Bacterial riboswitches and RNA thermometers: Nature and contributions to pathogenesis

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Abstract

Bacterial pathogens are always challenged by fluctuations of chemical and physical parameters that pose serious threats to cellular integrity and metabolic status. Sudden deprivation of nutrients or key metabolites, changes in surrounding pH, and temperature shifts are the most important examples of such parameters. To elicit a proper response to such fluctuations, bacterial cells coordinate the expression of parameter-relevant genes. Although protein-mediated control of gene expression is well appreciated since many decades, RNA-based regulation has been discovered in early 2000s as a parallel level of regulation. Small regulatory RNAs have emerged as one of the most widespread and important gene regulatory systems in bacteria with rare representatives found in Archaea and Eukarya. Riboswitches and thermostensors are cis-encoded RNA regulatory elements that employ different mechanisms to regulate the expression of related genes controlling key metabolic pathways and genes of temperature relevant proteins including virulence factors. The extent of RNA contributions to gene regulation is not completely known even in well-studied models such E. coli and B. subtilis. In depth understanding of riboswitches is promising for opportunity to discover a narrow spectrum antibacterial drugs that target riboswitches of essential metabolic pathways.

1. Introduction

Shifting of pathogenic bacterial species from an environmental niche to a living host or vice versa is a challenging condition to acclimate to. Changes in nutrients availability, pH, and temperature are the most important parameters to be monitored constantly. Dramatic changes of these parameters may lead to deleterious effects on cellular physiology or resources wasting in synthesis of already available metabolites. To prevent such problems, bacteria have established a panel of signaling networks to coordinate gene expression programs to respond to its surroundings. Proteins play the major role in signals sensing and mounting the regulatory responses by acting as signal receivers at membrane sensors and cytoplasmic effector molecules activate or repress transcription of relevant genes. It is well-appreciated that proteins coordinate gene expression at various phases, at transcription, translation, or post-translational levels. For two decades, bacterial RNA-based regulatory strategies are being discovered in accelerating fashion (reviewed elsewhere [1–3]). A plethora of RNA elements were found not to encode for proteins or proteins synthesizing machinery, but to execute regulatory functions in controlling gene expression. Such non-coding RNA elements reside in the intergenic regions of open reading frames (ORFs).

Regulatory RNAs are classified into two major categories; cis-encoded elements which are located mostly upstream genes they regulate and trans-encoded elements that are transcribed from other locations in the genome. Pathogenic lifestyle of some bacterial species prioritized the sensing and responding to changes of physical and chemical signals especially key metabolites and temperature to avoid synthesizing available metabolites or proteins in the absence of their substrates. Riboswitches and RNA thermometers (RNATs) are regulatory elements contained within the 5′-untranslated region (5′-UTRs) of bacterial mRNA transcripts for genes they regulate at the transcriptional and translational levels. The tertiary structure of such leader sequences are formed or

Abbreviations: Flavin mononucleotide; SAM, S-adenosylmethionine; SAH, 5-adenosylhomocysteine; SD, Shine-Dalgarno; AdoCbl, adenosylcobalamine; RNA polymerase; RNAT, RNA thermometer; TPP, Thiamine pyrophosphate; RBS, Ribosomal Binding Site; ORFs, open reading frames; 5′-UTRs, 5′-untranslated region; CSPs, Cold Shock Proteins.

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2. Riboswitches

2.1. Structure and secondary foldings

Bacterial riboswitches reside mostly at the 5′ untranslated regions (UTRs) of metabolic and transport genes which they regulate in cis-fashion after direct binding of a specific metabolite ligand [19–23]. Typical riboswitch sequence contains two functional domains, the aptamer and the expression platform with a region of overlap called switching sequence between the two domains [24]. Folding of the aptamer into distinctive secondary and tertiary structures, result in scaffolding of the ligand docking site. The expression platform responds to ligand-induced folding at the aptamer region by adopting the functioning structure which interfaces with the transcription or translation processes of downstream sequences to elicit a regulatory response.

