Small Non-Coding RNAs in Salmonella–Host Interactions

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Salmonella species infect hosts by entering phagocytic and non-phagocytic cells, causing diverse disease symptoms, such as fever, gastroenteritis, and even death. Therefore, *Salmonella* has attracted much attention. Many factors are involved in pathogenesis, for example, the capsule, enterotoxins, *Salmonella* pathogenicity islands (SPIs), and corresponding regulators. These factors are all traditional proteins associated with virulence and regulation. Small non-coding RNAs (sRNAs) have also been reported to function as critical regulators. *Salmonella* has become a model organism for studying sRNAs. sRNAs regulate gene expression by imperfect base-pairing with targets at the post-transcriptional level. sRNAs are involved in diverse biological processes, such as virulence, substance metabolism, and adaptation to stress environments.

Keywords: sRNAs ; Salmonella ; host ; interactions

1. Introduction

Salmonella enterica is one of the leading causes of foodborne gastroenteritis worldwide. The two most important serovars of *Salmonella* are *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis), which cause non-typhoid salmonellosis infections [1]. As an intracellular zoonotic pathogen, *Salmonella* regularly infects hosts. It enters the stomach and intestinal lumen of the host after ingestion of contaminated food, causing gastroenteritis in both humans and animals as well as typhoid fever in mice. *Salmonella* must survive within the acidic environment of the stomach and penetrates the gut barrier via M cells in Peyer's patches of the intestine [2]. *Salmonella* invades the cell membrane and forms *Salmonella*-containing vacuoles (SCVs) with the help of a Type III secretion system (T3SS) encoded by *Salmonella* pathogenicity islands (SPIs) [3]. After that, macrophages engulf the bacteria and kill them to resist infection by producing reactive nitrogen species (RNS) and reactive oxygen species (ROS) [4]. Interestingly, *Salmonella* employs sophisticated strategies to survive and replicate inside phagocytic and non-phagocytic cells, causing serious diseases in humans and animals.

The small non-coding RNAs (sRNAs), which are known to be involved in the regulation of gene expression, have a length of 50–500 nucleotides and have been found in various bacteria, for example, *Escherichia coli*, *Listeria monocytogenes*, and *S.* Typhimurium [5][6]. Based on their mode of base-pairing, they are classified into cis- and trans-encoded sRNAs. Cis-encoded sRNAs are transcribed from the same loci as the mRNAs on the opposite strand of DNA and bind to their cognate mRNA targets with perfect complementarity, resulting in either transcriptional termination or translational initiation. Trans-encoded sRNAs interact with multiple mRNA targets through imperfect complementation [6][7]. Gene expression is usually regulated by trans-acting sRNAs at the post-transcriptional level [8]. The functions of more than half of the trans-acting sRNAs require the chaperone protein Hfq, which plays an important role in regulation by stabilizing sRNAs and mediates their interaction with the trans-encoded target mRNAs of host cells, leading to repression of translation or acceleration of mRNA decay [9]. *S.* Typhimurium expresses hundreds of sRNAs, many of which are activated under special stress and virulence conditions, suggesting that sRNAs are an important component of regulatory networks controlling gene expression in bacteria during host infection [10].

sRNAs regulate many physiological processes in bacteria, including metabolism, iron homeostasis, quorum sensing, outer membrane protein biosynthesis, and the regulation of virulence genes [11][12]. In recent years, attention has been focused on the functions of sRNAs in bacteria–host interactions. To establish a successful infection, *Salmonella* must first resist the acidic environment and oxidative stress, adhere to and invade non-phagocytic cells, and finally evade host immunity to survive inside macrophages [13]. sRNAs play integral roles in bacterial stress responses, promote intracellular survival, and modulate host immune responses [9][14]. In this entry, researchers summarize the roles of sRNAs in the interaction between *Salmonella* and host cells (see **Table 1** for a summary of sRNAs), aiming to understand the roles of sRNAs upon

host cell infection, provide an overview of the functional mechanisms of sRNAs, and provide ideas to improve host resistance to *Salmonella* infection.

Table 1. Summarized characteristics of sRNAs during *Salmonella*–host interactions.

