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*

Running title: RsmA, RsmC and FlhDC regulate *sdhEygfX* in *Serratia*

Hannah G. Hampton[1,§], Matthew B. McNeil[1,#,§], Thomas J. Paterson[1], Blair Ney[1,#], Neil R. Williamson[2], Richard A. Easingwood[3], Mihnea Bostina[1,3], George P.C. Salmond[2] and Peter C. Fineran[1]*

[1]Department of Microbiology and Immunology, University of Otago, PO Box 56, Dunedin 9054, New Zealand. [2]Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK. [3]Otago Centre for Electron Microscopy, University of Otago, PO Box 56, Dunedin 9054, New Zealand.

[#]Present addresses: MBM, Infectious Disease Research Institute, Seattle, Washington, United States of America. BN, Faculty of Education, Science, Technology & Mathematics, University of Canberra, ACT 2601, Australia.
[§]These authors contributed equally to this work.
*For correspondence. E-mail. peter.fineran@otago.ac.nz; Tel. +64 (0)3 479 7735; Fax. +64 (0)3 479 8540.

Keywords: prodigiosin; motility; secondary metabolism; antibiotic; CRISPR-Cas; SDH5/SdhAF2

Subject category: Regulation

Word count: 5531

Abbreviations: Cas; CRISPR associated, CRISPR; clustered regularly interspaced short palindromic repeats, crRNA; CRISPR RNA, FAD; flavin adenine dinucleotide, FRD; fumarate reductase, MU; Miller Units, PAM; protospacer adjacent motif, SDH; succinate dehydrogenase, TCA; tricarboxylic acid.

ABSTRACT

SdhE is required for the flavinylation and activation of succinate dehydrogenase and fumarate reductase. In addition, SdhE is conserved in proteobacteria (α, β and γ) and eukaryotes. Although the function of this recently characterised 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 were 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 utilised endogenous CRISPR-Cas 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*, *rsmC*, or overexpression of FlhDC, led to increased prodigiosin, biosurfactant, swimming and swarming. Consistent with the modulation of *sdhE* by motility regulators, we demonstrate that SdhE and fumarate reductase 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 prodiginines due to their anticancer, immunosuppressant, antimicrobial and antimalarial properties (Williamson *et al.*, 2006; Williamson *et al.*, 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.*, 2003; Thomson *et al.*, 2000), cyclic-di-GMP signalling (Fineran *et al.*, 2007; Williamson *et al.*, 2008), phosphate availability (Gristwood *et al.*, 2009; Slater *et al.*, 2003), carbon source (Fineran *et al.*, 2005a) and stationary phase (Wilf & Salmond, 2012), among others. A number of the DNA-binding transcriptional regulators of prodigiosin, including SmaIR (LuxIR-type quorum sensing system), Rap (regulator of antibiotic and pigment) and PigP, also co-ordinately control the ability to undertake swimming and swarming motility (Fineran *et al.*, 2005b; Williamson *et al.*, 2008). The ability to swarm involves cell elongation and requires the synthesis of flagella and biosurfactant (Jarrell & McBride, 2008; Williamson *et al.*, 2008). Interestingly, the co-ordinate production of the biosurfactant and pigment is important for *Serratia* 39006 to elicit prodigiosin-dependent antibiotic killing of competing bacteria – a potentially important physiological role for this red pigment during movement of the bacterium into new niches (Williamson *et al.*, 2008).

During our studies into the regulation of secondary metabolism in *Serratia* 39006, SdhE (formerly YgfY) was identified, due to transposon insertions within the *sdhEygfX* operon that reduced prodigiosin biosynthesis (Fineran *et al.*, 2005b; McNeil *et al.*, 2012). In Enterobacteriaceae, *sdhE* is co-transcribed with *ygfX*, which encodes a membrane protein of unknown function that interacts with SdhE (McNeil *et al.*, 2013). 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 tricarboxylic acid (TCA) cycle (McNeil *et al.*, 2012; McNeil *et al.*, 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 flavin adenine dinucleotide (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; McNeil *et al.*, 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 *ygfX* 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; McNeil *et al.*, 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; McNeil *et al.*, 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-Cas system to generate chromosomal point mutations.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Tables S1 and S2, respectively. *Serratia* sp. ATCC 39006 (Fineran *et al.*, 2013) and *Escherichia coli* strains were grown at 30°C and 37°C, respectively. Bacteria were grown in Lysogeny Broth (LB: 5 g l^{-1} yeast extract, 10 g l^{-1} bacto tryptone and 5 g l^{-1} NaCl), minimal medium (0.1% w v^{-1} $(NH_4)_2SO_4$, 0.41 mM $MgSO_4$, 0.2% w v^{-1} glucose, 40 mM K_2HPO_4, 14.7 mM KH_2PO_4, pH 6.9–7.1) at 180 rpm, or on LB agar supplemented with 1.5% (w v^{-1}) agar (LBA) (Miller, 1972). Growth (OD_{600}) and absorbance was measured in a Jenway 6300 spectrophotometer (Staffordshire, UK). When required, media were supplemented with antibiotics at final concentration, as follows: kanamycin 50 μg ml^{-1} (Km), ampicillin 100 μg ml^{-1} (Ap), streptomycin 50 μg ml^{-1} (Sm) and chloramphenicol 25 μg ml^{-1} (Cm). 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 ** and less than 0.05 *.

Movement of mutations via generalised transduction. When required, mutations were moved by generalised transduction between strains to generate single, double and triple mutants. For these transductions, phage ϕOT8 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-Tn*5lacZ1*) (Fineran *et al.*, 2005b) was performed by conjugation with *E. coli* BW20767 harbouring the Tn-DS1028*uidA* delivery plasmid pDS1028*uidA* (Ramsay *et al.*, 2011). Cultures of the *sdhEygfX*::mini-Tn*5lacZ1* mutant and *E. coli* BW20767 donor were grown overnight in LB and 20 µl of each were mixed, pelleted by centrifugation, resuspended in 40 µl LB, spotted onto LBA and incubated for 6 h at 30°C. Following conjugation, the resulting mating patches were resuspended in 1 ml of LB and 100 µl aliquots of a 1 in 4 dilution were plated onto LBA containing Km, Cm and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (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. For quantitative assessment of *sdhEygfX*::*lacZ* expression throughout growth in different mutant backgrounds, standard β-galactosidase assays were used as described previously and expressed as Miller units (MU) (Przybilski *et al.*, 2011).

Arbitrary PCR. Transposon insertion sites of mutants of interest were mapped using arbitrary PCR, as previously described (Fineran *et al.*, 2005a; Jacobs *et al.*, 2003). Briefly, PCR was performed on colony DNA using a random primer mix (PF106, PF107 and PF108) and a Tn-DS1028*uidA* specific primer (PF225 or PF338, hybridising 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 µl aliquot of undiluted purified DNA from the first PCR with an adapter primer (PF109) that binds to the 5' ends of PF106-PF108, and a nested Tn-DS1028*uidA* specific primer (either PF226 or PF294). The resulting mix of PCR fragments was purified and sequenced with the nested Tn-DS1028*uidA* specific primer. The Tn-DS1028*uidA* insertion site and orientation was determined by aligning with the *Serratia* 39006 genome (Fineran *et al.*, 2013).

Phenotypic assays. Prodigiosin production was assessed as previously described (Slater *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 of growth. For swimming assays, bacterial cultures were grown overnight in 5 ml LB. The OD_{600} was adjusted to 0.2 and 3 µl was spotted onto a tryptic swimming agar (5 g l^{-1} NaCl, 10 g l^{-1} tryptone, 0.3% w v^{-1} agar) plate. Plates were incubated at 30°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 *et al.*, 2008). To measure surfactant production, overnight cultures of bacteria were adjusted to an OD_{600} of 0.2 and 5 µl spotted onto LBA plates solidified with 0.75% (w v^{-1}) agar. Plates were incubated for ~16 h at 30°C and surfactant production determined by the diameter (mm) of the clear ring surrounding the bacterial colony (Williamson *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 β-galactosidase assays on complemented strains, appropriate cultures were grown overnight in 5 ml LB. The OD_{600} was adjusted to give a

starting OD_{600} of 0.02 in 25 ml of LB with 1 mM IPTG. Strains were grown at 30°C at 180 rpm and OD_{600} and β-galactosidase activity measured at 12 h.

Generation of RsmC, RsmA and FlhDC expression plasmids. Plasmids for expression of RsmA, RsmC and FlhDC were constructed as follows. Firstly, the genes were amplified by PCR using *Serratia* 39006 genomic DNA as template and primer pairs PF786 and PF787 for *rsmC*, PF788 and PF789 for *rsmA* and PF796 and PF797 for *flhDC*. The forward primers contained a ribosome binding site and EcoRI sites. The reverse primers contained HindIII sites, except PF797, which had an XmaI site. PCR products were digested with the appropriate enzymes and ligated to pQE-80LoriT that had been previously cut with the same endonucleases. *E. coli* DH5α was transformed with the ligations and plasmids were verified by sequencing. Plasmids were introduced into *Serratia* 39006 by conjugation using either *E. coli* SM10 λpir or S17-1 λpir donors and minimal medium or appropriate antibiotics to counter-select the donors. The nature of transconjugants was confirmed by antibiotic resistance and PCR.

