Chapter 9

Temperature-Dependent Regulation of Bacterial Gene Expression by RNA Thermometers

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Additional information is available at the end of the chapter

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Abstract

RNA thermometers (RNATs) are cis-encoded regulatory elements that modulate translational efficiently in response to environmental temperature. Since their initial discovery, numerous RNATs have been identified and characterized, with the majority of currently known RNATs present in a wide variety of bacterial species. RNATs repress translation at relatively low temperatures by physically preventing binding of the ribosome to the regulated transcript by incorporating the Shine-Dalgarno sequences (and/or start codon) into an inhibitory structure. As the environmental temperature increases, the inhibitory structure within the RNAT is destabilized and the repression of translation initiation is gradually relieved. With the development of identification techniques, the rate at which RNATs are identified, and the understanding of the molecular mechanisms governing their regulator function, has grown exponentially. With the ever-increasing number of characterized RNATs, broad families of these regulators have now been identified. It has also become abundantly clear that RNATs influence several essential physiological processes. This chapter aims to summarize the current knowledge of bacterial RNATs, with special emphasis placed on the molecular mechanisms underlying RNAT function, experimental techniques used to identify and characterize RNATs, families of bacterial RNATs, as well as biological processes controlled by RNATs, and future directions of the field.

Keywords: RNA thermometer, ribo-regulator, gene regulation, heat shock response, virulence factors

1. Introduction

Whether it is within a host or within the non-host environment, bacteria experience frequent, and often extreme, changes within their immediate environment. In order to survive and thrive under different environmental conditions, bacteria have evolved various systems that function to sense changes in environmental conditions and mediate rapid adaptation in response to the

specific change. One condition that varies between the different environments encountered by pathogenic and non-pathogenic bacteria alike is temperature. Environmental temperature has direct effects on several fundamental biological processes, including proper folding of proteins and optimum activity of enzymes. To counteract the potentially detrimental effects of altered temperature, bacteria have evolved several strategies to respond to changes in environmental temperature, including specific heat shock and cold shock responses. Moreover, for pathogenic bacteria, a change of environmental temperature is a critical cue that can indicate entry into the host and/or progression of the disease process within an infected host. In order to establish and progress an infection, bacteria not only need to efficiently adapt to changing environmental conditions but also need to precisely regulate the production of specific virulence factors — processes that are dependent on the ability of bacteria to sense specific changes in environmental conditions, including temperature.

One method of sensing alterations in environmental temperature is through changes in the secondary structure of RNA molecules. Double-stranded regions within a given RNA molecule tend to dissociate into single-stranded structures with an increase in environmental temperature. The temperature at which half of the population of a given double-stranded RNA molecule is in the single-stranded conformation is defined as the Tm, a feature that is commonly used as a measurement for the stability of a given structure within an RNA molecule [1]. Due to its propensity to change conformation, an RNA structure that has a relatively low Tm is more responsive to changes in environmental temperature, a feature that facilitates its potential to act as a molecular thermosensors [2].

It is well established that translational efficiency is affected by the secondary structure of an RNA transcript, particularly that of the region containing the ribosome-binding site and/or start codon [3]. It was not until 1989, however, that the first *cis*-encoded temperature-responsive RNA regulatory element was identified [4]. Since that time, the rate at which temperature-responsive *cis*-encoded regulatory RNA elements have been identified, and the concurrent understanding of how they function to control target gene expression has grown exponentially — a statement that is particularly true of temperature-sensing RNA regulatory elements in bacteria. Based on their innate responsiveness to changes in environmental temperature, regulatory RNA elements that function to modulate the translational efficiency for the transcript in which they are housed in response to alterations in temperature have been termed "RNA thermometers" (RNATs) [5]. Unlike metabolites-binding riboswitches, the activity of RNAT is not modulated by the absence or presence of a ligand [6,7]. The regulatory function of an RNAT relies solely on its innate chemical nature, which dictates the differential stability of a specific inhibitory structure at different environmental temperatures.

With the ever-increasing number of characterized RNATs, variability within this class of regulators is now coming to light. While the majority of RNATs are composed of sequences within the 5′ untranslated region (5′ UTR) of the regulated gene, some have now been shown to be composed, at least in part, of sequences within the coding region of the regulated transcript or by sequences within the coding region of a preceding gene within a polycistronic transcript [8,9]. In addition, the number of stem loops composing different RNATs varies, ranging from one in the simplest RNATs to five in the most complex RNATs [10,11]. Despite the variability among RNATs, they all share several basic fundamental features. Identifying

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and understanding the functional contribution of features conserved among characterized RNATs, as well as those that vary among this class of regulators, has and will continue to inform the foundational knowledge of the biological functions and chemical nature of these ubiquitous regulators. This chapter focuses on bacterial RNATs and provides a comprehensive summary of the current state of knowledge of RNATs, with emphasis given to discussions of the molecular mechanism underlying RNAT function, experimental techniques used to identify and characterize RNATs, families of bacterial RNATs, as well as the biological processes controlled by RNATs, and future directions of the field.

2. Molecular mechanism underlying the regulatory function of RNA thermometers

The molecular mechanism underlying the regulatory activity of RNATs is exquisitely simple, mediated entirely by temperature-induced structural changes within a target mRNA molecule. The currently proposed model of the molecular mechanism underlying RNAT function is that of a zipper [5]. More specifically, at relatively low "non-permissive" temperatures, an inhibitory structure is formed within the RNAT, at least in part, by binding of Shine-Dalgarno (SD) sequences with upstream sequences within the regulated transcript. Once formed, the inhibitory structure functions to block translation initiation by physically preventing binding of the ribosome to the regulated transcript. With an increase of temperature to that within a permissive range, the base-pairs that stabilize the inhibitory structure within the RNAT gradually dissociate, the ribosome-binding site becomes increasingly exposed and translation proceeds (Figure 1) [7,12].

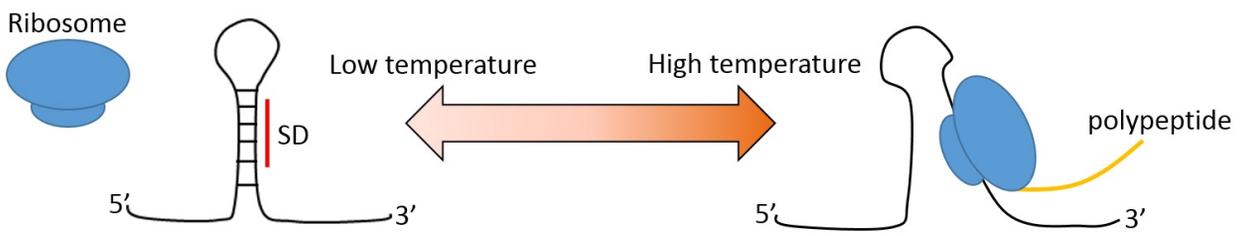

Figure 1. A schematic of the molecular mechanism underlying the regulatory function of RNA thermometers. In the figure, the hairpin structure indicates the inhibitory hairpin of an RNA thermometer, with the red line representing the region of Shine-Dalgarno (SD) sequence or ribosome-binding site. At relatively low, or non-permissive, temperatures, the formation of the inhibitory hairpin inhibits translation from the transcript by preventing binding of ribosome to the SD sequences. As the environmental temperature increases to the permissive range, the inhibitory structure dissociates, giving the ribosome access to the SD sequence and thus permitting translation.

