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sRNAs and the virulence of *Salmonella enterica* serovar Typhimurium

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The combination of genomics and high-throughput cDNA sequencing technologies has facilitated the identification of many small RNAs (sRNAs) that play a central role in the post-transcriptional gene regulation of *Salmonella enterica* serovar Typhimurium. To date, most of the functionally characterized sRNAs have been involved in the regulation of processes which are not directly linked to virulence. Just five sRNAs have been found to affect the ability of Salmonella to replicate within mammalian cells, but the precise regulatory mechanisms that are used by sRNAs to control Salmonella pathogenicity at the post-transcriptional level remain to be identified. It is anticipated that an improved understanding of sRNA biology will shed new light on the virulence of Salmonella.

Introduction

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a well-characterized enteropathogen which causes both gastroenteritis and serious systemic infections. In humans, salmonellosis is mainly contracted by the ingestion of contaminated food or water. The annual cost of Salmonella infection in the US is estimated to be US$3 billion.[1] Thus, Salmonella continues to have a big impact upon human life, and the control of this bacterium remains a significant challenge for the food industry.

The fact that Salmonella bacteria have been found in a number of different sites in the body during infection and at different stages of food processing reflects the ability of the microbes to thrive in many environmental conditions. Salmonella can sense its environment and rapidly adapt to changing conditions, a process which is mediated by regulation at the transcriptional, post-transcriptional and translational levels. The key players involved in this adaptation process are transcription factors and nucleoid-associated proteins, as well as the more recently identified regulatory small RNAs (sRNAs). Though the first evidence for the existence of bacterial sRNAs was reported in 1967,[2] most of the discoveries of bacterial sRNAs have only occurred in the last decade. The identification of sRNAs in Enterobacteriaceae initially focused on non-pathogenic strains of *Escherichia coli*.[3-6] The strategy for finding sRNAs involved bioinformatic screens that were validated by experimental approaches using transcriptomic tools such as tiling microarrays and high-throughput cDNA sequencing (RNA-seq).[7] To date, hundreds of sRNAs have been identified in bacteria, but roles in virulence have only been elucidated for a minority. RNAIII of *Staphylococcus aureus* was the first regulatory sRNA shown to be involved in bacterial pathogenicity by targeting at least five mRNAs that encode virulence factors.[8-10] Other examples of virulence-associated sRNAs have been described in a recent review.[11]

Here we survey the sRNAs identified in *S.* Typhimurium to date and discuss our current understanding of the role of sRNAs in the control of virulence. We then focus on the regulation of these sRNAs and their target mRNAs.

Small RNAs in bacteria. sRNAs are stable and abundant transcripts of about 50–500 nucleotides in length which are usually non-coding and exhibit a regulatory function. Post-transcriptional gene regulation by sRNAs may occur in different ways by base-pairing interaction with a target RNA resulting in different outcomes or by directly binding to proteins to modulate their function.[12-14] Two distinct classes of sRNAs have been identified: *trans*-encoded RNAs which are transcribed from intergenic regions of the genome, and *cis*-encoded RNAs which are encoded on the strand complementary to coding sequences or the 5' or 3' untranslated region (5' UTR, 3' UTR) of transcripts.[15-17] The family of *trans*-encoded sRNAs usually requires the chaperone Hfq to stabilize the often imperfect base-pairing interaction with target mRNA.[18] In contrast, *cis*-encoded sRNAs possess a region of perfect complementarity to their target mRNA and Hfq is not needed for target binding. It is now clear that sRNAs are involved in many key physiological processes including anaerobic growth, nutrient availability, iron homeostasis and the response to oxidative, envelope and osmotic stress.[9,19-23]

Insights from sRNA research in Salmonella. Much of the initial investigation of riboregulation by sRNAs involved non-pathogenic *E. coli* strains as a model. The more recent use of Salmonella as a model organism allows us to ask new questions about sRNAs involved in virulence in a variety of infection models, in the context of a well-established array of genetic tools. Next to *E. coli*, Salmonella is now the best-characterized model of sRNA-mediated regulation in Gram-negative bacteria. To

