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Small RNAs in major foodborne pathogens: from novel regulatory activities to future applications

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Abstract

Small regulatory RNAs (sRNAs) are involved in post-transcriptional control of important cellular processes and contribute to the success of a pathogen. Here, we use studies primarily selected from *Salmonella enterica* and *Listeria monocytogenes* to illustrate the current status of sRNA biology in important foodborne pathogens. We discuss how the regulatory activities of sRNAs can be affected by base pairing RNAs known as “sponge RNAs”, or by RNA-binding proteins, such as the newly discovered sRNA chaperone ProQ. Furthermore, we highlight recent findings for sRNAs with regulatory roles during infection, some of which are present in multiple copies, designated “sibling sRNAs”. Importantly, knowledge on sRNA-mediated regulation can be exploited for biotechnological applications, such as in generating gene knockdowns to promote desired traits.
Introduction

Non-coding RNAs serve as regulators of gene expression in bacteria, most often through interactions with other RNA molecules, and influence important cellular processes such as metabolism, stress responses and virulence [1]. In the major foodborne pathogens *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes*, one class of non-coding RNAs, the small regulatory RNAs (sRNAs), has been studied intensively, predominantly in the context of bacterial infection, and several examples of sRNAs that control the expression of virulence genes at the post-transcriptional level are known [2]. Here, we review the latest discoveries in sRNA biology in important foodborne pathogens, with special emphasis on regulatory functions of sRNAs that aid adaptation to host-specific niches. Additionally, we highlight regulatory mechanisms employed by sRNAs, as well as accessory interacting factors, which hold potential for use in synthetic biology and other areas of biotechnology.

**Hfq-binding sRNAs – recent discoveries**

The largest group of sRNAs in bacteria acts by direct base pairing to specific mRNAs, leading to either inhibition or enhancement of protein expression [1]. *Cis*-acting sRNAs form fully complementary interactions with their target mRNAs, whereas *trans*-acting sRNAs are only partly complementary to their partner mRNAs. In Gram-negative bacteria, the interaction between a *trans*-acting sRNA and its targets often relies on an RNA chaperone, such as Hfq, which promotes sRNA-mRNA duplex formation, whereas in Gram-positive bacteria, a role for Hfq in sRNA-mediated control is less clear [3]. Yet, Hfq contributes to stress tolerance and virulence in both Gram-positive *L. monocytogenes* [4] and Gram-negative *Salmonella* [5]. In early studies, the RNA-binding property of Hfq was successfully used
as means to identify sRNAs, such as LhrA in *L. monocytogenes* and GcvB in *Salmonella* [6,7]. Although LhrA and GcvB differ with respect to origin, size and nucleotide sequence, they both rely on Hfq for stability and regulatory activity [8,9]. During growth in rich medium, LhrA accumulates upon entry into stationary growth phase and affects the expression of nearly 300 genes, half of which belong to the regulon of the general stress sigma factor, σ^B^ [10]. GcvB, on the other hand, is mainly expressed during exponential growth in rich medium and its regulon is highly enriched with genes encoding amino acid- and peptide transporters and amino acid biosynthesis proteins [11]. Both sRNAs use specific seed sequences to pair with complementary sites within the 5´-untranslated region (5´ UTR) of their target mRNAs (Figure 1a and 1b). Intriguingly, recent findings demonstrate that sRNAs themselves are targets of regulation by other transcripts acting as “RNA sponges” or “anti-sRNAs”. One example from *Salmonella* involves a small Hfq-binding RNA, SroC, that derives from processing of the *gltIJKL* mRNA and antagonizes the activity of GcvB by direct base pairing (Figure 2) [12•]. The *gltIJKL* mRNA itself is a target of GcvB, thus, SroC and GcvB together form a feed-forward loop that increases the expression of *gltIJKL*, and moreover, de-represses other targets in the GcvB regulon [12•]. A search for Hfq-binding sRNAs in the enterohemorrhagic *Escherichia coli* (EHEC) transcriptome identified the anti-sRNA AgvB, which is encoded from a bacteriophage-derived region and targets an EHEC sRNA homologous to GcvB [13•]. In an Hfq-dependent manner, AgvB base pairs with the seed region of GcvB, blocking the interactions between GcvB and its target mRNAs [13•]. Interestingly, a growth experiment in bovine terminal rectal mucus, mimicking the preferred colonization site of EHEC in cattle, showed that a strain lacking the anti-sRNA had reduced competitive fitness compared to wild-type, confirming that AgvB is important for niche adaptation in EHEC [13•]. Transcripts acting to
regulate the stability and/or activity of sRNAs are likely to be discovered in other foodborne pathogens as well; indeed, several pairs of interacting sRNAs have been predicted in *L. monocytogenes* [14], however, the functional relevance (if any) of such sRNA-sRNA interactions remains to be investigated.

