



# Recent advances and future trends of riboswitches: attractive regulatory tools

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## Abstract

Bacterial genomes contain a huge amount of different genes. These genes are spatiotemporally expressed to accomplish some required functions within the organism. Inside the cell, any step of gene expression may be modulated at four possible places such as transcription initiation, translation regulation, mRNA stability and protein stability. To achieve this, there is a necessity of strong regulators either natural or synthetic which can fine-tune gene expression regarding the required function. In recent years, riboswitches as metabolite responsive control elements residing in the untranslated regions of certain messenger RNAs, have been known to control gene expression at transcription or translation level. Importantly, these control elements do not prescribe the involvement of protein factors for metabolite binding. However, they own their particular properties to sense intramolecular metabolites (ligands). Herein, we highlighted current important bacterial riboswitches, their applications to support genetic control, ligand-binding domain mechanisms and current progress in synthetic riboswitches.

**Keywords** Aptamer domain · Expression platform · Gene expression · mRNA 5'-UTR · Riboswitches

## Introduction

More developments in gene regulation reveal that natural and synthetic RNA-based elements have been demonstrated as key components applied in the diverse genetic control (Henkin 2008). Among them, riboswitches have received attention as attractive sensor–actuator hybrids that can control gene expression in response to intracellular metabolites concentration. Such regulatory RNA sequences are typically found in the 5'-untranslated regions of messenger RNAs, where they control gene expression by binding to small specific metabolites normally called 'effectors' (Berens and Suess 2015; Wang et al. 2015). Comparatively to other regulatory RNA elements, riboswitches directly interact with their effectors without requisition of intermediate factors

which suggest them to be one of the oldest regulatory systems (Alexey et al. 2003).

Riboswitches regulate several metabolic pathways including the biosynthesis of vitamins (e.g. riboflavin, thiamin and cobalamin) and the metabolism of methionine, lysine and purines (Barrick and Breaker 2007). Results obtained using a riboswitch sensor to examine coenzyme B12 metabolism and transport in *Escherichia coli* indicated that riboswitch based sensors can provide valuable information on intracellular small molecule concentrations that can be employed in the study of related cellular processes (Fowler et al. 2010). From these results, potential research on ligand concentrations has been carried out and it was reported that high ligand concentrations are needed to effectively regulate gene expression (Garst et al. 2011). However, additional research on this query revealed that in the most cases an exaggerated ligand concentration can cause the closures in one way or another of the expression (Khani et al. 2018). Even though most known riboswitches occur in bacteria, functional TPP riboswitch have been discovered in plants, some fungi and have also been predicted in archaea (Sudarsan 2003). Additionally, monitoring conditional gene expression by a tetracycline-riboswitch showed an influence on translation initiation in archaea (Demolli et al. 2014). Nowadays, metabolite-binding mRNAs are possibly involved in

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eukaryotic gene regulation and some riboswitches might be representatives of an ancient form of genetic control. This explains why recent researchers are in battle to intensify the transfer of riboswitch regulatory systems to mammalian cell culture and preferably to transgenic animals and plants (Berens and Sues 2015; Sudarsan 2003). Again, an intriguing research on cis-acting regulatory elements of genes has demonstrated that riboswitches are not only exclusively found in non-pathogenic bacteria, but also found in pathogenic species such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Vibrio cholera*, *E. coli* and *Pseudomonas aeruginosa* (Miotto et al. 2012; Ramesh 2015; Wang et al. 2017).

Mandal et al. (2003) reported that most of riboswitches found in *Bacillus subtilis* sense and respond to small target molecules which play a crucial role in central metabolic pathways of some organisms. These findings emphasized the influence of riboswitches in diverse living systems especially in *B. subtilis*. Whether artificial or natural, the function of riboswitches to switch on or off the gene expression remains a big challenge. The persistent challenge yet observed in gene regulation has deeply encouraged biologist engineers to worry about the existence of cis-acting regulatory elements affecting regulation in the genome and how they can be synthetically engineered to respond to the endogenous ligands. This further suggests that riboswitches can be used for metabolic control, especially in novel and synthetic cell factory for biofuels and chemicals production. Several researchers in this field explored about riboswitches mechanisms and their effect on downstream coding sequence in host cells. However, up to now, a query of understanding sophisticated phenomena of sensing and responding on intra or extracellular molecules known as ligands still contributes a limiting factor on gene expression.

In this manuscript, we focused on mentioning important riboswitches currently found in bacteria, their related mechanisms as well as their diversified applications. We also briefly describe other riboswitches found in other microorganisms and end up with some model perspectives in the future.

### General mechanisms of discovered riboswitches

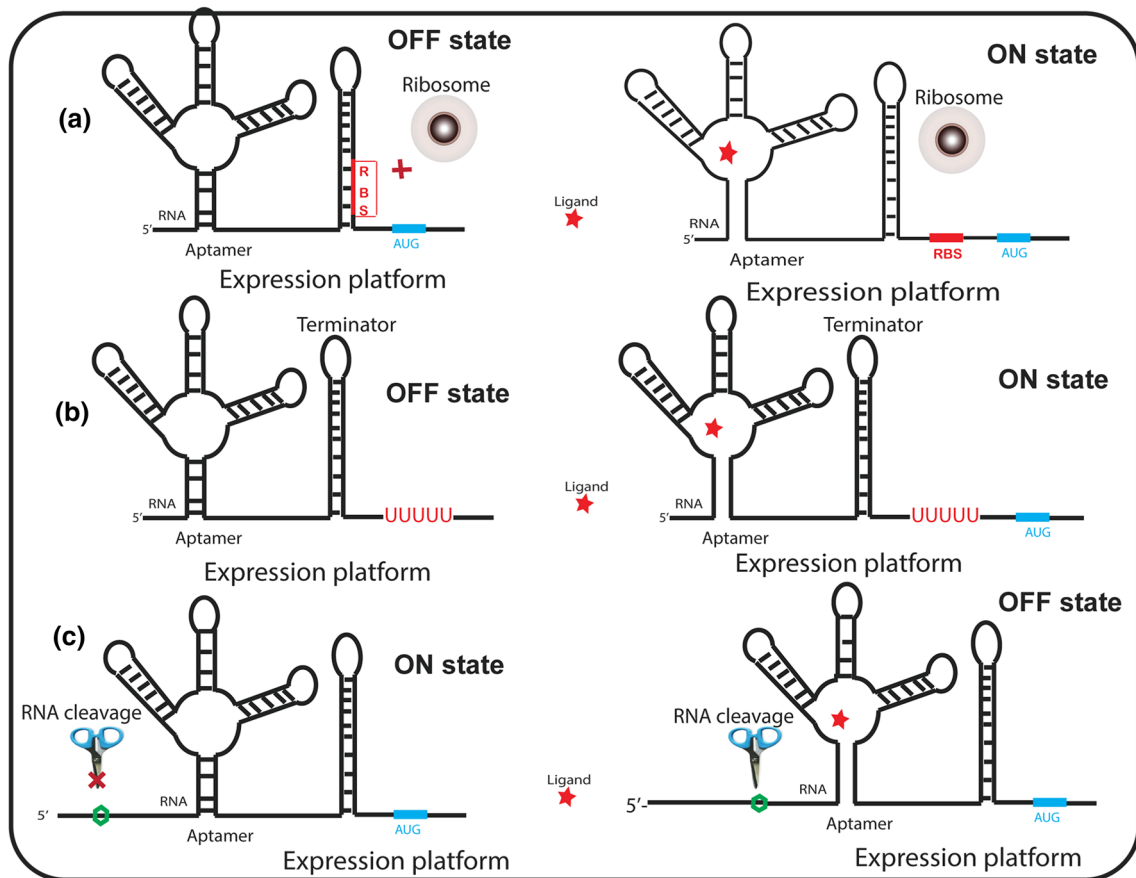
Among proposed riboswitches mechanisms, transcription termination, translation initiation, and RNA cleavage are the common mechanisms frequently found in bacterial species (Fig. 1).