Function in Infection	Serotype	sRNA	Description	Target Gene/Protein	Reference
Resisting AcidEnvironment	*S.* Typhimurium SB300	DsrA	Trans-coded	*rpoS*	[15]
	S. Typhimurium	RyeC	Trans-coded	*ptsi*	[16]
	S. Typhimurium	6S RNA	Trans-coded	*citGXFED*, *nuo* operon	[17]
Adhering and Invading to Non-Phagocytic Cells	*S.* Enteritidis 50336	STnc640	Trans-coded	*fimA*	[18][19]
	S. Typhimurium	MicC	Trans-coded	OmpD	[20][21]
	S. Enteritidis	InvR	Trans-coded	OmpD	[22]
	S. Typhimurium	IsrJ	Trans-coded	SptP	[23]
	Salmonella	IsrM	Trans-coded	HilE	[24]
	S. Enteritidis 50336	RyhB-1, RyhB-2	Trans-coded	*sipA*, *sopE*	[25]
	S. Typhimurium	InvS	Trans-coded	PrgH, FimZ	[26]
	Salmonella	PinT	Trans-coded	*hilA*, *rtsA*	[27]
	S. Enteritidis 2472	Sal-1	Trans-coded	iNOS	[28]
Resisting Oxidative Stress	*S.* Typhimurium	OxyS	cis-coded	*ompX*	[29]
	S. Typhimurium	CyaR	Trans-coded	*ompX*	[29]
	S. Typhimurium	MicA	Trans-coded	*ompX*	[29]
	S. Typhimurium *YK5104*	RaoN	Trans-coded	*ldhA*	[29]
	S. Typhimurium	RyhB-1, RyhB-2	Trans-coded	-	[23][30]
	S. Typhimurium	IsrC	cis-coded	*msgA*	[23]
	S. Typhimurium	IsrN	cis-coded	*STM2765*	[23]
	S. Typhimurium	OxyS	cis-coded	-	[23]
Survivalin Macrophages	*S. Typhi*	RfrA, RfrB	Trans-coded	-	[31]
	S. Typhimurium	RyhB-1, RyhB-2	Trans-coded	*fumA*, *sdhD*	[32]
	S. Typhimurium	IsrM	Trans-coded	SopA	[24]
	S. Typhimurium *YK5104*	RaoN	Trans-coded	-	[30][33]
Regulating Inflammatory Cytokines of Hosts	*S.* Typhimurium	PinT	Trans-coded	IL-8, SOCS3	[34][35]

2. sRNAs' Functions in *Salmonella*–Host Interactions

2.1. sRNAs Regulate Resistance to the Acidic Environment

Acid stress is one of the most important stresses that *Salmonella* must overcome to colonize the host. *Salmonella* may encounter acidic environments such as the stomach, and acid exposure also occurs after invading the intestinal mucosa [13][36]. Interestingly, *Salmonella* has evolved a precise response known as the acid tolerance response (ATR), which promotes the survival of acid-adapted cells at low pH levels [36]. The alternative sigma factor RpoS, which is a global regulator of gene expression when bacteria suffer from starvation or other stress conditions, is required for *Salmonella* resistance to acid stress [37]. Recently, sRNAs have been identified as major regulators of acid stress response networks. The 87-nucleotide sRNA DsrA, which is present in both *Salmonella* and *E. coli*, is a regulator of acid resistance. DsrA expression can be induced in *S.* Typhimurium in a minimum essential medium, with maximum induction at pH 3.1. Deletion of *dsrA* reduced the effectiveness of the ATR, resulting in a lower survival rate in acidic environments [38]. Although how DsrA modulates the acid stress response is still unclear, some evidence has proven that DsrA could regulate the translation of RpoS and the expression of some acid resistance genes [15][39][40]. DsrA activates the translation of RpoS by base-pairing with the upstream leader portion of *rpoS* mRNA in *S*. Typhimurium [15]. Mutations in *dsrA* decreased the expression of RpoS in exponential and stationary phases of *E. coli* [39]. The mRNA levels of multiple acid resistance genes in the *hdeAB*, *gadAX*, and *gadBC* operons were increased with DsrA overproduction in *E. coli* [40]. The above reports are useful for further study of the mechanism of acid resistance regulation through DsrA. Besides the acid stress response, DsrA enhances the invasive phenotype of *S.* Typhimurium by repressing the translation of the histone-like nucleoid protein (H-NS) [15][38]. Moreover, deletion of *dsrA* reduced the motility, adhesion, and invasion efficacy of *S*. Typhimurium [38]. Another example is the sRNA RyeC, which is the antitoxin component of a type I toxin–antitoxin (TA) system that is encoded by a small ORF and is associated with extremely high toxicity [41]. Overexpression of the trans-encoded RyeC in *S.* Typhimurium during the ATR inhibits the expression of the target PtsI, which is a subunit of a major carbohydrate transporter, through direct interaction at the post-transcriptional level, resulting in a reduced ATR in *Salmonella* [16].