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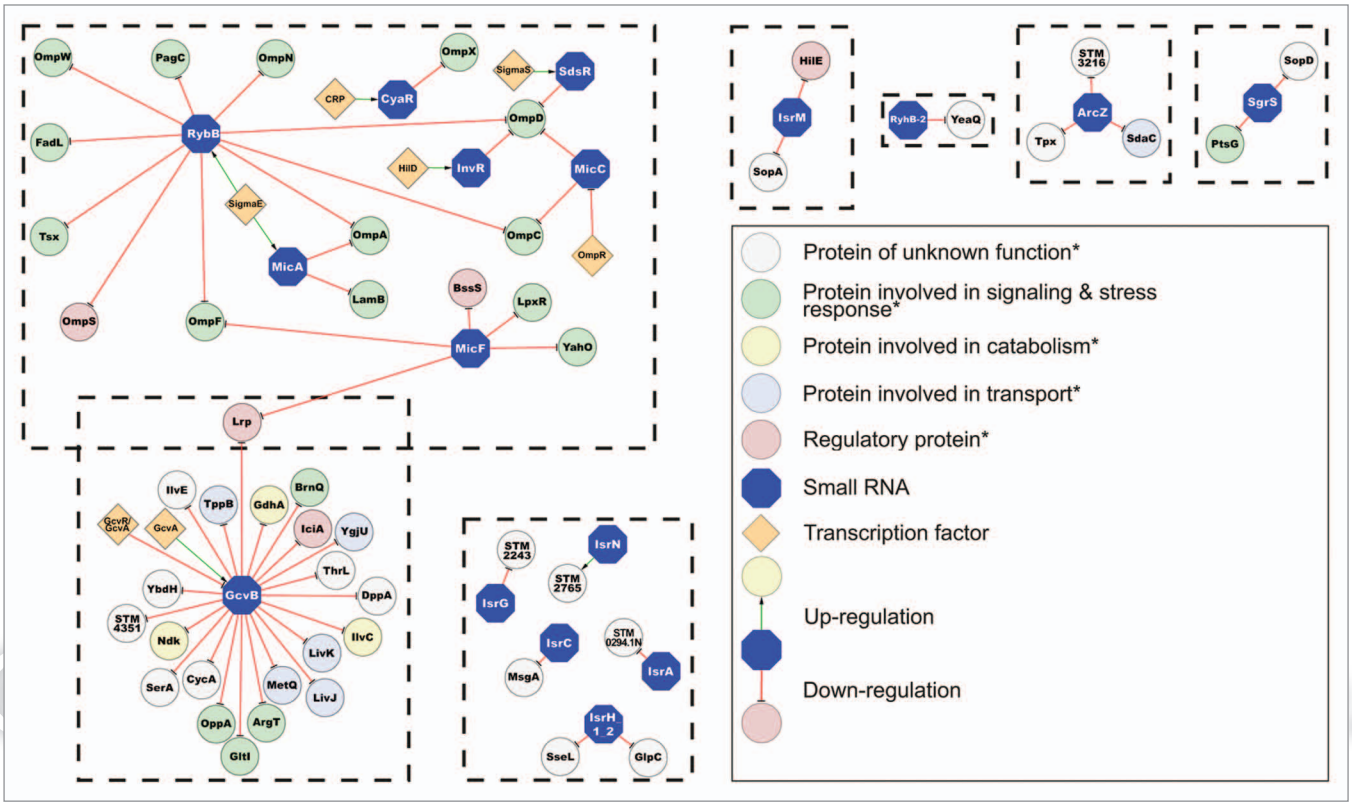

Figure 1. An overview of published small RNA regulatory networks in *S.* Typhimurium.[24,25,29,32,33,36,37,45,56,57,74,93-98]

date, the largest sRNA regulon has been identified in Salmonella, with the GcvB sRNA controlling expression of ~1% of the S. Typhimurium genome (**Fig. 1**).[24,25] These studies expand our view on the biological significance of sRNAs, establishing them as global gene regulators.