Riboswitches are often conceptually divided into two parts: an aptamer domain and an expression platform. The receptor (aptamer) domain directly binds the small target molecule (effector), and the expression platform or regulatory domain undergoes structural changes in response to the changes in the aptamer and communicates this response to

other gene expression components of the cell (Garst et al. 2011). Like engineered aptamers, each natural aptamer serves as a molecular sensor embedded within the riboswitch, where it selectively senses and recognizes its corresponding target molecule within the complex sea of other metabolites (Bastet et al. 2011; Mandal and Breaker 2004). The expression platform is what regulates gene expression by typically shut down gene expression in response to the small molecule, but some turn it on (Garst et al. 2011) (Fig. 2).

In this context, the conformational changes in the riboswitch aptamer domain are used to couple ligand binding to the folding of a downstream structural switch in the expression platform that in turn instructs the transcriptional initiation and elongation of mRNA, translational initiation, and presumably even the stability and splicing of mRNA transcript machinery (Berens et al. 2015; Mandal and Breaker 2004; Serganov and Nudler 2013). Transcription interference or possibly antisense action, dual transcription and translation control, and ligand-dependent self-cleaving ribozyme action were reported but they may be prevalent nonetheless (Breaker 2012). Previous study has demonstrated that binding alone does not determine the effectiveness of a compound to regulate the activity of a riboswitch. Therefore, different aspects of an interaction of the effector with the RNA must be preserved for a chemical analog to be able to also regulate the riboswitch (Trausch and Batey 2014). Reviewed in “single-molecule studies of riboswitch folding”, the author showed that magnesium seems to promote folding intermediates and/or fully folded states that are competent to bind ligand (Savinov et al. 2014). Recently discovered riboswitch classes have surprisingly complex mechanisms for regulating gene expression and new high-resolution structural models of these RNAs provide insight into the molecular details of metabolite recognition by natural RNA aptamers (Berens et al. 2015). Besides, structural studies have revealed that riboswitches, which bind a variety of small molecule metabolites, including purine bases, *S*-adenosylmethionine, amino acids and cofactors, may contain a complicated tertiary architecture that facilitates their function. Hence, these structures guide the messenger RNA to adopt one of two mutually exclusive forms, dictating the outcome of transcription or translation. Another highly structured messenger RNA element known as the viral internal ribosomal entry site, able to manipulate the ribosome and take over from the function of initiation factors by promoting gene expression has been identified (Batey 2006).

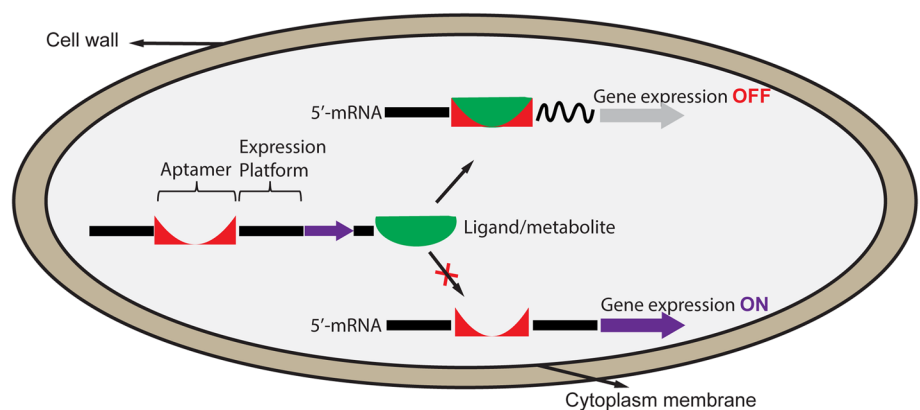
Among the discovered riboswitches, guanine was the first riboswitch to show the possibility of being engineered through point mutation which tends its molecular specificity to bind to adenine (Kim et al. 2007). Basing on metabolite-binding mechanisms of natural riboswitches which exist in different bacteria, more than six elements (*glmS*, *gcvT*,



**Fig. 1** Established or proposed mechanisms of riboswitch-mediated gene regulation. **a** Translation activation in the presence of ligand. The ribosome binding site (RBS) is sequestered in the absence of ligand. Upon ligand binding, the RNA undergoes a conformational shift, revealing the RBS and enabling translation. **b** Transcription continues in the presence of ligand. In the absence of ligand, an

intrinsic terminator stops transcription. Ligand binding induces a conformational shift that forms an anti-terminator, enabling expression of the downstream genes. **c** RNA cleavage in the presence of ligand slows down gene expression. The *glmS* riboswitch functions by a ligand-dependent splicing mechanism, whereby protein expression is diminished in the presence of the ligand

**Fig. 2** The general organization of riboswitch RNAs in bacteria mRNAs. Binding of the target ligand to the aptamer domain destabilizes a stable conformation of the expression platform that results in changing gene expression for the downstream gene(s). The gene is expressed in a normal way in the absence of ligand



*ydaO\_yuaA*, *ykkC\_yxkD*, *ykoK*, and *yybP\_ykoY*) within the *B. subtilis* genome that exhibit characteristics of riboswitch function have been identified and added on what are currently present with the well-defined functions. Further researches revealed that these motifs are not only existed

in *B. subtilis* but also exhibit an extensive sequence and secondary-structure conservation among many other bacterial species and occur upstream of related gene (Barrick et al. 2004). In previous years, research studies confirmed *glmS* (Watson and Fedor 2011) and *ydaO* motives as *glmS*

and ATP riboswitches (Watson and Fedor 2012) respectively. Moreover, coenzyme-B<sub>12</sub>, thiamin pyrophosphate, S-adenosyl methionine, flavin mononucleotide, purine, lysine, glycine, and preq1 riboswitches are considered as potential riboswitches often found in bacteria as well as in other microorganisms. These riboswitches will be described in detail below.

### Coenzyme-B<sub>12</sub> riboswitch structure and gene-control function

Cobalamin riboswitch (B<sub>12</sub>-element), is a natural RNA device which binds adenosylcobalamin to regulate cobalamin biosynthesis and transport of cobalamin and similar metabolites, and other genes (Nahvi et al. 2004).

