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Rcs signaling-activated transcription of *rscA* induces strong anti-sense transcription of upstream *fliPQR* flagellar genes from a weak intergenic promoter: regulatory roles for the anti-sense transcript in virulence and motility

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Summary

In *Salmonella enterica*, an activated Rcs signaling system inhibits initiation of transcription of the *flhD* master operon. Under these conditions, where motility is shut down, microarray experiments showed an increased RNA signal for three flagellar genes - *fliPQR* - located upstream of *rscA*. We show here that it is the anti-sense (AS) strand of these genes that is transcribed, originating at a weak promoter in the intergenic region between *fliR* and *rscA*. RcsA is an auxiliary regulator for the Rcs system, whose transcription is dependent on the response regulator RcsB. Rcs-activated rightward transcription, but not translation, of *rscA* is required for stimulation of leftward AS transcription. Our results implicate a combined action of RcsB and *rscA* transcription in activating the AS promoter, likely by modulating DNA superhelicity in the intergenic region. We show that the AS transcript regulates many genes in the Rcs regulon, including SPI-1 and SPI-2 virulence and stress-response genes. In the wild-type strain the AS transcript is present in low amounts, independent of Rcs signaling. Here, AS transcription modulates complementary sense RNA levels and impacts swarming motility. It appears that the flagellar AS transcript has been co-opted by the Rcs system to regulate virulence.

Introduction

The Rcs phosphorelay signaling system was first identified by its role in the positive regulation of capsular polysaccharide or colanic acid synthesis in *Escherichia coli*, but is now known to control a vast genetic program (Majdalani and Gottesman, 2005; Wang *et al.*, 2007). This system is present exclusively in the family *Enterobacteriaceae* (Huang *et al.*, 2006). Pathways regulated by the Rcs system include maintenance of cell wall integrity, cell division, stationary phase sigma factor activity, motility, and virulence. The specific signals that activate the Rcs system are not known, although the general physiological signals that trigger the system are accepted to be osmotic or other membrane stresses acting through the outer membrane lipoprotein RcsF or the inner membrane protein IgaA, upstream of the hybrid kinase RcsCD (Cano *et al.*, 2002; Castanie-Cornet *et al.*, 2006; Dominguez-Bernal *et al.*, 2004; Laubacher and Ades, 2008; Majdalani *et al.*, 2005). The response regulator RcsB associates with an unstable protein RcsA to bind to an RcsAB box in the promoter region of several genes; not all genes require RcsA for regulation, suggesting that RcsB can bind independently of RcsA (Wang *et al.*, 2007; Wehland and Bernhard, 2000). RcsB is required for *rscA* transcription (Wehland and Bernhard, 2000). The Rcs system is expected to be most activated in a sessile state, where capsular polysaccharide helps cells survive drying

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(Ophir and Gutnick, 1994), and is apparently important for later stages of biofilm formation (Danese *et al.*, 2000; Prigent-Combaret *et al.*, 1999). A transition from a sessile to a motile state, which is proficient in the intestinal phase of host invasion, requires that the Rcs system to be deactivated, since motility and SPI-1 virulence are negatively regulated by this system. Once inside the host, however, the Rcs system likely interacts with other two-component signaling systems to selectively turn on the SPI-2 virulence pathway required for intracellular proliferation and for systemic infection (Wang *et al.*, 2007).

Flagellar biogenesis in *Salmonella enterica* involves expression of more than 50 genes that are transcribed in operons of three temporal classes (Chilcott and Hughes, 2000). The class 1 genes are included in the master operon *flhDC*, whose proteins direct transcription of class 2 promoters which encode structural and assembly proteins required for the biosynthesis of the base of the flagellar structure. FliA (σ^{28}) is made at this stage and regulates class 3 transcription which produces the functional flagellar filament. In a mutant *Salmonella* strain that is near-maximally activated for the Rcs system, motility is completely inhibited (Wang *et al.*, 2007). Under these conditions, RcsB was observed to inhibit transcription of the master operon through an RcsB box downstream of the *flhDC* promoter; RcsA appeared to have no effect on *flhDC* transcription. Even though the flagellar regulon was turned off, microarray experiments showed a strong positive signal for three class 2 genes *fliPQR*, whose products are part of the Type III secretion apparatus that directs export of most extra-cytoplasmic flagellar proteins. These elevated RNA levels were dependent on RcsB, but not on RcsA (Wang *et al.*, 2007).

We show in this study that the positive RNA signal observed for *fliPQR* in the Rcs-activated strain is due to anti-sense (AS) transcription of these genes, and that the AS transcript plays a regulatory role in both the Rcs and motility pathways, apparently by different mechanisms. We describe novel features involved in induction of the AS transcript, and propose a model to explain how RcsB might participate in this process. Throughout, 'RcsB' refers to its phosphorylated form, which functions as a transcriptional regulator.

Results

An activated Rcs system promotes anti-sense transcription of *fliPQR*

The flagellar *fliPQR* genes are located at the end of the *fliL* operon, upstream of *rcsA* (Fig. 1A). We had reported a large RNA signal for these genes in a strain carrying a point mutation in *igaA* (*igaA**), where the Rcs system is highly activated (Wang *et al.*, 2007). As determined by microarray analysis, RNA levels for *fliR* were elevated 10–50 fold, *fliQ* 3–15 fold and *fliP* 1–3 fold (log-stationary phase), the induction being dependent on RcsB. Since the methods employed in the Wang *et al.* study did not distinguish between sense (S) and anti-sense (AS) transcription, a 2-step RT-PCR reaction was carried out as described under Experimental Procedures.

In a wild-type strain probed for *fliQ* and *fliR* along with the upstream gene *fliM*, an S transcript was readily detected for *fliM* (Fig. 1B, left panel). S-*fliQ* was present in lower amounts, and S-*fliR* was undetectable under the same RT-PCR conditions. A weak AS signal (marked with a dot) was present for both *fliQ* and *fliR*, but not for *fliM*.

In the *igaA** strain QW119, where flagellar transcription is inhibited at the class 1 level, S transcripts were not detected for any of the three genes (Fig. 1B, right panel). However, induced levels of AS transcripts were seen for *fliQ* and *fliR*. As a control, we also monitored the downstream gene *rcsA*; only the S transcript was detected for this gene in QW119, showing that AS transcription in this strain is specific to the flagellar genes. These results

show that the transcription data reported for these genes in the earlier study represents AS transcription (see Fig. 3 in Wang *et al.*, 2007).

To ascertain that AS transcription observed in the *igaA** mutant is through activation of the RcsC kinase, a mutant *rscC* allele (*rscC**) reported to signal constitutively, was tested (Garcia-Calderon *et al.*, 2005). AS-*fliR* induction was seen in this strain as well (Fig. 1C).

