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SYNTHETIC BIOLOGY OF SMALL RNAs AND RIBOSWITCHES

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Abstract

In bacteria and archaea, small RNAs (sRNAs) regulate complex networks through antisense interactions with target mRNAs mediated by chaperone proteins, and riboswitches regulate gene expression in cis based on the ability to bind small molecule ligands. Although our understanding and characterization of these two important regulatory RNA classes is far from complete, these RNA-based mechanisms have proven useful for a wide variety of synthetic biology applications. Besides classic and contemporary applications in the realm of metabolic engineering and orthogonal gene control, this review also covers newer applications of regulatory RNAs as biosensors, logic gates, and tools to determine RNA-RNA interactions. A separate section focuses on critical insights gained and challenges posed by fundamental studies of sRNAs and riboswitches that should aid future development of synthetic regulatory RNAs.

1. INTRODUCTION: COMPARISON OF REGULATORY MECHANISMS OF sRNAs AND RIBOSWITCHES

RNAs have been known to perform a vast amount of regulatory functions in bacteria and archaea. Small RNA (sRNA) and riboswitches are two extensively studied classes of regulatory RNAs. sRNAs are trans-acting RNA elements between ~50-500 nucleotides in length that are either independently transcribed or processed from a non-target mRNA, and contain imperfect complementarity to the target messenger RNA (mRNA) to perform post-transcriptional regulatory functions. On the contrary, riboswitches are cis-regulatory structured RNA elements in the untranslated regions of mRNAs, capable of regulating downstream gene expression through small molecule ligand-induced conformational switching. These regulatory RNAs have revealed the precise and sophisticated nature of natural gene regulatory networks and have inspired efforts to mimic these mechanisms and functions by engineering RNA tools for an increasing number of synthetic biology applications.

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There have been several recent reviews on synthetic biology of sRNAs and riboswitches that have focused on one regulatory RNA class and mainly have been addressed to the synthetic biology field (1–6). Our approach in this review is to provide comparisons between these two classes of regulatory RNAs in bacteria, in terms of their regulatory features, current challenges to their paradigms, and synthetic biology and biotechnology applications. We hope this review sparks productive dialogue and creative exchange of ideas between synthetic biologists and researchers working on the two natural classes.

1.1. Initial discoveries and regulatory models of sRNAs and riboswitches

Several important discoveries and technical advances paved the way for understanding the importance of RNA elements in cellular gene regulation (Figure 1). Regulatory sRNAs were first described as chromosomally encoded noncoding RNAs called antisense RNAs (asRNA) capable of regulating expression of mRNA targets in *Escherichia coli* (9). Initially, sRNAs were discovered from [³²P]orthophosphate-labeled total RNA analysis via polyacrylamide gel electrophoresis (4.5S, 6S, Spot42, tmRNA and RNase P RNA), found as cloned genomic fragments that modulated expression levels of a particular target (MicF, DicF, and DsrA), or identified by conditions that suggested function (CsrB and OxyS) (9–11). In contrast to sRNAs, riboswitches are embedded within the untranslated regions (UTRs) of mRNAs and thereby cannot be separately isolated as independent transcripts or genes. Instead, two contemporaneous advances set the stage for riboswitch discovery. First, in vitro evolution from random sequence libraries showed that short RNA sequences were capable of binding small molecules selectively (7, 8), and these “aptamers” could even allosterically regulate ribozyme activity (12, 13). Second, there was genetic evidence for regulatory sequences within the 5′ UTRs of biosynthetic pathway genes that was responsible for feedback inhibition by the metabolite product (14–16). Discovery that these 5′ UTRs contained complex RNA structures capable of direct binding to adenosylcobalamin (B₁₂), thiamine pyrophosphate (TPP), and flavin mononucleotide (FMN) led to the coining of the term “riboswitches” for these metabolite-sensing regulatory RNA elements (14, 17–20).

