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REVIEW

RNA-based mechanisms of virulence control in *Enterobacteriaceae*

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ABSTRACT

Enteric pathogens of the family *Enterobacteriaceae* colonize various niches within animals and humans in which they compete with intestinal commensals and are attacked by the host immune system. To survive these hostile environments they possess complex, multilayer regulatory networks that coordinate the control of virulence factors, host-adapted metabolic functions and stress resistance. An important part of these intricate control networks are RNA-based control systems that enable the pathogen to fine-tune its responses. Recent next-generation sequencing approaches revealed a large repertoire of conserved and species-specific riboregulators, including numerous *cis*- and *trans*-acting non-coding RNAs, sensory RNA elements (RNA thermometers, riboswitches), regulatory RNA-binding proteins and RNA degrading enzymes which regulate colonization factors, toxins, host defense processes and virulence-relevant physiological and metabolic processes. All of which are important cues for pathogens to sense and respond to fluctuating conditions during the infection. This review covers infection-relevant riboregulators of *E. coli, Salmonella, Shigella* and *Yersinia*, highlights their versatile regulatory mechanisms, complex target regulons and functions, and discusses emerging topics and future challenges to fully understand and exploit RNA-based control to combat bacterial infections.

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Introduction

The large family of *Enterobacteriaceae* includes various harmless commensals but also many well-characterized enteric pathogens such as *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and *Yersinia enterocolitica*/*Yersinia pseudotuberculosis*. They colonize and replicate within the intestinal tract of mammals and have the ability to invade deeper tissues. Enteric pathogens of these species are frequently cycling between different environmental and animal reservoirs, and the human host. To adapt to these rapidly changing virulent, commensal and saprophytic lifestyles, they evolved numerous survival strategies that enable them (i) to adjust the expression of host-specific colonization factors and other virulence-relevant traits, (ii) to reprogram their metabolism in response to the changing temperature and availability of nutrients and ions, and (iii) to control their stress responses and overall physiology to encounter hazardous conditions experienced in- and outside their hosts. Complex regulatory networks coordinate the spatiotemporal expression of their survival strategies. Over the last decades numerous conserved and species-specific regulatory proteins have been identified in enteric pathogens that are implicated in virulence control networks. However, recent advances in high-throughput sequencing approaches now allow us to profile entire RNA landscapes with single nucleotide resolution. This has led to the discovery of numerous regulatory non-coding RNAs. Obviously, control of virulence is much more complex than ever imagined and often occurs on multiple layers of post-transcriptional control.

The discovered riboregulation control processes involve riboregulators such as *cis*- and *trans*-acting non-coding RNA elements (ncRNAs), RNA thermometers, and riboswitches, as well as regulatory RNA-binding proteins and RNA degrading or modifying enzymes. Many of which respond to fluctuating environmental conditions encountered during the course of infection. This review will focus on the mechanistic actions of endogenous RNA-based processes by these regulators, which serve as crucial components of virulence control networks in enteric pathogenic *Enterobacteriaceae*.

Molecular mechanisms underlying RNA-based control of virulence

The molecular mechanisms underlying RNA-based control of virulence gene expression are very versatile (Fig. 1), but can share remarkable similarities between related species. Sensory and regulatory RNA elements operate at different levels of gene expression, ranging from the regulation of transcription and translation, control of RNA conformation, RNA stability, adjustment of replicon copy numbers, and the modulation of protein binding to RNAs/DNAs and activity.[1,2]

Recent advances in next generation sequencing technologies have provided us with large repertoires of newly identified ncRNAs of different enteric pathogens.[3-9] ncRNAs of *Enterobacteriaceae* are generally 50-400 nucleotides in lengths and are

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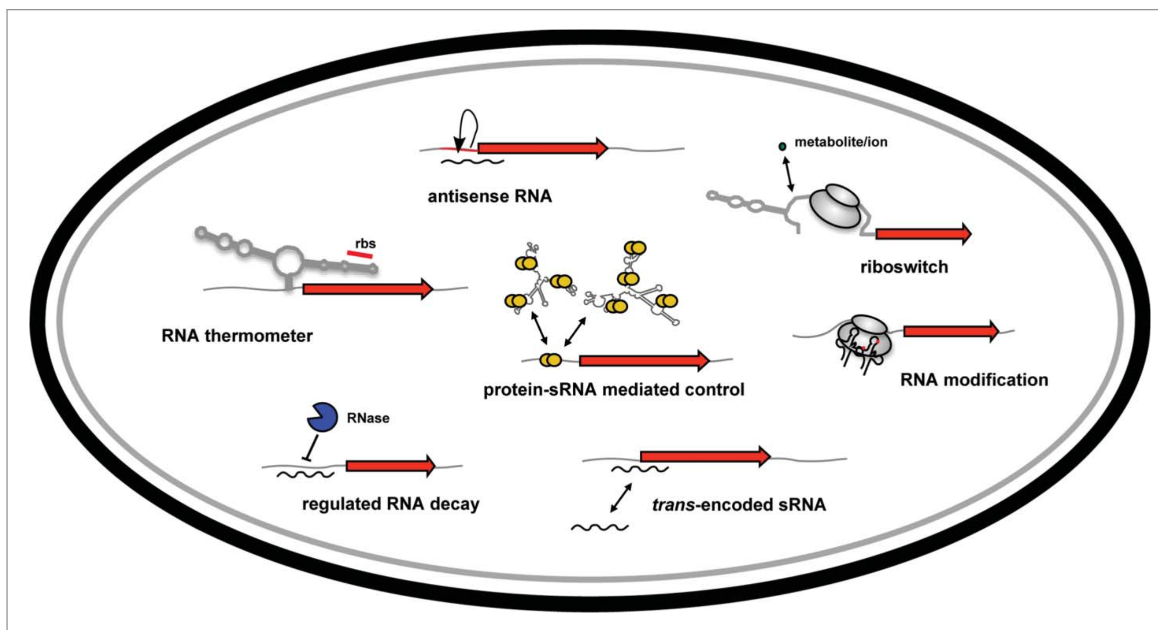

Figure 1. Overview of RNA-based control mechanisms employed by enteric pathogens of the family *Enterobacteriaceae*. mRNA translation can be controlled by RNA thermometers and by riboswitches within the 5′-UTR of target mRNAs in response to temperature or metabolites. Transcription, translation and/or stability of target transcripts can be modulated by *cis*-encoded asRNAs or *trans*-encoded ncRNAs. The RNA-binding protein CsrA modulates mRNA expression by interfering with translational initiation. The CsrB and CsrC RNAs counteract its activity. RNases control processing and degradation of ncRNAs and target transcripts. RNA-modifying enzymes change the efficiency of translation.

usually not translated. The vast majority of ncRNAs basepairs with one or multiple target mRNAs and influences distinct or numerous processes important for housekeeping functions, virulence regulation and the response to environmental challenges. A subset of the identified ncRNAs is encoded on the opposite strand of the regulated target mRNA (*cis*-encoded antisense RNAs/asRNAs) or is transcribed from a distant gene (*trans*-encoded ncRNA). The asRNAs are mostly encoded opposite to the un-translated regions of a gene and lead to autonomous RNA-RNA interactions that block ribosome-binding and translation. Alternatively, RNA-RNA duplex formation can affect the stability of the targeted mRNA through alterations of its secondary structure.[3] The *trans*-encoded ncRNAs act mainly by base-pairing to ribosome-binding sites or start codons of their target mRNAs to which they usually have only limited complementarity.[10] Most of the characterized *trans*-encoded ncRNAs interact with multiple target mRNAs with the assistence of the RNA chaperone Hfq [11] and act in concert with RNases that cleave the target mRNA and control its half-life.[12,13]

Other types of riboregulators are RNA thermometers,[14,15] and riboswitches.[16] These sensory RNA elements are found in the 5′-untranslated regions (5′-UTRs) or within intergenic regions, and control expression of the downstream gene through structural remodeling of the RNA segment. RNA thermometers typically respond to sudden thermal changes, a key signal sensed by enteric pathogens to detect host entry and activate relevant virulence programs. Currently several RNA thermometers of pathogens are known which act as thermo-responsive RNA zippers that control translation via RNA structure destabilization or alteration. They all form thermo-sensitive stem-loop structures at moderate (environmental) temperatures (‘closed’ conformation) in which the ribosome-binding site is hidden within the stem of the hairpin structure. The stem segment constitutes the thermosensing stretch of the RNA element. It typically includes internal bulges and/or non-canonical base pairs that decrease its stability and make it more susceptible to thermo-triggered zipper-like melting upon host entry. Gradual opening of the double-stranded segment of the RNA thermometer (‘open’ conformation) renders the ribosome-binding site accessible for the 30S subunit to initiate translation. This simple thermo-responsive mechanism allows a very rapid, extremely precise (range within the 1°C scale) and low/no energy-consuming adjustment of virulence factor synthesis without the need for additional factors and costly feedback loops to prevent uncontrolled hyper-induction.