Key mechanistic findings from studies in Salmonella have advanced our basic understanding of sRNA-mediated regulation in bacteria. The distinct modular structure of sRNAs, including the highly conserved target binding region (also referred as to the "seed" region), was demonstrated for the σ^E-dependent sRNA RybB in Salmonella. Fusion of this seed region to an unrelated sRNA backbone permitted full repression of the RybB regulon.[26-28]

Current techniques for the discovery of sRNA targets. To complement the identification of new sRNAs, several methods allowing the discovery of sRNAs targets have been developed in Gram-negative bacteria. For *trans*-encoded sRNAs, the base-pairing interaction with mRNAs is imperfect and often requires Hfq. One example is the Hfq-associated sRNA MicC sRNA which silences S. Typhimurium *ompD* mRNA and only requires a ⩽ 12-bp RNA duplex within the CDS (codons 23–26) for repression.[29] Different bioinformatic tools allow the prediction of the binding regions of sRNA and mRNA by combining comparative genomics with a search for certain physical parameters. TargetRNA calculates optimal hybridization scores between an sRNA and all mRNAs in the genome.[30] IntaRNA is a method for the prediction of interactions between two RNAs based on minimization of an extended hybridization energy.[31] Although most of these software tools can confirm previously known findings, they should be considered as predictive tools that often produce false-positive results and require experimental validation.

As the interaction of *cis*-encoded sRNAs involves a perfect match, the identification of their targets is more straightforward. IsrA is a *cis*-encoded RNA present on the complementary strand to the STM0294.1 gene, which encodes a protein with no clear functional annotation. Padalon-Brauch et al. have shown that IsrA is expressed during exponential phase, osmotic stress, peroxide stress and cold shock, and downregulated during stationary phase (**Fig. 2**). The expression of STM0294.1 shows the opposite pattern to that of IsrA, and so it has been suggested that IsrA could regulate transcription of STM0294.1.[32]

Pulse-expression of sRNAs has been developed as an efficient method to identify mRNA targets, because classical genetic approaches often result in quite subtle phenotypes for sRNA mutants.[33,34] The technique involves the rapid overproduction of an sRNA followed by the use of a microarray to identify bacterial transcripts that were bound by the sRNA and subsequently degraded by an RNaseE-dependent mechanism.[33] This approach has been used to elucidate large regulons of as many as 50 genes controlled by a single sRNA (**Fig. 1**).

The regulatory interaction between sRNAs and candidate mRNA targets must be confirmed within living bacterial cells, and can be done with a GFP-based two-plasmid reporter system. The ablation of GFP fluorescence by expression of the sRNA confirms the direct effect of an sRNA upon its mRNA target.