Though responsive to temperature, RNAT-mediated regulation is not an all-or-nothing regulation but rather the shifting of an equilibrium towards an open or closed configuration depending on temperature [5]. Furthermore, mutagenesis-based experimentation has clearly demonstrated that it is the altered stability of the inhibitory structure rather than the primary sequence that plays the most critical role in the regulatory function of RNATs [13].

Several features differentiate RNATs from metabolites-binding riboswitches, a superficially related class of ribo-regulators. Firstly, as demonstrated by UV and NMR spectroscopy assays, the temperature-induced destabilization of the inhibitory structure within a given RNAT is a gradual and reversible process [7,14]. As a result of these fundamental features, RNATs mediate a graded response to temperature as opposed to an "on/off" type of regulation that is often associated with riboswitch-mediated regulation. Secondly, unlike riboswitches, structural changes within the RNAT are not mediated by an interaction with a small molecule or other cellular component, a foundational feature confirmed by *in vitro* structural analyses [7]. While the regulatory activity of RNATs is not responsive to the presence or absence of a ligand, Mg^{2+} has been found to facilitate the regulatory function of some RNATs [15]. Specifically, Mg^{2+} has been shown to affect the stability and thus the temperature responsiveness of the inhibitory structure of some RNATs, a feature that is fundamentally different than small molecule-induced switching between mutually exclusive structures as is seen with riboswitches.

While many advances have been made in recent years, several questions remain regarding the details of the molecular mechanism(s) underlying the activity of RNATs. For example, several studies investigating the regulatory mechanism of RNATs focus exclusively on the hairpin containing the SD sequence. As a consequence, the impact of additional structural features within an RNAT, particularly that of commonly observed upstream hairpins, remains largely unknown. Additionally, a recent study revealed that, for at least a subset of RNATs, the ribosome can bind to the SD sequence of the regulated transcript even at non-permissive temperatures when the inhibitory structure would be present [16]. Finally, while the current model of regulation invokes nothing more than temperature in mediating the structural changes that underlie the regulatory activity of RNATs, the role of additional factors, including that of the ribosome itself, remains the subject of active investigation.

3. Identification of RNA thermometers

3.1. *In silico* predictions

Given that the function of an RNAT is dependent on its structure, the identification of a new RNAT often starts with *in silico* analyses aimed at predicting secondary structure and the Gibbs free energy released by folding of known transcripts at different temperatures. Such predictions are often generated using freely available web-based programs, such as Mfold and are typically carried out individually for each transcript under investigation [17]. The identification of putative RNATs has been facilitated by the generation of a searchable database that contains the predicted structures within the untranslated regions of bacteria transcripts (RNA-SURIBA) [18]. Additionally, web servers such as RNAtips and RNAthermsw are now available, which calculate the folding energy of a given RNA molecule under varied temperatures [19,20]. Together, these databases and programs can be utilized to predict the presence of a putative RNAT within a given transcript or genome. While *in silico* approaches have proven powerful in the identification of many RNATs, they are limited in that they are only predic-

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tions. Additionally, all currently available *in silico* prediction tools are based on our current understanding of identified RNATs and therefore may not recognize novel types of RNATs with unique structural features. As the number of characterized RNATs continues to grow, so will our ability to accurately predict their presence in sequenced transcripts.

3.2. Identification with experimental approaches

Regardless of the approach used to predict the existence of a functional RNAT, the thermosensing regulatory activity of each putative element must be validated experimentally. There are several lab-based approaches currently being utilized to demonstrate the functionality of newly identified RNATs. One way in which the thermoresponsive regulatory activity of a putative RNAT is tested is to clone the element being investigated between a constitutive or an arabinose-inducible plasmid promoter and a reporter gene (e.g., *lacZ* or *gfp*) on a plasmid [10,11,21]. By introducing the constructed reporter plasmid into a bacterial strain and measuring the relative amounts of both the reporter transcript and reporter protein following growth of the strain at different temperatures, the functionality of the putative RNAT under investigation can be accessed. Specifically, if the putative RNAT is functional, production of the reporter protein will increase with the rise of temperature, while the relative levels of reporter transcript will not vary. Such experimental investigations, along with mutagenesis analysis, have been utilized to demonstrate the functionality of several newly identified RNATs [11,22–24] (Figure 2).

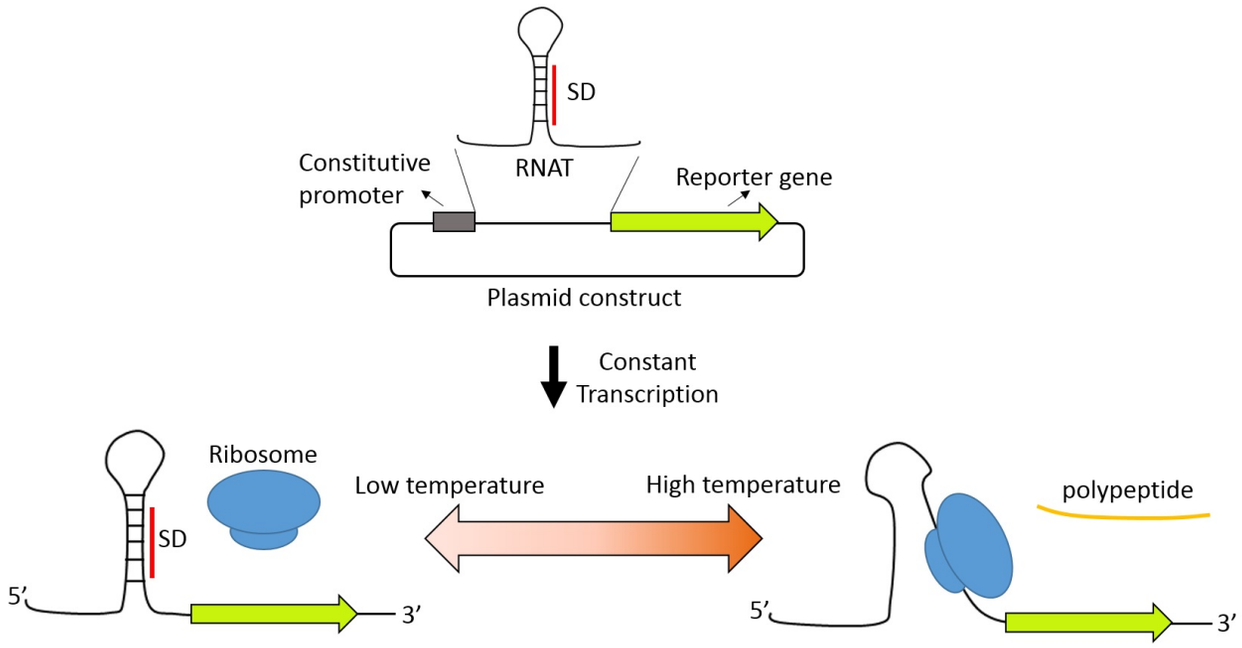

Figure 2. *In vivo* reporter plasmid-based assay used to experimentally test the thermoresponsive regulatory activity of a putative RNA thermometer. The reporter plasmid is constructed by cloning the inhibitory hairpin of a putative RNA thermometer between a constitutive or arabinose-induced plasmid promoter and a reporter gene. If the RNA thermometer is functional, it is expected that, following the introduction of the reporter plasmid into a bacterial strain and the growth of that strain at different temperatures, the relative amounts of the reporter transcript will be constant, while the relative levels of the reporter protein will be regulated in response to temperature.

