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A complex network of small non-coding RNAs regulate motility in *Escherichia coli*

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Summary

Small Hfq-dependent non-coding regulatory RNAs (sRNAs) that alter mRNA stability and expression by pairing with target mRNAs have increasingly been shown to be important in influencing the behaviour of bacteria. In *Escherichia coli*, *flhD* and *flhC*, which encode the master regulator of flagellar synthesis, are co-transcribed from a promoter that is regulated by multiple transcription factors that respond to different environmental cues. Here, we show that the 5′ untranslated region (5′ UTR) of the *flhDC* mRNA also serves as a hub to integrate additional environmental cues into the decision to make flagella. Four sRNAs, ArcZ, OmrA, OmrB and OxyS, negatively regulated and one sRNA, McaS, positively regulated motility and *flhDC* expression by base-pairing with the 5′ UTR of this mRNA. Another sRNA, MicA, positively regulated motility independent of regulation of *flhDC*. Furthermore, we demonstrate that the regulation of motility by the ArcB/A two component system is in part due to its regulation of ArcZ. *flhDC* is the first mRNA that has been shown to be both positively and negatively regulated by direct pairing to sRNAs. Moreover, both positive regulation by McaS and negative regulation by ArcZ require the same binding site in the *flhDC* mRNA.

Introduction

Flagellar-based motility is important for the behaviour and pathogenesis of bacteria. A mutation that disrupts flagellar synthesis in uropathogenic *Escherichia coli* caused a defect in the colonization of kidneys in a mouse animal model (Lane *et al*., 2007); flagellar mutants of *Salmonella enterica* serovar Typhimurium (Stecher *et al*., 2008) and of *Vibrio cholerae* (Lee *et al*., 2001) were defective in colonization of the small intestine in a mouse animal model.

The process of flagellar synthesis has been extensively studied in both *E. coli* and *S. enterica* serovar Typhimurium. Complete flagellar synthesis requires more than 60 genes located in multiple operons (reviewed in Chilcott and Hughes, 2000). The expression of the genes within these operons is highly regulated at the transcriptional and post-translational level by several different feedback loops (reviewed in Chevance and Hughes, 2008). This complex regulatory cascade serves two purposes. First, regulation provides temporal coordination of gene expression so that the appropriate flagellar genes are expressed at the proper stage in flagellar assembly. Secondly, this extensive degree of regulation ensures that this large macromolecular structure is made only when it is beneficial for the bacteria to swim.

At the top of this regulatory cascade is the master regulator of flagellar synthesis, which is encoded by two genes, *flhD* and *flhC*, which form a two-gene operon. *flhD* and *flhC* are transcribed from a sigma 70 promoter; many different global transcription factors, including OmpR, LrhA, Crp, H-NS and the RcsAB complex, have been implicated in regulating their expression (reviewed in Soutourina and Bertin, 2003). FlhD and FlhC form a heterotetramer of homodimers ($FlhD_2C_2$), which activates the transcription of the sigma 70 dependent class II promoters that encode the genes for hook and basal body synthesis, the flagellar sigma factor σ^F (FliA), and the flagellar anti-sigma factor FlgM. σ^F subsequently initiates transcription of the class III genes, which primarily encode proteins involved in the construction of the flagellar filament and the flagellar cap, and proteins involved in chemotaxis (reviewed in Chilcott and Hughes, 2000). The activity of the $FlhD_2C_2$ master regulator is also regulated by at least two inhibitors, FliT (Aldridge *et al*., 2010) and YdiV (Wada *et al*., 2011), which block $FlhD_2C_2$ from binding to Class II promoters.

Because flagellar synthesis is highly regulated at the transcriptional level and post-translational level, we thought that it would also be regulated at the post-transcriptional level by small non-coding RNAs (sRNAs). Approximately 30 Hfq-binding sRNAs have been identified in *E. coli* (Argaman *et al*., 2001; Wassarman *et al*.,

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2001; Chen *et al*., 2002; Vogel *et al*., 2003; Zhang *et al*., 2003). The transcription of many of these sRNAs is highly regulated by well-studied global regulators. Once transcribed, these sRNAs bind to the RNA chaperone Hfq, which mediates the pairing of these sRNAs to near-complementary sequences in target mRNAs (reviewed in Waters and Storz, 2009). Binding of the sRNA to the mRNA can lead to an increase or decrease in the stability and/or translation of the mRNA.

Here, we report that regulation of motility and *flhDC* by sRNAs is at least as complex as that seen with other levels of regulation. Five sRNAs act as negative regulators and two act as positive regulators, including McaS, recently described by Thomason *et al*. (Thomason *et al*., 2012). Our work demonstrates that the *flhDC* transcriptional regulators are subject to direct negative regulation as well as positive regulation, the first such example. This regulation of *flhD* and *flhC* expression by many different sRNAs, in addition to the extensive transcriptional regulation, gives the cell the ability to integrate many more environmental and physiological cues into the decision as to whether or not to make flagella. In addition, we find that sRNA-mediated regulation provides a positive feed-forward loop, reinforcing regulation at other levels.

Results

Studies by us (De Lay and Gottesman, 2009) and by others (Papenfort *et al*., 2009; Monteiro *et al*., 2012; Thomason *et al*., 2012) have suggested that Hfq-binding sRNAs play a role in the transition of *E. coli* and *S. enterica* serovar Typhimurium from biofilms to a motile, planktonic state and back. These transitions are complex and undoubtedly reflect regulation of multiple targets. One set of important targets in determining these behaviours are the genes that regulate motility. We directly tested the role of sRNAs in the regulation of motility in *E. coli*, using a library of plasmids, each expressing a different Hfq-binding sRNA (Mandin and Gottesman, 2010). Overnight cultures of a derivative of MG1655 harbouring the vector pBR-plac or a derived plasmid expressing one of 26 different sRNAs were spotted on motility plates containing ampicillin and IPTG to induce expression of the sRNA and incubated at 25°C for 24 h (Fig. 1). In agreement with experiments in *S. enterica* serovar Typhimurium (Papenfort *et al*., 2009; Monteiro *et al*., 2012), expression of ArcZ from a plasmid eliminated the motility of *E. coli*. Expression of OmrA also eliminated motility, while strains overexpressing OmrB, GadY or OxyS showed significantly reduced motility compared with a strain harbouring an empty vector (Fig. 1). While not as significant, expression of SdsR reduced the motility of strain NRD688 by one-third (Fig. 1). In contrast, a strain expressing MicA travelled 1.5 times the distance of the same strain harbouring an empty vector (Fig. 1). Consistent with the results of Thomason *et al*. (2012), expression of McaS drastically increased the motility of *E. coli* (4.3-fold). Therefore, eight sRNAs, almost 1/3 of those tested, had substantial effects on *E. coli* motility.

Multiple sRNAs negatively regulate the expression of FlhD and FlhC at the post-transcriptional level

The changes in motility shown in Fig. 1 could reflect regulation by the sRNAs at many stages of flagellar synthesis or activity. However, if this regulation is physiologically relevant, it would be most efficient for the cell to regulate at the top of the flagellar synthesis cascade. The *flhDC*-encoding mRNA has a conserved 5′ untranslated leader (5′ UTR) of 198 nt (Wei *et al*., 2001). The global regulator

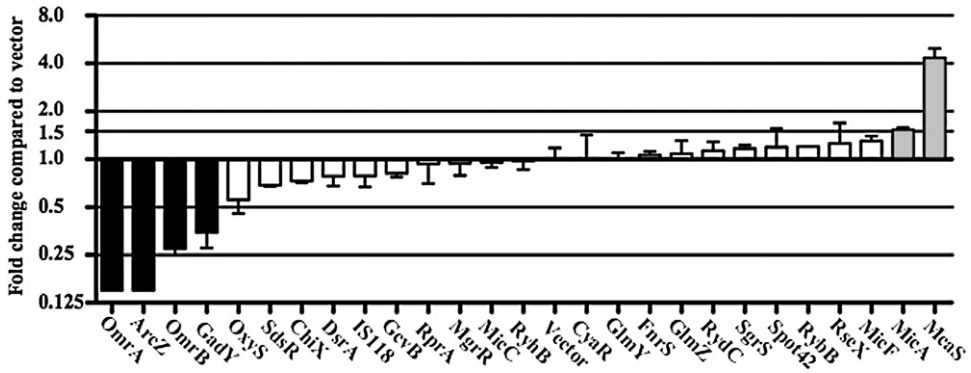

Fig. 1. Motility assays on strains expressing sRNAs from a plasmid-based sRNA library. Overnight cultures of strain NRD688 harbouring pBR-plac (vector) or a derivative expressing one of 26 different sRNAs from a *lac*-based promoter were spotted on top of a TB plate containing agar at a final concentration of 0.25%. After 16 h of incubation at 25°C, the diameter of motility was measured and the fold change relative to the strain harbouring the vector control was calculated. These results represent the average fold change relative to the vector control from three independent experiments. The black bars indicate expressed sRNAs that reduced motility by more than twofold and the grey bars represent expressed sRNAs that increased motility by more than 50%.

CsrA was shown to bind to the leader of the *flhD* mRNA and stimulate the translation of this message (Wei *et al*., 2001). We first generated a translational *lacZ* fusion to *flhD* under the control of the *araBAD* promoter, as previously described (Mandin and Gottesman, 2009). The resulting strain, NRD688, has a translational fusion that contains the entire 198 nt 5′ UTR and the first 9 codons of *flhD* fused to the 10th codon of *lacZ*.