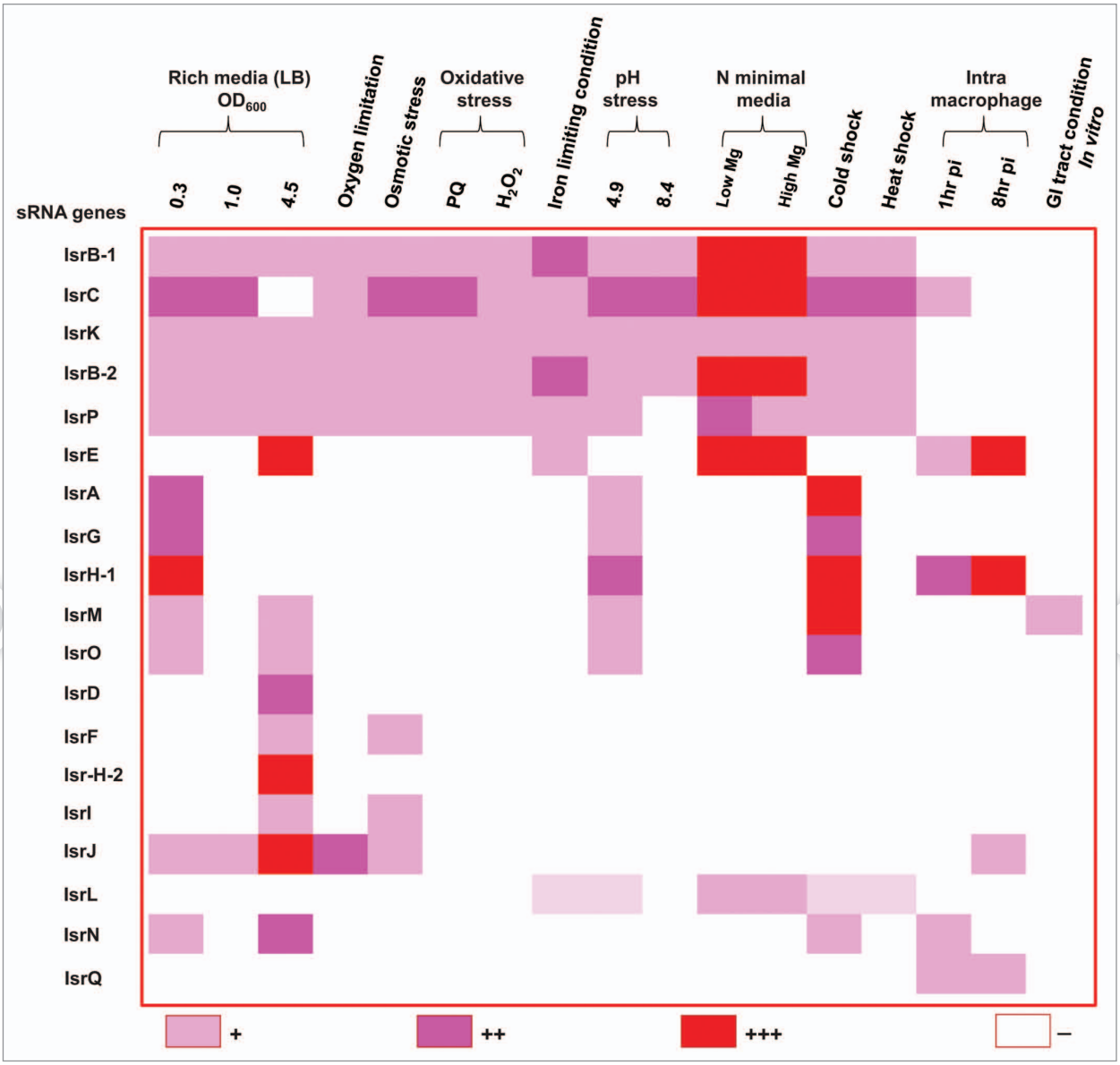

Figure 2. Differential expression of S. Typhimurium island-encoded sRNAs during growth under various stress conditions determined by northern blot analysis[32] or RT-PCR.[57] Expression levels are shown as high, medium, low and no expression.[32] Cultures of S. Typhimurium were grown under different conditions (described from left-hand side): Cells grown in LB to an OD_{600} of 0.3, 1 and 4.5; Oxygen limitation—overnight growth without agitation in 50 mL Falcon tubes to an OD_{600} of 0.9; Osmotic stress—cells grown in LB containing elevated (0.5 M) NaCl levels for 30 min; Oxidative stress using 0.2 mM paraquat (PQ) and 1 mM hydrogen peroxide (H_2O_2); Iron limiting conditions—addition of 0.2 mM 2,2′ dipyridyl; pH stress – LB at pH 4.9 (adjusted with HCl) and LBK media at pH 8.4; N min low Mg and N min high Mg – N minimal media with 10 μM $MgCl_2$ (low magnesium) and 10 mM $MgCl_2$ (high magnesium); Cold shock at 15°C and heat shock at 42°C; Intra-macrophage 1h and 8h – within activated J774.A1 macrophage cells assayed using gentamycin protection assay;[32] In vitro conditions resembling the gastrointestinal tract.[57]

This is measured accurately by flow cytometry, on agar plates or by determining the GFP protein levels by western blotting.[35]

The attribution of the role of a particular sRNA can be complicated by functional redundancy as it is well known that several sRNAs can silence the same target mRNA. For example, MicC, RybB, InvR and SdsR all negatively regulate the *ompD* gene (**Fig. 1**).[26,29,36,37] In this case, the effect of deleting one sRNA could be masked by the action of the remaining three sRNAs, and it may be necessary to delete all the sRNAs regulating a particular pathway to observe a clear phenotype. Another example of functional redundancy is the Csr system which modulates carbon metabolism and also regulates SPI1 and SPI2 expression through HilD.[38] It comprises two sRNAs, CsrB and CsrC and the RNA chaperone CsrA.[39,40] Although the single mutants Δ*csrB* or Δ*csrC* are not impaired in their ability to infect epithelial cells, a Δ*csrB* Δ*csrC* mutant shows a significant invasion defect. While complementation with either CsrB or CsrC leads only to partial restoration of wild-type levels of invasion, the presence of both

