RNA-based mechanisms of virulence control in *Enterobacteriaceae*

Ann Kathrin Heroven, Aaron M. Nuss and Petra Dersch*

Department of Molecular Infection Biology, Helmholtz Centre for Infection Research, Braunschweig, Germany

*Corresponding author:

Petra Dersch
Dept. of Molecular Infection Biology,
Helmholtz Centre for Infection Research
38124 Braunschweig
Germany
Phone: +49-531-6181-5700
FAX: +49-531-6181-5709
e-mail: petra.dersch@helmholtz-hzi.de

Running title: Riboregulators controlling virulence in *Enterobacteriaceae*

Keywords: regulatory RNAs, riboswitches, RNA thermometers, CsrA, regulated RNA degradation, gene regulation, virulence

Summary

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 the 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.

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 *Entero-bacteriaceae*.

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 with 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 usually not translated. The vast majority of ncRNAs basepairs with one or multiple target mRNAs and influences distinct or numerous processes important for house-keeping 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. Ribo-

126 switches typically regulate expression of protein-encoding mRNAs, but recently they
127 have also been shown to control the expression of non-coding RNAs and regulate
128 binding of proteins, e.g. the ribonuclease E (RNase E) or the transcriptional termi-
129 nation factor Rho to nascent RNAs [17, 18]. Riboswitches sense different metal ions
130 (e.g. Mg^{2+}, Mn^{2+}, fluoride), metabolites (e.g. vitamin B12, S-adenosylmethionine
131 (SAM), thiamine pyrophosphate, flavin mononucleotide) or signaling molecules (e.g.
132 c-di-GMP). Binding of these molecules to an evolutionary conserved ligand binding
133 segment (sensor region) leads to the formation of an alternative molecule-bound
134 RNA conformation which influences expression of the coding sequences located
135 downstream. The set of regulated downstream genes mainly includes biosynthesis
136 and transport systems of the equivalent or related metabolites [19]. Hence, the cognate
137 riboswitch ligand does not only act as the stimulating substance, it often represents
138 the end product of the controlled metabolic pathway and is implicated in feed back
139 control.

140 Besides riboregulators, several global, highly conserved transcription factors and
141 RNA chaperones have been shown to participate in RNA-based control mechanisms
142 of virulence [7, 20]. One important player in the coordinated control of regulatory RNA
143 function is the RNA chaperone Hfq. The Hfq protein was originally identified in *E. coli*
144 many decades ago as a host factor essential for the replication of the RNA phage Qβ
145 and has since then been shown to be crucial for virulence of many enteric pathogens
146 [21]. Mutants of enteropathogenic *Salmonella, E. coli, Yersinia* and *Shigella* species
147 have pleiotropic phenotypes. Lack of Hfq leads to reduced growth rates, altered
148 utilization of nutrients and major changes of the metabolic profile, and dramatically
149 alters expression of pathogenicity factors and virulence-relevant traits which impairs
150 virulence [21]. Hfq is ubiquitous and highly conserved among the *Enterobacteriaceae*
151 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 *Entero-bacteriaceae* 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 two 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 two 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 the adhesion of the bacteria 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 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 inhibits 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 two 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 the 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 control 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), 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 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. pseudotuberculosi*s 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 three 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 re-cognize 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 down-regulation 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 the 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

385 experimental approaches, including recent RNA-Seq analyses, led to the
386 identification of numerous ncRNAs (> 200) which are expressed or even activated
387 during the infection of murine and human cells [65, 77-79]. Those ncRNAs might
388 contribute to the regulation of pathogenicity factors during infection, but so far very
389 little is known about their role in virulence. Among the ncRNAs which are strongly up-
390 regulated within macrophages and/or human epithelial cell are the iron-regulated
391 ncRNAs RyhB1/RfrA and RyhB2/RfrB/IsrE (also found to be up-regulated in
392 *Salmonella* Typhi within macrophages) [80], as well as the stress-induced ncRNAs
393 OxyS, OmrA, OmrB, MicA, MicF, MicL/RyeF and RybB. Expression changes of these
394 conserved ncRNAs seem to reflect iron-limitation, reactive oxygen species and
395 multiple other stressors experienced within the hostile vacuolar compartment of host
396 cells (see also below). In addition, several other less-characterized ncRNAs were up-
397 regulated in macrophages (STnc440/PinT, STnc470 and STnc3750) and/or human
398 epithelial cells (STnc440/PinT, MgrR, IsrH), whereas others were down-regulated
399 (DapZ, STnc270/InvR) corresponding to the induction of the SPI-2 and repression of
400 the SPI-1 encoded genes [65, 78, 79, 81, 82]. The highest induced 80 nt ncRNA PinT is
401 encoded on a *Salmonella*-specific horizontally acquired locus. It was found to be
402 controlled by the SPI-2 activating two-component system PhoP/PhoQ which is crucial
403 for intracellular survival and replication of *Salmonella* [83 79, 84]. Most strikingly, this
404 ncRNA was also previously identified as a potential virulence factor of *Salmonella* in
405 a genome-wide *in vivo* mutagenesis screen (called TraDIS) in pigs and cattle [85]. A
406 dual RNA-Seq time-course of *Salmonella*-infected cells from humans and pigs,
407 subsequent validation, and pulse-induced expression of PinT demonstrated that this
408 ncRNA represses transcription of the SPI-2 genes very early after host cell invasion.
409 Repression seems to occur upstream of the SPI-2 master regulator SsrB indepen-
410 dent of PhoP/PhoQ and HilD, and seems to involve the metabolic global regulator

411 CRP as signal transmitter for PinT-mediated activation of SPI-2 [79, 86]. Interestingly, in
412 addition to a premature activation of SPI-2, the transcripts of the secreted SPI-1
413 effectors SopE and SopE2 were significantly de-repressed in *Salmonella pinT*
414 mutants within macrophages. This strongly suggests that PinT plays a crucial role in
415 the transition from extracellular to intracellular life-style after host cell invasion. The
416 elegant dual RNA-Seq approach by Westermann *et al*. further revealed that PinT-
417 mediated influence on SPI-1 and SPI-2 genes resulted in a differential regulation of
418 key regulators of the JAK-STAT signaling pathway and chemokine secretion (e.g.
419 SOC3, STAT3, IL-8) [79]. Another example of a regulatory RNA that is implicated in the
420 control of intracellular *Salmonella* is the antisense RNA LesR-1. The *lesR-1* gene is
421 encoded on the pSLT virulence plasmid and is preferentially expressed in non-
422 growing dormant bacteria residing within fibroblasts. Direct interaction of the 3'-end of
423 the asRNA to the PSLT047 transcript results in a significant reduction of the
424 PSLT047 protein level, and a deletion of *lesR-1* impaired virulence in a mouse
425 infection model [87]. Another recently discovered RNA-based mechanism of *S.*
426 Typhimurium impacts the expression of SPI-2 and the plasmid-encoded *spv* genes
427 needed for intracellular survival and propagation inside the macrophages of the liver
428 and spleen [88, 89]. The mutational inactivation of the PNPase gene (*pnp*) resulted in an
429 up-regulation of SPI-2 and the *spv* genes and this altered the pathogenesis of the
430 infection. *Salmonella pnp* mutants established more frequently persistent infections in
431 Balb/c mice compared to the wild-type which caused mainly acute systemic
432 infections [73]. The exact mechanism is still unknown, but elimination of the *spvR*
433 regulator gene inhibited expression of the *spv* gene cluster and affected growth also
434 in the absence of *pnp*, indicating that PNPase acts upstream or at the level of SpvR
435 [90].
436

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 the 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 two 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 four 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

462 infected with this closed thermometer variant survived the infection without any
463 disease symptoms [95].