**Expanding the family of global sRNA chaperones**

With the technological advance in large-scale transcriptomics, the number of published sRNA candidates has increased tremendously, now counting several hundred in both *L. monocytogenes* and *Salmonella* [14-17]. The fast discovery of sRNAs greatly encouraged the recent development of novel approaches to accelerate their functional characterization [18•-20]. One of these methods, the Grad-seq approach, enabled the identification of a novel class of structured sRNAs in *Salmonella* and the RNA-binding protein, ProQ, associated with them [21••]. The ProQ protein, which is absent in Firmicutes, holds a lot in common with Hfq [22,23]. Like Hfq, ProQ has a profound effect on global gene expression and acts to stabilize most of its sRNA partners [21••,22]. The first example of ProQ-dependent sRNA-mediated control of gene expression was recently described in *Salmonella* [24•]. The sRNA RaiZ base pairs in *trans* with the ribosome-binding site (RBS) of the *hupA* mRNA encoding a histone-like protein, HU-α. Binding of ProQ to RaiZ stabilizes the sRNA, however, ProQ also assists RaiZ in preventing ribosome loading on *hupA* mRNA [21••,24•]. Collectively, these findings position ProQ as the second global sRNA chaperone in *Salmonella* and likely other bacteria [18•,21••-24•]. Curiously, RaiZ also interacts with Hfq, but this interaction has no effect on sRNA stability or RaiZ-mediated control of *hupA* [24•]. Whether RaiZ acts to control a subset of targets in an Hfq-dependent manner still remains a possibility. Notably, similar findings were done for members of the LhrC sRNA
family in *L. monocytogenes*: although originally identified as Hfq-binding sRNAs [7], all target genes characterized so far are regulated by LhrC in an Hfq-independent manner [25•,26], suggesting that another RNA chaperone could be involved. Remarkably, a novel sRNA-binding protein, SpoVG, was recently identified in *L. monocytogenes* by a genetic approach [27•]. This protein interacts with sRNAs *in vitro* [27•], including Rli31, which contributes to lysozyme resistance and pathogenesis in mice [28], and importantly, deletion of *spoVG* was found to rescue the phenotypes of an *rli31* mutant [27•]. In general, mutations in *spoVG* cause diverse phenotypes, indicating a possible role as a global gene regulator in *L. monocytogenes*, but it still remains to be shown if SpoVG is required for sRNA-mediated regulation [27•]. Adapting methods like Grad-seq to *L. monocytogenes* and other foodborne pathogens will potentially accelerate the identification of novel RNA-binding proteins and their interacting sRNAs in these organisms.

**Sibling sRNAs with regulatory roles during infection**

A number of studies in *L. monocytogenes* and *Salmonella* have analyzed the expression of sRNA candidates during intracellular replication or under infection-relevant conditions [14-17,29•,30]. Both *L. monocytogenes* and *Salmonella* are able to invade and replicate inside a broad range of cell types, however, while *L. monocytogenes* escapes the vacuole to replicate in the host cytosol, *Salmonella* modifies the host compartment to allow its survival and replication within the vacuole [31]. Inside several cell lines, the sibling sRNAs RyhB-1 and RyhB-2 are among the most highly induced sRNAs in *Salmonella* [17,30,32••,33] and have been shown to contribute to the replication of *Salmonella enterica* serovar Typhi inside macrophages [34]. Their exact intracellular function is yet to be elucidated, but extracellular studies have implicated RyhB-1 and -2 in growth cessation during iron
starvation and in survival under acid shock, oxidative or nitrosative stress [30,35-37]. As similar stress conditions are encountered inside macrophages, RyhB-1 and -2 may contribute to intracellular survival by aiding the adaptation to these hostile environmental conditions. The *E. coli* RyhB homologue contributes to iron homeostasis when iron is limiting by repressing the translation of several non-essential, iron-containing proteins and enhancing the expression of siderophore synthesis [38]. Several lines of evidence indicate that RyhB-1 and -2 function in a similar manner in *Salmonella* [35,39,40], still, direct base pairing has only been confirmed for the *iroN* mRNA target, encoding a receptor for the Fe³⁺-bound siderophore salmochelin, which is positively regulated by the RyhB siblings [41•]. Typically, sRNA-mediated translational activation involves a structural change in the 5’ UTR, which renders the RBS accessible, and thus facilitates translation initiation [42], as exemplified by the intracellularly induced sRNA Rli27 in *L. monocytogenes* (Figure 1c) [15,29•]. Interestingly however, RyhB-1 and -2 activate *iroN* translation via a novel mechanism, where sRNA-mRNA base pairing increases the accessibility to a second RBS close to the 5’ terminus of *iroN* mRNA (Figure 1d) [41•]. The RyhB siblings share a sequence with perfect homology covering 1/3 of their length [30], which includes the sequence known to base pair with the *iroN* mRNA. However, despite their equal base pairing potential, RyhB-1 was found to have a larger impact on *iroN* expression than RyhB-2, which was ascribed to lower expression of RyhB-2 under the experimental conditions [41•]. In fact, transcriptional control of the siblings varies, which, together with potential base pairing interactions outside the shared sequence, may underlie their partly overlapping regulons and different phenotypic contributions (Figure 3a) [30,34,36,37,43].
Sibling sRNAs with regulatory roles under infection-relevant conditions have also been described in *L. monocytogenes*. The LhrC family of sRNAs includes the highly homologous LhrC1-5 [7] as well as Rli22 and Rli33-1, which are both structurally and functionally related to the LhrCs, but present lower homology [44]. The seven siblings are expressed from individual promoters, of which *rli22* and *lhrC1-5* are positively regulated by the two-component system LisRK that responds to cell envelope stress [25*,44], whereas σ^B^ controls the expression of *rli33-1* (Figure 3b) [15,44]. All seven sRNAs are induced upon exposure to whole human blood [14], and six of them are highly expressed during intracellular replication in macrophages [15]. Strikingly, Rli22 was the only member found to be expressed when *L. monocytogenes* resides in the intestinal lumen of mice [14], pointing out that additional factor(s) may contribute to the expression of this sRNA [44]. The differential expression of sRNA siblings may enable the bacterium to integrate numerous stimuli into the control of the LhrC regulon, and further suggests that siblings could possess unique regulatory functions under the conditions where they are specifically produced. To date, three mRNA targets, all negatively regulated by the LhrCs, have been identified: *lapB*, encoding a cell wall anchored virulence adhesin; *oppA*, encoding a substrate-binding protein of an oligopeptide transporter; and *tcsA*, encoding a CD4^+^ T cell-stimulating antigen [25*,26]. Initial base pairing to the 5’-end of these target mRNAs occurs via CU-rich motifs in the LhrC siblings. Each sRNA has 2-3 motifs, further adding to the multiplicity and complexity of target regulation by the LhrC family (Figure 3b) [25*,26,44].