We grew cultures of the strain expressing the *flhD′–′lacZ* fusion and harbouring the vector pBR-plac or a plasmid expressing each one of the 26 different sRNAs and measured the amount of fusion protein produced by β-galactosidase activity assays. In a wild-type strain, expression of OmrA, ArcZ and OxyS reduced the expression of the *flhD′–′lacZ* translational fusion by more than twofold (Fig. 2A). In a strain deleted for *arcZ*, *omrA*, *omrB* and *sdsR*, expression of the six sRNAs that reduced motility by the greatest amount (OmrA, ArcZ, OxyS, SdsR, OmrB and GadY) still reduced expression of the fusion by more than twofold (Fig. 2B). The increased effectiveness of these six sRNAs in regulating the fusion in this quadruple mutant indicates that one or more of the sRNAs deleted in this strain was affecting expression; the level of the fusion in the presence of the vector was increased 1.5-fold by deleting these sRNAs. The quadruple deletion also increased motility (data not shown). While the effects of deleting the genes for these sRNAs are not dramatic (or they probably would have been identified as regulators of motility years ago), expression of these sRNAs is not likely to be particularly high under the assay conditions. No other sRNAs showed significant negative regulation of *flhD′–′lacZ*.

MicA, which upregulated motility by 1.5-fold upon being expressed from a plasmid, had no significant effect on the *flhD′–′lacZ* fusion (Fig. 2A), indicating that the role of MicA in increasing motility is likely to be independent of FlhD

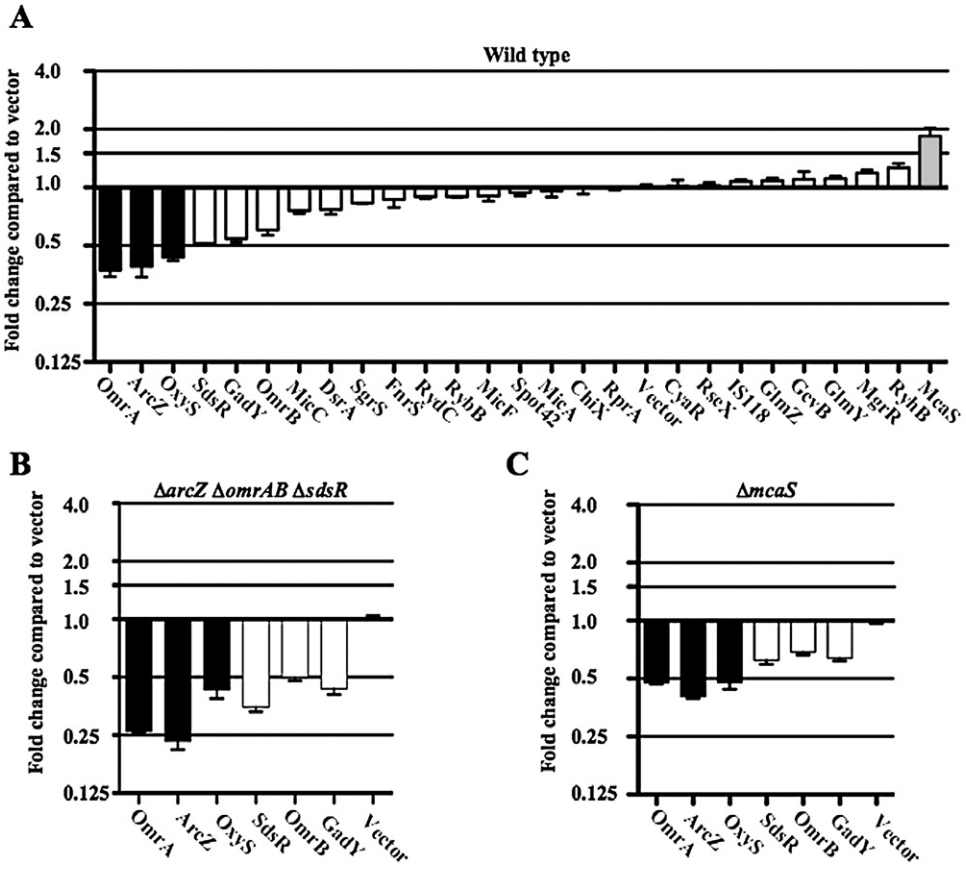

Fig. 2. Analysis of the effect of expressing sRNAs on a *flhD′–′lacZ* translation fusion. Overnight cultures of strain NRD688 harbouring a *flhD′–′lacZ* translation fusion (A), a derivative harbouring deletions of *arcZ*, *omrA*, *omrB* and *sdsR* (NRD765) (B), or a deletion of *mcaS* (NRD857) (C) and carrying either pBR-plac (vector) or a derivative plasmid that expresses a particular sRNA was diluted 200-fold in fresh LB medium containing ampicillin (100 mg l^{-1}), IPTG (100 μM), and arabinose (0.01%). After the culture was incubated at 37°C at 250 r.p.m. for 6 h, the OD_{600} of each culture was measured and a β-galactosidase assay was performed as described by Miller (1972). The experiments were performed in triplicate. The black bars indicate expressed sRNAs that reduced motility by more than twofold and the grey bars represent expressed sRNAs that increased motility by more than 50%. The level of expression with the vector control was 225 Miller units for NRD688 (A), 334 Miller units for NRD765 (B) and 172 Miller units for NRD857 (C).

and FlhC. However, it is possible that MicA could target a sequence in the *flhDC* mRNA that was not present in the fusion. In agreement with the results of Thomason *et al*. (Thomason *et al*., 2012), expression of McaS from a plasmid led to an increase in the expression of the *flhD′–′lacZ* fusion (Fig. 2A).

McaS was shown to positively regulate the expression of FlhD by direct base-pairing with the *flhDC* 5′ UTR in an Hfq-dependent manner (Thomason *et al*., 2012). Highly expressed sRNAs have been shown to block other sRNAs from regulating their targets by outcompeting them for the limiting pool of Hfq (Hussein and Lim, 2011; Moon and Gottesman, 2011; Olejniczak, 2011). Therefore, it was possible that the negative regulation of the *flhD′–′lacZ* fusion by some of the sRNAs was by blocking activation by McaS, by outcompeting McaS for Hfq binding or otherwise interfering with McaS action. If so, deleting *mcaS* would eliminate the effect of these sRNAs. This was tested (Fig. 2C). Deleting *mcaS* slightly reduced expression of the *flhD′–′lacZ* fusion, but did not interfere with the ability of these sRNAs to regulate the fusion (Fig. 2C; compare with Fig. 2A). Thus, the negative post-transcriptional regulation of *flhD* was not primarily due to interference with positive regulation by McaS.

We examined the sequence of the 5′ UTR of the *flhDC* mRNA and the sequence of each of the negatively regulating sRNAs for potential regions of base-pairing using the NUPACK software (Zadeh *et al*., 2011) and by visual inspection. Two regions of the *flhDC* leader were identified as potential pairing sites for OxyS, ArcZ, OmrA and OmrB (Fig. 3). One region (A site), close to the ribosome binding site, was predicted to interact with all four of these sRNAs; a second predicted pairing site for ArcZ was farther upstream (B site), and partially overlapped a region of predicted pairing for OmrA. Site B also overlaps one of the two sites that McaS pairs with (Fig. 3). Experiments investigating these predictions are described below.