To further validate the functionality of a predicted RNAT, *in vitro* analysis such as structure probing assays can be utilized to directly investigate the impact of varied temperature on the secondary structure of the element under investigation [22,25]. The principle underlying structure probing-based analyses is that specific RNA-digesting enzymes cleave RNA molecules based on the presence of specific secondary structures and/or primary sequences. For example, RNase T1 cleaves immediately 3′ to a single-stranded guanine, while RNase V1 cleaves double-stranded RNA in a sequence-independent manner. Briefly, to perform a structure probing analysis, the putative RNAT under investigation is synthesized by *in vitro* transcription and then radiolabeled at the 5′ end. Next, the labeled RNA molecule is subjected to partial digestion with various RNA-digesting enzymes separately, and the generated fragments visualized by electrophoresis in a denaturing polyacrylamide gel. By completing this analysis at different temperatures it is possible to determine the impact of environmental temperature on the global structure of the RNA molecule (Figure 3).

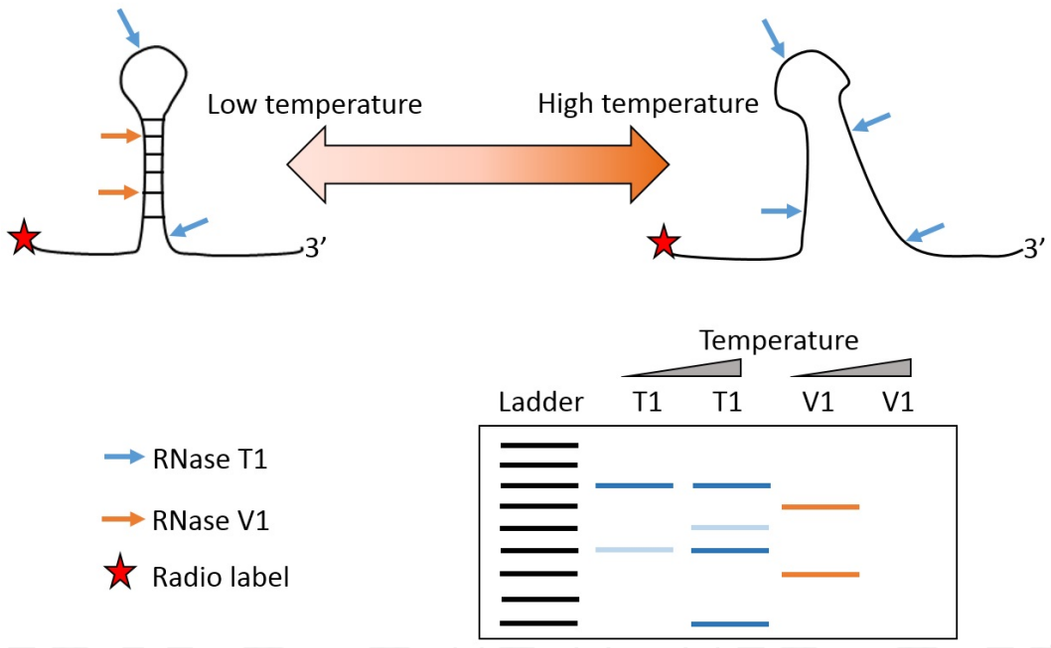

Figure 3. Structure probing assay used to experimentally determine the secondary structure of a putative RNA thermometer. An *in vitro* transcribed putative RNA thermometer is represented as the hairpin structure in this figure. Following radioactive end-labeling (indicated by a red star), the molecule is subject to partial digestions with different RNA-digesting enzymes and the resulting products are visualized by electrophoresis. In this figure, enzyme RNase T1 and RNase V1 are presented as examples of RNA-digesting enzymes, which cut, respectively, immediately 3′ to a single-stranded guanine and at double-stranded RNA in a sequence-independent manner. If the putative RNA thermometer under investigation changes conformation in response to alterations in temperature, an increase of environmental temperature would destabilize the inhibitory hairpin, resulting in a different pattern of radiolabeled fragments following digestion with the RNA degrading enzymes.

Moreover, techniques that study the physical properties of an RNA molecule, such as the nuclear magnetic resonance (NMR) spectroscopy and UV melting analysis, can be utilized to investigate the detailed base-pairing and their changes in response to temperature, thus revealing structural information as well as the molecular basis of thermosensing [7,26].

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Together, these experimental analyses provide information about the dynamics of the inhibitory structure of a putative RNAT.

In addition to studies aimed at characterizing temperature-dependent changes in secondary structure, the regulatory activity of putative RNATs can be verified using a toe-printing assay, an *in vitro* analysis designed to directly assess the ability a ribosome to assemble and bind to the SD sequence contained on a given RNA molecule [22,27]. In the case of a functional RNAT, it would be predicted that ribosomal binding occurs at permissive temperatures, when the inhibitory structure is absent, but does not occur at non-permissive temperatures, when the inhibitory structure is present. Briefly, a putative RNAT is synthesized by *in vitro* transcription and incubated at a given temperature with a mixture of ribosome subunits and methionine conjugated tRNAs. If an SD sequence is available, a stable initiation complex will form on the RNA molecule, the presence of which is detected by reverse transcription using a radiolabeled primer that binds the RNA molecule downstream to the ribosome-binding site. The presence of the initiation complex will hinder the progression of the reverse transcriptase and thus result in the formation of a relatively short radiolabeled cDNA product. If the initiation complex cannot be formed, in this case because the SD sequences are occluded by the formation of an inhibitory structure within the putative RNAT, reverse transcription will not be blocked and a relatively long radiolabeled cDNA product will be generated. By completing toe-printing assays at different temperatures, the impact of temperature on the ability of the ribosome to interact with a putative RNAT can be directly determined. In the case of a functional RNAT, it would be expected that a relatively short cDNA product will be formed at permissive temperatures when the transcription initiation complex can form and that a relatively long cDNA product will be formed at non-permissive temperatures when assembling of the translation initiation complex is blocked by the formation of the inhibitory structures of the RNAT (Figure 4).

3.3. RNA structuromics

Recently, a combination of experimental and next-generation high-throughput techniques have been used to identify the structures of every RNA molecule within a single organism, collectively termed the "RNA structurome" [28]. Structuromic analyses performed at various temperatures have the potential to reveal a massive amount of information that will directly lead to the discovery of potentially expansive numbers of temperature-responsive regulatory RNA elements including RNATs [2]. The structurome of *Saccharomyces cerevisiae* and that of mice nuclear transcriptome were generated using parallel analysis of RNA structure (PARS) and fragmentation sequencing (Frag-seq), respectively [29,30]. The general experimental procedure that reveals the structurome of an organism includes two main steps: 1) structural probing of a certain transcriptome by specific RNA-digesting enzymes or chemicals that differentially cleave or modify RNA molecules based on the presence of specific secondary structures and 2) high-throughput sequencing analysis of the cDNA libraries generated from the digested/modified transcriptome. The structural probing portion of the analysis can be done either *in vitro* by treating the transcriptome harvested from the organism with structure- and sequence-specific RNA endonucleases or *in vivo* by cell-penetrating chemicals that modify