In contrast to RNA thermometers, bacterial riboswitches represent sensory RNA elements that respond to varying metabolite or metal ion concentrations. Riboswitches typically regulate expression of protein-encoding mRNAs, but recently they have also been shown to control the expression of non-coding RNAs and regulate binding of proteins, e.g. the ribonuclease E (RNase E) or the transcriptional termination factor Rho to nascent RNAs.[17,18] Riboswitches sense different metal ions (e.g., Mg^{2+}, Mn^{2+}, fluoride), metabolites (e.g., vitamin B12, S-adenosylmethionine (SAM), thiamine pyrophosphate, flavin mononucleotide) or signaling molecules (e.g. c-di-GMP). Binding of these molecules to an evolutionary conserved ligand binding segment (sensor region) leads to the formation of an alternative molecule-bound RNA conformation which influences expression of the coding sequences located downstream. The set of regulated downstream genes mainly includes biosynthesis and transport systems of equivalent or related metabolites.[19] Hence, the cognate riboswitch ligand does not only act

as the stimulating substance, it often represents the end product of the controlled metabolic pathway and is implicated in feed back control.

Besides riboregulators, several global, highly conserved transcription factors and RNA chaperones have been shown to participate in RNA-based control mechanisms of virulence.[7,20] One important player in the coordinated control of regulatory RNA function is the RNA chaperone Hfq. The Hfq protein was originally identified in *E. coli* many decades ago as a host factor essential for the replication of the RNA phage Qβ and has since then been shown to be crucial for virulence of many enteric pathogens.[21] Mutants of enteropathogenic *Salmonella, E. coli, Yersinia* and *Shigella* species have pleiotropic phenotypes. Lack of Hfq leads to reduced growth rates, altered utilization of nutrients and major changes of the metabolic profile, and dramatically alters expression of pathogenicity factors and virulence-relevant traits which impairs virulence.[21] Hfq is ubiquitous and highly conserved among the *Enterobacteriaceae* where it is implicated in many ncRNA-based control processes, and the molecular mechanisms used by this central RNA manipulator to modulate gene expression are very diverse.[22,23] Hfq can act as a matchmaker from ncRNA-mRNA duplexes leading to translational repression, due to steric hindrance of ribosome binding, or to translational activation through disruption of repressive secondary structures. Moreover, Hfq can protect target mRNAs from ribolysis by (i) direct binding or (ii) assistance of ncRNA binding to RNase cleavage sites within the target mRNA.[11] Vice versa, Hfq can directly interact with RNases such as RNase E and the target ncRNA or mRNA to trigger formation of a degradosome-like complex that promotes RNA cleavage. Additionally, Hfq can promote 3′ – 5′ degradation of mRNAs by exoribonucleases through 3′-polyadenylation by the poly(A)polymerase.[24,25]

Another RNA-binding protein that is required for virulence of enteric pathogens is the CsrA protein that belongs to the post-transcriptional carbon storage regulator (Csr) sytem. Similar to Hfq, CsrA is highly conserved between different *Enterobacteriaceae* and is annotated in many other bacterial genomes.[26,27] CsrA has originally been identified in *E. coli* as a regulator of glycogen biosynthesis. Work over the last 2 decades has revealed that it also plays a crucial role in the regulation of virulence genes and converges them into complex regulatory networks with numerous metabolic functions, stress responses as well as cellular and physiological processes.[26,27] CsrA typically interacts with successive GGA motifs located in single-stranded RNA elements of the 5′-UTRs of its target mRNA, which usually includes the GGA motif of the ribosome-binding site. First, sequence-based computational approaches as well as systemic evolution of ligands by exponential enrichment (SELEX) and currently also RIP-Seq (RNA immunoprecipitation high-throughput sequencing) and CLIP-Seq (crosslinking immunoprecipitation high-throughput sequencing) analyses were performed to define CsrA targets and CsrA binding sites in individual pathogens. The vast majority of identified binding sites contains GGA motifs within loops of predicted hairpin structures.[28-30] CsrA binding to these sites results in the repression of ribosome association and prevents translation initiation and/or transcription elongation, and in many cases also increases the general turnover of the mRNA.[27] However, a few examples exist in which CsrA binding to a target mRNA activates gene expression. In this case it has been shown that CsrA stabilizes the target mRNA by protection of the transcript against cleavage by RNases.[31] Function of the CsrA regulator protein is controlled by a distinct set of ncRNAs, the so-called Csr-type RNAs.[26,27] This type of RNAs shows low sequence conservation. However, all members fold into complex secondary structures with multiple RNA hairpins of which the majority contains a GGA motif within single-stranded loops with conserved base-pairing flanking regions (CAGGA(U/A/C) G).[27,32,33] Pathogenic *E. coli, Salmonella* and *Yersinia* species encode at least 2 CsrA-sequestering RNAs, CsrB and CsrC,[27,34],[35] which carry several of the high-affinity CsrA-binding sites. This enables them to bind multiple CsrA homodimers and to sequester/titrate them away from their specific positions on their natural mRNA targets.[32]

Long-time focus on transcription initiation generated a view that prokaryotic gene expression is primarily controlled at the ‘birth’ of an mRNA, mRNA processing and degradation was mostly considered to be responsible for the fast functional trimming and turnover of transcripts. Several ribonucleases (RNases) involved in mRNA turnover were identified and the classical principles how they mediate mRNA degradation in prokaryotes are known. However, recent studies showed that the level and activity of individual RNases as well as the composition of the RNA degrading machinery can vary significantly under different growth/infection conditions.[36,37] Moreover, there is a burgeoning list of examples that RNase activity can be modulated by adaptor proteins, and a plethora of small non-coding RNAs have been discovered which can confer target selectivity to prokaryotic RNases.[38] Furthermore, the cellular localization of the RNA-degrading complexes seems to vary under different environmental conditions, and a fascinating new observation of spatial distribution patterns of the microbial mRNA species strongly suggests that microbes organize mRNA decay in time and space.[39,40] Our knowledge about the environmental signals and regulators that control mRNA decay is still in its infancy, but recent studies have undoubtedly shown that ‘regulated ribolysis’ is a crucial control mechanism for the expression of virulence factors in enteric pathogens.

The aim of the following chapter is to highlight the importance of RNA-mediated regulatory mechanisms for host-pathogen interactions and other virulence-relevant processes, dictating the progress of an intestinal infection. A selection of the most important virulence processes under RNA-based control and implicated sensory and regulatory control factors are summarized in Table 1.

Riboregulation of enterobacterial virulence

RNA-based control of colonization factors

Control of adhesion

An early step in the pathogenesis of enteric pathogens is their adhesion to the intestinal epithelial layer. The ability of certain bacteria to adhere to host cells is of fundamental importance for a successful colonization since they have to compete with the intestinal microflora and resist the flushing action of the intestinal peristalsis. For this purpose bacterial pathogens

Table 1. Examples of virulence processes under RNA-based control.

