped the 3’ end of the \textit{Cm}-resistance cassette. The following primer pairs were used to construct the fragments: left hand fragment, PF817 + PF1289; right hand fragment, PF822 + PF1299. The Cm-resistance cassette was constructed using PF432 + PF433, with pTRB32 as a template. All three fragments were cloned into pPF39S with BamHI and XbaI, cloned into pBluescript II KS\textsuperscript{+} and confirmed by sequencing. Deletion constructs were cloned from pBluescript II KS\textsuperscript{+} into pKNG101 using BamHI and XbaI. Deletion mutants were generated using an allelic exchange strategy with a sucrose selection protocol similar to that described elsewhere (Fineran \textit{et al}, 2005a; Kaniga \textit{et al}, 1991). Putative deletion mutants were sucrose resistant, \textit{Cm} resistant, non-motile on tryptic swimming agar, and were confirmed by PCR and sequencing.

\textbf{Construction of an \textit{flhC} point mutant using endogenous CRISPR-Cas targeting.} The \textit{Serratia} 39006 strain contains a type I-F CRISPR-Cas system (Fineran \textit{et al}, 2013). A plasmid (pPF704) for the expression of a CRISPR RNA (crRNA) designed to target \textit{flhc} was constructed using primers PF6163 and PF6140. PF6163 had a 19 bp sequence at the 3’ end that overlapped the 3’ end of PF6140. This overlap generated a 106 bp PCR product that contained two repeats of the \textit{Serratia} 39006 type I-F system separated by a 32 bp spacer targeting an internal region of the \textit{flhc} gene. The targeted protospacer region was chosen based on a GG protospacer adjacent motif (PAM) consensus. The product was digested with EcoRI and SalI, ligated into pBAD30 and confirmed by sequencing. The vector was used to transform chemically competent \textit{Serratia} HSP4246 (sdhEyg\textit{F}::mini-Tn\textit{5lacZ}21) and \textit{ΔflhDC::Cm} strains. Following heat shock, cells were recovered in, and plated on, LB and LBA, respectively, both supplemented with glucose (0.2 \%, w/v) to repress expression of the targeting plasmid. Transformants containing the targeting plasmid were grown for ~16 h in 5 ml LB with glucose (0.2 \%, w/v) to repress or arabinose (0.1 \%, w/v) to induce the crRNA expression required for chromosomal targeting. Surviving colonies that were potential mutants were screened by PCR using PF817 and PF822. One strain was selected for further work and the \textit{flhc}-targeting plasmid was cured by growth without antibiotic selection, resulting in strain PCF185. The \textit{flhc} mutant did not swim and could be complemented with plasmid-encoded \textit{flhDC} (pPF3516). Complementation allowed generalized transduction by the flagellum-dependent phage, \textit{PHO8}, of further mutations into the \textit{flhc} mutant. Resulting strains were cured of pPF3516.

\textbf{Cryo-electron microscopy.} A 4 \(\mu\)l aliquot of overnight culture grown in LB containing the strains analysed [WT and \textit{rsmC}\textit{RsmC} (PFC174)] was applied to a glow-discharged Quantifoil 2/2 grid (Quantifoil Micro Tools), blotted and frozen in liquid ethane using a KF80 plunge freezing device (Reichert). Grids with the frozen specimen were loaded into a 914 cryo holder (Gatan) and viewed using a 2200FS cryo transmission electron microscope (JEOL) with an omega filter. Zero-loss images were recorded at microscope magnifications of either \(×8000\) or \(×15000\) using SerialEM software (University of Boulder, Colorado, USA) controlling a TVIPS F416 camera (Tietz Video and Image Processing Systems).