After synthesis of aptamer, it undergoes a folding pathway in order to achieve its effective configuration. Sequences and structural studies of many bacterial riboswitches have deciphered the molecular architecture of aptamers at atomic levels. The folding events of riboswitch follow the common principles governing other RNA molecules [25]. Various RNA structural configurations have been reported in riboswitches including helices, loops, and bulges. The configuration of these loops and turns is dictated by the sequence motifs of nucleotides. Such motifs can interact with each other to form higher level of packing. These structural motifs include, but not limited to, GA3 tetraloop, kink-turns (K-turns), kissing-loop (KL), sarcin-ricin loops, T-loops, and pseudoknots which facilitate the global folding of RNA molecules (reviewed in Ref. [25]). Disrupting the sequences of these structural themes renders or markedly hinders riboswitch function [26–32]. Real-time folding of an aptamer is determined by sophisticated techniques other than crystallography, which shows the final configuration of the interrogated molecule. Local folding of the purine riboswitch, xpt-ppuX of B. subtilis was tracked over time by a chemical footprinting technique called Selective 2′-Hydroxyl Acylation analyzed by Primer Extension (SHAPE) that exploits the attacking reactivity of N-methylisatoic anhydride (NMIA) against the 2′-hydroxyl groups of aptamer nucleotides [31]. This technique revealed conformational changes of nucleotides at the binding pocket in response to ligand binding in time window. Folding dynamics of the TPP riboswitch, thinM of E. coli in the presence and absence of the ligand (thiamine pyrophosphate) and Mg2+ ions have also been studied at the molecular basis by another imaging technique called single-molecule Fluorescence Resonance Energy Transfer (smFRET) [33]. In smFRET technique, the targeted parts of the aptamer are labeled differently and folding transitions are correlated with changes in the detection smFRET value. The same study shows high degree of plasticity and dynamics of riboswitch parts configurations as a result of ligand docking.

Having its binding site organized, the aptamer domain can specifically bind the proper metabolite with a great discrimination power against closely related compounds. For instance, adenine riboswitch achieves ~10,000-fold level of discrimination between adenine and guanine [34,35], however, lysine riboswitch has at least 5000-fold level of discrimination between lysine and ornithine; amino acids that differ in their R group by a single methylene group [24]. The virtue of high specificity is attributed to the fact that all functional groups and polar parts of the ligand are engaged in interactions with the nucleobases of the binding pocket, in some instances, mediated by positive ions. The experimental proofs of high selectivity came initially from synthetic aptamers designed to sense different ligand metabolites with affinity and specificity [36]. Strikingly, these synthetic elements failed to exhibit the discrimination power of naturally occurring counterparts. This is not surprising because natural aptamers have been and continue to be sharpened by persistent and stringent evolutionary constraints for billions of years.

2.2. Mechanism of genetic regulation

The conformational structures of bacterial riboswitches are triggered by folding in response to ligand binding which directly modulate gene transcription either to seize or to proceed through formation of terminator or antiterminator structures respectively [24]. Interestingly, riboswitches in Gram-positive bacteria exert their action most commonly via transcriptional inhibition, while translation inhibition is the frequent mechanism in Gram-negative due to Shine-Dalgarno (SD) sequence sequestration. The preference of transcriptional arrest mechanism in Gram-positive may be linked to the fact that their genomes are embedded with large biosynthetic operons where more resources would be wasted if a full-length mRNA is synthesized.

Despite the fact that premature transcription termination is the most common mechanism employed by riboswitches [6], transcription activation, translation initiation inhibition, and ribozyme-like cleavage mechanisms have also been documented in certain riboswitch classes [37]. Such diversity in mechanisms, alongside with protein-mediated mechanisms, enables bacterial cells to finely tune its metabolic status and pathogenic lifestyle by disrupted in response to chemical or physical signals which lead to activation or inhibition of downstream genes [4]. Such RNA regulators exert their regulatory effects without obligate involvement of other factors. It is hard to accept as true that the number of riboswitches in Bacillus subtilis outpaces the number of validated metabolite-binding proteins coordinating gene expression [5].

Riboswitches participate in regulation of diverse cellular physiological and their ligands range from diverse metabolites of different molecular weights to uncharged tRNAs and ions. Such regulatory RNA elements can sense a plethora of cellular metabolites such as amino acids and their derivatives [lysine, glycine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH)], carboxydrates [glucosamine6-phosphate (Glcn6P)], coenzymes [flavin mononucleotide (FMN), thiamin pyrophosphate (TPP), coenzyme B12], nucleobases and their derivatives [adenine, guanine, cyclic di-GMP, cyclic di-AMP] [6,7], metal ions [Magnesium, Nickel, and Cobalt] [8,9], uncharged tRNA [10] and pH [11]. Temperature is another physical parameter that also found to be monitored by RNA elements called RNA thermometers (RNATs), which are considered by many authors as riboswitches [12,13].

The diversity of ligands and sensing RNA sequences has been exploited as criteria to classify riboswitches into currently ~40, different classes [24]. Indeed, not only the exact number of riboswitch classes is unknown, but also rough estimation is difficult to draw even in completely sequenced bacterial genomes [5]. Each class of riboswitches has a high degree of conserved nucleotides comprising the sensory domains in different bacterial species or, in some instances, among riboswitch variants of the same class in the same species. Bioinformatic studies and high throughput sequencing approaches accompanied by biochemical and genetic characterization continued to reveal the complexity and diversity of RNA-based gene regulation in various bacterial genomes.