To determine whether the AS transcript is also induced under physiological conditions known to trigger the Rcs system, we tested the effect of two reported conditions in *E. coli* – osmotic shock (Sledjeski and Gottesman, 1996), and growth at low temperatures in the presence of glucose and zinc (Hagiwara *et al.*, 2003). Only sucrose-induced osmotic shock resulted in induction of the Rcs system in *S. enterica*, as monitored by induction of transcription of *wcaA*, a colanic acid biosynthesis gene (Fig. 1C). AS-*fliR* transcription was induced under these conditions as well. Thus, production of the AS transcript is not an artifact of mutant backgrounds, and is seen under physiological conditions that activate the Rcs system as well.

In summary, an activated Rcs system promotes expression of an AS-*fliQR* transcript. In a wild-type strain, sense or mRNA levels of *fliQR* are much lower compared to those of the upstream gene *fliM*, with *fliR* being undetectable under similar PCR conditions.

The anti-sense transcript is present at low levels throughout the growth phase in a wild-type strain, independent of RcsB

Data in Fig. 1B showed trace amounts of an AS transcript for *fliQ* and *fliR* even in wild-type cells. To better detect this transcript and test if it arises from the low-level activity of RcsB reported in this strain (Wang *et al.*, 2007), the amount of RNA used in RT-PCR reactions was increased 5-fold along with increased cycle numbers, and the S and AS transcripts for *fliR* were monitored throughout the growth cycle of wild-type and isogenic $\Delta rcsB$ strains (Fig. 2). Both S- and AS-*fliR* transcripts could be detected under these conditions. In the wild-type strain the AS transcript was observed throughout the growth cycle, in higher amounts than the S-*fliR* transcript (Fig. 2A). An essentially similar pattern of AS-*fliR* expression was seen in the $\Delta rcsB$ strain (Fig. 2B), suggesting that RcsB activity does not make a large contribution to the AS-*fliR* transcript levels during this phase. This result is consistent with earlier gene expression as well as motility data showing only a low activity of the Rcs system under these conditions (Wang *et al.*, 2004; Wang *et al.*, 2007). Thus, the AS-*fliR* transcript in the wild-type strain is largely RcsB-independent. This transcript was unaffected in a $\Delta rpoS$ strain, showing that the stationary phase sigma factor RpoS is not responsible for its transcription (data not shown). In contrast to the continued presence of the AS transcript, the S-*fliR* transcript began to decline after 4 hrs in both strains, consistent with the pattern of class 2 gene expression reported for the wild-type strain in microarray experiments (Wang *et al.*, 2004). In these experiments, the non-class 2 RNA expression pattern reported for *fliQ* and *fliR* can now be explained as due to AS RNA.

The anti-sense transcript originates in the *fliR-rscA* intergenic region

Northern blots were performed to detect the length of the AS transcript in the *igaA** strain. The expected length of a *fliPQR*-sized transcript is ~1870 nt. When a 790 nt sense strand sequence of *fliR* was used as probe, five separate bands were detected, the most prominent of these being ~500 nt (Fig. 3A). The largest band of ~1500 nt was detected only upon a longer exposure of the blot. It is possible that the smaller bands are processed products of a larger precursor. A similar band pattern was seen in the wild-type, which required very long exposures for detection (data not shown).

To map the start of the AS transcript, primer extension was performed using a complementary sense-strand primer (*a-fliR-PE1*; see Fig. 3C). Two bands positioned at 50 and 51 nucleotides from the end of *fliR* TAA were observed (Fig. 3B). This places the transcript start site to the right of the RcsAB box in the intergenic between *fliR* and *rcaA* (referred to henceforth as intergenic or RA region) (Fig. 3C). Consistent with these results, RT-PCR reactions using a common *fliR* primer (*fliR-F3*) and various primers in the RA region generated efficient products with RA-R2 and RA-R2a primers which include the RcsAB box, but not RA-R2b, RA-R2c or RA-R3 primers (Fig. 3D).

Possible candidates for a class 1 or σ^{70} promoter for AS-*fliR* could be -10 atAT/-35 aaGtaA (marked as AS-*fliR* -10 and -35 in Fig. 3C; matches to the promoter consensus indicated by upper case letters), or -10 TATccT/-35 aaaACA (this alternate -10 is 3 bp to the left of the marked AS-*fliR* -10 in Fig. 3C). A promoter search program (<http://www.bimas.cit.nih.gov/molbio/proscan/>) did not recognize either promoter, nor found any strong candidates for alternative promoters in the RA region. To ascertain the promoter location, we mutated all six base pairs in the marked -10 region (which would also affect the alternate -10), as well as inserted the strong *rrnB* T1T2 terminator just outside the *S-fliR* stop codon (see Table 1). These manipulations resulted in either loss or strong diminution of the AS-*fliR* transcript, confirming the general promoter location of the AS transcript (Fig. 3E). We designate this promoter P_{AS-*fliR*}.

In summary, the AS-*fliPQR* transcript observed in an Rcs-activated strain is transcribed from a sub-optimal promoter P_{AS-*fliR*} located in the RA region. The transcript is long and appears to be processed. The weak nature of P_{AS-*fliR*} is consistent with low AS transcript levels observed in the wild-type strain (Figs. 1 and 2).

Induction of anti-sense transcription is dependent on *rcaA* transcription but not RcsA

The RcsAB box in the RA region is located 100 bp upstream of P_{rcaA} (Fig. 4A; see also Fig. 3C). This box is reported to bind both RcsB and RcsA in *E. coli* (Wehland and Bernhard, 2000). RcsB is required for *rcaA* transcription, while RcsA serves as an autoregulator. Wang *et al.*, 2007 reported that deletion of *rcaB* or of the RcsAB box (Δ RA1-48) in the *igaA** strain strongly diminished both *rcaA* and *fliPQR* (AS) transcripts. This is shown again for AS-*fliR* in Fig. 4B. Note that *rcaA* transcription is eliminated in the Δ rcaB strain, but is still detectable in the RcsAB box mutant (Δ RA 1-48). An internal deletion that removed most of *rcaA*, resulted in an increase in remnant *rcaA* RNA levels, but no significant difference in AS-*fliR* (Fig. 4B; the *rcaA* probe in these experiments is to an upstream untranslated region of the transcript). Thus, RcsA itself is not required for AS transcription. To test if transcription of *rcaA* is required, a 25 bp deletion was generated that removed the *rcaA* promoter P_{rcaA} (see Fig. 4A). The deletion abolished the *rcaA* transcript, with a concomitant loss of the AS-*fliR* transcript (Fig. 4B). The P_{rcaA} deletion did not alter P_{AS-*fliR*}, as judged by similar basal levels of AS-*fliR* in the Δ P_{rcaA} mutation in the wild-type strain (Fig. 4C). Mutation of the -10 region (P_{AS-*fliR*}[#]) abolished the AS transcript in the wild-type strain, as it did in the *igaA** strain (see Fig. 3E), showing a similar origin of the transcript in both strains. While loss of *rcaA* transcription abolished the AS transcript, the reverse was not the case i.e. *rcaA* transcription (which is dependent on RcsB binding to the RcsAB box) was unaffected in the P_{AS-*fliR*}[#] mutant (data not shown). In summary, induction of AS transcription requires the RcsAB box, RcsB, and *rcaA* transcription, but not RcsA.