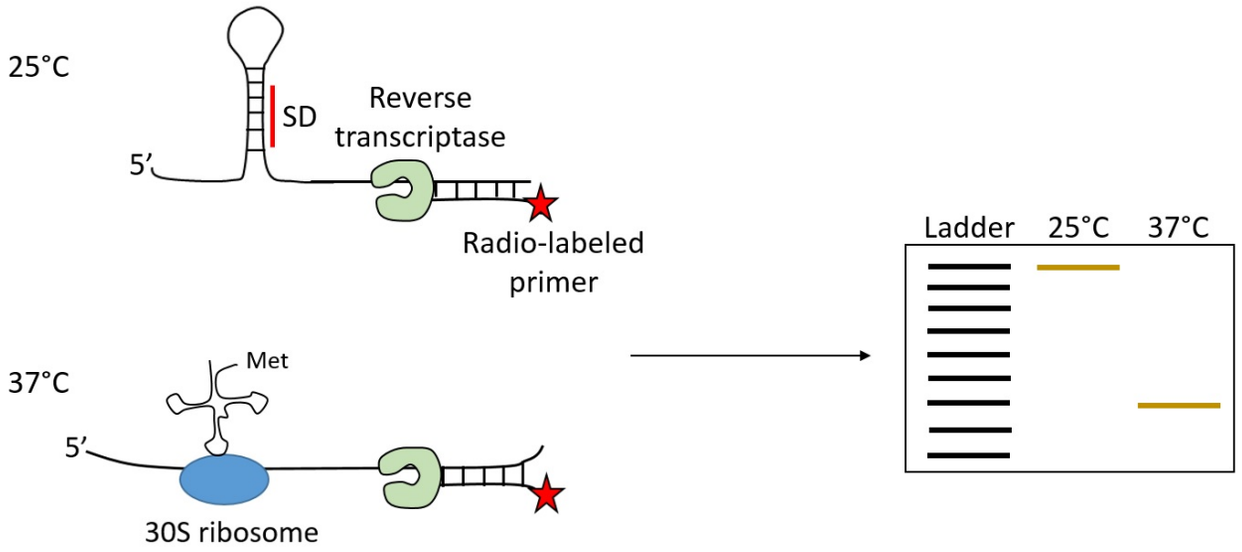

Figure 4. Toe-printing assay used to experimentally determine differential binding of the ribosome to a putative RNA thermometer at different temperatures. An *in vitro* transcribed putative RNA thermometer is incubated with a mixture of ribosome subunits and methionine-conjugated tRNA under different temperatures and then used as template in a reverse transcription reaction with a radiolabeled primer that binds downstream of the SD sequence. If the ribosome differentially binds the transcript, as would be predicted for that containing a functional RNA thermometer, reverse transcription would be expected to be hindered by the presence of the bound ribosome under permissive temperature (37°C in this example), thus producing a relatively short radiolabeled cDNA product. At non-permissive temperatures (25°C in this example), however, the ribosome would not be bound and reverse transcriptase would be expected to process to the end of the transcript generating a relatively long radiolabeled cDNA product.

or cleave single-stranded RNA bases within the cells [28,31]. This new experimental approach not only provides structural information of RNAs in physiological context but also evades the disadvantages of current *in silico* and *in vitro* analyses. Specifically, *in silico* prediction of RNA secondary structure is dependent on the length of RNA molecule that has been chosen; the longer the sequence, the lower the reliability of the prediction [32]. Additionally, secondary structures characterized by *in vitro* experimental analysis carry the caveat that the structures may be different *in vivo*. It is expected that, by completion of RNA structuromic analyses in a variety of bacterial organisms, the recognized numbers and types of RNATs will grow dramatically, an advancement that is critical to revealing the full impact of RNATs in controlling the physiology and virulence of bacterial species.

4. Families of RNA thermometers

The thermosensing activity of an RNAT is largely dependent on the physical features of its secondary structure, specifically by those features that impact the stability, or the Tm, of the inhibitory hairpin. In addition to the base-stacking interactions and the hydration shell of an RNA helix, other critical features of RNATs include 1) the number and stability of hairpins that are formed within the element; 2) the presence of canonical and non-canonical base-pairing within the inhibitory structure; 3) the existence of internal loops, bulges, or mismatches within the formed structure(s); and 4) the extent of base-pairing between sequences composing

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the SD site and/or start codon with upstream sequences contained on the transcript. Each of these features can directly impact the stability of the inhibitory structure within a given RNAT, which in turn dictates the responsiveness of the element to temperature. Despite sharing a common basic regulatory mechanism, differences in RNATs display different secondary structures and other key features, differences that are now used to classify bacterial RNATs into families. The two currently recognized families of RNATs are ROSE-like RNATs (repression of heat shock gene expression) and FourU RNATs. RNATs composing each of these two main families, as well as a few unique RNATs, are discussed below.

4.1. ROSE-like RNA thermometers

ROSE-like elements were first identified as conserved *cis*-regulatory elements located in the regions between the promoters and start codons of genes encoding small heat shock proteins (sHsps) in *Bradyrhizobium japonicum*. and within a short time were reported in other *Rhizobium* species as well as in *Agrobacterium tumefaciens* [21,33–35]. The heat shock response is a highly conserved process among microorganisms, and while their numbers vary between organisms, small heat shock proteins play a critical role in preventing protein denaturation and aggregation under heat stress. Based on the conservation of the biological process as well as the conservation of the primary sequence and secondary structure of the 17 originally identified ROSE-like elements, bioinformatics-based techniques were used to predict ROSE-like elements in the 5′ UTRs of sHsp encoding genes from 120 different archaea and bacteria [21,36]. As a result of these studies, 27 additional ROSE-like elements were identified in 18 different α- and γ-proteobacteria species [36]. Likely as a result of the approaches used to identify them, nearly all ROSE-like elements identified to date control the production of factors involved in the heat shock response. However, as additional ROSE-like RNATs are identified and characterized, it is expected that the contribution of these regulatory elements will be expanded beyond the production of heat shock response and into other physiological processes. This notion is supported by the recent identification of a ROSE-like RNAT in *Pseudomonas aeruginosa* that controls the production of rhamnolipids, a virulence factor that functions to protect the pathogen against killing by the human immune system [37]. Only with additional studies will the potentially expansive role of ROSE-like thermometers in controlling the physiology and virulence of bacterial species be revealed.

The ROSE-like family is the most extensively studied family of RNATs, harboring approximately 70% of all RNATs identified to date. All RNATs within the ROSE-like family are housed with 5′ UTR regions that range from 60 nucleotides to more than 100 nucleotides in length and that form 2 to 4 hairpins [36,37]. Within these hairpins, the 5′-proximal hairpin generally acts to stabilize the secondary structure and facilitate the correct folding of the other hairpins, while the 3′-proximal hairpin contains the SD region of the regulated transcript [7]. The defining features of ROSE-like RNATs that contribute to their temperature-responsive regulatory function include 1) the presence of a conserved anti-SD sequence 5′-UYGCU-3′ (Y stands for a pyrimidine) in the 3′-proximal hairpin, and 2) a "bulged" guanine within the SD sequestering hairpin (Figure 5) [36]. As a feature shared by all ROSE-like elements, it has been proposed that the "bulged" guanine within the SD sequestering hairpin is essential for the

thermoresponsiveness of the regulatory element, a prediction that is supported by various mutagenesis-based experimental approaches and by NMR spectroscopy [7,38,39]. These studies have not only demonstrated that the "budged" guanine is essential for function but also revealed that the "bulged" guanine forms hydrogen bonds with the second guanine within the SD sequence of 5′-AGGA-3′. Additionally, towards the 3′ end of the SD site, two pyrimidines from the anti-SD strand form a triple-base pair with a uracil from the SD site with hydrogen bonds (Figure 5). The existence of two highly unstable pairs — a G-G pair and a triple-base pair — within the inhibitory hairpin of ROSE-like RNATs enables it to respond to the subtle changes of environmental temperature and thus to function as a temperature-sensitive regulatory element [7].