The aim of this review is to discuss the nature and characteristics of riboswitches and RNATs reported in bacterial pathogens (overt or opportunistic) and their regulatory contributions to pathogenesis to appreciate the roles and importance behind such elements to bacterial cell physiology. Roles of other ncRNA elements in virulence and pathogenicity are excellently reviewed elsewhere [15–18].
modulating relevant genes and their products. Heterogeneity of genetic control mechanisms is attributed to different ligand-induced structural reorganization of the aptamer region which consequently determines the folding pathway of the downstream expression platform region (Fig. 1). Such mutually exclusive secondary and tertiary structures are harnessed to control the expression of downstream coding sequence(s). A strong intrinsic terminator stem has two features; high G-C content followed by a run of U residues that cause RNA polymerase (RNAP) to stall transcription and dissociate from the DNA and nascent RNA stretch [38]. In ligand-free state of riboswitch working by transcription termination, aptamer nucleobases are engaged in certain base pairing that masks key bases necessary to construct the terminator stem (Fig. 1). In other words, some key bases of the terminator stem are incorporated in certain structural motifs, but ligand docking establishes new interactions between binding site’ nucleobases and downstream bases. These new interactions favor terminator stem formation. Similarly, in translation inhibiting mechanism, ligand binding causes the Shine-Dalgarno (SD) sequence or translation initiation site to be masked in stem formation.

For a typical riboswitch to achieve optimally its regulatory goal, it should bind the correct ligand, adopt the final and stable conformation of ligand-bound status, and arrest the transcriptional machinery before the RNAP passes the 3’ boundary of the riboswitch. Global folding in timescale of adenine riboswitches, pbuE of B. subtilis, and add of Vibrio vulnificus, has been determined to be around 1 s using smFRET and force spectroscopy [29,40]. Interestingly, both techniques demonstrated that tertiary structure play a key role in pre-organizing the binding pocket before ligand binding.

Considering that the elongation rate of bacterial RNAP is around 45 nucleotides per second at 25 °C, transcription of such aptamers would require around 1.5 s, thereby transcription and folding should occur within the same time as expected for a cotranscriptional process. Experimental studies on purine and FMN riboswitches suggest that at 1 μM intracellular concentration of ligand, 10 s at least would be required for an aptamer to bind the ligand correctly [41,42]. If transcription machinery progresses at constant rate for this duration, a segment of nearly 500 nucleotides would be produced downstream the riboswitch terminus. How cells resolve this problem? One of the solutions is the programmed pause sites located within the expression platform domain that effectively decreasing transcription rate allowing time for the aptamer to fold.

**Fig. 1.** Diverse regulatory strategies of riboswitch elements [39]. Structurally, a riboswitch element precedes a coding sequence and comprises of two domains; a sensor part called an aptamer (corresponding to red bar) and an output domain called an expression platform (brown bar). In a transcription repressor riboswitches (a), binding of ligand induces conformational changes to trap the antiterminator sequence thus forming a terminator loop. However, translation inhibiting riboswitches sequester the SD sequence and/or start codon AUG in response to ligand binding (b). In transcription activation, ligand binding prevents the formation of terminator loop (c), while translation permissive riboswitches free SD sequence and/or start codon AUG for ribosomal units loading (d). Complementary sequences are color coded.
properly [43]. It is also worth pointing out that different riboswitch sequences will require different amounts of time to fold properly before binding the target ligand. Of note, other factors also play important roles in this regards include; speed of folding, RNA structural stability, and ligand concentration inside bacterial cell (R.R. Breaker, personal communication).

Transcription termination, via intrinsic structures, and translation inhibition are the two previously known mechanisms of riboswitches regulation. However, recently, a new general mechanism was demonstrated in Mg$^{2+}$-sensing (mgtA) and FMN-sensing riboswitches in Salmonella Typhimurium and E. coli respectively [44]. Interestingly, mgtA and ribB riboswitches lack typical intrinsic transcription terminator sequence [45–47] and shuts off the transcription process by Rho-dependent mechanism [44]. Rho factor is a homohexameric RNA helicase that binds to nascent RNA and translocates along the strand to reach the RNAP and cause disassociation of the newly synthesized mRNA from elongation complex at specific cytosine-rich sequence [48]. Surprisingly, few riboswitches show additional novel mechanism of gene regulation by acting in trans. In principle, premature termination of transcription produces short segments of RNA corresponding to the riboswitches sequence. Additionally, aptamer segments also produce by RNase cleavage of transcriptionally generated riboswitches full segments [49]. An intriguing question is; what is the fate(s) of these RNA segments? Degradation and recycling of building blocks is one of these fates. However, it was found that high aptamer concentration effectively maintain homeostasis by titration of ligand metabolites, in other words, by decreasing its cellular free concentrations [50]. A subsequent study detected an interesting finding that, in certain cases, these segments act in trans similarly to small regulatory RNAs [51]. This study showed, for the first time, that two S-adenosylmethionine (SAM) riboswitches (SreA and SreB) in Listeria monocytogenes target several mRNA transcripts of virulence genes through base pairing leading to translation inhibition and mRNA degradation (see contribution to pathogenicity).