Species	RNA/RNase/RNA-modifying enzyme	Mechanism	Virulence function	References
E. coli	AfaR	trans-encoded ncRNA destabilizes the afaD mRNA by binding to the 5′-UTR	Regulation of expression of afimbrial adhesins of the Afa family in ExPEC	64
	CsrABC	CsrA: RNA binding protein, CsrB/CsrC: trans-encoded CsrA-binding RNAs CsrA binds to the LEE4 mRNA, overexpression of CsrA represses expression of the LEE1-5 transcripts through a reduction of GrlA and Ler in EPEC CsrA binds to the 5′-UTR of the pga mRNA encoding biofilm matrix components CsrA directly binds and stabilizes the flhDC transcript	Global control of pathogenesis (colonization, immune resistance), stress responses and metabolism. Expression of type 1 fimbriae and Pef fimbriae. Control of c-di-GMP synthesis Important for pedestal formation and for membrane depolarization of epithelial cells, in EHEC, Control of biofilm formation and motility	26,27,31,52
	GadY	trans-encoded ncRNA interacts with 3′-UTR of the gadY transcript in the gadX-gadY mRNA which stimulates processing into stable gadX and gadY transcripts	Glutamate-dependent extreme acid resistance	136
	GcvB	trans-encoded ncRNA Hfq-dependent a 30 nt stretch of G/U residues of GcvB recognize extended C/A elements overlapping the ribosome binding site in some targets acts by direct antisense interaction with the csgD 5′-UTR	Repression of curli biogenesis. Regulation of ABC transporters and amino acid biosynthesis genes	44
	GlmY, GlmZ	trans-encoded ncRNAs destabilization of mRNA transcripts and facilitation of translation	Control of pathogenesis, regulation of the LEE4 and LEE5 operons, the LEE-encoded effector EspFu and the non-LEE-encoded effector NleA in EHEC, promote expression of the curli adhesin, repress tryptophan metabolism genes, and promote acid resistance	46-48,53
	McaS	trans-encoded ncRNA resembles CsrB and sequestrates CsrA, represses in collaboration with Hfq expression of the transcriptional activator gene csgD and activates the flhDC gene	Repression of curli biogenesis, Activation of flagella synthesis	44
	OmrA, OmrB	trans-encoded ncRNAs sibling ncRNA encoded in tandem, act by direct antisense interaction with the respective 5′-UTR, Hfq-dependent, highly redundant functions	Regulation of curli formation, motility and iron sequestration. Regulation of outer membrane proteins implicated in iron metabolism/ uptake (CirA, FecA, FepA) and protein degradation (OmpT)	44
	sRNA103	trans-encoded ncRNA activates expression of the transcriptional activator gene fimZ	Expression of type 1 fimbriae and the filament protein EspA of the T3SS in EHEC	48
	RyhB	trans-encoded ncRNA acts through direct base-pairing with target mRNAs, Hfq-dependent	Regulation of iron metabolism and other iron-containing proteins, production of siderophores	121
	5′-UTR-chuA	FourU RNA thermometer	Iron uptake in EPEC	132
	GidA	tRNA-modifying enzyme	Regulation of the cytotoxic necrotizing factor 1 (CNF1)	69
	VacC	tRNA-guanine glycolase	Enhances expression of the Shigella T3SS effectors IpaB, IpaC and IpaD, reduces amounts of VirG important for cell-to-cell spreading of EIEC	67
Salmonella	CsrABC	CsrA: RNA binding protein, CsrB/CsrC: CsrA-binding RNAs CsrA directly binds and stabilizes the flhDC and the fliA transcript	Regulation of expression of type 1 and Pef fimbrae, regulation of SPI-1 gene expression via translational repression of the SPI-1 regulator HilD, control of motility and biofilm formation, regulation of c-di-GMP synthesis	49,50,54
	IsrJ	unknown	Regulation of invasion and SPI-1 effector translocation	65
	IsrM	trans-encoded ncRNA reduces mRNA stability by binding to the 5′-UTR of its target mRNA	Control of SPI-1 gene expression by regulation of the global SPI-1 regulator HilE and the effector SopA	66
	LesR-1	antisense RNA interacts with the 3′-UTR of PSLT047 modulating translation rate	Control of virulence in mice	87
	RydC	trans-encoded ncRNA folds as a pseudoknot and interacts with Hfq	Control of the yejABEF operon which interferes with MHCI presentation, counteracts antimicrobial peptides and promotes survival and proliferation within the host. Control of the curli adhesin through repression of the major curli regulator CsgD;	44
	PinT/STnc440	trans-encoded ncRNA Hfq-dependent	Repression of SPI-2 genes and SPI-1 effector genes (sopE, sopE2), manipulation of host cell pathways to promote replication, important for the transition from the extracellular to the intracellular state, influences regulators of the JAK-STAT signaling pathway (e.g. STAT3), IL-8	65,79,85

(*Continued on next page*)

Table 1. (Continued).

Species	RNA/RNase/RNA-modifying enzyme	Mechanism	Virulence function	References
			production, and mitochondria localization and functions. Colonization in pigs and cattle	
	RaoN	trans-encoded ncRNA	Induced under oxidative stress and nutrient limitation, important for survival in macrophages, controls expression of the lactate dehydrogenase gene ldhA	135
	RyhB-1, RyhB-2	trans-encoded ncRNAs Hfq-dependent	Iron homeostasis, oxidative and acidic stress, intracellular growth, redundant functions but RyhB-2 targets some motility genes (flgJ, cheY, and fliF) that are not regulated by RyhB1, and RyhB1 influences safA, acnB expression but not RyhB2	129,130
	SgrS	trans-encoded ncRNA Hfq-dependent	Resistance against phosphosugar stress	60,61
	GidA, MnmE	tRNA-modifying enzymes	Active induction of the T3SS genes invAEG, spaPQ and prgHJ. Control of the oxidoreductase YghA and thiol peroxidase Tpx Important for cell invasion, survival in macrophages and mouse virulence	68
	PNPase	Polynucleotide phosphorylase	Influence on the expression of the AgfA fibers, the SPI-1 invasion genes, as well as the spv and SPI-2 genes important for macrophage survival, controls the ncRNAs RyhB, SgrS, CsrB and CsrC	4,12,73,90,105
Shigella	CsrABC	CsrA: RNA binding protein, CsrB/CsrC: CsrA-binding RNAs	Regulation of attachment and invasion via control of the regulators VirF and VirB	55
	RnaG	antisense RNA to icsA, expression leads to premature termination of icsA transcription	Control of host colonization by repression of the IcsA invasion protein	62,63
	RyhB	trans-encoded ncRNA acts through direct base-pairing with target mRNAs, Hfq-dependent	Regulation of invasion and cell-to-cell spreading via control of VirB synthesis, acid resistance	123-125,127
	5′-UTR-shuA	FourU RNA thermometer	Iron uptake	126,132
	RNase R	ribonuclease	Synthesis of the T3SS effector proteins IpaB, IpaC and IpaD	72
	VacC	tRNA-guanine glycolase tRNA-modifying enzyme	Enhances expression of the Shigella T3SS effectors IpaB, IpaC and IpaD, reduces amounts of VirG important for cell-to-cell spreading	67
Yersinia	CsrABC	CsrA: RNA binding protein, CsrB/CsrC: CsrA-binding RNAs	Contribute to attachment and invasion via regulation of global regulators of invasion factors (e.g.(e.g., InvA, PsaA/pH6 antigen), Control of biofilm formation and motility Regulation of c-di-GMP synthesis	26,35
	Ysr29	trans-encoded ncRNA	Specific to the Y. pseudotuberculosis strain IP32953, important for the virulence in a mouse model of Yersiniosis, represses synthesis of glutathione-S transferase (GST) and activates production of RpsA, OmpA and GroEL	5,6,134
	Ysr35	trans-encoded ncRNA	Survival in a Yersiniosis mouse model	5
	Ysr141	trans-encoded ncRNA acts through base-pairing with the yopJ 5′-UTR	Regulation of T3SS components (YopE, YscF, YopK, YopJ) and the T3SS activator LcrF	5,117
	RyhB-1, RyhB-2	Hfq-dependent trans-encoded ncRNA	Iron homeostasis	122,131
	YopD/LcrH	YopD: RNA-binding protein,interacts with 30S particle of the ribosome LcrF: YopD chaperone YopD-LcrH protein complex binds to the 5′-UTR of target transcripts preventing translation and/or enhancing degradation	Important for T3SS, Yop effector injection, survival of phagocyte attacks	96,97,99
	LcrQ (YscM1, YscM2)	cooperates with YopD-LcrH complex	Important for T3SS, Yop effector injection, survival of phagocyte attacks	98
	5′-UTR-lcrF	FourU RNA thermometer	Control of pathogenesis (T3SS regulation and Yop effector secretion), survival of phagocyte attacks	94,95
	SsrA (tmRNA) /SmpB	SsrA: aminoacylated SsrA RNA mimicking a tRNA or a mRNA SmpB: RNA binding protein that interacts with SsrA	Important for virulence (regulation of T3SS expression, survival of phagocyte attacks), activation of LcrF expression	114
	YbeY	single-strand specific endoribonuclease	Important for virulence (regulation of T3SS expression, survival of phagocyte attacks), regulation of LcrF expression	110
	PNPase RNase E	Ribonucleases part of the degradosome, regulated RNA degradation	Influence on the expression and activity of T3SS, Yop effector injection into host cells, survival of phagocyte attacks. Degradation of the hmsT and pgaABCD transcript, allows rapid regulation of c-di-GMP synthesis and biofilm production	100-103

