Microbiology Spectrum
American Society for Microbiology Press

# RNA Thermometers in Bacterial Pathogens

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**ABSTRACT** Temperature variation is one of the multiple parameters a microbial pathogen encounters when it invades a warm-blooded host. To survive and thrive at host body temperature, human pathogens have developed various strategies to sense and respond to their ambient temperature. An instantaneous response is mounted by RNA thermometers (RNATs), which are integral sensory structures in mRNAs that modulate translation efficiency. At low temperatures outside the host, the folded RNA blocks access of the ribosome to the translation initiation region. The temperature shift upon entering the host destabilizes the RNA structure and thus permits ribosome binding. This reversible zipper-like mechanism of RNATs is ideally suited to fine-tune virulence gene expression when the pathogen enters or exits the body of its host. This review summarizes our present knowledge on virulence-related RNATs and discusses recent developments in the field.

## INTRODUCTION

Temperature is an environmental cue that affects essentially every cellular process. To cope with sudden temperature changes, all living cells closely survey their ambient temperature through numerous sensory mechanisms, which involve regulatory proteins, changes in membrane fluidity, and impacts on DNA topology and RNA structures (1, 2). Most of these mechanisms were initially discovered in studies of the heat shock response, which protects the cell from serious damage after a drastic shift to high temperatures. However, it is now established that subtle temperature changes already induce cellular responses. One process that involves reversible temperature changes is the entry and exit of mammalian pathogens into and from the host. A temperature of ~37°C serves as a very good indicator to the bacterium that it is in a mammalian host. Various mechanisms regulating gene expression in response to host body temperature have been discovered, with some involving regulatory proteins and others utilizing sensory and regulatory RNAs. In this review, the main focus will be on RNA-mediated mechanisms; however, when the regulation involves a multicomponent regulatory network, protein-dependent regulatory events will be discussed.

A common regulatory principle acting in response to temperature in bacterial pathogens is translational control by RNA thermometers/thermosensors (RNATs). RNATs are elements usually located in the 5′ untranslated region (UTR) of mRNAs. They operate by changing their secondary structures in response to temperature fluctuation. Due to the close proximity to the protein-coding region, a change in the RNA secondary structure exerts a major effect on translation efficiency of the downstream gene. Generally, at low temperature (<30°C), an RNAT forms a stable structure masking the ribosome binding site (RBS) and blocking translation

**Received:** 20 November 2017, **Accepted:** 22 January 2018, **Published:** 6 April 2018

**Editors:** Gisela Storz, Division of Molecular and Cellular Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD; Kai Papenfort, Department of Biology I, Microbiology, LMU Munich, Martinsried, Germany

**Citation:** Loh E, Righetti F, Eichner H, Twittenhoff C, Narberhaus F. 2018. RNA thermometers in bacterial pathogens. *Microbiol Spectrum* 6(2):RWR-0012-2017. doi:10.1128/microbiolspec.RWR-0012-2017.

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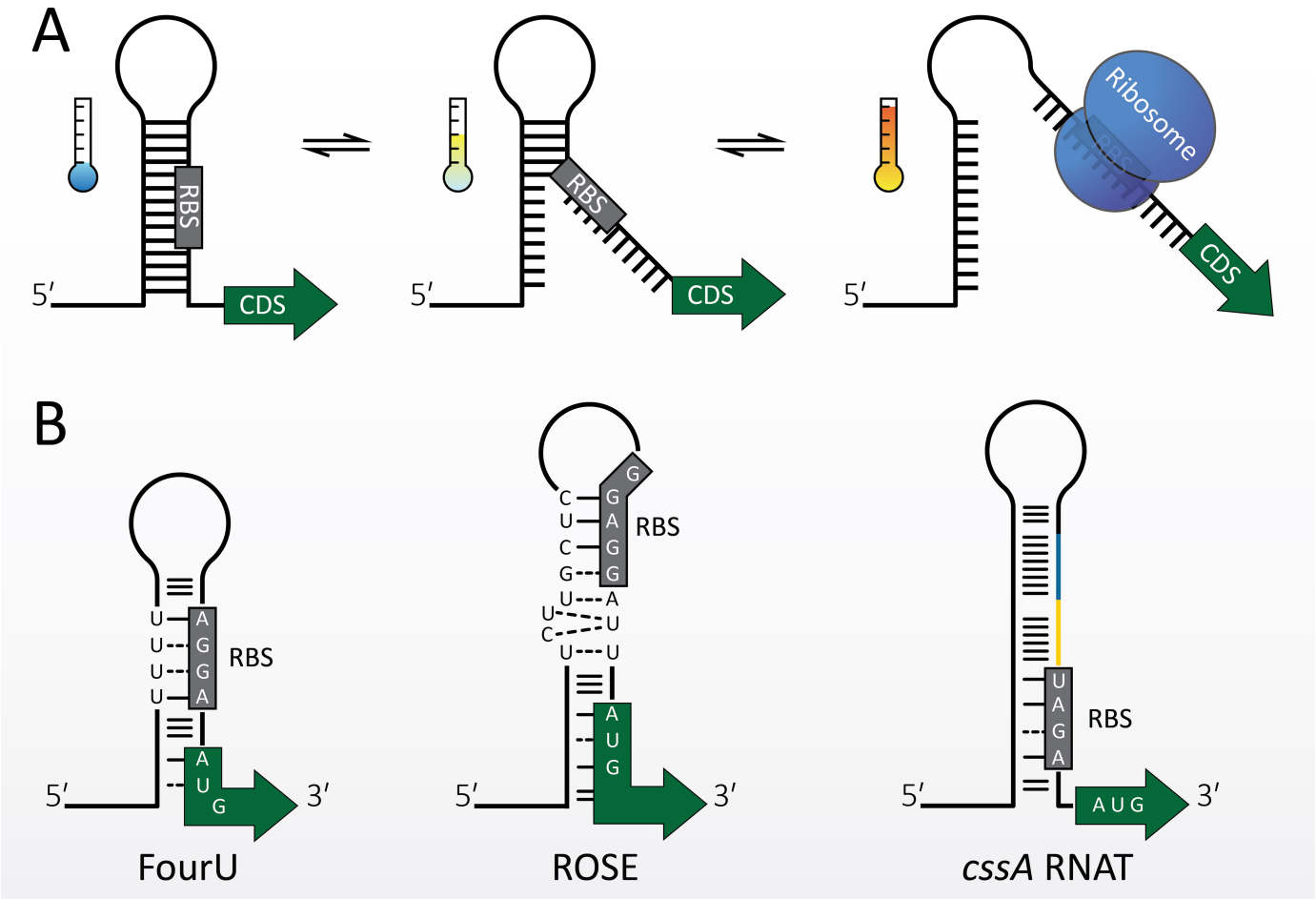
of the downstream gene. Upon encountering higher temperature, such as the host body temperature of 37°C, the higher thermodynamic energy weakens the RNAT structure, liberating the previously inaccessible RBS and allowing translation initiation (Fig. 1A).