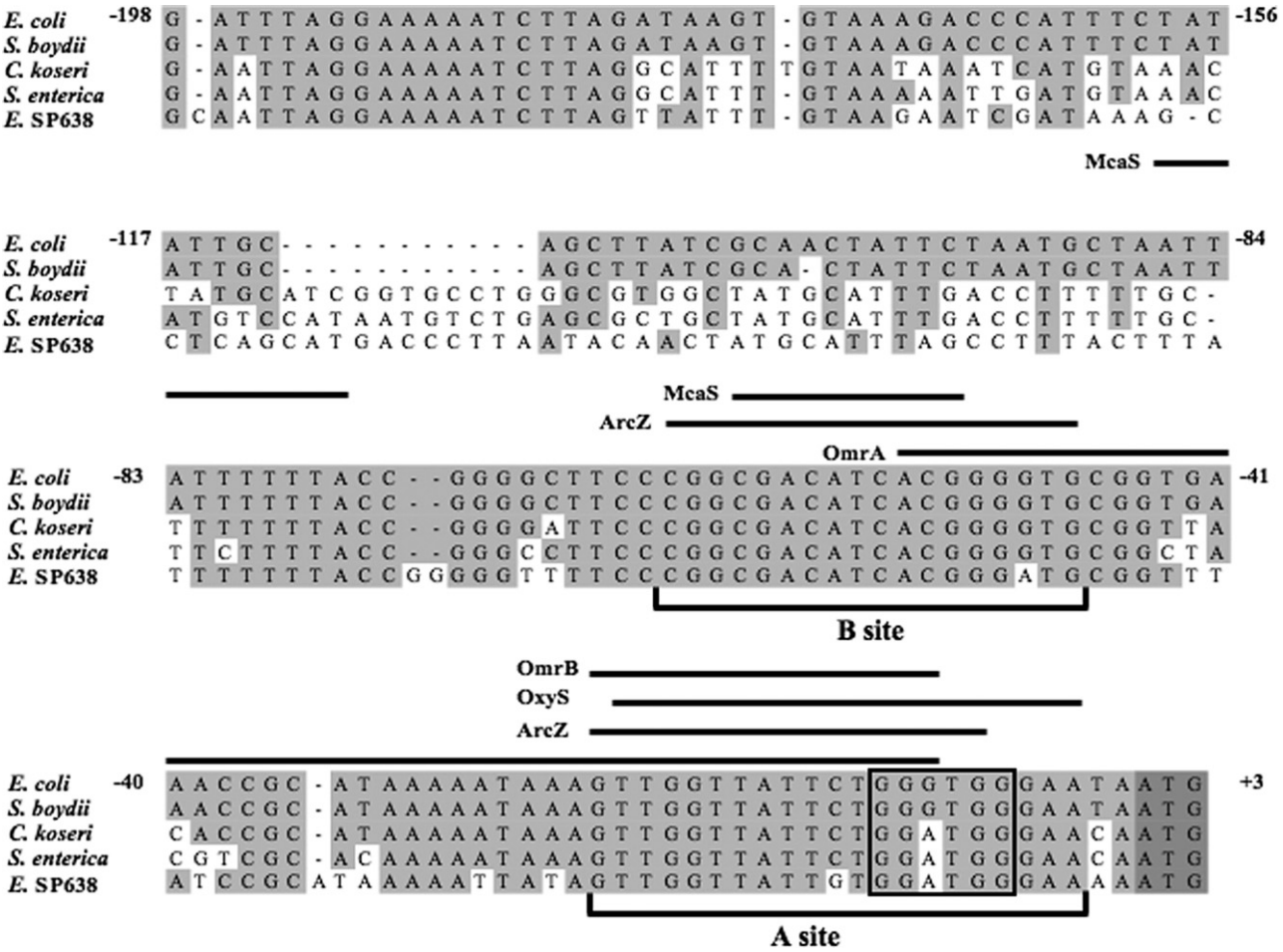

Fig. 3. Alignment of the 5′ UTR and the start codon of *flhD*. DNA sequence identity is shaded in grey, the start codon is highlighted in dark grey, and the putative ribosome binding site is boxed. The bars above the sequence indicate the region predicted to pair with the indicated sRNA. *S. boydii*, *Shigella boydii* Sb227; *C. koseri*, *Citrobacter koseri* ATCC BAA-895; *S. enterica*, *Salmonella enterica* serovar Typhimurium LT2; *E.* SP638, *Enterobacter* species 638.

We were unable to find regions of significant base-pairing between GadY and *flhD* mRNA, and GadY was still able to regulate a set of mutant derivatives of the A site of the *flhD′–′lacZ* fusion (Fig. S1), suggesting that GadY does not pair with this region of the *flhD* mRNA. While computational approaches failed to predict pairing between the *flhDC* mRNA and SdsR, visual inspection identified a potential region of pairing (Fig. S2A). However, mutations that should completely disrupt this pairing between SdsR and *flhDC* had only a modest effect on regulation, and restoring the predicted pairing did not improve regulation (Fig. S2B and C). Altogether, these results suggest that the negative regulation of the *flhDC* mRNA by GadY and SdsR may not be through direct base-pairing.

OxyS negatively regulates flhD and flhC by direct base-pairing

OxyS is a 109 nt RNA; its transcription is activated by OxyR in response to oxidative stress (Altuvia *et al*., 1997). OxyS was shown to negatively regulate the expression of the transcriptional regulators FhlA and RpoS (Altuvia *et al*., 1997). The negative regulation of FhlA expression by OxyS results from direct base-pairing between the encoding mRNA and two regions of OxyS (Altuvia *et al*., 1998; Argaman and Altuvia, 2000), while the negative regulation of RpoS appears to be indirect, via titration of Hfq (Zhang *et al*., 1998; Moon and Gottesman, 2011).

To determine whether OxyS regulated the *flhDC* mRNA at the post-transcriptional level via direct base-pairing, we generated mutations in the predicted pairing regions in both the *flhD′–′lacZ* fusion (mut3A) and in OxyS (OxyS-mut3*) (Fig. 4A). This region of OxyS, from +54 to +73, is located between the two regions of OxyS (+22 to +30 and +98 to +104) that pair with *flhA* (Argaman and Altuvia, 2000). The region from +54 to +59 is in the loop of a short stem-loop; the region from +64 to +73 is predicted to be single-stranded. We tested the ability of the wild-type OxyS and the mutant form of OxyS to regulate wild-type and mutant *flhD′–′lacZ* fusions. Unexpectedly, OxySmut3* regulated the wild-type *flhD′–′lacZ* fusion slightly better than the wild-type OxyS (Fig. 4B), possibly reflecting alternative pairing of OxySmut3* with the *flhD* leader (Fig. S3). However, the wild-type OxyS was not able to effectively regulate the *flhD*–mut3A fusion, while OxySmut3*, containing compensatory mutations for *flhD*–mut3A, was able to regulate this mutant fusion even better than it was able to regulate the wild-type *flhD′–′lacZ* fusion (repression to 24% of the vector control, compared with 38% of the vector control for the wild-type target; Fig. 4B). These results support direct pairing of OxyS with the 5′ UTR of *flhDC*. In addition, this work identifies a new region of OxyS involved in pairing with targets.

OmrA and OmrB negatively regulate flhD and flhC by direct base-pairing

As mentioned above, expression of OmrA reduced expression of the *flhD′–′lacZ* fusion by 2.7-fold; the paralogue of OmrA, OmrB, reduced expression by 1.7-fold.

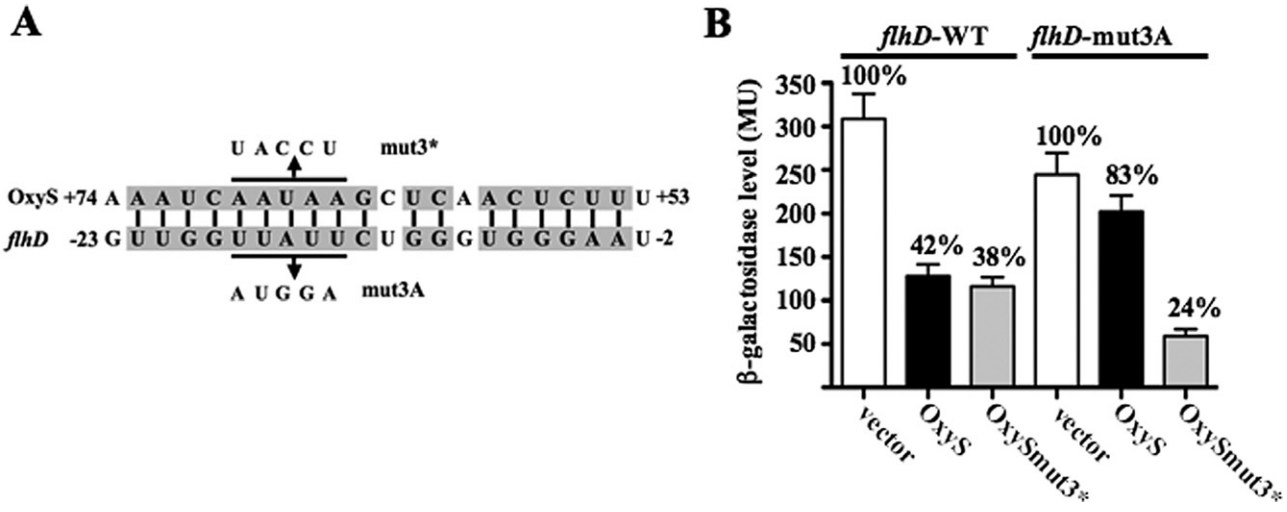

Fig. 4. Analysis of OxyS interaction with *flhD*.
A. The predicted region of base-pairing between OxyS and the 5′ UTR of the *flhD* mRNA, the substitutions present in OxySmut3* and the compensatory mutations in the *flhD* leader (mut3A) are shown. The *flhD′–′lacZ* strains contain deletions of *omrAB*, *sdsR* and *arcZ*.
B. Overnight cultures of NRD765 (*flhD′–′lacZ* fusion; *flhD*–WT) or NRD800 [*flhD*(T-18A A-16G T-15G T-14A)′–′*lacZ* fusion; *flhD*–mut3A] harbouring pBR-plac (vector), pBR-plac-OxyS (wild-type *oxyS*; OxyS), or pNRD440 (*oxyS* A65T A66C T67C A69T; OxySmut3*) were diluted 200-fold into LB liquid medium containing ampicillin, arabinose, and IPTG and incubated at 37°C for 6 h. β-Galactosidase assays were performed in triplicate. The percentage of β-galactosidase activity relative to the activity of the strain harbouring the empty vector (set at 100%) is indicated above each bar.

OmrA and OmrB are encoded in tandem in the chromosome and are transcribed in response to activation of the EnvZ/OmpR two component system (Guillier and Gottesman, 2006). Once transcribed, these sRNAs downregulate several outer membrane proteins, including OmpT and CirA, and at least two transcriptional regulators of cell surface proteins, OmpR and CsgD (Guillier and Gottesman, 2006; 2008; Holmqvist *et al*., 2010). The sequences of OmrA and OmrB are almost completely identical at the 5′ end, but diverge after the 21st nucleotide (Fig. 5A and 5B). The previously described targets of OmrA and OmrB have all been shown to pair with this conserved 5′ end (Guillier and Gottesman, 2006; 2008; Holmqvist *et al*., 2010). The predicted pairing between OmrA and the *flhD* leader that we identified is quite extensive (Fig. 5A). OmrB, which does not regulate *flhD* as effectively as OmrA, has far less predicted base-pairing with the *flhD* leader (Fig. 5B).