Construction of an Δ*flhDC*::Cm mutant. The Δ*flhDC*::Cm deletion plasmid (pPF595) was made by overlap extension PCR. Using *Serratia* 39006 genomic DNA as a template, the left hand fragment contained a 3' 20 bp sequence that overlapped the 5' end of the Cm resistance cassette, whilst the right hand fragment contained a 5' 20 bp sequence that overlapped the 3' end of the Cm resistance cassette. The following primer pairs were used to construct the left hand fragment (PF817 + PF1289) and the right hand fragment (PF822 + PF1299). The Cm resistance cassette was constructed using (PF432 + PF433) with pTRB32 as a template. All three fragments were joined using overlap PCR with primers PF817 and PF822. The resulting overlap product was digested with BamHI and XbaI, cloned into pBluescript II KS(+) and confirmed by sequencing. Deletion constructs were cloned from pBluescript II KS(+) into pKNG101 using BamHI and XbaI. Deletion mutants were generated using an allelic exchange strategy with a sucrose selection protocol similar to that described previously (Fineran *et al.*, 2005a; Kaniga *et al.*, 1991). Putative deletion mutants were sucrose resistant, Cm resistant, non-motile on tryptic swimming agar, and were confirmed by PCR and sequencing.

Construction of an *flhC* point mutant using endogenous CRISPR-Cas targeting. The *Serratia* 39006 strain contains a type I-F CRISPR-Cas system (Fineran *et al*., 2013). A plasmid (pPF704) for the expression of a crRNA designed to target *flhC* was constructed using primers PF1639 and PF1640. PF1639 had a 19 bp sequence at the 3' end that overlapped the 3' end of PF1640. This overlap generated a 106 bp PCR product that contained two repeats of the *Serratia* 39006 type I-F system separated by a 32 bp spacer targeting an internal region of the *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 *Serratia* HSPIG46 (*sdhEygfX*::mini-Tn5*lacZ1*) and Δ*flhDC*::Cm strains. Following heat shock, cells were recovered in, and plated on, LB and LBA respectively, both supplemented with glucose (0.2% w v^{-1}) to repress expression of the targeting plasmid. Transformants containing the targeting plasmid were grown ~16 h in 5 ml of LB with glucose (0.2% w v^{-1}), 1 ml of this was pelleted and washed twice with PBS (phosphate buffered saline) to remove any glucose. The pellet was resuspended in 1 ml of PBS and then a dilution series was plated onto media containing arabinose (0.1% w v^{-1}) 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 *flhC*-targeting plasmid was cured by growth without antibiotic selection, resulting in strain PCF185. The *flhC* mutant did not swim and could be complemented with plasmid-encoded *flhDC* (pPF516). Complementation allowed generalised transduction by the flagellum-dependent phage, ϕOT8, of further mutations into the *flhC* mutant. Resulting strains were cured of pPF516.

Cryo-electron microscopy. A 4 µL aliquot of overnight cultures grown in LB containing the strains analysed (WT and $rsmC_{pro}$ (PCF174)) was applied to a glow-discharged Quantifoil 2/2 grid (Quantifoil Micro Tools GmbH, Jena, Germany), blotted and frozen in liquid ethane using a Reichert KF80 plunge freezing device (C. Reichert AG, Vienna, Austria). Grids with the frozen specimen were loaded into a Gatan 914 cryo holder (Gatan Inc, California, USA) and viewed using a JEOL 2200FS cryo transmission electron microscope (JEOL Ltd, Toyko, Japan) with an omega filter. Zero-loss images were recorded at microscope magnifications of either 8,000 x or 15,000 x using SerialEM software (University of Bolder, Colorado, USA) controlling a TVIPS F416 camera (Tietz Video and Image Processing Systems GmbH, Gauting, Germany).

RESULTS

Identification of regulators of the *sdhEygfX* operon. To identify genes that affect the expression of *sdhEygfX*, a random transposon mutagenesis was performed in *Serratia* 39006 that contained a chromosomal *sdhEygfX*::*lacZ* transcriptional fusion. Mutants were screened for altered β-galactosidase activity relative to the control strain and mutants of interest were identified by arbitrary PCR and 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 a gene with similarity to *rsmC* (regulator of secondary metabolism C) from *Pectobacterium carotovorum* and *P. atrosepticum* (formerly *Erwinia carotovora* subsp. *carotovora* and subsp. *atroseptica*, 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, swarming 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; McNeil *et al.*, 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 3-fold 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; Cui *et al.*, 2008; Shih *et al.*, 1999). However, no study to date has examined the role of *rsmC* outside of the *Pectobacterium* genus. Therefore, we examined prodigiosin synthesis in the $rsmC_{pro}$ mutant. The transposon insertion resulted in an ~4-fold 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 swarming, 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 OD_{600} 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 (Fig. 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 $FlhD_4C_2$ 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 hypothesised 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), demonstrating that FlhDC activates *sdhEygfX*. In contrast, deletion of *flhDC* caused only a subtle reduction, if any, in *sdhEygfX* expression (Fig. 4b). Since both RsmA and RsmC inhibit FlhDC (Chatterjee *et al.*, 2009; Williamson *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 *sdhEygfX* caused by FlhDC overexpression (Fig. 4a). We could not identify a putative FlhDC binding site upstream of *sdhEygfX*, suggesting that FlhDC activation is indirect. In summary, FlhDC activates *sdhEygfX* expression.

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 *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 *rsmA* and *rsmC* deletion. In reciprocal experiments, deletion of *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 hypothesised 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 if 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 marker-less 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 (protospacer adjacent motif) required for DNA cleavage (Almendros *et al*., 2012; Vercoe *et al*., 2013). The strategy relied on expression of the repeat-spacer-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 Δ*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. atrosepticum*, 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 ϕOT8 (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 if the RsmC- and RsmA-dependent regulation of *sdhEygfX* acted through FlhDC, *sdhEygfX* transcription was assessed in various mutant backgrounds. As observed previously, mutation of *rsmA* led to increased *sdhEygfX* expression (Fig. 7a). However, mutation of *flhC* in the *rsmA* mutant partially abolished the RsmA-dependent repression of *sdhEygfX* expression seen in the single *rsmA* mutant (Fig. 7a). Therefore, RsmA negatively regulates *sdhEygfX* in an FlhDC-dependent manner (Wilf *et al.*, 2013; Williamson *et al.*, 2008), while also inhibiting *sdhEygfX* via an FlhDC-independent pathway. The FlhDC-dependent pathway is supported by our recent RNA-seq and qRT-PCR analyses of 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 *flhDC* mutant (Chatterjee *et al.*, 2009). In conclusion, the regulation of the *sdhEyfgX* operon by RsmC occurs in an FlhDC-dependent manner, whereas RsmA has both FlhDC-dependent and -independent effects on *sdhEyfgX* transcription.

DISCUSSION

This study has investigated the regulation of the *sdhEygfX* operon in *Serratia* 39006. We identified an overlapping pathway involving the post-transcriptional regulators, RsmA and RsmC that 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 colonisation 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 stabilises 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 (CsrB 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, swarming 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 proteomic study in *Serratia* 39006 showed that an *rsmA* mutant produced increased flagellar components and many prodigiosin biosynthetic proteins were elevated (Wilf *et al.*, 2013). Our *rsmA* data is consistent with these studies, but also demonstrate another route for RsmA and FlhDC (via *sdhEygfX*), by which additional control of metabolism (via SDH and FRD (McNeil *et al.*, 2012; McNeil *et al.*, 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 *Pectobacterium* genus. RsmC is exclusive to Enterobacteriaceae, being mainly present in the *Pectobacterium* and *Dickeya* genera. 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 antagonises its function (Chatterjee *et al.*, 2009). Mutation of *rsmC* causes 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; Cui *et al.*, 2008; Shih *et al.*, 1999). In agreement, RsmC repressed swimming, biosurfactant production and swarming 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 floatation. Similarly, RsmA displays inverse control of swimming and gas vesicle morphogenesis (Ramsay *et al.*, 2011), which conceivably might be occurring via FlhDC. To our knowledge we also provide the first evidence 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 *sdhEygfX* control pathway are currently unknown. However, it is likely that RsmA and RsmC inhibit FlhDC to reduce the expression of *sdhEygfX* under conditions where motility, prodigiosin or maximal SDH or FRD activity are not required. Importantly, *sdhEygfX* 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)). This would sequester RsmA and lead to elevated *sdhEygfX* (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; McNeil *et al.*, 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, makes it the favoured CRISPR-Cas type for genome-editing. However, in those bacteria with few, or no, 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 characterised. 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.

Acknowledgements. This work was supported by the Marsden Fund, Royal Society of New Zealand (RSNZ) to PCF and a Strategic Grant from the Otago School of Medical Sciences (OSMS) to MB. HGH was supported by a University of Otago Doctoral Scholarship, MBM by a Division of Health Sciences Career Development Post-doctoral Fellowship, BN by a Dean's Prestigious Summer Scholarship from the OSMS and PCF was supported by a Rutherford Discovery Fellowship (RSNZ). NRW and GPCS were supported by Biotechnology and Biological Sciences Research Council (BBSRC), UK awards to the GPCS laboratory. We thank members of the Fineran and Cook laboratories for helpful discussions, Tim Blower for plasmid pTRB32 and for critically reading the manuscript.

REFERENCES

Almendros, C., Guzman, N. M., Diez-Villasenor, C., Garcia-Martinez, J. & Mojica, F. J. (2012). Target motifs affecting natural immunity by a constitutive CRISPR-Cas system in *Escherichia coli*. *PloS one* **7**, e50797.

Blaut, M., Whittaker, K., Valdovinos, A., Ackrell, B. A., Gunsalus, R. P. & Cecchini, G. (1989). Fumarate reductase mutants of *Escherichia coli* that lack covalently bound flavin. *Journal of Biological Chemistry* **264**, 13599-13604.