How might transcription of *rcaA* promote anti-sense transcription? Several studies have demonstrated that the process of transcription can enhance negative DNA supercoiling (Liu and Wang, 1987; Wang and Lynch, 1993). An advancing polymerase generates positive supercoils in the DNA template ahead of it and negative supercoils behind it. Negative supercoiling generally stimulates transcription (Gralla, 1996), a well-known example being

activation of an inactive *leu-500* promoter in *S. enterica* and *E. coli* by negative supercoiling of DNA templates (Chen *et al.*, 1992; Lilley and Higgins, 1991; Tan *et al.*, 1994; Wu and Fang, 2003). To test if the activation of $P_{AS-fliR}$ is due to increased negative supercoiling generated in the wake of transcription from P_{rcsA} , a *topA* null mutation was generated in the wild-type strain, and transcription from both $P_{AS-fliR}$ and P_{rcsA} was monitored (Fig. 4D). The *topA* mutation is viable in *Salmonella* and produces increased negative DNA supercoiling (Richardson *et al.*, 1984). Since changes in supercoiling can have positive effects on transcription generally, we also constructed a *topA-rcsB* double mutant, to distinguish topology-related changes from RcsB-related changes. As controls, we monitored the behavior of two other genes - *wcaA* and *fliM* – which are regulated positively and negatively, respectively, by RcsB. RNA was isolated at stationary phase, where the Rcs system is expected to be slightly active, although not as active as in the *igaA** strain (see Figs. 1 and 2). This is evident in the *wcaA* control, where there is only a small difference between transcript levels in the *topA* versus *topA rcsB* strain. The increase in *wcaA* transcript levels in the *topA* strains compared to wild-type can therefore be attributed to a positive effect of increased negative supercoiling on this promoter. *fliM* transcription (from the *fliL* promoter; see Fig. 1A) is also stimulated in the *topA* strains, and the stimulation is enhanced in the *topA rcsB* strain, consistent with the negative regulation of the master flagellar operon by RcsB. Thus, both *wcaA* and *fliM* transcripts, respond positively to changes in DNA topology in the *topA* strain, independent of RcsB.

By contrast, *rcsA* transcript levels increase significantly in the *topA* mutant alone (Fig. 4D, top panel). Increased negative supercoiling itself is not sufficient for this stimulation, as seen by its absence in the *topA rcsB* strain. This suggests that supercoiling and RcsB have a synergistic effect on *rcsA* transcription. The stimulation is not seen in the *topAΔP_{rcsA}* strain, confirming that the induced *rcsA* transcript originates from P_{rcsA} in the *topA* strain.

Like *rcsA*, *AS-fliR* levels also increase in the *topA* mutant (Fig. 4D, second panel); the increase depends on RcsB and on transcription from P_{rcsA} (compare *topA* with *topA rcsB* and *topAΔP_{rcsA}*). However, while *rcsA* levels are induced ~5–6 fold in the *topA* strain, the increase in *AS-fliR* is modest (~1.5 fold). We note that the decrease in *AS-fliR* levels in the *topA rcsB* mutant compared to wild-type is consistent with the small contribution of RcsB to *AS-fliR* levels in wild-type cells (compare *AS-fliR* in Fig. 2A and 2B at 6 hr and 8 hr).

We draw several conclusions from these results. First, P_{rcsA} is stimulated by increased negative DNA supercoiling, but only in the presence of RcsB. Second, increased negative DNA supercoiling itself is not sufficient to activate transcription from $P_{AS-fliR}$, which is still dependent on transcription from P_{rcsA} and on RcsB. Third, relative *AS-fliR* levels (compared to *rcsA*) are not as high in the *topA* strain as that seen in the *igaA** strain. Thus, maximal stimulation of $P_{AS-fliR}$ requires higher activity of RcsB. A model rationalizing these results is presented under 'Discussion', where we propose that RcsB binding to the RcsAB box traps negative supercoils generated by transcription from P_{rcsA} , stimulating both P_{rcsA} and $P_{AS-fliR}$.

A regulatory role for the anti-sense transcript in the Rcs pathway

To test if the AS transcript participates in regulation of genes in the Rcs regulon, a microarray analysis was performed, comparing RNA from the *igaA** strain with that from three different *igaA** derivatives carrying disruptions that eliminated the anti-sense transcript as determined by RT-PCR experiments. These were QW422 ($\Delta fliR::kan$, *igaA**), QW471 ($\Delta fliQR$, *igaA**) and QW556 ($\Delta RA1-48$, *igaA**) (see Fig. 3E and Fig. 4B). The microarray data showed a similar transcription profile for all three strains. Differentially regulated genes belonging to the Rcs regulon and common to all three pairwise combination of strains analyzed are shown in Table 2. Similar results were also seen for QW674

($P_{AS-fliR}^{\#}$, $igaA^*$) (data not shown). None of the affected genes are reported to be regulated by RcsA (Wang *et al.*, 2007). The largest functionally related group of genes to be negatively regulated by the AS transcript i.e. genes whose expression increased in the AS minus strains compared to the parent AS plus strain, were virulence genes belonging to SPI-1 (*hil*, *inv*, *prg*, *sic*, *sop*) and SPI-2 (*ssa*, *sse*, *ssr*) (SPI is short for ‘*Salmonella* pathogenicity island’) (Galan, 1999; Waterman and Holden, 2003). Other regulated genes are listed in their alphabetical or numerical order. Among genes with a known function, *cadB* was strongly negatively regulated (>30 fold). The *cadBA* operon encodes a lysine decarboxylase (CadA) and a lysine-cadaverine antiporter (CadB); *cadB* is an essential component of the acid tolerance response in *Salmonella* (Park *et al.*, 1996). Known genes that were strongly positively regulated (10–150 fold) include *aceAB*, *manXYZ*, and *ompF*. The *ace* operon encodes enzymes for the glyoxylate shunt pathway, required for growth of *E. coli* on acetate or fatty acids as the sole carbon source (Maloy and Nunn, 1982); *aceA* is required for persistence of *Salmonella* in mice (Fang *et al.*, 2005). The *manXYZ* operon encodes a sugar transporter of the phosphoenol pyruvate-dependent phosphotransferase system (Plumbridge, 1998), and plays a role in organic solvent tolerance in *E. coli* (Okochi *et al.*, 2007). *ompF* is a major outer membrane porin that is regulated in response to various stresses (Begic and Worobec, 2006; Pratt *et al.*, 1996; Batchelor *et al.*, 2005). *ompF* translation is known to be regulated by the *micF* anti-sense RNA (Guillier *et al.*, 2006). A seven-member *ego* operon of unknown function was also strongly induced.