Like many temperature-responsive mechanisms, RNATs were discovered as regulatory elements in the heat shock response. In *Escherichia coli*, an RNAT with a very complex secondary structure involving >200 nucleotides (nt) of the coding region regulates the synthesis of its heat shock master regulator Sigma-32 ($\sigma^{32}$, RpoH) (3). As a result, RpoH protects the bacterium through upregulation of a large group of heat shock proteins such as molecular chaperones and proteases. In many bacteria, the translation of a particular class of heat shock proteins termed small heat shock proteins provides the first line of defense against heat-induced protein aggregation. The synthesis of these proteins is under the direct control of an RNAT in their mRNA (4). Later, it emerged that RNATs are equally well suited for mounting a virulence response when a pathogen experiences a rise in temperature upon ingestion by a mammalian host (5). Acting through a posttranscriptional mechanism, RNATs allow a rapid response as the mRNA—although in an inactive conformation—is already present when the bacterium encounters its mammalian host. An instantaneous melting of the RNA structure followed by ribosome binding and translation initiation enables the immediate production of virulence factors.

Since the discovery of a virulence-related RNA thermosensor, similar elements have been identified in

**FIGURE 1** (A) RNATs are structural elements located within the 5′ UTR of protein-coding mRNAs and control its translation by operating as reversible molecular zippers that mask or unmask the RBS in response to temperature changes. (B) Three examples of RNAT secondary structures: FourU element of *S. enterica agsA*, ROSE element of *Bradyrhizobium japonicum hspA*, and the 8-bp tandem repeats of *N. meningitidis cssA* (blue and yellow lines indicate repeats). CDS, coding sequence.



the translation initiation region of many other bacterial genes directly or indirectly related to virulence (6, 7). All currently reported virulence-associated RNATs are summarized in Fig. 2 and Table 1. Interestingly, the nucleotide sequences and secondary structures of these thermosensors are very diverse and poorly conserved. Only a few recurring nucleotide motifs have been observed (Fig. 1B) (for reviews, see references 8 and 9). One example is the FourU motif, composed of four uridines that base-pair with the Shine-Dalgarno (SD) sequence. It is present in various virulence and heat shock thermometers (10–12). The diversity of RNAT sequences and structures suggests that the conceptually simple principle of sequestering parts of the RBS by complementary base pairing has evolved multiple times independently during evolution. In the following sections, the currently known virulence-associated RNATs will be described and grouped according to their function in virulence.

## CONTROL OF MASTER REGULATORS OF VIRULENCE

### *Listeria monocytogenes*—Two Regulatory RNAs Team Up

Controlling the virulence master regulator with an RNAT is a strategy adopted by at least three different human pathogens. The first virulence-related RNAT was discovered upstream of the *prfA* gene in *Listeria monocytogenes* (5). This Gram-positive, rod-shaped, and nonsporulating bacterium is commonly found in decaying soil and in food. In humans, *L. monocytogenes* is responsible for causing meningitis, meningoencephalitis, fetal infection, and neonatal abortion. The disease termed listeriosis is common among individuals with weakened immune systems, pregnant women, and the elderly. *L. monocytogenes* possesses the ability to cross the host's intestinal, fetal placental, and blood-brain barrier. In addition, *L. monocytogenes* is a serious threat to food production industries as it can tolerate high concentrations of salt and acidic conditions and is able to multiply at refrigeration temperatures.

To survive and thrive, *L. monocytogenes* alters its gene expression rapidly in adaptation to new environments. The expression of major listerial virulence factors is regulated by its transcriptional regulator PrfA. Due to its pivotal role in the bacterium's survival, *prfA* expression itself is tightly regulated through multiple levels: transcriptional, translational, and posttranslational through the availability of glutathione and other factors (13–15).

Temperature plays a decisive role in the expression of *prfA*. Translation is controlled by an unusually long RNAT located within the 5′ UTR of the mRNA. Outside of the host and at temperatures such as 26°C, the RNA