Bowden, S. D., Hale, N., Chung, J. C., Hodgkinson, J. T., Spring, D. R. & Welch, M. (2013). Surface swarming motility by *Pectobacterium atrosepticum* is a latent phenotype that requires O antigen and is regulated by quorum sensing. *Microbiology* **159**, 2375-2385.

Bycroft, B. W., Maslen, C., Box, S. J., Brown, A. & Tyler, J. W. (1987). The isolation and characterisation of (3R,5R)-and (3S,5R)-carbapenem-3-carboxylic acid from *Serratia* and *Erwinia* species and their putative biosynthetic role. *J Chem Soc Chem Commun* **21**, 1623-1625.

Cecchini, G., Schroder, I., Gunsalus, R. P. & Maklashina, E. (2002). Succinate dehydrogenase and fumarate reducatse from *Escherichia coli*. *Biochimica et Biophysica Acta* **1553**, 140-157.

Chatterjee, A., Cui, Y. & Chatterjee, A. K. (2009). RsmC of *Erwinia carotovora* subsp. *carotovora* negatively controls motility, extracellular protein production, and virulence by binding FlhD and modulating transcriptional activity of the master regulator, FlhDC. *J Bacteriol* **191**, 4582-4593.

Chavez, R. G., Alvarez, A. F., Romeo, T. & Georgellis, D. (2010). The physiological stimulus for the BarA sensor kinase. *J Bacteriol* **192**, 2009-2012.

Chevance, F. F. & Hughes, K. T. (2008). Coordinating assembly of a bacterial macromolecular machine. *Nature Reviews Microbiology* **6**, 455-465.

Cobb, R. E., Wang, Y. & Zhao, H. (2015). High-Efficiency Multiplex Genome Editing of Streptomyces Species Using an Engineered CRISPR/Cas System. *ACS synthetic biology* **4**, 723-728.

Cohen-Ben-Lulu, G. N., Francis, N. R., Shimoni, E. & other authors (2008). The bacterial flagellar switch complex is getting more complex. *The EMBO journal* **27**, 1134-1144.

Cui, Y., Mukherjee, A., Dumenyo, C. K., Liu, Y. & Chatterjee, A. K. (1999). *rsmC* of the soft-rotting bacterium *Erwinia carotovora* subsp. *carotovora* negatively controls extracellular enzyme and harpin(Ecc) production and virulence by modulating levels of regulatory RNA (*rsmB*) and RNA-binding protein (RsmA). *J Bacteriol* **181**, 6042-6052.

Cui, Y., Chatterjee, A., Yang, H. & Chatterjee, A. K. (2008). Regulatory network controlling extracellular proteins in *Erwinia carotovora* subsp. *carotovora*: FlhDC, the master regulator of flagellar genes, activates *rsmB* regulatory RNA production by affecting *gacA* and *hexA* (*lrhA*) expression. *J Bacteriol* **190**, 4610-4623.

Dy, R. L., Pitman, A. R. & Fineran, P. C. (2013). Chromosomal targeting by CRISPR-Cas systems can contribute to genome plasticity in bacteria. *Mobile genetic elements* **3**, e26831.

Evans, T. J., Crow, M. A., Williamson, N. R., Orme, W., Thomson, N. R., Komitopoulou, E. & Salmond, G. P. (2010). Characterization of a broad-host-range flagellum-dependent phage that mediates high-efficiency generalized transduction in, and between, *Serratia* and *Pantoea*. *Microbiology* **156**, 240-247.

Fineran, P. C., Everson, L., Slater, H. & Salmond, G. P. (2005a). A GntR family transcriptional regulator (PigT) controls gluconate-mediated repression and defines a new, independent pathway for regulation of the tripyrrole antibiotic, prodigiosin, in *Serratia*. *Microbiology* **151**, 3833-3845.

Fineran, P. C., Slater, H., Everson, L., Hughes, K. & Salmond, G. P. (2005b). Biosynthesis of tripyrrole and beta-lactam secondary metabolites in *Serratia*: integration of quorum sensing with multiple new regulatory components in the control of prodigiosin and carbapenem antibiotic production. *Mol Microbiol* **56**, 1495-1517.

Fineran, P. C., Williamson, N. R., Lilley, K. S. & Salmond, G. P. (2007). Virulence and prodigiosin antibiotic biosynthesis in *Serratia* are regulated pleiotropically by the GGDEF/EAL domain protein, PigX. *J Bacteriol* **189**, 7653-7662.

Fineran, P. C., Iglesias Cans, M. C., Ramsay, J. P. & other authors (2013). Draft Genome Sequence of *Serratia* sp. Strain ATCC 39006, a Model Bacterium for Analysis of the Biosynthesis and Regulation of Prodigiosin, a Carbapenem, and Gas Vesicles. *Genome announcements* **1**. e01039-13.

Fineran, P. C. & Dy, R. L. (2014). Gene regulation by engineered CRISPR-Cas systems. *Current opinion in microbiology* **18**, 83-89.

Fineran, P. C., Gerritzen, M. J., Suarez-Diez, M., Kunne, T., Boekhorst, J., van Hijum, S. A., Staals, R. H. & Brouns, S. J. (2014). Degenerate target sites mediate rapid primed CRISPR adaptation. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E1629-1638.

Gristwood, T., Fineran, P. C., Everson, L., Williamson, N. R. & Salmond, G. P. (2009). The PhoBR two-component system regulates antibiotic biosynthesis in *Serratia* in response to phosphate. *BMC microbiology* **9**, 112.

Hao, H.-X., Khalimonchuk, O., Schraders, M. & other authors (2009). *SDH5*, a Gene Required for Flavination of Succinate Dehydrogenase, Is Mutated in Paraganglioma. *Science* **325**, 1139-1142.

Huang, S., Taylor, N. L., Ströher, E., Fenske, R. & Harvey Millar, A. (2013). Succinate dehydrogenase assembly factor 2 is needed for assembly and activity of mitochondrial complex II and for normal root elongation in Arabidopsis. *The Plant Journal* **73**, 429-491.

Iverson, T. M., Luna-Chavez, C., Cecchini, G. & Rees, D. C. (1999). Structure of the *Escherichia coli* Fumarate Reductase Respiratory Complex. *Science* **284**, 1961-1966.

Jacobs, M. A., Alwood, A., Thaipisuttikul, I. & other authors (2003). Comprehensive transposon mutant library of Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 14339-14344.

Jarrell, K. F. & McBride, M. J. (2008). The surprisingly diverse ways that prokaryotes move. *Nature Reviews Microbiology* **6**, 466-476.

Jiang, W., Bikard, D., Cox, D., Zhang, F. & Marraffini, L. A. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature biotechnology* **31**, 233-239.

Kaniga, K., Delor, I. & Cornelis, G. R. (1991). A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* **109**, 137-141.

Kim, H. J. & Winge, D. R. (2013). Emerging concepts in the flavinylation of succinate dehydrogenase. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1827**, 627-636.

Li, Y., Lin, Z., Huang, C., Zhang, Y., Wang, Z., Tang, Y. J., Chen, T. & Zhao, X. (2015). Metabolic engineering of Escherichia coli using CRISPR-Cas9 meditated genome editing. *Metabolic engineering* **31**, 13-21.

Maklashina, E., Cecchini, G. & Dikanov, S. A. (2013). Defining a direction: electron transfer and catalysis in Escherichia coli complex II enzymes. *Biochim Biophys Acta* **1827**, 668-678.

McNeil, M. B., Clulow, J. S., Wilf, N. M., Salmond, G. P. & Fineran, P. C. (2012). SdhE is a conserved protein required for flavinylation of succinate dehydrogenase in bacteria. *J Biol Chem* **287**, 18418-18428.

McNeil, M. B. & Fineran, P. C. (2013). Prokaryotic assembly factors for the attachment of flavin to complex II. *Biochim Biophys Acta* **1827**, 637-647.

McNeil, M. B., Iglesias-Cans, M. C., Clulow, J. S. & Fineran, P. C. (2013). YgfX (CptA) is a multimeric membrane protein that interacts with the succinate dehydrogenase assembly factor SdhE (YgfY). *Microbiology* **159**, 1352-1365.

McNeil, M. B., Hampton, H. G., Hards, K. J., Watson, B. N., Cook, G. M. & Fineran, P. C. (2014). The succinate dehydrogenase assembly factor, SdhE, is required for the flavinylation and activation of fumarate reductase in bacteria. *FEBS letters* **588**, 414-421.

Miller, J. (1972). Experiments in Molecular Genetics, 3 edn. NY: Cold Spring Harbour Laboratory.

Oh, J. H. & van Pijkeren, J. P. (2014). CRISPR-Cas9-assisted recombineering in *Lactobacillus reuteri*. *Nucleic acids research* **42**, e131.

Paruchuri, D. K. & Harshey, R. M. (1987). Flagellar variation in *Serratia marcescens* is associated with color variation. *J Bacteriol* **169**, 61-65.

Przybilski, R., Richter, C., Gristwood, T., Clulow, J. S., Vercoe, R. B. & Fineran, P. C. (2011). Csy4 is responsible for CRISPR RNA processing in *Pectobacterium atrosepticum*. *RNA Biology* **8**, 517-528.

Ramsay, J. P., Williamson, N. R., Spring, D. R. & Salmond, G. P. (2011). A quorum-sensing molecule acts as a morphogen controlling gas vesicle organelle biogenesis and adaptive flotation in an enterobacterium. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 14932-14937.