What is the role of LhrC regulation during infection? Presently, there is no simple answer to this question, but it should be noted that the seven siblings act as repressors of cell envelope-associated proteins with virulence functions. Since surface proteins are recognized by the immune system, the
regulatory action by the LhrCs may be seen as an attempt to evade immune detection. In addition, LhrC1-5 and Rli33-1 contribute to infection of macrophages [15,26], suggesting a role for these sRNAs in the intracellular environment. Curiously, the LhrC regulon comprises genes encoding amino acid and oligopeptide transporters, and branched chain amino acid biosynthesis proteins, indicating that upon phagocytosis, a transient decrease in the uptake and biosynthesis of amino acids may be beneficial to *L. monocytogenes* [26]. Notably, recent studies demonstrated that a decrease in branched chain amino acid availability leads to an increase in virulence gene expression [45,46]. Hence, the LhrC family may well be part of the complex gene regulatory network that controls the immune evasion and virulence strategies of *L. monocytogenes* in response to various host-based cues.

**When traditional assays fall short: unraveling sRNA regulation during infection by dual RNA-seq**

Despite the wealth of information on sRNA candidates and their expression patterns, relating these data to a functional role of sRNAs during infection is not completely straightforward. For instance, despite robust induction of the sRNA PinT in intracellular *Salmonella* [17,32••], disruption of *pinT* has no significant effect in cell culture, mice or chicken models of infection [32••,47]. Still, *pinT* contributes to the fitness of *Salmonella* during colonization of cattle and pigs [47], underlining the importance of choosing a suitable animal model and, furthermore, confirming that PinT actually contributes to pathogenesis in some niches. Recently, the role of PinT during intracellular infection was unraveled in an impressive dual RNA-seq study, which simultaneously mapped the transcriptome of both *Salmonella* and the epithelial host cell over the course of infection [18*,32••]. The authors showed that PinT, through base pairing with several target genes, contributes to fine-tuning of the transition between two major virulence programs required for *Salmonella* to switch from invasion to
intracellular replication [32••]. Most fascinating, the expression of PinT also has a large impact on the host transcriptome resulting in modulation of the host immune response, which facilitates the establishment of an intracellular niche that supports survival and replication of Salmonella [32••]. Dual RNA-seq analysis of host-pathogen interactions is a powerful approach to elucidate the biological roles of gene products during infection, which may not have a phenotype in “standard” infection assays [18*,48*]. By looking at the bacterium and host cell simultaneously, the technique offers unprecedented insight into how both organisms are affected by the expression of an sRNA, and how the sRNA contributes to the fitness of the pathogen.