Table 1A. Thirty four *S*. Typhimurium sRNAs that are not required for murine virulence*

sRNA name (Alternative name)	First reported in	Upstream gene	Downstream gene	Relevant references
ArcZ (SraH, RyhA)	*E. coli*	*yhbL*	*acrB*	74, 99
CsrB	*E. coli*	*yqcC*	*syd*	100, 40
CyaR (RyeE)	*E. coli*	*yegQ*	SL2113	93
DsrA	*E. coli*	*yodD*	*yedP*	71, 75
GcvB (IS145)	*E. coli*	*gcvA*	*ygdI*	101, 24, 25
GlmY (SroF, tke1)	*E. coli*	*yfhK*	*purG*	102, 103
GlmZ (SraJ, k19, RyiA)	*E. coli*	yifK	*hemY*	102, 103
InvR (STnc270)	*Salmonella*	*invH*	SL2880	37
IsrB-1 (IS092)	*Salmonella*	SL0946	SL0947	32
IsrC (IS102)	*Salmonella*	*envF*	*msgA*	32
IsrE (RyhB-2, RfrB)	*Salmonella*	SL1208	*yeaQ*	32
MicA (SraD)	*E. coli*	*luxS*	*gshA*	104, 33, 96, 105
MicC (IS063, tke8)	*E. coli*	*nifJ*	*ynaF*	29
MicF	*E. coli*	*ompC*	*yojN*	106, 107, 95
MicM (RybC, ChiX, SroB)	*E. coli*	*ybaK*	*ybaP*	63, 108
MntS (RybA)	*E. coli*	*ybiP*	*mntR*	5
OmrA (RygB)	*E. coli*	*aas*	*galR*	27
OmrB (t59, RygA, SraE)	*E. coli*	*aas*	*galR*	27
RprA (ISO83)	*E. coli*	*ydiK*	*ydiL*	72, 75
RybB (p25)	*E. coli*	SL0845	SL0846	5, 33, 109, 28, 26
RydB (tpe7, IS082)	*E. coli*	*ydiH*	SL1302	5
RydC (IS067)	*E. coli*	SL1568	*cybB*	4, 110
SdsR (RyeB, tpke79)	*E. coli*	SL1806	SL1807	111, 36
RyfA (tp1, PAIR3)	*E. coli*	SL2496	*sseB*	5
RyhB (RyhB-1, SraI, IS176, RfrA)	*E. coli*	*yhhX*	*yhhY*	5
SgrS (RyaA)	*E. coli*	*yobN*	*leuD*	112, 94, 45
SibC (t27, RygC, QUAD1c)	*E. coli*	*ygfA*	*serA*	113,114
SibD (tp8, RygD, C0730)	*E. coli*	*yqiK*	*rfaE*	113,114
Spot42 (spf)	*E. coli*	*polA*	*yihA*	115,116
SraA (psrA/t15)	*E. coli*	*clpX*	*lon*	3
SraB (pke2)	*E. coli*	SL1126	*yceD*	3
SraF (tpk1, IS160, PRE-element)	*E. coli*	*yceD*	*ygjT*	3, 117
SraL (RyjA)	*E. coli*	*soxR*	SL4203	3
SroC	*E. coli*	*gltJ*	*gltI*	63

Target mRNAs of some sRNAs are shown in **Figure 1**. *See ref. 61 for details of virulence experiments.

sRNAs expressed *in trans* is necessary to rescue the invasion defect, illustrating the difficulties in assigning virulence-associated functions to sRNAs.

