Title:

Applications and limitations of regulatory RNA elements in synthetic biology and biotechnology.

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Running Headline:

RNA elements in synthetic biology.

Summary:

Synthetic biology requires the design and implementation of novel enzymes, genetic circuits, or even entire cells, which can be controlled by the user. RNA-based regulatory elements have many important functional properties in this regard, such as their modular nature and their ability to respond to specific external stimuli. These properties have led to the widespread exploration of their use as gene-regulation devices in synthetic biology. In this review, we focus on two major types of RNA elements: riboswitches and RNA thermometers (RNATs). We describe their general structure and function, before discussing their potential uses in synthetic biology (e.g. in the production of biofuels and biodegradable plastics). We also discuss their limitations, and novel strategies to implement RNA-based regulatory devices in biotechnological applications. We close with a description of some common model organisms used in synthetic biology, with a focus on the current applications and limitations of RNA-based regulation.

Keywords:

Synthetic biology, biotechnology, RNA elements, riboswitches, RNA thermometers (RNATs), *Ralstonia sp.*, Cyanophyta.

1. Introduction

Synthetic biology is a field that employs artificial biological tools to better understand and manipulate complex biological systems, metabolic pathways, structures, and functions. Synthetic biology aims to integrate bioengineering and biology to synthesize modified or novel biological complexes—such as enzymes, genetic circuits, or entire cells—that are not normally found in a given biological system (Serrano 2007; Rodrigues and Rodrigues 2018). Conversely, systems biology studies existing, natural biological systems to better understand how they work. Synthetic biology can benefit from the knowledge generated by systems biology and expands on or optimizes this knowledge by modifying natural pathways or creating novel structures that enable the implementation of desired metabolic pathways and products. Thus, synthetic biology contributes to the understanding of fundamental biological processes and it is contributing to promising future applications in the fields of bioenergetics, biofuels, and bioremediation, as well as the development of living 'chemical factories' and vectors for gene therapy (Serrano 2007; Villa et al. 2018).

Gene expression has been successfully controlled at the level of transcription by promoters for quite some time, such as the arabinose inducible promoter in the pBAD series of plasmids (Guzman et al. 1995), which is still used to this day. Although they function well in *Escherichia coli*, some inducible promoter-based expression systems, such as the P_{lac}/lacI system, do not function in other bacteria, such as *Ralstonia eutropha* (Fukui et al. 2011). Other issues with inducible promoters include leaky expression (i.e. the protein is expressed even in the absence of induction). For example, the T7 expression system is capable of expressing high levels of proteins, with the trade off that they are prone to leaky expression (Giacalone et al. 2006). Inducible promoters may also require other factors to function adequately, such as the *lacY* gene (encoding the lactose permease) needed for proper isopropyl β-D-1-thiogalactopyranoside (IPTG) induction (Hansen et al. 1998).

In contrast, riboswitches are excellent candidates to modulate gene expression in a ligand-dependent manner without the involvement of organism-specific promoter sequences or factors (Tucker and Breaker 2005; Scott et al. 2010; Ma et al. 2014). RNA-based regulatory elements have many important functional properties that have led to the widespread exploration of their potential use as regulatory tools in synthetic biology (Kushwaha et al. 2016). For instance, they are theoretically modular in nature (i.e. their sequence allows the formation of discrete structures that can be independently controlled) and they can be 'tuned' to respond to external stimuli (Vazquez-

Anderson and Contreras 2013). In this review, we will focus on two of the major types of RNA elements: riboswitches and RNA thermometers (RNATs). We will discuss their potential uses in synthetic biology, some practical limitations, and novel strategies to implement riboswitch- or RNAT-based regulatory devices in biotechnological applications. We will close with a brief description of some model organisms commonly used in synthetic biology, with a particular focus on the current applications and limitations of RNA elements in biotechnological applications.

2. Riboswitches

Riboswitches are RNA-based regulatory tools that specifically modulate the expression of a gene or operon. Riboswitches hold great potential as regulatory elements in synthetic biology and biotechnological applications, as they are (theoretically) modular and independent of protein factors and chaperones (Tucker and Breaker 2005; Scott et al. 2010; Ma et al. 2014; Villa et al. 2018). Although riboswitches can be bioengineered with relative ease, and they hold great promise in various applications (e.g. bioenergetics, biofuels, therapy, bioremediation, and biochemical engineering) (Etzel and Morl 2017; Hallberg et al. 2017; Villa et al. 2018), their practical applications are currently limited. So far, synthetically engineered riboswitches have been employed mostly for proof-of-concept studies, using reporter genes like green fluorescent protein (GFP) in model microbes like *Escherichia coli, Bacillus subtilis, and Saccharomyces cerevisiae* to demonstrate functionality (Topp et al. 2010; Etzel and Morl 2017; Hallberg et al. 2017). In this section, we discuss various potential uses for riboswitches, common challenges, and possible solutions for their biotechnological applications.

2.1. Riboswitch structure-function

Riboswitches have two main components: an ‘aptamer domain’ and an ‘expression platform’. The aptamer domain binds tightly to a specific metabolite (or ligand). Riboswitches are named based on their target ligands. For example, the theophylline and SAM II riboswitches bind specifically to theophylline and S-adenosyl methionine (SAM), respectively. A riboswitch is a *cis*-acting RNA structure that is generally found in the 5’ untranslated region (5’ UTR) of an mRNA, with only the thiamin pyrophosphate (TPP) riboswitch known to exist in the 3’ UTR (Wachter et al. 2007). These elements are divided into two groups based on their structural features: (i) pseudoknot-like S-

Adenosylmethionine (SAM II) riboswitches (Gilbert et al. 2008) and (ii) three-way junctions with distal tertiary contacts, such as the TPP riboswitch (Serganov et al. 2006). However, other exceptional tertiary structures have been discovered in some riboswitches, such as a four-way junction in SAM I (Eschbach et al. 2012), a tertiary docking interface in yybP-ykoy (Price et al. 2015), an inverted junction that has a single aptamer in THF with two metabolite binding sites (Trausch et al. 2011; Peselis and Serganov 2014), and inter-domain interactions in glycine riboswitches (Huang et al. 2010). All members of a riboswitch family recognize the same ligand and the family can be classified based on their folding features. For example, the SAM riboswitch family members all bind the SAM ligand, but the SAM I (Eschbach et al. 2012), SAM II (Gilbert et al. 2008), and SAM III (Lu et al. 2008) classes have a four-way helical junction, classic pseudoknot, and a three-way folding features, respectively. The expression platform changes its structural conformation in response to binding of the metabolite to the aptamer domain. The refolding of the expression platform in response to the binding of the specific ligand is dose-dependent and can either activate or repress the expression of the downstream open reading frame (ORF).

This regulation can be exerted at the level of transcription (Blouin et al. 2011), translation, or (less often) alternative splicing (Wachter et al. 2007). Most riboswitches regulate the transcription of a gene—either they prematurely terminate transcription by forming an intrinsic (Rho-independent) terminator structure (Figure 1A), or they form an alternative 'anti-terminator' structure to enable transcription of the full mRNA (Stewart and van Tilbeurgh 2012) (Figure 1B). Other riboswitches control the initiation of translation by sequestering (e.g. SAM II riboswitch) (Haller et al. 2011) or exposing (e.g. theophylline riboswitch) (Cui et al. 2017) the Shine-Dalgarno (SD) ribosome-binding site (RBS) to repress (Figure 1C) or activate (Figure 1D) gene expression, respectively. Lastly, some riboswitches have been found to regulate splicing in prokaryotes or eukaryotes (Lee et al. 2010; Chen et al. 2011; Li and Breaker 2013). In bacteria—for instance, in *Clostridium difficiles* strains—the cyclic-di-guanosine monophosphate (GMP) II riboswitch responds to GMP II in a dose-dependent manner to promote the self-splicing of group I introns (Lee et al. 2010; Chen et al. 2011). To our knowledge, only the TPP riboswitch has been discovered in eukaryotes so far (Li and Breaker 2013). The TPP riboswitch modulates alternative splicing in a fungus (*Neurospora crassa*), leading to the removal of an inhibitory upstream open reading frame (uORF) from the 5' UTR (Cheah et al. 2007). In plants, the TPP riboswitch alters splicing and

alternative 3' UTR lengths of its mRNA by either occluding or exposing a splicing site at a low or high concentration of TPP, respectively (Wachter et al. 2007).