\textbf{RESULTS}

\textbf{Identification of regulators of the \textit{sdhEygF}\textit{X} operon.} To identify genes that affect the expression of \textit{sdhEygF\textit{X}}, a random transposon mutagenesis was performed in \textit{Serratia} 39006 that contained a chromosomal \textit{sdhEygF}\textit{X}::\textit{lacZ}\textit{2} transcriptional fusion. Mutants were screened for altered \(\beta\)-galactosidase activity relative to the control strain, and mutants of interest were identified by arbitrary PCR and

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sequencing. Transposon insertions that affected *sdhEygfX* expression were mapped to two distinct genomic regions – upstream of *rsmA* (regulator of secondary metabolism A) (Williamson et al., 2008), also known as *csrA* (carbon storage regulator A) in *E. coli*, and with a gene similarity to *rsmC* (regulator of secondary metabolism C) from Pectobacterium carotovorum and Pectobacterium atrosepticum (formerly *Erwinia carotovora* subsp. carotovora and subsp. *atrosepticum*, respectively) (Cui et al., 1999; Shih et al., 1999). The *rsmC* gene has also been alternatively termed *hexY* (hyperproduction of exoenzymes Y) in *P. atrosepticum* (Bowden et al., 2013; Shih et al., 1999), but is unrelated to the *rsmC* RNA methyltransferase gene of *E. coli*. The analyses of these mutants are discussed in more detail in the following sections.

**RsmA represses *sdhEygfX* expression**

In *Serratia* 39006, RsmA is a pleiotropic regulator, and mutation of *rsmA* results in increased prodigiosin synthesis, swimming and surfactant production (Williamson et al., 2008). One transposon insertion mapped 206 bp upstream of *rsmA* (denoted as *rsmA*pro) and caused an increase in *sdhEygfX* expression (Fig. S1). The transposon insertion in the *rsmA*pro mutant disrupted or reduced the production of RsmA, since the increased prodigiosin phenotype was consistent with the elevated pigment in an *rsmA* mutant (Williamson et al., 2008) and could be complemented by plasmid-encoded RsmA (Fig. S2). Independently, we isolated a transposon mutant that mapped within *rsmA* (Fig. 1a). To further investigate the role of *rsmA* in *sdhEygfX* regulation, we used this gene disruption mutant. We constructed an *rsmA*, *sdhEygfX::lacZ* double mutant and assessed *sdhEygfX* expression. The *rsmA* mutation caused up to a >2-fold increase in *sdhEygfX* expression (Fig. 1b). To confirm that the effect of the *rsmA* mutation was due to the absence of RsmA, the strain was complemented by plasmid-encoded RsmA, which restored *sdhEygfX* to levels observed in the WT background containing an empty vector control (Fig. 1c). Previously, we demonstrated increased prodigiosin production when SdhE and YgfX were overexpressed (McNeil et al., 2012, 2013), which is consistent with the elevated *sdhEygfX* expression and pigment levels in *rsmA* mutants (Fig. S2) (Williamson et al., 2008). In conclusion, RsmA negatively affects *sdhEygfX* expression in addition to its roles in secondary metabolism and motility (Wilf et al., 2013; Williamson et al., 2008).

**RsmC represses *sdhEygfX* expression**

Two independent transposon insertions were identified upstream of a gene encoding a small 14.5 kDa protein with similarity to RsmC from Pectobacterium spp. (Cui et al., 1999; Shih et al., 1999). One mutant with a transposon insertion 23 bp from the translational start of *rsmC* was selected for further work (termed *rsmC*pro) (Fig. 1d). In the *rsmC*pro background, *sdhEygfX* expression was increased throughout growth, with up to threefold elevation (Fig. 1e).

The increased *sdhEygfX* transcription in the *rsmC*pro background was restored to WT levels by expression of RsmC from a plasmid in trans (Fig. 1f), confirming that the transposon insertion had disrupted the synthesis of RsmC. Therefore, RsmC negatively affects *sdhEygfX* expression in *Serratia*.