2.3. Tandem architecture

Composite riboswitches come in two versions (Fig. 2), two-aptamer-one expression platform riboswitches (e.g. Glycine riboswitches in Vibrio cholerae and Bacillus anthracis) and two complete riboswitches reside adjacent to each other within the same mRNA transcript (TPP riboswitches in Bacillus anthracis). Tandem arrangement of binding domains produces more complex characteristics of interacting with ligand metabolites and gene expression control. The two-aptamer version either possesses the same specificity for a particular ligand or the two aptamers differ markedly in their target metabolites. The latter case was found in metE mRNA in Bacillus clausii tandem riboswitches sensing SAM and Adenosylcobalamin (AdoCbl or coenzyme B$_{12}$) [52]. The double riboswitches version was also characterized biochemically and genetically in TPP riboswitches located in the 5'-UTR of tenA operon in Bacillus anthracis encoding for thiaminase II involved in the regeneration of the thiamine [53].

Similarly to all riboswitches, interactions of binding domains with cognate ligands rely on many chemical strategies including hydrogen bonding, van der Waals, electrostatic, and stacking interactions [54]. Intradomain interactions have been proposed in glycine riboswitches, gcviF, of V. cholerae [55] and the saprophytic species B. subtilis [54]. Having two-aptamer one expression platform architecture, these riboswitches are neither independent nor cooperative however. The benefit of such architecture was a topic of debate until recently. Indeed, studies of ligand binding kinetics revealed a sigmoidal curve which was interpreted as indications of cooperative nature between the aptamers [55]. Nucleotide Analog Interference Mapping (NAIM) approaches and mutagenesis studies suggest cooperative nature inferred by specific interaction between specific regions of both aptamers [55]. In fact, these studies have employed and characterized glycine riboswitches lacking P0 double helix linker at the 5' end of the riboswitch (Fig. 2a). Hence, the earlier observed cooperativity was an artifact of the truncated constructs used for biochemical characterization. The discovery of this k-turn motif precludes the proposed cooperativity between aptamers in glycine riboswitches from V. cholerae [56]. This small segment base pairs with a small stretch of ribonucleotides connecting the two aptamers (Fig. 2a). A revised model for ligand binding in such riboswitches was proposed explaining that aptamers dimerization, rather than double ligand–site occupancy, is crucial for high ligand binding affinity. In other words, dimerization and stability of the second aptamer results from ligand binding in the first aptamer [56]. These in vitro analyses have been supported very recently by evidences from in vivo interrogation of Glycine riboswitch folding behavior [57].

Tandem T-box riboswitches (regulating amino acid metabolic genes) are known in several pathogenic species; B. anthracis, C. perfringens, and M. pneumoniae [58]. In order to trigger the expression of amino acids metabolic operons, high levels of uncharged t-RNAs are required to saturate all copies of T-boxes upstream the operon. What advantage has been gained from harboring identical successive riboswitches? This, presumably, would ensure nearly complete repression of the subsequent operon through the combined action of both riboswitches with a nearly half-ligand concentration needed for single riboswitch to reach the same outcome (transcription termination). TPP Riboswitches were found in tandem as in tenA RNAs encoding thiaminase (EC. 3.5.99.2) in Bacillus anthracis [53]. TPP riboswitches in tenA are composed of two complete versions of riboswitches linked by a stretch of repetitive U residues. These tandem riboswitches control gene transcription termination independently as a natural Boolean NOR logic gate, where ligand docking to either aptamer yields an output of

![Fig. 2. Tandem architecture of riboswitches. (A) Glycine (Gly) riboswitch from Bacillus with a double-aptamer and single expression platform [52]. The newly characterized kink-turn (P0) is depicted in red. This configuration is found in many glycine riboswitches in Gram-positive species as well as in V. cholerae. (B) Two similar complete TPP riboswitches from B. anthracis and (C) two different complete riboswitches; SAM and Adenosylcobalamin (AdoCbl) precede methionine metabolic gene in Bacillus [52]. Expression platforms sequences are colored in blue.](image-url)
gene regulation. NOR logic function does not require that the two riboswitches influence each other [52]. Such conclusion was drawn from genetic experiments in which mutated and wild-type tandem TPP riboswitch was fused with beta-galactosidase reporter gene in various combinations and tested at different concentrations of thiamine, the precursor of TPP. Furthermore, the absence of the second riboswitch was found negligible and one riboswitch was sufficient to impart transcription termination of the reporter gene construct in transformed B. subtilis [53].