Similar to other riboswitches, it is comprised of a conserved aptamer domain and an expression platform which broadly changes with evolution. Also, this riboswitch controls gene expression by transcription termination and translation control mechanisms. At the transcriptional level, the binding of a ligand (B<sub>12</sub>) to the aptamer domain triggers the formation of an anti-terminator stem or an intrinsic terminator stem which favors RNA polymerase to block transcription before the formation of the coding portion of mRNA. Translation control is conveyed by ligand binding to the aptamer domain which influences the formation of an anti-anti-RBS (ribosome-binding site) stem and an anti-RBS stem, resulting in an allosteric change along the expression platform and stop translation initiation (Mandal and Breaker 2004; Vitreschak 2003).

Recently, through genome mining applied in *Klebsiella pneumoniae*, a sequence was identified by bioinformatics and proposed to be a B<sub>12</sub> riboswitch regulated by a coenzyme B<sub>12</sub>. Further, in-line probing experiments were used to explore the structural rearrangement along the RNA sequence while Isothermal Titration Calorimetry (ITC) confirmed the thermodynamic parameters of the interaction between the riboswitch and its metabolite. Consequently, the interaction of coenzyme B<sub>12</sub> with the *butB* riboswitch of *K. pneumoniae* found to be the exothermic process with a 1:1 binding stoichiometry and binding affinities ranging from log KA = 6.73 ± 0.02 at 15 °C to log KA = 6.00 ± 0.09 at 30 °C (Palou-Mir et al. 2016).

Moreover, research revealed that *btuB* riboswitch from *E. coli*, once binds to its ligand, coenzyme B<sub>12</sub> undergoes a conformational change and takes part in the gene regulation of an outer membrane B<sub>12</sub>-transport protein in *E. coli*.

This RNA is characterized by its selective high-affinity binding to coenzyme B<sub>12</sub> and by the structural rearrangement it undergoes upon this interaction (Gallo and Sigel 2018). Very recently, the distribution analysis of different riboswitches families in cyanobacteria genomes was achieved. Significantly, the observation analysis highlighted

four classes of riboswitch plentiful in cyanobacteria where B<sub>12</sub> (Cob)/AdoCbl/AdoCbl-variant being the most abundant. This research also confirmed a large number of genes regulated by riboswitches which may assist in the elaborating diversity among cyanobacteria species (Singh et al. 2018). To the best of our knowledge on this type of riboswitch, further studies, which take B<sub>12</sub> riboswitch mechanisms into account, will need to be undertaken in other B<sub>12</sub> producing bacteria. Additional study was carried out in other cyanobacteria to know the effect of 5'-UTR sequences on genes involved in methionine metabolism, which thus led to the identification and validation of a cobalamin riboswitch, with potential transcriptional regulation, in the 5'-UTR of the *metE* gene from *Synechococcus* sp. strain PCC 73109. Crucially, this discovery provided a novel genetic tool for controlling gene expression responsible for de novo methionine biosynthesis in this cyanobacterium (Perez et al. 2016).

### Thiamin pyrophosphate riboswitches

TPP is the active form of thiamine (vitamin B<sub>1</sub>), an essential coenzyme synthesized by coupling of pyrimidine and thiazole moieties in bacteria (Hallera et al. 2013). With latest technological advances in gene regulatory networks, scientists demonstrated that thiamin biosynthesis and transport, as well as transport of similar metabolites are governed by TPP riboswitches which bind thiamin pyrophosphate (TPP) in its aptamer domain and alter conformation in expression platform of 5'-UTR region of corresponding gene (Winkler and Breaker 2003). However, this is particularly interesting given the fact that it is the only riboswitch found so far in eukaryotes and nowadays can be found within all three kingdoms (McRose et al. 2014; Moldovan et al. 2018; Zhang et al. 2017). To date, this riboswitch is known as a highly conserved RNA secondary structure and researchers consider its domain as a direct contributor to the mechanism of regulating genes by modulating transcription termination, translation initiation, or even (in eukaryotes) alternative RNA splicing mediated translational regulation (Henkin 2008; Savinov et al. 2014). Moreover, this regulatory RNA sequence serves as a riboswitch that binds directly to thiamine pyrophosphate (TPP) to regulate gene expression through a variety of mechanisms in archaea, bacteria and eukaryotes (McRose et al. 2014; Moldovan et al. 2018; Zhang et al. 2017). Furthermore, in a more recent advance, Singh et al. (2018) showed that TPP riboswitches are distributed across 290 cyanobacterial genomes. This research stressed with a clear evidence that TPP riboswitches reside in upstream of *thiC*, which encodes an essential thiamine biosynthesis enzyme, regulate mainly *thiC* gene in cyanobacteria. Many hypothetical genes were also mentioned as riboswitch regulated for this family. Apart from *thiC* gene often regulated by TPP riboswitch in terrestrial cyanobacteria, aquatic marine and



aquatic fresh water cyanobacteria showed the presence of TPP riboswitch in the upstream of their various genes (Singh et al. 2018). These discoveries may have a potential promising application and offer a crucial evidence for future exploitations of more regulatory RNA sequences governing various metabolic pathways in cyanobacteria.

### SAM riboswitch (S-box)

Other class of riboswitches, “S-box (SAM-I)” has been primarily found in low-G + C gram-positive bacteria where they are involved in the regulation of sulfur metabolism. These riboswitches, once bound to S-adenosyl methionine (SAM), they undergo a regulation of methionine, SAM biosynthesis and transport as well (Huang et al. 2012). Until now, three kinds of these riboswitches are known: (1) SAM-I which is widespread located in bacteria especially in *B. subtilis*, (2) SAM-II uniquely found in alpha-, beta- and a few  $\gamma$ -proteobacteria and (3) S<sub>MK</sub> box which only found in the order lactobacillales where it regulates gene expression at the level of translation initiation (Hickey and Hammond 2014; Wilson et al. 2011). The *metX* SAM-II riboswitch, firstly identified in the Sargasso Sea metagenome, is thought to regulate gene expression at the translational level by sequestering the SD sequence of its associated gene in an H-type pseudoknot, with pseudoknot formation being promoted by ligand binding (Savinov et al. 2014). To date, there are no obvious similarities among these three varieties of the riboswitch regarding to the sequence or structure. A fourth variety, SAM-IV and SAM-I riboswitches, seems to have a similar ligand-binding core, except in riboswitch scaffold. SAM-SAH riboswitches bind both SAM and SAH with similar affinities. Since they are still found in a position to regulate genes encoding methionine adenosyltransferase, it was proposed that only their binding to SAM is physiologically relevant (Mandal and Breaker 2004).