The microarray data were validated by RT-PCR using *hilC* and *aceA* as representative genes that show negative and positive regulation, respectively (Fig. 5). The expression of these genes in the $igaA^*$ strain compared with its isogenic $P_{AS-fliR}^{\#}$ mutant derivative is consistent with repression of *hilC* and activation of *aceA* by AS-*fliR*.

To test if the AS transcript regulates gene expression in the absence of an activated Rcs system, we cloned *fliPQR* on a plasmid, with a P_{BAD} promoter positioned to the right of *fliR* driving the AS transcript ($pfliPQR^{BAD}$). The expression profile of a wild-type strain carrying $pfliPQR^{BAD}$ (QW610) showed a consistent repression (1.5 – 5.5 fold) of virulence genes belonging to SPI-1 (*hil*, *inv*, *prg*, *sic*, *sop*) and SPI-4 (STM4257 operon). The *rtsAB* genes, which encode regulators of the SPI-1 genes (Ellermeier and Schlauch, 2003), were also repressed (data not shown). These data were validated for *hilC* using RT-PCR (data not shown).

In summary, the AS transcript regulates a large number of genes in the Rcs regulon in both a positive and negative manner. The largest functional blocks of genes showing negative regulation belong to the SPI-1 and SPI-2 virulence pathways. Genes showing >10-fold regulation are involved in various stress responses. SPI-1 genes were regulated by the AS transcript even when expressed from a plasmid in the absence of Rcs signaling. Genes for colanic acid synthesis, which are the major target of the Rcs system (Majdalani and Gottesman, 2005), were not regulated by the AS transcript.

Gene regulation in the Rcs pathway is eliminated in AS-*fliR* stop codon mutants

A fairly long potential ORF (either 210 or 255 aa) is present in AS-*fliR*, while short ORFs are found in AS-*fliQ* and AS-*fliP* (40 and 75 aa, respectively) (Fig. 5A). The 255 aa AS-*fliR* ORF starts in Val, while the others all start in Met. A BLAST search revealed that in the closely related organisms *E. coli* and *Shigella*, there is a stop codon in the middle of their AS-*fliR* ORF. However, we found a 253 aa ORF in the AS-*fliR* strand from *Burkholderia pseudomallei*; this ORF has 37% amino acid identity to the *S. enterica* AS-*fliR*. To test if AS-*fliR* might be translated, we created FLAG epitopes at either the N- or C-terminal regions of the putative anti-FliR ORF. However, no protein bands were detected in Western blots probed with anti-epitope antibody, suggesting that the RNA is either not translated, or

the translated product is unstable. To distinguish between these possibilities, we created two stop codons in AS-*fliR* ORF at two different locations (Fig. 5A) and monitored their effect on gene regulation in the *igaA** strain (QW700 and QW702 in Table 1) by RT-PCR of the representative genes *hilC* and *aceA* genes. Like the P_{AS-*fliR*}[#] mutant, the stop codon mutants also showed relief of repression of *hilC* and suppression of activation of *aceA*, even though unlike the P_{AS-*fliR*}[#] mutant, AS-*fliR* expression was not abolished (Fig. 5B). Microarray data for these mutants were consistent with these results (data not shown). These data implicate a translated product of AS-*fliR* in regulating gene expression, although we were unable to detect such a product.

The anti-sense transcript plays a regulatory role in motility

To test if the AS transcript observed in wild-type cells (Fig. 2) plays a role in motility, we tested the effect of absence of this transcript on both swimming and swarming. Three strains not making the AS transcript – carrying the mutant promoter P_{AS-*fliR*}[#], the terminator insertion (AS-*fliR*::T), or a 40 bp deletion that selectively removed the P_{AS-*fliR*} promoter as well as the start site of the AS transcript (Δ RA 49–88) – were examined. All three mutant strains showed a moderate inhibition of swimming (~10%), but a more severe effect on swarming motility (Fig. 5C). The more pronounced effect on swarming could be due to the development dynamics of the swarmer colony, in which motility initiates only after a substantial lag. The growth rates of all these strains in LB broth were similar to wild-type (data not shown). The stop codon mutants, which do not affect AS-*fliR* transcription did not affect motility, in contrast to their effect on gene regulation in the *igaA** strain (Fig. 5B). These results implicate a role for the AS-RNA, and not its translation product, in regulation of motility.

To test if the decrease in motility associated with the loss of the AS transcript might be explained by a decrease in levels of the S transcript, S- and AS-*fliR* transcripts were analyzed in the AS transcript mutants. All three mutants showed ~1.5–2.5 fold increase in S-*fliR* levels (Fig. 5D). This surprising result i.e. increased S-RNA but decreased motility, implicates the AS transcript in either decreasing the stability of the S transcript, or AS transcription in interfering with S transcription. The negative impact on motility of increased S-RNA levels may suggest that the specific stoichiometry of FliPQR proteins, which are part of a multi-component type III secretion apparatus, is critical for flagella biogenesis. Indeed, providing extra copies of *fliQR* from a plasmid (p^L-*fliQR*) had a negative effect on both swimming and swarming motility of the wild-type strain (data not shown).

In summary, AS transcription from P_{AS-*fliR*} decreases complementary S transcript levels and plays a positive role in motility. This regulation is not via a translated product of AS-RNA.

Discussion

In this work we report the discovery of an anti-sense (AS) flagellar *fliPQR* transcript induced by the Rcs signaling system. We show that the transcript originates in the intergenic region between *fliR* and *rscA*, at a weak promoter adjacent to P_{rscA}. Strong induction of leftward transcription from the P_{AS-*fliR*} promoter is due to RcsB-controlled rightward transcription of *rscA*. The Rcs system, which shuts down motility, appears to have exploited a translated product of the anti-sense transcript to regulate virulence and other stress-related genes. In a wild-type strain, AS transcription contributes to improving motility by a mechanism that controls levels of the complementary sense-strand RNA. A diagram summarizing our main findings is presented in Fig. 6, where we propose a model for RcsB function as discussed below.

Anti-sense regulation

Anti-sense RNAs were first discovered in controlling processes such as phage lysogeny, plasmid DNA replication, and DNA transposition in *E. coli* (Brantl, 2007). A common feature of these systems is that the anti-sense RNAs are *cis*-encoded, i.e. they are transcribed from the same loci, but in an anti-sense direction from the target RNA. Their perfect complementarity to large segments of the target RNA leads to the formation of stable double-stranded RNA structures. These RNAs are generally small (~50 to 300 nt), are not translated, display highly structured stem-loops, and bind their target or sense RNAs to regulate gene expression in many different ways including transcription attenuation, translation inhibition, mRNA degradation and mRNA stabilization. Recent studies have identified many sRNAs or small regulatory RNAs in *E. coli*, many of which act in *trans* by base-pairing to activate or repress translation or to destabilize mRNAs (Storz *et al.*, 2004). Base-pairing between these regulatory RNAs and their target mRNAs is not always perfect, and requires the Hfq protein which likely functions as an RNA chaperone to increase RNA unfolding or local target RNA concentration in order to stabilize the partial duplexes formed with their target RNA(s) (Brennan and Link, 2007). Non-coding RNAs are abundant in eukaryotes, varying in size from 200 bp to >10,000 bp (Wilusz *et al.*, 2009). While the regulatory mechanisms involving many of the eukaryotic small RNAs are known (e.g. micro and siRNAs), those of the long RNAs are still enigmatic.

