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MicroReview

A rough guide to the non-coding RNA world of *Salmonella*

Jörg Vogel*
RNA Biology Group, Max Planck Institute for Infection Biology, Charitéplatz 1, D-10117 Berlin, Germany.

Summary

***Salmonella* species are enterobacterial pathogens that have been exceptionally well investigated with respect to virulence mechanisms, microbial pathogenesis, genome evolution and many fundamental pathways of gene expression and metabolism. While these studies have traditionally focused on protein functions, *Salmonella* has also become a model organism for RNA-mediated regulation. The present review is dedicated to the non-coding RNA world of *Salmonella*: it covers small RNAs (sRNAs) that act as post-transcriptional regulators of gene expression, novel Salmonella *cis*-regulatory RNA elements that sense metabolite and metal ion concentrations (or temperature), and globally acting RNA-binding proteins such as CsrA or Hfq (inactivation of which cause drastic phenotypes and virulence defects). Owing to mosaic genome structure, some of the *Salmonella* sRNAs are widely conserved in bacteria whereas others are very specific to *Salmonella* species. Intriguingly, sRNAs of either type (CsrB/C, InvR, SgrS) facilitate cross-talk between the *Salmonella* core genome and its laterally acquired virulence regions. Work in *Salmonella* also identified physiological functions (and mechanisms thereof) of RNA that had remained unknown in *Escherichia coli*, and pioneered the use of high-throughput sequencing technology to identify the sRNA and mRNA targets of bacterial RNA-binding proteins.**

Introduction

Proteins were long regarded as the only relevant players in the control of bacterial gene expression. However, the recent discoveries of unexpected numbers of small non-coding RNAs (sRNAs) and *cis*-encoded RNA control elements have challenged the above perception. Bacterial sRNAs are typically 50–250 nucleotides in length, generally untranslated and encoded in the 'empty' intergenic regions (IGRs) of bacterial chromosomes (Vogel and Sharma, 2005). Their synthesis is tightly regulated and often induced by a specific stress or virulence condition. Most sRNAs function as regulators by base-pairing with *trans*-encoded mRNAs, and thereby either repress or activate target genes at the post-transcriptional level. There are also some sRNAs that specifically modulate protein functions (Majdalani *et al*., 2005; Storz *et al*., 2005).

Whereas sRNAs exert control over *trans*-encoded targets, other non-coding RNAs regulate gene expression in *cis*. Besides the well-known class of antisense RNAs encoded opposite to mRNA genes (Wagner *et al*., 2002), *cis*-control includes the new classes of riboswitches and RNA thermometers. Riboswitches are highly structured RNA elements found in 5′ untranslated regions (5′ UTRs) of metabolic genes. A riboswitch commonly senses the metabolite that is synthesized or taken up by the downstream encoded protein(s), and thereby facilitates feedback regulation at the transcriptional or translational level; regulation is brought about by a programmed shift in RNA structure in response to binding of the metabolite. RNA thermometers are local structure elements that sequester the ribosome binding site (RBS) of an mRNA; structure formation is highly responsive to environmental temperature, and can thereby control mRNA translation in a temperature-dependent manner.

The systematic identification and functional analyses of non-coding RNAs were largely pioneered by work on non-pathogenic model bacteria (Majdalani *et al*., 2005; Storz *et al*., 2005). For example, a variety of approaches (Vogel and Sharma, 2005; Altuvia, 2007) have identified more than a hundred chromosomally encoded sRNAs in *Escherichia coli*, a number not yet matched in any other bacterium. Similarly, riboswitches have been mostly studied in *Bacillus subtilis*; ~5% of all genes of this soil bacterium might be controlled by RNA-based metabolite sensing (Mandal *et al*., 2003).

Yet, there are well-characterized non-coding RNA regulators in pathogenic bacteria, e.g. the 514 nt RNAIII of *Staphylococcus aureus*, which controls many virulence factor mRNAs, or an RNA thermometer that controls *prfA*

Accepted 17 October, 2008. *For correspondence. E-mail vogel@mpiib-berlin.mpg.de; Tel. (+49) 30 28460-265; Fax (+49) 30 28460-244

mRNA encoding a key transcription factor of virulence genes in *Listeria monocytogenes* (Romby *et al*., 2006; Toledo-Arana *et al*., 2007). Moreover, following the success of sRNA discovery in *E. coli*, bacterial pathogens are now being scrutinized to discover new sRNA regulators (Livny and Waldor, 2007).

Salmonella enterica serovar Typhimurium is one of the pathogens in which non-coding RNA functions have been studied extensively. This workhorse of research into enterobacterial pathogenesis is in many ways paradigmatic of the larger group of Salmonellaea; for simplicity, it will be referred to as *Salmonella* unless specified otherwise. *Salmonella* is a Gram-negative bacterium closely related to *E. coli* K12. Unlike the latter, non-pathogenic species, *Salmonella* invades and replicates in eukaryotic cells and causes disease in a variety of mammalian and non-mammalian hosts. For infection, *Salmonella* relies upon a range of laterally acquired virulence regions, the so-called *Salmonella* pathogenicity islands (SPIs). Of these, SPI-1 and SPI-2 encode type 3 secretion systems (T3SS), which translocate effector proteins to facilitate either invasion of non-phagocytic cells (SPI-1) or survival within macrophages (SPI-2). The secreted effectors are encoded by SPI-1 or SPI-2, by other minor SPIs, or by individual genes scattered throughout the *Salmonella* chromosome.

Both the evolutionarily close relationship with *E. coli* and the pathogen-specific aspects make *Salmonella* an attractive candidate for RNA research. First, many *Salmonella s*RNAs were originally identified in *E. coli*; whether these sRNAs serve identical functions in conserved general pathways or evolved new ones more relevant to the pathogenic lifestyle of *Salmonella* is a fascinating question. Second, > 25% of the total genetic material has been laterally acquired since *Salmonella* diverged from *E. coli* (Porwollik and McClelland, 2003). Do these *Salmonella*-specific regions contain new sRNAs or other RNA elements whose function would be missed in *E. coli*, and do RNA factors interconnect expression of the *Salmonella* core genome and virulence regions at the post-transcriptional level? Third, the auxiliary proteins (nucleases, RNA chaperones) typically involved in RNA-based circuits are highly conserved between the two species. Thus, RNA work in *Salmonella* can often take advantage of knowledge and mutants available for *E. coli*.