**RsmC represses prodigiosin synthesis, swimming, swarming and biosurfactant production**

In *Pectobacterium* spp., *rsmC* mutants are pleiotropic with increased swimming, swarming and production of surfactant and plant cell wall degrading enzymes (Bowden et al., 2013; Chatterjee et al., 2009; Cui et al., 1999, 2008; Shih et al., 1999). However, no study to date has examined the role of *rsmC* outside of the genus *Pectobacterium*. Therefore, we examined prodigiosin synthesis in the *rsmC*pro mutant. The transposon insertion resulted in an approximately fourfold increase in prodigiosin production in the *rsmC* mutant (Fig. 2a). The elevated pigment phenotype was complemented by plasmid-encoded RsmC (Fig. 2b). Thus, RsmC is a newly identified protein involved in the control of prodigiosin synthesis in *Serratia*. Next, the role of RsmC in motility in *Serratia* 39006 was assessed. The *rsmC*pro mutant had increased swimming and swarming compared with that of the WT, and the expression of RsmC in trans in the *rsmC*pro mutant complemented these phenotypes (Fig. 2c–f). Swarming in *Serratia* 39006 requires the production of a biosurfactant, the synthesis of which requires RhlA (Williamson et al., 2008). Consistent with the enhanced swimming, biosurfactant production was elevated in the *rsmC*pro mutant compared with that seen in the WT (Fig. 2g) and this effect could be complemented (Fig. 2h). In these complementation assays, the overexpression of RsmC did not significantly affect the OD600 when compared with the WT control. The same trend was observed when measuring the ability of surfactant to influence surface tension in drop-collapse assays (Fig. S3).

Cryo-electron microscopy revealed that the *rsmC*pro mutant was elongated and hyper-flagellated, compared with the WT (Figs 3 and S4) – features typical of swarming cells. We detected abundant gas vesicles in the WT (Fig. 3a), consistent with our earlier work (Ramsay et al., 2011). Gas vesicles are buoyancy organelles that assist bacterial flotation towards air–liquid interfaces in aquatic niches (Ramsay et al., 2011). No gas vesicles were detected in the *rsmC*pro mutant, indicating that RsmC enhances flotation and inhibits swarming (Fig. 3b). In conclusion, RsmC negatively affects prodigiosin synthesis, swimming, swarming and biosurfactant production, and is required for gas vesicle production in *Serratia*.

**FlhDC activates *sdhEygfX* expression**

A common feature of RsmA and RsmC is that they control the master regulator of flagella biosynthesis (FlhDC) (Chatterjee et al., 2009; Williamson et al., 2008). In
Enterobacteriaceae, the FlhD$_4$C$_2$ complex is produced from the flhDC operon, and activates a cascade of flagellar and chemotaxis gene expression (Chevance & Hughes, 2008). In Serratia 39006, rsmA mutants have elevated levels of the flhDC regulator and rhlA biosurfactant mRNAs (Wilf et al., 2013; Williamson et al., 2008). To control motility in Pectobacterium, RsmC directly interacts with, and inhibits, the FlhDC protein complex (Chatterjee et al., 2009). Therefore, we hypothesized that RsmA and RsmC may act through FlhDC, and that this master regulator could affect sdhEygfX expression. Indeed, expression of FlhDC from a plasmid in trans elevated sdhEygfX transcription (Fig. 4a),