**FIGURE 2** Virulence-associated RNATs in bacterial pathogens. For details, see text and Table 1.

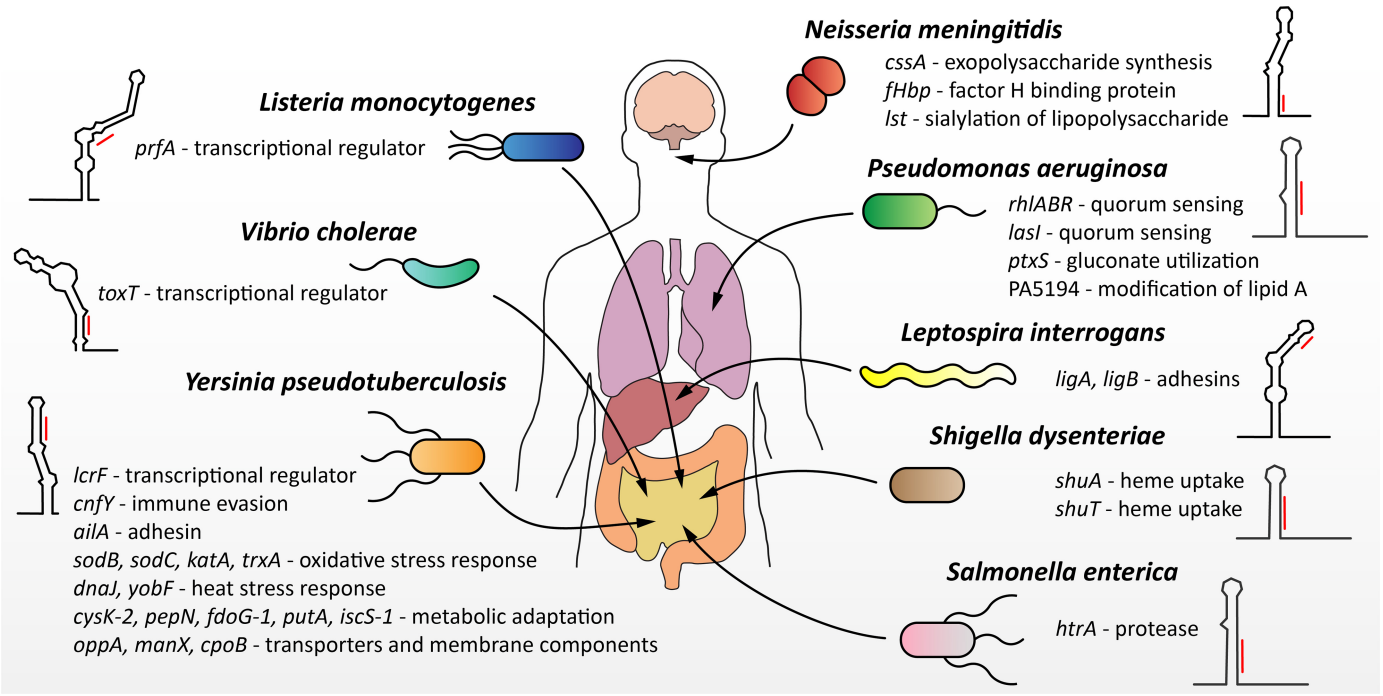


**TABLE 1** Summary of currently known virulence-associated RNATs in bacterial pathogens

| Regulated gene | Gene function | Role in pathogenicity | Organism | Characteristics of the RNAT | Experimental evidence | Reference(s) |
|---|---|---|---|---|---|---|
| *prfA* | Transcriptional regulator | Activates transcription of major virulence factors | *L. monocytogenes* | Single stem-loop structure occluding the RBS<br>Two prematurely terminated SAM riboswitches base-pair to the RNAT region, inhibiting translation | *In vitro* characterization via cross-linking, structure probing, and ribosome toeprinting assays<br>Reporter gene studies<br>Virulence assays in a mouse infection model | 5, 16, 17 |
| *toxT* | Transcriptional regulator | Activates transcription of the toxin-coregulated pilus and the cholera toxin | *V. cholerae* | Single stem-loop structure containing a FourU element | *In vitro* characterization via enzymatic structure probing and ribosome toeprinting assays<br>Reporter gene studies<br>Virulence assays in a mouse infection model | 12 |
| *lcrF* | Transcriptional regulator | Activates transcription of plasmid-encoded virulence factors, including the T3SS apparatus, *Yersinia* outer protein genes, and regulatory factors | *Y. pseudotuberculosis* | The intergenic region of the cotranscribed genes *yscW* and *lcrF* folds into two hairpin structures; the second contains a FourU element | *In vitro* characterization via enzymatic structure probing and ribosome toeprinting assays<br>Reporter gene studies<br>Virulence assays in a mouse infection model | 11, 29 |
| *cnfY* | Cytotoxic necrotizing factor | Toxin that modulates the host cell cytoskeleton, increases inflammatory responses, protects the bacteria from attacks of innate immune effectors, and enhances the severity of a *Yersinia* infection | *Y. pseudotuberculosis* | Single stem-loop structure partially occluding the RBS | Ectopic reporter gene studies | 32 |
| *ailA* | Adhesin | Mediates the attachment and killing of neutrophils, increases the delivery of Yops, and reduces the complement-mediated immune response | *Y. pseudotuberculosis* | Single stem-loop structure occluding the RBS | *In vitro* characterization via enzymatic structure probing and ribosome toeprinting assays<br>Ectopic reporter gene studies | 32 |
| *sodB* | Cytoplasmic superoxide dismutase | Protects from superoxide by converting it into hydrogen peroxide and water | *Y. pseudotuberculosis* | Two stem-loop structures; the second partially occludes the RBS | Ectopic reporter gene studies | 32 |
| *sodC* | Periplasmic superoxide dismutase | Protects from superoxide by converting it into hydrogen peroxide and water | *Y. pseudotuberculosis* | Two stem-loop structures; the second partially occludes the RBS | Ectopic reporter gene studies | 32 |
| *katA* | Catalase | Protects from hydrogen peroxide by converting it into water and oxygen | *Y. pseudotuberculosis* | Two stem-loop structures; the second partially occludes the RBS | Ectopic reporter gene studies | 32 |
| *trxA* | Thioredoxin | Reduces oxidized proteins and other molecules | *Y. pseudotuberculosis* | Two stem-loop structures; the second partially occludes the RBS | Ectopic reporter gene studies | 32 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *dnaJ* | DnaK cochaperone | Prevents protein aggregation and assists protein folding, in particular during stress conditions | *Y. pseudotuberculosis* | Complex architecture with base-paired AUG | Ectopic reporter gene studies | 32 |
| *yobF* | Putative heat shock protein | Unknown function | *Y. pseudotuberculosis* | Single stem-loop structure occluding the RBS | Ectopic reporter gene studies | 32 |
| *cysK-2* | Cysteine synthase A component | Enzyme involved in the synthesis of cysteine | *Y. pseudotuberculosis* | Single stem-loop structure occluding the RBS | Ectopic reporter gene studies | 32 |
| *pepN* | Aminopeptidase N | Involved in the degradation of peptides generated by protein breakdown | *Y. pseudotuberculosis* | Two stem-loop structures; the second partially occludes the RBS | Ectopic reporter gene studies | 32 |
| *fdoG-1* | Formate dehydrogenase | Catalyzes the oxidation of formate to carbon dioxide, donating the electrons | *Y. pseudotuberculosis* | Complex architecture with partially base-paired RBS | Ectopic reporter gene studies | 32 |
| *putA* | $\Delta^1$-Pyrroline-5-carboxylate dehydrogenase | Transcriptional repressor and membrane-associated enzyme involved in proline degradation | *Y. pseudotuberculosis* | Two stem-loop structures; the second partially occludes the RBS | Ectopic reporter gene studies | 32 |
| *iscS-1* | Component of cysteine desulfurase | Enzyme involved in the synthesis of Fe-S cluster | *Y. pseudotuberculosis* | Complex architecture with partially base-paired RBS | Ectopic reporter gene studies | 32 |
| *oppA* | Oligopeptide ABC transporter periplasmic binding protein | Uptake of oligopeptides | *Y. pseudotuberculosis* | Complex architecture with partially base-paired RBS | Ectopic reporter gene studies | 32 |
| *manX* | Component of mannose PTS[a] permease | Subunit of the mannose PTS permease | *Y. pseudotuberculosis* | Three stem-loop structures; the third partially occludes the RBS | Ectopic reporter gene studies | 32 |
| *cpoB* | Component of Tol-Pal cell envelope complex | Periplasmic protein involved in the coordination of peptidoglycan synthesis and cell division | *Y. pseudotuberculosis* | Four stem-loop structures; the fourth partially occludes the RBS | Ectopic reporter gene studies | 32 |
| *cssA* | UDP-*N*-acetylglucosamine 2-epimerase | Polysialic acid capsule biosynthesis, which confers resistance to immune system detection | *N. meningitidis* | Single stem-loop structure occluding the RBS | *In vitro* characterization via enzymatic structure probing and ribosome toeprinting assays<br>Ectopic reporter gene studies | 41, 44 |
| *fHbp* | Factor H binding protein | Confers protection against complement defense | *N. meningitidis* | Single stem-loop structure occluding the RBS | Ectopic reporter gene studies | 41 |
| *lst* | Sialyltransferase | Sialylates surface lipooligosaccharide | *N. meningitidis* | Single stem-loop structure occluding the RBS | Ectopic reporter gene studies | 41 |
| *ligA/ligB* | Surface-exposed lipoprotein | Binds human complement factor H and C4-binding proteins | *L. interrogans* | Single stem-loop structure occluding the RBS | Ribosome toeprinting assays<br>Ectopic reporter gene studies | 57 |
| *rhlR* | Transcriptional regulator | Mediates the QS response by controlling the expression of multiple genes, including virulence factors | *P. aeruginosa* | ROSE element located within the 5′ UTR of *rhlABR* mRNA and controlling RhlR synthesis through a polar effect | Reporter gene studies | 63 |