Research into *Salmonella* was never alien to aspects of non-coding RNA. For example, it revealed a novel mechanism of translational control by a *Salmonella* phage P22-encoded antisense RNA (Ranade and Poteete, 1993), or identified one of the first virulence phenotype of the ubiquitous tmRNA system (Julio *et al*., 2000). It also contributed much to understanding of *cis*-antisense control of extrachromosomal replication, e.g. of the pSLT plasmid essential for *Salmonella* virulence (Torreblanca *et al*., 1999), and identified a physiological role of Hfq (Brown and Elliott, 1996), a protein that since emerged as a key facilitator of the action of sRNAs.

In this review, we will focus on sRNAs and *cis*-encoded RNA control elements of *Salmonella*, and give an overview of their numbers and functions. Some of the molecules covered here have also been well investigated in *E. coli* or other bacteria; space constraints do not permit to extensively discuss this overlap.

Phenotypes of hfq *mutants implicate sRNAs as important regulators in* Salmonella

Perhaps the strongest evidence that sRNAs serve important functions in *Salmonella* stems from work on the RNA chaperone, Hfq. This protein preferentially binds A/U-rich single-stranded regions of RNA, and is required for both the intracellular stability of many regulatory sRNAs and their annealing with target mRNAs (Valentin-Hansen *et al*., 2004).

The numbers of phenotypes and deregulated genes observed in an *hfq* deletion mutant of *Salmonella* surpass those reported for any other pathogen. Deletion of *hfq* has for long been known to impair the expression of σ^S (Brown and Elliott, 1996), a general stress sigma factor essential for *Salmonella* virulence in mice (Fang *et al*., 1992). More recently, the *hfq* mutation was shown to attenuate the ability of *Salmonella* to invade epithelial cells, to secrete virulence factors, to infect mice and to survive inside cultured macrophages (Sittka *et al*., 2007). Loss of Hfq function also abrogates *Salmonella* motility, and deregulates > 70 abundant proteins. The latter includes the accumulation of outer membrane proteins (OMPs), which in turn cause chronic activation of the σ^E-mediated envelope stress response (Bang *et al*., 2005; Figueroa-Bossi *et al*., 2006; Sittka *et al*., 2007; 2008; Bossi *et al*., 2008). Moreover, Hfq was implicated in the control of *Salmonella* gene expression changes induced by the low-gravity condition experienced during spaceflight (Wilson *et al*., 2007).

Recent transcriptomic analysis revealed that Hfq controls, directly or indirectly, the expression of almost a fifth of all *Salmonella* genes, including genes in several horizontally acquired pathogenicity islands (SPI-1, -2, -4, -5), two sigma factor regulons and the flagellar gene cascade (Sittka *et al*., 2008). Given that Hfq primarily acts in concert with sRNAs, many of the above phenotypes in *Salmonella* may be attributable to loss of gene regulation by Hfq-associated sRNAs.

Salmonella *expresses many sRNAs of mosaic origin*

At least 70 sRNAs have been identified in *Salmonella*, examples of which are shown in Fig. 1. Almost half of these are encoded by genes that were originally