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

482 Another study analyzing the post-transcriptional regulation of T3SS showed that *Y.*
483 *pseudotuberculosis* expressing a dominant negative variant of RNase E (a *rne*
484 knock-out is lethal) or a PNPase mutant secreted only a reduced amount of the YopE
485 effector protein [100-102]. Counter-intuitively, a Δ*pnp Yersinia* mutant possessed in-
486 creased levels of all three T3SS-encoding and several *yop* transcripts, demonstrating
487 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 the 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 the 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 up-regulated 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 endo-ribonuclease 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 towards 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

566 virulence. Very little is known about Ysr35, but significant compromised survival of a
567 Ysr35 mutant in a Yersiniosis mouse model indicates that this ncRNA is important for
568 *Yersinia* adaptation to its host [5]. Ysr141 is an unstable ncRNA, which is encoded on
569 the *Yersinia* virulence plasmid on the opposite strand within the intergenic region of
570 *yopH* and a putative transposase [5, 117]. Expression of Ysr141 stimulates the
571 production of multiple T3SS/Yop proteins (e.g. YopE, YscF, YopK) as well as their
572 main activator LcrF, and seems to control *yopJ* mRNA translation through
573 basepairing with its 5'-UTR [117].
574

575 ## RNA-based control of virulence-associated traits

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

578 Over the last decades many regulatory RNAs and riboswitches have been dis-
579 covered as important regulators affecting myriad aspects of bacterial stress re-
580 sponses, ion homoeostasis, metabolism, motility and other physiological properties,
581 which also influence bacterial virulence. In particular riboregulators that are con-
582 served among the well-characterized *Enterobacteriaceae* and implicated in the con-
583 trol of primary and secondary metabolic pathways have been characterized in more
584 detail. The identified RNA-controlled mechanisms and their action at the interface of
585 bacterial metabolism and virulence control have been recently summarized in several
586 comprehensive review articles [4, 118-120] and are thus not in the focus of this article.
587

588 *Ion homeostasis.*

589 One of the most important and best-characterized small ncRNA is RyhB found in all
590 enteric *E. coli, Salmonella, Shigella*, and *Yersinia* species. The RyhB ncRNAs are
591 activated following iron scarcity and are negatively regulated by the ferric uptake

592 regulator Fur [121, 122]. The RyhB ncRNAs regulate iron homeostasis by (i) inhibiting the
593 translation of transcripts encoding non-essential iron-containing proteins under iron
594 starvation conditions in order to liberate iron for essential iron-dependent cellular
595 functions and by (ii) up-regulation of the synthesis of iron-chelating molecules
596 (siderophores) to scavenge iron under iron-limiting conditions as experienced during
597 infection [121]. RyhB is highly similar between *E. coli* and *Shigella* and impacts
598 conserved, but also species-specific virulence genes [123-125]. One of which is the
599 virulence regulator VirB controlling the expression of the IcsP protease, which limits
600 the production of the actin polymerizing IcsA protein [125, 126]. Upon cell entry, iron
601 levels become limiting and relieve Fur-mediated repression of RyhB transcription,
602 which in turn increase host actin polymerization and cell-to-cell spreading through
603 activation of the VirB-IcsP-IcsA cascade [124], and promotes acid resistance by a so far
604 unknown mechanism [127].

605 Interestingly, both *Salmonella* and *Yersinia* possess two RyhB homologs, RyhB1
606 and RyhB2, recently termed sibling ncRNAs, which are slightly differentially regulated
607 in response to iron and nutrient starvation [65, 122, 128]. Both ncRNAs have additional
608 redundant functions such as protecting the pathogen against oxidative and acidic
609 stress [129, 130], and they are both highly induced and important for the intracellular
610 growth of *Salmonella* [65, 77-80] (see also above/below). However, it appears that
611 sequence differences at the 5'-end of the ncRNAs could account for some
612 differences in the regulatory targets of the RyhB RNAs. For instance, Kim *et al*. have
613 shown that RyhB2 of *Salmonella* targets some motility genes (*flgJ, cheY*, and *fliF*)
614 that are not regulated by RyhB1 and vice versa, RyhB1 influences *safA* and *acnB*
615 expression, but not RyhB2 [129]. In *Yersinia*, the region that mediates target gene
616 recognition is highly conserved between both RyhB ncRNAs, indicating regulatory
617 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

670 the 3'-UTR of the *gadX* transcript. This interaction stimulates processing of the *gadX*
671 gene after the stop codon resulting in more stable *gadX* and *gadW* transcripts [136].
672

673 ## Concluding remarks

674 The rapidly increasing amount of transcriptomic data obtained from next-generation
675 sequencing approaches and tiling microarrays combined with sophisticated bio-
676 informatics tools provided us with a vast number of transcribed but non-translated
677 sensory and regulatory RNA element implicated in the fine-tuning of physiological
678 and cellular processes important for pathogenesis. The diversity of their physiological
679 role, the complexity and accuracy of their molecular function as well as their central
680 role in the coordinated regulatory network of virulence-relevant processes illustrates
681 their regulatory potential and relevance. The huge amount of riboregulators pre-
682 sented in this review not only demonstrates that RNA-based control mechanisms
683 represent a crucial additional level of regulation, as they allow a less-energy
684 consuming and faster control of gene expression, it also shows that it enables the
685 bacteria to fine-tune and coordinate their responses to environmental changes in a
686 more rapid and sensitive fashion. The majority of conserved ncRNAs of *Entero-*
687 *bacteriaceae* contribute to the regulation of metabolism and stress responses, but
688 these 'core' ncRNAs were also hijacked to regulate mRNAs of horizontally acquired
689 virulence factor through Hfq and base-pairing or other conserved global RNA-binding
690 regulators, e.g. CsrA. In addition, many species or even strain-specific ncRNAs have
691 evolved which modulate more specific virulence processes of the pathogen to
692 promote optimal adaptation to its host niches. The combined set forms an impressive
693 number of versatile, programmable and highly efficient RNA-based regulators. As
694 outlined in the review by Papenfort & Vanderpool [137], no special characteristics seem
695 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 within host cells and/or