Conservation of small RNAs between Salmonella and *E. coli*. The last decade witnessed increasing numbers of sRNAs being discovered in *E. coli* and Argaman et al. reported that about 24 sRNAs were conserved between Salmonella and *E. coli*.[3,41] This important finding prompted the use of conservation analysis to discover new sRNAs in *E. coli*.[5] The advent of bacterial whole genome sequencing and the use of RNA-seq led

Table 1B. Five sRNAs involved in virulence of *S.* Typhimurium

sRNA name	Target mRNA	Role in virulence	References
IsrJ		Control of effector protein production	32
IsrM	*hilE*, *sopA*	Modulates the expression of SPI1 proteins via *hilE*; downregulates SopA	57
IstR	*tisAB*	SOS induced toxic peptide – Inhibits growth allowing DNA repair	62,61
OxyS	Regulates about 40 genes; including *rpoS*	Inhibits alternate stress adaptation pathways during oxidative stress	20,32,61
SroA	Riboswitch element of the *thiBPQ* operon	Putative import of Thiamine and Thiamine pyrophosphate	61

to the discovery of the widespread nature of sRNAs, many of which were found to be highly conserved in intergenic regions in bacteria. Approximately 400 sRNAs have now been predicted in about 70 microbial genomes, including those of the *Escherichia*, *Shigella* and Salmonella genera,[42] and comparative analyses of the genomes of different Salmonella serovars and *E. coli* have shown the levels of sRNA conservation. Recent studies have reported similar levels (48–67%) of conservation among Salmonella and *E. coli* species.[43,44] The large number of non-conserved small RNAs suggests that species-specific sRNAs could have specialized roles in pathogenicity. However, even highly conserved sRNAs were shown to regulate species-specific virulence factors as demonstrated by Papenfort et al.[45] In this study, the Salmonella-specific effector protein SopD was shown to be regulated by the ancestral sRNA SgrS, which is found in both pathogenic and non-pathogenic species.

Expression profiles of S. Typhimurium sRNAs and their role during infection. S. Typhimurium and *E. coli* diverged from a common ancestor about 100–130 million years ago and share about 71% of their genetic information.[46-48] *S.* Typhimurium possesses a unique set of attributes that allow it to survive in the hostile environments associated with each stage of animal infection, and to colonize different intracellular niches within mammalian cells. For instance, once ingested, this bacterium must first cope with an increase in temperature followed by the acidic environment of the stomach. In the intestine, the microorganism is subjected to increased osmolarity, a decrease in oxygen tension, bile and competition with the intestinal microbiota.[49] *S*almonella can subsequently enter and proliferate within non-phagocytic and phagocytic cells, where the pathogen resists intracellular defense mechanisms such as antimicrobial peptides, the acidification of the Salmonella-containing vacuole (SCV) and the production of reactive oxygen and nitrogen species. In response to these stressful conditions, S. Typhimurium must quickly modulate its transcriptional profile, raising the possibility that the rapid gene regulation mediated by sRNAs would be particularly relevant.[50] Monitoring sRNA expression could reveal patterns of induction relevant to the strategies used by Salmonella to survive within host cells.

An sRNA involved in Salmonella virulence was first reported in the year 2000.[51] Deletion of the bi-functional transfer-mRNA (tmRNA), which rescues ribosomes stalled on defective mRNAs (reviewed in ref. 52), resulted in an avirulent Salmonella mutant in mouse infections. Binding of tmRNA to stalled ribosomes requires the small protein SmpB which has been shown to be important for proliferation of Salmonella in macrophages.[53,54]

In an attempt to find *S.* Typhimurium specific sRNAs that had not already been characterized in *E. coli*, the Altuvia lab used a computational approach to identify and validate 19 new sRNAs located in intergenic regions of the Salmonella pathogenicity islands (SPIs).[32] The sRNA expression was monitored by northern blot analysis both in media mimicking infection-relevant stress conditions and directly inside macrophages. Many of the island-encoded sRNAs were induced in conditions including stationary phase growth, in minimal medium, upon temperature shock, acidity and oxidative stress (**Fig. 2**). In macrophages, expression of IsrC and IsrN was induced early during infection and then decreased as the infection progressed, similar to the results shown earlier for OxyS in *E. coli*.[20,55] Conversely, IsrE (RyhB2), RyhB1 and IsrH showed increased levels of expression later during infection. The differential expression patterns suggest a role for sRNAs at different stages of infection. In contrast, IsrH has recently been shown to be downregulated during infection of fibroblasts, in which wild-type Salmonella is non-replicative.[56]

Two of the island-encoded sRNAs, IsrJ and IsrM, were found to be particularly important for Salmonella proliferation within non-phagocytic cells and/or macrophages.[32,57] IsrJ is upregulated under conditions which promote invasion of epithelial cells and is positively regulated by HilA, the central transcriptional activator of SPI1. The deletion of *isrJ* results in a less invasive mutant strain that is impaired for translocation of the effector protein SptP, which is required for remodelling the host cell cytoskeleton after bacterial entry.[32,58]