2.2. Riboswitch applications

Riboswitches have a short aptamer sequence, which binds to small molecules that are simple to incorporate into a metabolic circuit. These handling characteristics make riboswitches a promising potential tool to regulate gene expression in various applications, such as gene therapy, bioremediation, and the production of antibacterial drugs and biofuels (Etzel and Morl 2017; Hallberg et al. 2017; Villa et al. 2018).

Firstly, riboswitches have been adapted to regulate antibiotic resistance genes. For example, Feng et al. constructed a synthetic riboswitch containing a theophylline-specific aptamer that, when bound to theophylline, leads to the formation of a *cis*-acting ribozyme that degrades the riboswitch-controlled mRNA (Feng et al. 2011). The mRNA in question encodes a β-lactamase, which inactivates β-lactam antibiotics (Fisher et al. 2005). On the other hand, an intensive multiple sequence alignment led to the identification of a *Pseudomonas fluorescens* riboswitch that senses aminoglycoside antibiotics. The riboswitch in question was confirmed to control the expression of two antibiotic resistance genes—aminoglycoside acetyltransferase (AAC) and aminoglycoside adenyl transferase (AAD) (Jia et al. 2013)—in a dose-dependent manner, and to induce the expression of a β-galactosidase reporter fusion by 2.5- to 3.2-fold (He et al. 2013).

Secondly, riboswitches have been highlighted as a potential tool for bioremediation (Breaker et al. 2017). Nelson et al. demonstrated that the ykkC riboswitch binds to the guanidine moiety (Nelson et al. 2017). A large amount of guanidine, which denatures protein structures by interacting with the peptide backbone to promote unfolded conformations (Jha and Marqusee 2014), has been released into the natural environment from the industrial production of, for example, plastics, explosives, and automobile airbags (Breaker et al. 2017). Bioremediation could be carried out by a microbe engineered to express urea carboxylases (Kanamori et al. 2004). Urea carboxylases have been shown to catabolize both guanidine and urea, with a 40-fold higher affinity for guanidine (Nelson et al. 2017). Therefore, the guanidine-sensing ykkC riboswitch is a potential candidate for regulating bioremediation, by ensuring that the carboxylase is expressed in the presence of environmental guanidine (ranging from 0.1 to 1 mol l^{-1}) (Nelson et al. 2017).

Moreover, existing riboswitches can be engineered to regulate the expression of proteins of interest, such as the theophylline-responsive riboswitch, which is a potential candidate to regulate enzymes required for biofuel production in cyanobacteria (Ma et al. 2014). For instance, the theophylline riboswitch could enable theophylline-dependent expression of the acyl-ACP thioesterase (t*esA*) gene. TesA from *E. coli* has been used to increase production of free fatty acids (a pre-cursor to biodiesel) in *Saccharomyces cerevisiae* (Runguphan and Keasling 2014). By maintaining low levels of thioesterase during initial culturing—when free fatty acid production could limit growth—and inducing its expression after a sufficient cell mass has been produced, an engineered theophylline riboswitch could further improve biodiesel production.

Beyond simple gene expression, riboswitches have been successfully used to alter both metabolism and behavior of bacteria. For example, lysine biosynthesis utilizes a multistep metabolic pathway that competes with other metabolic pathways that require citrate (Zhou and Zeng 2015). To improve lysine biosynthesis, a lysine riboswitch was used to shut down citrate synthesis in order to favour lysine biosynthesis over that of the competing pathways (Zhou and Zeng 2015). For behavioral control of bacteria, theophylline riboswitches controlling genes involving mobility of chemotactic bacteria were used to control cellular mobility in a ligand dependent manner while directing the cells along a theophylline containing path (Topp and Gallivan 2007). Theophylline riboswitches have also been used to regulate pre-RNA splicing *in vitro*. For this, engineered theophylline riboswitches were placed on the 3' splice site of a pre-mRNA that repressed its splicing ability once theophylline was introduced (Kim et al. 2005).

Synthetic riboswitches are also promising future tools for the external regulation of therapeutic genes ('transgenes') transferred by replication-deficient adenoviruses for therapeutic applications (Ketzer et al. 2012; Ho et al. 2016). The idea is to introduce a theophylline-responsive riboswitch into the 5' and 3' UTRs of the transgene (encoding the secreted chemokine CCL5) prior to its introduction into the patient by an adenoviral vector; expression of the transgene can then be repressed up to 11-fold by theophylline (Ketzer et al. 2012).

Finally, riboswitches have been useful when screening for different traits and directed evolution, such as selecting for lysine production using lysine riboswitches. This was done by having a lysine riboswitch controlling an antibiotic resistance gene while applying a selective pleasure (the antibiotic), allowing one to screen for and select high lysine-producing cells and enzyme mutations (Yang et al. 2013; Wang et al. 2015). Other riboswitches, such as theophylline,

flavin mononucleotide (FMN), and c-di-GMP riboswitches—all controlling GFP expression—have successfully been used to screen enzyme libraries of caffeine demethylase, vitamin B2 producing bacterial strains, and c-di-GMP-metabolizing enzymes, respectfully (Michener and Smolke 2012; Gao et al. 2014; Meyer et al. 2015).

2.3. Limitations of synthetic riboswitches and potential mitigations

Although it is been almost two decades since riboswitches were discovered (Nahvi et al. 2002), their applications have been largely limited to model bacteria such as *E. coli* and have rarely been applied in industrially relevant organisms or to regulate the expression of industrially useful genes. Instead, they have mostly been used in proof-of-concept studies or as fluorescently-tagged biosensors (Topp et al. 2010; Etzel and Morl 2017; Hallberg et al. 2017). The current section discusses several reasons why riboswitches have yet to reach their full biotechnological potential.

The lack of successful riboswitch applications outside of basic research raises doubts about whether they are simple 'plug and play' devices that enable dose-dependent control over gene expression. Recent studies have assessed riboswitch modularity (Folliard et al. 2017) in different genetic contexts. For instance, by replacing its original open reading frame (ORF) with a new one, the secondary or tertiary structure of an aptamer domain can be subtly disrupted and thus lose its ligand specificity (Folliard et al. 2017). This calls into question the functional modularity of riboswitches. To overcome this loss of fidelity when attempting to change the genetic context of a riboswitch, several groups have fused the gene of interest downstream of the first several dozen nucleotides of the riboswitch's native ORF, rather than directly downstream of the riboswitch itself, thus stabilizing the native structure of the riboswitch (Winkler et al. 2002; Dixon et al. 2012). However, the optimal length of this linker varies with every riboswitch and each new genetic context, leading to the formation of fusion proteins of varying length. An N-terminal fusion will often affect the stability, solubility, localization, or functionality of the protein of interest—especially in the case of enzymes (Chant et al. 2005; Park et al. 2015). Recently, Folliard et al. developed a mechanism to mitigate this issue, termed a 'riboattenuator', which has allowed them to successfully enhance the modularity of riboswitches and circumvent the inclusion of a 5' fusion, increasing protein production by ~1000 fold (Folliard et al. 2017). The riboattenuator has a second RBS, sequestered by a local hairpin, which is directly downstream of the stop codon of the riboswitch-controlled ORF (Figure 2). If a ribosome initiates translation from the upstream RBS—

which will be controlled by the ligand and riboswitch, as usual—it will elongate, translating the riboswitch's native ORF until it reaches the stop codon. The helicase activity of the ribosome will unwind the hairpin structure sequestering the second RBS (Figure 2). An important feature of these attenuators is the stop codon, the last nucleotide of which is the first nucleotide of the start codon for the gene of interest. Thus, the ribosome is poised to dissociate from the upstream ORF and immediately re-initiate translation at the downstream start codon, producing the protein of interest without any N-terminal fusion (Folliard et al. 2017).