usually express a set of different adhesion factors (**adhesins**) that mediate tight association to intestinal epithelial cells (Fig. 2). Adhesion can be mediated by pili/fimbriae or afimbrial adhesive surface proteins.[41] Both types of adhesive structures can be regulated by ncRNA-mediated control mechanisms. For example, multiple ncRNAs have been identified in *E. coli* to control expression of curli fimbriae (Fig. 2A). They are involved in attachment of the pathogens to host cells as well as abiotic surfaces and are important for biofilm formation.[42,43] Six different regulatory ncRNAs repress curli production in response to specific environmental changes by downregulation of the LuxR-type regulator CsgD. The ncRNAs McaS, RydC and GcvB block CsgD synthesis dependent on carbon limitation, nutrient uptake and amino acid supply. The ncRNAs OmrA/OmrB inhibit CsgD production under high osmolarity and RprA under membrane stress conditions.[44] They all bind to specific regions within the 5′-UTR of the *csgD* mRNA, which was described as a hub for signal transduction of multiple ncRNAs and reduce CsgD translation initiation.[45] The 2 related regulatory RNAs GlmY and GlmZ activate the translation of glucosamine-6-phosphate synthetase (GlmS), an enzyme important for cell wall biosynthesis.[46,47] Recently, they have also been shown to promote expression of genes encoding the curli adhesin in enterohemorrhagic *E. coli* (EHEC).[48] Another EHEC-specific ncRNA (sRNA103) was identified directly downstream of the Shiga toxin-encoding stx_{2b} gene, which regulates synthesis of the transcriptional regulator FimZ, that controls expression of type 1 fimbriae.[48] Moreover, the regulatory RNAs CsrB and CsrC, which control the abundance and activity of the RNA-binding protein CsrA, seem to influence the expression of type 1 fimbriae, but also the Pef fimbriae in *S.* Typhimurium.[49,50] Interestingly, the 5′-UTR of the type I fimbrial *fimAICDGF* polycistronic mRNA is highly abundant. It titrates CsrA from other transcripts (molecular sponge),

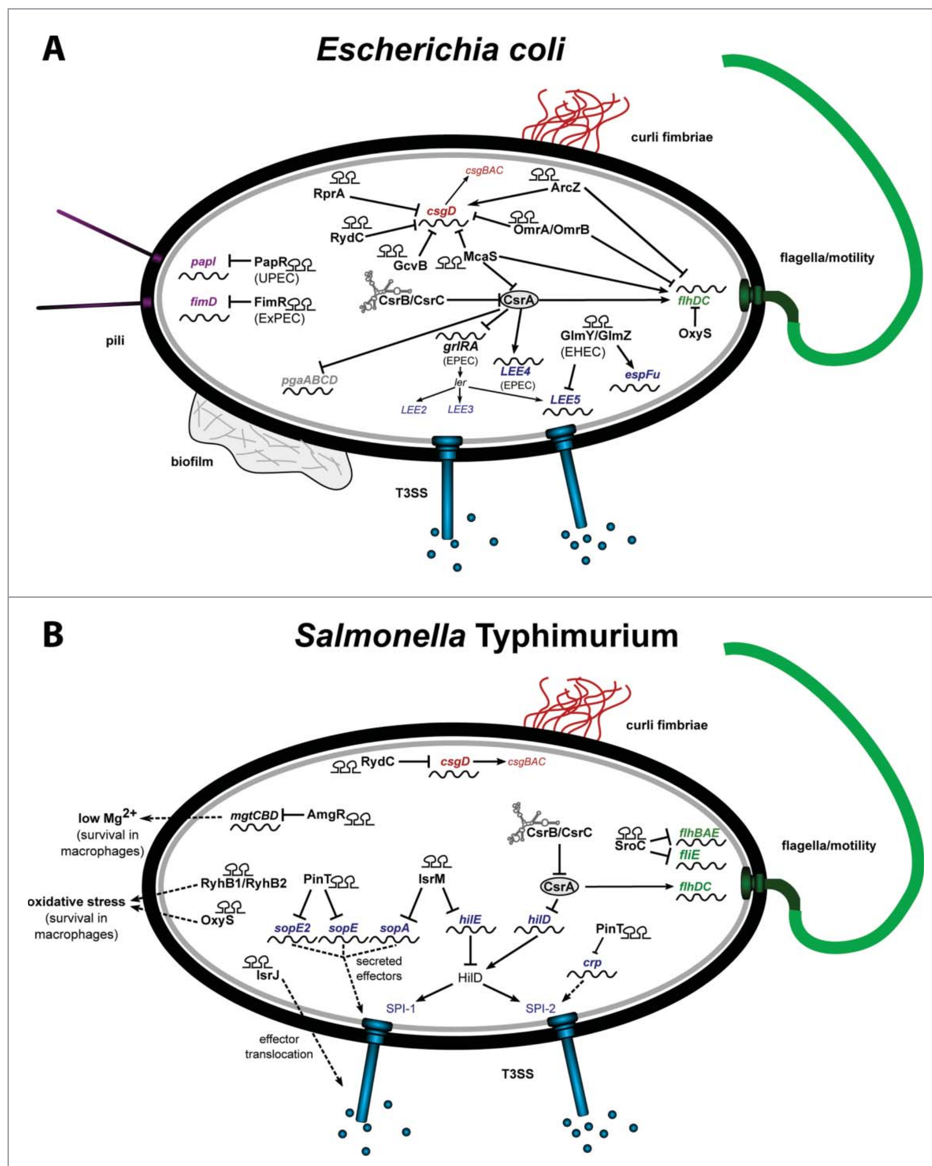

Figure 2. RNA-based control mechanism of *E. coli* and *Salmonella*. (A) Riboregulators controlling the colonization factors of pathogenic *E. coli* important for efficient cell adhesion and invasion. (B) Riboregulators coordinating the expression of *Salmonella* pathogenicity factors, responsible for cell entry, intracellular persistence and proliferation. The riboregulators are given in black and the target genes in green for flagella biosynthesis, in red for curli formation, in purple for pili production, and in blue for T3SS genes encoded on the pathogenicity islands LEE of EHEC/EPEC (A) or SPI-1 and SPI-2 of *Salmonella* (B).

including the *pefA* mRNA which is positively regulated by CsrA.[49,50] This regulatory system promotes a hierarchical control for expression of different types of fimbriae in response to surrounding conditions dedicated to modulate cell adhesion strength and/or specificity.

Regulation of pedestal formation

Enteropathogenic *E. coli* (EPEC) and EHEC strains, which cause attaching and effacing (A/E) lesions characterized by disruption of the intestinal microvilli, form special actin-filled membranous protrusions. These structures termed 'pedestals' emanate beneath the cell-attached bacteria and are important for pathogenicity. All genes required for pedestal formation are encoded in five 5 polycistronic operons in the pathogenicity island *LEE* (locus for enterocyte effacement).[51] In a recent study, the Csr system was shown to regulate pedestal formation.[52] Purified CsrA protein was found to bind to the *LEE4*-mRNA leader encoding the translocator exporter SepL, and the secreted translocators EspA, EspB and EspD of the type III secretion system (T3SS) machinery that promotes injection of effector molecules to initiate pedestal formation by the host cell. CsrA-binding to the leader segment enhances the steady-state transcript and protein levels. In contrast to single-copy expression, multi-copy expression of CsrA globally repressed expression of the *LEE* locus (operons *LEE*1-5), most likely through a reduction of GrlA protein levels. Low GrlA amounts reduce Ler protein levels, which in turn results in a down-regulation of all *LEE*-encoded operons.[52] Moreover, the GlmZ/Y ncRNAs play a role in EHEC pathogenicity as they affect pedestal formation. Both ncRNAs control *LEE*4 and *LEE*5 transcripts and the secreted effector EspFu.[53]

Control of invasion

Following the colonization of the intestinal epithelial tract, the majority of the enteric pathogens penetrates and transmigrates through the intestinal epithelium to colonize subepithelial tissues. This invasion process is generally associated with the synthesis of outer membrane proteins, termed **invasins** that promote active invasion into epithelial cells. Several ncRNAs have recently emerged as key regulators of these invasion proteins in *S. flexneri, Y. pseudotuberculosis* and *S.* Typhimurium. Striking examples are the homologous ncRNAs CsrB and CsrC in *Shigella, Salmonella* and *Yersinia*, which positively regulate the expression of the primary invasion genes in all 3 pathogens. In *Salmonella*, CsrB and CsrC together with CsrA control the translation of the transcriptional activator HilD.[54] HilD promotes expression of the *Salmonella* pathogenicity island 1 (SPI-1) encoded invasion genes through the master regulator HilA [27,54] (Fig. 2B).