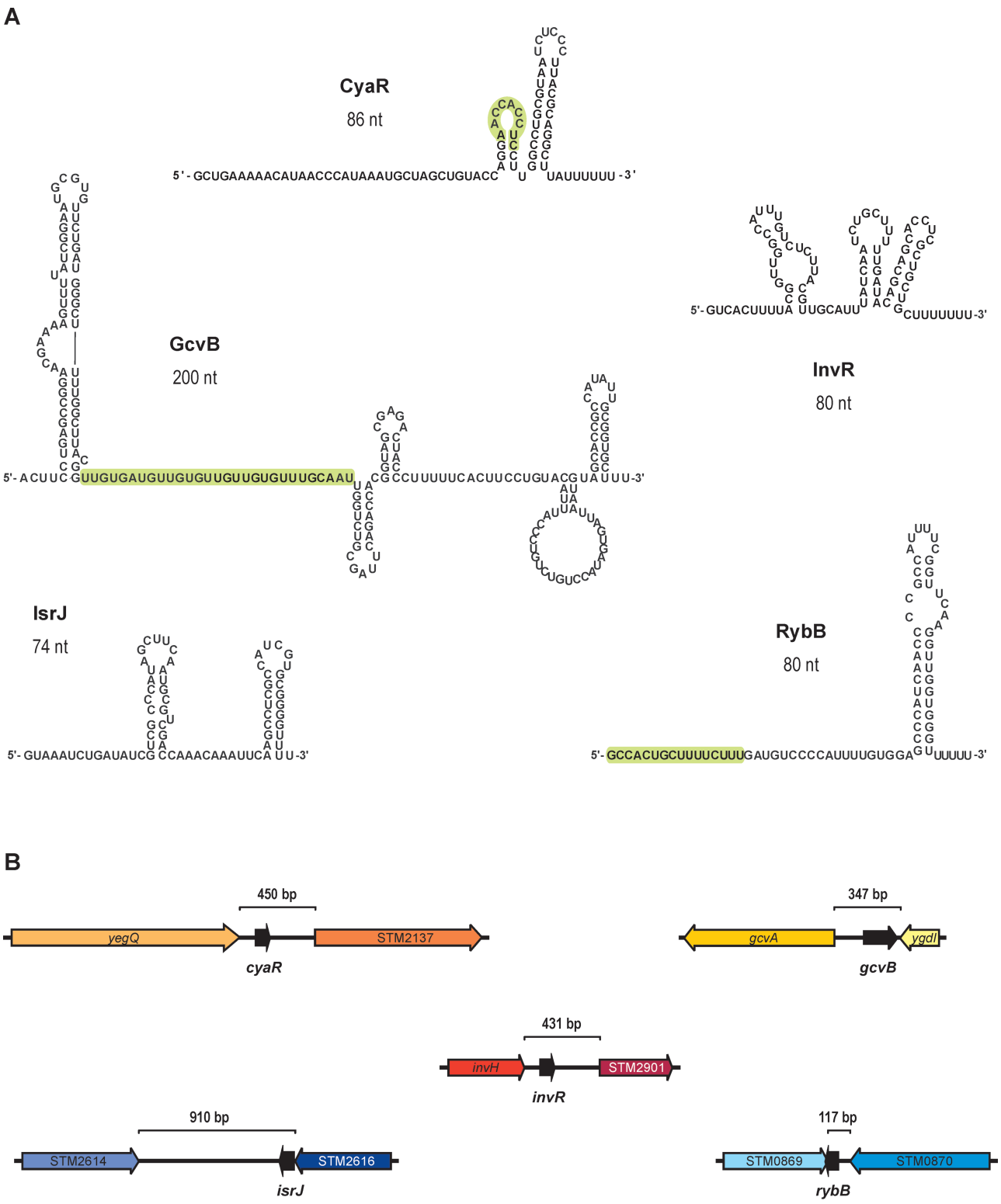

Fig. 1. Examples of *Salmonella* sRNAs and their genomic location.
A. Secondary structures are based on *in silico* prediction of CyaR and IsrJ (Papenfort *et al.*, 2008; S. Altuvia, pers. comm.) or *in vitro* chemical probing of GcvB, InvR and RybB sRNAs (Pfeiffer *et al.*, 2007; Sharma *et al.*, 2007; Bouvier *et al.*, 2008). Shadowed nucleotides of the CyaR, GcvB or RybB sRNAs denote residues to interact with target mRNAs.
B. Location of sRNA genes in the IGRs (drawn to scale) of the *Salmonella* chromosome (*Salmonella typhimurium* strain LT2). Neighbouring genes and the size of the IGRs are given for orientation.

described in *E. coli* (Hershberg *et al*., 2003); the genomic co-ordinates of the corresponding *Salmonella* genes were compiled by Papenfort *et al*. (2008). Expression in *Salmonella* was confirmed either in the course of functional analysis, e.g. of the CsrB/C, CyaR, GcvB, MicA, RybB, RyhB and SgrS sRNAs described further below, or by a global approach that used high-throughput sequencing of Hfq-associated RNA (Sittka *et al*., 2008).

Two parallel studies (Pfeiffer *et al*., 2007; Padalon-Brauch *et al*., 2008) took biocomputational approaches to identify *Salmonella*-specific *s*RNAs. Pfeiffer *et al*. (2007) searched for 'orphan' pairs of σ^{70}-type promoters and ρ-independent transcription terminators in the IGRs of the *Salmonella* LT2 genome, and predicted 46 candidates (STnc10 through STnc460) of sRNA genes that were absent from *E. coli* K12, but mostly present in the early branching *Salmonella* species, *S. bongori*. This screen discovered the first sRNA from an enterobacterial pathogenicity island, i.e. the 80 nt InvR RNA (Fig. 1) that is expressed from the invasion gene locus, SPI-1 (Pfeiffer *et al*., 2007).

Padalon *et al*. (2008) explored the genetic islands of *Salmonella*, i.e. those IGRs that were > 100 bp and showed < 80% identity to their most similar sequence in *E. coli* K12. The predictions, which were largely based upon orphan ρ-independent terminators, resulted in 28 sRNA candidate genes. Expression of 19 sRNAs, now denoted Isr (A, B, etc.) for island-encoded sRNA, was verified by Northern blot analysis of a large panel of growth conditions reminiscent of the environments encountered by *Salmonella* upon host cell infection. In addition, several validated sRNAs were shown to be differentially expressed upon *Salmonella* infection of macrophages.

The function of these sRNAs is as yet to be elucidated. A significant number of them overlap with the 5′ or 3′ ends of open reading frames, and modulate the expression of these flanking open reading frames or are in turn affected by those same genes. IsrJ sRNA (74 nt) encoded between STM2614 and STM2616 is a very promising candidate for the study of sRNA-mediated control of virulence: IsrJ synthesis requires the major SPI-1 transcription factor, HilA and an *isrJ* mutant is impaired in host cell invasion and effector translocation (Padalon-Brauch *et al*., 2008).

A network of sRNAs controls the biogenesis of Salmonella *OMPs*

Regulation of OMP biogenesis is the best understood function of enterobacterial sRNAs (Guillier and Gottesman, 2006; Vogel and Papenfort, 2006), and is tightly linked to two prominent phenotypes of *Salmonella hfq* mutants, i.e. overproduction of OMPs and concomitant constitutive induction of the σ^E-controlled envelope stress response (Figueroa-Bossi *et al*., 2006; Sittka *et al*., 2007; Bossi *et al*., 2008). Figure 2A shows the currently known network of Hfq-dependent sRNAs that act to repress *Salmonella omp* genes at the post-transcriptional level. The expression of these sRNAs is controlled by diverse transcription factors, i.e. σ^E, HilD, Crp and OmpR, and activated by a specific stress or growth condition.

The 80 nt RybB sRNA (Fig. 1), which is transcriptionally induced by the envelope stress sigma factor, σ^E (Papenfort *et al*., 2006), is the most globally acting OMP regulator of *Salmonella* (Fig. 2A). It represses the synthesis of all major porins (OmpA/C/D/F) and many minor OMPs (Papenfort *et al*., 2006) by base-pairing with the 5′ UTRs or coding regions of *omp* target mRNAs; these RNA interactions are generally short (7–16 bp) and imperfect, and involve the conserved 5′ end of RybB sRNA (Bouvier *et al*., 2008; F. Mika *et al*., submitted). The 74 nt MicA sRNA is also activated by σ^E, and represses the mRNAs of two *Salmonella* porins, OmpA and LamB, by antisense pairing similarly to RybB (Udekwu *et al*., 2005; Figueroa-Bossi *et al*., 2006; Papenfort *et al*., 2006; Bossi and Figueroa-Bossi, 2007).