Tomsic et al. (2008) demonstrated that in case SAM binds to S-box leader RNA, there is a creation of an allosteric change which immediately induces the premature termination of transcription at the S-box leader region terminators. However, methionine, as one of the most valuable amino acids known, its metabolism regulation ought to be driven by S-box riboswitch which often regulates genes expression in s-box gene family. Similar to other techniques applied to the 5'-UTR to regulate downstream sequence, Lu et al. (2010) introduced mutations in the SAM-binding region resulting in loss of SAM binding and constitutive termination. Further, regarding the crucial role they play in medical pathogens to regulate the expression of survival and/or virulence essential genes, SAM riboswitches might be important targets for the development of new antimicrobial agents (Tausch et al. 2014). SAM-II riboswitch which is usually found in proteobacteria, is solely found in five cyanobacterial species

namely *Fischerella* PCC 9605, *Mastigocoleus testarum* BC008, *Obscuribacter phosphatis* Mle1 12, *ThermoSynchococcus* sp. NK55a and *Scytonema millei* VB511283, while SAM-I/IV-variant riboswitch was observed in only two cyanobacterial species namely *Gastranaerophilaceae* (Zag\_1) and *Gastranaerophilaceae* (Zag\_111). Recent findings regarding SAM riboswitches revealed that a gene belongs to the CBS domain family protein which is under the regulation of SAM-I/IV riboswitch is anticipated to play a major role fermentation (Singh et al. 2018). Taken together, these evidential results suggest that further screening and applications of this riboswitch for valuable compounds production in cyanobacteria could be advantage.

### Flavin mononucleotide riboswitch

Flavin mononucleotide riboswitch is a highly conserved RNA element frequently found in the 5'-UTR of prokaryotic mRNAs that encode for flavin mononucleotide (FMN) biosynthesis, transport proteins as well as transport of riboflavin, the nonphosphorylated precursor of FMN (Sklyarova and Mironov 2014). Similarly, it was well established that the phosphorylated form of roseoflavin can be converted into a roseoflavin mononucleotide which in turn competes with FMN for riboswitch binding and consequently causes FMN downregulation which subsequently results in cell growth challenges (Lee et al. 2014; Ott et al. 2014). It is well known that ligand binding properties (kinetics and affinity) of aptamer domain are the most important parameters to be considered when engineering riboswitches. In this way, a kinetic analyses of binding reaction between flavin mononucleotide and several natural mutations in aptamer domains of FMN-specific riboswitches were performed. To well execute this, a rational modification in the tuning regions was used to generate variants of synthetic riboswitches which subsequently provides further evidence that the gene expression can be controlled by rational adjustment of tuning regions (Rode et al. 2015). The experiments conducted by Pedrolli et al. (2012) demonstrated that *ribFMN* riboswitch of *S. davawensis* is the only promised model confided to discriminate between the closely similar flavins FMN and RoFMN and finally they showed an opposite response to these ligands. In *B. subtilis*, this riboswitch controls gene expression by causing premature transcription termination within the 5'-UTR of the *ribDEAHT* operon and precluding access to the ribosome-binding site of *ypaA* mRNA (Winkler and Breaker 2003). Moreover, an additional study conducted in *B. subtilis*, revealed that a *ypaA* gene which encodes riboflavin transporter presents in its 5'-UTR sequence a FMN riboswitch which binds flavin mononucleotide, then resulting in translation inhibition due to the sequestration of SD-sequence (Sklyarova and Mironov 2014). In cyanobacteria, FMN riboswitch plays a great role on genes belonging to

PIN domain superfamily, where it regulates tryptophanyl-tRNA synthetase and tRNA 2-selenouridine synthase (Singh et al. 2018).

### Purine riboswitches

Purine riboswitches are normally known as adenine and guanine riboswitches which control gene expression (Kim et al. 2009), purine metabolism and transport in the response to their purine ligand molecule (Delfosse et al. 2010). The expression platform consists of an anti-terminator hairpin that shares nucleotides with the alternatively-formed P1 hairpin of the aptamer. Guanine binding promotes folding of the aptamer domain over the anti-terminator, leading to transcription termination via an intrinsic terminator hairpin (Savinov et al. 2014). Different forms of purine riboswitches bind guanine, adenine or 2'-deoxyguanosine (2'-dG). Homologous types of purine riboswitches bind deoxyguanosine, but have more significant differences than a single nucleotide mutation (Marcano-Velazquez and Batey 2015). Research carried out in *B. subtilis*, revealed that the *pbuE* adenine riboswitch (A riboswitch) acts as a genetic switch which turns on transcription. In contrast, *xpt-pbuX* guanine riboswitch (G-riboswitch) found in *B. subtilis* appears to control more than five transcription units and acts as a genetic switch that turns 'off' transcription (Delfosse et al. 2010; Mandal and Breaker 2004).

Moreover, structure evaluation of purine riboswitches aptamer by X-ray, showed that these riboswitches are characterized by a compact fold in where the ligand forms a Watson-Crick base pair with residue 65 (Delfosse et al. 2010). Nowadays, Singh et al. (2018) affirmed the presence of this riboswitch in cyanobacteria; their results demonstrated that purine riboswitches control relevant genes for purine biosynthesis and salvage. These genes with upstream purine riboswitch are present in the pathway of purine de novo biosynthesis.

### Lysine riboswitch (L-box or LYS-element)

Lysine riboswitch also called L-box, is found in the leader sequence of bacterial mRNAs coding for proteins related to the regulation of biosynthesis, catabolism, and transport of lysine (Doerks et al. 2002; Wang et al. 2015). Similar to other riboswitches, its aptamer domain encapsulates the ligand and undergoes a conformational change hence regulating the enzyme activity upon interacting with lysine (Yang et al. 2013).

Lysine may exert its control by inducing premature transcription termination by an unknown mechanism (Kochhar and Paulus 1996). This proposition was emphasized by Rodionov et al. (2003) who demonstrated that mechanism of the lysine-specific riboswitch is similar to the other

metabolite-specific riboswitches found in *B. subtilis* and involves either transcriptional or translational attenuation in various groups of bacteria. Moreover, the *lysC* leader RNA not only uses some common mechanisms, like shape complementarity and metal ion assistance for lys binding and recognition, but also makes nucleotide-specific interactions for ligand identification. These interactions differ from the mechanism used by the glycine riboswitch to recognize similar amino acid features. For this riboswitch, the unique arrangement of contacts for ligand recognition and gene regulation are frequently used (Wilson-Mitchell et al. 2012).

Mutations applied within the lysine-responsive riboswitch play great importance in the acquisition of resistance to antimicrobial lysine analogs. This indicates that lysine induces only limited and local conformational changes upon ligand binding (Garst et al. 2008). However, the knowledge about genes driven by this riboswitch activity has remained undiscovered for many years. Mukherjee et al. (2017) analyzed about 2785 bacterial gene sequences of which 468 were natural lysine riboswitches that regulate almost all biosynthetic and transporter genes in *Firmicutes* and *Gammaproteobacteria*. This research confirmed that lysine riboswitches are relatively rare in all other prokaryotic phyla where if it is present they are primarily found upstream to operons containing many lysine biosynthesis genes. In recent years, research has demonstrated that the regulation of biosynthesis and transport of amino acid in cyanobacteria is controlled by lysine riboswitch. For instance, d-hydantoinase, Holliday junction DNA helicase subunit (RuvA) and an amino acid carrier protein showed in their upstream sequence the presence of lysine riboswitches (Singh et al. 2018). Even though these discoveries brought many benefits in cyanobacteria gene regulation, further investigations based on molecular evolution are also needed on this riboswitch to better understand its effect on in vivo expression of these genes.