3. RNA thermometers

The activities of enzymes and many biological molecules are affected by changes in ambient temperature. Drastic fluctuations of temperature pose a serious threat to cell physiology, therefore, bacterial cell has to monitor and respond efficiently and rapidly to such heat perturbations. Elevated temperature causes protein denaturation, weakening structural integrity of DNA and RNA, and increase fluidity of plasma membrane to an extent that disrupts its selective permeability. In contrast, decrease in temperature leads to dramatic decrease of enzymes activities, membranes rigidification, and formation of stable RNA structures that obscure or interfere with transcription and translation leading eventually to growth arrest. Different physiological responses are mounted to face such physiological deleterious alterations among which is an RNA-based immediate response. Accumulation of denatured proteins serves as a signal that trigger the expression of hundreds genes employed to respond to and limit the heat-triggered damages to maintain normal metabolism. Three categories of genes are subjected to thermoregulation; heat shock genes, cold shock genes, and virulence genes.

Prompt change in temperature is most efficiently sensed and transduced by RNA structures, called RNA Thermometers (RNATs) because other signal transduction systems, protein- or DNA-mediated, require much time to come into effect. The virtue of RNATs immediate response is attributed to their mode of regulation in which translation initiation of existing mRNA is controlled in temperature-dependent manner. It has been reported that as minor as 1 °C variation of temperature is detected by such RNA-based system [59]. However, the mechanism employed by RNATs to detect such subtle variations of temperature is still largely unknown. The exploitation of mismatch pairing in the effector hairpins of RNATs seems to be the major strategy. Pathogenic bacterial species of environmental origin seem to depend on RNATs to confirm that a living host has been reached in order to initiate virulence factors production.

These complex RNA structures are mostly found in the 5’-UTR of temperature-responsive genes, and alter their conformation between two distinct structures in response to temperature [59]. In low temperature, the “closed” configuration is adopted, in which the SD sequence and/or AUG codon are occluded due to base pairing with complementary nucleotides in the same element. Melting of these secondary structures results in the alternative “open” conformation exposing SD sequence and/or AUG to the ribosome [60]. Although the first RNAT element was found in plague agent, Yersinia pestis, in 1993 [61], the best understood RNAT is the thermometer of prfA transcript in Listeria monocytogenes controlling, with other activators, the synthesis of PrfA protein, a transcriptional global activator of virulence genes [62]. This thermosensor (127 nt.) folds into hairpin structure at low temperature to inhibit translation initiation, but the hairpin stem is melted upon increase of temperature to 37 °C [63]. In general, these riboswitches are highly dynamic motifs in a narrow temperature ranges to modulate heat and cold shock response proteins [64,65]. To clarify the fundamentals of RNAT-based regulation, two classes of these thermosensors are discussed herein.

3.1. High temperature RNA thermosensors

3.1.1. ROSE elements

Repression Of heat Shock gene Expression (ROSE) is the most common class of RNATs encountered in rhizobia, alphaproteobacteria, and gammaproteobacteria [66-68]. ROSE elements range from 60 to >100 nucleotides (nt.) upstream the coding sequences of mRNA coding for heat shock chaperone proteins. Structurally, ROSE elements are usually composed of 2-4 hairpins (Fig. 3a). The hairpin of the 5’-end is structurally stable irrespective of the temperature [69], whereas the 3’-end hairpin looses its tertiary and secondary structures gradually as temperature increase, much like a zipper. The SD sequence is trapped by base pairing with conserved U(U/C)GCU motif in the 3’-end hairpin which is the functional part of the RNAT. The other hairpins, however, are thought to confer scaffolding purposes and ensure proper folding of the ROSE element [70,71]. In vivo analysis of ROSE elements, it was proved that at 30 °C, the ribosomal binding site (RBS) at the 3’-end hairpin is masked but partial liberation of SD sequence occurs at 37 °C, whereas an increase of temperature to around 42 results in full liberation of RBS leading to complete translation of downstream gene in a zipper like fashion [70].