*(continued)*

**TABLE 1** Summary of currently known virulence-associated RNATs in bacterial pathogens *(continued)*

| Regulated gene | Gene function | Role in pathogenicity | Organism | Characteristics of the RNAT | Experimental evidence | Reference(s) |
|---|---|---|---|---|---|---|
| *lasI* | Acylhomoserine lactone synthase | Enzyme involved in the synthesis of an effector molecule that triggers the QS response | *P. aeruginosa* | ROSE-like element | Reporter gene studies | 63 |
| *ptxS* | Transcriptional regulator | Controls gluconate transport and usage | *P. aeruginosa* | Two stem-loop structures; the second partially occludes the RBS | *In vitro* characterization via enzymatic and chemical structure probing<br>Reporter gene studies | 66 |
| PA5194 (*lpxT*) | Putative lipid A 1-diphosphate synthase | Enzyme putatively involved in the phosphorylation of the lipid A | *P. aeruginosa* | Two stem-loop structures; the second contains a FourU-like element that partially occludes the RBS | *In vitro* characterization via enzymatic and chemical structure probing<br>Reporter gene studies | 66 |
| *shuA* | Heme uptake protein A | Outer membrane receptor required for heme uptake and iron scavenging | *S. dysenteriae* | Short single stem-loop containing a FourU element | Reporter gene studies | 70 |
| *shuT* | Heme uptake protein T | Periplasmic heme binding protein | *S. dysenteriae* | Single stem-loop structure occluding the RBS and part of the AUG start codon | *In vitro* characterization via enzymatic structure probing<br>Reporter gene studies | 71 |
| *htrA* | Protease | Periplasmic protease involved in protein quality control and stress response | *S. enterica* | FourU element | Reporter gene studies and extensive *in vitro* characterization via chemical structure probing (SHAPE) | 83, 84 |

[a]PTS, phosphotransferase system.

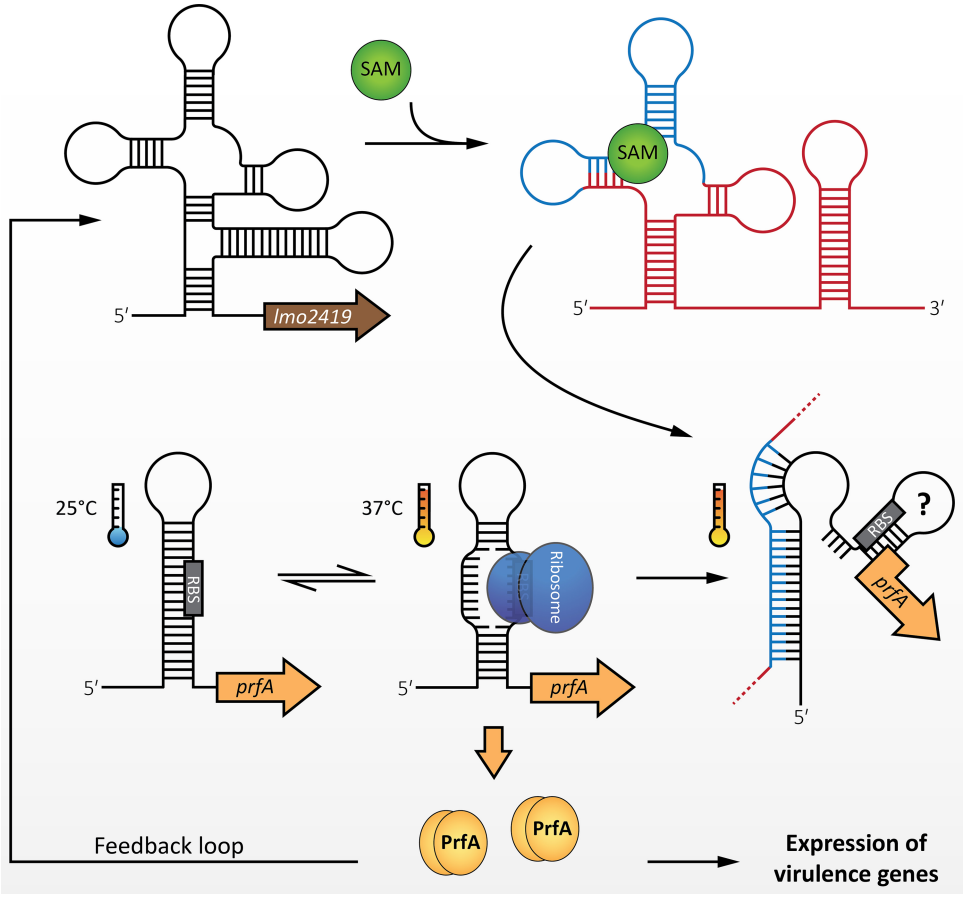
forms a stem-loop masking the RBS and blocking translation. During transition to the human host temperature of 37°C, the stem-loop structure unwinds, thus allowing translation of the *prfA* mRNA, as demonstrated through structure probing (5). Reporter gene and toeprint experiments showed that the first 20 codons within the *prfA* mRNA form a flexible hairpin-loop required for ribosome binding and efficient translation (16).

In addition to sensing temperature, the *prfA* RNAT is able to directly base-pair with a prematurely terminated *S*-adenosylmethionine (SAM) riboswitch. Two *trans*-acting SAM riboswitch-derived small RNAs have been demonstrated to be able to interact with the *prfA* 5′ UTR to inhibit translation (17). It has been speculated that upon base pairing, the noninteracting nucleotides of the *prfA* mRNA form an alternative structure occluding the RBS. Interestingly, this interaction between SAM riboswitch and *prfA* 5′ UTR is only observed at 37°C and not at low temperature, possibly due to the inaccessible *prfA* thermosensor stem-loop (Fig. 3). The intracellular concentration of SAM could play a role in controlling *prfA* expression during infection, especially during growth within the intestine, where PrfA-controlled virulence factors are not required (17). Overall, the *prfA* RNAT poses as a unique example as it is able to sense changes in temperature and SAM concentration for its efficient translation and activation of virulence factors.

## *Vibrio cholerae*—From the Water and Back into the Water

*Vibrio cholerae* is a facultative human pathogen and the causative agent of the diarrheal disease cholera. The bacterium resides in aquatic environments and is well adapted to the many challenges in this habitat (18). It experiences temperature fluctuations due to seasonal changes, after transmission from the environment into a

**FIGURE 3** Synthesis of the virulence regulator PrfA in *L. monocytogenes* is regulated by an RNAT in the 5′ UTR of *prfA* and a *trans*-acting SAM riboswitch element. *L. monocytogenes* detects temperature increase to 37°C as the signal of host entry, and as a consequence, PrfA is synthesized. A prematurely terminated riboswitch produced in the presence of SAM inhibits the translation of *prfA* via base pairing with its 5′ UTR.



human host, and when shed from the host back into the environment. Within the small intestine, *V. cholerae* initiates a virulence program that is dependent on its transcriptional regulators ToxR and ToxT (19). This program ultimately results in the production of two ToxT-induced factors: the toxin-coregulated pilus, required for intestinal colonization, and the cholera toxin, responsible for the profuse watery diarrhea.