tissue [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-

748 specific chemical cleavage is combined with RNA-Seq have been developed which
749 allow detailed RNA probing and structural profiling *in vivo* [143]. These methods not
750 only enable us to identify RNA thermometers and RNA riboswitches on the system-
751 level, they will also allow us to follow the dynamic of these processes in the context
752 of an infection and compare RNA structuromes from different clinical isolates to
753 identify functionally important differences in RNA structures (riboSNitches).
754 (v) Recently, a bacterial RNA has been shown to use a *cis*-acting signal to change its
755 localization within the bacterial cell in response to environmental changes [39, 40].
756 Development of fluorescent labels for direct tracking of RNA *in vivo* will give novel
757 insights into the spatial organization of (s)RNAs (e.g. association of an ncRNA to
758 hyper-structures such as translational and degradation machineries) and dynamic
759 changes of ncRNA transport in the context of an infection (e.g. during secretion of
760 effectors upon host cell contact).
761 (vi) RNA modifications are known to manipulate the stability and interaction of RNAs
762 with interacting proteins. Transcriptional profiling employing different RNA-Seq-based
763 technologies now enable us to identify intrinsic modified nucleotides and investigate
764 their regulatory potential.
765 (vii) Another tempting question is how we could exploit ncRNAs or associated RNA-
766 based control systems for therapeutic applications to disrupt host-pathogen
767 interaction. One possibility is the discovery of small molecule inhibitors or design of
768 tailor-made compounds for a particular ncRNA or riboswitch [144, 145]. In fact, an
769 effective riboswitch inhibitor (ribocil) and an aptamer-based riboswitch blocker have
770 recently been identified [144, 146]. A pitfall in this approach is that many *trans*-encoded
771 ncRNAs are non-essential or have partially redundant function and are per se not
772 very well suited as drug targets. Consequently, central riboregulators such as CsrA
773 or global transcriptional or post-transcriptional regulators such as Crp or Hfq which

774 influence many RNA-mediated control systems seem to be much more promising
775 targets for the design of novel diagnostics and therapeutic measures.

776

777 Acknowledgments

778 We thank Dr. Martin Fenner for discussion and Claudia Munzel for critical reading of
779 the manuscript. German Research Foundation provided funding to Petra Dersch
780 under the grant number DE616/4 and DE616/5 for the analysis of sensory and
781 regulatory RNAs and host-adapted metabolism of *Yersinia*. Petra Dersch is further
782 supported by the German Center for Infection Research under grant number DZIF-
783 TTU 06.801.

784

785 References

786 1. Waters LS, Storz G. Regulatory RNAs in bacteria. Cell 2009; 136:615-28.
787 2. Gottesman S, Storz G. Bacterial small RNA regulators: versatile roles and
788 rapidly evolving variations. Cold Spring Harb Perspect Biol 2011; 3.
789 3. Caldelari I, Chao Y, Romby P, Vogel J. RNA-mediated regulation in pathogenic
790 bacteria. Cold Spring Harb Perspect Med 2013; 3:a010298.
791 4. Papenfort K, Vogel J. Small RNA functions in carbon metabolism and virulence
792 of enteric pathogens. Front Cell Infect Microbiol 2014; 4:91.
793 5. Koo JT, Alleyne TM, Schiano CA, Jafari N, Lathem WW. Global discovery of
794 small RNAs in *Yersinia pseudotuberculosis* identifies *Yersinia*-specific small,
795 noncoding RNAs required for virulence. Proc Natl Acad Sci U S A 2011;
796 108:E709-17.
797 6. Koo JT, Lathem WW. Global discovery of small noncoding RNAs in pathogenic
798 *Yersinia* species. Adv Exp Med Biol 2012; 954:305-14.

7. Nuss AM, Heroven AK, Waldmann B, Reinkensmeier J, Jarek M, Beckstette M, et al. Transcriptomic profiling of Y*ersinia pseudotuberculosis* reveals reprogramming of the Crp regulon by temperature and uncovers Crp as a master regulator of small RNAs. PLoS Genet 2015; 11:e1005087.
8. Landstorfer R, Simon S, Schober S, Keim D, Scherer S, Neuhaus K. Comparison of strand-specific transcriptomes of enterohemorrhagic *Escherichia coli* O157:H7 EDL933 (EHEC) under eleven different environmental conditions including radish sprouts and cattle feces. BMC Genomics 2014; 15:353.
9. Raghavan R, Groisman EA, Ochman H. Genome-wide detection of novel regulatory RNAs in *E. coli*. Genome Res 2011; 21:1487-97.
10. Storz G, Vogel J, Wassarman KM. Regulation by small RNAs in bacteria: expanding frontiers. Mol Cell 2011; 43:880-91.
11. Vogel J, Luisi BF. Hfq and its constellation of RNA. Nat Rev Microbiol 2011; 9:578-89.
12. Viegas SC, Pfeiffer V, Sittka A, Silva IJ, Vogel J, Arraiano CM. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. Nucleic Acids Res 2007; 35:7651-64.
13. Saramago M, Barria C, Dos Santos RF, Silva IJ, Pobre V, Domingues S, et al. The role of RNases in the regulation of small RNAs. Curr Opin Microbiol 2014; 18:105-15.
14. Kortmann J, Narberhaus F. Bacterial RNA thermometers: molecular zippers and switches. Nat Rev Microbiol 2012; 10:255-65.
15. Steinmann R, Dersch P. Thermosensing to adjust bacterial virulence in a fluctuating environment. Future Microbiol 2013; 8:85-105.
16. Serganov A, Nudler E. A decade of riboswitches. Cell 2013; 152:17-24.

824 17. Mellin JR, Cossart P. Unexpected versatility in bacterial riboswitches. Trends
825 Genet 2015; 31:150-6.
826 18. Peselis A, Serganov A. Themes and variations in riboswitch structure and
827 function. Biochim Biophys Acta 2014; 1839:908-18.
828 19. Henkin TM. Riboswitch RNAs: using RNA to sense cellular metabolism. Genes
829 Dev 2008; 22:3383-90.
830 20. Papenfort K, Vogel J. Regulatory RNA in bacterial pathogens. Cell Host
831 Microbe 2010; 8:116-27.
832 21. Chao Y, Vogel J. The role of Hfq in bacterial pathogens. Curr Opin Microbiol
833 2010; 13:24-33.
834 22. Sobrero P, Valverde C. The bacterial protein Hfq: much more than a mere
835 RNA-binding factor. Crit Rev Microbiol 2012; 38:276-99.
836 23. Wagner EG. Cycling of RNAs on Hfq. RNA Biol 2013; 10:619-26.
837 24. Morita T, Maki K, Aiba H. RNase E-based ribonucleoprotein complexes:
838 mechanical basis of mRNA destabilization mediated by bacterial noncoding
839 RNAs. Genes Dev 2005; 19:2176-86.
840 25. Hajnsdorf E, Regnier P. Host factor Hfq of *Escherichia coli* stimulates
841 elongation of poly(A) tails by poly(A) polymerase I. Proc Natl Acad Sci U S A
842 2000; 97:1501-5.
843 26. Heroven AK, Bohme K, Dersch P. The Csr/Rsm system of *Yersinia* and related
844 pathogens: A post-transcriptional strategy for managing virulence. RNA Biol
845 2012; 9.
846 27. Vakulskas CA, Potts AH, Babitzke P, Ahmer BM, Romeo T. Regulation of
847 Bacterial Virulence by Csr (Rsm) Systems. Microbiol Mol Biol Rev 2015;
848 79:193-224.