Expression of heat shock genes occurs when sigma factor 32 (σ32), assists RNAP to identify their promoters. In turn, genes of σ32, rpoH, in E. coli, are controlled by four different promoters which respond to different environmental signals [72] alongside with ROSE-like RNATs at the level of transcription and translation respectively. Unlike other RNATs, the RBS of rpoH is not completely occluded, but partially exposed [12,13]. Nevertheless, its translation is prevented at non-stress temperature by inhibitory structure created by folding of two distinct segments in the coding sequence that obscure ribosome units loading. At high temperature the inhibitory structure is weakened and ribosome entry becomes feasible [12,72]. Two of sigma 32- activated genes, IbpA and IbpB encode for heat shock proteins participating in multichaperone network that stabilizes heat-denatured proteins have been found to possess a 96-nt ROSE-like RNATs at their 5’-UTRs [73-75].

3.1.2. Four uredine thermometers

A stretch of four uredines (a.k.a. fourU) was firstly found at the 5’-UTR of agsA gene in Salmonella enterica serovar Typhimurium. AgsA gene encodes for Aggregation Suppression A (AgsA) protein [76]. Several heat shock and virulence genes were also found to harbor this sequence which exploits the strategy of mismatch base pairing that would be disrupted easily in response to temperature increase. Such sequence sequesters the SD sequence and control translation initiation of mRNA at low temperatures. The 5’-UTR containing the fourU element folds into two stem-loops; the 5’-hairpin is heat stable, as in ROSE elements (Fig. 3b), while the second hairpin containing the fourU is temperature-sensitive [76]. At high temperatures (40s °C), the mismatch pair A29:G52 is easily disrupted due to their weak bonding and the melting of the fourU hairpin ensues. However, the entire melting of the stem-loop is prevented by the stable base pair G34:C60 at the top of the stem. Substitution of A29:G52 with a typical C-G base pair results in complete repression of the gene in vivo due to difficulty in opening the stem [76]. On the other hand, substitution of G34:C60 by A-U pair decreases the melting point by 5 °C and expressed the downstream gene at non-stress conditions. As in riboswitches, Mg2+ ions participate in tertiary structure stabilization of the 3’-hairpin [77]. Additionally, translation of htrA gene in Salmonella Typhimurium, encoding for periplasmic serine protease (a.k.a. Do or DegP protein), is controlled by a fourU-type RNAT composed of only one
around intracellular macromolecules (for example, DNA, RNA and proteins), with a possible effect on their functionality [59]. Purple shaded bases are Mg$^{2+}$ aggregation suppressing A (melted at 37°C) regulated by thermosensitive histone-like protein that only when [85].

Antibacterial immune response [84] and type III secretion system LcrF is a global transcriptional activator of many virulence genes of the intergenic region of two-gene operon, [87]. The best characterized representative of cold RNATs is the 160 nucleotides 5′-UTR of cspA in E. coli. Similar to riboswitch, the arrangement of this thermosensor results from mutually-exclusive structures. At 10°C, the RNAT is stable and adopted a secondary structure with exposed SD sequence. Whereas at 37°C, stem-loop formation traps the ribosome binding site which limits translation process. Interestingly, the functional sequence of cspA RNAT in E. coli extended into ~60 nucleotides of the downstream coding sequence [65].

3.2. Cold RNA thermosensors

Cold Shock Proteins (CSPs) are protective proteins produced under cold conditions to mitigate the damage or harmful effects at membranes and undesirable RNA structures that impede translation initiation and elongation [80]. In L. monocytogenes, three genes encoding CSPs are preceded by in-built relatively long (198–363 nt.) sequences predicted to have hairpin structures [81]. This pathogen endures refrigerator temperature which reflects its physiological ability to acclimate to such low temperature. In 4°C incubation, the expression levels of these genes elevate by 4–27-fold compared to incubation at 37°C [81]. The best characterized representative of cold RNATs is the 160 nucleotides 5′-UTR of cspA in E. coli. Similar to riboswitch, the arrangement of this thermosensor results from mutually-exclusive structures. At 10°C, the RNAT is stable and adopted a secondary structure with exposed SD sequence. Whereas at 37°C, stem-loop formation traps the ribosome binding site which limits translation process. Interestingly, the functional sequence of cspA RNAT in E. coli extended into ~60 nucleotides of the downstream coding sequence [65].