**Biotechnological applications of “basic knowledge” on bacterial sRNAs**

sRNAs are not limited to pathogenic species and numerous candidates are indeed found in biotechnologically relevant species such as Clostridium acetobutylicum [49] and Lactococcus lactis [50], in which they may be involved in controlling stress responses and metabolism. Additionally, sRNAs may also be considered a great biotechnological toolbox in synthetic biology. For instance, base pairing antisense RNAs have successfully been employed to knockdown selected genes in organisms such as the wine-associated lactic acid bacterium Oenococcus oeni [51] and the butanol-producing C. acetobutylicum [52], which are difficult to genetically engineer. Under some conditions, it might even be advantageous to knockdown, rather than knockout, gene expression; for example, a double knockdown strategy was used to screen 18 E. coli strains for the influence of genetic background on phenol production and tolerance [53], a task that would have been very time-consuming using knockouts. Moreover, antisense knockdown allows for transient target regulation by controlling the sRNA expression, or partial target knockdown by controlling the strength of interaction
between an sRNA and its target. The latter naturally occurs in *E. coli* where RyhB partially represses *cysE*, encoding an essential serine acetyltransferase, thereby redirecting serine flux into siderophore synthesis when iron is limiting [54]. Compared to protein-based regulation of pathway fluxes, RNA-based regulation may have a faster recovery time [55]. Besides, when considering base pairing sRNAs, the relationship between sequence and function is often more predictable. Based on knowledge about sRNA regulation in model bacteria such as *E. coli*, Cho and Lee [52] rationally designed a synthetic sRNA based on the scaffold of an *E. coli* sRNA to knockdown translation of a target gene in *C. acetobutylicum* as a proof-of-concept. Even synthetic sRNAs targeting multiple mRNAs simultaneously are being developed to coordinate the expression of key enzymes in a pathway [56]. Thus, studying the diverse regulatory mechanisms employed by sRNAs and the multiple contributing factors in different species, lays the foundation for future utilization of RNA-based regulation in biotechnological processes.

**Conclusions**

Most of our current knowledge on sRNAs in foodborne pathogens derives from studies in *L. monocytogenes* and *Salmonella*, aiming to explore the importance of sRNAs during infection. Indeed, several examples of sRNAs with roles in virulence control have been uncovered, some of which are highlighted in this review. In future studies, we suggest that functional analyses of sRNAs are extended to include conditions encountered by foodborne pathogens in the external environment (e.g. in soil [57]) or during food production and storage. Furthermore, hundreds of sRNAs are waiting to be characterized in other important foodborne pathogens, such as *Campylobacter jejuni* [58,59]. The use of RNA-seq-based methodologies promises to provide rapid and global insights into the
regulatory functions of these sRNAs. Ultimately, such studies would stimulate the discovery of novel players in RNA-based regulation in bacteria, which could be exploited for biotechnological applications.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:
• of special interest
•• of outstanding interest

1. Waters LS, Storz G: Regulatory RNAs in bacteria. Cell 2009, 136:615-628.

2. Oliva G, Sahr T, Buchrieser C: Small RNAs, 5' UTR elements and RNA-binding proteins in intracellular bacteria: impact on metabolism and virulence. FEMS Microbiol Rev 2015, 39:331-349.

3. Bouloc P, Repoila F: Fresh layers of RNA-mediated regulation in Gram-positive bacteria. Curr Opin Microbiol 2016, 30:30-35.

4. Christiansen JK, Larsen MH, Ingmer H, Sogaard-Andersen L, Kallipolitis BH: The RNA-binding protein Hfq of Listeria monocytogenes: role in stress tolerance and virulence. J Bacteriol 2004, 186:3355-3362.

5. Sittka A, Pfeiffer V, Tedin K, Vogel J: The RNA chaperone Hfq is essential for the virulence of Salmonella typhimurium. Mol Microbiol 2007, 63:193-217.

6. Sittka A, Lucchini S, Papenfort K, Sharma CM, Rolle K, Binnewies TT, Hinton JC, Vogel J: Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. PLoS Genet 2008, 4:e1000163.

7. Christiansen JK, Nielsen JS, Ebersbach T, Valentin-Hansen P, Sogaard-Andersen L, Kallipolitis BH: Identification of small Hfq-binding RNAs in Listeria monocytogenes. RNA 2006, 12:1383-1396.

8. Nielsen JS, Lei LK, Ebersbach T, Olsen AS, Klitgaard JK, Valentin-Hansen P, Kallipolitis BH: Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in Listeria monocytogenes. Nucleic Acids Res 2010, 38:907-919.

9. Sharma CM, Darfeuille F, Plantinga TH, Vogel J: A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. Genes Dev 2007, 21:2804-2817.