849 28. Dubey AK, Baker CS, Romeo T, Babitzke P. RNA sequence and secondary
850 structure participate in high-affinity CsrA-RNA interaction. Rna 2005; 11:1579-
851 87.
852 29. Kulkarni PR, Jia T, Kuehne SA, Kerkering TM, Morris ER, Searle MS, et al. A
853 sequence-based approach for prediction of CsrA/RsmA targets in bacteria with
854 experimental validation in *Pseudomonas aeruginosa*. Nucleic Acids Res 2014;
855 42:6811-25.
856 30. Holmqvist E, Wright PR, Li L, Bischler T, Barquist L, Reinhardt R, et al. Global
857 RNA recognition patterns of post-transcriptional regulators Hfq and CsrA
858 revealed by UV crosslinking in vivo. EMBO J 2016; 35:991-1011.
859 31. Yakhnin AV, Baker CS, Vakulskas CA, Yakhnin H, Berezin I, Romeo T, et al.
860 CsrA activates *flhDC* expression by protecting *flhDC* mRNA from RNase E-
861 mediated cleavage. Mol Microbiol 2013; 87:851-66.
862 32. Babitzke P, Romeo T. CsrB sRNA family: sequestration of RNA-binding
863 regulatory proteins. Curr Opin Microbiol 2007; 10:156-63.
864 33. Liu MY, Gui G, Wei B, Preston JF, 3rd, Oakford L, Yuksel U, et al. The RNA
865 molecule CsrB binds to the global regulatory protein CsrA and antagonizes its
866 activity in *Escherichia coli*. J Biol Chem 1997; 272:17502-10.
867 34. Kulkarni PR, Cui X, Williams JW, Stevens AM, Kulkarni RV. Prediction of CsrA-
868 regulating small RNAs in bacteria and their experimental verification in *Vibrio*
869 *fischeri*. Nucleic Acids Res 2006; 34:3361-9.
870 35. Heroven A, Bohme K, Rohde M, Dersch P. A Csr-type regulatory system,
871 including small non-coding RNAs, regulates the global virulence regulator RovA
872 of *Yersinia pseudotuberculosis* through RovM. Mol Microbiol 2008; 68:1179-95.
873 36. Garneau NL, Wilusz J, Wilusz CJ. The highways and byways of mRNA decay.
874 Nat Rev Mol Cell Biol 2007; 8:113-26.

875 37. Silva IJ, Saramago M, Dressaire C, Domingues S, Viegas SC, Arraiano CM.
876 Importance and key events of prokaryotic RNA decay: the ultimate fate of an
877 RNA molecule. Wiley Interdiscip Rev RNA 2011; 2:818-36.
878 38. Lalaouna D, Simoneau-Roy M, Lafontaine D, Masse E. Regulatory RNAs and
879 target mRNA decay in prokaryotes. Biochim Biophys Acta 2013; 1829:742-7.
880 39. Buskila AA, Kannaiah S, Amster-Choder O. RNA localization in bacteria. RNA
881 Biol 2014; 11:1051-60.
882 40. Nevo-Dinur K, Govindarajan S, Amster-Choder O. Subcellular localization of
883 RNA and proteins in prokaryotes. Trends Genet 2012; 28:314-22.
884 41. Pizarro-Cerda J, Cossart P. Bacterial adhesion and entry into host cells. Cell
885 2006; 124:715-27.
886 42. Pawar DM, Rossman ML, Chen J. Role of curli fimbriae in mediating the cells of
887 enterohaemorrhagic *Escherichia coli* to attach to abiotic surfaces. J Appl
888 Microbiol 2005; 99:418-25.
889 43. Barnhart MM, Chapman MR. Curli biogenesis and function. Annu Rev Microbiol
890 2006; 60:131-47.
891 44. Bordeau V, Felden B. Curli synthesis and biofilm formation in enteric bacteria
892 are controlled by a dynamic small RNA module made up of a pseudoknot
893 assisted by an RNA chaperone. Nucleic Acids Res 2014; 42:4682-96.
894 45. Boehm A, Vogel J. The *csgD* mRNA as a hub for signal integration via multiple
895 small RNAs. Mol Microbiol 2012; 84:1-5.
896 46. Urban JH, Vogel J. Two seemingly homologous noncoding RNAs act
897 hierarchically to activate glmS mRNA translation. PLoS Biol 2008; 6:e64.
898 47. Gopel Y, Luttmann D, Heroven AK, Reichenbach B, Dersch P, Gorke B.
899 Common and divergent features in transcriptional control of the homologous

900 small RNAs GlmY and GlmZ in *Enterobacteriaceae*. Nucleic Acids Res 2011;
901 39:1294-309.
902 48. Gruber CC, Sperandio V. Global analysis of posttranscriptional regulation by
903 GlmY and GlmZ in enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun
904 2015; 83:1286-95.
905 49. Teplitski M, Al-Agely A, Ahmer BM. Contribution of the SirA regulon to biofilm
906 formation in *Salmonella enterica* serovar Typhimurium. Microbiology 2006;
907 152:3411-24.
908 50. Sterzenbach T, Nguyen KT, Nuccio SP, Winter MG, Vakulskas CA, Clegg S, et
909 al. A novel CsrA titration mechanism regulates fimbrial gene expression in
910 *Salmonella typhimurium*. EMBO J 2013; 32:2872-83.
911 51. McDaniel TK, Kaper JB. A cloned pathogenicity island from enteropathogenic
912 *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12.
913 Mol Microbiol 1997; 23:399-407.
914 52. Bhatt S, Edwards AN, Nguyen HT, Merlin D, Romeo T, Kalman D. The RNA
915 binding protein CsrA is a pleiotropic regulator of the locus of enterocyte
916 effacement pathogenicity island of enteropathogenic *Escherichia coli*. Infect
917 Immun 2009; 77:3552-68.
918 53. Gruber CC, Sperandio V. Posttranscriptional control of microbe-induced
919 rearrangement of host cell actin. MBio 2014; 5:e01025-13.
920 54. Martinez LC, Yakhnin H, Camacho MI, Georgellis D, Babitzke P, Puente JL, et
921 al. Integration of a complex regulatory cascade involving the SirA/BarA and Csr
922 global regulatory systems that controls expression of the *Salmonella* SPI-1 and
923 SPI-2 virulence regulons through HilD. Mol Microbiol 2011; 80:1637-56.
924 55. Gore AL, Payne SM. CsrA and Cra influence *Shigella flexneri* pathogenesis.
925 Infect Immun 2010; 78:4674-82.