The AS transcript described in this study is long and is processed (Fig. 3). We surmise that the regulatory function of AS-*fliPQR* is encoded mainly in AS-*fliQR*, since AS-*fliP* is not present in high levels in the *igaA** strain (Wang *et al.*, 2007), and is not detectable in the wild-type strain (our unpublished data). We will therefore refer to this regulatory transcript as AS-*fliQR*. AS-*fliR* has a 255 aa ORF that spans nearly the entire *fliR* gene. Interruption of this ORF with two separate stop codon mutants abolished the AS RNA-dependent Rcs regulation in the *igaA** strain (Table 2 and Fig. 5B), but did not affect motility in the wild-type strain (Fig. 5C), suggesting that the transcript appears to exert its regulatory role in distinctly different ways in the Rcs and motility pathways.

In the Rcs pathway, a translation product of AS-*fliR* appears to regulate a diverse set of genes. However, FLAG epitopes engineered at either the N- or C-terminal regions of the anti-FliR ORF failed to detect any protein. This suggests that the product is either processed or unstable. We note that RcsA, the auxiliary regulator in the Rcs pathway, is also unstable (Majdalani and Gottesman, 2005). The anti-FliR regulator can apparently function independently of RcsB, since overexpression of the AS-RNA from a plasmid in the absence of an activated Rcs system, was seen to regulate a subset of virulence genes common to those regulated in the *igaA** strain.

In the motility pathway, the sense or mRNA of the promoter-distal genes *fliQR* in the *fliL* operon shows lower abundance compared to mRNA of the promoter-proximal genes (see Fig. 1B). This could be due to the lack of an obvious Rho-independent terminator after *fliR* (Malakooti *et al.*, 1994), increasing the susceptibility of the 3' end of the *fliL-R* transcript to degradation. However, absence of the AS-*fliQR* transcript increases S-*fliR* levels (Fig. 5D), suggesting that 3' end of the *fliL-R* transcript may be targeted for degradation by the AS-RNA. Failure of the stop codon mutants in AS-*fliR* ORF to affect motility (Fig. 5C), suggests that the observed AS-RNA regulation is not dependent on translation of the AS transcript. An RNA-dependent mechanism for degradation could include direct base-pairing between the S and AS transcripts. Alternatively, AS transcription could create a DNA structure in the intergenic region that releases the S transcript immediately from the DNA template, shortening its life. Or, S transcript levels might be controlled by interference of S transcription from RNA polymerase traveling in the AS direction. The decrease in motility associated with increase in S-*fliR* levels upon loss of the AS transcript (Fig. 5C,D), suggests

that the stoichiometry of these proteins is critical to flagellar assembly. FliO, FliP, FliQ and FliR are integral membrane proteins located within the MS ring; they function in concert with two other membrane proteins FlhA and FlhB to form the Type III secretion apparatus for extracytoplasmic flagellar components (Chevance and Hughes, 2008). The numbers of these components and their mechanism of assembly are currently unknown.

Transcription, DNA topology and RcsB

Transcription and DNA topology are mutually linked (Wang and Lynch, 1993). Liu and Wang formulated the “twin-supercoiled-domain” model to explain the enhanced local negative DNA supercoiling associated with transcription (Liu and Wang, 1987). They hypothesized that as the size of the growing RNA transcript increases, it becomes progressively more difficult for the transcribing complex to rotate around the DNA double helix, reaching a point where it is energetically more feasible for DNA to rotate around its own helical axis to generate a transient positively supercoiled domain in front of the RNA polymerase and a transient negatively supercoiled domain behind it. DNA gyrase rapidly converts the positive supercoils into negative supercoils (Wang, 1996), which are thought to stabilize the formation of open transcription complexes by changing DNA twist (Gartenberg and Wang, 1992; Travers and Muskhelishvili, 2007). A striking stimulation of transcription-coupled DNA supercoiling has been demonstrated *in vitro* in the presence of site-specific DNA binding proteins such as the λ O protein, or the Gal or Lac repressors (Leng and McMacken, 2002). Such nucleoprotein complexes have been proposed to act as barriers by either wrapping or bending DNA in a manner that hinders diffusion of DNA supercoils, helping to localize transcription-generated supercoils in the vicinity of their recognition sequences.

The location of RcsB binding sites on DNA differs in promoters that do and do not require RcsA (Majdalani and Gottesman, 2005). RcsB sites are located immediately adjacent to the -35 region of promoters that do not require RcsA, and there is evidence for co-operative binding of RcsB and RNA polymerase at such promoters. In promoters that are modulated by RcsA, RcsB binding sites are located 50–100 bp upstream of the promoters, and the mechanism of activation from these distant sites is not known. From the results reported in this study, where (a) increased negative supercoiling and RcsB were observed to act synergistically to activate transcription from P_{rcsA} (Fig. 4D), (b) maximal activation of $P_{AS-fliR}$ was dependent on transcription from P_{rcsA} , a highly activated RcsB, as well as the RcsAB box (compare data in Fig. 4B and 4D), and (c) low-level transcription of *rcsA* was observed even in the absence of the RcsAB box (Fig. 4B; see also Fig. 3C in Wang et al., 2007), we propose the following model for transcription activation by RcsB in the *fliR-rcsA* intergenic region (Fig. 6): 1) RcsB contributes to a low-level stimulation of transcription from P_{rcsA} , independent of the RcsAB box. 2) RcsB binding to the RcsAB box enhances transcription from P_{rcsA} by slowing the diffusion of *rcsA* transcription-generated supercoils. 3) Strong binding of RcsB to the RcsAB box acts as a barrier against dissipation of the negative supercoils generated by *rcsA* transcription, producing a feed-forward loop that increases negative supercoiling even further, activating $P_{AS-fliR}$. 4) The affinity of RcsA for the RcsAB box may be modulated in response to the altered DNA topology to autoregulate this process.

The curious placement of *rcsA*

Finally, we find it curious that *rcsA* is the lone gene in the Rcs signaling pathway that is positioned downstream of the *fliL* operon, while three other *rcs* genes are located together elsewhere on the genome. We speculate that the original role of AS transcription was to regulate *fliQR* mRNA levels from its *cis* locus. We suggest that this transcript was later co-opted by the Rcs system for *trans* regulatory functions. We note that genes for the

biosynthesis of colanic acid, which are the major target of the Rcs system and thought to be required for survival outside the host, are not regulated by the AS transcript. Instead, AS-regulated genes include those required for host virulence or other stress responses. These data might also argue for a later appearance of the *trans* AS regulation in the evolutionary history of this organism.