In *Yersinia*, the CsrABC system controls the expression of the primary cell invasion factor InvA and the afimbrial adhesin PsaA through a regulatory cascade implicating the LysR-type regulator RovM and the virulence master regulator RovA.[26,35] Moreover, the CsrABC system was also shown to contribute to the attachment and invasion of *S. flexneri* into cultured cells and to cell-to-cell spreading.[55] Although much less is known about the molecular mechanism, it has been demonstrated that similar to *Yersinia* and *Salmonella* influence on invasion was due to decreased expression of the *S. flexneri* virulence factor regulators VirF and VirB, resulting in decreased production of the *Shigella* invasion plasmid antigens (Ipa).[55,56] In summary, the conserved Csr system is generally located upstream of the species-specific regulator cascades controlling expression of the invasion genes.

Besides the global post-transcriptional control system CsrABC, also other more specific conserved regulatory RNAs can influence the host cell invasion process of *Enterobacteriaceae*. One example is the ncRNA SgrS, which controls a metabolic stress response (phosphosugar stress resistance) that occurs upon accumulation of certain glycolytic intermediates, e.g., glucose-6-P.[57,58] Upregulation of SgrS leads to translation repression and destabilization of several transcripts of metabolic genes and sugar transport, e.g. the *ptsG* and *manXYZ* mRNAs.[59] Besides all metabolic targets, in *S.* Typhimurium SgrS is also recruited to the *sopD* mRNA, encoding an important T3SS effector of SPI-1 that contributes to host cell invasion.[60,61] This allows *Salmonella* to adjust expression of its invasion factors according to the metabolic status of the pathogen. Notably, SgrS is highly selective and does not recognize the duplicated sibling mRNA *sopD2* found in some *Salmonella* isolates. The *sopD2* messenger only differs in a single nucleotide within the SgrS binding region.[60]

In addition, the following species-specific regulatory RNAs control host cell uptake of the enteric pathogens. An antisense RNA called RnaG was discovered to control expression of the *icsA/virG* mRNA, encoding the IcsA/VirG invasin of *S. flexneri*. This protein is crucial for cell internalization and cell-to-cell spreading as it induces host actin polymerization, which propels the pathogen from one cell into another.[62,63] The RnaG ncRNA can directly bind to the *icsA* transcript via kissing complexes. This alters the mRNA structure and promotes premature transcriptional termination. Moreover, close convergent location of the *rnaG* and *icsA* promoter results in a reduced transcription of the *icsA* gene through promoter interference, until the activator VirF is produced to enhance *icsA* transcription.[62,63] RnaG is most likely synthesized during the initial stages of the infection when *Shigella* first reaches sub-epithelial tissues to prevent premature production of IcsA, which may lead to unwanted immune responses.

Furthermore, an Hfq-dependent *trans*-acting regulatory RNA AfaR (SQ109) of pathogenic *E. coli* was lately characterized which regulates expression of afimbrial adhesins of the Afa family.[64] AfaR interacts with the 5′-UTR of the *afaD* invasin mRNA. This initiates RNase E-mediated degradation of the transcript, leading to downregulation of AfaD-VIII invasin production in pathogenic *E. coli*.[64] A computational screen and experimental verification identified several unique ncRNA genes encoded within genetic islands in *S.* Typhimurium.[65] Of those a 74 nt ncRNA IsrJ was shown to be activated by the major regulator HilA of the SPI-1 virulence genes and to be required for host cell invasion and effector translocation.[65] Another *trans*-acting ncRNA, IsrM, is implicated in expression of the SPI-1-encoded virulence genes. *Salmonella* deficient for *isrM* is impaired in its ability to invade and replicate in human cells and to colonize ileum and spleen of infected mice.[66] IsrM targets the 5′-UTR of the mRNA of the SPI-1 effector SopA and the mRNA of HilE, a global regulator of bacterial evasion genes. This demonstrates that the pathogenicity island-encoded

ncRNAs seem to function as a distinct class of specific virulence regulators that significantly contribute to pathogenicity.

It is worth mentioning that tRNA-modifying enzymes have been found to play a potential role in the expression of colonization factors of *S. enterica* and *S. flexneri*. One example is the VacC protein of *S. flexneri*, which is homologous to a tRNA-guanine transglycosylase of *E. coli*. A *vacC* mutant of *Shigella* and entero-invasive *E. coli* (EIEC) is characterized by lower levels of the T3SS proteins IpaB, IpaC and IpaD that are essential for *Shigella* and EIEC invasion into host cells, and by reduced amounts of VirG, a protein important for cell-to-cell spreading.[67] A more detailed analysis indicated that this phenotype is caused by a downregulation of the major virulence regulator VirF.

A more comprehensive investigation was performed with the interacting tRNA modifying enzymes GidA and MnmE. Knock-out mutations in the equivalent genes were shown to significantly reduce the colonization of *S.* Typhimurium in liver and spleen. This effect can be explained in part by the fact that several colonization genes, including the T3SS genes *invAEG, spaPQ* and *prgHJ* important for host cell invasion, were down regulated in the attenuated mutants. However, the overall contribution of both tRNA modifying enzymes to host tissue colonization is certainly greater as they also control expression of several proteins promoting the survival of *Salmonella* under the stressful conditions experienced within host macrophages, i.e. the oxidoreductase YghA, and the thiol peroxidase Tpx.[68] In this context it is further notable that a study by Yu *et al.*[69] reported that the tRNA modifying enzyme GidA inhibits translation of the cytotoxic necrotizing factor 1 (CNF-1). CNF-1 is an important toxin in meningitis-causing *E. coli* K1, uropathogenic *E. coli* strains but also enteric *E. coli* isolates.[70,71] It is likely that tRNA modifying enzymes help the pathogen to fine-tune the synthesis of highly energy-consuming virulence factors under stressful conditions.

Another RNA-based mechanism by which bacteria can alter gene expression to promote host colonization is the regulation of RNA stability. In fact, the ribonuclease R (RNase R) of *S. flexneri* has been reported to be required for the synthesis of the effector proteins IpaB, IpaC and IpaD.[72] In *S.* Typhimurium the polynucleotide phosphorylase (PNPase) affects the levels of a subset of virulence mRNAs, in particular those encoding the AgfA fibers and the invasion genes of SPI-1.[73] However, the precise molecular mechanisms how the RNases target these virulence genes remain to be elucidated.

RNA-mediated suppression of host stresses and immune responses

Regulatory ncRNAs and RNases with impact on intracellular survival and replication of Salmonella