The disruption of the aptamer structure of a riboswitch, and thus its ligand-binding function, simply by altering the genetic context raises the question of the stability of secondary and tertiary riboswitch structures, especially *in vivo*. Chen et al. conducted a full analysis of structural conformation changes of the SAM II riboswitch from the unbound to ligand-bound form (Chen et al. 2012). Their study showed that the well-organized, natively folded structure of the SAM II riboswitch requires both Mg^{2+} and SAM II. The cofactor, Mg^{2+}, promotes the formation of tertiary interactions in the SAM II binding-pocket of the aptamer region. The intermediate structures after Mg^{2+} are bound in equilibrium between SAM II binding-competent and -incompetent states. Therefore, there is a significant chance that the riboswitch will fail to properly control gene expression even in the presence of both ligands (Chen et al. 2012). It will be necessary to increase the stability of the ligand-binding conformations of a given riboswitch—for instance, through the introduction of point mutations—in order to ensure the maximal response of the riboswitch to its cognate ligand and thus increase its efficacy in biotechnological or industrial applications.

The ligands of riboswitches are small molecules and metabolites, such as nucleotides and metal ions. These metabolites—for instance, cobalamin metabolites (Polaski et al. 2017)—are also bound by certain proteins and used as co-factors (Romine et al. 2017). Multiple riboswitches can be found in the same bacterium (Mandal et al. 2003) and could hypothetically compete with one another, or with other cellular components, for ligand-binding *in vivo*. Consequently, the expression of the gene of interest may not be controlled by its cognate ligand in the expected dose-dependent manner. For future applications, the selection of a riboswitch should not be based solely on riboswitch functionality but should also account for the presence of the metabolite in existing biological pathways in the host organism, which can interfere with the regulation of the gene of interest. Ideally, the riboswitch's ligand should be absent altogether, allowing for a greater degree

of control, although this may prove impractical. Additionally, an intensive analysis of metabolites is needed to test whether the ligand in question is involved in chemical reactions. For instance, the ligand cobalamin contains cobalt metal at the center of its structure, which is linked to four pyrrole groups by nitrogen bonds. Cobalamin comprises different chemical forms of vitamin B12 that are classified based on the upper axial ligand of the cobalt ion (Polaski et al. 2017). Those chemical forms (with their upper axial ligands) are cyanocobalamin, hydroxocobalamin, methylcobalamin, and adenosylcobalamin (Polaski et al. 2017). Both methylcobalamin and adenosylcobalamin are biologically active forms of B12 and the oxidation of the cobalt results in different charged compounds: hydroxocobalamin (3+) and methyladenosine (1+) (Polaski et al. 2017). Small sequence differences among structurally related riboswitches can affect their ligand specificity and therefore their regulatory behavior. Polaski and colleagues have demonstrated, using a mutagenic approach both *in vitro* and *in vivo*, that ligand-binding specificity of the cobalamin riboswitch can be altered such that it responds to either methylcobalamin or adenosylcobalamin (Polaski et al. 2017). Another study has revealed that the aptamer of the tetrahydrofolate (THF) riboswitch from Firmicutes can bind to two chemical forms of folate (Trausch et al. 2011). This strategy of using an aptamer that binds blindly to chemical variants of the same metabolite works well when the riboswitch recognizes faces or chemical moieties of substituents that are not involved in chemical reactions. However, when a greater ligand specificity is required, it may be necessary to edit the sequence of the riboswitch to enable it to recognize subtle chemical differences, as demonstrated for the cobalamin riboswitch (Polaski et al. 2017).

Riboswitch functionality has been described as a simple expression response to different doses of a metabolite. However, it is still unclear just how quickly ligand binding leads to activation or repression of gene expression. One kinetic model suggests that the speed of transcription can determine riboswitch function (Wickiser et al. 2005). Another study suggests that transcriptional pausing at the translational start site is necessary, during transcription, for the aptamer to fold into its functional structure before the ligand can bind (Chauvier et al. 2017). During this pause or lag, the metabolite is waiting to access the newly transcribed aptamer. In the case of riboswitches that bind to toxic ligands, such as S-adenosylhomocysteine (Wang et al. 2008), fluoride (Baker et al. 2012), heavy metals (Furukawa et al. 2015), and azaaromatic compounds (Li et al. 2016), a long lag could be harmful to the cell. Furthermore, it has been shown that aptamer folding depends on physiological conditions (Chen et al. 2012; Reuss et al. 2014).

For instance, Reus et al. have demonstrated that, at low concentrations of Mg^{2+}, tertiary interactions are not formed, preventing a riboswitch from binding tetracycline (Reuss et al. 2014). Therefore, a given riboswitch might not function optimally *in vivo* when the concentrations of various osmolytes, salts, and divalent cations (particularly Mg^{2+}) are suboptimal. This raises the question of whether we know all the physiological parameters that can affect riboswitch functionality *in vivo*. Moreover, the dependence of riboswitch functionality on physiological factors like pH, temperature, metals, and ions might limit the transferability of riboswitches among different organisms. Indeed, the exhaustive study of a given riboswitch under a variety of physiologically relevant conditions—such as that conducted by (Robinson et al. 2014)—is paramount in order to transfer its applications from the laboratory to industry.

Most engineered riboswitches are based on the systematic evolution of ligands by exponential enrichment (SELEX). This is an *in vitro* technique in which aptamers are selected from a library of possible sequences; those sequences that bind the desired ligand with the highest affinity are enriched over many cycles of amplification (Tuerk and Gold 1990). The pool of sequences has a constant, known sequence at their 5' and 3' ends to which primers can anneal. The pool is exposed to the ligand and the unbound sequences are eliminated by affinity chromatography, after which the remaining sequences are PCR amplified and subjected to another round of selection with more stringent wash conditions. This process is reiterated until a sequence is discovered with a sufficiently high binding affinity for the ligand. Although SELEX works well to design a synthetic aptamer with a high binding affinity for the desired ligand, it does not ensure that the aptamer will not bind, nor the riboswitch will respond to, other metabolites. A more recent technique called RNA-aptamers-in-droplets (RAPID) screens possible metabolites *in vivo* that can be sensed by a given aptamer (Abatemarco et al. 2017). New bioinformatics and *in vivo* techniques, such as RAPID, are required to screen for cellular metabolites that can interfere with the functionality of a riboswitch *in vivo* and will advance the biotechnological applications of riboswitches as well as increase their transferability among bacterial hosts (Abatemarco et al. 2017).