### *glmS* riboswitch

The *glmS* is a unique riboswitch not only known as gene regulator, but also as nucleic acid catalyst (Fei et al. 2014), and this regulation is monitored through a self-cleavage activity (Davis et al. 2011). Here, the reaction requires the hydrated  $Mg^{2+}$  which is simultaneously cleaved in the presence of glucosamine-6-phosphate (GlcN6P) (Barrick et al. 2004; Winkler et al. 2004). Alternatively, the *glmS* ribozyme adopts a compact double pseudoknot tertiary structure, with two strictly packed helical stacks (Cochrane et al. 2007).

It has been demonstrated that genetic switches may have functioned as metabolite sensors in primitive organisms, and other researchers have suggested that modern cells retain some of these ancient hereditary control systems (Barrick et al. 2004). In this case, *glmS* riboswitch shows a unique mechanism compared to other riboswitch classes studied

which utilize ligand-induced changes in expression platform sequences to regulate numerous gene expression processes. Further, this research has emphasized that ribozyme acts as a metabolite responsive genetic switch that cleaves itself and represses the *glmS* gene whose activity depends on binding in the active site of the ribozyme and sufficient concentration of glucosamine-6-phosphate. This makes the amine to function as a general acid and electrostatic catalyst (Barrick and Breaker 2007; Ferre-D'Amare 2011).

Apart from GlcN6P, other researches revealed that *glmS* riboswitch can respond to multiple metabolites but not triggered by a conformational change. To provide evidence on this, the comparison of inhibitor apo, and activator which bound this ribozyme structures were further carried out and revealed that the active site is preorganized even in the absence of the effector (Butler et al. 2011; Cochrane et al. 2007; Watson and Fedor 2011).

A 246-nt sequence found in 5'-UTR of the *glmS* gene in *B. subtilis* explains the cleavage at a specific site in the RNA (Barrick and Breaker 2007; Barrick et al. 2004). Further, the ribozyme is pre-folded but inactive in the absence of GlcN6P, meaning that it has evolved strict dependence on the small exogenous molecule. To elucidate this, research carried out on the use of coenzyme by the *glmS* ribozyme–riboswitch demonstrated that replacement of the ribozyme by catalytically compromised mutant results in abrogation of sporulation by *B. subtilis*. In this research, the in vitro study on self-cleaving domain of the *glmS* mRNA further demonstrated that a ribozyme can be designed to function as a multiple-turnover catalyst (Ferre-D'Amare 2011). Recent findings regarding reporter gene experiments in *B. subtilis* confirmed that this riboswitch integrates positive and negative chemical signals in its natural biological context (Watson and Fedor 2011). The current discovery demonstrates that *glmS* riboswitch resides in cyanobacteria with a function of controlling amidohydrolase, thioredoxin and cytochrome c biogenesis protein (CcdA) expression (Singh et al. 2018).

## Glycine riboswitch

Glycine riboswitch binds glycine to regulate glycine metabolism genes, including the use of glycine as an energy source (Butler et al. 2011). This natural regulator belongs among the earliest known riboswitches and has been known in various bacteria including *Streptomyces griseus* (Mandal et al. 2004; Tezuka and Ohnishi 2014). So far, glycine-sensing riboswitch found in *B. subtilis* works as a rare genetic on switch for the *gcvT* gene, which codes for proteins that form the glycine cleavage system. For this riboswitch, a leader sequence was recognized but the linker interaction was not well characterized. Mostly, these riboswitches combine two ligand-binding domains which function cooperatively to

more closely approximate a two-state genetic switch which is important to ensure the excess glycine. Here, there are used efficiently to provide carbon flux by means of the citric acid cycle even to maintain adequate amounts of the amino acid for protein synthesis (Mandal et al. 2004). In addition, an effect on gene expression regulation by a putative glycine riboswitch located in the 5'-UTR of a sodium: alanine symporter family (SAF) protein gene residing in *A. Streptococcus pyogenes* strain 591 (serotype M49) was evaluated. Hence, the evaluation of the presence or absence of glycine pointed out that high glycine concentrations downregulate gene expression whereas less concentration of glycine led to the production of a full-length transcript. Finally, this research concluded that putative glycine riboswitch in *S. pyogenes* strain 591 (serotype M49) represses expression of the SAF protein gene and the downstream putative cation efflux protein gene in the presence of high glycine concentrations (Khani et al. 2018). Similarly, within this year, seven cyanobacterial genomes namely *Leptolyngbya* PCC 7375, *Mastigocoleus testarum* BC008, *Halothece* PCC 7418, *Myxosarcina* sp. GI1, *Prochlorococcus* sp. scB243\_498P3, *Scytonema millei* VB511283 and *Tolypothrix campylonemoides* VB511288 have been mined and confirmed to have glycine riboswitch where aspartate racemase, aspartyl/asparaginyl beta-hydroxylase encoding genes are glycine riboswitch regulated (Singh et al. 2018).

## PreQ1 riboswitches

7-Aminomethyl-7-deazaguanine (PreQ1) have been known as the smallest riboswitches which sense mRNA domains. This class of riboswitch binds to pyrrolopyrimidine to regulate genes involved in the biosynthesis or transport of precursor queuosine, universally found in four RNA tRNAs (Kang et al. 2014; McCown et al. 2014; Meyer et al. 2008). Two entirely distinct classes of PreQ1 riboswitches are known: PreQ1-I and PreQ1-II (Rieder et al. 2010). The binding domain of PreQ1-I riboswitches are unusually small among naturally occurring riboswitches and, divalent cations are required for its high-affinity binding. It was found that the P4 helix in PreQ1 class I and flanking adenine residues play crucial and unexpected roles to control pseudoknot formation and, in turn, to sequester the Shine–Dalgarno sequence (Kang et al. 2014). PreQ1-II riboswitches mainly found in the genera *Streptococcus* and *Lactococcus*, can also be found in *B. subtilis* (Petrone et al. 2011), but their response mechanism remains undetermined at the molecular and biophysical level (Rieder et al. 2010). A study conducted by comparing the free-state 3D structure of the *T. tengcongensis* and *B. subtilis* PreQ1 riboswitch, revealed that *T. tengcongensis* PreQ1 riboswitch remains the same in its bound state whereas *B. subtilis* PreQ1 riboswitch showed a remarkable change.