To test whether these predictions for base-pairing between OmrA or OmrB and the *flhD* mRNA were correct, a series of mutations were made in the plasmid-encoded *omrA* and *omrB* genes (Guillier and Gottesman, 2008), in the regions common to both sRNAs and predicted to pair with the *flhD* mRNA. In all of these tests, the wild-type and mutant forms of OmrA and OmrB were assayed for the ability to regulate the *flhD′–′lacZ* fusion in a strain harbouring deletions of *omrA*, *omrB*, *sdsR* and *arcZ*. In this assay, OmrA expression resulted in a 70% reduction in

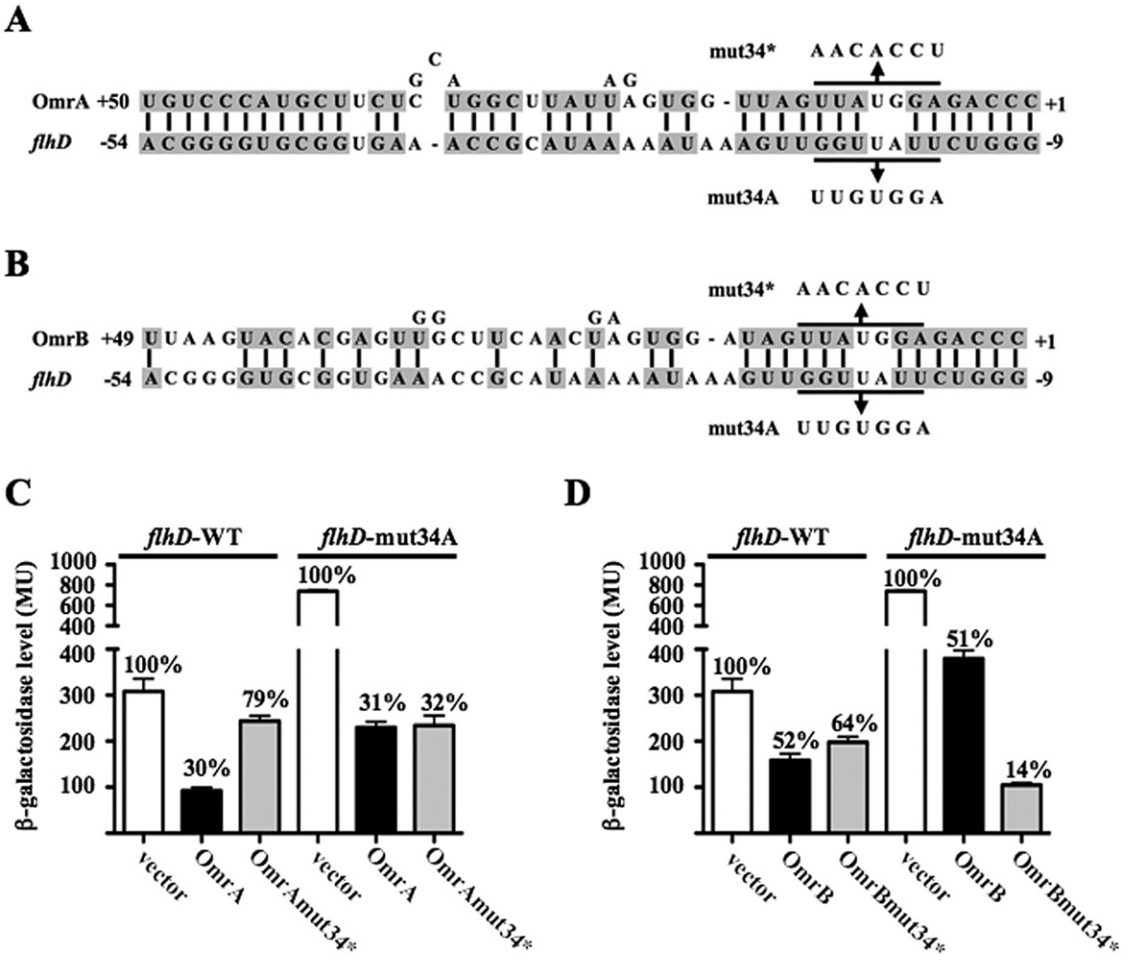

Fig. 5. Analysis of OmrA and OmrB interaction with *flhD*.
A. The predicted region of pairing between OmrA and the 5′ UTR of the *flhD* mRNA. The substitutions in *flhD*–mut34A and OmrAmut34* are indicated. The *flhD′–′lacZ* strains contain deletions of *omrAB*, *sdsR* and *arcZ*.
B. The predicted base-pairing between OmrB and the 5′ UTR of the *flhD* mRNA. The substitutions present in *flhD*–mut34A and OmrBmut34* are indicated.
C. Overnight cultures of NRD765 (*flhD′–′lacZ* fusion; *flhD*–WT) or NRD855 [*flhD*(G-20T G-19T T-18G A-16G T-15G T-14A)′–′*lacZ* fusion; *flhD*–mut34A] harbouring pBR-plac (vector), pBR-plac-OmrA (wild-type *omrA*; OmrA), or pNRD441 (*omrA* A6T G7C G8C T9A A10C T11A T12A; OmrAmut34*) were diluted, grown and assayed as described in Fig. 4.
D. Overnight cultures of NRD765 (*flhD*–WT) or NRD855 (*flhD*–mut34A) harbouring pBR-plac (vector), pBR-plac-OmrB (wild-type *omrB*; OmrB), or pNRD442 (*omrB* A6T G7C G8C T9A A10C T11A T12A; OmrBmut34*) were diluted, grown, and assayed as described in Fig. 4.

the expression of the wild-type *flhD′–′lacZ* fusion; mut34* abrogated much of this regulation (Fig. 5C). The results for OmrB followed a similar pattern (Fig. 5D). Thus, introduction of these mutations in OmrA or OmrB disrupted the ability of these sRNAs to fully regulate this fusion.

If this pairing is critical for regulation, compensatory mutations in the 5′ UTR of the *flhD′–′lacZ* fusion should restore regulation by OmrAmut34* or OmrBmut34*. A strain containing the compensating mutations in the fusion was generated and the ability of the wild-type and mutant OmrA and OmrB to regulate this fusion was tested. As shown in Fig. 5, the results with both OmrA and OmrB are consistent with direct pairing. Introduction of compensating mutations in the *flhD′–′lacZ* fusion (*flhD*–mut34) increased the ability of OmrAmut34* or OmrBmut34* to regulate the *flhD′–′lacZ* fusion (Fig. 5C and D). The wild-type sRNAs were as effective in regulating the *flhD*–mut34A as they are for the wild-type *flhD′–′lacZ* fusion. These results may be explained by the existence of alternative pairing sites for OmrA and OmrB in the *flhDC* leader. Alternatively, the extensive base-pairing between these sRNAs and the *flhDC* leader may make it difficult to disrupt regulation by mutating only one region of the target mRNA.

We also tested two additional mutants of OmrA (OmrAmut2* and OmrAmut3*; Fig. S4A), and OmrB (OmrBmut2* and OmrBmut3*; Fig. S4C) predicted to disrupt base-pairing. These Omr mutants have been shown to be defective for regulation of other targets (Guillier and Gottesman, 2008). All four sRNA mutants had reduced ability to regulate the wild-type *flhD′–′lacZ* fusion (Fig. S4B and D). Compensating mutations for OmrAmut2* and OmrBmut2* reduced the basal level of expression and were not effective in either disrupting regulation with the wild-type sRNA or restoring regulation for the mutants (Fig. S4B and D). However, compensating mutations for OmrAmut3* and OmrBmut3* in the *flhD′–′lacZ* fusion improved the ability of these mutant sRNAs to regulate, compared with their activity on a wild-type *flhD′–′lacZ* fusion (Fig. S4B and D). Altogether, these results suggest that OmrA and OmrB regulate the *flhD* mRNA by direct base-pairing, but that alternative binding modes may be possible.

ArcZ negatively regulates flhD and flhC by direct base-pairing to a region upstream of the ribosome binding site

ArcZ decreased both motility and *flhD′–′lacZ* fusion expression as strongly as did OmrA (Figs 1 and 2). ArcZ is transcribed as a 121 nt RNA, which is subsequently processed to generate a smaller, stable RNA that consists of the last 56 nt of the original transcript (Argaman *et al*., 2001; Papenfort *et al*., 2009; Mandin and Gottesman, 2010). In *E. coli*, ArcZ has been shown to positively regulate *rpoS* mRNA translation as a result of direct base-pairing between a region spanning the first 26 nt of this smaller, processed sRNA (nt 66–91 of the full-length transcript), and a region in the leader of the *rpoS* mRNA (Mandin and Gottesman, 2010). In *S. enterica* serovar Typhimurium, this region of ArcZ has also been shown to be involved in pairing with the *sdaC*, STM3216 and *tpx* mRNAs, resulting in negative regulation of these targets (Papenfort *et al*., 2009). In addition, overexpression of ArcZ in *S. enterica* serovar Typhimurium led to a threefold reduction in the level of the mRNAs encoding *flhD* and *flhC* (Papenfort *et al*., 2009) and reduced motility (Papenfort *et al*., 2009; Monteiro *et al*., 2012). This is fully consistent with our observation of a reduction in expression of the *flhD′–′lacZ* translational fusion upon ArcZ expression. The first 16 nt of the processed ArcZ, nucleotides 66–81, could potentially pair with either of two conserved regions within the 5′ UTR of *flhD* (Figs 3 and 6A). One potential pairing region is located from –7 to –23 relative to the start codon and will be referred to here as the A site; the second pairing region, the B site, is located from –47 to –64 relative to the start codon. Interestingly, the B site overlaps one of the two sites McaS pairs with (Thomason *et al*., 2012; shown in Fig. 3).