A popular question about riboswitches is whether they exist in eukaryotes. As discussed above, only one example (the TPP riboswitch of plants and fungi) has been discovered so far (Li and Breaker 2013), but it has been suggested that other riboswitches may have evolved in eukaryotes (Sudarsan et al. 2003). New bioinformatics tools might enable the discovery of other

eukaryotic riboswitches in the years to come. A bioinformatics approach has been used to find new secondary and tertiary models of RNA structure (Klein et al. 2001); similar approaches could be used to expand the structural repertoire of riboswitches. A deeper understanding of the structures and regulatory mechanisms of riboswitches will facilitate their regulatory applications in synthetic biology, leading to new developments in medicine and biotechnology.

3. RNA thermometers (RNATs)

3.1. RNAT structure-function

Another major form of RNA-based regulation is called 'thermosensing', exemplified in bacteria by RNA thermometers (RNATs), also called thermosensors or thermoswitches. Similar to the translational riboswitches discussed above, RNATs are regions within an mRNA that undergo a change in secondary structure, triggering a rapid activation or repression of translation initiation. Rather than responding to a ligand, however, RNATs respond to temperature (Kortmann and Narberhaus 2012; Krajewski and Narberhaus 2014). Bacteria can exploit RNATs to respond to stresses triggered by both heat and cold, enabling the efficient translation of proteins required to adapt to either heat- or cold-stress. As for the translational riboswitches described above, the RNAT response centers on the exposure of the Shine-Dalgarno ribosome-binding sequence of an mRNA. Structural changes leading to sequestration of the RBS repress translation, while exposure of the RBS allows the 30S ribosomal subunit to bind the mRNA and initiate translation (Figure 3). Binding of the ribosome to the mRNA can further alter its secondary structure, enhancing the mRNA-ribosome interaction (Meyer et al. 2017). Simple RNATs comprise relatively small structures (e.g. hairpins) that melt at elevated temperatures, while more complex RNATs shift between alternative structures comprising relatively large regions (Kortmann and Narberhaus 2012); the latter is important for those RNATs that activate translation in response to decreased temperature, such as those controlling many bacterial cold-shock proteins. In either case, RNATs are remarkably sensitive, able to detect changes on the order of 1-2°C and rapidly activate or repress translation accordingly (Rinnenthal et al. 2011).

The importance of RNATs for naturally occurring genetic circuits is illustrated by two examples. The 'FourU' thermosensors of the Gram-negative pathogen *Salmonella typhimurium* (Rinnenthal et al. 2011)—so-named for four consecutive uridines that base-pair with a portion of

the SD sequence—enable the bacterium to adapt upon entering a mammalian host. When the cell's temperature rises from an ambient temperature to one approaching 37°C, this RNAT melts to allow the rapid initiation of translation of pathogenic proteins. Similarly, in the Gram-positive pathogen *Listeria monocytogenes*, the translation of *prfA* mRNA is controlled by an RNAT. PrfA, a master transcriptional activator of virulence proteins, is translationally repressed by the RNAT hairpin structure at a lower temperature (less than approximately 30°C), but this hairpin melts—and PrfA translation commences—at 37°C (Johansson et al. 2002). Another type of RNAT commonly regulates the bacterial heat-shock response. Known as ROSE (repression of heat-shock gene expression) elements, these RNATs regulate the translation of mRNAs encoding heat-shock proteins (HSPs) (Kortmann and Narberhaus 2012). The stem-loop structure of a given ROSE element melts at temperatures above its particular 'on/off' temperature, exposing the RBS and allowing a rapid activation of HSP translation from pre-existing mRNA. Conversely, the translational competence of HSP mRNAs is lost due to the re-formation of the hairpin structure when the temperature of the cell decreases towards optimal levels, allowing the production of HSPs to be rapidly halted. As continued production of HSPs in the absence of heat stress can be detrimental to the cell, both the activating and repressing functions of the RNAT are important (Kortmann et al. 2011).

3.2. Potential RNAT applications

RNATs have been genetically engineered in an attempt to provide insight into RNAT evolution. A particular focus has been the 'on/off' temperature (i.e. the exact temperature, or range of temperatures, at which the RNAT structure melts or reforms in order to activate or repress translation) and the degree to which this can be altered by changing the nucleotide sequence of the RNAT. For instance, a single nucleotide substitution in a FourU RNAT can alter the on/off temperature by 5 to 11°C, while variations in the concentration of Mg^{2+} from 1 to 2 mmol l^{-1} can shift the melting point by about 3°C (Rinnenthal et al. 2011). Such manipulations of RNAT on/off temperatures suggest that their evolutionary adaptation is rapid, as a single base substitution would enable the expression of a given protein to be regulated in a temperature-sensitive fashion over a relevant temperature range. RNATs have also been manipulated in order to modify the range over which they respond to temperature, which can vary from a gradual response over a wide range

(akin to a rheostat) to a sharp on/off response over a very narrow temperature range (Neupert et al. 2008).

Most natural RNATs respond over a wider range of temperatures, but it might be useful to engineer RNATs with sharper on/off responses for biotechnological applications. For instance, it would be extremely useful to rapidly and reversibly activate the expression of a gene of interest in an organism of choice simply by increasing the temperature by a few degrees. An obvious advantage of this approach is that it avoids the addition of potentially costly ligands. Additionally, avoiding the use of ligands to control a genetic circuit circumvents many of the limitations described above for riboswitches, such as the potential complication of cellular metabolites mimicking the ligand and interfering with the desired regulation. Indeed, RNATs are being used as the basis for designing a variety of temperature-sensing tools for the regulation of protein expression (Neupert et al. 2008; Kortmann et al. 2011; Hoynes-O'Connor et al. 2015; Sen et al. 2017; Rodrigues and Rodrigues 2018). However, as for riboswitches, these designs have thus far been limited to reporter proteins like β-galactosidase (Waldminghaus et al. 2008) and green fluorescent protein (Hoynes-O'Connor et al. 2015). Moreover, RNATs are not necessarily limited to translational regulation, as at least one group has successfully exploited RNAT structures to create regulatory elements that control transcription termination in a temperature-dependent manner (Rossmanith et al. 2018). The modular nature of riboswitches and thermosensors have even been exploited to create hybrid regulatory devices, capable of responding to both temperature and ligand concentration and modulating both transcription and translation of a gene of interest (Rossmanith and Narberhaus 2016). Such hybrid 'thermoswitches' would theoretically provide much more stringent regulation of a gene of interest in a biotechnological context because both temperature and ligand concentration would need to be modulated in order for expression or repression to occur.

3.3. Limitations of synthetic RNATs and potential mitigations

Like riboswitches, engineering RNAT-regulated genes for synthetic biology might be subject to certain limitations. For instance, the RNAT controlling PrfA translation in *L. monocytogenes* has been fused to various reporter genes, but optimal expression upon switching to the activating temperature (37°C) requires roughly the first 20 codons of the native *prfA* mRNA (Loh et al. 2012). Thus, as for riboswitches, genetic context will likely have to be considered in the design of any

useful RNAT-fusion mRNAs. As for riboswitches, the riboattenuator approach (Folliard et al. 2017) might prove useful for ensuring the production of a protein of interest that lacks any unwanted N-terminal fusions. Moreover, as the structure and melting temperature of RNATs can be affected by osmolytes, salts, and divalent cations (Rinnenthal et al. 2011; Gao et al. 2017), the optimal growth conditions for a given synthetic RNAT-gene fusion will require careful consideration. As optimal SD sequences can vary from one organism to the next (Ma et al. 2002), RNATs that function optimally in one species might have to be re-engineered before use in the desired organism. This is important, as many of the current synthetic applications of RNATs are based on the *E. coli* heat-shock-response or are initially designed and tested in *E. coli* (Neupert et al. 2008; Hoynes-O'Connor et al. 2015; Rodrigues and Rodrigues 2018) or *S. enterica* (Rinnenthal et al. 2011). Recently, a set of RNATs has been designed for use in the PURExpress cell-free protein synthesis system (Sadler et al. 2018), potentially providing a new set of tools to exploit temperature as a rapid trigger for *in vitro* protein expression.