10. Nielsen JS, Larsen MH, Lillevad EM, Bergholz TM, Christiansen MH, Boor KJ, Wiedmann M, Kallipolitis BH: A small RNA controls expression of the chitinase ChiA in Listeria monocytogenes. PLoS One 2011, 6:e19019.
11. Sharma CM, Papenfort K, Pernitzsch SR, Mollenkopf HJ, Hinton JC, Vogel J: Pervasive post-transcriptional control of genes involved in amino acid metabolism by the Hfq-dependent GcvB small RNA. *Mol Microbiol* 2011, 81:1144-1165.

12. Miyakoshi M, Chao Y, Vogel J: Cross talk between ABC transporter mRNAs via a target mRNA-derived sponge of the GcvB small RNA. *EMBO J* 2015, 34:1478-1492.

- In this study, the authors discover a mechanism of mRNA cross talk, where the mRNA-derived sRNA SroC in *Salmonella* base pairs with the sRNA GcvB. The sRNA-sRNA interaction triggers degradation of GcvB by RNase E, which alleviates GcvB-mediated repression of its multiple target genes involved in amino acid synthesis and transport, including SroC’s parental mRNA. This study represents the first example of sponge-mediated cross-talk that affects several physically unlinked mRNAs from the same regulon.

13. Tree JJ, Granneman S, McAteer SP, Tollervey D, Gally DL: Identification of bacteriophage-encoded anti-sRNAs in pathogenic *Escherichia coli*. *Mol Cell* 2014, 55:199-213.

- In this research paper, Tree et al. use UV-induced RNA-protein crosslinking in order to identify Hfq-binding sites in the transcriptome of EHEC. Multiple Hfq-binding sRNAs were discovered; the majority was encoded from bacteriophage-derived regions. One of the most abundant sRNAs identified was AgvB, which functions as an anti-sRNA that antagonizes GcvB. The results demonstrated that AgvB provided EHEC with a growth advantage in bovine rectal mucus, indicating a role of this anti-sRNA for niche adaptation.

14. Toledo-Arama A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Balestrino D, Loh E, Gripenland J, Tiensuu T, Vaitkevicius K, et al.: The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature* 2009, 459:950-956.

15. Mraheil MA, Billion A, Mohamed W, Mukherjee K, Kuenne C, Pischimaro J, Krawitz C, Retey J, Hartsch T, Chakraborty T, et al.: The intracellular sRNA transcriptome of *Listeria monocytogenes* during growth in macrophages. *Nucleic Acids Res* 2011, 39:4235-4248.

16. Kröger C, Colgan A, Srikumar S, Handler K, Sivasankaran SK, Hammarlof DL, Canals R, Grissom JE, Conway T, Hokamp K, et al.: An infection-relevant transcriptomic compendium for *Salmonella enterica* Serovar Typhimurium. *Cell Host Microbe* 2013, 14:683-695.

17. Srikumar S, Kroger C, Hebrard M, Colgan A, Owen SV, Sivasankaran SK, Cameron AD, Hokamp K, Hinton JC: RNA-seq Brings New Insights to the Intra-Macrophage Transcriptome of *Salmonella Typhimurium*. *PLoS Pathog* 2015, 11:e1005262.
18. Saliba AE, S CS, Vogel J: New RNA-seq approaches for the study of bacterial pathogens. *Curr Opin Microbiol* 2017, 35:78-87.

* This review provides an excellent overview of novel RNA-seq-based approaches for studies on sRNA-mediated control in bacterial pathogens.

19. Barquist L, Vogel J: Accelerating Discovery and Functional Analysis of Small RNAs with New Technologies. *Annu Rev Genet* 2015, 49:367-394.

20. Hor J, Vogel J: Global snapshots of bacterial RNA networks. *EMBO J* 2017, 36:245-247.

21. Smirnov A, Forstner KU, Holmqvist E, Otto A, Gunster R, Becher D, Reinhardt R, Vogel J: Grad-seq guides the discovery of ProQ as a major small RNA-binding protein. *Proc Natl Acad Sci U S A* 2016, 113:11591-11596.

* The authors discover the conserved RNA-binding protein ProQ in *Salmonella*, associated with a distinct class of structured sRNAs, using the Grad-seq method. This discovery positions ProQ as a global sRNA-binding protein in bacteria.

22. Olejniczak M, Storz G: ProQ/FinO-domain proteins: Another ubiquitous family of RNA matchmakers? *Mol Microbiol* 2017.

23. Attaiech L, Glover JN, Charpentier X: RNA Chaperones Step Out of Hfq's Shadow. *Trends Microbiol* 2017, 25:247-249.

24. Smirnov A, Wang C, Drewry LL, Vogel J: Molecular mechanism of mRNA repression in trans by a ProQ-dependent small RNA. *EMBO J* 2017.

* This study investigates the mode-of-action of the ProQ-dependent sRNA RaiZ in *Salmonella*. Through detailed analyses of ProQ-RaiZ binding specificity and ProQ-mediated stability of the sRNA, the authors provide evidence that ProQ acts as an sRNA chaperone.