The σ^E response counteracts the accumulation of unfolded OMPs in the periplasm, and the role of MicA and RybB within this regulon is to halt OMP synthesis when porin production threatens outer membrane homeostasis (Papenfort *et al*., 2006). Hfq is mandatory for the stability and function of the two sRNAs (Papenfort *et al*., 2006; Viegas *et al*., 2007; Sittka *et al*., 2008) and, therefore, the loss of MicA/RybB-mediated *omp* mRNA repression might partly explain the chronic σ^E stress observed in *Salmonella hfq* mutants (Figueroa-Bossi *et al*., 2006; Sittka *et al*., 2007). In more general terms, the analyses of MicA and RybB introduced novel approaches to sRNA target identification in *Salmonella*, i.e. sRNA pulse-expression combined with global transcriptome profiling (Papenfort *et al*., 2006), and chromosomal mutagenesis of an sRNA gene (Bossi and Figueroa-Bossi, 2007), both of which will help unravel other sRNA-based circuits.

Similar to InvR sRNA, which will be covered further below, the ~86 nt CyaR sRNA regulates a single OMP (Fig. 2A). CyaR targets the *ompX* mRNA encoding a small abundant porin that is highly overproduced in *hfq* mutants (Sittka *et al*., 2007). Until very recently, sRNA regulators were known for virtually every abundant porin except OmpX (Guillier and Gottesman, 2006; Vogel and Papenfort, 2006). Yet, an Hfq association of *ompX* mRNA in both *Salmonella* and *E. coli* (Zhang *et al*., 2003; Sittka *et al*., 2008) correctly predicted the existence of a cognate Hfq-dependent sRNA shared by these two species. CyaR was then identified in a 'reverse target search' approach by screening 35 *Salmonella* strains with mutations inactivating conserved sRNA genes for effects on OmpX synthesis (Papenfort *et al*., 2008).

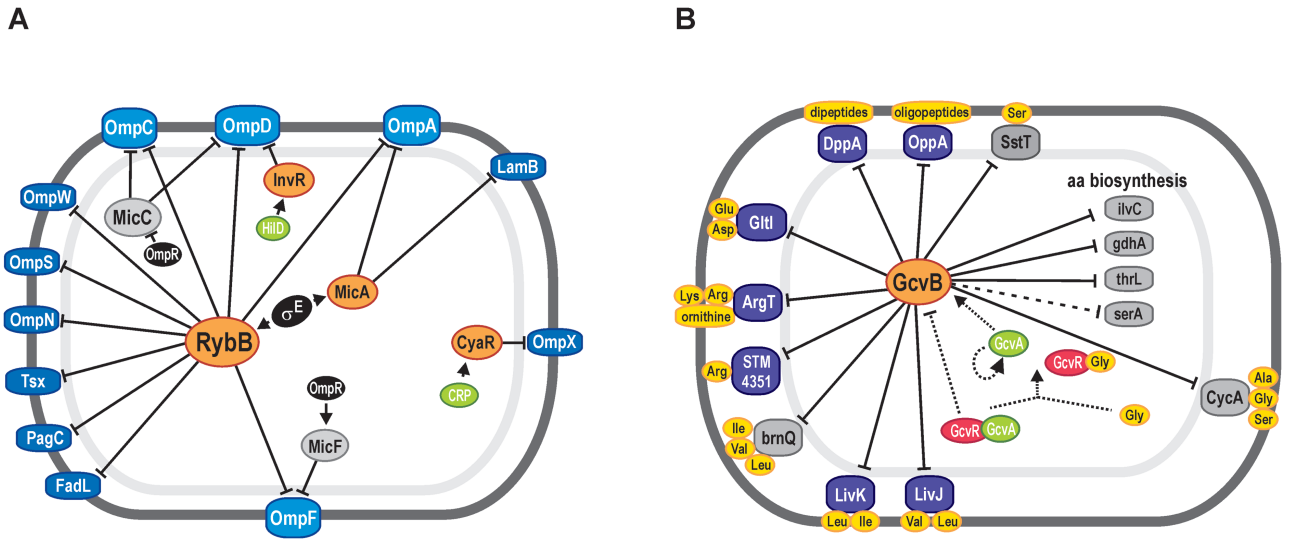

Fig. 2. sRNA-mediated control of extracytoplasmic protein production in *Salmonella*.
A. An emerging network of sRNAs that control OMP (blue circles) expression in *Salmonella*. Regulatory *s*RNAs are shown in orange circles within a schematic drawing of a *Salmonella* cell (the thick black or grey lines indicate outer or inner membrane respectively). Transcription factors of sRNA genes that are associated with membrane perturbation (OmpR, σ^E) are shown in black circles, whereas those of general (Crp) or virulence (HilD) regulons are shown in green circles. See text for more details on the OMP targets, and the input signals of the network.
B. GcvB sRNA represses mRNAs of the periplasmic substrate proteins of many ABC transporters involved in amino acid uptake. The seven targets that have been validated by *in vitro* structure probing in Sharma *et al.* (2007) are shown as blue circles; grey circles denote additional proteins of transport systems or with roles in amino acid biosynthesis, and whose synthesis has been shown to be directly regulated by GcvB in *Salmonella* (C.M. Sharma *et al.*, in preparation). The preferred amino acid, di- or oligopeptide substrates of relevant periplasmic binding proteins are shown in yellow circles. Control of the *gcvB* gene by two transcription factors (GcvR, GcvA) of the glycine cleavage system is indicated based on extensive work in *E. coli* by the Stauffer laboratory (see e.g. Urbanowski *et al.*, 2000).

Two features of CyaR are particularly interesting. First, *cyaR* expression is tightly controlled by the cyclic AMP receptor protein, CRP, which represents a link between porin repression and nutrient availability. Second, the target interaction regions of sRNAs are typically single-stranded. In CyaR, however, a conserved RNA hairpin featuring a C-rich apical loop (Fig. 1A) acts to sequester the Shine–Dalgarno sequence of *ompX* mRNA and to inhibit translational initiation.