Richter, C., Chang, J. T. & Fineran, P. C. (2012a). Function and regulation of clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR associated (Cas) systems. *Viruses* **4**, 2291-2311.

Richter, C., Gristwood, T., Clulow, J. S. & Fineran, P. C. (2012b). In vivo protein interactions and complex formation in the *Pectobacterium atrosepticum* subtype I-F CRISPR/Cas System. *PloS one* **7**, e49549.

Romeo, T., Vakulskas, C. A. & Babitzke, P. (2013). Post-transcriptional regulation on a global scale: form and function of Csr/Rsm systems. *Environmental microbiology* **15**, 313-324.

Selle, K. & Barrangou, R. (2015). Harnessing CRISPR-Cas systems for bacterial genome editing. *Trends in microbiology* **23**, 225-232.

Shih, Y. L., Harris, S. J., Borner, G., Rivet, M. M. & Salmond, G. P. (1999). The *hexY* genes of *Erwinia carotovora* ssp. *carotovora* and ssp. *atroseptica* encode novel proteins that regulate virulence and motility co-ordinately. *Environmental microbiology* **1**, 535-547.

Slater, H., Crow, M., Everson, L. & Salmond, G. P. (2003). Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. *Mol Microbiol* **47**, 303-320.

Takeuchi, K., Kiefer, P., Reimmann, C., Keel, C., Dubuis, C., Rolli, J., Vorholt, J. A. & Haas, D. (2009). Small RNA-dependent expression of secondary metabolism is controlled by Krebs cycle function in *Pseudomonas fluorescens*. *J Biol Chem* **284**, 34976-34985.

Thomson, N. R., Crow, M. A., McGowan, S. J., Cox, A. & Salmond, G. P. (2000). Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. *Mol Microbiol* **36**, 539-556.

Tong, Y., Charusanti, P., Zhang, L., Weber, T. & Lee, S. Y. (2015). CRISPR-Cas9 Based Engineering of Actinomycetal Genomes. *ACS synthetic biology* **4**, 1020-1029.

Vakulskas, C. A., Potts, A. H., Babitzke, P., Ahmer, B. M. & Romeo, T. (2015). Regulation of bacterial virulence by Csr (Rsm) systems. *Microbiology and molecular biology reviews : MMBR* **79**, 193-224.

Vercoe, R. B., Chang, J. T., Dy, R. L. & other authors (2013). Cytotoxic chromosomal targeting by CRISPR/Cas systems can reshape bacterial genomes and expel or remodel pathogenicity islands. *PLoS genetics* **9**, e1003454.

Wiedenheft, B., van Duijn, E., Bultema, J. B. & other authors (2011). RNA-guided complex from a bacterial immune system enhances target recognition through seed sequence interactions. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 10092-10097.

Wilf, N. M. & Salmond, G. P. (2012). 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. *Microbiology* **158**, 648-658.

Wilf, N. M., Reid, A. J., Ramsay, J. P. & other authors (2013). RNA-seq reveals the RNA binding proteins, Hfq and RsmA, play various roles in virulence, antibiotic production and genomic flux in *Serratia* sp. ATCC 39006. *BMC genomics* **14**, 822.

688 **Williamson, N. R., Fineran, P. C., Leeper, F. J. & Salmond, G. P. (2006)**. The biosynthesis and regulation of
689 bacterial prodiginines. *Nature Reviews Microbiology* **4**, 887-899.

690
691 **Williamson, N. R., Fineran, P. C., Gristwood, T., Chawrai, S. R., Leeper, F. J. & Salmond, G. P. (2007)**.
692 Anticancer and immunosuppressive properties of bacterial prodiginines. *Future microbiology* **2**, 605-618.

693
694 **Williamson, N. R., Fineran, P. C., Ogawa, W., Woodley, L. R. & Salmond, G. P. (2008)**. Integrated
695 regulation involving quorum sensing, a two-component system, a GGDEF/EAL domain protein and a post-
696 transcriptional regulator controls swarming and RhlA-dependent surfactant biosynthesis in *Serratia*.
697 *Environmental microbiology* **10**, 1202-1217.

698
699 **Yankovskaya, V., Horsefield, R., Tarnroth, S., Luna-Chavez, C., Miyoshi, H., Lager, C., Byrne, B.,**
700 **Cecchini, G. & Iwata, S. (2003)**. Architecture of Succinate Dehydrogenase and Reactive Oxygen Species
701 Generation. *Science* **299**, 700-704.

702

703

704

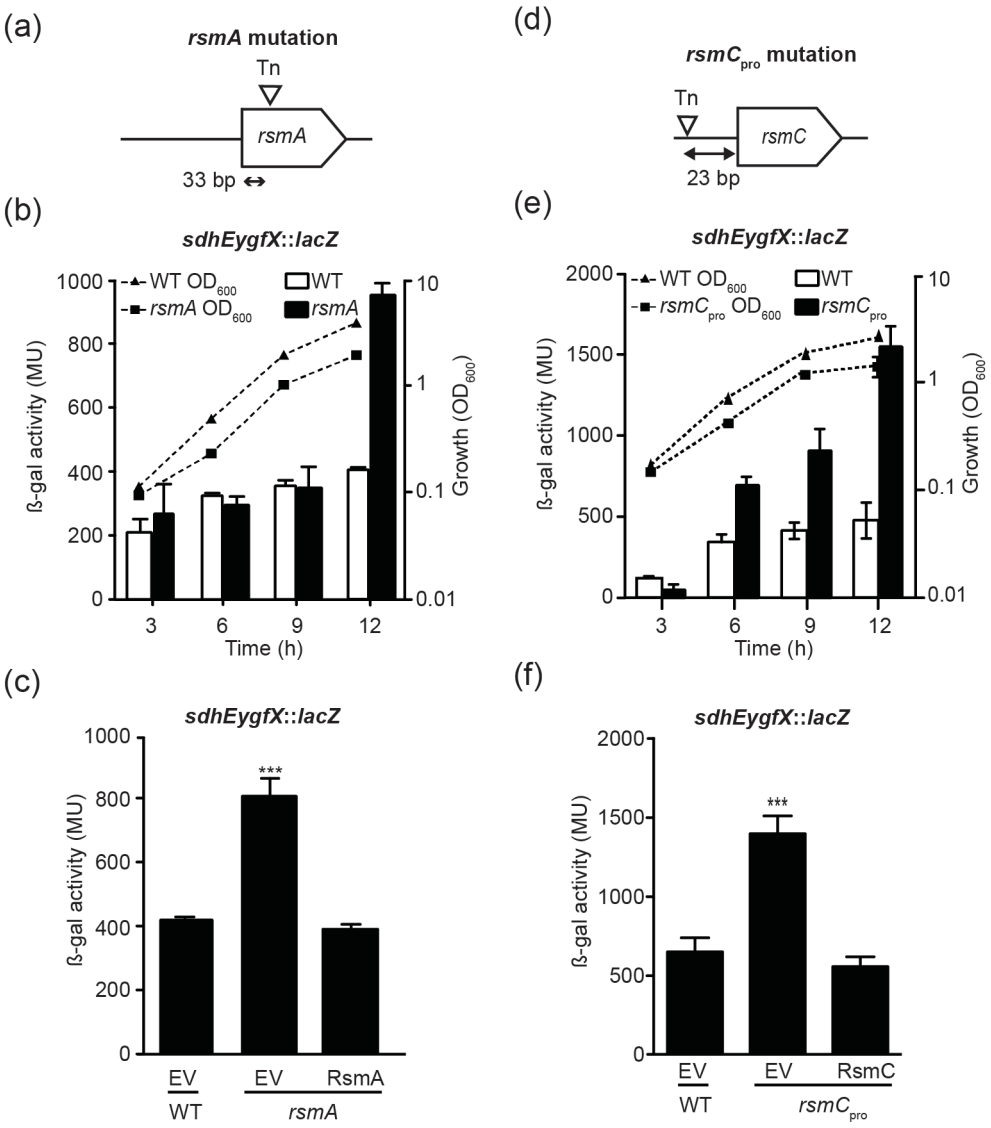
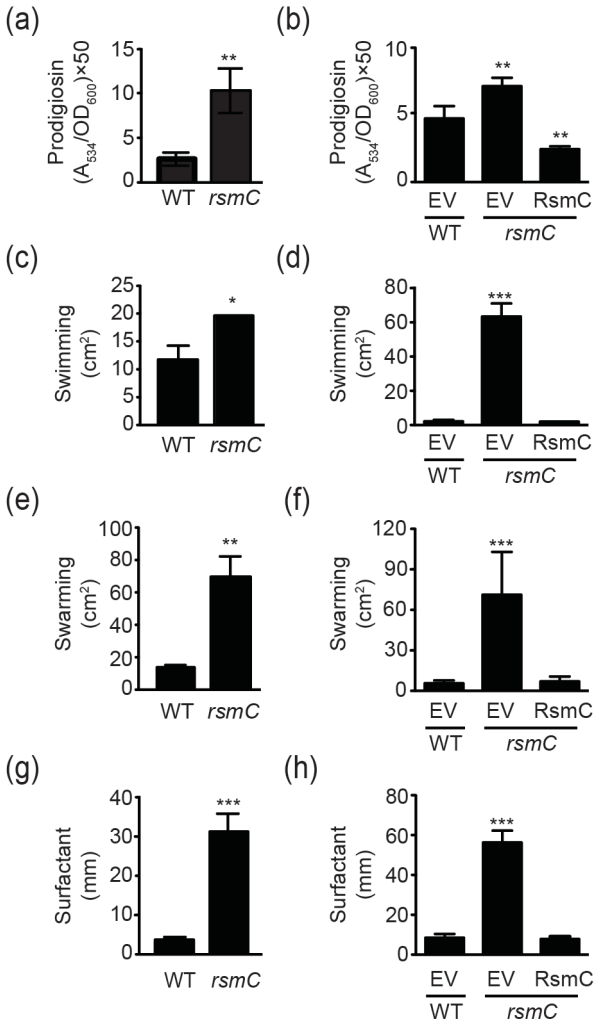