We generated a set of site-directed mutants of *arcZ* in the plasmid pBR-plac-ArcZ in the region predicted to pair with the two different regions of *flhD*, as well as one mutant, ArcZmut1*, outside of this region (Fig. S5A). Wild-type or mutant ArcZ sRNAs were assayed in strains harbouring either an *flhD′–′lacZ* or an *rpoS′–′lacZ* fusion (Fig. S5C and D), and the level of sRNA was assayed by Northern blot analysis (Fig. S5B). All of the mutants generated showed defects in regulation of the *flhD′–′lacZ* fusion (Fig. S5C). Three of the mutants, ArcZmut1*, ArcZmut5* and ArcZmut6*, had reduced levels of processed sRNA, and were defective for regulation of both fusions. ArcZmut34* and ArcZmut3*, a mutant carrying three of the five substitutions present in ArcZmut34* (Fig. S5A), were expressed at significant levels, stimulated *rpoS* expression by twofold or more, but were defective in negative regulation of the *flhD′–′lacZ* fusion (Fig. S5C and D).

To test potential base-pairing of ArcZ with *flhD* and the role of pairing at either the A or B site, compensating mutations were created in each site (*flhD*mut3A, *flhD*mut3B and *flhD*mut34B) and tested with wild-type and appropriate mutant ArcZ derivatives (Figs 6 and S6). We note that the pairing of ArcZ at site B is more extended (8 adjacent nucleotide pairs versus 5 at A site; A site is also primarily G:U base pairs). When expressed from a plasmid, the wild-type ArcZ did not effectively regulate the mutant *flhD*–mut34B fusion (expression of the fusion was reduced to 71% of the vector control), while ArcZmut34*

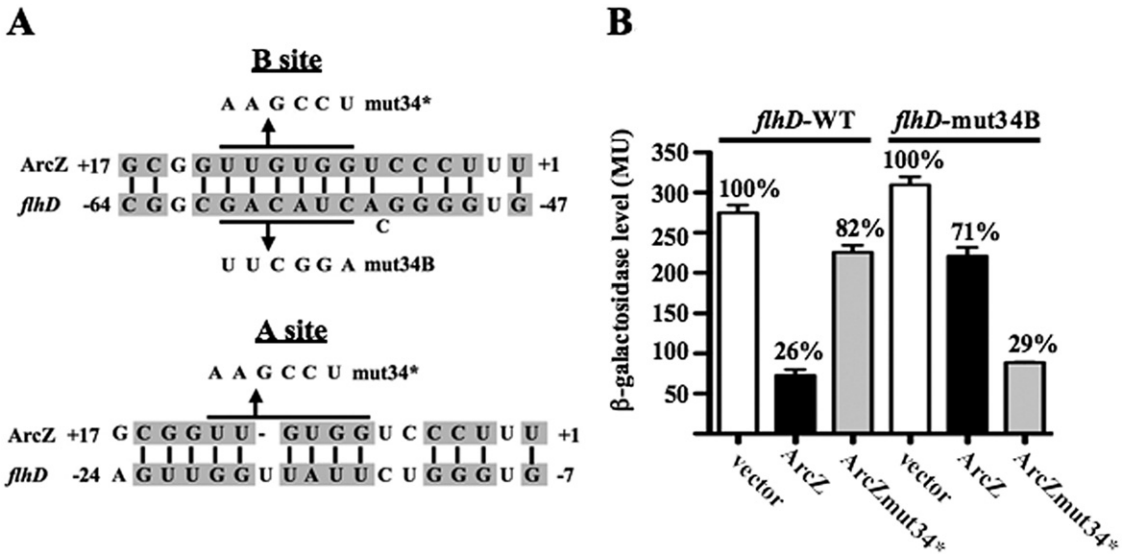

Fig. 6. Analysis of the interaction of ArcZ and *flhD*.
A. The predicted base-pairing between ArcZ and two regions in the 5′ UTR of the *flhD* mRNA (B site and A site) and the substitutions present in ArcZmut34* and the *flhD*–mut34B fusion. These strains are deleted for the chromosomal *arcZ* gene.
B. Overnight cultures of NRD741 (*flhD*–WT) or NRD883 [*flhD*(G60T A-59T A-57G T-56G C-55A)′–′*lacZ* fusion; *flhD*–mut34B] harbouring pBR-plac (vector), pBR-plac-ArcZ (ArcZ), or pNRD436 (ArcZmut34*) were diluted, grown, and assayed as for Fig. 4.

was able to regulate this mutant fusion as effectively as the wild-type sRNA regulated the wild-type fusion (Fig. 6B). A less dramatic change at site B, mut3B, also interfered with the ability of wild-type ArcZ to regulate, and regulation by an ArcZ with a compensating mutation (ArcZmut3*) was slightly better (Fig. S6). Mutation of site A did not interfere with the ability of wild-type ArcZ to regulate (*flhD*–mut3A, Fig. S6). Mutation of both sites (mut3AB) was regulated poorly by wild-type ArcZ but improved with the compensating mutation in ArcZ, ArcZmut3* (Fig. S6). Altogether, these results demonstrate that site B is critical for pairing while site A is not.

Interestingly, the B site mutation (mut34B) in the *flhDC* leader that disrupted negative regulation by the wild-type ArcZ also completely eliminated the ability of the McaS to positively regulate this fusion (Fig. S7A and B). This result confirms that the B site is also critical for the positive regulation of *flhDC* by McaS (Thomason *et al*., 2012).

The regulation of motility by ArcZ is mediated in part by the regulation of flhD

We have demonstrated that the overexpression of ArcZ from a plasmid eliminates the motility of *E. coli* (Fig. 1). Moreover, we have shown that ArcZ negatively regulates the expression of FlhD and FlhC, which form the master regulator of flagellar synthesis, by pairing with the *flhDC* mRNA (Fig. 6). However, given the complexity of the regulatory circuits affecting motility, it was unclear whether the negative regulation of *flhDC* fully explains the effect on motility or whether ArcZ might act in other ways as well.

To address this issue, we examined the effects of mutations in *arcZ* on motility, and took advantage of the specificity mutations tested in Fig. 6 to directly assess the importance of pairing with the *flhDC* mRNA. When expressed from a plasmid, ArcZmut34* was not able to downregulate motility (Fig. 7A); therefore, all targets of ArcZ important for motility regulation are resistant to this mutation. This mutant was partially active for regulation of RpoS (Fig. S5D). We then replaced the sequence upstream of *flhD* at the normal chromosomal site with the mut34B mutation. Expression of the wild-type ArcZ reduced the motility of the strain carrying *flhD*34B by slightly less than twofold (Fig. 7A). Since expression of ArcZ in a strain wild-type for *flhD* eliminated motility, this suggests that the reduction in *flhD* expression by ArcZ contributes significantly to the reduced motility. However, it is likely that *flhD* is not the only target important for motility; if it were, we would have expected no or very little reduction of motility in the *flhD*34B strain by wild-type ArcZ. Reinforcing this interpretation is the observation that overproducing ArcZmut34* in the *flhD*34B strain only modestly decreased motility (presumably now repressing *flhDC* but not the additional targets).

Next, we wanted to determine whether or not the expression of ArcZ from its native promoter on the chromosome had an effect on motility. ArcZ is regulated by the ArcB/ArcA two-component system (Mandin and Gottesman, 2010). Under anaerobic conditions, ArcB, the sensor