Translation of the *toxT* mRNA is inhibited at environmental temperatures through a FourU-type thermometer that pairs with the AGAG of the SD sequence (12). Liberation of the SD sequence and binding of the ribosome at 37°C were demonstrated by structure probing and toeprint experiments. Most importantly, *V. cholerae* strains carrying point mutations that strengthen the *toxT* thermometer structure and prevent its opening at 37°C were unable to colonize the mouse intestine and to produce CT. These findings provided compelling evidence for the relevance of the temperature-responsive RNA element in disease development.

Upon returning to the water reservoir through fecal contamination, the reversible nature of this zipper-like RNA structure is expected to diminish ToxT production. *V. cholerae* closes the transmission cycle by inducing a genetic program that depends on the signaling molecule cyclic-di-GMP and favors a sessile lifestyle and biofilm formation (20).

## *Yersinia* Species—Temperature Sensing at Multiple Levels

The third example of an RNAT-controlled virulence transcription factor derives from *Yersinia* species and is best studied in *Yersinia pseudotuberculosis*. This foodborne pathogen infects humans and animals, causing enteritis and lymphadenitis. It is closely related to *Yersinia pestis*, the causative agent of bubonic and pneumonic plague. *Y. enterocolitica* is another human pathogen belonging to the *Yersinia* genus and infects the gastrointestinal tract, leading to enteritis and diarrhea.

Despite adopting diverse lifestyles and infection strategies, all three *Yersinia* species possess a similar virulence plasmid encoding the type 3 secretion system (T3SS) apparatus, *Yersinia* outer protein genes (*yop*), and regulatory factors (21, 22). Yops are either components of the translocation pore or effector proteins, which are injected into target eukaryotic cells via the T3SS. Yop effectors inhibit phagocytosis and the innate and adaptive immune responses by disrupting several signaling systems and interfering with the host cytoskeleton assembly (23).

*Y. pseudotuberculosis* is an excellent model system to study the correlation between temperature and virulence, as many of its genes are under temperature control. A transcriptomic study revealed that >300 genes are differentially regulated in response to temperature fluctuations typical of warm-blooded host infection (24). For most of these genes, the detailed regulatory mechanisms remain unknown.

The best-studied temperature-controlled gene is *lcrF* (*virF* in *Y. enterocolitica*), coding for a transcription factor responsible for the expression of the majority of the plasmid-encoded virulence factors (25). It has long been observed that the expression of *Yersinia* virulence factors is thermoregulated in response to a transition from a colder external environment to the host body temperature of 37°C (22). This regulation is mediated by LcrF, which is produced at high temperature due to a multilayered control cascade (Fig. 4) (26, 27).

The first level of temperature control acts on transcription of the *lcrF* gene, which is repressed by YmoA, a histone-like protein that binds to the *yscW-lcrF* promoter at low environmental temperature (11, 22). A temperature increase causes alteration of the DNA architecture, resulting in the liberation of the *yscW-lcrF* promoter and subsequent increase in transcription efficiency. Moreover, YmoA is stable at low temperature, while it is rapidly degraded by Clp and Lon proteases at 37°C (11, 28). In addition to temperature, other environmental signals are involved in the regulation of *lcrF* transcription (Fig. 4). These include oxygen stress via the IscR regulator, extracytoplasmic stress via the Rcs phosphorelay system, and the CpxA-CpxR two-component system (26, 27).

It was hypothesized in 1993 that the thermoregulation of *Y. pestis lcrF* occurs at the translational level due to an RNA structure sequestering the ribosome binding region (29). Only recently, experimental evidence was reported for a functional FourU-type RNAT upstream of *lcrF* in *Y. pseudotuberculosis* (11). In contrast to the RNATs discussed above, this thermosensor is not positioned in a 5′ UTR but located within the 124-nt-long intergenic region of the cotranscribed *yscW* and *lcrF* genes (Fig. 4). Its functionality is context independent, as it confers temperature regulation regardless of whether it is present in the full-length bicistronic transcript or as a short, isolated fragment. The *lcrF* thermometer consists of two hairpin structures. The second hairpin contains the FourU motif that occludes the SD region, thus impairing translation at 25°C. Strains of *Y. pseudotuberculosis* carrying stabilizing or destabilizing mutations in the RNAT that alter its thermosensing ability are either noninfectious or attenuated in a murine infection model, showing that a functional thermosensor is critically important for virulence.

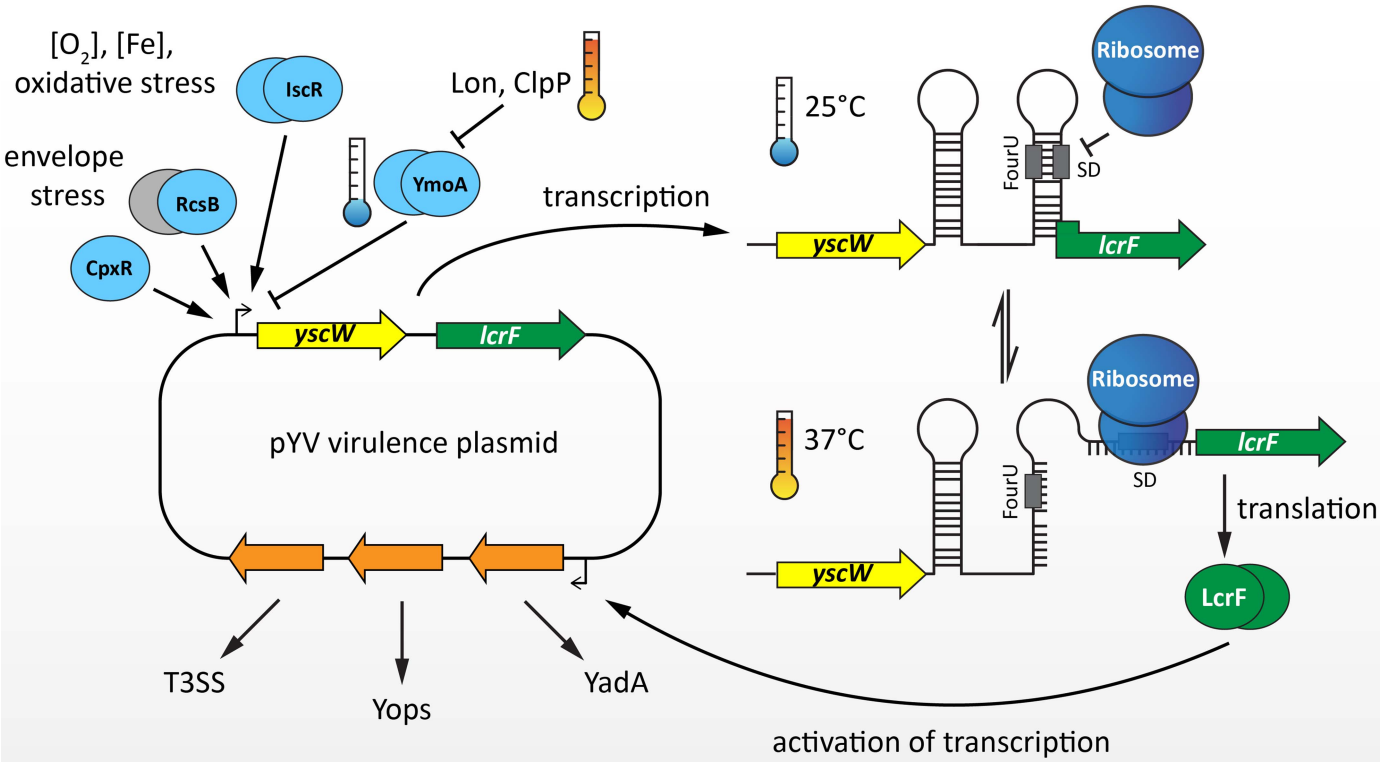



**FIGURE 4** Environmental regulation of *lcrF* expression in *Yersinia* species. Multiple stimuli are integrated and influence LcrF synthesis on the transcriptional and translational level. Temperature affects both transcription and translation of *lcrF*, via the histone-like protein YmoA and the *cis*-encoded FourU RNAT, respectively.