4. Common model organisms in synthetic biology

4.1. *Escherichia coli*

E. coli is one of the most widely studied and well-characterized model organisms and many tools for synthetic biology, including those based on riboswitches or RNATs, are often created and implemented in *E. coli* (Neupert et al. 2008; Topp et al. 2010; Berens and Suess 2015; Hoynes-O'Connor et al. 2015; Rodrigues and Rodrigues 2018). This section will discuss a few of the more interesting applications of synthetic riboswitches that have been tested in *E. coli*, as well as their implications.

Synthetic riboswitches have proven useful in practical screening methods for the detection of molecules. For example, utilizing computer programing, riboswitches capable of responding to flavonoids (specifically, naringenin) have been designed, developed, and tested in *E. coli*. The cells were able to respond to the flavonoid by expressing GFP in the presence of naringenin, allowing for a mechanism to screen for the presence of this flavonoid (Xiu et al. 2017). Research into the relationship between flavonoids and cancer is conflicting, as certain flavonoids (e.g. quercetin) have been shown to be potentially carcinogenic (Rietjens et al. 2005) while others (e.g. luteolin) have been shown to be anti-carcinogenic (Seelinger et al. 2008). Various flavonoids (both

cancer-causing and cancer-preventing) are found in food stuff (Justesen et al. 1997; Nair et al. 1998), which suggests a need for reliable screening. Considering that computer modeling has already proved successful in creating riboswitches that respond to specific flavonoids (Xiu et al. 2017), it is feasible to design riboswitches that could respond to either carcinogenic or anti-carcinogenic flavonoids, providing a reliable mechanism to screen for the types of flavonoids found in various food stuff.

Creative mechanisms using riboswitches have been developed that can alter the behavior of an organism without a need to genetically engineer the organism itself. To accomplish this, artificial cells were developed that produce a phospholipid vesicle containing isopropyl β-D-1-thiogalactopyranoside (IPTG) (Lentini et al. 2014). The artificial cells also contain a theophylline riboswitch that, in the presence of theophylline, induces translation of α-hemolysin (α-HL), which is a pore-forming protein (Lentini et al. 2014). In the presence of theophylline, α-HL is produced, which then permeates the IPTG-containing vesicle, causing the IPTG to be released. Co-culturing *E. coli* with the artificial cells allows the *E. coli* to respond to IPTG (*via* a plasmid containing a *lac* promoter) and, therefore, indirectly respond to the theophylline (Lentini et al. 2014). In this example, the *E. coli* harbored a plasmid, and so might still be considered genetically modified. Nonetheless, this approach could allow the manipulation of cellular behavior while avoiding the direct genetic manipulation of the organism itself. Such artificial cells have interesting applications in medicine. For example, it was suggested that, rather than using engineered bacteria to treat for the presence of *Pseudomonas aeruginosa* in patients suffering from cystic fibrosis, artificial cells could be designed to respond to chemicals in the biofilm of *P. aeruginosa* which would trigger the artificial cells to release agents designed to clear the biofilm (Lentini et al. 2014).

Although *E. coli* is an ideal organism to work within a basic research laboratory, it may not always be the most appropriate organism for a given biotechnological application. For instance, its sensitivity to high levels of certain fatty acids (Marounek et al. 2003; Royce et al. 2013) may be a detriment to biofuel production, its pathogenic potential (Kaper et al. 2004) may preclude medical applications, and comparing it to photosynthetic organisms such as Cyanophyta (Wang et al. 2012) makes it less attractive with regards to renewable energy production. Below, we discuss two model organisms with great potential for these and other biotechnological applications.

491

492 **4.2. Cyanophyta**

493 Cyanophyta, or cyanobacteria, is an extremely useful phylum of organisms for practical
494 biotechnological applications due to its ability to photosynthesize (Wang et al. 2012). Most
495 organisms, if producing a value-added compound, require a carbon source to convert into the
496 desired resource. Cyanophyta has the advantage of fixing carbon from the air, allowing for the
497 highly economical production of a resource while removing a greenhouse gas (CO_2) from the
498 atmosphere (Wang et al. 2012). Surprisingly little research has been conducted with the goal of
499 utilizing synthetic RNA elements in Cyanophyta. Research has shown that riboswitches can be
500 used in Cyanophyta to control the expression of GFP and other reporter genes (Ma et al. 2014;
501 Ohbayashi et al. 2016; Higo et al. 2017), as well as a toxic protein, SacB (Ma et al. 2014). However,
502 to our knowledge, nothing has been implemented beyond proof-of-concept experiments.

503 An intense area of research is the potential use of Cyanophyta for the production of
504 renewable clean energy (Shuba and Kifle 2018). For instance, the generation of biodiesel requires
505 the production of free fatty acids (FFA) which are then chemically converted into a useable
506 biodiesel (Machado and Atsumi 2012). One approach to increase FFA production (and therefore
507 biofuel yields) in Cyanophyta is to introduce and express a modified *tesA* gene taken from *E. coli*
508 (Liu et al. 2011). The TesA enzyme is responsible for hydrolyzing acyl-CoA (and to a lesser extent,
509 acyl-APC) to generate FFAs (Cho and Cronan 1993). Translational control could help regulate
510 and enhance this process. For example, an inducible promoter could be used to generate a high
511 concentration of mRNA transcripts encoding *tesA* under the control of an inducible riboswitch or
512 an RNAT. Once the 'stockpile' of mRNA transcripts is made, and cells have grown to achieve a
513 sufficient biomass, the riboswitch can be induced by addition of its cognate ligand (or the RNAT
514 by a shift in temperature), causing a massive spike in TesA protein production.

515 Cyanophyta can also be used in the synthetic chemical industry, as in the production of
516 ethylene (Wang et al. 2012). However, the production of ethylene from Cyanophyta has
517 encountered challenges, as increasing the production rate of ethylene has resulted in unstable
518 batches of the engineered Cyanophyta (*Synechococcus*) and an overall decrease in ethylene
519 production (Takahama et al. 2003). It has been suggested that enhancement of ethylene
520 biosynthesis is necessary for the reliable and sustainable production of ethylene (Wang et al. 2012),

which is something that synthetic riboswitches or RNATs may be able to provide by the strategy described above for TesA production.

4.3. *Ralstonia sp.*

Ralstonia sp. (which has undergone numerous name changes) is an industrially relevant genus of bacteria due to several attributes, such as heavy metal resistance (Nies 1999; Tibazarwa et al. 2000; Goris et al. 2001), bioplastic production (Khanna and Srivastava 2005; Menezes et al. 2014), resistance to extreme conditions (Mijnendonckx et al. 2012), and its ability to break down urea (Ammann and Reed 1967). Despite its slew of useful applications, *Ralstonia sp.* has hosted (to our knowledge) no research on implementing synthetic riboswitches or RNATs. To our knowledge the only research on riboswitches in *Ralstonia sp.* is the characterization of a naturally occurring riboswitches (Rodionov et al. 2003; Edwards et al. 2010).

One of the biggest reasons for *Ralstonia's* industrial relevance is its ability to produce poly-β-hydroxybutyric acid (PHB) in large quantities. PHB is a polyhydroxyalkanoate (PHA), which is a biodegradable polymer used in the production of bioplastics (Khanna and Srivastava 2005; Chen 2009; Budde et al. 2011). Currently, the biggest issue with large-scale PHB production is the high cost of production when compared to alternative petrochemical plastics (Khanna and Srivastava 2005). One way to reduce the cost of PHB production would be to have tight control over the expression of proteins involved in the PHB biosynthetic pathway. For example, overproduction of proteins important in PHB production—such as PHB granule-associated proteins (PGAPS) or PHA synthase (Chen 2009; Pfeiffer and Jendrossek 2012)—could increase the PHB synthesis rate and increase cost efficiency.