926 56. Adler B, Sasakawa C, Tobe T, Makino S, Komatsu K, Yoshikawa M. A dual
927 transcriptional activation system for the 230 kb plasmid genes coding for
928 virulence-associated antigens of *Shigella flexneri*. Mol Microbiol 1989; 3:627-35.
929 57. Vanderpool CK. Physiological consequences of small RNA-mediated regulation
930 of glucose-phosphate stress. Curr Opin Microbiol 2007; 10:146-51.
931 58. Bobrovskyy M, Vanderpool CK. The small RNA SgrS: roles in metabolism and
932 pathogenesis of enteric bacteria. Front Cell Infect Microbiol 2014; 4:61.
933 59. Bobrovskyy M, Vanderpool CK. Diverse mechanisms of post-transcriptional
934 repression by the small RNA regulator of glucose-phosphate stress. Mol
935 Microbiol 2016; 99:254-73.
936 60. Papenfort K, Podkaminski D, Hinton JC, Vogel J. The ancestral SgrS RNA
937 discriminates horizontally acquired *Salmonella* mRNAs through a single G-U
938 wobble pair. Proc Natl Acad Sci U S A 2012; 109:E757-64.
939 61. Papenfort K, Sun Y, Miyakoshi M, Vanderpool CK, Vogel J. Small RNA-
940 mediated activation of sugar phosphatase mRNA regulates glucose
941 homeostasis. Cell 2013; 153:426-37.
942 62. Giangrossi M, Prosseda G, Tran CN, Brandi A, Colonna B, Falconi M. A novel
943 antisense RNA regulates at transcriptional level the virulence gene *icsA* of
944 *Shigella flexneri*. Nucleic Acids Res 2010; 38:3362-75.
945 63. Tran CN, Giangrossi M, Prosseda G, Brandi A, Di Martino ML, Colonna B, et al.
946 A multifactor regulatory circuit involving H-NS, VirF and an antisense RNA
947 modulates transcription of the virulence gene *icsA* of *Shigella flexneri*. Nucleic
948 Acids Res 2011; 39:8122-34.
949 64. Pichon C, du Merle L, Lequeutre I, Le Bouguenec C. The AfaR small RNA
950 controls expression of the AfaD-VIII invasin in pathogenic *Escherichia coli*
951 strains. Nucleic Acids Res 2013; 41:5469-82.

65. Padalon-Brauch G, Hershberg R, Elgrably-Weiss M, Baruch K, Rosenshine I, Margalit H, et al. Small RNAs encoded within genetic islands of *Salmonella typhimurium* show host-induced expression and role in virulence. Nucleic Acids Res 2008; 36:1913-27.

66. Gong H, Vu GP, Bai Y, Chan E, Wu R, Yang E, et al. A *Salmonella* small non-coding RNA facilitates bacterial invasion and intracellular replication by modulating the expression of virulence factors. PLoS Pathog 2011; 7:e1002120.

67. Durand JM, Okada N, Tobe T, Watarai M, Fukuda I, Suzuki T, et al. *vacC*, a virulence-associated chromosomal locus of *Shigella flexneri*, is homologous to *tgt*, a gene encoding tRNA-guanine transglycosylase (Tgt) of *Escherichia coli* K-12. J Bacteriol 1994; 176:4627-34.

68. Shippy DC, Fadl AA. tRNA modification enzymes GidA and MnmE: potential role in virulence of bacterial pathogens. Int J Mol Sci 2014; 15:18267-80.

69. Yu H, Kim KS. mRNA context dependent regulation of cytotoxic necrotizing factor 1 translation by GidA, a tRNA modification enzyme in *Escherichia coli*. Gene 2012; 491:116-22.

70. Knust Z, Blumenthal B, Aktories K, Schmidt G. Cleavage of *Escherichia coli* cytotoxic necrotizing factor 1 is required for full biologic activity. Infect Immun 2009; 77:1835-41.

71. Lemonnier M, Landraud L, Lemichez E. Rho GTPase-activating bacterial toxins: from bacterial virulence regulation to eukaryotic cell biology. FEMS Microbiol Rev 2007; 31:515-34.

72. Cheng ZF, Zuo Y, Li Z, Rudd KE, Deutscher MP. The *vacB* gene required for virulence in *Shigella flexneri* and *Escherichia coli* encodes the exoribonuclease RNase R. J Biol Chem 1998; 273:14077-80.

978 73. Clements MO, Eriksson S, Thompson A, Lucchini S, Hinton JC, Normark S, et
979 al. Polynucleotide phosphorylase is a global regulator of virulence and
980 persistency in *Salmonella enterica*. Proc Natl Acad Sci U S A 2002; 99:8784-9.
981 74. Jones BD, Ghori N, Falkow S. *Salmonella typhimurium* initiates murine infection
982 by penetrating and destroying the specialized epithelial M cells of the Peyer's
983 patches. J Exp Med 1994; 180:15-23.
984 75. Vazquez-Torres A, Jones-Carson J, Baumler AJ, Falkow S, Valdivia R, Brown
985 W, et al. Extraintestinal dissemination of *Salmonella* by CD18-expressing
986 phagocytes [In Process Citation]. Nature 1999; 401:804-8.
987 76. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW.
988 Internalization of *Salmonella* by macrophages induces formation of
989 nonreplicating persisters. Science 2014; 343:204-8.
990 77. Ortega A, Gonzalo-Asensio J, Garcia-Del Portillo F. Dynamics of *Salmonella*
991 small RNA expression in non-growing bacteria located inside eukaryotic cells.
992 RNA Biol 2012; 9.
993 78. Srikumar S, Kroger C, Hebrard M, Colgan A, Owen SV, Sivasankaran SK, et al.
994 RNA-seq brings new insights to the intra-macrophage transcriptome of
995 *Salmonella* Typhimurium. PLoS Pathog 2015; 11:e1005262.
996 79. Westermann AJ, Forstner KU, Amman F, Barquist L, Chao Y, Schulte LN, et al.
997 Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions.
998 Nature 2016; 529:496-501.
999 80. Leclerc JM, Dozois CM, Daigle F. Role of the *Salmonella enterica* serovar Typhi
1000 Fur regulator and small RNAs RfrA and RfrB in iron homeostasis and
1001 interaction with host cells. Microbiology 2013; 159:591-602.
1002 81. Pfeiffer V, Sittka A, Tomer R, Tedin K, Brinkmann V, Vogel J. A small non-
1003 coding RNA of the invasion gene island (SPI-1) represses outer membrane

protein synthesis from the *Salmonella* core genome. Mol Microbiol 2007; 66:1174-91.

82. Chao Y, Papenfort K, Reinhardt R, Sharma CM, Vogel J. An atlas of Hfq-bound transcripts reveals 3' UTRs as a genomic reservoir of regulatory small RNAs. EMBO J 2012; 31:4005-19.

83. Prost LR, Miller SI. The Salmonellae PhoQ sensor: mechanisms of detection of phagosome signals. Cellular Microbiology 2008; 10:576-82.

84. Groisman EA. The pleiotropic two-component regulatory system PhoP-PhoQ. Journal of Bacteriology 2001; 183:1835-42.

85. Chaudhuri RR, Morgan E, Peters SE, Pleasance SJ, Hudson DL, Davies HM, et al. Comprehensive assignment of roles for *Salmonella typhimurium* genes in intestinal colonization of food-producing animals. PLoS Genet 2013; 9:e1003456.

86. Chen ZW, Hsuan SL, Liao JW, Chen TH, Wu CM, Lee WC, et al. Mutations in the *Salmonella enterica* serovar Choleraesuis cAMP-receptor protein gene lead to functional defects in the SPI-1 Type III secretion system. Vet Res 2010; 41:5.

87. Gonzalo-Asensio J, Ortega AD, Rico-Perez G, Pucciarelli MG, Garcia-Del Portillo F. A novel antisense RNA from the *Salmonella* virulence plasmid pSLT expressed by non-growing bacteria inside eukaryotic cells. PLoS One 2013; 8:e77939.