4. Contributions of riboswitches and RNATs to pathogenicity

Though the aforementioned examples of RNATs reside in the 5′-UTRs of either monocistronic or polycistronic mRNAs, new levels of RNAT-based sophisticated loci and regulation were documented in human pathogens yersinia and Neisseria meningitidis [82,83]. In Yersinia pestis, an RNAT at the 5′-UTR of lacF gene was identified in the intergenic region of two-gene operon, yscW-lacF operon [82]. LacF is a global transcriptional activator of many virulence genes including outer membrane proteins (Yops) employed for evasion of antibacterial immune response [84] and type III secretion system [85–87]. Furthermore, transcription of the whole operon is also regulated by thermosensitive histone-like protein that only when melted at 37°C allows RNAS to read-through the operon [82,88]. LacF RNAT of human pathogenic yersinia is composed of two conserved hairpins that sequester SD sequence in its 3′ end loop by a typical fourU motif. The partially occluded start AUG codon in secondary structure is liberated at 37°C when G-C mismatches of SD/fourU are disrupted [82].

In Bartonella henselae, the causative agent of Cat-scratch disease, and also in other species of Bartonella, the coding sequences of numerous transcriptional regulators, trp family, are preceded by putative riboswitches named Brt1-9, with unidentified ligands [89]. From biochemical experiments, it is likely that in the absence of its ligand, Brt1 terminates the transcription of the ensuing trp 1 sequence. Trp 1 is a helix-turn-helix DNA binding protein that positively regulates badA gene transcription [89]. badA is a major virulence surface protein involved in autoaggregation, biofilm formation, and phagocytosis inhibition [90,91]. Interestingly, besides being a putative riboswitch, experimental evidence inferred a possible trans-acting mechanism for Brt1 to downregulate badA by base paring with badA mRNA transcript [89].

Ecologically, Listeria monocytogenes is a widely distributed species that survives primarily as a saprophytic species in soil [92]. The ability of L. monocytogenes to switch to pathogenic lifestyle upon ingestion by a susceptible human host is mediated by the activation of number of genes collectively known as virulence genes. Products of virulence genes are implicated in host cell invasion, intracellular growth, and spread to neighboring cells [93]. The majority of such virulence factors, thus far, are known to be regulated by PrfA protein, a transcriptional activator, which harbors a thermometer regulatory sequence [92]. After successful access to human host, increase of temperature serves as a confirmation signal to the listerial cell to switch to parasitism. Intriguingly, PrfA biosynthesis is subjected to regulation by a thermometer located in the 5′-UTR. At 30°C, this regulatory region folds into hairpin secondary structure masking the SD sequence which only freed from the hairpin if temperature elevated to 37°C when RNA secondary structures denatured [51]. At 37°C, the virulence factors modulate the pathogenicity after the thermometer permits the translation of PrfA mRNA [92]. In presence of SAM, SreA riboswitch responds by transcription termination of methionine metabolic genes. The produced stretch of RNA corresponding to the 5′-UTR of SreA engages in base pairing with the sequence upstream the SD sequence of prfA mRNA transcript at 37°C. This trans-acting regulatory effect of SreA does not take place at 30°C, because the SD sequence is incorporated in a thermometer stem [51].

Other examples in which a riboswitch plays a role in regulating virulence factors are reported in Enterococcus faecalis [94] and also in Listeria monocytogenes [95]. In L. monocytogenes, the eut operon encoding for ethanolamine utilization is regulated by two-
component regulatory system (EutVW). In this protein system, EutW is the membrane sensor kinase and EutV is its associate DNA-binding response regulator. EutV functions as antiterminator via disrupting intrinsic terminator hairpins produced in the Rli55 segment during 5'-end transcription of the operon [96–98]. The enzymes of ethanolamine utilization pathway require vitamin B12 as a coenzyme. If ethanolamine is available (as in the intestines during infection), EutW signals the EutV to aid transcription of the operon. However, if B12 is absent, Rli55 is fully transcribed with its terminator loops leading to EutV sequestration and failure to express eut genes. Yet binding of B12 to its riboswitch at the 5'-UTR of Rli55 masks the sequences that sequester EutV allowing for eut operon to be expressed [95]. Such integrative multifaceted (protein and RNA) regulation emphasizes the idea that pathogenic species have engineered efficient systems to express pathogenicity-related genes only under correct optimal conditions. Ethanolamine is abundant molecule in the intestines of vertebrates and its utilization was found to be part of listerial pathogenesis in mice model [98].

Shigella dysenteriae has an outer membrane heme receptor, ShuA, important for acquisition of iron from complex molecules in human body. Nutrient iron is required for pathogenicity initiation and progression [100,101]. ShuA transcription is regulated by an iron-dependent transcriptional repressor, Fur [102,103] while its translation is subjected to fourU RNAT located in its 300 nucleotides 5'-UTR [104]. In iron-poor condition, ShuA is synthesized only at 37 °C when translation of ShuA mRNA is permitted, however, at low temperature; fourU RNAT occludes the SD sequence regardless of iron concentration.