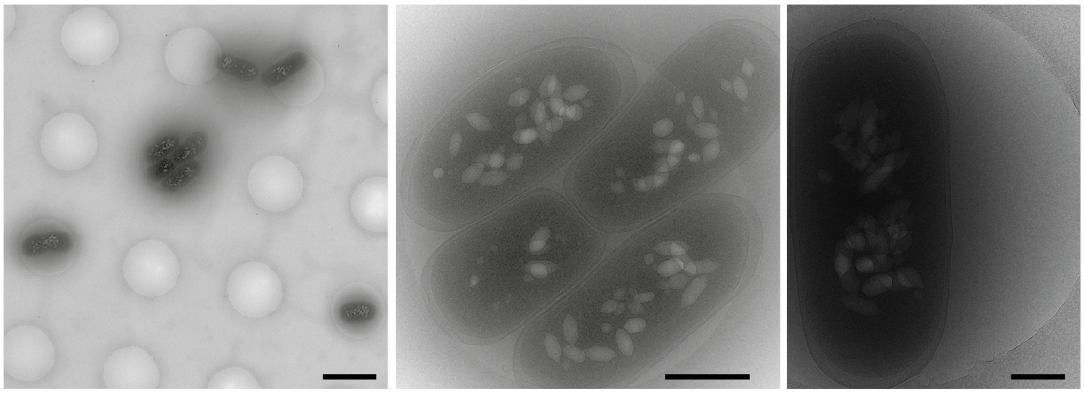
Fig. 1. RsmA and RsmC are negative regulators of *sdhEygfX* expression. (a) Schematic of the transposon insertion within *rsmA* (strain NW64). (b) β-galactosidase activity of the *sdhEygfX*::*lacZ* fusion in a WT strain (HSPIG46) or *rsmA* mutant strain (NW67) background. (c) Complementation of *sdhEygfX*::*lacZ* 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* (*rsmC*$_{pro}$; strain PCF174). (e) β-galactosidase activity of the *sdhEygfX*::*lacZ* fusion in a WT background (strain HSPIG46) or in the presence of the *rsmC*$_{pro}$ 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 *rsmC*$_{pro}$ mutant (PCF175) backgrounds. Data shown are the means ± SD (*n*=3).



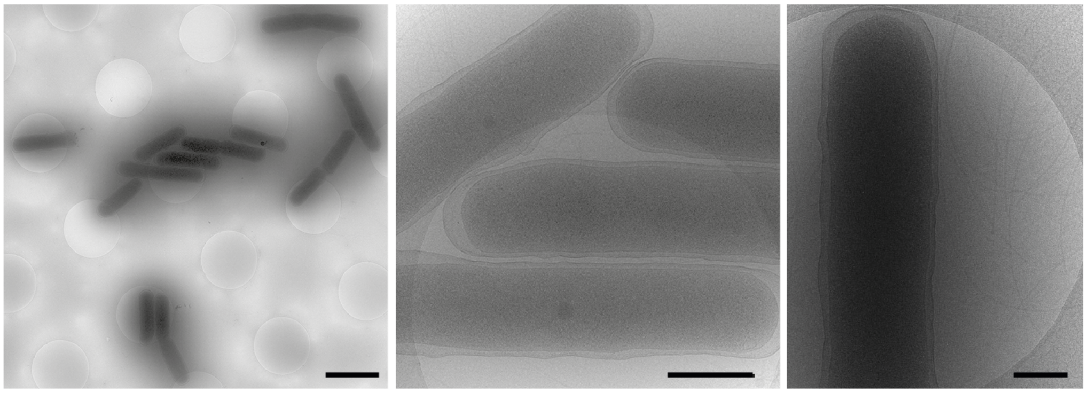
718

719 **Fig. 2.** RsmC is a negative regulator of prodigiosin production and motility. (a, b) Prodigiosin
720 production (at 12 h), (c, d) swimming, (e, f) swarming and (g, h) surfactant production in the
721 WT and *rsmC*$_{pro}$ (PCF174) strains. Where relevant complementation is shown using either an
722 empty vector (EV) control (pQE-80LoriT) or a plasmid expressing RsmC (pPF512). Data
723 shown are the means ± SD (*n*=3).

(a) **WT**

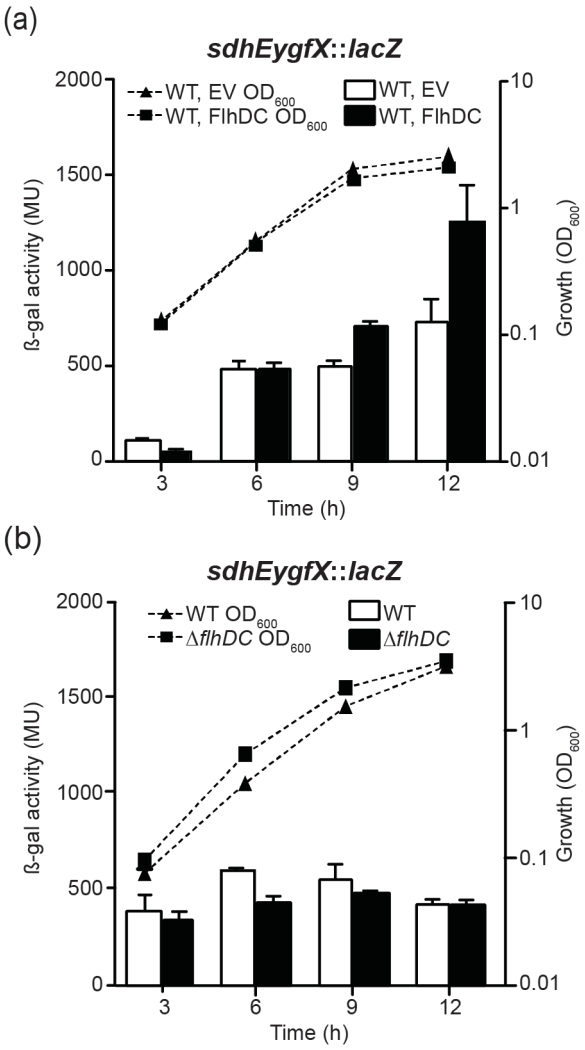


(b) ***rsmC***



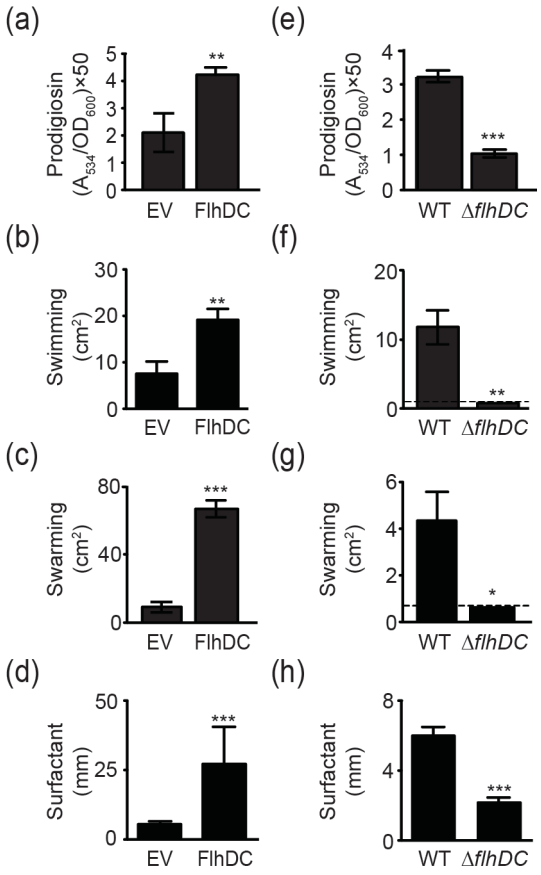
724

725 **Fig. 3.** The *rsmC* mutant is elongated, hyper-flagellated and does not contain gas vesicles.
726 Cell morphology by cryo-TEM in the (a) WT and (b) $rsmC_{pro}$ (PCF174) strains. Scale bars
727 are 2 μm (left panel), 500 nm (centre), and 200 nm (right panel). See Fig. S4 for higher
728 resolution images.