Experimental Procedures

Bacterial strains, growth conditions and motility assays

Strains are listed in Table 1. Bacteria were grown either in L-broth (LB) base (20g/liter), or in M63 minimal medium for experiments involving either pBAD33 plasmid derivatives or for inducing osmotic shock (see below). Strains carrying pBAD33 constructs were first grown in M63-Glycerol overnight and subsequently diluted 1:100 in M63-Arabinose for induction. M63 was prepared according to the recipe from Molecular Cloning (Sambrook *et al.*, 1989). Final concentration of carbon sources (glycerol, glucose or arabinose) in M63 was 0.2%. For preparation of RNA samples, cultures grown in LB were harvested at OD₆₀₀ of 0.6 (~2.5 hr) for mid-log phase, and OD₆₀₀ of ~2.3 (6 hr) for stationary phase; cultures grown in M63 minimal medium reached OD₆₀₀ of 0.6 at ~4 hrs. Strains carrying the *topA* mutation grew more slowly than wild-type. The initial inoculum was therefore increased ~3-fold for *topA* strains, in order for both strains to reach a similar OD₆₀₀ at 6hr. Swim plates were solidified with 0.3% agar, swarm plates with 0.6% Eiken agar (Wang *et al.*, 2007).

To mimic physiological conditions that activate the Rcs system, two previously reported protocols were followed. In one, wild-type cells were grown in EB (1.8% Eiken broth, 0.4% glucose, 50 mM MOPS (pH 7.0), 1 mM ZnCl₂) at 20°C (Hagiwara *et al.*, 2003). Control cells were incubated in LB at 37°C. Cultures were harvested at OD₆₀₀ of 0.6 for RNA extraction. In the second protocol, wild-type cells were incubated in M63-glucose at 30°C for 4 hrs, and sucrose was added to a final concentration of 15% (Sledjeski and Gottesman, 1996). Control cultures received an equal volume of M63 salts. After one hour, cells were harvested for RNA extraction. Eiken agar and broth were purchased from Eiken Chemical Co., Japan.

Deletion or insertion of genes and regulatory regions was achieved by the onestep mutagenesis procedure (Datsenko and Wanner, 2000) as described (Wang *et al.*, 2005). The extent of coding or regulatory sequences deleted is provided in Suppl. Table 2. Point mutations and insertions were introduced on the chromosome by first amplifying DNA fragments from genomic template DNA using appropriate primers containing the mutations (see Suppl. Table 1), followed by replacement of a *tetRA* cassette previously inserted into the relevant region, by selecting recombinants for Tet sensitivity as described (Wang *et al.*, 2007). Design of the anti-sense stop codon 2 mutation ensured that the sense strand amino acid code was unaltered. The 426 bp *rrnB* terminator fragment (Orosz *et al.*, 1991) was amplified from pBAD33 (Guzman *et al.*, 1995), and inserted 10 bp after the stop codon of *fliR*. FLAG epitopes at the N- and C terminus of the putative 210 aa anti-*fliR* ORF were constructed by a similar *tetRA* replacement method. All constructs were confirmed by DNA sequencing. Mutant combinations were constructed by P22 transduction where appropriate. FLAG antibodies were purchased from Sigma.

Plasmids

pfliPQR^{BAD} contains DNA from ATG of *fliP* to TAA of *fliR*, cloned into pBAD33 such that the anti-sense strand was driven by the P_{BAD} promoter, with no specific promoter driving the sense strand (Guzman *et al.*, 1995). Primers for PCR amplification of this region included *SphI* and *XbaI* restriction sites, respectively, for ligating into similar sites on

pBAD33. In p^L*fliQR*^{BAD}, the *fliL* promoter was introduced to drive sense strand expression of *fliQR*.

Northern blots and Primer extension

QW119 was grown in LB medium for 6 hrs. RNA was extracted using MasterPureTM RNA Purification Kit (Epicentre, MD). DNA contamination was removed by using DNA-freeTM (Ambion, TX). The Northern blot preparation followed the instruction manual of NorthernMax[®]-Gly Kit (Ambion, TX). Briefly, 15 µg total RNA was loaded to a 1% agarose gel, which was run at 65 volts for 2 hrs and transferred to a positively charged nylon membrane. An RNA probe covering nearly the entire *fliR* coding sequence was synthesized with Strip-EZ[®] RNA T7 Kit (Ambion, TX) with addition of αP³²-dUTP, using primers *fliR*-T7 and RA-R1 (see Suppl. Table 1) and *fliR* DNA template.

Primer extension for mapping the start of the transcript was performed using Primer Extension System from Promega according to the instruction manual, using the primer a-*fliR*-PE1. DNA sequence reactions were run alongside using the same primer, and the *fmoI*[®] DNA cycle sequencing system from Promega.

Microarray experiments and RT-PCR

RNA preparation and microarray analyses were done as described (Wang *et al.*, 2004). RT-PCR was performed as described (Wang *et al.*, 2007) using a one-step RT-PCR kit (Qiagen). However, in order to distinguish the sense and anti-sense transcript, a strategy of “two-step RT-PCR” was used: the reverse transcription step was done first with one primer (the forward primer for detecting the anti-sense strand or the reverse primer for the sense strand) with cycling condition of 50°C for 35 min and 95°C for 15 min. After the initial reverse transcription, the other primer was added to the reaction tube to perform regular PCR amplification using the following steps: 94°C for 15 s, 55 °C for 25 s and 72 °C for 45 s. RNA amounts and cycling numbers varied depending on the RNA abundance of target genes. All samples were normalized using 30S ribosomal RNA. Primer pairs were designed to amplify ~150–350 bp of DNA. DNA bands were quantitated using Bio-Rad Gel-doc QuantityOne.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Anti-sense regulation of *fliPQR* by an activated Rcs signaling system

A. Schematic showing the *fliL* operon and *rcsA*. Length of the regions corresponding to the individual genes within the operon are drawn approximately to scale with respect to each other. Arrows indicate transcription start. The square box in the intergenic region between *fliR* and *rcsA* is the RcsAB box, not drawn to scale.

B. RT-PCR results for RNA isolated from wild-type and QW119 (*igaA**) strains. Primer pairs (listed in Suppl. Table 1) were designed to anneal to either the sense (S) or anti-sense (AS) strands of RNA from indicated genes. RNA for the wild-type strain was isolated at mid-log phase where flagellar gene expression is maximal, whereas that for QW119 was isolated at stationary phase where activity of the Rcs system is maximal. 40 ng of RNA were used for PCR reactions, with 26 cycles of amplification. M, size markers $\times 100$ bp.

C. RT-PCR results for RNA isolated from QW698, which contains a constitutively active RscC kinase allele we refer to as *rcsC**. Reaction conditions as in B.

D. RT-PCR results for RNA isolated after subjecting wild-type cells to osmotic stress by addition of 15% sucrose (Suc) as described under Experimental Procedures. The S transcript was monitored for *wcaA*. 200 ng of RNA were used for PCR reactions, with 29 cycles of amplification.