Recently developed deep sequencing-based RNA structure-probing approaches enabled the identification of RNATs on a transcriptome-wide scale. By parallel analysis of RNA structures (PARS) the conformations of all detectable RNA species extracted from a bacterial culture can be mapped (30, 31). The analysis of conformational changes in response to temperature changes provided insights into the dynamic RNA structurome of *Y. pseudotuberculosis*. At least 16 novel RNATs have been discovered by PARS. They regulate genes for two major virulence factors, oxidative stress protection, and metabolic functions (32, 33). Several of these candidates are currently under investigation.

## CONTROL OF IMMUNE EVASION AND EXTRACELLULAR FACTORS

### *Neisseria meningitidis*—Three RNATs To Undermine the Host Immune Response

The capsular polysaccharide of the obligate human pathogen *Neisseria meningitidis* is the major virulence factor of the bacterium. A variety of at least 13 different capsular structures has so far been identified and is used for classification of meningococcal serogroups. Six serogroups are associated with outbreaks, namely, A, B, C, W-135, X, and Y (34, 35). Isolates from the nasopharynx of carriers often are unencapsulated, while *N. meningitidis* isolates found in the circulatory or central nervous system possess capsule (36). The carbohydrate layer protects the bacterium from complement-mediated killing; and antimicrobial peptides are less effective, thus enabling the bacteria to survive intracellularly (37, 38). On the contrary, overproduction of capsule in the nasopharynx could mask adhesins important for attaching to epithelial cells and consequently impede its colonization ability (39).

Production of this capsular polysaccharide involves multiple steps and is energy demanding. The *css* locus, coding for capsule production, is highly conserved among the same serogroup (40). An RNAT has been discovered that modulates the translation of proteins involved in capsular biosynthesis (Fig. 1B). When *Neisseria* resides in the nasopharynx, where the temperatures range from 32 to 34°C, the *cssA* RNAT adopts a closed structure that covers the RBS (41–43). As a consequence, less capsular polysaccharide is produced, and thus adhesins are

more exposed, allowing better attachment to epithelial cells.

Upon dissemination into the circulatory system, the temperature rises to 37°C and the *cssA* RNAT stem-loop becomes more flexible, allowing ribosomes to bind, thus increasing the production of capsule (41). Through nuclear magnetic resonance (NMR) and SHAPE (selective 2′-hydroxyl acylation analyzed by primer extension) analyses, the *cssA*-RNAT structure was shown to start melting from top down at lower temperatures. The RBS, located at the bottom of the stem-loop, becomes partially accessible only at 37°C (44).

It is noteworthy that two tandem repeats of 8 nt in the 5′ UTR of the *cssA* RNAT are important for its functionality. Absence of one of these repeats leads to increased capsule biosynthesis even at the thermal conditions of the nasopharynx due to a weaker RNA secondary structure (41). Through gradual accessibility of the RBS, *N. meningitidis* fine-tunes capsule production in order to colonize and/or not detach from the epithelial cells but also to evade immune responses. Hence, the delicate and reversible RNAT control mechanism can be involved in virulence but also is important for colonization as a commensal.

Another RNAT-controlled immune evasive factor of *N. meningitidis* is the surface-located factor H binding protein (fHbp). Factor H is a negative regulator of the innate immune system's alternative complement pathway. Neisserial fHbp is able to bind to the host factor H, thus supporting C3b inactivation and C3-convertase degradation, ultimately blocking membrane attack complex formation and lysis (45, 46). During an invasive infection, *N. meningitidis* is exposed to this host protection mechanism; thus, binding factor H enables the bacterium to survive in human blood (47, 48). The *fHbp* RNAT possesses two anti-RBSs within the coding region. In the slightly colder nasopharynx, the bacterium is not in direct contact with such defenses and therefore downregulates fHbp (49). In addition, fHbp is surface exposed and highly immunogenic; therefore, reducing its presentation during colonization is a necessary consequence to not provoke an immune cell response.

The production of the sialyltransferase Lst, which modifies the lipopolysaccharide molecules on the outer membrane of *N. meningitidis*, is also directly controlled by an RNAT (41). Sialylation of lipopolysaccharide molecules protects *N. meningitidis* from complement-mediated serum killing as well as phagocytic killing by neutrophils.

Infection with viral pathogens, such as influenza virus, could lead to local inflammation (temperature increases in the nasopharynx) and recruitment of immune effectors onto the surface. Temperature therefore acts as a warning signal to *N. meningitidis*, enhancing its defense against human immune killing. Thus, *N. meningitidis* poses as a unique example where the bacterium has three independently evolved RNATs to counteract immune killing. In addition, *N. meningitidis* is currently the only known obligate human pathogen, with no host other than humans or external habitat, to possess RNAT-mediated virulence gene regulation.

## *Leptospira interrogans*—Two Identical RNATs To Colonize the Host

*Leptospira interrogans* is a Gram-negative, obligate aerobic spirochete bacterium that possesses periplasmic flagella. These spirochetes, as well as other pathogenic members of the genus *Leptospira*, are the causative agents of the globally endemic zoonotic infection termed leptospirosis. The most common mammalian reservoir of *L. interrogans* is the rodent, where it starts its life cycle within the renal tubules. Transmission usually occurs through the rodents' urine coming in contact with abraded human skin, eyes, or mouth. Human patients usually experience a sudden onset of fever as the bacteria disseminate within their circulatory system and replicate within the internal organs. This multifaceted pathogen encounters various environmental signals and has evolved sensing mechanisms to modulate its gene expression to facilitate its survival within hosts.

Such regulation is exemplified in the expression of surface-exposed lipoproteins allowing the bacterium to bind to various host proteins such as fibronectin. The *L. interrogans* Lig proteins encoded by *ligA* and *ligB* have previously been shown to facilitate colonization of the bacterium among components of the host extracellular matrix (50–54). In addition, both Lig proteins are able to directly bind human complement factor H and C4-binding protein (55), avoiding complement-mediated killing. Lig proteins were also shown to bind to other complement proteins such as C3b and C4b (56).