Implementing riboswitches and RNATs in *Ralstonia sp.* could also prove to be invaluable in bolstering its bioremediative properties. Due to its heavy metal resistance (Nies 1999; Tibazarwa et al. 2000; Goris et al. 2001), *Ralstonia sp.* has the potential to be heavily involved with bioremediation. Riboswitches exist that bind to nickel and cobalt (Furukawa et al. 2015), both of which *Ralstonia eutropha* can tolerate well (Tibazarwa et al. 2000), and *R. eutropha* has also been shown to be capable of biosorption of nickel and other heavy metals (Fereidouni et al. 2009). Implementing this type of riboswitch would allow *R. eutropha* to directly respond to the presence of nickel, cobalt, or any other heavy metals to which new riboswitches could be designed to

551 respond, allowing for direct control over possible enhancements of *R. eutropha's* bioremediative
552 mechanisms.

553 An interesting area of research regarding *Ralstonia sp.* focuses on its potential uses in the
554 space industry, as it has been suggested that *Ralstonia sp.* could benefit long-term space missions.
555 For instance, *Ralstonia sp*. can use urea as a nitrogen source for growth (Ammann and Reed 1967),
556 and its ability to produce bioplastics could provide space missions a convenient source of
557 renewable plastics on the go (Menezes et al. 2014)—especially if their production could be
558 controllably enhanced *via* such tools as riboswitches. Another potential advantage is that certain
559 *Ralstonia* species are resistant to extreme conditions—for instance, the bacterium is found on the
560 international space station (ISS) and was found to be resistant to concentrations of silver (0.5-4
561 μmol l^{-1}) that are substantially higher than what is found in the drinking water aboard the ISS, as
562 well as many antibiotics (Mijnendonckx et al. 2012). These properties allow it to grow in space
563 more easily than other bacteria, with the disadvantage that *Ralstonia* species have contaminated
564 multiple areas on the ISS, including the water systems, air, and various surfaces (Mijnendonckx et
565 al. 2012). Although *Ralstonia sp.* is not, to our knowledge, pathogenic to humans, certain strains
566 are pathogenic to plant hosts (Denny 2006), and for long-term space travel, it is suggested that
567 plants would be highly beneficial for life support (Ferl et al. 2002; Wheeler 2010). Another issue
568 with the presence of *Ralstonia sp.* on the ISS is that they often contain mobile DNA (such as
569 plasmids) which could help bacterial stains to adapt to the environment onboard the ISS (Leys et
570 al. 2009). Therefore, there is a risk of horizontal gene transfer (Thomas and Nielsen 2005) of these
571 mobile genetic elements into other bacteria (potentially pathogenic to humans) which could help
572 them survive in space. Furthermore, when organisms are used for long-term space travel they are
573 exposed to a multitude of stresses such as pH changes, hypoxia, temperature changes, and
574 radiation. These stresses, in tandem with the length of long-term space travel, may induce genetic
575 instability and mutations. It is therefore important to detect and counter any genetic changes that
576 may accumulate in an organism during long-term space travel (C. Lasseur 2010). *Ralsontia*
577 *metallidurans* has been shown to adapt to many of the extreme conditions that space travel presents
578 by increasing the expression of proteins involved in responding to oxidative stress, carbon
579 limitation, DNA damage, and more (Leys et al. 2009). For any mutations that do arise, a 'kill
580 signal' could be engineered into the bacteria under the control of a riboswitch, allowing an easy,
581 inducible mechanism to eliminate any problematic bacteria. Riboswitches could also provide

582 control over *Ralsontia's* capability to break down urea, thus providing enhanced waste
583 management.
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585
586 **5. Future Perspectives**
587 Practical applications of synthetic biology are often limited to relatively simple tasks that may
588 involve only model organisms (Topp et al. 2010; Etzel and Morl 2017; Hallberg et al. 2017). Once
589 a constructed circuit is placed into cells, the synthetic tool can have unintended effects on the host,
590 fail to work as expected (Cookson et al. 2009), and may not be versatile enough to be employed
591 in a variety of bacterial species (Robinson et al. 2014). *E. coli* has played an important role in the
592 development of synthetic biology tools for the insertion and control of rationally designed circuits.
593 However, the future of synthetic biology requires robust organisms that can allow the increasingly
594 sophisticated synthetic physiological circuits to proceed from the laboratory into fieldable
595 technologies (Adams 2016). For instance, it has been shown that the employment of *E. coli* to
596 produce biofuel is limited by the toxicity of the generated fuel, which reduces bacterial growth
597 (Foo et al. 2014; Haft et al. 2014). *R. eutropha* is a likely candidate for biofuel production due to
598 its tolerance to toxicity and ability to store carbon and energy for the production of value-added
599 chemicals (Lu et al. 2016). Unfortunately, it is not simple to employ a toolkit developed in one
600 bacterial chassis (such as *E. coli*) in another organism. The regulation of gene expression and of
601 synthetic circuits are host-specific because of, for example, the different DNA-binding specificities
602 of homologous RNA polymerases (Cheetham and Steitz 2000; Werner 2012) and transcription
603 factors across different organisms (Perez and Groisman 2009). Therefore, DNA-based synthetic
604 biology tools cannot be widely transferred to other bacteria.
605 RNA-based tools for the regulation of gene expression can theoretically overcome such
606 limitations, as they can be transcribed from host-encoded transcriptional machinery and do not
607 require protein co-factors or chaperones (Tucker and Breaker 2005; Scott et al. 2010; Ma et al.
608 2014). Instead, RNA elements rely primarily on the structural features implicit to their nucleotide
609 sequence in order to respond to specific ligands or changes in temperature, and this modularity
610 gives riboswitches and RNATs considerable potential for biotechnology applications (Ceres et al.
611 2013). A thorough investigation of the structure and function of RNA elements in a variety of
612 model organisms, under a wide variety of conditions (e.g. pH, temperature, salt, and metal

concentrations), will bolster their applicability in synthetic biology in general and in the biotechnology industry in particular.

Acknowledgments

This work was funded by the Campus Alberta Innovates Program and the Alberta Ministry of Economic Development and Trade. J.C.N. was supported by an Alberta Innovates Technology Futures Graduate Scholarship.

Conflict of Interest

No conflict of interest declared.

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Table 1: Current and potential applications of RNA elements presented in this review.

Current Applications	RNA Element	Description
Metabolism & Behavioral Regulation	Lysine Riboswitch	Used to improve lysine biosynthesis by favouring its biosynthetic pathway over that of other competing biosynthetic pathways (Zhou and Zeng 2015).
	Theophylline Riboswitch	Directing mobility of bacteria to specific locations using theophylline in a ligand-dependent manner (Topp and Gallivan 2007).
		Regulation of pre-mRNA splicing (Kim et al. 2005).
Screening for traits	Lysine Riboswitch	Lysine riboswitch controlling antibiotic resistance to screen for *E. coli* strains with higher lysine fermentation efficiency (Yang et al. 2013; Wang et al. 2015).
	Theophylline Riboswitch	Screening for mutations that increased caffeine demethylase activity (Michener and Smolke 2012).
	FMN Riboswitch	Isolate bacterial variants that produce increased amounts of vitamin B2 (Meyer et al. 2015).
	c-di-GMP Riboswitch	Biosensor for screening of multiple redundant c-di-GMP metabolic enzymes (Gao et al. 2014).
Regulation of genes	Theophylline Riboswitch	Increasing bacteria susceptibility to the antibiotics (Feng et al. 2011).
		Granting inducible expression systems to bacterial species that otherwise lacked such systems (Villa et al. 2018).
	Aminoglycoside Riboswitch	Increasing bacteria resistance to antibiotics (He et al. 2013; Jia et al. 2013).