Salmonella strains that passed the intestinal epithelial layer are engulfed and taken up by phagocytic cells of the innate immune system such as macrophages.[74,75] *S.* Typhimurium evades macrophage killing by secreting effector proteins encoded on *Salmonella* pathogenicity island 2 (SPI-2), that generate a special protective membrane-bound compartment, the *Salmonella*-containing vacuole (SCV). In the SCV *Salmonella* replicates and is able to form persister cells.[76] Use of computational and experimental approaches, including recent RNA-Seq analyses, led to the identification of numerous ncRNAs (> 200) which are expressed or even activated during the infection of murine and human cells.[65,77-79] Those ncRNAs might contribute to the regulation of pathogenicity factors during infection, but so far very little is known about their role in virulence. Among the ncRNAs which are strongly up-regulated within macrophages and/or human epithelial cell are the iron-regulated ncRNAs RyhB1/RfrA and RyhB2/RfrB/IsrE (also found to be upregulated in *Salmonella* Typhi within macrophages),[80] as well as the stress-induced ncRNAs OxyS, OmrA, OmrB, MicA, MicF, MicL/RyeF and RybB. Expression changes of these conserved ncRNAs seem to reflect iron-limitation, reactive oxygen species and multiple other stressors experienced within the hostile vacuolar compartment of host cells (see also below). In addition, several other less-characterized ncRNAs were up-regulated in macrophages (STnc440/PinT, STnc470 and STnc3750) and/or human epithelial cells (STnc440/PinT, MgrR, IsrH), whereas others were downregulated (DapZ, STnc270/InvR) corresponding to the induction of the SPI-2 and repression of the SPI-1 encoded genes.[65,78,79,81,82] The highest induced 80 nt ncRNA PinT is encoded on a *Salmonella*-specific horizontally acquired locus. It was found to be controlled by the SPI-2 activating 2-component system PhoP/PhoQ which is crucial for intracellular survival and replication of *Salmonella*.[83] [79,84] Most strikingly, this ncRNA was also previously identified as a potential virulence factor of *Salmonella* in a genome-wide *in vivo* mutagenesis screen (called TraDIS) in pigs and cattle.[85] A dual RNA-Seq time-course of *Salmonella*-infected cells from humans and pigs, subsequent validation, and pulse-induced expression of PinT demonstrated that this ncRNA represses transcription of the SPI-2 genes very early after host cell invasion. Repression seems to occur upstream of the SPI-2 master regulator SsrB independent of PhoP/PhoQ and HilD, and seems to involve the metabolic global regulator CRP as signal transmitter for PinT-mediated activation of SPI-2.[79,86] Interestingly, in addition to a premature activation of SPI-2, the transcripts of the secreted SPI-1 effectors SopE and SopE2 were significantly de-repressed in *Salmonella pinT* mutants within macrophages. This strongly suggests that PinT plays a crucial role in the transition from extracellular to intracellular life-style after host cell invasion. The elegant dual RNA-Seq approach by Westermann *et al.* further revealed that PinT-mediated influence on SPI-1 and SPI-2 genes resulted in a differential regulation of key regulators of the JAK-STAT signaling pathway and chemokine secretion (e.g., SOC3, STAT3, IL-8).[79] Another example of a regulatory RNA that is implicated in the control of intracellular *Salmonella* is the antisense RNA LesR-1. The *lesR-1* gene is encoded on the pSLT virulence plasmid and is preferentially expressed in non-growing dormant bacteria residing within fibroblasts. Direct interaction of the 3′-end of the asRNA to the PSLT047 transcript results in a significant reduction of the PSLT047 protein level, and a deletion of *lesR-1* impaired virulence in a mouse infection model.[87] Another recently discovered RNA-based mechanism of *S.* Typhimurium impacts the expression of SPI-2 and the plasmid-encoded *spv* genes needed for intracellular

survival and propagation inside the macrophages of liver and spleen.[88,89] The mutational inactivation of the PNPase gene (*pnp*) resulted in an up-regulation of SPI-2 and the *spv* genes and this altered the pathogenesis of the infection. *Salmonella pnp* mutants established more frequently persistent infections in Balb/c mice compared to the wild-type which caused mainly acute systemic infections.[73] The exact mechanism is still unknown, but elimination of the *spvR* regulator gene inhibited expression of the *spv* gene cluster and affected growth also in the absence of *pnp*, indicating that PNPase acts upstream or at the level of SpvR.[90]

Post-transcriptional control mechanisms of Yersinia *T3SS/Yop-mediated defense against immune cells*

Also pathogenic yersiniae are confronted with phagocytic immune cells such as neutrophils, macrophages and dendritic cells after entry of the sub-epithelial, gut-associated lymphatic tissues. In order to resist phagocytosis by professional phagocytes and induce apoptosis of the host immune cells they express a virulence plasmid-encoded Ysc-Yop T3SS to inject multiple effector molecules, the Yops.[91,92] During infection it is a prerequisite for *Yersinia* to tightly control the expression of the secretion system, as it requires a large energetic effort of the bacteria to fuel the production of the components of this injectisome and promote effector translocation. Several RNA-based control mechanisms have already been characterized which are part of a complex, multi-layered regulatory network that controls the expression and synthesis of the *Yersinia* T3SS.[93] Many years ago Hoe *et al.* [94] published a report in which they predicted post-transcriptional regulation of the major transcriptional activator (LcrF/VirF) of T3SS/Yop expression. New results by Böhme *et al.* [95] further showed that translation of the *lcrF* mRNA is controlled through a FourU RNA thermometer, a thermo-responsive secondary structure formed of 2 stem-loops within the intergenic region of the *yscW-lcrF* transcript. The first stem-loop enhances the stability of the second hairpin which includes a stretch of 4 uridines (FourU) base-paired with the AGGA sequence of the *lcrF* ribosome binding site.[95] Structure-probing and toe-printing analysis further demonstrated thermo-induced melting and partial opening of the second stem-loop which allows ribosome binding at 37°C, but not at moderate temperatures. The importance of the RNA thermometer for the control of *Yersinia* virulence was proven with mutant variants of the *lcrF* RNA thermometer that prevent melting of the thermometer at host temperature. Mice infected with this closed thermometer variant survived the infection without any disease symptoms.[95]

Another intriguing observation is that a complex of the translocon protein YopD and its secretion chaperone LcrH controls the translation of a cohort of *ysc*/*yop* mRNAs. The YopD-LcrH complex was found to bind mRNAs in the 5′-UTRs and promotes translation repression most likely by blockage of the ribosome binding site or/and by enhancing the degradation of the *ysc/yop* transcripts.[96,97] Translational repression is eliminated when intracellular YopD levels decrease as a result of activated secretion. AU-rich regions including multiple AUAAA sequence motifs in the proximity of the ribosome binding site appear to support YopD-LcrH complex binding, but this alone does not seem to be sufficient to confer YopD-LcrH mediated translational repression.[97,98] Furthermore, LcrQ (YscM1 and YscM2 in *Y. enterocolitica*) was shown to participate in the post-transcriptional control of the *ysc/yop* genes. The molecular mechanism is unclear, but it has been proposed that it may associate with the YopD-LcrH complex to repress *ysc/yop* translation.[98] A recent study further showed that YopD also associates with 30S ribosomal particles in an LcrH-dependent fashion.[99] This suggests that transient interaction of the YopD-LcrH-LcrQ/YscM complex on the *ysc/yop* mRNA with the 30S particle might affect translational initiation by perturbing the formation of the 30S complex before the 50S particle binds to assemble the ribosome.

Another study analyzing the post-transcriptional regulation of T3SS showed that *Y. pseudotuberculosis* expressing a dominant negative variant of RNase E (a *rne* knock-outknockout is lethal) or a PNPase mutant secreted only a reduced amount of the YopE effector protein.[100-102] Counter-intuitively, a Δ*pnp Yersinia* mutant possessed increased levels of all 3 T3SS-encoding and several *yop* transcripts, demonstrating that the PNPase affects the expression and activity of the T3SS by distinct mechanisms.[101] This is in clear contrast to the situation in *Salmonella* in which increased T3SS expression levels matched with a corresponding increase in the T3SS-mediated increase of bacterial invasion [73] (see above). RNase E and PNPase both associate with the RNA helicase RhlB and the glycolytic enzyme enolase (Eno) to form the RNA degradosome, a large multi-protein complex or hyper-structure controlling RNA degradation in *Yersinia* and other enteric pathogens.[103,104] Most interestingly, blockage of T3S could be restored in the *pnp* mutant strain by expressing the S1 domain of PNPase and RNase E.[101] The S1 domain is characterized by a distinctive β-barrel core, which binds to nucleic acids, carbohydrates or is involved in protein-protein interactions. Use of different truncated S1 domains further demonstrated that especially residues 50-65, forming the conserved oligonucleotide binding cleft, play an important role in controlling Yop secretion, but are not involved in the alteration of T3SS/Yop expression levels.[101] This finding indicated that the S1 domain of PNPase or RNase E might bind an mRNA or ncRNA that modulates T3SS-mediated Yop secretion.