Fig. 2. Presence of the anti-sense transcript through the time course of growth in a wild-type strain

A. RT-PCR results for *AS-* and *S-fliR* transcripts monitored through 8 hrs of growth in LB in the wild-type strain. 200 ng of RNA were used for PCR reactions, with 29 cycles of amplification.

B. As in A except in an isogenic $\Delta rcsB$ mutant QW434.

Fig. 3. Length and origin of the anti-sense *fliPQR* transcript

A. Northern blot of RNA prepared from QW119, hybridized to a 790 nt sense *fliR* RNA probe, prepared as described under Experimental Procedures (lane 2). Lane 1, size markers $\times 1000$ nt. The segment of the blot around the 1500 nt marker was exposed for a longer time.

B. Primer extension results, where the primer a-*fliR*-PEI (see C) was used for annealing to the anti-sense *fliR* transcript. T and A are DNA sequencing reactions using the same primer. Arrows indicate extension products.

C. DNA sequence of the intergenic region. Nucleotides are numbered starting after the TAA codon of *fliR*. Primers used in the experiments in B and C are indicated with half arrowheads below the sequence. *fliR*-F3 actually hybridizes to the 61–82 nt region upstream of *fliR* stop codon (not shown; see Suppl. Table 1). 3 dots, stop codon for *fliR*; box, RcsAB binding region identified in *E. coli* (Francez-Charlot *et al.*, 2003); double arrows, transcription start sites identified in B; –10 and –35 regions of a putative antisense or *AS-fliR* promoter (see text) and of the *rcsA* promoter are underlined; single dot, *rcsA* transcription start site (Ebel and Trempey, 1999). The translation start site for *rcsA* would be at nucleotide 282 i.e. 134 nucleotides downstream from the transcription start site (not shown).

D. RT-PCR results for RNA isolated from QW119 and probed with a fixed primer *fliR*-F3 paired with five different primers in the indicated *fliR*-*rcsA* intergenic region in C.

E. *AS-fliR* levels monitored by RT-PCR in strains carrying indicated mutations. * indicates *igaA** mutant background. *AS-fliR::T* contains an insertion of the 426 bp *rrnB* terminator next to the *S-fliR* stop codon. *P_{AS-fliR}*[#] is a promoter mutant where the –10 ATATAT has been changed to CGCGCG. PCR conditions in D and E as in Fig. 1B.

Fig. 4. Dependence of induction of anti-sense transcription on *rcsA* transcription

A. Schematic diagram showing regulatory elements in the *fliR* – *rcsA* intergenic region. These include the stop codon TAA of *fliR*, the RcsAB binding box, and the divergent promoters $P_{AS-fliR}$ and P_{rcsA} . Other symbols as in Fig. 1A.

B. RT-PCR results for *AS-fliR* and *rcsA* RNA isolated from *igaA** and its indicated derivative strains. An upstream untranslated region of *rcsA* (see Suppl. Table 1), which is still present in the $\Delta rcsA$ strain, was used for monitoring *rcsA* RNA levels in these strains. RNA was isolated at stationary phase. 40 ng of RNA were used for PCR reactions, with 26 cycles of amplification.

C, D. RT-PCR results for *AS-fliR*, *rcsA*, *wcaA* and *fliM* in wild-type, *topA::kan* ($\Delta topA$), and their indicated derivative strains. RNA was isolated from these strains at 6 hr. 200 ng of

RNA were used for PCR reactions, with 29 cycles of amplification for all except *fliM* reactions, which used 100 ng RNA and 28 cycles.

Fig. 5. Differential effects of AS-RNA on regulation of genes in the Rcs pathway and motility

A. Putative ORFs in the anti-sense strand of *fliPQR*. Open boxes show sense strand proteins, with their amino acid (aa) lengths indicated on top. Dark boxes indicate antisense or AS-ORFs starting with Met, and the light box the AS-ORF starting in Val. The aa lengths of these putative ORFs are shown below the boxes. c' and n' FLAG refer to FLAG epitopes engineered into the 210 aa AS-*fliR* ORF at the indicated locations. Numbers on the bottom refer to nucleotides, with 1 marking the start of the AS transcript as determined in Fig. 3B. Position of stop codons introduced into AS-*fliR* are marked.

B. RT-PCR validation of the microarray data in Table 2 for *hilC* and *aceA* using as control the AS-*fliR* defective mutant *P_{AS-fliR}[#]*. The AS-*fliR* stop codon mutants show a similar loss of regulation of *hilC* and *aceA* even though their AS-*fliR* RNA levels are unaffected. 200 ng RNA/29 cycles of amplification were used for *hilC* and *aceA*, and 40 ng RNA/26 cycles for AS-*fliR* in RT-PCR reactions.

C. Swimming and swarming motility of wild-type and indicated promoter (Δ RA49–88 and *P_{AS-fliR}[#]*), terminator (*AS-fliR::T*), and stop codon (*AS-fliR_{stop1}*) mutant strains. Plates were incubated at 37°C for 4 hr.

D. S- and AS-*fliR* RNA in wild-type and indicated AS-RNA mutant derivative strains. RNA was isolated at 6 hr. RT-PCR conditions as in Fig. 2.

Fig. 6. Summary of regulatory interactions in the *fliR-rcsA* intergenic region, and model for how *RcsB* functions as a transcription activator. Steps 1–3 are described in text. Wavy lines, transcripts; solid arrows, positive regulation; dotted arrow, proposed intermediate; Θ , increased negative DNA supercoiling; $\perp$, negative regulation.