The Δ*isrM* mutant showed a broad virulence defect, with reduced invasion of epithelial cells, lower intracellular replication/survival in macrophages, and reduced growth in the ileum and spleen of mice.[57] IsrM post-transcriptionally represses the expression of virulence factors *hilE* and *sopA*. Most SPI1 genes are negatively regulated by HilE through sequestration of HilD, the major transcriptional activator of SPI1, while SopA is a secreted effector protein that is involved in causing inflammation and diarrhea.[59,60] IsrM therefore aids in choreographing the expression of virulence factors.

Another study to identify sRNAs required for *S.* Typhimurium virulence focused on 37 sRNAs that are conserved in both *E. coli* and S. Typhimurium. Single small RNA deletion mutants were tested by competitive index in the murine infection model. A key finding of this study was that 34 of the tested 37 sRNAs did not play a role in Salmonella virulence (**Table 1A**).[61] Only two sRNA mutants, Δ*sroA* and Δ*istR* gave a reproducible attenuated phenotype in mice, with a reduced ability to compete with the wild-type strain (**Table 1B**). The IstR sRNA, which was originally identified in *E. coli* by the Altuvia lab in 2004, inhibits the synthesis of an SOS-induced toxic peptide.[62] The SroA RNA is assumed to result from attenuated transcription of a riboswitch element of the *thiBPQ* mRNA that codes for proteins involved in thiamine uptake,[63,64] but its function remains unclear. In contrast, one strain lacking the OxyS sRNA was shown to be hypervirulent. OxyS, a member of the OxyR regulon, is upregulated by micromolar levels of peroxide and coordinates the cellular response to oxidative stress.[65]

AmgR is a 1.2 Kb antisense transcript encoded on the complementary strand to the *mgtCBR* operon. The *mgtC* gene encodes a protein necessary for Salmonella to survive within macrophages, to grow in low Mg^{2+} environments and for virulence in mice.[66] PhoQ, the kinase in the PhoPQ two component regulatory system, senses low levels of Mg^{2+} and the response regulator PhoP induces transcription of the *mgtCBR* operon. AmgR regulates expression of the *mgtCBR* operon by de-stabilizing the *mgtC* and *mgtB* transcripts in an RNaseE-dependent manner. An *amgR* mutant strain was found to be more virulent than the wild-type strain in mice. AmgR is PhoP-dependent and PhoP directly binds the *amgR* promoter, leading to amgR expression in low Mg^{2+} conditions. Therefore, PhoP has an apparently paradoxical effect on *mgtC* expression as it directly activates both *mgtC* and *amgR*, but AmgR has a repressive effect on *mgtC*. This regulatory mechanism may have evolved to titrate the levels of MgtC expressed at appropriate times during infection.[67]

The published roles of sRNAs in the virulence of S. Typhimurium are summarized in **Tables 1A and 1B**, and it is likely that the list of sRNAs that are required for infection will increase in the future. The expression profiles derived from northern blot and RT-PCR analyses of 19 island-encoded sRNAs are shown in **Figure 2**, and it is apparent that the levels of sRNAs vary in different environmental conditions. The recent profiling of 13 sRNAs during infection of fibroblasts showed that the levels of sRNAs varied during an infection time-course.[56] The levels of regulatory sRNAs within bacterial cells are likely to give clues to their function, and so expression profiling should be a useful discovery tool in the future.

RpoS and Salmonella virulence. The alternative sigma factor RpoS (σ^{38}) plays a key role in Salmonella infection and is required for full virulence of S. Typhimurium.[68,69] Specifically, RpoS is important for persistence in lymphoid organs, such as the spleen and liver, and for the initial stages of infection in murine Peyer's patches.[69] RpoS also activates the plasmid-borne *spvR* and *spvABCD* genes, which are required for intracellular growth and systemic infection in mice and humans.[70]

In *E. coli*, the translation of RpoS is repressed by OxyS[68] and the sigma factor is positively regulated by 3 Hfq-dependent sRNAs, namely DsrA, ArcZ and RprA, which act by relieving the inhibitory secondary structure that prevents *rpoS* translation.[71-73] This type of regulation is conserved, but is less pronounced in Salmonella, questioning the significance of DsrA, ArcZ and RprA for Salmonella virulence.[74,75] Further study of the function of the RpoS sigma factor in *S.* Typhimurium is required, and may lead to the identification of more links with sRNA biology.