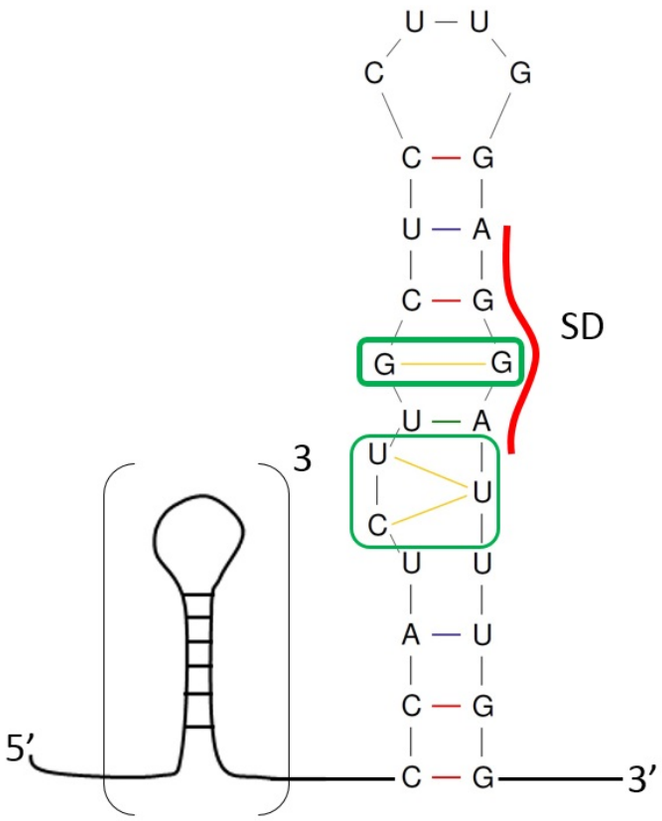

Figure 5. Structural features of the ROSE-like family of RNA thermometers, demonstrated by a schematic of the *hspA* RNA thermometer of *B. japonicum*. Within the four hairpins of *hspA* RNA thermometer, only the conserved structural features in the 3′ proximal hairpin are shown in detail, with the varied number of upstream hairpins indicated by the general hairpin structure in parentheses. The red line indicates the location of the SD sequence, while the conserved G-G pairing and triple-base pair are highlighted by the green boxes.

4.2. FourU RNA thermometers

FourU RNATs, so named due to the presence of four consecutive uracil residues within the SD sequestering inhibitory hairpin, represent the second family of currently identified RNATs. First identified in *Salmonella enterica,* a total of eight FourU RNATs have now been identified and characterized in a variety of bacterial species [10,11,22,40–42]. Unlike ROSE-like RNATs,

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only two characterized FourU RNATs function to control the production of a heat shock-related factor [10,22]. Instead, the majority of characterized FourU RNATs (*toxT* from *Vibrio cholera*, *lcrF*/*virF* from *Yersinia* species, as well as *shuA* from *S. dysenteriae* and its homologous gene *chuA* from some pathogenic *E. coli*) function to regulate the production of virulence factors in response to alterations in environmental temperature [11,40,41].

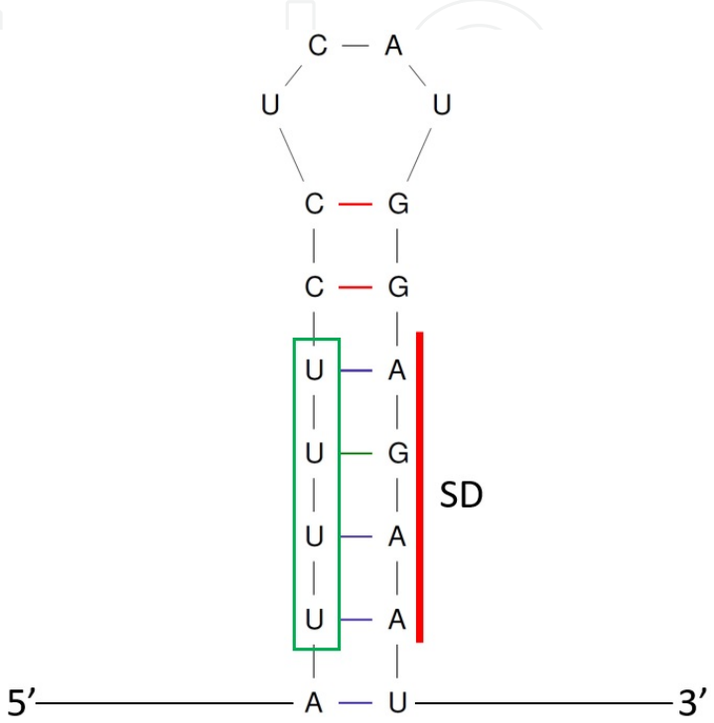

Figure 6. Structural features of the FourU family of RNA thermometers, demonstrated by a schematic of the *shuA* RNA thermometer of *S. dysenteriae*. Only the conserved portion of the inhibitory hairpin of *shuA* RNA thermometer is shown in this figure. The red line indicates the location of the SD sequences, while a green box indicates the location of the conserved four consecutive uracil residues.

The structural features of FourU RNATs are largely varied. For example, the length of the 5′ UTRs in which FourU RNATs are housed ranges from as short as 40 nucleotides (*htrA* from *E. coli* and *Salmonella*) to more than 280 nucleotides (*shuA* from *Shigella dysenteriae*) in length [10,11]. Additionally, the number of hairpins varies from a single hairpin with internal loops (*toxT* from *Vibrio cholerae*) to five hairpins, including an inhibitory hairpin with no internal loops (*shuA* from *S. dysenteriae*) [11,40]. Despite these differences, there are also several key features shared within FourU RNATs. The first shared feature is the presence of four consecutive uridine residues that form canonical A-U and/or non-canonical G-U base-pairs with SD sequences on the regulated transcript (Figure 6). Additionally, for RNATs within the FourU family, the SD sequestering hairpin is generated by no less than 5 continuous base-pairs, and often displaying conserved destabilizing features including the presence of relatively few G-C pairs, as well as internal mismatches or loops within the inhibitory structures. Likely due to the innate stability of the inhibitory hairpin within FourU RNATs, features destabilizing the inhibitory structure increase the responsiveness of FourU RNATs to temperature alterations, and disruption of these features result in altered thermosensing abilities. In the studies of each

characterized FourU RNAT, mutagenesis analyses that introduce G-C base-pairs into the inhibitory structure result in the expected stabilization and, importantly, loss of thermosensing activity by the regulatory element [11,22,40]. NMR spectroscopy analysis has been utilized to study the dynamics of the inhibitory hairpin within the *agsA* FourU RNAT [15]. Specifically, a point mutation that introduces a C-G base-pair at the previously mismatched position adjacent to the SD region increased the melting temperature of the hairpin by 11°C. Additionally, two Mg^{2+} binding sites were found in the *agsA* FourU thermometer hairpin and it was demonstrated that Mg^{2+} functions to stabilize the inhibitory structure [15]. The degree to which these important features are conserved among members of the FourU RNAT family will be revealed only after additional members are identified and experimentally characterized. Such experimentation will not only define the FourU RNAT family of regulators but will also advance our ability to identify new FourU RNATs.