The expression of both *ligA* and *ligB* is temperature dependent and is controlled by two identical RNATs (57). These RNATs are predicted to form an extended secondary structure of two stem-loops, with the second loop occluding the RBS. Due to difficulties in the genetic manipulation of *L. interrogans*, all molecular studies on *lig* RNATs have so far been conducted in *E. coli*. The RNATs were shown to control expression in a temperature-dependent manner both *in vivo* and *in vitro*. Various point and deletion mutations within the 5′ UTR confirmed that the nucleotides predicted to be positioned opposite

to the RBS are crucial for translation efficiency (57). In addition to responding to temperature, Lig protein production is upregulated during osmotic stress (58). However, the precise mechanisms of how the osmolarity and temperature signals are integrated and coordinated remain unknown.

## QUORUM SENSING AND IRON ACQUISITION

### *Pseudomonas aeruginosa*—RNATs Coordinate Lifestyle Decisions

*Pseudomonas aeruginosa* is an opportunistic pathogen that causes nosocomial infections, especially in immunocompromised patients. It is the primary cause of mortality in cystic fibrosis patients, where the bacterium establishes chronic lung infections and forms biofilms that are difficult to eradicate due to intrinsic antibiotic resistance (59). *P. aeruginosa* possesses a remarkably versatile lifestyle and is capable of proliferating in diverse environments such as soil, water, and animal and plant tissues.

Posttranscriptional temperature responses in *P. aeruginosa* rely on various ROSE (repression of heat shock gene expression)-like RNATs. In addition to the FourU motif, ROSE elements constitute a second class of moderately conserved RNATs (60). The NMR structure of the founding member of this family revealed a temperature-labile hairpin with several unusual base pairs that form a network of weak hydrogen bonds and thereby facilitate opening of the structure (61) (Fig. 1B). As in many other proteobacteria, the small heat shock gene *ibpA* is under the control of a ROSE element in *P. aeruginosa* (62).

Two additional ROSE-like RNATs in *P. aeruginosa* are responsible for regulating key components of the quorum sensing (QS) cascade (63). QS is a communication mechanism used by bacteria to sense the population density and adjust their lifestyle accordingly. *Pseudomonas* uses this strategy to modulate metabolism, biofilm formation, motility, competition with other commensal microorganisms, and, importantly, expression of several virulence factors, including pyocyanin, rhamnolipid biosurfactants, and the cytotoxic PA-IL lectin (64).

Three interconnected regulatory cascades govern the *P. aeruginosa* QS response, with RhlR acting as one of the master transcriptional regulators. The gene *rhlR* can be cotranscribed in a tricistronic operon, *rhlABR*, together with two genes involved in the biosynthesis of rhamnolipids. A ROSE element in the 5′ UTR of the first open reading frame, *rhlA*, controls the expression of *rhlR* through a polar effect on transcription elongation of the operon. At 30°C, the ROSE element inhibits translation of *rhlA*. As a consequence, transcription is prematurely terminated due to the formation of possible RNA structures that fold within the *rhlA* coding region. Consequently, the downstream *rhlB* and *rhlR* genes are not expressed. At 37°C, the ROSE structure melts, *rhlA* is translated, and transcription of the full-length tricistronic operon is enabled. Concomitantly, the *rhlA* 5′ UTR was identified as the target of the small RNA NrsZ (65). NrsZ is expressed under nitrogen limitation and binds to the anti-SD region of the *rhlA* 5′ UTR, liberating the RBS and thus promoting translation. The interplay between the RNAT-mediated thermoregulation of *rhlABR* and the small RNA action in response to nitrogen deprivation has not yet been investigated.

Another thermoregulated gene is *lasI*, coding for an enzyme involved in the synthesis of an important effector molecule that triggers the QS response (63). The RBS is partially occluded in the predicted structure of the *lasI* 5′ UTR in a ROSE-like conformation. The regulatory potential of the *lasI* RNAT was verified in reporter gene studies; however, the temperature-dependent accumulation of LasI only leads to a minor increase in the production of the effector molecules and expression of the associated virulence traits.

To identify additional RNATs in *P. aeruginosa*, a genetic screening approach was developed and led to the identification of four candidates that responded to a temperature shift from 28 to 37°C (66). One of the regulated genes codes for PtxS, a transcription factor that controls gluconate transport and usage; and another codes for PA5194, an ortholog of *E. coli* LpxT, which phosphorylates lipid A. The structure of the *ptxS* RNAT does not resemble any conserved class of RNATs, while PA5194 contains a partial FourU motif comprising three uridines that pair with the SD sequence. Interestingly, the predicted structure of the 5′ UTR of *E. coli lpxT* might also form a stem-loop that involves the ribosome binding region. The physiological role of the newly identified *P. aeruginosa* RNATs remains to be elucidated. Interestingly, PtxS has been shown to bind to PtxR, which regulates the expression of ToxA, an important virulence factor (67). Moreover, modifications of the lipopolysaccharide, such as the phosphorylation of lipid A, are known to impact its stability and endotoxicity in several pathogenic bacteria (68).

### *Shigella*—Host Temperature Facilitates Iron Acquisition

*Shigella* species are Gram-negative, facultative anaerobic, pathogenic enterobacteria that cause dysentery in

humans. Throughout the infection process, pathogens experience numerous environmental changes, among them iron limitation. Freely available iron is scarce in the human body because most of it is complexed by heme and other iron-binding proteins. To cope with this situation, *Shigella* species employ several iron acquisition systems to take up ferric iron ($Fe^{3+}$), heme, or ferrous iron ($Fe^{2+}$) (69). These systems are regulated at the transcriptional level by Fur, an iron-binding repressor protein. Since heme is an iron source that is typically encountered in the host but not in the environment, the expression of heme acquisition systems should ideally be coordinated with the presence in a mammalian host. This is in fact the case in *Shigella dysenteriae* and in enteropathogenic *E. coli* strains, where translation of *shuA* and *chuA*, respectively, is under dual control, namely, by the Fur regulator and an RNAT (70). ShuA and its ortholog ChuA are outer membrane receptors for heme. Toward the 3′ end of their ~300-nt-long 5′ UTR, the *shuA* and *chuA* transcripts are able to fold into a short and fairly simple hairpin structure that can be classified as FourU thermometer. It confers temperature-dependent translation control. The isolated RNAT region is sufficient for this regulation, and dysregulated expression by stabilization and destabilizing point mutations supported that it acts in a zipper-like manner. Whether other environmental signals are integrated by the rest of the long 5′ UTR remains an interesting open question.

The periplasmic binding protein ShuT is another component of the *Shigella* heme uptake system, and expression of the *shuT* gene is also under transcriptional control by the Fur regulator and under translational control by an RNAT (71). Interestingly, this RNAT has nothing in common with the *shuA* thermosensor. The full-length *shuT* 5′ UTR is only 42 nt long and folds into a single hairpin that differs from the *shuA* RNAT in both sequence and structure. The existence of two different RNATs in the same physiological pathway suggests that temperature-regulated acquisition of the host-specific iron source is fundamentally important and that it can easily be achieved by the evolution of RNA-based thermosensors.

