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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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.

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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 pairing 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 *Imo0514* 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].

Translational inhibition

(a)

(b)

Translational enhancement

(c)

(d)

GcvB regulon: amino acid/peptide uptake

(a)

(b)