In V. cholerae, the causative agent of life-threatening diarrheal cholera disease, the production of cholera toxin (CT) and toxin-coregulated pilus (TCP) occurs when the bacterium reaches human intestines [105]. The successful arrival to host is confirmed by rise in temperature from less than 20 °C of the contaminated water to the 37 °C of the body. The mentioned toxin and pilus are the important major virulence factors for pathogenicity [reviewed in Ref. [106]]. The expression of these factors is subjected to transcriptional activator ToxT. The ToxT genes harbor a fourU RNAT element that masks SD sequence under low temperature conditions [107].

5. Evolution of riboswitches and RNATs

Before evolutionary emergence of DNA as genetic material, life thought to have relayed on RNA for the mission. Furthermore, life of the RNA world might have depended on RNA for catalysis of chemical reactions and for modulation of biological processes, at least the essential ones. RNA viruses are considered as evidences to support the hypothesis of RNA being the only medium of genetic material [108–110]. On the other hand, ribozymes are thought to have been the biocatalysts in the RNA life, much like modern enzymes before protein existence. This is supported by the discovery of a carbohydrate-sensing riboswitch element that employ self-cleavage mechanism. The key metabolites thought to have been existed are amino acids and nucleobases, from which modern co-enzyme may have evolved. Despite the fact that primordial RNA world hypothesis is controversial, at least currently, successive discoveries in the arena of RNA continue to provide testimonials one after another. Riboswitches are robust evidences that RNA has the ability to selectively bind specific molecules under low concentration with great discrimination power, much like contemporary metabolite-binding proteins. If riboswitches are real fossils from ancient RNA life, it is remarkable that they have retained conserved sequences during billions of years of evolutionary pressure to serve in modern organisms. Indeed, TPP riboswitches have been found in the three domains of life and representatives of different ribozymes are distributed in the three domains of life and viruses.

On the other hand, an intriguing question is that; if modern RNATs are remnants of the primordial RNA world, then what were they employed for in the absence of DNA or protein molecules? If their existence in RNA world is proven, then their contributions were likely to regulate the expression and protection of genetic material (RNA) resembling the modern roles in the modern DNA-protein world. The catalysis capabilities of RNA molecules (such as ribozymes) are affected by temperature raise which necessitated the existence of a protective/regulatory aid mostly fulfilled by RNATs.

Bioinformatic analyses continue to identify not only new classes but also variants of already characterized classes [111–114]. A new computational study employing multiple-sequence alignments, atomic-resolution structural information, and riboswitch gene association have identified many variants that changed their ligand specificities much like protein enzymes [114]. Some variants completely lost their ability to bind the primary ligand and the ligands of such variants are still unknown. Such changes are direct results of mutations in key nucleotides at the binding pockets reflecting continuous genome evolution. From gene regulation standpoint, the associated genes with such variants are most probably relying on other more effective regulatory mechanisms beside the original riboswitch versions.

6. Conclusions and perspectives

Riboswitches and RNATs are important RNA-based regulators that respond to key metabolites and temperature, respectively, via different mechanisms and to different extent. Some riboswitches precede a single gene while others control a polycistronic transcript encoding more than one gene. Unlike other noncoding RNA elements that are devoted to interact with other RNA transcripts via restricted typical base pairing, riboswitches and RNAT are more developed to compete with proteins to bind chemical compounds and sense temperature shifts, respectively, to coordinate gene expression and cope up with pathogenic lifestyle. The distribution and levels of complexity reflect the paramount role of RNA domains to participate in maintenance of cell physiology and homeostasis. The extent of RNA-based regulatory networks is far from being fully appreciated. In occasions, some riboswitches have been found to interact with other riboregulators that control different physiological process. Cells relay on riboswitches and RNA thermosensors to avoid synthesizing unnecessary proteins or to induce the immediate production of others in a much rapid response than protein-mediated one.

Levels of RNA-based regulation of cell physiology are getting expanded and sophisticated. Understanding of simple prokaryotic model systems will provide the knowledge and principles to explore higher eukaryotic RNA regulatory networks. Additionally, discovery of selective drugs that compete with ligand to bind riboswitch is a promising approach to fight pathogenic species and lowering the burden of antibiotics resistant infections. The continuous sequencing and annotation of bacterial genomes will aid the research to delve deeply into mechanistic, biological function, and evolutionary questions regarding riboswitches and RNA thermosensors. Additionally, characterization of a riboswitch upstream the coding sequence of a hypothetical gene helps in deciphering the identity and function of that gene product.

Conflicts of interest

The author declares the absence of any actual or potential
conflict of interest.

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