Potential Applications	RNA Element	Details
Production of Compounds	Theophylline-responsive Riboswitch	Inducible production of biofuels and other relevant compounds (Ma et al. 2014).
	RNAT (temperature response)	Rapid response to temperature for the production of compounds without the use of costly ligands (Neupert et al. 2008; Kortmann et al. 2011; Hoynes-O'Connor et al. 2015; Sen et al. 2017; Rodrigues and Rodrigues 2018).
Biosensor	Flavonoid Riboswitch	Detection of flavonoid contamination (Xiu et al. 2017).
Medical	Theophylline Riboswitch	Inducible delivery of medicine (Lentini et al. 2014).
	Unspecified Riboswitch	Gene therapy regulation (Ketzer et al. 2012; Ho et al. 2016).
Bioremediation	ykkC riboswitch (Guanidine Response)	Responds to the environmental toxin guanidine to break it down (Nelson et al. 2017).
Space Travel	Unspecified Riboswitch	Could be used in a bioreactor or in the production of compounds during space flight (suggested in this review).

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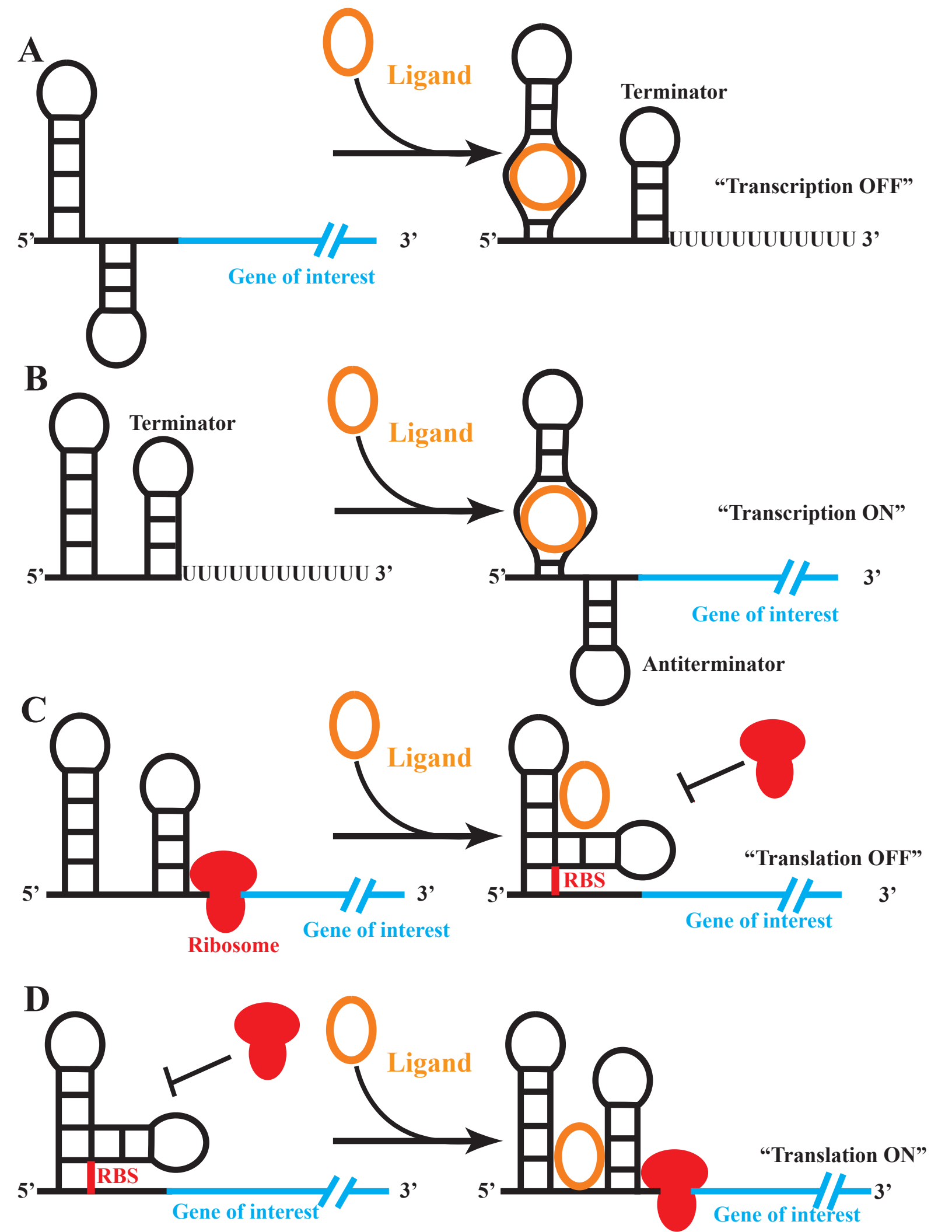
Figure 1. Schematic representation of mechanisms employed by riboswitches to regulate gene expression. Riboswitches can broadly be classified as those that regulate transcription termination **(A, B)** or translation initiation **(C, D)** in order to repress **(A, C)** or activate **(B, D)** gene expression. Riboswitches repress transcription of a gene by forming a Rho-independent 'terminator' hairpin structure that leads to premature transcriptional termination **(A)**. Conversely, riboswitches facilitate transcription of a gene by forming alternative 'anti-terminator' secondary structures that prevent the formation of the terminator structure, allowing transcription of the entire message **(B)**. Alternatively, riboswitches repress the initiation of mRNA translation by sequestering the Shine-Dalgarno (SD) ribosome binding sequence (RBS) in a secondary structure, occluding the small (30S) ribosomal subunit **(C)**. Conversely, exposure of the RBS allows the 30S ribosomal subunit to bind **(D)**. In all cases, the secondary and tertiary structures of the riboswitch (represented as black stem-loops) are controlled by binding of a specific ligand (orange oval). The SD is highlighted in red, while the gene of interest is blue. The large and small ribosomal subunits are represented as red ovals.

Figure 2. Schematic representation of the riboattenuator mechanism to modulate riboswitch fidelity. The 'translation on' riboswitch structure is located in its native genetic context, i.e. upstream of the first several dozen nucleotides of its native ORF (black). The riboattenuator (highlighted by the dashed green rectangle) is a stem-loop structure that includes the stop codon of the riboswitch's ORF (TAA; purple) immediately followed by a Shine-Dalgarno RBS (red) and the gene of interest (blue). The stop codon and RBS are normally sequestered by the riboattenuator, repressing translation of the gene of interest (I). When the ligand (orange oval) binds the aptamer domain of the riboswitch, the first RBS is exposed and the ribosome can translate the first ORF (II). The helicase activity of the ribosome then unwinds the attenuator hairpin (III), exposing the second RBS and allowing the immediate re-initiation of translation of the gene of interest (IV). Note that a riboattenuator can also be coupled to a 'translation off' type riboswitch (not shown).

Figure 3. Schematic representation of a simple RNA thermometer. At lower temperatures, a stem-loop structure (black) sequesters the Shine-Dalgarno RBS (red). At higher temperatures (e.g. 37ºC), the stem-loop structure 'melts', unveiling the RBS and allowing the small (30S) ribosomal subunit to bind and initiate translation of the gene of interest (blue).