729

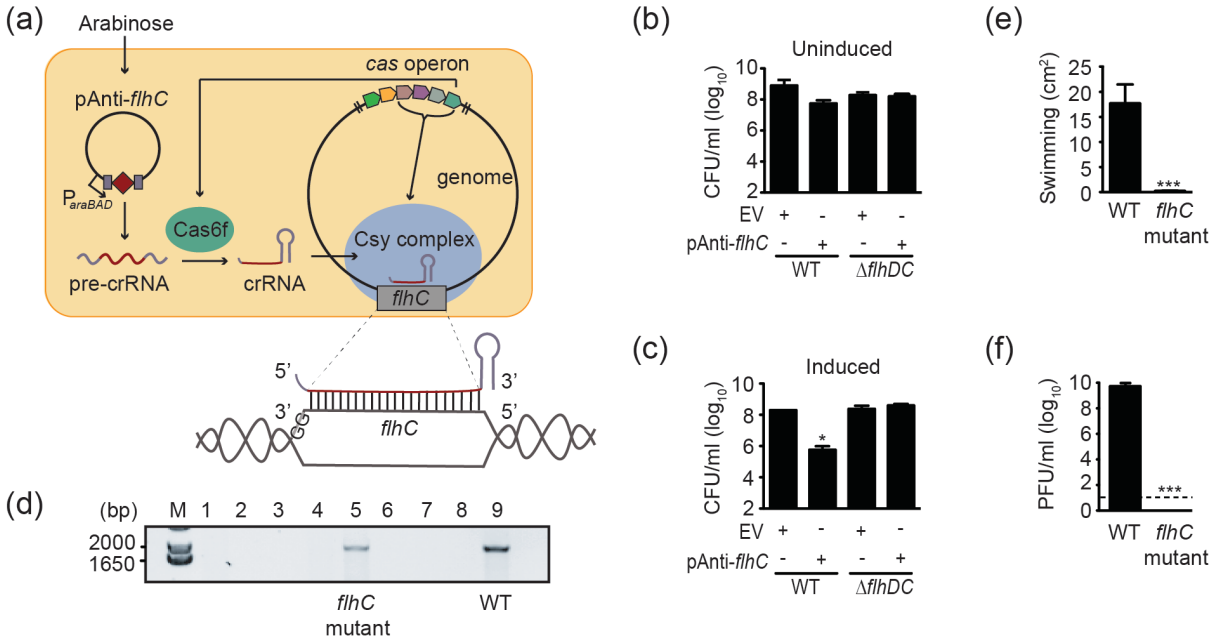
730 **Fig. 4.** FlhDC activates *sdhEygfX* expression. (a) β-galactosidase activity of the
731 *sdhEygfX*::*lacZ* fusion in a WT background (strain HSPIG46) with either an empty vector
732 (pQE-80LoriT) or FlhDC (pPF516). (b) β-galactosidase activity of the *sdhEygfX*::*lacZ* fusion
733 in a WT background (strain HSPIG46) or in the *flhDC* mutant (Δ*flhDC*::Cm). Data shown are
734 the means ± SD (*n*=3).



735

736 **Fig. 5.** FlhDC activates prodigiosin production and motility. (a) Prodigiosin production (at 12
737 h), (b) swimming, (c) swarming and (d) surfactant production in a WT with either an empty
738 vector (EV; pQE-80LoriT) or a plasmid expressing FlhDC (pPF516). (e) Prodigiosin
739 production, (f) swimming, (g) swarming and (h) surfactant production in the WT or in the
740 *flhDC* mutant (Δ*flhDC*::Cm). Data shown are the means ± SD (*n*=3). Where shown, dashed
741 lines represent the limits of detection.

742



743

744 **Fig. 6.** Generation of an *flhC* mutant using endogenous CRISPR-Cas targeting. (a) Schematic
745 of the pAnti-*flhC* plasmid (pPF704) and the endogenous type I-F CRISPR-Cas system in
746 *Serratia* 39006. Colony forming units (CFU) of WT or Δ*flhDC*::Cm strains containing the
747 pAnti-*flhC* plasmid (pPF704) in (b) uninduced and (c) induced conditions. (d) PCR screening
748 of the *flhDC* locus using primers PF817 and PF822. Empty lanes are due to strains with
749 deletions larger than the *flhDC* operon. The band in the *flhC* mutant lane is the same size as
750 the band in the WT control lane, but contains a GG to GA PAM mutation. (e) Swimming and
751 (f) ϕOT8 phage infection of the resulting *flhC* mutant (PCF185). Dashed line in (f) represents
752 the limit of detection. Data shown are the means ± SD (*n*=3).

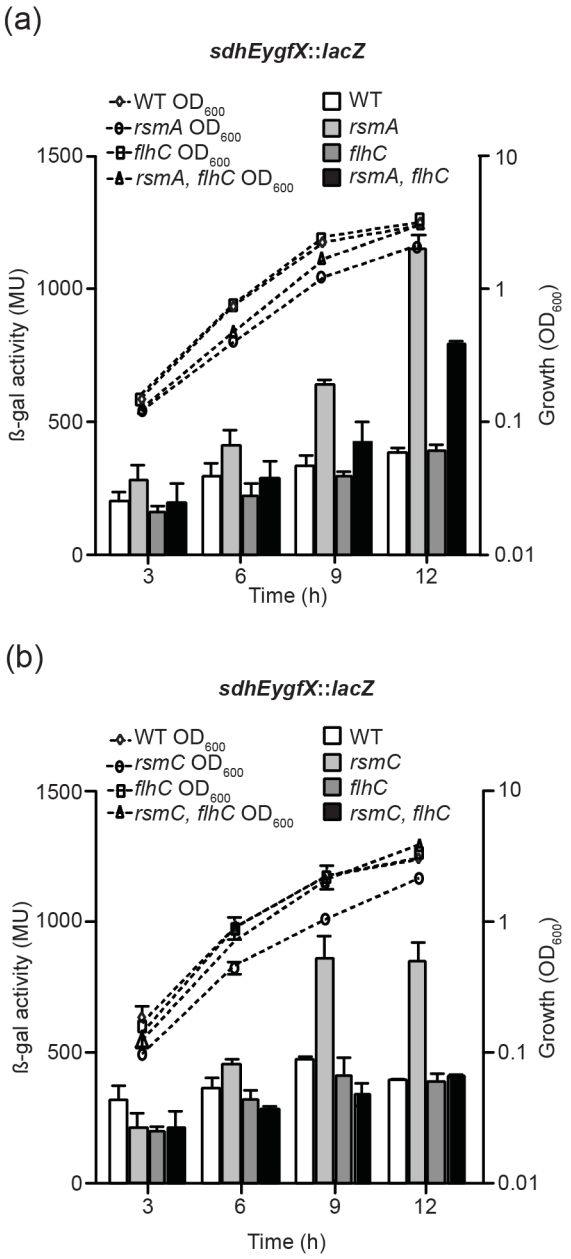
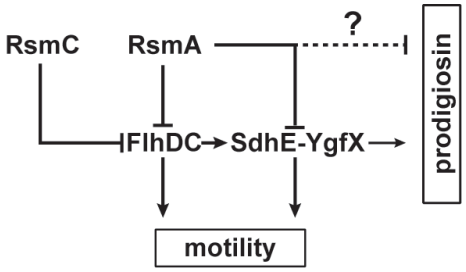


Fig. 7. RsmA and RsmC repress *sdhEygfX* transcription via *flhDC*. (a) The β-galactosidase activity of *sdhEygfX*::*lacZ* fusion was assessed in WT (HSPIG46), *rsmA* (NW67), *flhC* (PCF185) and *rsmA, flhC* (PCF186) backgrounds. (b) The β-galactosidase activity of *sdhEygfX*::*lacZ* fusion was assessed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



760

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

766

SUPPLEMENTARY MATERIAL

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*

Hannah G. Hampton[1,§], Matthew B. McNeil[1,#,§], Thomas J. Paterson[1], Blair Ney[1,#], Neil R. Williamson[2], Richard A. Easingwood[3], Mihnea Bostina[1,3], George P.C. Salmond[2] and Peter C. Fineran[1]*

[1]Department of Microbiology and Immunology, University of Otago, PO Box 56, Dunedin 9054, New Zealand. [2]Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK. [3]Otago Centre for Electron Microscopy, University of Otago, PO Box 56, Dunedin 9054, New Zealand.

[#]Present addresses: MBM, Infectious Disease Research Institute, Seattle, Washington, United States of America. BN, Faculty of Education, Science, Technology & Mathematics, University of Canberra, ACT 2601, Australia.

[§]These authors contributed equally to this work.

*For correspondence. E-mail. peter.fineran@otago.ac.nz; Tel. +64 (0)3 479 7735; Fax. +64 (0)3 479 8540.