4.3. Additional types of RNA thermometers

It is important to note that not all characterized RNATs fit neatly into one of the two main families: ROSE-like and FourU. While all RNATs are thought to share a basic zipper-like thermosensing mechanism, several identified RNATs differ from those composing the main families in critical features, including primary sequence and/or secondary structure, features that impact the regulatory activity of these elements. It is the identification and characterization of the details of the molecular mechanisms underlying each of these additional types of RNATs that will expand our understanding of foundational principles governing RNA-mediated thermosensing.

In some RNATs, base-pairing involving the SD sequence is not complete but instead is disrupted by mismatches or "bulged" nucleotides, a feature also noted for ROSE-like elements. For example, the inhibitory structure within the RNATs that control the production of two putative lipoproteins LigA and LigB in *Leptospira interrogans* have identical nucleic acid sequences that include a mismatch of an adenine and a guanine within the SD sequestering hairpin [43]. Genes *hspX* and *hspY* that encode sHsps in *Pseudomonas putida* are also regulated by RNATs that contain one or two A·G mismatches disrupting the otherwise continued base-pairing of the SD region [8]. For these RNATs, further investigation is needed to understand the direct impact of the apparently conserved feature of mismatched or bulged sequences within the inhibitory structure on the regulatory activity of these elements.

Although lacking the presence of four consecutive uracil residues, two RNATs are similar to FourU RNATs in that they display more than 5 continuous base-pairs within the SD region of their inhibitory hairpins: one RNAT controls the production of an sHsp (Hsp17) from *Synechocystis* sp. PCC 6803, while the other controls the production of *Salmonella* GroES, a component of protein chaperon machinery [23,44]. RNAT-mediated regulation of *hsp17* is important for the survival of *Synechocystis* under heat stress, because Hsp17 not only prevents denatured proteins from aggregation but also protects the integrity of cellular membranes [45,46]. The 5′ UTR of *hsp17* has a single hairpin with an internal asymmetric loop [23]. In the SD sequence-binding region, instead of four uracils as seen in the FourU thermometer, the *hsp17* RNAT has a sequence of 5′-UCCU-3′ that forms four canonical pairs with the SD

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sequence, including two G-C pairs. The remaining base-pairs in the inhibitory hairpin are mainly A-U pairs with two non-canonical G-U pairs. As the most stabled base-pairs within the *hsp17* RNAT, these two G-C base-pairs contribute to the stability and thus the inhibitory function of the hairpin. Other features such as the asymmetric internal loop and low ratio of G-C base-pairs destabilize the inhibitory structure, features that together enable the hairpin to dissociate with the increase of temperature. For the inhibitory hairpin of the *groES* RNAT, it has a mismatch of an adenine and a guanine that destabilizes this structure. While the secondary structure and temperature-responsive regulatory function of the *groES* RNAT has only been experimentally characterized in *Salmonella* and *E. coli*, this RNAT and its regulated factor, a necessary chaperon for proper folding of cellular components, are well conserved in enterobacteria [44].

For some RNATs, the function and stability of the inhibitory hairpin are impacted by base-pairing with sequences other than those within the SD region. For example, in the 5′ UTR of *prfA* from *Listeria monocytogenes*, a major portion of the SD region and the start codon are confined within internal loops and thus are partially single-stranded [47]. It has been demonstrated, however, that the hairpin within the *prfA* 5′ UTR containing the SD region and start codon does function as an RNA thermometer, an activity that is dependent on base-pairs that are located upstream of the SD site, which function to stabilize the unusually long hairpin. Another example of sequences other than those within the SD region that directly impact the regulatory function of an RNAT is the repeated nucleotide sequence of 5′-UAUACUUA-3′ in the RNAT of *cssA* from *Neisseria meningitides* [24]. These 8-nucleotide sequences are located upstream of the SD region and enable the RNAT to sense mild changes of environmental temperature, which is important for the survival of *N. meningitides.* As an opportunistic pathogen that colonizes only humans, it is important that *N. meningitidis* can sense and respond to a mild increase of temperature, as would be encountered during a fever response.

A unique example among currently identified RNATs is the one that controls the expression of *rpoH* in *E. coli* [9]. Binding of the ribosome to the SD region within the *ropH* transcript is facilitated by a sequence (named downstream box) located between the SD site and the start codon [48]. The *rpoH* RNAT inhibits translation via embedding this downstream box in the junction region of three stem loops instead of forming base-pairs within a single inhibitory hairpin as is the usual conformation in RNATs [9]. As the environmental temperature increases, two stems that paired with the downstream box melt at the junction position exposing the downstream box as a single strand, a conformation that facilitates ribosome binding to the transcript.

Lastly, there are currently three characterized RNATs that are located within intergenic regions of a polycistronic transcripts: *ibpB* from *E. coli*, *lcrF* from *Yersinia* species, and *hspY* from *P. putida* [8,41,49]. Their location within polycistronic transcripts differentiates these three RNATs from all others found in the 5′ UTR of monocistronic or polycistronic transcripts.

Although they display key features that differ from those possessed by RNATs in the ROSE-like or FourU families, many of the unique RNATs highlighted above are conserved between several bacterial species. There is little doubt that as additional bacterial RNATs are identified and characterized, commonalities will emerge and additional families will be recognized.

5. Bacterial processes controlled by RNA thermometers

The regulation of gene expression in response to changes in environmental temperature is important for survival of all bacteria and for virulence of pathogenic bacteria. RNATs have been found to confer efficient temperature-dependent regulation onto the expression of bacterial genes encoding factors involved in two critically important bacterial processes —heat shock response and virulence. In the following section, each of these two critical biological processes will be briefly introduced and the role that RNATs play in facilitating them will be discussed.

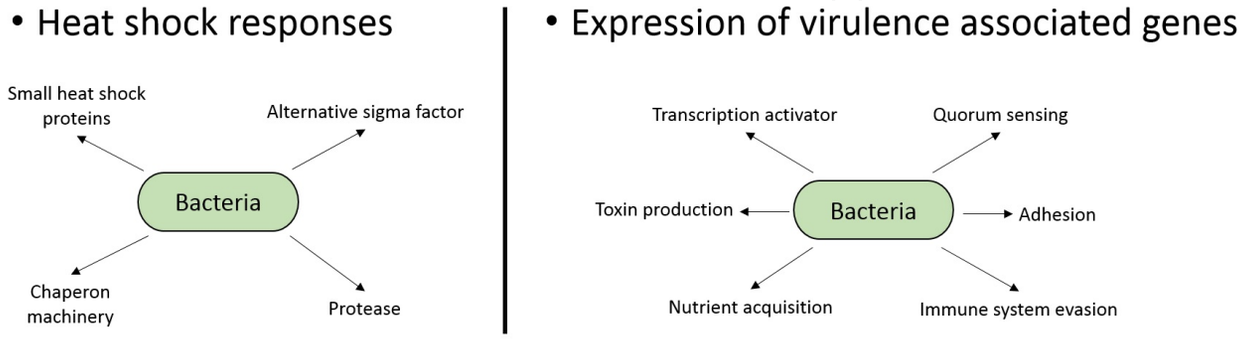

Figure 7. Key processes are controlled by RNA thermometers in bacteria. The influence of RNA thermometers on the bacterial heat shock response and on bacterial virulence is indicated by highlighting the different groups of genes whose expression is directly regulated by an RNA thermometer.