Further discussions and evaluation on some regulation functions for these riboswitches and the unfolding of their aptamers by all-atom molecular dynamic simulation indicated that they have similar unfolding or folding pathways and ligand-binding processes. Beyond this, only what has been noticed about their difference is the folding intermediate states. Preferably, the results obtained from this evaluation were suggested to be used to understand the regulation mechanism of different riboswitches with free-state 3D structures similar to their bound states (Gong et al. 2012). Moreover, to deepen elegantly way, the study concerned ligand-free conformation mechanisms of PreQ1 aptamer domains for *B. subtilis* has been conducted. As results, an overall 1.5  $\mu$ s all-atom molecular dynamics simulations were used and showed a state stability of ligand-free aptamers supported by a folded P1-L3 with an open binding pocket (Gong et al. 2014). In the last 2 years, rational re-engineering of a transcriptional silencing PreQ1 riboswitch was then introduced in *B. subtilis* resulting in the identification of an orthogonal riboswitch-ligand pairing that effectively repressed the transcription of selected genes in *B. subtilis*. Among these candidate riboswitches, the resulting artificial riboswitch, M1 (C17U), responded to the diamino analog of PreQ0 (DPQ0) and effectively repressed transcription of *mreB*, a gene for cell morphology in *B. subtilis*, upon addition of 2 mM DPQ0 (Robinson et al. 2016; Wu et al. 2015).

Furthermore, apart from *B. subtilis*, research carried out on mycobacteria revealed that these organisms do not have *preQ1* biosynthetic genes, this absence pushed researchers to study whether *preQ1* could be used as an exogenous non-metabolite ligand to control riboswitches in mycobacteria. Hence, PreQ1 riboswitches were assayed and successfully drove PreQ1-dependent repression of a green fluorescent protein reporter in *Mycobacterium smegmatis*. Above all, researchers, by practicing engineering approaches on naturally occurring PreQ1 riboswitches, they have not only extended the tools available for inducible gene regulation in mycobacteria but also uncovered new behavior of these riboswitches (Van Vlack et al. 2017). In very recent years, the curiosity of exploring the importance of riboswitch on cyanobacteria genes regulation accelerated scientists to conduct experiments on this riboswitch and results led to the confirmation that PreQ1-I riboswitches in cyanobacteria are thinly dispersed with only 14 genomes showing their presence. This research has further showed that the largest number of riboswitch regulated genes are present in *Microcystis aeruginosa* NIES 843 and *Trichodesmium erythraeum* IMS 101 while only single gene was found to be regulated under PreQ1-II (pre-queuosine) riboswitch in *Leptolyngbya* sp. The PreQ1-II riboswitch also showed its present upstream of soluble [2Fe-2S] ferredoxin gene in *Leptolyngbya* sp. (Singh et al. 2018).

Based on chronological discoveries of regulatory RNA elements involved in gene regulation, it has been confirmed that highly structured domains residing within non-coding regions of certain bacterial mRNAs serve as metabolite-responsive genetic switches. Yet, early 2004, around seven classes of riboswitch and related mechanisms have been experimentally demonstrated. A few years later until now, with considerable progress made in this field, different classes of natural and synthetic riboswitches together with their related application were discovered. In the above text, we reviewed nine studied natural riboswitches in detail (Table 1). However, there are still some other riboswitches especially the synthetic ones showing potential application in gene regulation (Table 2). In the next section, we will discuss progresses in synthetic riboswitches.

### Progress in synthetic riboswitches

Artificial devices such as synthetic riboswitches which own similar properties as natural riboswitches, have shown potential in introducing unnatural phenotypic perturbation due to their synthetic traits which are distinct from that of innate metabolism (Lee and Oh 2015). Heretofore, two remarkable limitations should be carried away to allow researchers to build a beneficial riboswitch. The first one is the lack of a large number of alternative aptamers as ligand-binding modules and the second is the large discrepancy between the in vitro affinities of aptamers for their ligands and some cases the high concentrations of ligand needed in vivo to flip the corresponding switches (Berens et al. 2015). More elegant solution to these problems is that different synthetic riboswitches that display deficient background levels of gene expression in the absence of the desired ligand and robust increases in expression when it is present have been suggested (Lynch et al. 2007). Contrariwise, the construction of synthetic regulators of transcription and translation has failed repeatedly. Importantly, over the past few years, engineered riboswitches most often targeting translation initiation, either by controlling access to the ribosomal binding site through helix slippage or by sequestering its sequence were reported in bacteria (Suess et al. 2004). However, a more in-depth, accessible and understandable design is quite imperative to discover and combat the inefficacy of riboswitches towards regulation of metabolic systems (Fig. 3).

A continuous incentive on this query underlined the possibility of performing selections for novel synthetic riboswitches which function similarly both in vitro and in vivo (Mishler and Gallivan 2014), and different previous researches revealed that riboswitches for translational control could be designed successfully. Furthermore, an in-silico pipeline for the rational design of synthetic riboswitches that regulate gene expression at the transcriptional level have been reported (Wachsmuth et al. 2013; Wittmann and Suess