2.2. sRNAs Regulate Adhesion to and Invasion of Non-Phagocytic Cells

Salmonella initiates infection of the host by attaching to the intestinal mucosal surface and subsequently adhering to and invading non-phagocytic cells. Adhesion to and invasion of host cells are crucial steps in *Salmonella* infection. Many adhesive structures such as fimbrial and non-fimbrial proteins were found in *Salmonella*, including type I fimbriae, autotransporter adhesins, and the outer membrane protein OmpD [1][42][43][44].

2.2.1. sRNAs Regulate the Expression of a Fimbrial Subunit

FimA is the major fimbrial subunit of Type 1 Fimbriae in *Salmonella*, and it is important for adhesion to enterocytes and colonization of the intestine [18]. STnc640, which is an Hfq-binding sRNA identified in *S.* Typhimurium by deep sequencing [45], is also present in *S.* Enteritidis and upregulates the expression of *fimA*. Although STnc640 regulates the expression of *fimA*, it decreases the adhesion ability of *S.* Enteritidis to human colorectal adenocarcinoma epithelial cells (Caco-2) and attenuates the virulence of *S.* Enteritidis in chickens [19]. STnc150, another sRNA of *S.* Typhimurium, downregulates the protein expression of FimA by base-pairing with the 5'-end of *fimA* mRNA. Deletion of STnc150 enhanced the adhesion ability of *S.* Typhimurium to macrophages and reduced LD50 in mice [46].

2.3. sRNAs Regulate Resistance to Oxidative Stress within Cells

After entry into phagocytic cells, *Salmonella* faces oxidative stress in the cytoplasm. Phagocytic cells have two important antimicrobial systems, the NADPH phagocyte oxidase (phox) and inducible nitric oxide synthase (iNOS) pathways, which produce ROS and RNS, respectively. ROS play important roles in the early host response to infection, and RNS limit bacterial survival in host cells [47]. As a result, the ability of bacteria to survive the oxidative stress inside hosts is the key to induce pathogenicity. Interestingly, bacteria have involved mature mechanisms to promote survival and replication inside host cells. Recently, sRNAs in *Salmonella* have been demonstrated to have irreplaceable functions in resisting oxidative stress inside host cells. *S.* Enteritidis strain SE2472 can use mammalian atypical miRNA processing machinery to cleave sRNA into a ~22-nt miRNA-like RNA fragment, Sal-1, which facilitates the intracellular survival of invaded bacteria by targeting cellular iNOS, attenuating the iNOS-mediated antimicrobial ability of human colonic epithelial cells. sRNAs are important in the resistance to oxidative stress inside host cells [28].

OxyS is a stable and abundant sRNA that was first identified in *E. coli*. It has been proven that OxyS helps bacteria adapt to the mutagenic effects of hydrogen peroxide (H_2O_2) and protects *E. coli* against oxidative damage [48][49]. Moreover, OxyS is strongly activated when *S.* Typhimurium resides within J774 macrophages, suggesting that SPI-encoded sRNAs

play significant roles in the network that regulates the stress response within the macrophage environment [23]. A recent report showed that OxyS could positively regulate the mRNA levels of the porin-encoding gene *ompX* under H_2O_2-induced stress in *S.* Typhimurium, and sRNAs CyaR and MicA positively regulate *ompA* mRNA levels under H_2O_2-induced stress [29]. To sum up, OxyS provides important assistance for bacteria to resist oxidative stress.

Some sRNAs also target virulence genes under oxidative stress to promote survival and replication in macrophages. RaoN is a sRNA encoded in the *cspH-envE* intergenic region on SPI-11. The expression of RaoN is increased under oxidative stress with nutrient-limiting conditions in vitro. It has been shown that the lactate dehydrogenase gene *ldhA*, whose inactivation and overexpression both render *Salmonella* more sensitive to oxidative stress, was upregulated in the *raoN* knockout mutant. Deletion of *raoN* impedes *Salmonella* survival and replication in macrophages [30][33].

RyhB and its homologs are key regulators of iron homeostasis in *E. coli* and *Salmonella* [31][50]. As an iron-regulatory sRNA, RyhB downregulates a large number of transcripts encoding iron-using proteins when facing iron deficiency and modulates the usage of intracellular iron. Beyond that, RyhB-1 and RyhB-2 expression is induced upon exposure to H_2O_2 [23]. RyhB deletion mutants (Δ*ryhB-1*, Δ*ryhB-2*, and Δ*ryhB-1*/Δ*ryhB-2*) displayed increased levels of intracellular ROS and a growth defect when treated with H_2O_2 in iron-rich or iron-deficient conditions. OxyR upregulated the expression of *ryhB-1* and *ryhB-2* through direct interaction with their promoter region when *Salmonella* was treated with H_2O_2 [51]. The above illustrates that RyhB-1 and RyhB-2 are necessary for *Salmonella* to resist oxidative stress in the intracellular environment.