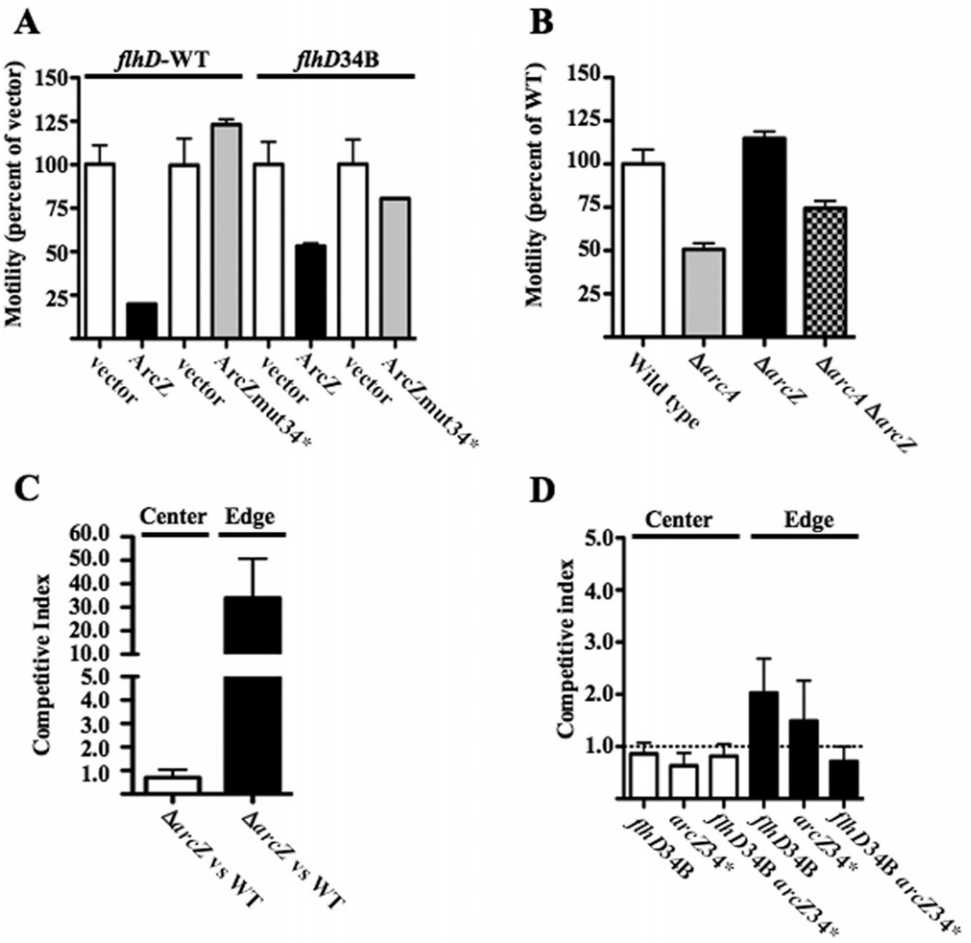

Fig. 7. The role of ArcZ regulation of FlhD expression in motility.
A. Overnight cultures of MG1655 (*flhD*–WT) or NRD892 (*flhD* G60T A-59T A-57G T-56G C-55A; *flhD*34B) harbouring pBR-plac (vector), pBR-plac-ArcZ (ArcZ), or pNRD436 (ArcZmut34*) were spotted on top of a TB motility plate containing ampicilllin and IPTG and incubated at 30°C for 6 h.
B. Overnight cultures of NRD688 or the derived strains NRD741 (Δ*arcZ*), NRD929 (Δ*arcA*), NRD930 (Δ*arcA* Δ*arcZ*) were spotted on TB plate containing agar at a final concentration of 0.25%, incubated at 30°C for 23h. The diameter of motility after incubation was then measured and the percent motility relative to the vector control (A) or wild-type strain (B) was calculated.
C. Overnight cultures of NRD688 (WT) or a Δ*arcZ::tet* derivative NRD741 (Δ*arcZ*) were mixed in a 1:1 ratio and spotted on a TB plate containing agar at a final concentration of 0.25% and incubated at 30°C for 16h.
D. Overnight cultures of the *lac*⁻ derivative of MG1655 (NRD948; WT) and the *lac*⁺ strains NRD892 (*flhD* G60T A-59T A-57G T-56G C-55A; *flhD*34B), NRD915 (*arcZ* G73T G74C T75C T77A T78A; *arcZmut*34*), or NRD919 (*flhD*34B *arcZmut*34*) were mixed in a 1:1 ratio and spotted on a TB plate containing agar at a final concentration of 0.25% and incubated at 30°C for 6.5 h. The mutant competed against the wild-type strain is listed below each bar. Similar results were obtained in competition experiments when the wild-type strain was *lac*⁺ and the mutant strain was *lac*⁻. For (C) and (D), the competitive index or ratio of the mutant to wild-type cells recovered at each position divided by the ratio of mutant cells over the number of wild-type cells was calculated as described in *Experimental procedures*. Note the difference in incubation times for motility assays for MG1655 derivatives (6–6.5 h in A and D) and derivatives of NRD688 (derived from MG1655) (16–23 h in B and C); NRD688 swims significantly more slowly. An IS element has been found upstream of *flhDC* in MG1655, and has been shown to increase motility (Barker *et al*., 2004). The strains in (A) and (D) contain this IS element, while those in (B) and (C) do not, providing an explanation for the motility differences. These differences make direct comparisons between (C) and (D) difficult.

kinase of this two-component system, phosphorylates ArcA, which then represses ArcZ transcription. The kinase activity of ArcB is inhibited under aerobic conditions by oxidized quinones (reviewed in Malpica *et al*., 2006). As a result, ArcZ is best expressed under aerobic conditions. Under anaerobic conditions, an *arcA* deletion mutant expresses ArcZ at a higher level (Mandin and Gottesman, 2010). Prior to this work, others have shown in *S. enterica* serovar Typhimurium that deletion of *arcA* reduced motility and decreased the expression of flagellar genes (Kato *et al*., 2007; Evans *et al*., 2011). We first examined the effect of an *arcA* and/or an *arcZ* deletion on expression of the *flhD′–′lacZ* fusion. As shown in Fig. S8, little difference in expression of the *flhD* translational fusion was observed between the wild-type strain and a derived *arcA*, *arcZ* or *arcA arcZ* deletion strain. However, we found that

a deletion of *arcA* led to a twofold decrease in motility, whereas deletion of *arcZ* resulted in a 15% increase in motility (Fig. 7B). The decreased motility of the *arcA* deletion strain was partially suppressed by introduction of an *arcZ* deletion (51% of wild-type motility for an *arcA* deletion strain, versus 74% of wild-type motility for an *arcA arcZ* double mutant; Fig. 7B).

To further determine the significance of the effect of the *arcZ* deletion on motility, we carried out a motility competition experiment between a wild-type strain and an *arcZ* deletion strain. Overnight cultures of a wild-type strain and the *arcZ* deletion mutant were mixed in a 1:1 ratio and spotted on motility plates. After incubation, cells were recovered from the inoculation site and the leading edge of swimming, and the ratio of mutant and wild-type cells at the two sites relative to the input ratio of mutant cells and wild-type cells spotted on the plate (the competitive index) was determined. In these competition experiments, we found that 34 times more *arcZ* deletion cells than wild-type cells were detected at the edge of swimming, while samples from the inoculation site gave nearly equal numbers of wild-type and *arcZ* mutants (Fig. 7C). Altogether, these results demonstrate that the regulation of motility by ArcA is in part mediated by ArcZ (Fig. 7B), and that chromosomally encoded levels of ArcZ have a significant repressing effect on motility (Fig. 7C).

If the effect of chromosomally expressed ArcZ on motility is primarily through its regulation of *flhDC* expression, we should be able to abrogate and then restore regulation by using appropriate pairing mutants. We first compared the motility of a wild-type strain to a strain harbouring the *flhDmut34B* mutation using motility competition experiments. In these competition experiments, roughly the same number of wild-type and *flhD34B* mutant cells were recovered at the inoculation site (competitive index of almost 1; Fig. 7D). However, more than twice as many *flhD34B* mutant cells, not effectively regulated by either ArcZ (Fig. 6B) or McaS (Fig. S7B), were recovered at the edge of the plate as wild-type cells (Fig. 7D).

Next, we introduced the *arcZmut34** mutation (G73T, G74C, T75C, T77A, T78A substitutions) into the copy of *arcZ* in the chromosome of a wild-type strain or a strain harbouring the *flhD34B* mutation. We then competed the wild-type strain against the *arcZmut34** mutant or the *arcZmut34* flhD34B* double mutant. In all of these competition experiments, approximately the same number of wild-type and mutant cells was recovered at the inoculation site (Fig. 7D, centre). However, the *arcZ*34* mutant strains (in which regulation by *arcZ* should be lost) were recovered 1.5 times more than the wild-type strain at the leading edge of swimming (Fig. 7D). When pairing of *flhD* with ArcZ was restored, in the *flhD*34B *arcZmut*34* double mutant, roughly the same number of wild-type cells and double mutant cells was recovered at the leading edge of swimming when they were competed against each other (Fig. 7D). Altogether these results demonstrate that at normal levels of expression, ArcZ has a significant effect, negatively regulating motility by regulation of *flhDC*.

Discussion

In *E. coli*, many surface proteins and/or transcriptional regulators of surface proteins are regulated by one or more sRNAs. All of the major porins are targets for negative regulation by one or more sRNAs (reviewed in Guillier *et al*., 2006). CsgD, a transcriptional regulator necessary for synthesis of curli, important in biofilm formation, is negatively regulated by at least five sRNAs (reviewed in Boehm and Vogel, 2012). Moreover, Thomason *et al*. recently reported that the *flhDC* mRNA, encoding the master regulator of flagellar synthesis, is positively regulated by the sRNA McaS (Thomason *et al*., 2012).

Here we have shown, using a library of plasmids, each expressing an Hfq-dependent sRNA, that *flhDC* expression and motility are negatively regulated by six sRNAs, ArcZ, OmrA, OmrB, OxyS, SdsR and GadY, and positively regulated by one sRNA, McaS (Fig. 8). An additional sRNA, MicA, positively regulates motility, but does not affect the expression of *flhDC*, indicating that it regulates motility at some other step. ArcZ, OmrA, OmrB and OxyS negatively regulate *flhDC* expression through direct base-pairing with the 5′ UTR of *flhDC*. Not only is this the first example of an mRNA that is both positively and negatively regulated by pairing with sRNAs, but this is also the first example of a positively regulating sRNA, McaS, and a negatively regulating sRNA, ArcZ, sharing a binding site on the same mRNA. The extensive regulation of *flhDC* expression by sRNAs in addition to the considerable regulation of expression at the transcriptional level allows the cell to integrate many environmental cues into the decision to make flagella (Fig. 8).