Why PNPase influences T3SS/Yop expression in the opposite manner is less clear.[101] It is assumed that the yersiniae prepare and/or readjust the T3SS/Yop expression profiles through the removal of unnecessary T3SS transcripts prior or after the attack of immune cells to minimize the energetic burden.[103] The molecular mechanism by which PNPase controls T3SS/Yop expression is also unknown. However, PNPase was shown to control several regulatory RNAs including RyhB and SgrS, known to be important to control virulence-relevant metabolic traits in *Salmonella*.[4,105] It is thus tempting to speculate that the degradosome could also protect ncRNAs of *Yersinia* implicated in the expression of the T3SS machinery. In fact, the RNA chaperone Hfq is required for the expression of many *Yersinia* RNAs,[5,7,106] and regulates expression of PNPase.[107] Hfq was found to play a critical role in *Yersinia* virulence (i.e. phagocytosis resistance, intracellular survival, growth within mice organs) by participating in the regulation of the expression of T3SS effector proteins.[108,109] The abundance of all tested Yop

effector proteins was decreased in the absence of Hfq, although the *yop* transcript levels remained unchanged. This together indicates that Hfq- and PNPase/RNaseE-dependent ncRNAs participate directly or indirectly through interactions with a T3S/Yop regulator in the control of Yop effectors. In fact, a recent study of our group further showed that several antisense RNAs are expressed from the virulence plasmid opposite of important T3SS genes, including *ypkA, yopD, lcrV*, and *yscC*.[7] All these T3SS-associated proteins were upregulated in a Δ*pnp* mutant of *Y. pseudotuberculosis*, suggesting that PNPase negatively regulates the expression of the T3SS machinery, e.g. through the asRNAs. In addition to RNase E and PNPase, another ribonuclease, YbeY, was shown to repress *ysc/yop* expression in *Y. enterocolitica* through a down-regulation of VirF/LcrF levels and manipulates many other virulence-related features.[110] How this RNase influences expression of the virulence determinants is unclear. It is likely that it implicates a more general gene control process as this RNase is usually implicated in the processing of the 16S rRNA and ribosome biogenesis as well as in the late-stage 70S ribosome quality control.[111,112] It generally recognizes defective 30S ribosomal particles and functions together with RNase R or PNPase to remove non-functional 70S ribosomes. YbeY acts as a single-strand-specific endoribonuclease that is able to degrade rRNA and mRNA and has an impact on ncRNAs.[111,113]

Intriguingly, also the unique bacterial translational control system, composed of the small stable RNA A (SsrA/tmRNA/10Sa/sR022/Yp-sR31) and the small RNA-binding protein B (SmpB), affects expression of the *ysc/yop* genes.[114] This system rescues stalled ribosomes from incomplete transcripts, a process called *trans*-translation, to maintain the bacterial translational machinery in a fully operational state.[115] The SmpB protein interacts with the amino-acylated SsrA RNA mimicking a tRNA and mRNA, and this complex enters the empty site of a stalled ribosome.[116] A *trans*-peptidation reaction then links the unfinished peptide chain to SsrA, and SsrA replaces the aberrant mRNA. This promotes translation of an additional 11 amino acid residue tag until the apparatus reaches a built-in stop codon marking the polypeptide for degradation by bacterial proteases.[115] A detailed study addressing the function of the SsrA-SmpB tagging and ribosome rescue system in *Y. pseudotuberculosis* demonstrated that loss of both genes renders the bacteria sensitive to sublethal antibiotic concentrations, less efficient in their cytotoxicity toward macrophages, and avirulent in the oral mouse infection model.[114] This phenotype was consistent with the observation that expression of the key regulator of the *ysc/yop* gene LcrF was significantly reduced in a *ssrA-smpB* deficient mutant and could not be activated under secretion-inducing conditions.[114]

Although the precise molecular mechanisms and the interplay of the different post-transcriptional regulatory steps in the complex multi-layered network of T3SS/Yop expression and synthesis are far from understood, they clearly demonstrate that this pathogen resides in an energy-balanced stand-by position that prepares the pathogen for translocation of effectors immediately upon host cell contact. The regulatory circuits allow only low-level transcription, but no translation of the *lcrF* and *ysc/yop* mRNAs in the absence of host cell contact. However, under secretion conditions, tight coupling between the transcription, translation, RNA degradation and secretion machineries enables the pathogen to immediately upregulate synthesis and export of the virulence determinants.

In contrast to the previous described riboregulators, the ncRNAs Ysr35 and Ysr141 are *Yersinia*-specific ncRNAs, which have also been found to contribute to virulence. Very little is known about Ysr35, but significant compromised survival of a Ysr35 mutant in a Yersiniosis mouse model indicates that this ncRNA is important for *Yersinia* adaptation to its host.[5] Ysr141 is an unstable ncRNA, which is encoded on the *Yersinia* virulence plasmid on the opposite strand within the intergenic region of *yopH* and a putative transposase.[5,117] Expression of Ysr141 stimulates the production of multiple T3SS/Yop proteins (e.g. YopE, YscF, YopK) as well as their main activator LcrF, and seems to control *yopJ* mRNA translation through basepairing with its 5′-UTR.[117]

RNA-based control of virulence-associated traits

Control of host-adapted metabolism and ion homeostasis through small RNAs and riboswitches

Over the last decades many regulatory RNAs and riboswitches have been discovered as important regulators affecting myriad aspects of bacterial stress responses, ion homeostasis, metabolism, motility and other physiological properties, which also influence bacterial virulence. In particular riboregulators that are conserved among the well-characterized *Enterobacteriaceae* and implicated in the control of primary and secondary metabolic pathways have been characterized in more detail. The identified RNA-controlled mechanisms and their action at the interface of bacterial metabolism and virulence control have been recently summarized in several comprehensive review articles [4,118-120] and are thus not in the focus of this article.

Ion homeostasis

One of the most important and best-characterized small ncRNA is RyhB found in all enteric *E. coli, Salmonella, Shigella*, and *Yersinia* species. The RyhB ncRNAs are activated following iron scarcity and are negatively regulated by the ferric uptake regulator Fur.[121,122] The RyhB ncRNAs regulate iron homeostasis by (i) inhibiting the translation of transcripts encoding non-essential iron-containing proteins under iron starvation conditions in order to liberate iron for essential iron-dependent cellular functions and by (ii) upregulation of the synthesis of iron-chelating molecules (siderophores) to scavenge iron under iron-limiting conditions as experienced during infection.[121] RyhB is highly similar between *E. coli* and *Shigella* and impacts conserved, but also species-specific virulence genes.[123-125] One of which is the virulence regulator VirB controlling the expression of the IcsP protease, which limits the production of the actin polymerizing IcsA protein.[125,126] Upon cell entry, iron levels become limiting and relieve Fur-mediated repression of RyhB transcription, which in turn increase host actin polymerization and cell-to-cell spreading through activation of the VirB-IcsP-IcsA cascade,[124] and promotes acid resistance by a so far unknown mechanism.[127]

Interestingly, both *Salmonella* and *Yersinia* possess 2 RyhB homologs, RyhB1 and RyhB2, recently termed sibling ncRNAs,

which are slightly differentially regulated in response to iron and nutrient starvation.[65,122,128] Both ncRNAs have additional redundant functions such as protecting the pathogen against oxidative and acidic stress,[129,130] and they are both highly induced and important for the intracellular growth of *Salmonella* [65,77-80] (see also above/below). However, it appears that sequence differences at the 5′-end of the ncRNAs could account for some differences in the regulatory targets of the RyhB RNAs. For instance, Kim *et al.* have shown that RyhB2 of *Salmonella* targets some motility genes (*flgJ, cheY*, and *fliF*) that are not regulated by RyhB1 and vice versa, RyhB1 influences *safA* and *acnB* expression, but not RyhB2.[129] In *Yersinia*, the region that mediates target gene recognition is highly conserved between both RyhB ncRNAs, indicating regulatory redundancy, but only RyhB1 is stabilized by Hfq and is slightly more sensitive to alterations of degradosome factors.[122,131] Differences in the 5′-end of the RNAs may result in these differences in stability. Both ncRNAs are strongly expressed in the infected tissues, but a *ryhB1/ryhB2* double mutant has no major influence on the colonization and dissemination of the pathogen, indicating that other systems with redundant function(s) can compensate for the loss of the ncRNAs.[122]

Another riboregulator implicated in iron homeostasis is the FourU RNA thermometer located within the 5′-UTR of the *Shigella* heme uptake system *shuA* and in the orthologous gene *chuA* in pathogenic *E. coli*.[132] Transcription of the heme transporter is subject to iron-dependent repression by the Fur protein and translation is under control of the thermo-responsive RNA thermometer, ensuring that ShuA synthesis only occurs when *Shigella* encounters heme as a potential iron source in the human body.[132]

Stress response

Multiple ncRNAs have been identified which are implicated in general and specific stress responses. Several of them are expressed in all well-characterized enteric pathogens of the family *Enterobacteriaceae*, i.e. OxyS, 6S RNA, FnrS, ArcZ, MicF, OmrA, OmrB, RybB, RprA, DrsA, SgrS, SraL, RyhB, (RyhB1, RyhB2). Their physiological role has recently been summarized in a review by Michaux *et al.* [133] They confer resistance against acid, oxidative and osmotic stress, cell envelope perturbations and nutrient starvation/stress, which are experienced by the pathogen during the different stages of the infection (i.e., oxidative and nutrient stress within phagosomal compartments in host cells, and acid and osmotic stress in the mammalian gastrointestinal tract). Many of these conserved ‘core’ ncRNAs are implicated in the primary metabolism, ion/nutrient sensing and transport, and participate in the fine regulation of cellular processes important to adapt to environmental changes. However, some of them were also found to control species-specific virulence factors (e.g. GlmY/Z, SgrS, ArcZ), which are explained in more detail in the individual virulence chapters.