5.1. Heat shock response

The primary effect of increased temperature on bacteria is the resulting denaturation of temperature-sensitive proteins. Similar to regulatory RNA molecules, the function of a protein is strictly dependent on its structure, a feature that can be impacted by environmental temperature. Increased environmental temperature can result in partial or complete denaturation of a protein, resulting in a stable but often non-functional molecule [50]. In addition to the denaturation of proteins, high temperature is also associated with disruption of the bacterial cell membrane as well as damage to DNA molecules [46,51]. As a result of these effects, increased environmental temperature can be lethal to bacterial life and thus represents a stress that must be overcome.

In order to facilitate responsive adaptation to a rise in environmental temperature, bacteria express several genes that encode for factors that function to protect the organism from the detrimental effects generated by heat, collectively termed the heat shock response [52]. The main components of the heat shock response include 1) alternative sigma factors that direct the transcription of other heat shock responding genes; 2) heat shock proteins (Hsps), such as protein chaperon machinery that facilitate the proper folding of other proteins; 3) small Hsps (sHsps) that have multiple functions including preventing the formation of protein aggregates and protecting the integrity of cellular membrane; and 4) enzymes that degrade denatured proteins, repair damaged DNA, and more.

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Understanding the molecular mechanisms underlying the temperature-dependent regulation of factors that facilitate the bacterial heat shock response is a major focus of ongoing investigations; the discovery of RNATs is rooted in these important studies. Since the identification of an RNAT that regulates the expression of a small heat shock protein (HspA from *B. japonicum*) and the heat shock alternative sigma factor σ^{32} (RpoH from *E. coli*), many other players in the heat shock response have been found to be regulated by RNATs, including chaperon component (GroES from *Salmonella*), heat-induced protease (HtrA from *E. coli*), and other small heat shock proteins [7,8,29,35] (Figure 7). Regulation of heat shock response is a complex process that involves the regulation of multiple factors at different steps of gene expression. That said, it seems that temperature-dependent regulation by RNAT is a fundamental regulatory mechanism that coordinately influences nearly all types of heat shock response factors. Given the high degree of conservation seen between heat shock responses factors produced by a wide variety of living organisms, temperature-dependent regulation mediated by RNATs is expected to be present in many organisms, including eukaryotic systems [52]. A finding that directly supports this prediction is that of a secondary structure within the 5′ UTR of *Drosophila* Hsps encoding mRNAs that functions to regulate translation from the transcript in response to environmental temperature [53]. The full extent of RNATs in controlling heat shock response in bacteria and beyond is yet to be revealed.

5.2. Virulence-associated genes of pathogenic bacteria

Once within the body of the host, and throughout the course of a natural infection, pathogenic bacteria face several challenges, including but not limited to 1) the need to adhere to host cells, 2) the need to evade killing by the host immune system, and 3) the need to acquire essential nutrients. To overcome these challenges and progress of an infection, bacteria produce specific virulence factors. As the production of virulence factors is most beneficial to an invading bacterium when it is within the host, several levels of regulation are often employed to ensure that the production of these important factors occurs only when the bacteria is within an environment that resembles that encountered within the infected host. RNATs are involved in regulating the production of a variety of virulence factors in several species of pathogenic bacteria, ensuring that these factors are most efficiently produced at the relatively high temperatures encountered within the infected host (Figure 7).

The expression of many virulence-associated genes is controlled by protein-based regulation, specifically that carried out by transcriptional regulators. Interestingly, RNATs have been found to directly control the production of three transcriptional activators that, in turn, function to control the expression of virulence-associated genes: *prfA* from *L. monocytogenes*, *lcrF* from *Y. pestis*, and *toxT* from *V. cholera* [40,41,47]. Another regulatory system that controls the expression of multiple virulence factors is quorum sensing. To date, one gene whose product is involved in quorum sensing-dependent modulation of virulence gene expression has been found to be regulated by an RNAT; this gene is *lasI* from *P. aeruginosa* [37]. RNATs within *lcrF* and *toxT* are FourU RNATs, while the RNATs controlling the expression of *prfA* and *lasI* have currently unique structure.

RNATs have also been implicated in controlling the expression of virulence-associated genes that encode factors involved in adhesion and immune evasion. For example, three virulence-associated genes in *N. meningitis* have been found to be regulated by RNATs: *cssA,* a gene encoding a factor involved in capsule production; *fHbp,* a gene encoding a factor H binding protein; and *lst,* a gene encoding a factor required for modifications of lipopolysacccharides [24]. In *L. interrogans*, *ligA* and *ligB*, two genes encoding putative lipoprotein, are also regulated by RNATs [43]. Additionally *P. aeruginosa rhlA,* a gene encoding an enzyme required for the synthesis of rhamnolipid, a compound that can prevent killing of the bacteria by host immune system, is regulated by an RNAT [37]. Except for *rhlA* RNAT, which is a member of the RSOE-like family, these other RNATs mentioned above have unique structures and thus are not members of the ROSE-like or FourU families of regulators.

To date, two genes involved in the acquisition of essential nutrients have been shown to be regulated by RNATs: *S. dysenteriae shuA*, a gene encoding an outer membrane heme-binding protein, and its homologous gene *chuA* in pathogenic *E. coli* [11]. Translation of *shuA*/*chuA* is controlled by a FourU RNAT located in the relative large 5′ UTR of the corresponding gene. Production of ShuA or ChuA facilitates the utilization of iron from heme, a potential source of essential iron found only within the relatively warm environment of the infected host [54].

For many pathogenic bacteria, the transmission from one host to the next involves exposure to different environments with different temperatures. The expression of many virulence-associated genes is influenced by environmental temperature, a signal that varies between the host and non-host environments. With an increasing number of virulence-associated genes that are now known to be regulated by the activity of RNATs, it is possible that temperature-dependent regulation mediated by RNATs will emerge as one of the basic regulatory strategies utilized by pathogenic bacteria. The full and potentially expansive role that RNATs play in controlling virulence of pathogenic bacteria is yet to be revealed.

6. Future directions

Although RNA-dependent regulation of gene expression has been a topic of active investigation for decades, investigations of RNATs are much more recent, with less than 100 RNATs having been identified to date (Table 1). Of note, RNATs vary in key structural features and influence different essential physiological processes.

RNAT type	Organism	Gene	Function of the gene	Reference
ROSE-element	*Agrobacterium tumefaciens*	*hspAT1* & *hspAT2*	Small heat shock protein	Balsiger *et. al.* 2004 [33]
	Bartonella henselae	*ibpA2*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]

RNAT type	Organism	Gene	Function of the gene	Reference
	Bartonella quintana	*ibpA2*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Bradyrhizobium japonicum	*hspA, hspB, hspD, hspE,& hspH*	Small heat shock protein	Narberhaus *et. al.* 1998 [35]; Münchbach *et. al.* 1999 [34]
	Bradyrhizobium sp. *(Parasponia)*	*hspAP, hspCP, hspDP,& hspEP*	Small heat shock protein	Nocker *et. al.* 2001 [21]
	Brucella suis	*ibpA* & *hspA*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Caulobacter crescentus	CC2258 & CC3592	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Erwinia carotovora	*ibpA* & *ibpB*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Escherichia coli	*ibpA* & *ibpB*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]; Waldminghaus *et. al.* 2009 [39]; Gaubig *et. al.* 2011 [49]
	Mesorhizobium loti	*mll2387, mll3033, mlr3192,& mll9627*	Small heat shock protein	Nocker *et. al.* 2001 [21]
	Pseudomonas aeruginosa	*ibpA*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]; Krajewski *et. al.* 2013 [38]
	Pseudomonas putida	*ibpA*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]; Krajewski et. al. 2013 [38]
	Pseudomonas syringae	*PSPT02170*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
		ibpA	Small heat shock protein	Krajewski *et. al.* 2013 [38]
	Rhizobium sp. strain NGR234	*hspAN* & *hspCN*	Small heat shock protein	Nocker *et. al.* 2001 [21]
	Rhodopseudomonas palustris	*RPA0054* & *hspD*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Salmonella typhimurium	*ibpA* & *ibpB*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]