**Fig. 1.** RsmA and RsmC are negative regulators of sdhEygfX expression. (a) Schematic of the transposon insertion within rsmA (strain NW64). (b) β-Galactosidase activity of the sdhEygfX::lacZ fusion in a WT strain (HSPIG46) or rsmA mutant strain (NW67) background. (c) Complementation of sdhEygfX::lacZ expression by expression of RsmA (pPF513) or an empty vector (EV) control (pQE-80LoriT) in WT (HSPIG46) or rsmA mutant (NW67) backgrounds. (d) Schematic representation of the location of the transposon insertion upstream of rsmC (rsmCpro; strain PCF174). (e) β-Galactosidase activity of the sdhEygfX::lacZ fusion in a WT background (strain HSPIG46) or in the presence of the rsmCpro mutation (strain PCF175). (f) Complementation of sdhEygfX::lacZ expression by expression of RsmC (pPF512) or an empty vector (EV) control (pQE-80LoriT) in WT (HSPIG46) or the rsmCpro mutant (PCF175) backgrounds. Data shown are the means ± SD (n=3). MU, Miller units.
demonstrating that FlhDC activates \textit{sdhEygfX}. In contrast, deletion of \textit{flhDC} caused only a subtle reduction, if any, in \textit{sdhEygfX} expression (Fig. 4b). Since both RsmA and RsmC inhibit FlhDC (Chatterjee \textit{et al.}, 2009; Williamson \textit{et al.}, 2008), there is very little active FlhDC in the WT background during growth in broth, which is likely to explain the stronger effect on \textit{sdhEygfX} caused by FlhDC overexpression (Fig. 4a). We could not identify a putative FlhDC-binding site upstream of \textit{sdhEygfX}, suggesting that FlhDC activation is indirect. In summary, FlhDC activates \textit{sdhEygfX} expression.

\textbf{FlhDC activates prodigiosin, swimming, swarming and biosurfactant production}

Since RsmA and RsmC inhibit prodigiosin synthesis and motility, we examined the role of FlhDC on these phenotypes in \textit{Serratia} 39006. Expression of plasmid-encoded FlhDC resulted in increased prodigiosin biosynthesis, swimming, swarming and surfactant production in the WT background (Fig. 5a–d) – phenotypes associated with \textit{rsmA} and \textit{rsmC} deletion. In reciprocal experiments, deletion of \textit{flhDC} caused the opposite effects, a decrease in pigment...
production, and swimming, swarming and biosurfactant synthesis were undetectable (Fig. 5e–h). Therefore, FlhDC activates prodigiosin production and motility. The role of FlhDC in regulating both motility and \( sdhEygfX \) suggested that SdhE might play a part in motility. Deletion of \( sdhE \) resulted in reduced swimming compared with the WT (Fig. S5), whereas \( ygfX \) had no discernable effect (McNeil et al., 2012). In \( E. coli \), FRD associates with the flagella switch complex and is required for aerobic motility (Cohen-Ben-Lulu et al., 2008). In \( Serratia \) 39006, \( frdABCD \) mRNA is detected during aerobic growth (Wilf et al., 2013), so we hypothesized that SdhE activates FRD to influence swimming. Indeed, maximal swimming required both FRD and SdhE (Fig. S5), most likely due to SdhE-dependent flavinylation of FrdA and activation of FRD (McNeil et al., 2014).

**Generation of a missense \( flhC \) mutant using endogenous type I-F CRISPR-Cas targeting**

To test whether the reduced \( sdhEygfX \) expression elicited by RsmA and RsmC required FlhDC, double and triple mutants were required. Since the \( flhDC, rsmA \) and \( rsmC \) mutants had the same resistance markers, we made an unmarked \( flhC \) mutant. The construction of markerless allelic exchange mutants in \( Serratia \) 39006 can be inefficient; therefore, we developed a new method based on CRISPR-Cas genome-editing. CRISPR-Cas systems are bacterial adaptive immune systems that use small RNAs to guide
protein complexes to complementary DNA and cause cleavage (Richter et al., 2012a). We previously showed that a strain with an existing type I-F CRISPR-Cas system could be exploited to generate large deletion mutations in the host chromosome (Dy et al., 2013; Vercoe et al., 2013). To exploit the type I-F CRISPR-Cas system present in Serratia 39006, a plasmid was generated with an inducible guide crRNA (a short 32 bp spacer sequence matching an internal region of \(flhC\)) between two 28 bp type I-F repeats (Fig. 6a) (Fineran et al., 2013). The targeted region (termed a protospacer) was flanked by a GG PAM required for DNA cleavage (Almendros et al., 2012; Vercoe et al., 2013). The strategy relied on expression of the repeat–spacerm-repeat RNA (termed a precursor-crRNA), crRNA generation by the host Cas6f (Przybilski et al., 2011) and formation of an endogenous Csy interference complex (Richter et al., 2012b; Wiedenheft et al., 2011). The interference complex should then target chromosomal \(flhC\), causing cell death and enabling the selection of \(flhC\) mutants that escape targeting (Vercoe et al., 2013).