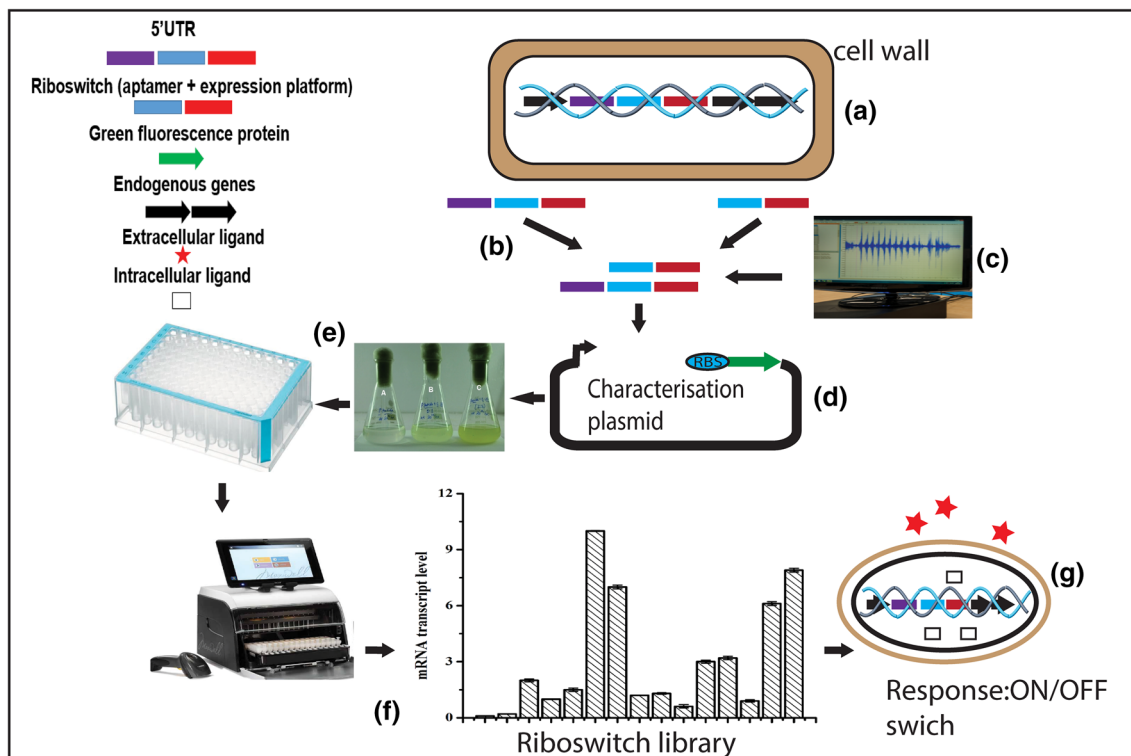


**Table 1** Properties of the nine known natural riboswitches often diversified in bacteria

Riboswitches	Size (bp)	Sensed ligands	Target genes	Target process	Applications	Organism	References
FMN	236	FMN	B2 synthesis and transport	Transcription termination or translation initiation	Antimicrobial drug targets	<i>B. subtilis</i> , <i>S. davawensis</i>	Lee et al. (2009), Pedrolli et al. (2012), Serganov and Nudler (2013), Vicens et al. (2011)
Glycine	87	Glycine	Citric acid cycle and protein synthesis	Transcription terminator	Inducible production, Boolean logic gates	<i>B. subtilis</i>	Butler et al. (2011), Mandal et al. (2004), Tezuka and Ohnishi (2014)
Lysine	87	Lysine	<i>lysC</i>	Transcription termination	Antimicrobial drug targets, Riboselector	<i>B. subtilis</i> , <i>E. coli</i>	Blount et al. (2007), Klein and Ferré-D'Amaré (2009), Yang et al. (2013)
PreQ1 I	46	PreQ1 I	Guanosine biosynthetic pathway	Transcription termination	Regulation of queuosine (Q) biosynthesis and transport	<i>B. subtilis</i>	Gong et al. (2012), Suddala et al. (2013)
Guanine	100	Guanine	Purine metabolism and transport	Transcription termination and antitermination	Antimicrobial drug targets	<i>S. aureus</i> , <i>B. subtilis</i>	Kim et al. (2009), Kim et al. (2007)
SAM-I	159	SAM-I	Sulfur metabolism and transport	Transcription termination	Gene expression control	<i>B. subtilis</i>	Huang et al. (2012), Tomsic et al. (2008)
TPP	158	TPP	B1 synthesis	Transcription termination or translation initiation	Antimicrobial drug targets, Boolean logic gates	<i>Bacillus anthracis</i> , <i>E. coli</i>	Bocobza et al. (2007), Sudarshan et al. (2005), Welz and Breaker (2007)
<i>glmS</i>	171	GlcN6P	GlcN6P synthetase	RNA cleavage	Antimicrobial drug targets	<i>B. subtilis</i>	Ferre-D'Amare (2011), Watson and Fedor (2011)
Cobalamin	90	Coenzyme B12	Transport of cobalamin compounds or metals	Transcriptional antitermination	Gene expression control	<i>B. subtilis</i>	Moore et al. (2014)

**Table 2** Other promising riboswitches applicable in bacteria

Riboswitch type	Potential application	Organism	References
Tandem <i>metE</i> riboswitch	Boolean logic gates	<i>Bacillus clausii</i>	Sudarsan et al. (2006)
Theophylline sensitive riboswitch	Gene expression control	<i>E. coli</i>	Jin et al. (2009)
Theophylline sensitive riboswitch	Boolean logic gates	<i>E. coli</i>	Topp and Gallivan (2007)
Theophylline sensitive riboswitch	Virus gene expression and replication control	–	Ketzer et al. (2014)
Theophylline	Gene expression control in cell-like system	–	Martini and Mansy (2011)
Theophylline	Control of bacterial cell behavior	<i>E. coli</i>	Topp and Gallivan (2007)
Glycosamine-6-pyrophosphate	Antimicrobial drug targets	<i>B. subtilis</i>	Lunse et al. (2014)
<i>ydaO</i> motif	Transcription termination of cell wall metabolism genes	<i>B. subtilis</i>	Watson and Fedor (2012)
Adenine	transcription termination of <i>ydhL</i> , <i>pbuE</i>	<i>B. subtilis</i>	Ramesh and Winkler (2014), Shin et al. (2014)
Magnesium	Transcription termination of magnesium transporter genes	<i>B. subtilis</i>	Garst et al. (2011)
Tryptophan	Posttranscription initiation of tryptophan metabolism and transport	<i>E. coli</i> , and <i>B. subtilis</i>	Babitzke and Gollnick (2001), Jang and Jung (2018)



**Fig. 3** Development procedure of new riboswitch. (a) Bacterial chromosome showing the location of natural riboswitch in 5'-UTR between two endogenous genes. (b) Scheme of synthetic riboswitch library construction from 5'-UTR, natural riboswitch (aptamer and expression platform) or simulated sequences. (c) In silico riboswitch sequence prediction. (d) Sequence engineering, plasmid construction,

library screening and (e) dilution of culture and set up ligand incubations followed by fluorescence measurement and expression level quantitation. (f) Results analysis and selection of the strongest riboswitch. (g) In vivo assay: ON or OFF switch response in accordance with internal or external ligand effect

2012). Here, a theophylline aptamer was used as a sensor to design an actuator part as RNA sequences that can fold into functional intrinsic terminator structures. Based on biochemical characterization observed during an *in silico* pipeline for the rational design of synthetic riboswitches, several of the designed constructs demonstrated ligand-dependent control of gene expression in *E. coli*, promising that it is possible to engineer riboswitches for both translational and transcriptional regulation.

De novo design of a synthetic riboswitch based on a connection of functional building blocks has been achieved. This riboswitch candidate should be evaluated by folding simulations to further tune the transcription termination in organisms (Wachsmuth et al. 2013).

Recently, different techniques based on screening or rational design methods to construct artificial riboswitches that function in either bacterial or eukaryotic translational systems were described (Ogawa 2014). Urgent studies on riboswitches have been conducted and results are now assured to be used in biosensor design. For instance, the combination of different permutations topology of the ligand sensing domain of natural riboswitches with *in vitro* selected fluorogenic aptamers proved to be a promising technology to enhance fluorescence turn-on and ligand binding affinity compared to the non-permuted topology. In the same way, SAM-I riboswitch circular permutation of the riboswitch ligand sensing domain also gives functional biosensors (Truong et al. 2018). Some azaaromatic molecules are bound by the RNA with nanomolar dissociation constants, and a subset of these ligands activate riboswitch-mediated gene expression in the cells. Based on this supposition, Lubelski et al. (2006) used a dual strategy of formulating ligand hypotheses based on rare gene associations and testing a variety of diverse chemical compounds for possible binding by *yjdf* motif RNAs. Results obtained demonstrated that *yjdf* motif RNAs are riboswitches that can regulate gene expression by binding to an unusually large diversity of polycyclic aromatic nitrogen-containing heterocycles, which are sometimes called PANHs or azaaromatics. From these findings, a profound research is needed to confirm the hypothesis raised up that these “azaaromatic riboswitches” might have evolved to bind natural member classes of large, planar, and hydrophobic compounds to activate production of the Yjdf protein, perhaps as a mechanism for detoxification. If true, azaaromatic riboswitches would be analogous to protein receptors that bind diverse polycyclic aromatic hydrocarbon (PAH) compounds and activate gene expression to regulate natural PAH production or to overcome PAH toxicity as seen in some bacterial species (Huillet et al. 2006). Few examples of protein sensor-regulators for flavonoids have been shown (Siedler et al. 2014; Wenzel et al. 2012). More recent research (Jang et al. 2017) develops artificial riboswitches that activate gene expression in response to

naringenin. The results showed a high response to naringenin and activated gene expression up to 2.91 fold. The reported naringenin riboswitches will be valuable tools in metabolic engineering of microorganisms for the production of flavonoids.