Hfq as a mediator of sRNA regulation. The Hfq protein is a key player in the global post-transcriptional regulatory network that facilitates the interactions of Salmonella sRNAs with target mRNAs.[18] Deletion of *hfq* in Salmonella gives rise to a non-motile strain which is highly attenuated in its ability to infect mice, invade epithelial cells, secrete virulence factors and to survive and proliferate within macrophages. These significant phenotypes suggest that Hfq interacts with a number of sRNAs which are involved in virulence.[54,76,77] As most *trans*-acting sRNAs depend upon Hfq to stabilize their binding to target mRNAs, the chaperone can facilitate the binding of an sRNA to its target mRNA and thereby prevent translation or induce target degradation. Hfq can also bring about positive regulation by recruiting an sRNA to its target binding site and thereby de-stabilizing secondary structures which inhibit target translation.[78,79] Additionally, Hfq can regulate sRNA levels independently from their mRNA targets by protecting sRNAs from endonucleolytic decay.[80] There are several suggestions as to how Hfq regulates sRNAs by controlling the base-pairing interaction between the sRNA and its target mRNA. The protein may act as a catalyst which increases the rate of complex formation between the *trans*-acting sRNA and an mRNA to stabilize the imperfect base-pairing between the two RNAs, as duplex formation in the absence of Hfq is relatively poor.[81,82]

In *E. coli*, Hfq has been demonstrated to interact with other RNA-associated proteins, such as PNPase, an exoribonuclease, and PAP, a poly(A) polymerase which may add an additional level of sRNA regulation by Hfq.[83] Recently, it was also suggested that Hfq plays a role in transcription termination in *E. coli* by associating with the transcription termination factor Rho.[84] Furthermore, limiting concentrations of Hfq can regulate sRNAs as the abundance of Hfq per cell remains fairly constant while the amount of its target sRNAs can increase under certain conditions.[85] Sequestration of Hfq can therefore serve to modulate sRNA function by creating competition for binding between different sRNAs. This was demonstrated in *E. coli* when overexpression of one sRNA led to a decrease in the accumulation of other sRNAs, as Hfq protein levels become limiting.[86] This method of regulation by Hfq was also suggested by the transcriptomic profile of a strain overexpressing ArcZ which showed some similarities to that of an Δ*hfq* mutant, indicating that an individual sRNA may displace other sRNAs from Hfq.[74]

Future prospects: Next-Generation Sequencing. RNA-seq is now the tool of choice for the discovery of novel small RNAs in bacteria.[77,87,88] The rapid reduction in the costs of RNA-seq will lead to increasing numbers of new sRNAs being identified in the near future. The addition of small RNA genes to existing genome

annotations will help to shed light on the complex nature of the bacterial transcriptome.

The recent publication of the transcriptional landscape of *S.* Typhimurium represents an important advance. An RNA-seq-based approach was used to identify the major transcriptional start sites and to define the motif of σ^{70}-dependent promoters.[44] About 140 sRNAs were found to be expressed at one stage of growth. The fact that 60 novel sRNAs were discovered in a single set of experiments suggests that next-generation sequencing-based methods will make a big impact upon the RNA world.

In future, technical advances promise to extend the applicability of RNA-seq for the monitoring of transcriptional changes in complex environments or from the very low (femtomolar) amounts of RNA obtained from infected animals.[89,90] Another challenge will be to simplify the identification of mRNA targets. Until now, pulse overexpression of a small RNA and subsequent monitoring of transcript levels using a microarray has been widely used to identify many mRNA targets,[91] but this is a labor-intensive approach. Sequence-based target prediction tools are available on the web, and as they become more effective they will be a valuable and cost-effective alternative to experimental approaches.[92]

Although many un-answered questions remain about the precise role of sRNAs during the infection process, it is likely that the burgeoning field of sRNA biology will have a great impact on our understanding of *Salmonella* pathogenicity.

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