Table S1. Strains used in this study

Strain	Genotype/Phenotype	Reference
Escherichia coli		
CC118 λ pir	*araD,* Δ*(ara, leu),* Δ*lacZ74, phoA20, galK, thi-1, rspE, rpoB, argE, recA1,* λ *pir*	(Herrero *et al.*, 1990)
DH5 α	F-, Δ80Δd*lacZ*M15, Δ (*lacZYA–argF*)*U169*, *endA1*, *recA1*, *hsdR17* ($r_K^- m_K^+$), *deoR*, *thi-1, supE44,* λ $^-$, *gyrA96*, *relA1*	Gibco/BRL
HH26	Marker exchange mobilization strain for conjugal transfer	(Grinter, 1983)
S17-1 λ pir	*recA*, *pro*, *hsdR*, *recA*::RP4-2-Tc::Mu, λ *pir*, Tmp^R, Sp^R, Sm^R	(de Lorenzo *et al.*, 1990)
SM10 λ pir	*thi-1, thr, leu, tonA, lacY, supE, recA*::RP4-2-Tc::Mu, λ pir, Km^R	(de Lorenzo *et al.*, 1990)
***Serratia* sp. ATCC 39006** *(all in LacA background)*		
LacA (referred to as "WT" in this study)	Lac$^-$ derivative of *Serratia* sp. ATCC 39006	(Thomson *et al.*, 2000)
Δ*sdhE*	Markerless deletion mutant of *sdhE*	(McNeil *et al.*, 2012)
Δ*frdABCD*::Cm	Deletion mutant of *frdABCD* replaced with Cm^R	(McNeil *et al.*, 2014)
Δ*sdhE*, Δ*frdABCD*::Cm	*sdhE* and *frdABCD* double mutant constructed via transduction of Δ*frdABCD*::Km into Δ*sdhE*, Km^R	(McNeil *et al.*, 2014)
Δ*flhDC*::Cm	Deletion mutant of *flhDC* replaced with a Cm^R	This study
Δ*flhDC*::Cm, *sdhEygfX*::*lacZ*	Δ*flhDC*::Cm, *sdhEygfX*::mini-Tn*5lacZ1*, constructed via transduction of *sdhEygfX*::mini-Tn*5lacZ1* into Δ*flhDC*::Cm, Cm^R, Km^R	This study
HSPIG46	*sdhEygfX*::mini-Tn*5lacZ1*, Km^R	(Fineran *et al.*, 2005)
NW64	*rsmA*::Tn-DS1028*uidA*, Cm^R	This study
NW67	*rsmA*::Tn-DS1028*uidA*, *sdhEygfX*::mini-Tn*5lacZ1*, Cm^R, Km^R; Tn insertion from NW64 transduced into HSPIG46	This study
PCF174	$rsmC_{pro}$::Tn-DS1028*uidA*, Cm^R; Tn insertion from original mutant transduced into LacA	This study
PCF175	$rsmC_{pro}$::Tn-DS1028*uidA*, *sdhEygfX*::mini-Tn*5lacZ1*, Cm^R, Km^R; Tn insertion from PCF174 transduced into HSPIG46	This study
PCF176	$rsmA_{pro}$::Tn-DS1028*uidA*, Cm^R; Tn insertion from original mutant transduced into LacA	This study
PCF177	$rsmA_{pro}$::Tn-DS1028*uidA*, *sdhEygfX*::mini-Tn*5lacZ1*, Cm^R, Km^R; Tn insertion from PCF176 transduced into HSPIG46	This study
PCF185	*flhC* mutant, *sdhEygfX*::mini-Tn*5lacZ1,* Km^R	This study
PCF186	*flhC* mutant, *rsmA*::Tn-DS1028*uidA*, *sdhEygfX*::mini-Tn*5lacZ1*, Cm^R, Km^R; Tn insertion from NW64 transduced into PCF185	This study
PCF187	*flhC* mutant, $rsmC_{pro}$::Tn-DS1028*uidA, sdhEygfX*::mini-Tn*5lacZ1*, Cm^R, Km^R	This study

Table S2. Plasmids used in this study

Plasmid Name	Description	Reference
pBAD30	Arabinose inducible expression vector, p15A replicon, Ap^R.	(Guzman *et al.*, 1995)
pBluescript II KS(+)	Cloning vector, ColE1 replicon, Ap^R	Stratagene
pDS1028uidA	Conjugative plasmid containing Tn-DS1028*uidA* (mini-Tn*5*-based), oriR6K, Cm^R, Tc^R	(Ramsay *et al.*, 2011)
pKNG101	Marker exchange suicide vector, *sacBR*, mobRK2, oriR6K, Sm^R	(Kaniga *et al.*, 1991)
pNJ5000	Mobilizing plasmid used in marker exchange, Tc^R	(Grinter, 1983)
pPF512	pQE-80LoriT containing *rsmC*, Ap^R	This study
pPF513	pQE-80LoriT containing *rsmA*, Ap^R	This study
pPF516	pQE-80LoriT containing *flhDC*, Ap^R	This study
pPF595	pBluescript II KS(+) with the Δ*flhDC*::Cm construct, Ap^R	This study
pPF596	pKNG101 with the Δ*flhDC*::Cm construct, Sm^R	This study
pPF704	pBAD30 containing *flhC* targeting fragment, Ap^R	This study
pQE-80LoriT	Expression vector derivative of pQE-80L with RP4 *oriT*, Ap^R	(Gristwood *et al.*, 2011)
pTRB30	Expression vector derivative of pQE-80L, Km^R	(Przybilski *et al.*, 2011)
pTRB32	Expression vector derivative of pQE-80L, Cm^R	Tim Blower; unpublished

Table S3. Oligonucleotides used in this study

Primer Name	Sequence (5'-3')	Notes	Restriction sites (bold)
PF106	GACCACACGTCGACTAGTGCNNNNNNNNNN AGAG	Arbitrary PCR primer 1	
PF107	GACCACACGTCGACTAGTGCNNNNNNNNNN ACGCC	Arbitrary PCR primer 2	
PF108	GACCACACGTCGACTAGTGCNNNNNNNNNN GATAC	Arbitrary PCR primer 3	
PF109	GACCACACGTCGACTAGTGC	Arbitrary PCR adapter primer	
PF209	TCGTCTTCACCTCGAGAAATC	F pQE-80LoriT MCS	
PF210	GTCATTACTGGATCTATCAACAGG	R pQE-80LoriT MCS	
PF213	CAACTTAACGTAAAAACAACTTCAGA	F pKNG101 MCS	
PF214	TACACTTCCGCTCAGGTCCTTGTCCT	R pKNG101 MCS	
PF217	CGACGTAAAACGACGGCCAGT	F pBluescript II (KS)+ MCS	
PF218	GGAAACAGCTATGACCATG	R pBluescript II (KS)+ MCS	
PF225	GATAATAAGCGGATGAATGGCAG	Tn-DS1028*uidA* out RHS	
PF226	CATAAGGGACTCCTCATTAAG	Tn-DS1028*uidA* nested primer out LHS	
PF294	CTTGCTCAATCAATCACCG	Tn-DS1028*uidA* nested primer out RHS	
PF338	ATCTGCATCGGCGAACTGAT	Tn-DS1028*uidA* out LHS	
PF432	TTT**GTCGAC**ATACCGGGAAGCCCTGGG	R Cm^R cassette	SalI
PF433	TTT**AAGCTT**AGGCGTTTAAGGGCACCA	F Cm^R cassette	HindIII
PF786	ATA**GAATTC**AGGAGGAATATAATGAGTCTG ATATTTGGGCAG	F *rsmC* with RBS	EcoRI
PF787	GAT**AAGCTT**TTAAGAAGCGAGGTGTGATG	R *rsmC*	HindIII
PF788	ATA**GAATTC**AGGAGGAATATAATGCTTATTT TAACTCGTCG	F *rsmA* with RBS	EcoRI
PF789	GAT**AAGCTT**TCAATAAGATGTTGGCTGAG	R *rsmA*,	HindIII
PF796	ATA**GAATTC**AGGAGGAATATAATGGGTACTT CTGAG TTACTTAAGC	F *flhDC* with RBS	EcoRI
PF797	GAT**CCCGGG**TCAGACTGCGTGTTTTACTTG	R *flhDC*	XmaI
PF817	ATA**GGATCC**TTCGTAATTGATAAGTGGTTTG	F Δ*flhDC*::Cm LHF	BamHI
PF822	GAT**TCTAGA**AATTCAACAATATAAGCGTCTG	R Δ*flhDC*::Cm RHF	Xba1
PF1298	ACGCCTAAGCTTAAAAATCTCATCCCGGCAA G	R Δ*flhDC*::Cm LHF	
PF1299	CGGTATGTCGACAAAGCCTGGTAGCGATTTA TAAG	F Δ*flhDC*::Cm RHF	
PF1639	TTT**GAATTC**GTTCACTGCCGTACAGGCAGCT TAGAAA*CGCCTGCAGATGCTCGAAAGTG*	F for *flhC* targeting (repeat underlined, partial spacer italic)	EcoRI
PF1640	AAA**GTCGAC**TTTCTAAGCTGCCTGTACGGCA GTGAAC*AACTGCGTTTCACTTTCGAGCATCTGC AG*	R for *flhC* targeting spacer (repeat underlined, partial spacer italic)	SalI

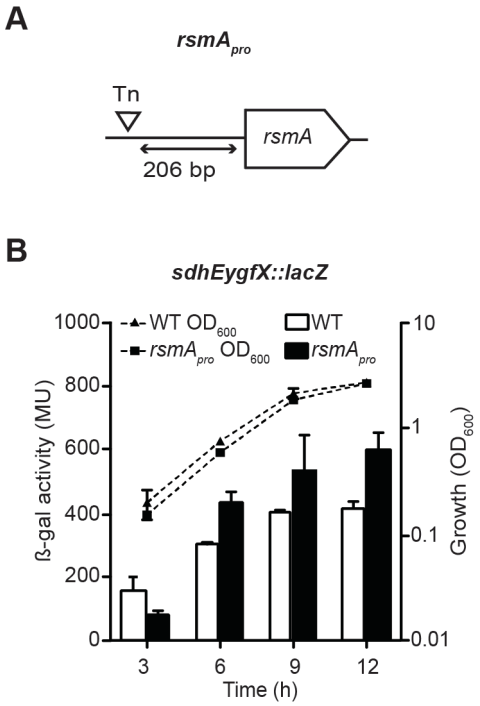


Figure S1. RsmA is a negative regulator of *sdhEygfX* expression. A. Schematic representation of the location of the transposon insertion upstream of *rsmA* (*rsmA*$_{pro}$; strain PCF176). **B.** β-galactosidase activity of the *sdhEygfX*::*lacZ* fusion in a WT background (strain HSPIG46) or in the presence of the *rsmA*$_{pro}$ mutation (strain PCF177). Data shown are the means ± SD (*n*=3).