25. Sievers S, Sternkopf Lillebaek EM, Jacobsen K, Lund A, Mollerup MS, Nielsen PK, Kallipolitis BH: A multicopy sRNA of *Listeria monocytogenes* regulates expression of the virulence adhesin *LapB*. *Nucleic Acids Res* 2014, 42:9383-9398.

* This study demonstrates that LhrC1-5 act as regulatory sRNAs in *L. monocytogenes* and identifies their first target gene (*lapB*). Evidence of LhrC1-5 regulation, virulence association and mechanism of action is provided.

26. Sievers S, Lund A, Menendez-Gil P, Nielsen A, Storm Mollerup M, Lambert Nielsen S, Buch Larsson P, Borch-Jensen J, Johansson J, Kallipolitis BH: The multicopy sRNA LhrC controls
expression of the oligopeptide-binding protein OppA in Listeria monocytogenes. RNA Biol 2015, 12:985-997.

27. Burke TP, Portnoy DA: *SpoVG Is a Conserved RNA-Binding Protein That Regulates Listeria monocytogenes Lysozyme Resistance, Virulence, and Swarming Motility*. MBio 2016, 7:e00240.

In this research paper, the authors investigate the role of SpoVG in *L. monocytogenes* and demonstrate that SpoVG interacts with sRNAs.

28. Burke TP, Loukitcheva A, Zemansky J, Wheeler R, Boneca IG, Portnoy DA: *Listeria monocytogenes is resistant to lysozyme through the regulation, not the acquisition, of cell wall-modifying enzymes*. J Bacteriol 2014, 196:3756-3767.

29. Quereda JJ, Ortega AD, Pucciarelli MG, Garcia-Del Portillo F: *The Listeria Small RNA Rli27 Regulates a Cell Wall Protein inside Eukaryotic Cells by Targeting a Long 5’-UTR Variant*. PLoS Genet 2014, 10:e1004765.

The sRNA Rli27 is shown to activate expression of the cell wall protein Lmo0514 during intracellular infection by targeting a long 5’ UTR variant of *lmo0514* mRNA. Both Rli27 and the long 5’ UTR variant of *lmo0514* mRNA are upregulated in intracellular *L. monocytogenes*.

30. Padalon-Brauch G, Hershberg R, Elgrably-Weiss M, Baruch K, Rosenshine I, Margalit H, Altuvia S: *Small RNAs encoded within genetic islands of Salmonella typhimurium show host-induced expression and role in virulence*. Nucleic Acids Res 2008, 36:1913-1927.

31. Cossart P, Sansonetti PJ: *Bacterial invasion: the paradigms of enteroinvasive pathogens*. Science 2004, 304:242-248.

32. Westermann AJ, Forstner KU, Amman F, Barquist L, Chao Y, Schulte LN, Muller L, Reinhardt R, Stadler PF, Vogel J: *Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions*. Nature 2016, 529:496-501.

In this impressive study, dual RNA-seq is used to map the transcriptomes of *Salmonella* and host cells during infection. The authors show that the sRNA PinT temporally controls virulence gene expression in *Salmonella* and affects the host transcriptome.

33. Ortega AD, Gonzalo-Asensio J, Garcia-del Portillo F: *Dynamics of Salmonella small RNA expression in non-growing bacteria located inside eukaryotic cells*. RNA Biol 2012, 9:469-488.
34. Leclerc JM, Dozois CM, Daigle F: Role of the Salmonella enterica serovar Typhi Fur regulator and small RNAs RfrA and RfrB in iron homeostasis and interaction with host cells. *Microbiology* 2013, 159:591-602.

35. Kim JN, Kwon YM: Genetic and phenotypic characterization of the RyhB regulon in *Salmonella Typhimurium*. *Microbiol Res* 2013, 168:41-49.

36. Calderon IL, Morales EH, Collao B, Calderon PF, Chahuan CA, Acuna LG, Gil F, Saavedra CP: Role of *Salmonella Typhimurium* small RNAs RyhB-1 and RyhB-2 in the oxidative stress response. *Res Microbiol* 2014, 165:30-40.

37. Calderon PF, Morales EH, Acuna LG, Fuentes DN, Gil F, Porwollik S, McClelland M, Saavedra CP, Calderon IL: The small RNA RyhB homologs from *Salmonella typhimurium* participate in the response to S-nitrosoglutathione-induced stress. *Biochem Biophys Res Commun* 2014, 450:641-645.

38. Salvail H, Masse E: Regulating iron storage and metabolism with RNA: an overview of posttranscriptional controls of intracellular iron homeostasis. *Wiley Interdiscip Rev RNA* 2012, 3:26-36.