Based on conservation of *E. coli* sRNA–target interactions, the MicC and MicF sRNAs (Mizuno *et al.*, 1984; Chen *et al.*, 2004) have been integrated into the OMP-regulatory network of *Salmonella* (Fig. 2A). Experimental screens similar to that for RybB targets (Papenfort *et al.*, 2006) have not only confirmed MicC and MicF action in *Salmonella*, but also identified OmpD as an additional MicC target (V. Pfeiffer *et al.*, in preparation). OmpD is the most abundant *Salmonella* porin, and its deregulation the primary cause of σ^E stress in *hfq* mutants (Bossi *et al.*, 2008). It should now come as no surprise that a total of three Hfq-dependent sRNAs (InvR, MicC, RybB; Fig. 2A) control OmpD synthesis at the post-transcriptional level.

Control of ABC transporter expression by GcvB sRNA

Some of the above sRNAs regulate the synthesis of multiple OMPs, i.e. proteins with a shared cellular destination. Similarly, the *Salmonella* homologue of *E. coli* GcvB sRNA (Urbanowski *et al.*, 2000) was shown to directly target seven mRNAs of periplasmic binding proteins of ABC uptake systems for amino acids and peptides (Sharma *et al.*, 2007 and Fig. 2B). The ability of the 200 nt GcvB sRNA to interact with multiple mRNAs is based upon a ~30 nt stretch of G and U residues (Fig. 1A); this G/U stretch recognizes extended C/A-rich elements in GcvB target mRNAs. The C/A elements overlap with the RBS of some targets, but are found far upstream of the start codon of others. In either case, the binding of GcvB prevents ribosome access to the mRNA, thus downregulating the target. Independent of GcvB action, the C/A elements have a stimulatory effect on ABC transporter mRNA translation (Sharma *et al.*, 2007). Thus, multiple mRNA targeting by GcvB seems to have evolved through hijacking a translational enhancer element shared by numerous mRNAs of periplasmic transporters.

The work on *Salmonella* GcvB revealed novel aspects of sRNA action (Sharma *et al.*, 2007). Not only did this sRNA commonly repress mRNAs at a 'non-canonical' position, i.e. much upstream of the RBS, but the same region in GcvB interacted with all identified target mRNAs. This region, i.e. the G/U element, is readily visible in alignments of GcvB homologues of a vast number of bacteria. Thus, an ultra-conserved region of the sRNA determines target mRNA regulation, and the recognition

of this principle remedies some of the previous difficulty of sRNA target prediction (Vogel and Wagner, 2007).

GcvB is specifically expressed in fast-growing *Salmonella* (Sharma *et al*., 2007), i.e. when nutrients are plentiful, and the repression of ABC transporter synthesis by GcvB may help optimize amino acid uptake under this condition. Recent work has revealed an extended post-transcriptional regulon of GcvB, which also includes repression *cycA* mRNA encoding the major glycine transporter (Fig. 2B and C.M. Sharma *et al*., in preparation). As the *gcvB* gene is controlled by transcription factors of the glycine cleavage system (Urbanowski *et al*., 2000), the downregulation of CycA synthesis by GcvB might facilitate a feedback loop in the expression of ABC transporters.

Small RNAs mediate cross-talk of the Salmonella *core genome and virulence regions*

The *Salmonella* invasion gene island, SPI-1, has been one of the most intensely studied bacterial virulence regions. Following its original discovery almost 20 years ago, both its overall sequence and its ~35 genes encoding the SPI-1 T3SS structural components, chaperones, effector proteins or transcription factors were subject to much scrutiny. Perhaps owing of the traditional focus on proteins, the SPI-1 gene encoding InvR RNA (Fig. 1 and Pfeiffer *et al*., 2007) remained unnoticed.

The *invR* gene, located at the right SPI-1 border, was presumably acquired along with the island very early at (or soon after) the divergence of *E. coli* and *Salmonella*. This sRNA gene is coexpressed with other SPI-1 genes under conditions that favour host cell invasion, and directly controlled by HilD, the protein that acts at the top of the SPI-1 transcription factor cascade (Fig. 3). The results of Hfq co-immunoprecipitation (co-IP) and deep sequencing analysis showed that InvR is the most abundant Hfq-associated RNA under conditions of host cell invasion (Pfeiffer *et al*., 2007; Sittka *et al*., 2008).

Despite what genomic origin and expression intuitively suggest, InvR is not directly involved in SPI-1 regulation or T3SS function. In contrast, InvR affects gene expression of the core *Salmonella* genome by acting as a post-transcriptional repressor of *ompD* mRNA (InvR targets the *ompD* coding region). The biological impact of this SPI-1-mediated repression of OmpD synthesis is not yet understood. However, one might speculate that, as *invR* is conserved in the early branching *S. bongori* species, the repression of the most abundant *Salmonella* porin by InvR might have aided the successful establishment of the membrane-spanning SPI-1 T3SS after horizontal acquisition in the *Salmonella* lineage (Pfeiffer *et al*., 2007). Note that OmpD overproduction destabilizes the envelope and abrogates SPI-1 effector secretion, and these phenotypes are successfully complemented by InvR expression (V. Pfeiffer and J. Vogel, unpublished).

IsrE sRNA (98 nt) represents another example of cross-talk function. It is an island-encoded paralogue of RyhB, a Fur-regulated sRNA of the *Salmonella* core genome (Vogel and Sharma, 2005; Ellermeier and Slauch, 2008; Padalon-Brauch *et al*., 2008). In *E. coli*, RyhB is an important regulator of iron homeostasis, and one of its function is the post-transcriptional repression of the core genome-encoded *sodB* mRNA under iron starvation conditions (Massé and Gottesman, 2002). In *Salmonella*, IsrE and RyhB act redundantly to downregulate *sodB* mRNA (Ellermeier and Slauch, 2008) and additively to slow down growth under iron-deplete conditions (Padalon-Brauch *et al*., 2008).