The anti-\(flhC\) plasmid was induced in the \(sdhEygfX::\lacZ\) background and a >100-fold reduction in viable count was detected compared with controls (Fig. 6b, c). To demonstrate targeting of \(flhC\), the experiments were performed in the \(\Delta flhDC\) strain that lacks the \(flhC\) target. Consistent with specific targeting, no reduction in viable count was observed in this strain (Fig. 6b, c). Survivors following genome-targeting were screened for the \(flhDC\) region by PCR. Of the ~500 colonies screened, the majority had deletions larger than the \(flhDC\) operon. This is consistent with our earlier study in P. aeruginosa, where large deletions of ~100 kb resulted from chromosomal targeting (Vercoe et al., 2013). Other mutations, such as those within the PAM or protospacer, allow escape from targeting (Fineran et al., 2014). Indeed, three mutants with an \(flhDC\) locus of WT size (example in Fig. 6d) were sequenced and contained a GG to GA PAM substitution, resulting in a missense FlhC A24V mutation. The targeting plasmid was cured from one strain. The FlhC A24V mutant was non-motile (Fig. 6e) and, as expected, resistant to the flagellum-dependent phage \(\Phi OT8\) (Fig. 6f) (Evans et al., 2010). To our knowledge, this generation of an unmarked \(flhC\) mutant is the first demonstration that endogenous type I-F CRISPR-Cas systems can be used to generate point mutants in bacterial chromosomes.

**RsmA and RsmC repress \(sdhEygfX\) expression via FlhDC**

To determine whether the RsmC- and RsmA-dependent regulation of \(sdhEygfX\) acted through FlhDC, \(sdhEygfX\) transcription was assessed in various mutant backgrounds. As
RsmA, RsmC and FlhDC regulate sdhEygfX in Serratia

Fig. 7. RsmA and RsmC repress sdhEygfX transcription via flhDC. (a) The β-galactosidase activity of the sdhEygfX::lacZ fusion was assessed in WT (HSPIG46), rsmA (NW67), flhC (PCF185) and rsmA, flhC (PCF186) backgrounds. (b) The β-galactosidase activity of the sdhEygfX::lacZ fusion was assessed in WT (HSPIG46), rsmC (PCF175), flhC (PCF185) and rsmC, flhC (PCF187) backgrounds. Data shown are the means ± SD (n=3).

An rsmA mutant, which revealed increased flhDC mRNA and mRNAs encoding other flagella proteins in the rsmA mutant compared with the WT (Wilf et al., 2013; Williamson et al., 2008).

The rsmC mutation resulted in increased sdhEygfX transcription that was entirely FlhDC-dependent (Fig. 7b). Importantly, mutation of flhC in the rsmC background caused the elevated sdhEygfX expression in the single rsmC mutant to return to levels observed in both the WT and flhC mutant (Fig. 7b). These observations are supported by previous work in P. carotovorum, where an rsmC mutation had no effect in an flhC mutant (Chatterjee et al., 2009). In conclusion, the regulation of the sdhEygfX operon by RsmC occurs in an FlhDC-dependent manner, whereas RsmA has both FlhDC-dependent and -independent effects on sdhEygfX transcription.

Fig. 8. Proposed model of regulation of sdhEygfX. RsmC binds FlhDC and inhibits its activity. RsmA negatively affects the levels of the flhDC mRNA and transcription of sdhEygfX. FlhDC activates the transcription of sdhEygfX, which promotes prodigiosin production and motility. FlhDC also affects motility independently of SdhE-YgfX and the pleiotropic regulator RsmA is also likely to function via additional pathways (dotted line).