39. Kim JN, Kwon YM: Phenotypic characterization of *Salmonella* RyhB-1 mutations that modulate target regulation. *Curr Microbiol* 2014, 69:212-217.

40. Ellermeier JR, Slauch JM: Fur regulates expression of the *Salmonella* pathogenicity island 1 type III secretion system through HilD. *J Bacteriol* 2008, 190:476-486.

41. Balbontin R, Villagra N, Pardos de la Gandara M, Mora G, Figueroa-Bossi N, Bossi L: Expression of IroN, the salmochelin siderophore receptor, requires mRNA activation by RyhB small RNA homologues. *Mol Microbiol* 2016, 100:139-155.

The sRNA homologues RyhB11 and RyhB-2 are shown to activate, to a different extent, the expression of the salmochelin siderophore receptor in *Salmonella*. The sRNAs target the 5´ UTR of *iroN* mRNA and act to stimulate 30S ribosomal subunit binding to an upstream site in the 5´ UTR. This is the first direct evidence confirming that RyhB-1 and -2 act via a base pairing mechanism in *Salmonella*.

42. Papenfort K, Vanderpool CK: Target activation by regulatory RNAs in bacteria. *FEMS Microbiol Rev* 2015, 39:362-378.

43. Kim JN, Kwon YM: Identification of target transcripts regulated by small RNA RyhB homologs in *Salmonella*: RyhB-2 regulates motility phenotype. *Microbiol Res* 2013, 168:621-629.
44. Mollerup MS, Ross JA, Helfer AC, Meistrup K, Romby P, Kallipolitis BH: Two novel members of the LhrC family of small RNAs in Listeria monocytogenes with overlapping regulatory functions but distinctive expression profiles. *RNA Biol* 2016, 13:895-915.

45. Lobel L, Sigal N, Borovok I, Ruppin E, Herskovits AA: Integrative genomic analysis identifies isoleucine and CodY as regulators of Listeria monocytogenes virulence. *PLoS Genet* 2012, 8:e1002887.

46. Lobel L, Sigal N, Borovok I, Belitsky BR, Sonenshein AL, Herskovits AA: The metabolic regulator CodY links Listeria monocytogenes metabolism to virulence by directly activating the virulence regulatory gene prfA. *Mol Microbiol* 2015, 95:624-644.

47. Chaudhuri RR, Morgan E, Peters SE, Pleasance SJ, Hudson DL, Davies HM, Wang J, van Diemen PM, Buckley AM, Bowen AJ, et al.: Comprehensive assignment of roles for Salmonella typhimurium genes in intestinal colonization of food-producing animals. *PLoS Genet* 2013, 9:e1003456.

48. Westermann AJ, Barquist L, Vogel J: Resolving host-pathogen interactions by dual RNA-seq. *PLoS Pathog* 2017, 13:e1006033.

This review provides useful information on how to design dual RNA-seq experiments, from RNA preservation to data interpretation. It also summarizes the possible applications for this technique.

49. Chen Y, Indurthi DC, Jones SW, Papoutsakis ET: Small RNAs in the genus Clostridium. *MBio* 2011, 2:e00340-00310.

50. van der Meulen SB, de Jong A, Kok J: Transcriptome landscape of Lactococcus lactis reveals many novel RNAs including a small regulatory RNA involved in carbon uptake and metabolism. *RNA Biol* 2016, 13:353-366.

51. Darsonval M, Msadek T, Alexandre H, Grandvalet C: The Antisense RNA Approach: a New Application for In Vivo Investigation of the Stress Response of Oenococcus oeni, a Wine-Associated Lactic Acid Bacterium. *Appl Environ Microbiol* 2015, 82:18-26.

52. Cho C, Lee SY: Efficient gene knockdown in Clostridium acetobutylicum by synthetic small regulatory RNAs. *Biotechnol Bioeng* 2017, 114:374-383.

53. Kim B, Park H, Na D, Lee SY: Metabolic engineering of Escherichia coli for the production of phenol from glucose. *Biotechnol J* 2014, 9:621-629.
54. Salvail H, Lanthier-Bourbonnais P, Sobota JM, Caza M, Benjamin JA, Mendieta ME, Lepine F, Dozois CM, Imlay J, Masse E: *A small RNA promotes siderophore production through transcriptional and metabolic remodeling*. Proc Natl Acad Sci U S A 2010, 107:15223-15228.

55. Hussein R, Lim HN: *Direct comparison of small RNA and transcription factor signaling*. Nucleic Acids Res 2012, 40:7269-7279.

56. Lahiry A, Stimple SD, Wood DW, Lease RA: *Retargeting a Dual-Acting sRNA for Multiple mRNA Transcript Regulation*. ACS Synth Biol 2017, 6:648-658.

57. Vivant AL, Garmyn D, Gal L, Hartmann A, Piveteau P: *Survival of Listeria monocytogenes in Soil Requires AgrA-Mediated Regulation*. Appl Environ Microbiol 2015, 81:5073-5084.