Multiple levels of regulation of motility by ArcA and ArcZ

The sRNA ArcZ is negatively regulated by the ArcB/ArcA two-component system. ArcB and ArcA have been shown to regulate multiple genes in response to changes in oxygen availability (Malpica *et al*., 2006). ArcZ has been previously described to positively regulate RpoS as well as to affect a variety of other targets (Papenfort *et al*., 2009; Mandin and Gottesman, 2010; Moon and Gottesman, 2011) and was also found to downregulate motility (Papenfort *et al*., 2009; Monteiro *et al*., 2012). A positive role for ArcA in regulating motility has previously been found (Kato *et al*., 2007; Evans *et al*., 2011). Our results confirm the positive role of ArcA. Deletion of *arcA* reduced motility by twofold; this was significantly but not fully

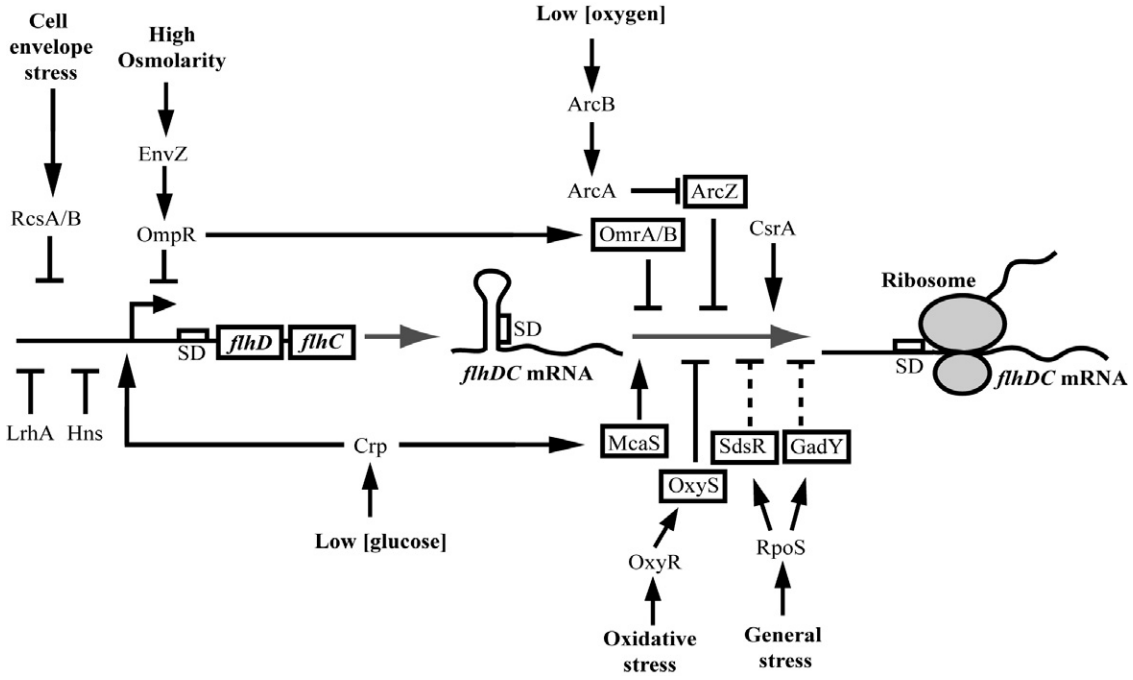

Fig. 8. Model of the regulation of FlhD and FlhC expression. Arrows and blocked arrows indicate positive and negative regulation respectively. Solid lines represent direct regulation and dashed lines indicate indirect regulation. sRNAs are boxed. SD, Shine–Dalgarno sequence.

overcome by deleting *arcZ* (Fig. 7B). Our experiments also demonstrate that ArcZ regulates motility at least in part through pairing with *flhD* (Fig. 7A and D). Therefore, we suggest that the previously observed effects of ArcA on motility and on the levels of genes downstream of *flhDC* (Kato *et al*., 2007; Evans *et al*., 2011) are in part explained by negative regulation of ArcZ by ArcA and the negative effects of ArcZ on *flhDC*.

However, it is clear that the effects of both ArcA and ArcZ are more complex. Since an *arcA arcZ* double deletion is still less motile than wild type (Fig. 7B), ArcA must have at least one target other than *arcZ* affecting motility. ArcZ is also likely to act on targets other than *flhD*, since overexpression of ArcZ virtually eliminated the motility of *E. coli* (Fig. 1), and was still able to reduce the motility of *E. coli* carrying an ArcZ-resistant *flhD* gene (Fig. 7A). ArcZ may regulate the expression of other genes by direct base-pairing and/or by outcompeting other sRNAs for Hfq binding.

ArcZ negatively regulates the expression of the *flhDC* mRNA through base-pairing with a region on the *flhDC* mRNA that also base-pairs with McaS, a sRNA that has been shown to positively regulate the expression of FlhD and FlhC (Thomason *et al*., 2012), but the effects of ArcZ are not simply due to blocking McaS action (Fig. 2C). The critical site for pairing with ArcZ is far upstream of the ribosome binding site (–47 to –64; Fig. 6A and B). Nevertheless, there are accumulating examples of other sRNAs that negatively regulate from a distant site, in some cases blocking ribosome binding by affecting an upstream translational enhancer (Sharma *et al*., 2007), and in other cases acting more indirectly. Spot42 regulates the expression of *sdhC* by recruiting Hfq to block ribosome binding (Desnoyers and Masse, 2012); possibly ArcZ pairing upstream could recruit Hfq to the AU-rich sequence upstream of the ribosome binding site (Fig. 3). MicC negatively regulates OmpD by MicC by inducing cleavage of the mRNA without blocking ribosome binding (Pfeiffer *et al*., 2009); this is also a possibility for ArcZ. Uncovering the mechanism by which pairing of ArcZ with the *flhDC* mRNA leads to decreased *flhDC* expression will be an interesting direction for future research.

As mentioned above, we have shown that the region of the *flhD* leader that serves as the critical site of pairing with ArcZ (Fig. 6B) is also one of the two sites that McaS binds (Fig. S7). As suggested by Thomason *et al*. (2012), it is possible that the single stranded region of McaS initially pairs with a region overlapping the ArcZ binding site on the *flhDC* mRNA and that this initial base-pairing facilitates the pairing of a second site on McaS with the second site on the *flhDC* mRNA, which would then facilitate the opening up of the structure of the *flhDC* mRNA to allow ribosome access. Such a model would explain how two sRNAs, pairing to the same region, can lead to

such different outcomes of regulation. Regardless, these results demonstrate that ArcZ and McaS are likely to compete with each other for the same binding site on the *flhDC* mRNA, and under conditions in which both sRNAs are expressed, the motility of the cell will be influenced by the outcome of this competition.

Input of signals via OxyR and EnvZ/OmpR and their downstream sRNAs

A second well-studied two-component system, EnvZ and OmpR, has previously been shown to negatively regulate motility by negatively regulating *flhDC* (Shin and Park, 1995). Direct binding of OmpR to the *flhDC* promoter has been demonstrated (Shin and Park, 1995). Two homologous sRNAs, OmrA and OmrB, are positively regulated by EnvZ and OmpR (Guillier and Gottesman, 2006). Both sRNAs negatively regulate motility (Fig. 1) and *flhDC* (Fig. 2A) when overexpressed. In this case, direct pairing of OmrA and OmrB with *flhDC* is near the ribosome binding site, and is likely to act by blocking ribosome access (Fig. 5). Thus, OmrA and OmrB reinforce the negative regulation of *flhDC* by the EnvZ/OmpR two-component system, in a coherent feed-forward loop, possibly allowing the cells to more rapidly shut down flagellar synthesis in response to an increase in osmolarity of the growth medium. The regulation of *flhDC* expression by OmrA and OmrB reinforces previous observations on the role of OmrA and OmrB in controlling the cell surface by controlling the expression of regulators of cell surface proteins CsgD (Holmqvist *et al*., 2010) and OmpR (Guillier and Gottesman, 2006; 2008).

One of the first Hfq-dependent sRNAs studied was OxyS, induced under oxidative stress and dependent upon OxyR, a LysR family regulator (Altuvia *et al*., 1997). Studies on OxyS revealed a number of phenotypes associated with its expression, some of them due to negative regulation of RpoS (Zhang *et al*., 1998); others are yet unexplained. OxyS directly pairs with and negatively regulates the transcriptional regulator FhlA (Altuvia *et al*., 1998; Argaman and Altuvia, 2000). Here we find that OxyS also negatively regulates *flhDC* expression by pairing near the ribosome binding site, at the same region as OmrA and OmrB (Fig. 4). The region in OxyS that pairs is located between the two regions that pair with *flhA*.

Expression of OxyS is expected to be high under oxidative stress conditions, while expression of ArcZ is expected to be relatively low anaerobically but higher for aerobic growth. Since both negatively regulate *flhDC*, the expectation is that these sRNAs will limit flagellar synthesis under high oxygen/oxidative stress conditions. This is consistent with published results demonstrating that flagellar expression is increased under anaerobic conditions (Landini and Zehnder, 2002).