The *Salmonella* core genome extensively controls the virulence regions at the transcriptional level, e.g. by two-component systems that time the expression of invasion

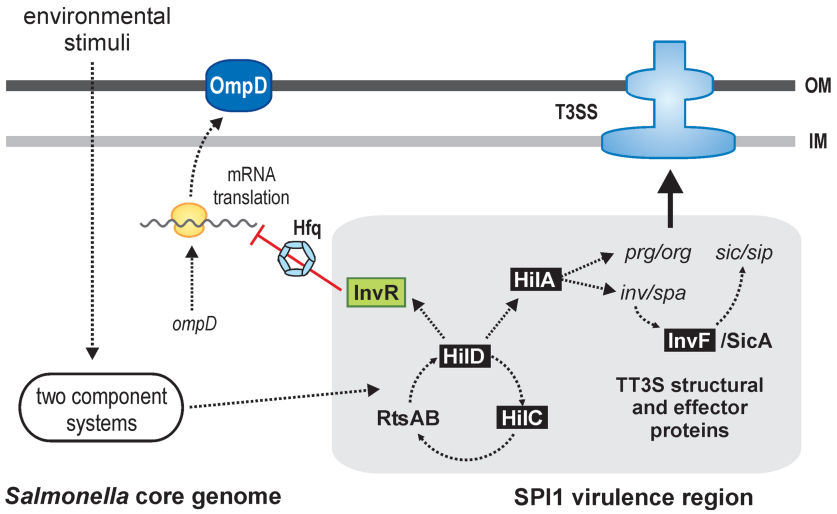

Fig. 3. Example of sRNA-mediated cross-talk of *Salmonella* virulence region with core genome expression. Proposed model of porin repression by InvR sRNA (Pfeiffer *et al*., 2007). Two component systems encoded by the *Salmonella* core genome sense environmental signals that lead to activation of the SPI-1 transcription factor cascade (HilD, HilC, HilA, InvF), and subsequently, to the expression of the SPI-1 T3SS. HilD also activates expression of the SPI-1-borne *invR* gene. Together with the RNA chaperone Hfq, InvR acts post-transcriptionally to repress synthesis of the major OMP, OmpD, which is encoded by the *Salmonella* core genome. One might speculate that at times the repression of the most abundant porin by InvR might have aided the successful establishment of the membrane-spanning SPI-1 T3SS after lateral acquisition in the *Salmonella* lineage.

genes. The case of InvR shows that such cross-talk also takes place post-transcriptionally, through an Hfq-dependent sRNA. If true, then it might also work the other way around, and core genome-encoded sRNAs might control horizontally acquired virulence factors.

In line with this prediction, SgrS sRNA, which acts to combat phosphosugar stress in *E. coli*, regulates the production of a secreted effector protein, SopD, in *Salmonella* (K. Papenfort *et al*., in preparation). Intriguingly, the same region of SgrS involved in antisense targeting of mRNAs relevant to phosphosugar stress, e.g. the *ptsG* mRNA, is reused to modulate protein synthesis from *sopD* mRNA. This suggests that bacteria selectively employ their existing repertoire of sRNA regulators of general stress responses to co-ordinate the expression of laterally acquired virulence genes.

Small RNAs antagonize the global regulator, CsrA

Cross-talk of the *Salmonella* core genome with virulence regions is also facilitated by the CsrB (360 nt) and CsrC (240 nt) sRNAs. However, these two conserved sRNAs do not directly act upon mRNAs, yet commonly sequester (through GGA sequence motifs) the abundant RNA-binding protein, CsrA, which in turn directly modulates mRNA translation (Babitzke and Romeo, 2007). CsrA is a global regulator of gene expression, and its absence deregulates > 8% of all *Salmonella* genes (Lawhon *et al*., 2003). By a yet unknown mechanism, CsrA also controls the SPI-1-borne invasion genes, and is ultimately required for successful host cell invasion (Altier *et al*., 2000). In this circuit, the pool of free CrsA protein needs to be tightly controlled, as is evident from the attenuated host cell invasion phenotype caused by both deletion and overexpression of *csrA* (Altier *et al*., 2000); this is where the Csr sRNAs come into play.

A double *csrB csrC* deletion mutant is markedly impaired in host cell invasion *in vitro* (Fortune *et al*., 2006) and ~100-fold attenuated in mouse infections (D. Becker and D. Bumann, pers. comm.), presumably due to hyperactivity of the CrsA protein. Intriguingly, however, the single *csrB* or *csrC* mutants do not show these phenotypes, suggesting redundancy of function, at least in the control of invasion gene expression.

The possible redundancy of *csrB* and *csrC* touches upon a key issue of the identification of sRNA functions in *Salmonella*. Thus far, none of the many global mutagenesis screens have discovered a sRNA gene as the cause of altered *Salmonella* virulence. However, these screens scoring the effects of individual gene disruption did not take into account that the function of individual sRNAs could be either backed up by another homologue, or masked by the action of a redundantly acting regulatory protein.

Riboswitches sense metabolite and magnesium concentrations

Many metabolic genes in bacteria are controlled at the RNA level by *cis*-acting riboswitches. *Salmonella* has been predicted to encode riboswitches for the sensing of cobalamin (vitamin B12), molybdenum cofactor, flavin mononucleotide and thiamine pyrophosphate (R. Breaker, pers. comm.). Of these, the cobalamin riboswitch located in the leader of *cob* operon mRNA has been exceptionally well investigated (Ravnum and Andersson, 2001 and references therein). The *cob* 5′ UTR is unusually long (462 nt) and highly structured. In the absence of cobalamin, the long-range interaction of an RNA element encoded approximately 100–250 bp upstream of AUG suppresses the formation of a short local RNA hairpin that would normally sequester the *cob* RBS to inhibit *cob* mRNA translation (Fig. 4A).

Cobalamin binding to the upstream RNA element disrupts the long-range interaction (Nahvi *et al*., 2004). As a result, the RBS hairpin forms and *cob* mRNA translation is switched off. The 25 *cob* operon genes, which are required for *de novo* synthesis of cobalamin, are translationally coupled. Thus, the RNA sensor of cobalamin located upstream of the first *cob* cistron can feedback-control the entire *cob*-encoded cobalamin synthesis pathway. The *btuB* mRNA encoding an OMP needed for import of extracellular cobalamin is feedback-regulated by a similar mechanism (Ravnum and Andersson, 1997; Nahvi *et al*., 2004).