RNAT type	Organism	Gene	Function of the gene	Reference
	Shewanella oneidensis	*ibpA*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Shigella flexneri	*ibpA* & *ibpB*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Sinorhizobium meliloti	*ibpA* & *b21295*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Vibrio cholerae	*hspA*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Vibrio parahaemolyticus	*hspA*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Vibrio vulnificus	*hspA*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Yersinia pestis	*ibpA* & *ibpB*	Small heat shock protein	Waldminghaus *et. al.* 2005 [36]
	Pseudomonas aeruginosa	*rhlA*	Enzymes involved in the production of biosurfactant rhamnolipids	Grosso-Becerra *et. al.* 2014 [37]
FourU element	*Escherichia coli*	*htrA*	Stress-responding periplasmic protease	Klinkert et. al. 2012 [10]
	Salmonella enterica	*agsA*	Small heat shock protein	Waldminghaus *et. al.* 2007 [22]
		htrAp3	Stress-responding periplasmic protease (transcribed from the 3rd promoter of the gene)	Klinkert et. al. 2012 [10]
	Escherichia coli (some strains)	*chuA*	Outer membrane heme-binding protein	Kouse *et. al.* 2013 [11]
	Shigella dysenteriae	*shuA*	Outer membrane heme-binding protein	Kouse *et. al.* 2013 [11]
	Vibrio cholerae	*toxT*	Transcriptional activator of virulence factors (including cholera toxin)	Weber et. al. 2014 [40]
	Yersinia pestis	*lcrF*	Transcriptional activator of multiple virulence genes	Böhme *et. al.* 2012 [41]; Hoe *et. al.* 1993 [42]
	Yersinia pseudotuberculosis	*virF (lcrF)*	Transcriptional activator of multiple virulence genes	Böhme *et. al.* 2012 [41]

RNAT type	Organism	Gene	Function of the gene	Reference
Additional types	*Escherichia coli*	*rpoH*	Heat shock alternative sigma factor σ^{32}	Morita *et. al.* 1999 [9]
	Pseudomonas putida	*hspX* & *hspY*	Putative small heat shock proteins (similar to hspA,B,C)	Krajewski et. al. 2014 [8]
	Salmonella typhimurium	*groES*	Component of protein chaperon machinery	Cimdins *et. al.* 2013 [44]
	Synechocystis sp. PCC 6803	*hsp17*	Small heat shock protein	Kortmann *et. al.* 2011 [23]
	Leptospira interrogans	*ligA* & *ligB*	Putative lipoproteins promote adhesion -virulence related	Matsunaga *et. al.* 2013 [43]
	Listeria monocytogenes	*prfA*	Transcription activator of virulence factors	Johansson *et. al.* 2002 [47]
	Neisseria meningitidis	*cssA*	Capsule biosynthesis	Loh *et. al.* 2013 [24]
		fHbp	Factor H binding protein	
		lst	Lipopolysaccharide modification	
	Pseudomonas aeruginosa	*lasI*	Quorum sensing –synthesis quorum sensing signal	Grosso-Becerra *et. al.* 2014 [37]

Table 1. Summary of currently identified RNA thermometers

Despite their differences, all currently characterized RNATs are thought to share the same basic zipper-like temperature-responsive molecular mechanism, based on which both experimental and therapeutic applications can be derived. For example, artificial RNATs that have only a single hairpin to perform the temperature-dependent inhibition of translation have now been designed [55]. These artificial RNATs can be used as genetic tools to manipulate target gene expression. In the aspect of applying knowledge of RNATs in developing therapeutics, it is conceivable that compounds can be developed that would specifically stabilize the inhibitory structure within a given RNAT, thus decreasing expression of this target gene. Utilizing such an approach to inhibit the production of an essential gene product or virulence factor could prevent or limit infections by a variety of pathogenic bacteria.

Future applications of RNATs as genetic tools and/or drug targets are dependent on an increased understanding of these ubiquitous regulatory elements. With the maturation and development of experimental techniques, we could identify additional RNATs and study the molecular mechanisms underlying their regulatory activity in even greater detail. Moreover, due to the fundamental roles of RNA in the biological world, there is a great potential that RNATs also exist in archaea and eukaryotes. Further investigation and characterization of the conserved features and mechanisms of RNATs along with an understanding of the function of their regulatory targets could provide insight into the complex evolution of gene regulation. With the rate at which advances have been made in the field of RNA-mediated regulation, and

specifically within the study of RNATs, there is no doubt that these and other important findings will be revealed sooner than later.

7. Nomenclature

cDNA library: The collection of single-stranded DNA products generated by reverse transcription using total RNAs isolated from an organism as templates.

Gibbs free energy: The thermodynamic potential of a system at a certain temperature and pressure. In this chapter, Gibbs free energy indicates the stability of a certain hairpin structure.

Heat shock response: The coordinated production of several proteins and other essential cellular components by a cell that work together to facilitate survival when the cell is exposed to an environmental temperature that is higher than its ideal surviving temperature.

Hydration shell: A shell-like structure formed by water molecules surrounding a molecule.

Melting temperature (Tm): The temperature at which half of the double-stranded molecules within a population assume a single-stranded conformation.

Quorum sensing: The coordinated regulation of bacterial gene expression in response to a secreted signal molecule that indicates the population density. When the signal molecule reaches a threshold amount, the cascade of signal-induced regulation occurs.

Riboswitch: A *cis*-encoded regulatory RNA element that functions to modulate target gene expression via switching between two mutually different secondary structures. Conformational changes within a riboswitch are induced by binding to a metabolite or other small molecule at a specific ligand-binding region.

RNA thermometer: A *cis*-encoded RNA element that represses translation via incorporation of the ribosome-binding site within an inhibitory hairpin at non-permissive temperatures. With increased temperature, the inhibitory structures within an RNA thermometer is destabilized, the ribosome-binding site is exposed, and translation proceeds.

Shine-Dalgarno (SD) sequence: Also known as the ribosome-binding site, the Shine-Dalgarno sequence is a sequence on an mRNA molecule to which the ribosome binds. Binding of a ribosome to an SD sequence on a transcript is necessary for the initiation of translation.

Sigma factor: A protein factor that facilitates the sequence-specific binding between an RNA polymerase and specific promoter regions on the DNA. Sigma factors are necessary for transcription initiation.

Structurome: The collective determination of the secondary structure of each transcript present in a given organism or cell type.

Transcriptome: The total population of RNA molecules present in a given organism or cell type.

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5′ Untranslated region (5′ UTR): The region of a protein-encoding transcript that is located upstream of the translation start site. The 5′ UTR thus does not containing amino acid-coding sequences but rather contains the ribosome-binding site and often houses regulatory elements, such as RNA thermometers.

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