88. Gulig PA, Danbara H, Guiney DG, Lax AJ, Norel F, Rhen M. Molecular analysis of *spv* virulence genes of the *Salmonella* virulence plasmids. Mol Microbiol 1993; 7:825-30.

89. Libby SJ, Lesnick M, Hasegawa P, Weidenhammer E, Guiney DG. The *Salmonella* virulence plasmid *spv* genes are required for cytopathology in human monocyte-derived macrophages. Cell Microbiol 2000; 2:49-58.

90. Ygberg SE, Clements MO, Rytkonen A, Thompson A, Holden DW, Hinton JC, et al. Polynucleotide phosphorylase negatively controls *spv* virulence gene expression in *Salmonella enterica*. Infect Immun 2006; 74:1243-54.
91. Cornelis GR, Wolf-Watz H. The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. Mol Microbiol 1997; 23:861-7.
92. Shao F. Biochemical functions of *Yersinia* type III effectors. Curr Opin Microbiol 2008; 11:21-9.
93. Schiano CA, Lathem WW. Post-transcriptional regulation of gene expression in *Yersinia* species. Front Cell Infect Microbiol 2012; 2:129.
94. Hoe NP, Goguen JD. Temperature sensing in *Yersinia pestis*: translation of the LcrF activator protein is thermally regulated. J Bacteriol 1993; 175:7901-9.
95. Böhme K, Steinmann R, Kortmann J, Seekircher S, Heroven AK, Berger E, et al. Concerted actions of a thermo-labile regulator and a unique intergenic RNA thermosensor control *Yersinia* virulence. PLoS Pathog 2012; 8:e1002518.
96. Anderson DM, Ramamurthi KS, Tam C, Schneewind O. YopD and LcrH regulate expression of *Yersinia enterocolitica* YopQ by a posttranscriptional mechanism and bind to *yopQ* RNA. J Bacteriol 2002; 184:1287-95.
97. Chen Y, Anderson DM. Expression hierarchy in the *Yersinia* type III secretion system established through YopD recognition of RNA. Mol Microbiol 2011; 80:966-80.
98. Cambronne ED, Schneewind O. *Yersinia enterocolitica* type III secretion: *yscM1* and *yscM2* regulate *yop* gene expression by a posttranscriptional mechanism that targets the 5' untranslated region of *yop* mRNA. J Bacteriol 2002; 184:5880-93.

1054 99. Kopaskie KS, Ligtenberg KG, Schneewind O. Translational regulation of
1055 *Yersinia enterocolitica* mRNA encoding a type III secretion substrate. J Biol
1056 Chem 2013; 288:35478-88.
1057 100. Rosenzweig JA, Weltman G, Plano GV, Schesser K. Modulation of *Yersinia*
1058 type three secretion system by the S1 domain of polynucleotide phosphorylase.
1059 J Biol Chem 2005; 280:156-63.
1060 101. Rosenzweig JA, Chromy B, Echeverry A, Yang J, Adkins B, Plano GV, et al.
1061 Polynucleotide phosphorylase independently controls virulence factor
1062 expression levels and export in *Yersinia* spp. FEMS Microbiol Lett 2007;
1063 270:255-64.
1064 102. Yang J, Jain C, Schesser K. RNase E regulates the *Yersinia* type 3 secretion
1065 systemros. J Bacteriol 2008; 190:3774-8.
1066 103. Rosenzweig JA, Chopra AK. The exoribonuclease polynucleotide
1067 phosphorylase influences the virulence and stress responses of *yersiniae* and
1068 many other pathogens. Front Cell Infect Microbiol 2013; 3:81.
1069 104. Norris V, Menu-Bouaouiche L, Becu JM, Legendre R, Norman R, Rosenzweig
1070 JA. Hyperstructure interactions influence the virulence of the type 3 secretion
1071 system in *yersiniae* and other bacteria. Appl Microbiol Biotechnol 2012; 96:23-
1072 36.
1073 105. De Lay N, Gottesman S. Role of polynucleotide phosphorylase in sRNA
1074 function in *Escherichia coli*. RNA 2011; 17:1172-89.
1075 106. Beauregard A, Smith EA, Petrone BL, Singh N, Karch C, McDonough KA, et al.
1076 Identification and characterization of small RNAs in *Yersinia pestis*. RNA Biol
1077 2013; 10:397-405.

1078 107. Mohanty BK, Maples VF, Kushner SR. The Sm-like protein Hfq regulates
1079 polyadenylation dependent mRNA decay in *Escherichia coli*. Mol Microbiol
1080 2004; 54:905-20.
1081 108. Schiano CA, Bellows LE, Lathem WW. The small RNA chaperone Hfq is
1082 required for the virulence of *Yersinia pseudotuberculosis*. Infect Immun 2010;
1083 78:2034-44.
1084 109. Geng J, Song Y, Yang L, Feng Y, Qiu Y, Li G, et al. Involvement of the post-
1085 transcriptional regulator of Hfq in *Yersinia pestis* virulence. PLoS One 2009;
1086 4:e6213.
1087 110. Leskinen K, Varjosalo M, Skurnik M. Absence of YbeY RNase compromises the
1088 growth and enhances the virulence plasmid gene expression of *Yersinia*
1089 *enterocolitica* O:3. Microbiology 2015; 161:285-99.
1090 111. Jacob AI, Kohrer C, Davies BW, RajBhandary UL, Walker GC. Conserved
1091 bacterial RNase YbeY plays key roles in 70S ribosome quality control and 16S
1092 rRNA maturation. Mol Cell 2013; 49:427-38.
1093 112. Grinwald M, Ron EZ. The *Escherichia coli* translation-associated heat shock
1094 protein YbeY is involved in rRNA transcription antitermination. PLoS One 2013;
1095 8:e62297.
1096 113. Pandey SP, Winkler JA, Li H, Camacho DM, Collins JJ, Walker GC. Central role
1097 for RNase YbeY in Hfq-dependent and Hfq-independent small-RNA regulation
1098 in bacteria. BMC Genomics 2014; 15:121.
1099 114. Okan NA, Bliska JB, Karzai AW. A Role for the SmpB-SsrA system in *Yersinia*
1100 *pseudotuberculosis* pathogenesis. PLoS Pathog 2006; 2:e6.
1101 115. Karzai AW, Roche ED, Sauer RT. The SsrA-SmpB system for protein tagging,
1102 directed degradation and ribosome rescue. Nat Struct Biol 2000; 7:449-55.