58. Taveirne ME, Theriot CM, Livny J, DiRita VJ: *The complete Campylobacter jejuni transcriptome during colonization of a natural host determined by RNAseq*. PLoS One 2013, 8:e73586.

59. Dugar G, Herbig A, Forstner KU, Heidrich N, Reinhardt R, Nieselt K, Sharma CM: *High-resolution transcriptome maps reveal strain-specific regulatory features of multiple Campylobacter jejuni isolates*. PLoS Genet 2013, 9:e1003495.
Figure captions

Figure 1. Regulatory mechanisms used by sRNAs – examples of negative and positive effects on translation.

(a) “Canonical” sRNA regulation: LhrA in *L. monocytogenes* represses translation of several target mRNAs by base paring in the vicinity of the RBS, thus preventing access of the 30S ribosomal subunit. The Hfq protein stabilizes LhrA and stimulates sRNA-mRNA duplex formation [7,8]. (b) GcvB represses translation of multiple mRNAs in *Salmonella* by base pairing to a region upstream of the RBS. Binding of GcvB blocks a CA-rich enhancer site, which acts to stimulate translation [9]. Consequently, base pairing results in translational repression even though the RBS is accessible. (c) In intracellular *L. monocytogenes*, the *lmo0514* gene is transcribed with a long 5' UTR, which forms an inhibitory secondary structure that sequesters the RBS. When the sRNA Rli27 base pairs with the 5' UTR, the inhibitory structure is relieved, and the RBS is freely accessible to the ribosome [29•]. (d) The RyhB siblings in *Salmonella* base pair with the 5' UTR of *iroN* mRNA, causing a structural change in the mRNA that exposes an enhancer site to which the 30S ribosomal subunit binds [41•]. The stimulating effect of RyhB on *iroN* expression depends on this enhancer sequence, but it is unknown whether 30S binding to the enhancer stabilizes the mRNA or facilitates 30S sliding into the RBS. Blue box, RBS; yellow ovals, ribosome; purple box, enhancer site; base pairing is indicated by thin lines.

Figure 2. Cross-talk between mRNAs within the *Salmonella* GcvB regulon.

The *gltIJKL* locus is transcribed as two transcripts: a long mRNA spanning the entire operon, and a short mRNA covering only *gltI*. Both are post-transcriptionally repressed by the sRNA GcvB. RNase E-
and Hfq-dependent processing of the short gltI transcript results in the formation of the sponge RNA SroC, which base pairs with GcvB in an Hfq-dependent manner, leading to RNase E-dependent degradation of GcvB. Thus, via SroC, the gltIJKL locus indirectly stimulates its own expression as well as expression of other GcvB targets (dashed arrow), which are involved in amino acid or peptide transport [12•].

Figure 3. Differential expression and regulation by sibling sRNAs.

Homologous sRNAs may be differentially expressed due to nucleotide differences in their promoter regions. External stimuli known to induce the expression of the RyhB siblings in Salmonella (a) and LhrC siblings in L. monocytogenes (b) are shown together with the transcriptional regulators (yellow ovals) involved; a question mark indicates that the signaling pathway responsible for the induction is presently unknown.

(a) Transcription of the RyhB siblings in Salmonella is controlled by a common set of transcriptional regulators; however, their strength of regulation is not identical for the two promoters (relative strength at each promoter is indicated by the thickness of the arrow). The RyhB siblings share a 33 nt sequence with perfect homology, which forms the basis of the regulation of shared targets [35,40,41•], whereas differences in the flanking sequences might explain the regulation of unique targets by either sRNA [43]. The thickness of the lines indicate the observed relative strength of regulation by the sibling sRNAs, which might reflect both differences in sRNA expression as well as the strength of interaction with mRNA targets. Dashed lines indicate that RyhB expression affects target mRNA level, but dependence on direct base pairing has not been examined further. Selected target
genes are shown to illustrate the differences, but mRNA levels of more genes are known to be affected by the RyhB siblings [35,37,43].

(b) The seven members of the LhrC family are expressed from separate promoters in four distinct loci on the *L. monocytogenes* chromosome giving rise to the highly homologous LhrC1-5 (>85% identical) as well as Rli22 and Rli33-1, which share less homology (indicated by the different colors of the sRNAs). All siblings contain two or three UCCC motifs (red) that are involved in the post-transcriptional repression of a common set of genes via base pairing [25*,26,44]. Solid lines indicate that an observed decrease in target mRNA levels in response to sRNA expression is supported by *in vitro* data demonstrating a direct interaction between sRNA and mRNA. A dashed line indicates that sRNA expression reduces mRNA levels *in vivo*, however, a direct interaction was not observed under the conditions used *in vitro* [44].