In the last few years, researches in synthetic biology and biotechnology have evolved rapidly, and their application for bioengineering are nowadays remarkably achieved to meet human needs. CRISPR technology is among the current useful biotechnology tool used in prokaryotic and eukaryotic organisms for gene editing intention even though they are some challenges remained to alleviate. Very recently, a combination of riboswitch and CRISPR development technology has been launched with the aim to functionalize synthetic sgRNA designs to enable inducible and spatiotemporal regulation of CRISPR-based genetic editors in response to cellular or extracellular stimuli. Interestingly, some riboswitches can be developed and adapted to control sgRNA activity by adding corresponding sequences to its 5' end to repress the guide sequence in the unbound confirmation and reconstitute the active confirmation after ligand binding. In this sense, current study on genome editing and transcriptional activation in mammalian cells was able to incorporate ligand-responsive self-cleaving catalytic RNAs (aptazymes) into guide RNAs resulting a developed set of aptazyme-embedded guide RNAs that enable small molecule-controlled nuclease-mediated genome editing and small molecule-controlled base editing, as well as small molecule-dependent transcriptional activation in mammalian cells (Tang et al. 2017). Also, the advantage of using sgRNA-based inducible systems for synthetic biology applications has been recently showcased in a study demonstrating the ability to rewire cellular pathways by CRISPR-TR with modified sgRNAs containing ligand-responsive riboswitches. These sgRNA ‘signal conductors’ employ a strand-displacement mechanism to transition between OFF and ON states and can be coupled to a variety of inducers and dCas9 effectors (Liu et al. 2016). Furthermore, aptamer-controlled hammerhead ribozymes (so-called aptazymes) have been shown to be a versatile platform for the engineering of novel gene regulators. However, the generation of these novel aptazymes requires a functional aptamer-ribozyme connection, which can be difficult to engineer. Interestingly, Wieland et al. (2012), defined and elaborated new methods and protocols for FACS-mediated aptazyme identification in bacteria and for engineering an aptazyme-based gene control system in mammalian cells.

Roßmanith and Narberhaus (2016) established three riboswitch-RNAT systems conferring dual regulation of transcription and translation depending on the two triggers ligand binding and temperature. This concept relied on designing thermoswitches by integration of a thermosensor into riboswitches from different classes (TPP and lysine)

as well as synthetic riboswitches (theophylline) in modular ways to gain functional regulatory elements. These thermoswitches respond to the cognate ligand at low temperatures and are turned into a continuous on-state by a temperature upshift. These findings might help scientists to deplore riboswitches and RNATs for engineering synthetic RNA regulators due to their modular behavior.

Most recently, engineered drug-inducible catalytically inactive Cpf1 nuclease has been used to effectively construct transcriptional repressors in bacteria and plants. Besides, when it is fused to transcriptional activation domains, the construct enabled to tune the expression of endogenous genes in human cells. It is in this sense that, a programmable ligand-controlled dAsCpf1 systems either by coupling crRNAs with engineered riboswitches or by fusing dAsCpf1 proteins with G protein-coupled receptors has been constructed. These approaches using tunable CRISPR–Cpf1-based transcription factors allow the regulation of the transcription of endogenous genes in response to diverse classes of ligands, thus constructing artificial signaling pathways with rewired cellular input–output behaviors (Liu et al. 2017; Tak et al. 2017). Referring to a crucial impact of environmental pH on metabolism and behavior of living cells, Pham et al. (2017) engineered a set of riboswitch-based pH-sensing genetic devices to allow the control of gene expression. A digital pH sensing system designed can use the analog-sensing characteristic of these devices for high-resolution recording of host cell exposure to the external pH levels. This valuable innovation could be used in multiple engineering aspects of host cell for improved tolerance to a narrow range of organic acids, bioremediation and a precocious phenotype for metabolic engineering.

## Concluding remarks and future perspectives

Riboswitches, regulatory RNA elements which control a wide set of metabolic pathways in bacteria are nowadays used as molecular tools for the precise control of gene expression in many genetic studies. They only consist of RNA molecules and are composed of aptamer domain (high affinity and specific binding of metabolites) and expression platform (regulation of gene expression). Their output effect can intervene in controlling transcription termination (kinetically controlled), translation initiation, RNA stability and protein stability. Natural riboswitches are considered as an ideal target for constructing synthetic regulatory systems for gene expression and some synthetic riboswitches have been achieved so far. Basing on their simple use without any involvement of intermediate factors, riboswitches could not only be manipulated for biosynthetic regulation and transport of cognate ligand, but also in various domains such as microbial food detection, antimicrobial drug targets,

antibiotic resistance, boolean logic gates as well as bacterial cell behavior as shown in Table 2. Interestingly, riboswitches demonstrated good functionality associated with both transcription initiation and translation initiation in gram positive bacteria especially in *B. subtilis* as well as in other microorganisms. However, riboswitches remain the rarest genetic parts hitherto less known but promising a greater importance in the regulation of chromosomal genes. Despite their insufficiency in the genome, the design of synthetic variants could be an interesting direction for future research.

In addition to their essential role in molecular engineering as well as in biochemical molecules production, their sequences may also be a requisite tool to be deeply explored in order to better understand drug resistance challenges, genetic-associated syndromes, cancerous genes regulation, designing of synthetic aptamers required for the detection of harmful substances in food microorganisms as well as the discovery of epigenetic related diseases. Moreover, the implementation to alleviate the above challenges, would require much concerted efforts from synthetic biologists and metabolic pathway engineers to deploy synthetic biology and genetic engineering techniques at the forefront. When these requisites are filled in, it will facilitate the use of these RNA molecules and understand their effect in different areas of life. It would be an essential matter to explore these regulatory tools and discover new intracellular ligands which could likewise be used as metabolites ligand sensing. Similarly, in-depth studies on the response of 5'-UTR to metabolites should be an essential focus in diversified organisms. Subjected to our critical analysis, the results outlined in this review point out the urgent need for multiple comprehensive studies using mathematical modelling and computational simulation as well as experimental practices to demonstrate the effect of RNA molecules during the expression of endogenous genes. Whether it is an artificial or natural, the function of riboswitches to regulate genes involved in metabolic pathways abide a big challenge. Researchers in this field have intensively delved into riboswitches mechanisms and their effect on downstream coding sequence in host cells, nevertheless, there is still a serious quest of understanding the sophisticated phenomena of sensing and responding on intra or extracellular molecules known as ligands which encompass limiting factors on gene expression.

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