116. Neubauer C, Gillet R, Kelley AC, Ramakrishnan V. Decoding in the absence of a codon by tmRNA and SmpB in the ribosome. Science 2012; 335:1366-9.
117. Schiano CA, Koo JT, Schipma MJ, Caulfield AJ, Jafari N, Lathem WW. Genome-wide analysis of small RNAs expressed by *Yersinia pestis* identifies a regulator of the Yop-Ysc type III secretion system. J Bacteriol 2014; 196:1659-70.
118. Oliva G, Sahr T, Buchrieser C. Small RNAs, 5' UTR elements and RNA-binding proteins in intracellular bacteria: impact on metabolism and virulence. FEMS Microbiol Rev 2015; 39:331-49.
119. Bobrovskyy M, Vanderpool CK, Richards GR. Small RNAs Regulate Primary and Secondary Metabolism in Gram-negative Bacteria. Microbiol Spectr 2015; 3.
120. Mika F, Hengge R. Small Regulatory RNAs in the Control of Motility and Biofilm Formation in E. coli and Salmonella. Int J Mol Sci 2013; 14:4560-79.
121. Masse E, Gottesman S. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc Natl Acad Sci U S A 2002; 99:4620-5.
122. Deng Z, Meng X, Su S, Liu Z, Ji X, Zhang Y, et al. Two sRNA RyhB homologs from Y*ersinia pestis* biovar microtus expressed i*n vivo* have differential Hfq-dependent stability. Res Microbiol 2012; 163:413-8.
123. Murphy ER, Payne SM. RyhB, an iron-responsive small RNA molecule, regulates *Shigella dysenteriae* virulence. Infect Immun 2007; 75:3470-7.
124. Africa LA, Murphy ER, Egan NR, Wigley AF, Wing HJ. The iron-responsive Fur/RyhB regulatory cascade modulates the *Shigella* outer membrane protease IcsP. Infect Immun 2011; 79:4543-9.

125. Broach WH, Egan N, Wing HJ, Payne SM, Murphy ER. VirF-independent regulation of *Shigella virB* transcription is mediated by the small RNA RyhB. PLoS One 2012; 7:e38592.
126. Fris ME, Murphy ER. Riboregulators: Fine-Tuning Virulence in *Shigella*. Front Cell Infect Microbiol 2016; 6:2.
127. Oglesby AG, Murphy ER, Iyer VR, Payne SM. Fur regulates acid resistance in *Shigella flexneri* via RyhB and *ydeP*. Mol Microbiol 2005; 58:1354-67.
128. Caswell CC, Oglesby-Sherrouse AG, Murphy ER. Sibling rivalry: related bacterial small RNAs and their redundant and non-redundant roles. Front Cell Infect Microbiol 2014; 4:151.
129. Kim JN, Kwon YM. Identification of target transcripts regulated by small RNA RyhB homologs in *Salmonella*: RyhB-2 regulates motility phenotype. Microbiol Res 2013; 168:621-9.
130. Calderon PF, Morales EH, Acuna LG, Fuentes DN, Gil F, Porwollik S, et al. The small RNA RyhB homologs from *Salmonella typhimurium* participate in the response to S-nitrosoglutathione-induced stress. Biochem Biophys Res Commun 2014; 450:641-5.
131. Deng Z, Liu Z, Bi Y, Wang X, Zhou D, Yang R, et al. Rapid degradation of Hfq-free RyhB in *Yersinia pestis* by PNPase independent of putative ribonucleolytic complexes. Biomed Res Int 2014; 2014:798918.
132. Kouse AB, Righetti F, Kortmann J, Narberhaus F, Murphy ER. RNA-mediated thermoregulation of iron-acquisition genes in *Shigella dysenteriae* and pathogenic *Escherichia coli*. PLoS One 2013; 8:e63781.
133. Michaux C, Verneuil N, Hartke A, Giard JC. Physiological roles of small RNA molecules. Microbiology 2014; 160:1007-19.

134. Martinez-Chavarria LC, Vadyvaloo V. *Yersinia pestis* and *Yersinia pseudotuberculosis* infection: a regulatory RNA perspective. Front Microbiol 2015; 6:956.
135. Lee YH, Kim S, Helmann JD, Kim BH, Park YK. RaoN, a small RNA encoded within *Salmonella* pathogenicity island-11, confers resistance to macrophage-induced stress. Microbiology 2013; 159:1366-78.
136. Tramonti A, De Canio M, De Biase D. GadX/GadW-dependent regulation of the *Escherichia coli* acid fitness island: transcriptional control at the *gadY-gadW* divergent promoters and identification of four novel 42 bp GadX/GadW-specific binding sites. Mol Microbiol 2008; 70:965-82.
137. Papenfort K, Vanderpool CK. Target activation by regulatory RNAs in bacteria. FEMS Microbiol Rev 2015; 39:362-78.
138. Park SY, Cromie MJ, Lee EJ, Groisman EA. A bacterial mRNA leader that employs different mechanisms to sense disparate intracellular signals. Cell 2010; 142:737-48.
139. van Opijnen T, Camilli A. Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. Nat Rev Microbiol 2013; 11:435-42.
140. Diard M, Garcia V, Maier L, Remus-Emsermann MN, Regoes RR, Ackermann M, et al. Stabilization of cooperative virulence by the expression of an avirulent phenotype. Nature 2013; 494:353-6.
141. Claudi B, Sprote P, Chirkova A, Personnic N, Zankl J, Schurmann N, et al. Phenotypic variation of *Salmonella* in host tissues delays eradication by antimicrobial chemotherapy. Cell 2014; 158:722-33.
142. Davis KM, Mohammadi S, Isberg RR. Community behavior and spatial regulation within a bacterial microcolony in deep tissue sites serves to protect against host attack. Cell Host Microbe 2015; 17:21-31.

143. Burgess DJ. RNA. Detailed probing of RNA structure in vivo. Nat Rev Genet 2015; 16:255.
144. Lee CH, Han SR, Lee SW. Therapeutic applications of aptamer-based riboswitches. Nucleic Acid Ther 2016; 26:44-51.
145. Blount KF, Breaker RR. Riboswitches as antibacterial drug targets. Nat Biotechnol 2006; 24:1558-64.
146. Howe JA, Wang H, Fischmann TO, Balibar CJ, Xiao L, Galgoci AM, et al. Selective small-molecule inhibition of an RNA structural element. Nature 2015; 526:672-7.

Figure Legends

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.

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).

1213 **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 *LEE*-4 mRNA, overexpression of CsrA represses expression of the *LEE*1-5 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-c*huA*	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 SPI1 gene expression via translational repression of the SPI1 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 SPI1 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 production, and mitochondria localization and functions Colonization in pigs and cattle	65, 79, 85
	RaoN	*trans*-encoded ncRNA	Induced under oxidative stress and nutrient limitation, important for survival in macrophages, controls expression of the lactate dehydrogenase gene *ldh*A	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	Enhances expression of the *Shigella* T3SS effectors IpaB, IpaC and IpaD,	67

		tRNA-modifying enzyme	reduces amounts of VirG important for cell-to-cell spreading	
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. 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