DISCUSSION

In this study, we have investigated the regulation of the sdhEygfX operon in Serratia 39006. We identified an overlapping pathway involving the post-transcriptional regulators RsmA and RsmC which repressed sdhEygfX expression by acting through the flagella master regulatory complex, FlhDC. FlhDC activated sdhEygfX transcription and the inhibitory effect of RsmC was dependent on flhDC. In contrast, RsmA repressed sdhEygfX in both an FlhDC-dependent and -independent manner (Fig. 8). Currently, it is not known how SdhE and YgfX influence pigment production. SdhE was initially identified as a gene neighbouring YgfX, a regulator of prodigiosin production, and the deletion of either, or both, of these genes results in a decrease in transcription of the prodigiosin biosynthesis operon (McNeil et al., 2012). The physiological role of prodigiosin has been an issue of debate (Williamson et al., 2006), but previous work uncovered an antibiotic effect that is elicited in a surfactant-dependent manner (Williamson et al., 2008). This led to a model whereby swarming and surfactant production may enable the local dispersal of the prodigiosin.
antibiotic to help *Serratia* in niche colonization and competition with other bacteria (Williamson et al., 2008). Our data show that RsmA, RsmC and FlhDC co-ordinately regulate motility, surfactant and antibiotic pigment production, which is consistent with the synergism between biosurfactant and prodigiosin (Williamson et al., 2008).

RsmA is a homologue of CsrA (carbon storage regulator) from *E. coli*, which is a post-transcriptional regulator that binds to the 5′ untranslated regions of mRNA and either represses translation (by occluding ribosome-binding sites) or stabilizes transcripts by blocking RNase E-dependent cleavage (Romeo et al., 2013; Vakulskas et al., 2015). As its nomenclature implies, CsrA affects carbon flux, but it is also a highly pleiotropic regulator that controls other processes, including motility and virulence. The mRNA-binding activity of CsrA can be out-competed by small antagonistic RNAs (CarB and CsrC) that fold into secondary structures generating binding sites in the single-stranded loops that sequester the CsrA partner (Romeo et al., 2013; Vakulskas et al., 2015). In *Serratia* 39006, rsmA mutants exhibit enhanced prodigiosin production, swimming and biosurfactant production (Williamson et al., 2008). Indeed, in an rsmA mutant the mRNA involved in biosurfactant synthesis (rhlA) was increased by ~60-fold and transcripts of the flhDC operon were ~8-fold higher than in the WT strain (Williamson et al., 2008). Furthermore, a recent RNA-seq and proteome study in *Serratia* 39006 showed that an rsmA mutant produced increased flagellar components and many prodigiosin biosynthetic proteins were elevated (Wilt et al., 2013). Our rsmA data are consistent with these studies, but also demonstrate another route for RsmA and FlhDC (via *sdhE*), by which additional control of metabolism (via SDH and FRD; McNeil et al., 2012, 2014) and motility may be modulated. Both *sdhE* and FRD mutants show reduced swimming, which echoes results with *E. coli*, where FRD binds to the flagellar switch, thereby impacting flagellar assembly and switching (Cohen-Ben-Lulu et al., 2008). Adjusting SdhE levels in response to different regulatory cues should allow the bacterium to ensure appropriate flavinylation/activation of FRD to fine-tune motility.

To our knowledge, this is the first study of RsmC outside of the genus *Pectobacterium*. RsmC is exclusive to Enterobacteriaceae, being mainly present in the genera *Pectobacterium* and *Dickeya*. However, some homologues exist in other genera (e.g. *Brenneria* and *Lonsdalea*). Despite its name, RsmC is not a bona fide member of the Rsm pathway, but controls some shared phenotypes. In *Pectobacterium* spp., RsmC directly binds FlhDC and antagonizes its function (Chatterjee et al., 2009). Mutation of *rsmC* causes increased swimming, swimming and production of surfactant and plant cell wall degrading enzymes (Bowden et al., 2013; Chatterjee et al., 2009; Cui et al., 1999, 2008; Shih et al., 1999). In agreement, RsmC repressed swimming, biosurfactant production and swimming in *Serratia* 39006. The *Serratia* 39006 rsmC mutant also produced longer, hyper-flagellated cells, but no gas vesicles. This shows that RsmC (and by inference FlhDC) inversely controls swimming and flotation. Similarly, RsmA displays inverse control of swimming and gas vesicle morphogenesis (Ramsay et al., 2011), which conceivably might be occurring via FlhDC.