A
Ligand
Terminator
"Transcription OFF"
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Gene of interest
5'
UUUUUUUUUUUU 3'
B
Terminator
Ligand
"Transcription ON"
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UUUUUUUUUUUU 3'
5'
3'
Gene of interest
Antiterminator
C
Ligand
"Translation OFF"
5'
3'
RBS
5'
3'
Ribosome
Gene of interest
Gene of interest
D
Ligand
"Translation ON"
RBS
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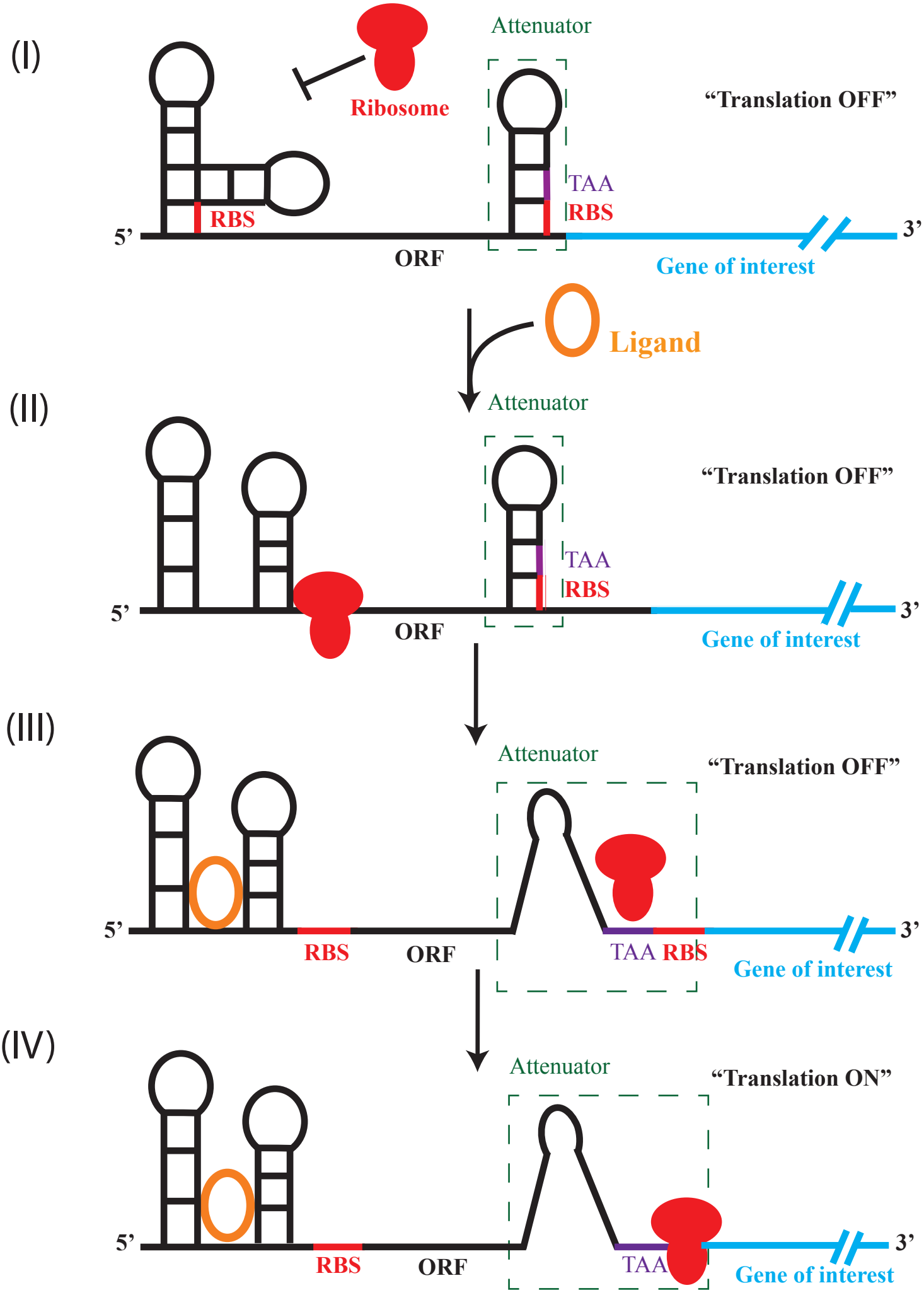
Gene of interest
Gene of interest


(I)
Attenuator
Ribosome
"Translation OFF"
TAA
RBS
RBS
5'
3'
ORF
Gene of interest
Ligand
(II)
Attenuator
"Translation OFF"
TAA
RBS
5'
3'
ORF
Gene of interest
(III)
Attenuator
"Translation OFF"
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TAA RBS
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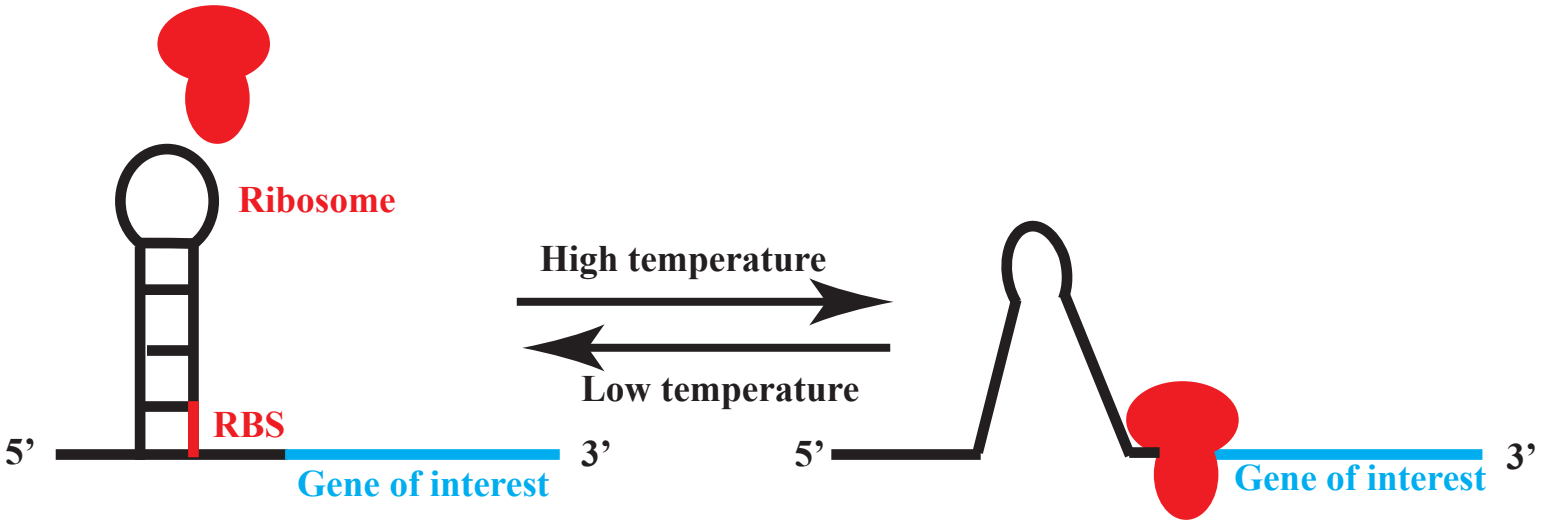
Gene of interest
(IV)
Attenuator
"Translation ON"
5'
RBS
ORF
TAA
3'
Gene of interest


Ribosome
RBS
5’
3’
Gene of interest
High temperature
Low temperature
5’
3’
Gene of interest