1214

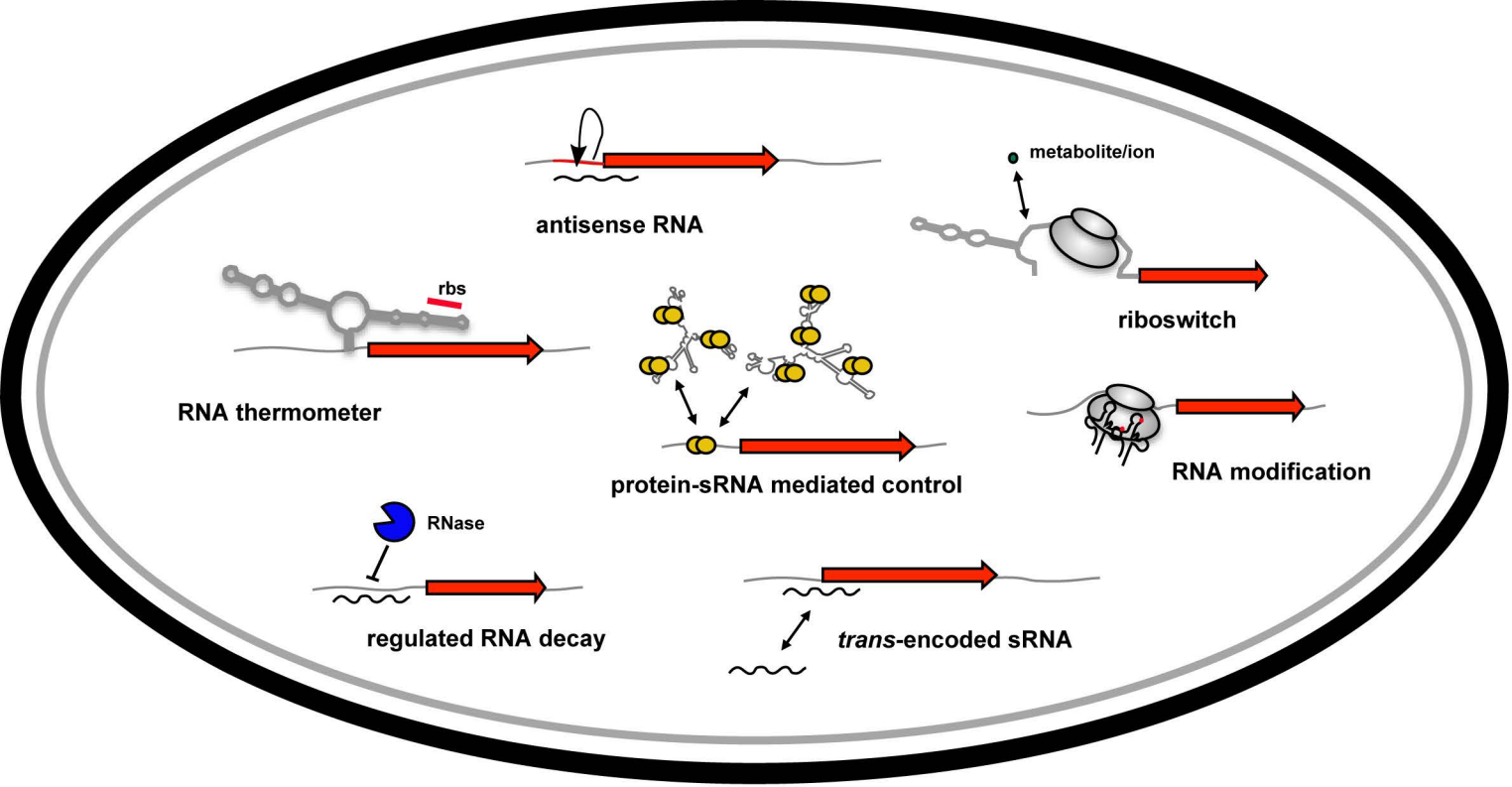

Figure 1 Heroven *et al.*, 2016

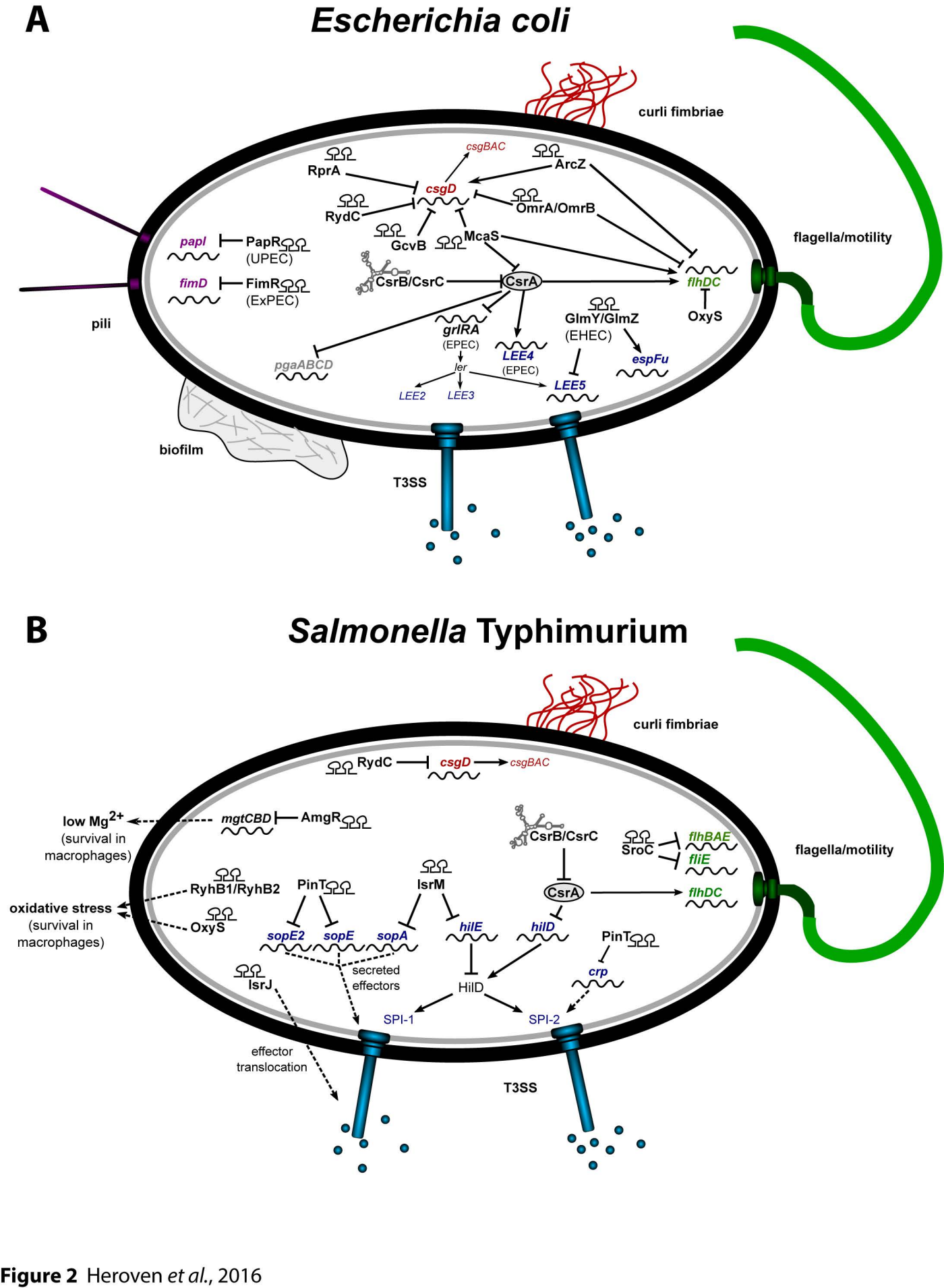

Figure 2 Heroven *et al.*, 2016