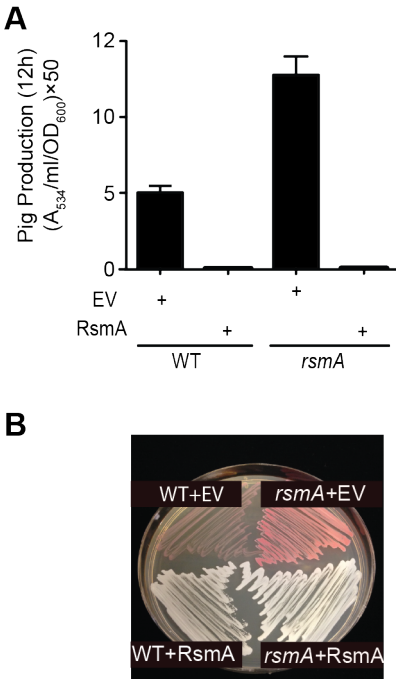


Figure S2. Prodigiosin production and complementation in the *rsmA*$_{pro}$ mutant. A Prodigiosin assay and **B** photo of prodigiosin production in WT and *rsmA*$_{pro}$ strain (PCF176) containing either an empty vector control (EV; pQE-80LoriT) or a plasmid expressing RsmA (pPF513). In (A) data shown are the means ± SD (*n*=3).

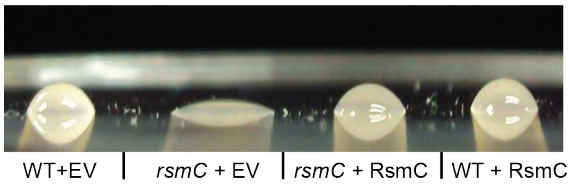


Figure S3. RsmC represses surfactant production. Drop collapse assay that qualitatively measures the surface tension effects of surfactant production in WT and *rsmC*$_{pro}$ (strain PCF174) containing either an empty vector control (EV; pQE-80LoriT) or a plasmid expressing RsmC (pPF512). The assay was performed as described previously (Williamson *et al.*, 2008).

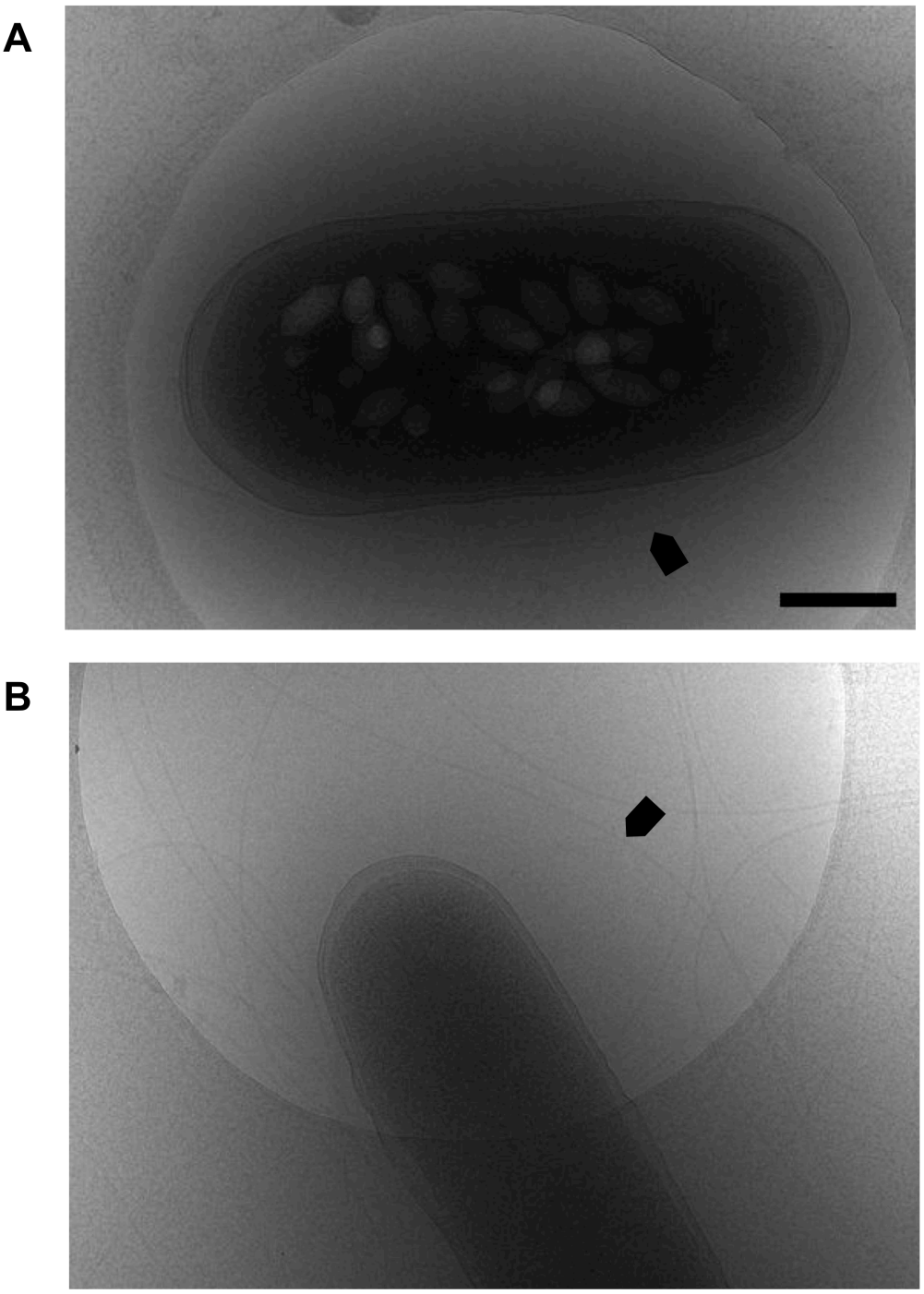


Figure S4. Cryo-Electron microscopy. Cell morphology of the WT (A) and *rsmC*$_{pro}$ (B) strains. Visible flagella are labeled by black arrowheads. Scale bar 200 nm.

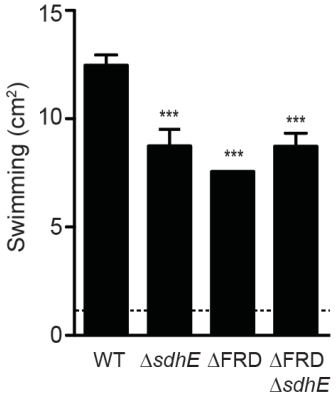


Figure S5. Both FRD and SdhE are required for maximal swimming. Swimming assay on WT, Δ*sdhE*, ΔFRD (Δ*frdABCD*::Cm), and Δ*sdhE*, ΔFRD (Δ*sdhE*, Δ*frdABCD*::Cm) mutants. Data shown are the means ± SD (*n*=3). The dashed line represents the limit of detection.

SUPPLEMENTARY REFERENCES

de Lorenzo, V., Herrero, M., Jakubzik, U. & Timmis, K. N. (1990). Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* **172**, 6568-6572.

Fineran, P. C., Slater, H., Everson, L., Hughes, K. & Salmond, G. P. (2005). Biosynthesis of tripyrrole and beta-lactam secondary metabolites in *Serratia*: integration of quorum sensing with multiple new regulatory components in the control of prodigiosin and carbapenem antibiotic production. *Mol Microbiol* **56**, 1495-1517.

Grinter, N. J. (1983). A broad host range cloning vector transposable to various replicons. *Gene* **21**, 133-143.

Gristwood, T., McNeil, M. B., Clulow, J. S., Salmond, G. P. & Fineran, P. C. (2011). PigS and PigP regulate prodigiosin biosynthesis in *Serratia* via differential control of divergent operons, which include predicted transporters of sulfur-containing molecules. *J Bacteriol* **193**, 1076-1085.

Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* **177**, 4121-4130.

Herrero, M., de Lorenzo, V. & Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* **172**, 6557-6567.

Kaniga, K., Delor, I. & Cornelis, G. R. (1991). A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* **109**, 137-141.

McNeil, M. B., Clulow, J. S., Wilf, N. M., Salmond, G. P. & Fineran, P. C. (2012). SdhE is a conserved protein required for flavinylation of succinate dehydrogenase in bacteria. *J Biol Chem* **287**, 18418-18428.

McNeil, M. B., Hampton, H. G., Hards, K. J., Watson, B. N., Cook, G. M. & Fineran, P. C. (2014). The succinate dehydrogenase assembly factor, SdhE, is required for the flavinylation and activation of fumarate reductase in bacteria. *FEBS letters* **588**, 414-421.

Przybilski, R., Richter, C., Gristwood, T., Clulow, J. S., Vercoe, R. B. & Fineran, P. C. (2011). Csy4 is responsible for CRISPR RNA processing in *Pectobacterium atrosepticum*. *RNA Biology* **8**, 517-528.

Ramsay, J. P., Williamson, N. R., Spring, D. R. & Salmond, G. P. (2011). A quorum-sensing molecule acts as a morphogen controlling gas vesicle organelle biogenesis and adaptive flotation in an enterobacterium. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 14932-14937.

Thomson, N. R., Crow, M. A., McGowan, S. J., Cox, A. & Salmond, G. P. (2000). Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. *Mol Microbiol* **36**, 539-556.

Williamson, N. R., Fineran, P. C., Ogawa, W., Woodley, L. R. & Salmond, G. P. (2008). Integrated regulation involving quorum sensing, a two-component system, a GGDEF/EAL domain protein and a post-transcriptional regulator controls swarming and RhlA-dependent surfactant biosynthesis in *Serratia*. *Environmental microbiology* **10**, 1202-1217.