Other identified ncRNAs involved in the regulation of stress responses are species- or even strain-specific. The ncRNA Ysr29 is specific to *Y. pseudotuberculosis* strain IP32953 and was found to contribute significantly to mortality in a mouse model for Yersiniosis.[5,6] Ysr29 was shown to repress the synthesis of glutathione-S transferase (GST) and activates production of RpsA, OmpA and GroEL.[5,6] As GST allows protection against the damage of oxidative stress, it has been suggested that this ncRNA, which is mostly induced at moderate temperatures, could be involved in the response to reactive oxygen species produced by insect vectors, e.g. flies and fleas upon infection.[134] In addition, the *Salmonella*-specific 200 nt long ncRNA (RaoN) encoded on SPI-11 between the *cspH* and *envE* locus was recently shown to be highly induced under oxidative stress conditions and nutrient limitation. Loss of the *raoN* gene resulted in high susceptibility against both of these stresses and reduced the survival of the pathogen in macrophages. RaoN controls the expression of the lactate dehydrogenase gene *ldhA*, and it is assumed that it promotes stress resistances at least to some extent through the generation of NAD^+ from NADH when converting pyruvate to lactate.[135] A multi-component glutamate-dependent acid resistance system (GadABC) is responsible for the extreme acid tolerance of enteric *E. coli* and *Shigella*. This system converts glutamate into γ-aminobutyric acid and exports the product in exchange of extracellular glutamate to consume intracellular H^+. Its expression is tightly regulated by the transcriptional activators GadE, GadX and GadW, and the regulatory RNA GadY encoded within the intergenic region of *gadX* and *gadW*.[136] The GadY ncRNA is induced upon nutrient starvation and acid stress through the alternative sigma factor σ^S and interacts with the 3′-UTR of the *gadX* transcript. This interaction stimulates processing of the *gadX* gene after the stop codon resulting in more stable *gadX* and *gadW* transcripts.[136]

Concluding remarks

The rapidly increasing amount of transcriptomic data obtained from next-generation sequencing approaches and tiling microarrays combined with sophisticated bioinformatics tools provided us with a vast number of transcribed but non-translated sensory and regulatory RNA elements implicated in the fine-tuning of physiological and cellular processes important for pathogenesis. The diversity of their physiological role, the complexity and accuracy of their molecular function as well as their central role in the coordinated regulatory network of virulence-relevant processes illustrates their regulatory potential and relevance. The huge amount of riboregulators presented in this review not only demonstrates that RNA-based control mechanisms represent a crucial additional level of regulation, as they allow a less-energy consuming and faster control of gene expression, it also shows that it enables the bacteria to fine-tune and coordinate their responses to environmental changes in a more rapid and sensitive fashion. The majority of conserved ncRNAs of *Enterobacteriaceae* contribute to the regulation of metabolism and stress responses, but these ‘core’ ncRNAs were also hijacked to regulate mRNAs of horizontally acquired virulence factor through Hfq and base-pairing or other conserved global RNA-binding regulators, e.g. CsrA. In addition, many species- or even strain-specific ncRNAs have evolved which modulate more specific virulence processes of the pathogen to promote optimal adaptation to its host niches. The combined set forms an impressive number of versatile, programmable and highly efficient RNA-based regulators. As outlined in the review by Papenfort & Vanderpool,[137] no special characteristics seem to define an ncRNA as an inhibitor or activator. Action of an ncRNA seems to depend on the target and how the ncRNA

interferes with its structure or recruited proteins that influence its stability or translation. The characterization of the molecular details of the individual control mechanisms will be key to understand the full potential of riboregulators.

Perspectives and future challenges

Considering the tremendous amount of sensory and regulatory RNAs identified in the different pathogenic *Enterobacteriaceae*, very little is known about their physiological relevance and molecular action. Only a very small number of ncRNAs has been functionally characterized and the direct and indirect targets of most regulatory RNA elements have yet not or only partially been identified. The analysis of their physiological role, including the characterization of the molecular interactions with their individual targets constitutes one of the great challenges that we are currently facing in our attempt to understand bacterial pathogenesis and virulence control.

In addition, there are many intriguing questions and unaddressed issues associated with the ncRNA elements:

(i) Recently, several regulatory and sensory RNAs were shown to encode small peptides (e.g. SgrS, *mgtA* leader), which participate in the sensory or regulatory process of the encoding ncRNA or 5′-UTR.[60,138] A screening of the bacterial ncRNAomes indicates that there are many more potential dual function ncRNAs. Up to date, their potential has been mostly disregarded because of the difficulty in the detection of small oligopeptides. A combination of next generation sequencing-based transcriptomics and novel proteomic tools optimized for small peptides will help to identify and characterize their biological activities and regulatory potential.

(ii) More sensitive RNA-Seq technologies now allow us to simultaneously profile the transcriptome of the pathogen and the infected host cells.[78,79] This gives us a catalogue of putative virulence-relevant riboregulators, but which of them are relevant for pathogenesis is still an open question. Based on the observed redundancy of their function more powerful tools are needed that allow high-throughput evaluation and exploration of their role during infection. Transposon-insertion sequencing (TraDIS) [85,139] or adaptation of the CRISPR-Cas technology for high-throughput use could be applied to construct single and multiple ncRNA-deficient mutants of pathogens and dissect the influence of sensory and regulatory RNAs on the overall fitness and pathogenicity. Another possibility to gain a systems-level view of ncRNA activity is to globally follow the dynamics of their structure, stability and translational dynamics of RNAs. Moreover, many enteric pathogens, including *Yersinia* and *Salmonella* form phenotypically distinct subpopulations in host niches [140-142] which demands new highly sensitive single-cell dual RNA-Seq protocols to characterize the different expression programs in individual bacterial cells during infection.

(iii) Although some prototypical control mechanisms of ncRNAs have emerged, it became also obvious that ncRNAs use numerous ways to influence gene expression. A future task will be to find global approaches that will allow us to comprehensively trace the regulatory networks and the target regulons of specific ncRNAs, identify proteins (e.g., RNA chaperones, degradation adaptors) involved in their regulatory activity and/or RNA degradation functions associated with their action. Another unsolved question is: What is the advantage of having sibling ncRNAs with redundant functions? It is possible that they differ in their regulatory outcome - target regulons and/or the strength of target regulation, which implies a slightly better fitness.

(iv) The function of sensory and regulatory RNA elements depends on the formation of a particular secondary and tertiary structure. New approaches in which structure-specific chemical cleavage is combined with RNA-Seq have been developed which allow detailed RNA probing and structural profiling *in vivo*.[143] These methods not only enable us to identify RNA thermometers and RNA riboswitches on the system-level, they will also allow us to follow the dynamic of these processes in the context of an infection and compare RNA structuromes from different clinical isolates to identify functionally important differences in RNA structures (riboSNitches).

(v) Recently, a bacterial RNA has been shown to use a *cis*-acting signal to change its localization within the bacterial cell in response to environmental changes.[39,40] Development of fluorescent labels for direct tracking of RNA *in vivo* will give novel insights into the spatial organization of (s)RNAs (e.g. association of an ncRNA to hyper-structures such as translational and degradation machineries) and dynamic changes of ncRNA transport in the context of an infection (e.g., during secretion of effectors upon host cell contact).

(vi) RNA modifications are known to manipulate the stability and interaction of RNAs with interacting proteins. Transcriptional profiling employing different RNA-Seq-based technologies now enables us to identify intrinsic modified nucleotides and investigate their regulatory potential.

(vii) Another tempting question is how we could exploit ncRNAs or associated RNA-based control systems for therapeutic applications to disrupt host-pathogen interaction. One possibility is the discovery of small molecule inhibitors or design of tailor-made compounds for a particular ncRNA or riboswitch.[144,145] In fact, an effective riboswitch inhibitor (ribocil) and an aptamer-based riboswitch blocker have recently been identified.[144,146] A pitfall in this approach is that many *trans*-encoded ncRNAs are non-essential or have partially redundant function and are per se not very well suited as drug targets. Consequently, central riboregulators such as CsrA or global transcriptional or post-transcriptional regulators such as Crp or Hfq which influence many RNA-mediated control systems seem to be much more promising targets for the design of novel diagnostics and therapeutic measures.

Disclosure of potential confllicts of interest

No potential conflicts of interest were disclosed.

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