## OTHER VIRULENCE-RELATED FUNCTIONS

### *Salmonella*—Heat Shock Thermometers Might Contribute to Virulence

Members of the genus *Salmonella* are motile, Gram-negative pathogenic bacteria and closely related to *E coli*. The genus consists of two members, namely, *S. bongori* and *S. enterica*, which can be subdivided into >2,400 serovars, based on their antigenic presentation (72). Infection via ingestion of contaminated food or liquids causes enteric diseases. *S. enterica* serovars Typhi and Paratyphi are restricted to the human host and responsible for a systemic disease termed enteric (typhoid) fever. Interestingly, nontyphoidal *Salmonella* serovars, such as *S. enterica* serovar Typhimurium or *S. enterica* serovar Enteritidis, have a broad host range and are causative agents of enteritis in immunocompetent as well as bacteremia in immunocompromised individuals (73, 74). Consequently, infection strategies of nontyphoidal *Salmonella* and typhoidal *S. enterica* species are different for the most part, although *S.* Typhi and *S.* Typhimurium species share 89% of their genes (75, 76).

*S. enterica* is a facultative intracellular pathogen that is able to survive and replicate in phagocytic and nonphagocytic cells (77). For this purpose, *S. enterica* has evolved a huge arsenal of virulence factors. Among these factors are two T3SSs (T3SS1 and T3SS2) that encode transport machineries for injection of effector proteins (78, 79). Among the numerous virulence-related factors is the high temperature requirement A (HtrA or DegP) protease, which plays a key role in protein quality control and stress response through refolding or degrading misfolded proteins in the periplasm (80). Mutants that carry a null mutation in *htrA* (and *htrA* homologs) have been created in several bacterial species and showed overlapping phenotypes, such as increased thermosensitivity or sensitivity to oxidative as well as osmotic stress (81). A deletion of *htrA* impairs growth of *S.* Typhimurium at higher temperatures (42°C), and additionally, the deletion strain is less viable in a mouse model compared to the wild type (82).

Translation of the *S.* Typhimurium *htrA* gene is controlled by a FourU RNAT that forms a single hairpin occluding the RBS at low temperatures (83). A recent SHAPE analysis systematically characterized the dynamics of the *htrA* thermometer at single-nucleotide resolution and confirmed the proposed hairpin structure, which unfolds in a cooperative fashion, with nucleotides from the upper and lower part of the stem gaining flexibility at a common transition temperature (84). Mutational analysis revealed three U-G base pairings, including two uridines of the FourU motif, to be essential for the thermometer functionality.

Several non-virulence-associated RNATs have also been identified and characterized in *Salmonella*. The small heat shock gene *agsA* (aggregation suppressing A) is regulated by the founding member of the FourU family (10) (Fig. 1B). The cooperative melting behavior of this

RNAT has been studied at nucleotide resolution by NMR, and magnesium binding to the FourU motif was found to stabilize the structure (85, 86). A recent in-cell SHAPE analysis revealed that only a small fraction of the RNA population undergoes structural changes after temperature upshift, and that subtle changes in the RNA helix are sufficient to increase translation efficiency. An active contribution of the ribosome to the melting process was proposed (87).

An RNAT upstream of the *groES* gene confers differential temperature control to the *groESL* heat shock operon coding for a major molecular chaperone system (88). In addition to these structurally and functionally characterized thermometers, a biocomputational approach predicted various new RNATs in the genomes of 25 *S. enterica* isolates (89). The identified potential hairpin structures were further analyzed by secondary structure prediction in order to retrieve the melting temperature at the physiological ionic strength (89). If and how these diverse RNAT candidates, often upstream of genes with unknown function, are involved in virulence processes in *Salmonella* remains to be determined.

## MUCH MORE TO COME?

The concept of RNAT-mediated translational control established almost 20 years ago (3, 90) has paved the way to the discovery of numerous heat shock and virulence genes under the control of such structured RNA elements (91). Challenges in the identification of new thermosensors are the poor conservation of RNAT sequences and structures as well as the existence of noncanonical base pairs in the temperature-responsive region, as was shown for the ROSE element (61). This makes genome-wide searches for temperature-regulated RNA structures difficult and often leads to false-positive candidates showing no temperature regulation *in vivo* (92). The implementation of algorithms able to predict temperature-modulated RNA structures such as RNAtips (temperature-induced perturbation of structure) or RNAthermsw (93, 94) has improved the reliability of computational approaches but nonetheless requires labor-intensive experimental validation of the candidates.

Fundamentally new strategies have recently been established to experimentally map temperature-sensitive RNA structures on a transcriptome-wide scale with nucleotide resolution. "RNA structuromics" approaches combine structure-probing protocols with the power of next-generation sequencing (95, 96). Typical PARS experiments have been performed at one given temperature, for example, in yeast (30) and *E. coli* (97). In this *in vitro* method, total RNA samples are partially digested *in vitro* with nucleases that have a preference for nucleotides in either paired or unpaired conformation. After adapter ligation, cDNA synthesis, and next-generation sequencing, the cleavage sites are mapped onto the transcriptome and PARS-guided structure calculations reveal the structure of thousands of transcripts. Extending the protocol to samples refolded at different temperatures allows the transcriptome-wide mapping of temperature-responsive RNA structures. Such PARTE (PARS with temperature elevation) experiments have been conducted with yeast (31) and *Y. pseudotuberculosis* (32). Thousands of temperature-sensitive RNA structures in yeast and hundreds in *Yersinia* promise the discovery of novel RNA thermosensors involved in the regulation of temperature-modulated processes. Although not all PARTE-derived RNAT candidates turned out to be true thermosensors *in vivo*, the success rate of this approach was ~75% (32). Given that only subtle changes in the dynamic RNAT structure can be sufficient to foster translation initiation, it is well possible that some temperature-responsive RNA structures will escape detection by global structure-probing approaches (87). Nonetheless, in *Y. pseudotuberculosis,* the PARTE approach revealed numerous functional RNATs upstream of bona fide virulence genes, as well as upstream of genes responsible for oxidative stress adaptation and various other metabolic pathways (32). These findings suggest that numerous cellular pathways in bacterial pathogens are under RNAT control to allow induction at host body temperature.

An interesting open question is whether RNAT-modulated virulence gene expression also occurs in eukaryotes such as fungal pathogens. Given the abundance of RNATs in bacterial pathogens (Fig. 2) and the presence of myriads of yeast mRNA structures that change their conformation with increasing temperature (31, 98), it might be rewarding to investigate the dynamic RNA structurome of microbial eukaryotic pathogens that transition between environmental temperatures and warm-blooded animals or humans.

In summary, the rapid temperature sensing through RNATs utilized by bacterial pathogens is an exquisite but simple concept. Due to the lack of sequence conservation, these RNA elements have independently evolved based on their functional secondary structures. The combination of structure probing and next-generation sequencing promises the discovery of more of these RNA elements in all kingdoms of life in the future.

## ACKNOWLEDGMENTS

E.L., F.R., and H.E. were supported by the Knut and Alice Wallenberg Foundation, Karolinska Institutet, Swedish Research Council grant 2014–2050, and the Swedish Foundation for Strategic Research. F.N. is supported by the German Research Foundation (NA 240/10-1).

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