We also provide the first evidence to our knowledge that RsmC and FlhDC differentially affect prodigiosin production. Interestingly, an earlier study reported that flagellin protein variation correlated with pigment variation in *Serratia marcescens* (Paruchuri & Harshey, 1987).

The signals that regulate this *sdhE* control pathway are currently unknown. However, it is likely that RsmA and RsmC inhibit FlhDC to reduce the expression of *sdhE* under conditions where motility, prodigiosin or maximal SDH or FRD activity are not required. Importantly, *sdhE* expression is still robust, even in the absence of FlhDC, and under both aerobic and anaerobic conditions, ensuring sufficient active SDH and FRD for metabolism (McNeil et al., 2014). The non-coding RNA antagonists of RsmA proteins are activated by the GacAS two-component signalling systems (Romeo et al., 2013; Vakulskas et al., 2015). The signals for GacAS systems appear to be intermediates of carbon metabolism, including acetate, and GacAS responds to intracellular levels of TCA cycle intermediates (α-ketoglutarate, succinate and fumarate) that we predict should signal increased activation of the associated metabolic pathways (Chavez et al., 2010; Takeuchi et al., 2009). Thus, it is possible that in *Serratia*, these TCA precursors/intermediates would up-regulate RsmB via GacAS signalling (PigQW in *Serratia*; Fineran et al., 2005b; Williamson et al., 2008). RsmB would sequester RsmA and lead to elevated *sdhE* (in FlhDC-dependent and -independent pathways). The increased SdhE would ensure activation of the TCA cycle and the electron transport chain through SDH and/or FRD flavinylation to support metabolism (McNeil et al., 2012, 2014). To date, it is not known what regulates RsmC.

Here, we have also developed and demonstrated the feasibility of using endogenous CRISPR-Cas targeting by type I systems to isolate point mutations in target genes. This is an extension of our previous work, which showed that large regions, such as entire pathogenicity islands, could be deleted (Vercoe et al., 2013). Despite the widespread uptake of Cas9 genome-editing in eukaryotes, few studies have explored CRISPR-Cas utility in bacteria (Selle & Barrangou, 2015) and almost all use the Cas9 technology (Cobb et al., 2015; Jiang et al., 2013; Li et al., 2015; Oh & van Pijkeren, 2014; Tong et al., 2015). The simplicity of the CRISPR-Cas9 system, and its ability to make double-stranded breaks without further degradation, make it the favoured CRISPR-Cas9 type for genome-editing. However, in these bacteria with few, if any, current genetic tools, exploiting endogenous CRISPR-Cas systems has considerable potential (Selle & Barrangou, 2015; Vercoe et al., 2013). For applications of endogenous CRISPR-Cas systems, type I are the most abundant and well characterized. A distinction from CRISPR-Cas9 is that the processive DNA degradation caused by Cas3 in type I systems typically causes large deletions (Vercoe et al., 2013). Indeed, the vast majority of mutants we generated in this study contained deletions of the *flhDC* region. Developing methods to control the extent of deletions,
either through mutagenesis of Cas proteins or by providing substrates for homology-directed repair, is essential to harness the potential of genome-editing and gene silencing using type I CRISPR-Cas systems (Fineran & Dy, 2014; Selle & Barrangou, 2015). Nevertheless, in this study, we successfully isolated three point mutations in flhC and so our results suggest strongly that further refinement of this CRISPR-Cas-based approach to bacterial mutagenesis could have a generic utility for precise engineering of prokaryotes – with implications from basic microbiology through synthetic biology to industrial, agricultural and medical translation.

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