2.4. sRNAs Regulate Survival in Macrophages

Though T3SS-1 effectors are translocated into host cells to promote invasion of diverse cell types, the expression of T3SS-2 (encoded by SPI-2) is mainly triggered after entering cells and results in translocation of effectors to manipulate the intracellular niche [44][52]. *Salmonella* suffers extreme stresses in macrophages because macrophages produce RNS and ROS to kill it [4]. Therefore, survival and replication of *Salmonella* within macrophages is essential for its pathogenicity in hosts. Recently, many sRNAs have been found to promote survival and replication of *Salmonella* in macrophages.

When *Salmonella* is present in the intracellular environment of macrophages, the expression of many sRNAs is induced. Transcriptome analysis of intra-macrophage *S.* Typhimurium showed that 88% of 280 sRNAs were expressed [53]. Compared to the early stationary phase (ESP) conditions, with high expression of SPI-1 genes, 34 sRNAs (including RyhB-1, RyhB-2, OxyS, MicF, and RybB) were upregulated, and 119 sRNAs were downregulated [52]. The expression of many SPI-encoded sRNAs is induced when *Salmonella* multiplies within macrophages. For example, the transcription of IsrC and IsrN is increased 7-fold within the first hour post-infection of J774 macrophages and declines as infection progresses. IsrC overlaps with its flanking gene *msgA* at the 3'-end and affects the expression of *msgA* (encoding a macrophage survival-related protein) in cis. This indicates that IsrC is important for *Salmonella* to survive in macrophages [23]. OxyS, an sRNA that responds to oxidative stress, shows the same expression pattern as IsrC and IsrN. OxyS levels increase 35-fold within the first hour of infection and decrease thereafter. OxyS is strongly activated by H_2O_2 and increases the H_2O_2 resistance of *Salmonella* in macrophages [23]. The transcript levels of IsrH, IsrE (RyhB-2), and its homolog RyhB-1 increase within the first hour of infection and then increase dramatically at 8 h post-infection. The induction of expression of these sRNAs suggests that these sRNAs play important roles in the survival and replication of *Salmonella* inside macrophages [23].

Besides regulating the invasion of epithelial cells and the response to oxidative stress, RyhB-1 and RyhB-2 also contribute to intracellular survival in macrophages. In *S. Typhi*, RyhBs (RfrA and RfrB) are regulated by the ferric uptake regulator (Fur). Expression of both is induced within the human monocyte cell line THP-1 at 24 h post-infection. They are crucial for *Salmonella* replication inside macrophages [31]. In *S.* Typhimurium, RyhB-1 and RyhB-2 are the most highly induced sRNAs within macrophages compared to ESP conditions [53]. They contribute to survival and proliferation inside RAW264.7 murine macrophages [32]. Deletion of RyhB-1 and RyhB-2 leads to an increase in ATP levels and a decrease in the NAD+/NADH ratio, resulting in more active metabolism of *S.* Typhimurium in macrophages. RyhB-1 and RyhB-2 affect the expression of SPI-1-related genes encoding the transcriptional regulators HilA, HilC, HilD, InvF, and RtsA, the effector proteins SipA, SipB, SopA, and SopB, and the tricarboxylic acid cycle-related genes *fumA* and *sdhD*, which encode iron-containing enzymes involved in energy metabolism within macrophages. Moreover, RyhB-1 and RyhB-2 directly downregulate the expression of *rtsB* (encoding an invasion chaperone) and *sicA* (encoding a regulatory protein). This suggests that the two sRNAs possess the ability to integrate a global response to multiple stresses encountered inside macrophages [32].

2.5. sRNAs Regulate the Expression of Inflammatory Cytokines in Host Cells

The innate immune system is essential to defend against bacterial pathogens [54]. SCVs induce the innate immune response through the interaction of pathogen-associated molecular patterns with Toll-like receptors in host cells, such as epithelial cells and macrophages. Upon this interaction, mitogen-activated protein kinases produce a signal, leading to secretion of a variety of cytokines, such as tumor necrosis factor and interleukin-8 (IL-8), which stimulate antibacterial responses and are beneficial for bacterial clearance [14][55][56]. However, *Salmonella* has evolved sophisticated methods to modify the host cells to meet their needs by regulating these inflammatory cytokines. This regulatory function of sRNAs of *Salmonella* has received increasing attention in recent years.

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