The flhDC 5′ UTR as a platform for translational regulation

Our results provide evidence for a direct interaction of multiple sRNAs with the 5′ UTR of *flhDC*. Other sRNAs (SdsR and GadY) may either act by direct pairing that we failed to detect, or act indirectly, for instance by affecting the action of other regulators, such as CsrA (Wei *et al*., 2001). Figure 8 outlines some of what we know about the various regulators and regulatory inputs that impinge on *flhDC* expression. There are multiple overlaps between signals at the transcriptional and post-transcriptional level, and it seems likely that other overlaps will be found.

RpoS has been reported to negatively regulate *flhDC* (Uchiyama *et al*., 2010); such negative regulation by a sigma factor must be indirect. Because RpoS stimulates synthesis of both SdsR (Fröhlich *et al*., 2012) and GadY (Opdyke *et al*., 2004), our observations provide an explanation for the negative regulation. OmpR acts both at the promoter and via sRNAs, and our results suggest this is a feed-forward loop, likely to act at levels in addition to *flhDC* to modulate motility. Similarly, ArcA stimulates motility, in part via ArcZ and in part independently of ArcZ, possibly via previously described regulation of the anti-sigma FliA (Kato *et al*., 2007). Cyclic AMP and CRP promote motility at multiple levels, including through stimulation of McaS synthesis (Thomason *et al*., 2012).

Integrating these multiple sRNAs and protein regulators is likely to significantly constrain evolution of the 5′ UTR of *flhDC*, and thus it is not surprising that the 198 nt leader is well conserved (Fig. 3). Once a single sRNA and the necessary Hfq binding site have been integrated into the leader, interactions of additional sRNAs may be easier to evolve, integrating yet more signals into the regulation of motility, a behaviour that has a profound effect on the lifestyle of bacteria. It seems likely that a similar multiplicity of regulatory sRNAs will be found affecting motility genes throughout bacterial species.

Experimental procedures

Bacterial strains and plasmids

All strains used in this study are derivatives of *E. coli* K-12 strain MG1655 and are listed along with all plasmids used in this study in Table S1. Primers and 5′ biotinylated probes used in this study are listed in Table S2 and were supplied by Integrated DNA Technologies. Transductions were performed using phage P1*vir* according to Miller (Miller, 1972). The coding sequence for *sdsR* was replaced with the zeocin resistance gene by λ Red recombinase-mediated gene replacements using a PCR product generated from the genomic DNA from NM1201 and the SdsRzeoKO For and Rev primers. The wild-type and mutant *lacZ* translational fusions to *flhD* under the control of the *araBAD* promoter were generated using the procedure developed by Mandin and Gottesman

(Mandin & Gottesman, 2009). PCR products containing the entire wild-type 5′ UTR or mutant versions of the 5′ UTR and the first 9 codons of *flhD* were generated from template genomic DNA from strain MG1655 using primer FlhDlacZ For and FlhDlacZ Rev, FlhDcompmut2A Rev, FlhDcompmut3A Rev, FlhDcompmut3B Rev, FlhDcompmut3AB Rev, FlhDcompmut34A Rev, or FlhDcompmut34B Rev, FlhDcompmut4A Rev and FlhDmutcomp5A Rev. The 5′ end of the forward primer has sequence homologous to the *araBAD* promoter and the 5′ end of the reverse primer has sequence homologous to *lacZ*. Each PCR product was then recombined into the chromosome of strain PM1205 by λ Red recombinase-mediated gene replacement to generate the *lacZ* translational fusion regulated by the *araBAD* promoter.

The chromosomal *flhD*34B mutation in the leader of *flhD* was introduced as follows. A cassette containing the toxin-encoding gene *ccdB* under the *araBAD* promoter and a kanamycin resistance gene (C. Ranquet, C. Pinel, N. Majdalani and J. Geiselmann, manuscript in preparation; deposit patent number: FR 11/60169, 08/11/2011, UJF/BGene) was amplified from template genomic DNA from NM570 (obtained from N. Majdalani) using the primers FlhDUTRccdB For and Rev, and introduced into the chromosome of MG1655 by λ Red recombinase-mediated gene replacement generating strain NRD846. The PCR product generated from MG1655 genomic DNA described above using the primers FlhDlacZ For and FlhDcompmut34B Rev was then used as a template for a second PCR reaction using primers FlhDUTR For and Rev. The *ccdB kan* cassette in the leader of *flhD* in strain NRD846 was then replaced with this PCR product by λ Red recombinase-mediated gene replacement. A successful recombinant (NRD892) was obtained by selection for growth in the presence of arabinose (1%) and verified by PCR and sequencing using primers FlhDUTRinschk For and Rev. To introduce the *arcZmut*34* mutation into the chromosome, the *ccdB kan* cassette that replaces *arcZ* in strain NM381 (obtained from N. Majdalani) was replaced by λ Red recombinase-mediated gene replacement using the arcZmut34comp oligonucleotide that contained this mutation. A successful recombinant (NRD888) was obtained by selection for growth in the presence of arabinose and verified by PCR and sequencing using primers ArcZseq For and Rev.

Plasmid pNRD419, pNRD422, pNRD423, pNRD428, pNRD432, pNRD433, pNRD435 or pNRD436 was generated by site-directed mutagenesis from the template pBR-plac-ArcZ using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and the primers ArcZmut1 For and Rev, ArcZmut2 For and Rev, ArcZmut3 For and Rev, ArcZmut4 For and Rev, ArcZmut5 For and Rev, ArcZmut6 For and Rev, ArcZmut7 For and Rev, or ArcZmut34 For and Rev. Plasmid pNRD441 or pNRD442 containing *omrA* or *omrB* with the A6T, G7C, G8C, T9A, A10C, T11A and T12A mutations was generated from the template plasmid pBR-plac-OmrAmut3* or pBR-plac-OmrBmut3* by site-directed mutagenesis as described above using the primers OmrAmut34 For and Rev or OmrBmut34 For or Rev primers. Plasmid pNRD440 containing *oxyS* with A65T, A66C, T67C and A69T mutations was generated from the template plasmid pBR-plac-OxyS by site-directed mutagenesis as described above using the primers OxySmut3 For and Rev. Plasmid pNRD434 or pNRD437 was generated by site-directed mutagenesis as described above using the template pBR-plac-SdsR and primers SdsRmut3 For and Rev or SdsRmut6 For and Rev.

Culture media and growth conditions

Strains were grown in Lennox broth (LB) or on agar plates. Antibiotics were used at the following concentrations: ampicillin, 100 mg l^{-1}; chloramphenicol, 15 mg l^{-1}; tetracycline, 25 mg l^{-1}; kanamycin, 25 mg l^{-1}; and zeocin, 25 mg l^{-1}. Arabinose, Isopropyl-β-D-thogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were used at a final concentration of 0.01%, 100 μM and 20 mg l^{-1}, respectively, unless otherwise indicated.

Motility assays

Overnight cultures of each strain were diluted 10-fold in fresh LB medium and then spotted on a tryptone broth (10 g l^{-1} of Tryptone and 5 g l^{-1} of NaCl) plate containing agar at a final concentration of 0.25%. For strains harbouring plasmids, the overnight cultures were spotted directly on the tryptone broth motility plates containing ampicillin and IPTG. The assays were performed at 25°C or 30°C.

For the motility competition assays, the OD_{600} of each overnight culture was measured and then the two strains being competed against each other were mixed in a 1:1 ratio. The mixed culture was spotted on a motility plate and incubated at 30°C for 6.5 h. A 100 μl sample was then taken from the inoculation site or the edge of swimming. The samples were serially diluted and plated on MacConkey lactose plates and the number of Lac^- and Lac^+ colonies were determined. These experiments were carried out with the *lac* mutation in each partner (*lac*$^-$ wild type versus *lac*$^+$ mutant or *lac*$^+$ wild type versus *lac*$^-$ mutant), to avoid any effects of the *lac* deletion. In some cases, strains were distinguished instead by the presence or absence of an antibiotic resistance marker. The diluted sample was plated on LB plates and the recovered strains were screened for resistance to the appropriate antibiotic. All data represents the ratio of mutant to wild-type cells recovered over the ratio of mutant to wild-type cells inoculated on the motility plates.

β-Galactosidase assays

Overnight cultures of each strain were diluted 200-fold into fresh LB medium containing IPTG, ampicillin, and arabinose and incubated at 37°C for 6 h while shaking at 250 rpm. Samples were removed, and a β-galactosidase assay was performed as described by Miller (1972).

RNA isolation and Northern blot analysis

Overnight cultures of each strain were diluted 200-fold into fresh LB medium containing IPTG, ampicillin, and arabinose and incubated at 37°C for 6 h while shaking at 250 r.p.m. Samples were removed, and RNA was extracted using the hot phenol method previously described by Massé *et al*. (2005). Northern blot analysis of ArcZ or SsrA was performed

by fractionating 3 μg of RNA on a Bio-Rad Criterion 10% Tris-borate-EDTA (TBE) urea polyacrylamide gel at 55 V after pre-running the gel at 55 V for 30 min. The fractionated RNA was transferred to a Bio-Rad Zeta-Probe GT membrane at 200 mA for 2 h in 0.5× TBE, and the RNA was subsequently cross-linked to the membrane by UV irradiation. The membrane was then probed with the indicated probe in ULTRAhyb solution (Ambion) overnight at 42°C, and then was developed using the Brightstar Biodetect kit (Ambion) according to the manufacturer's instructions.

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