TABLE 1

Strains and plasmids used

Strain/plasmid	Genotype ^a	Source (Ref.)
Strains		
14028	Wild type <i>S. enterica</i>	
QW119	<i>igaA</i> *	Wang et al 2007
QW422	$\Delta fliR::kan$, <i>igaA</i> *	This study
QW434	$\Delta rcsB$	Wang et al 2007
QW441	$\Delta rcsA$, <i>igaA</i> *	Wang et al 2007
QW442	$\Delta rcsB$, <i>igaA</i> *	Wang et al 2007
QW471	$\Delta fliQR$, <i>igaA</i> *	This study
QW555	$\Delta RA(49-88)$	This study
QW556	$\Delta RA(1-48)$, <i>igaA</i> *	This study
QW593	<i>anti-fliFLAG-R</i> , <i>igaA</i> *	This study
QW594	<i>anti-fliR-FLAG</i> , <i>igaA</i> *	This study
QW597	pBAD33	This study
QW610	<i>pfliPQR^{BAD}</i>	This study
QW605a	<i>p^LfliQR^{BAD}</i>	This study
QW663	$\Delta topA::kan$	This study
QW667	ΔP_{rcsA}	This study
QW668	$\Delta topA$	This study
QW669	<i>AS-fliR::T</i> (<i>T_{rrmB}</i> at bp 10 after <i>fliR</i> TAA)	This study
QW671	<i>P_{AS-fliR}[#]</i> (atatat @ -10 mutated to cgcgcg)	This study
QW672	ΔP_{rcsA} , <i>igaA</i> *	This study
QW673	<i>AS-fliR::T</i> , <i>igaA</i> *	This study
QW674	<i>P_{AS-fliR}[#]</i> , <i>igaA</i> *	This study
QW675	$\Delta topA::kan$, ΔP_{rcsA}	This study
QW676	$\Delta topA::kan$, $\Delta rcsB$	This study
QW695	$\Delta rpoS$	This study
QW698	SV4989 (<i>rcsC</i> P484L)	Garcia-Calderón et al 2005
QW699	<i>AS-fliR_{STOP2}</i> (186 th n.t. C changed to A)	This study
QW700	<i>AS-fliR_{STOP2}</i> , <i>igaA</i> *	This study
QW701	<i>AS-fliR_{STOP1}</i> (13 th n.t. A changed to T)	This study
QW702	<i>AS-fliR_{STOP1}</i> , <i>igaA</i> *	This study
Plasmids		
pBAD33	vector	Guzman et al 1995
<i>pfliPQR^{BAD}</i>	<i>fliPQR</i> in pBAD33	This study
<i>p^LfliQR^{BAD}</i>	<i>fliQR</i> in pBAD33	This study

^a* refers to the point mutation described in the indicated study. Δ followed by :: refers to gene deletion concomitant with substitution of indicated antibiotic resistance cassette. Primers used for various manipulations are listed in Supp. Table 1, and extent of deletions created in Suppl. Table 2. *RA* refers to the intergenic region between *fliR* and *rcsA*, and deletion numbers refer to base pairs to the 3' of *fliR* after the stop codon TAA. FLAG

subscripts refer to epitope tags placed either at the 5' or 3' end of the indicated genes. *L* and *BAD* superscripts refer to *fliL* or PBAD promoters driving sense or anti-sense transcription of the indicated genes, respectively. T refers to the 426 bp *rrnB* T1T2, # to the 6 bp substitution at -10 of AS promoter. The stop codon numbering refers to the putative anti-FliR ORF that starts in Val; G of GTA is 17th nt after TAA of *fliR*. See Experimental Procedures for details.

TABLE 2

Regulation of the Rcs regulon by anti-sense *flhPQR* RNA^a

Name ^b	Ratio ^c	SD	Ratio	SD
<u>Virulence genes negatively regulated</u>				
<i>avrA</i>	2.6	0.5		
			Others positively regulated (cont'nd)	
			<i>melA</i>	3.0 0.9
<i>hilC</i>	122	95	<i>narV (narZ)</i>	3.1 0.7
<i>hilD</i>	2.3	0.3	<i>nirB (nirB)</i>	2.2 0.6
<i>invC,F,G,I,spaO,S (invF)</i>	1.4–2.8	0.1–0.7	<i>ompF</i>	24 11.8
<i>prgH,I,K,orgA,STM2868 (prgH)</i>	2.0–4.9	0.3–1.1	<i>osmE</i>	2.5 0.4
<i>sicA,P,sipA–D,iacP,sptP (sicA)</i>	1.7–2.3	0.1–1.0	<i>pisG</i>	3.1 1.1
<i>sopA</i>	2.4	0.2	<i>rnf</i>	4.3 1.6
<i>sopB (sopB)</i>	4.8	1.4	<i>rpsP,rpmM,rmrD,rplS (rpsP)</i>	2.2–3.0 0.3–0.5
<i>ssaC (ssaB)</i>	1.6	0.1	<i>sodC</i>	2.0 0.7
<i>ssaJ,L (ssaC)</i>	2.5–4.3	0.2–0.9	<i>spvA</i>	4.5 1.7
<i>ssaA,B,ssaC (ssaA)</i>	1.7–2.1	0.1	<i>spvB</i>	2.6 0.8
<i>sseF,G (sseB)</i>	1.9–2.1	0.3–0.4	<i>spy</i>	2.2 0.4
<i>sseJ</i>	9.8	2.1	STM0950 (STM0951)	2.7 0.9
<i>ssrA,B (ssrA)</i>	1.6–2.6	0.2–0.6	STM1273	3.4 0.2
<u>Others negatively regulated</u>				
			STM1561	2.4 0.5
<i>cadB</i>	36.5	7.0	STM1633 (STM1633)	2.3 0.4
<i>cdd</i>	8.4	1.5	STM1638	7.3 2.0
<i>fabB</i>	2.5	0.1	STM4071	5.3 0.8
<i>ppk (ppk)</i>	2.4	0.3	STM4239,yjbJ (<i>dinF</i>)	3.2–5.3 0.7–1.6
<i>yfbA (yfbA)</i>	2.3	0.5	STM4242	3.6 0.6
STM1328	6.6	0.4	STM4510	5.6 1.5
STM2287	4.9	0.4	STM4519	5.4 2.4
<i>tdcE (tdcD)</i>	3.2	0.7	<i>yahoo</i>	5.6 1.7
<u>Others positively regulated</u>				
			<i>yaiA (aroL)</i>	2.8 1.0
<i>aceA–B (aceB)</i>	103–143	43–46	<i>yctE,STM1731 (yctE)</i>	3.5–3.9 1.6–1.9
<i>bax</i>	3.0	0.5	<i>yctF</i>	2.6 1.2
<i>chaB</i>	2.5	0.8	<i>ydeV</i>	5.6 0.8

Name ^b	Ratio ^c	SD	Ratio	SD
<i>dhfR</i>	3.6	2.2		7.0
<i>ecfB</i>	2.5	0.6		2.6
<i>ego, ydeY, Z, yneA-C, STM4080 (ego)</i>	2.5–20	0.1–4.6		2.2
<i>fbaB</i>	4.1	1.4		2.1
<i>galP</i>	2.2	0.6		2.3
<i>ggt</i>	5.6	5.0		2.0
<i>katE</i>	2.0	0.6		2.8
<i>malE</i>	2.3	0.5		2.5
<i>manX-Z (manX)</i>	7.3–13	1.5–4.9		2.7
				1.1

^aThe microarray data are for RNA harvested from cells at stationary phase.

^bGenes and open reading frames from the same operon are listed together. The first gene of the operon is indicated in parenthesis. ~ indicates intervening genes in the operon.

^cTranscription ratios are expressed such that the numbers are >1 for both positively and negatively regulated genes, because it is easier to compare fold regulation using whole numbers rather than fractions. Positive ratios were derived by dividing QW119 (*igaA**) values by either QW422 (*fliR::kan, igaA**) or QW556 (Δ RA 1–48, *igaA**) or QW471 (*fliQR, igaA**) values; negative ratios were reverse of these. The differentially regulated Rcs regulon genes common to all three pairs of strains are shown. The range indicates low and high values for individual genes within the operon. The ratios represent an average of 4 independent repeats. SD, standard deviation.