RNA switches of *Salmonella* are not limited to sensing metabolites and performing translational control. Work on the *mgtA* mRNA, which encodes an Mg^{2+} transporter, revealed that its 5′ UTR served as a RNA sensor of Mg^{2+} (Cromie *et al*., 2006). Depending on the intracellular Mg^{2+} concentration, the 5′ UTR of *mgtA* adopts different stem-loop structures that allow or prohibit transcriptional readthrough into the *mgtA* coding region (Fig. 4B). Considering that Mg^{2+} is commonly required for proper RNA folding, its discovery as the specific determinant of a RNA switch mechanism provided a radically new example of RNA-mediated control in bacteria. In the context of *mgtA*, the RNA-mediated control of transcription elongation by cytoplasmic Mg^{2+} complements the tight control of transcription initiation by extracytoplasmic Mg^{2+} facilitated by the PhoP/Q two-component system (Cromie *et al*., 2006). Thus, the same ligand is sensed in different cellular environments to regulate distinct steps in gene transcription. Note that Mg^{2+} also seems to influence *mgtA* mRNA decay in an RNase E-dependent manner (Spinelli *et al*., 2008).

Mg^{2+} is a key signal in *Salmonella* virulence gene expression (Ohl and Miller, 2001), and it is an intriguing possibility that Mg^{2+}-dependent RNA switches control additional *Salmonella* genes post the initiation of

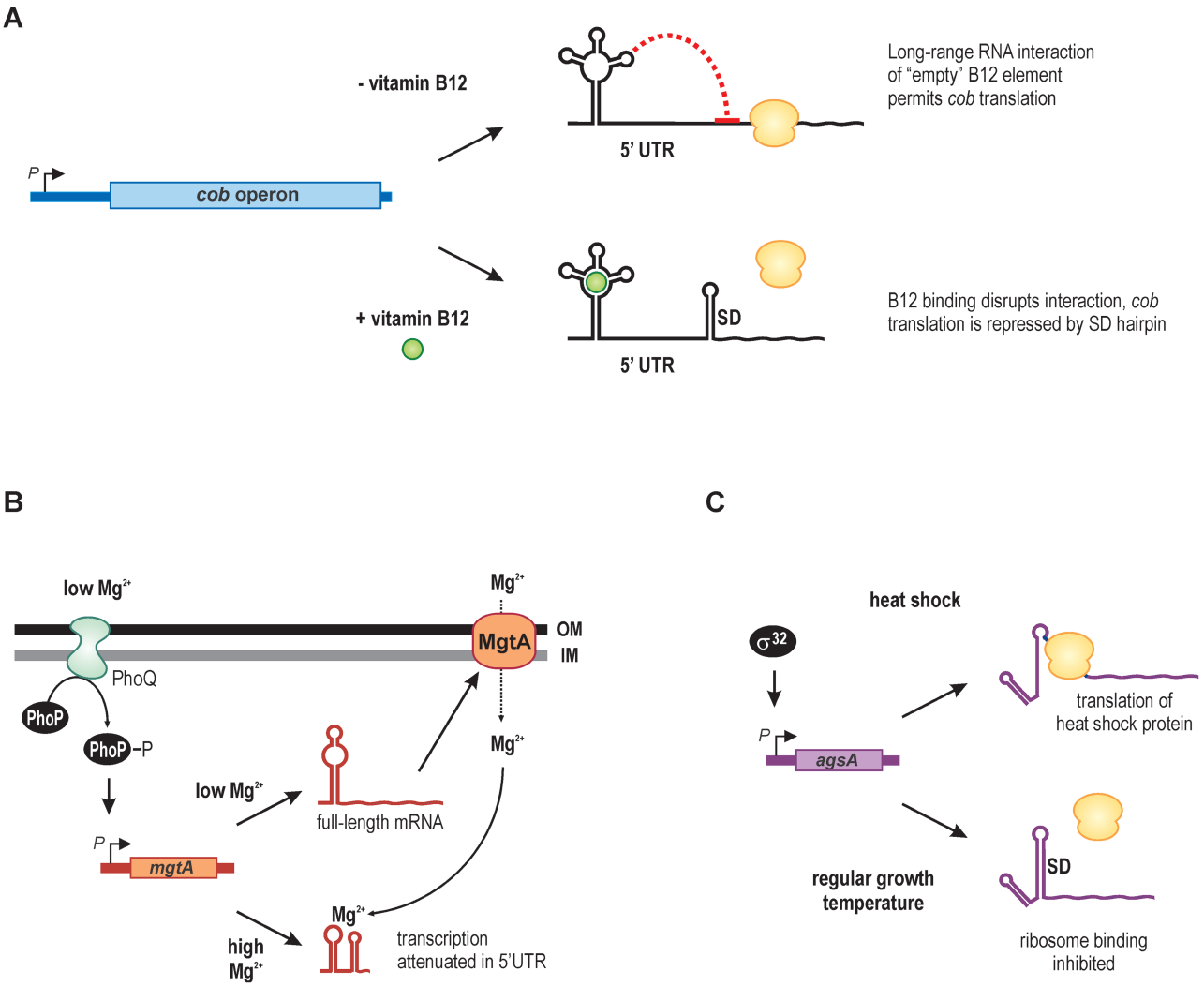

Fig. 4. Post-transcriptional control by *cis*-encoded RNA sensors in *Salmonella*.
A. A riboswitch mechanism for vitamin B12 (cobalamin) sensing by the first 5′ UTR of *cob* operon mRNA. Binding of cobalamin disrupts a long-range RNA interaction (red dotted line) that in the absence of cobalamin stabilizes a hairpin that sequesters the Shine–Dalgarno sequence of *cob*, and represses *cob* translation. The yellow structure denotes a ribosome.
B. A sensor of Mg^{2+} concentration resides in the 5′ UTR of *mgtA*, encoding a membrane-standing Mg^{2+} transporter. In low-Mg^{2+} environment (extracytoplasmic Mg^{2+}), the PhoPQ two-component systems directly activates *mgtA* transcription. However, if cytoplasmic Mg^{2+} are still high, Mg^{2+} binding to the *mgtA* 5′ UTR results leads to the formation of hairpin structure that attenuates *mgtA* transcription. Once the cytoplasmic Mg^{2+} concentration drops as well, the formation of an alternative stem-loop structure allows transcriptional readthrough into the *mgtA* coding region, and therefore MgtA protein synthesis. Internalization of Mg^{2+} by MgtA increase cytoplasmic Mg^{2+}, which in turn attenuates transcription of full-length *mgtA* mRNA. Adapted from Cromie *et al.* (2006).
C. Thermo-control of translation by an RNA thermometer contained in the 5′ UTR of *Salmonella agsA* mRNA that encodes a small heat shock protein. At regular growth temperature, a stable RNA hairpin sequesters the *agsA* Shine–Dalgarno to repress translation. Shift to non-permissive temperature activates both *agsA* transcription (by the heat shock factor, σ^{32}) and translation by partial melting of the Shine–Dalgarno hairpin.

transcription. More generally, we must determine whether other specialized riboswitches sense substances emitted by host cells in the course of infection.

Sensing temperature with RNA

Thermosensing as an input function in the control of *Salmonella* gene expression is well documented at the level of proteins, e.g. TlpA, a DNA-binding protein encoded by the *Salmonella* virulence plasmid (Hurme *et al.*, 1997). Yet, temperature is also sensed directly by *Salmonella* messengers, e.g. the *ibpA* and *agsA* mRNAs, which contain so-called RNA thermometers (Waldminghaus *et al.*, 2005; 2007).

The 5′ UTR of *agsA*, which encodes a small heat shock protein, contains one such RNA thermometer (Fig. 4C).

A stable hairpin blocks the Shine–Dalgarno sequence of *agsA* mRNA by base-pairing, and represses translation at normal growth temperature (37°C). A shift to a non-permissive temperature, i.e. heat shock (42°C), is predicted to open the hairpin, which is measurable by increased translation of an *agsA* reporter fusion. The results of *in vitro* RNA structure probing and 30S ribosome toeprinting experiments have fully supported this prediction, for example, showing that a stable translational initiation complex forms on *agsA* mRNA at 45°C but not at 30°C (Waldminghaus *et al*., 2007).

The *agsA* gene is also activated by heat shock at the transcriptional level. Thus, in contrast to the two-layer repression of *mgtA* by Mg^{2+} (see above), the *agsA* gene is *activated* by the same stimulus at both the DNA and the RNA level.

More roads to travel in the Salmonella *RNA world*

This brief review can only give a rough overview of the many roles that RNA may have in the control of *Salmonella* gene expression. For example, given that we have only glimpsed at Hfq functions, new pathways involving Hfq-dependent sRNAs will certainly continue to be discovered. High-throughput sequencing of Hfq-associated RNA species has identified > 700 mRNAs from diverse cellular pathways that interact with this protein *in vivo*, including many messengers from the *Salmonella* virulence regions (Sittka *et al*., 2008). This catalogue of putative sRNA targets provides departure points for improving *in silico* predictions and functional screens to identify new sRNA-based regulatory circuits. The method itself, which involves co-IP of RNA along with epitope-tagged Hfq protein and subsequent cDNA pyrosequencing, is general and should facilitate similar studies in many other pathogens. In *Salmonella*, the current Hfq co-IP data set is limited to one growth condition, and is definitely worth extending to more *Salmonella*-relevant stress conditions and infections of *in vitro* cultured cells or even animals.

A collection of *Salmonella* mutant strains for conserved sRNA genes (Papenfort *et al*., 2008) and for major factors relevant for sRNA processing (Viegas *et al*., 2007), combined with well-established methods to find and validate sRNA–target interactions in this organism (Papenfort *et al*., 2006; 2008; Bossi and Figueroa-Bossi, 2007), should also help test the extent to which sRNA functions in *Salmonella* are shared with other bacteria. There is evidence that even among *Salmonella* isolates, sRNA expression and thus function can radically differ. For example, the commonly used 14 028 s strain does not seem to produce the conserved DsrA and RprA sRNAs (Jones *et al*., 2006), two translational activators of *rpoS* mRNA in *E. coli* (Repoila *et al*., 2003). However, both sRNAs are produced and are functional as *rpoS* activators in the mouse-adapted SL1344 strain (Sittka *et al*., 2008 and K. Papenfort *et al*., in preparation).

The overall number of *Salmonella* sRNAs is likely to grow. First, most candidates identified in the screens by Pfeiffer *et al*. (2007) and Sittka *et al*. (2008) await experimental testing, and even the many growth conditions assayed by (Padalon-Brauch *et al*., 2008) might not have been sufficient to capture the expression of all currently predicted sRNAs. Second, several hundreds of *Salmonella* IGRs that correspond to sRNA candidate loci of *E. coli* (Hershberg *et al*., 2003; Livny *et al*., 2006) are yet to be validated in either of these two organisms. Essentially the same applies to riboswitches and RNA thermometers predicted in organisms closely related to *Salmonella*.

Last but not least, in a cellular environment, non-coding RNA is unlikely to be 'naked' and is more likely to be associated with RNA-binding proteins throughout its lifetime. Aside from the here-reviewed Hfq and CsrA proteins, work in *Salmonella* keeps discovering new involvements of RNA-binding proteins in post-transcriptional regulation. The latest examples of this include FljA, a protein long thought to be a transcription repressor in the flagellar gene expression cascade. It now turns out that FljA acts at the RNA level, by blocking the translation of *fliC* mRNA through specific binding to this messenger (Aldridge *et al*., 2006). Thus, the future analysis of *Salmonella* RNA-binding proteins has great potential to understand better RNA-based circuits in this model pathogen.

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