For most discovered sRNAs, interaction with its mRNA target involves repression of translation by antisense binding to mRNAs in a way that blocks the ribosome binding site (RBS) or altering mRNA stability by revealing an RNase E degradation site (Figure 2A) (4, 10, 11, 21). Likewise, the most widespread mechanisms of gene regulation for riboswitches are transcription termination or translation inhibition by RBS occlusion upon ligand binding, turning off gene expression (Figure 2B). Both rho-independent and rho-dependent transcription termination (22) mechanisms have been described for riboswitches, although the former is more readily identified by the intrinsic terminator stem (23). Regulation by sRNAs can also involve transcription repression, as in the case of the role of 6S RNA on the RNA Polymerase (RNAP) and the sigma factor σ^{70} (11, 24). However, there are examples in each class that turn on gene expression instead. sRNAs can activate translation by releasing a hairpin structure that blocks the RBS (as demonstrated by DsrA), enhance mRNA stability by blocking RNase E degradation sites, or suppress Rho-dependent transcription termination (Figure 2A) (10, 11, 21, 25, 26). Ligand binding to a riboswitch leading to gene activation by transcription anti-termination or translation activation has been demonstrated, with the first examples in the adenine riboswitch class (27) (Figure 2B). While riboswitches

that respond to metabolic end-products usually turn off genes, those that respond to signaling molecules (e.g. cyclic dinucleotides) are more likely to turn on genes (28, 29).

Studies of the initial exemplars revealed key aspects of the target binding mechanism for sRNAs and riboswitches that still comprise the canonical models for these two regulatory RNA classes. For sRNAs, two canonical key mechanistic binding aspects have been unveiled from initial *E. coli* studies of the sRNAs MicF and SgrS (30–32): (i) imperfect complementarity to the target region and (ii) short (~6 nucleotides of complementarity) required for proper binding and regulation (31). These small “seed” regions of required complementarity to targets (typically between 6–12 nucleotides in length), are often present in unstructured regions at the 5′ end of the RNA and are frequently the most conserved regions of sRNAs (30). Recent biophysical models have demonstrated that accessibility of these small regions to the target mRNA is critical for sRNA-mRNA antisense interaction (33). For riboswitches, their canonical model contains: (i) a ligand-binding aptamer domain and (ii) an expression platform for downstream gene regulation. Aptamer domains are evolutionary conserved in secondary structure and specific nucleotides involved in ligand binding or folding. However, expression platforms typically exhibit little sequence conservation due to variability in regulatory logic between genes/operons and regulatory mechanisms between organisms, including control of transcription, translation, splicing, and mRNA stability.

1.2. Discovery of sRNAs and riboswitches in the genomics era

Starting in 2001, deep-sequencing techniques rapidly expanded the collection of identified sRNAs and riboswitches in bacteria and archaea (11, 34–37) (Figure 1). The development of RNA-sequencing, which provides a strand-specific readout of the transcriptome, has been one of the most useful tools in finding novel sRNAs in a range of bacteria (11, 36–41) and adaptations of this technique, including differential RNA-seq (38, 42), have aided the specific genomic search for sRNAs. Despite the fact that some sRNAs maintain the same function across species, the high sequence divergence of sRNAs has challenged sRNA prediction and discovery via phylogenetic conservation (11). However, machine learning approaches have made advances towards predicting new members in broad classes of sRNAs in a diverse set of organisms (35, 43).

De novo riboswitch discovery has also been made possible by the sequencing of many microbial genomes. Early genome-scale computational riboswitch prediction works relied on comparative genomic analysis. An initial pairwise, BLAST-based analysis of intergenic regions (IGRs) of similar genes among 91 prokaryotic genomes resulted in discovery of 6 orphan riboswitch families that were eventually validated experimentally (44). Later, advances in RNA motif prediction algorithms led to a powerful pipeline that is structure-oriented and applicable to unaligned or even poorly conserved sequences. RNA motif prediction was integrated with RNA homology search to further refine structural alignments (45, 46). This pipeline was then expanded to be independent of protein-coding genes for clustering IGRs (47). To date, many of the predicted orphan riboswitches have had their ligands identified, making the total number of validated riboswitch classes reach around 40. However, the ligands of some rarer variants remain elusive, and this prediction pipeline may

fail to discover extremely rare riboswitch classes (48). Some alternative strategies, such as using RNA-seq and transcription start site profiling to discover potential regulatory elements in the 5'-UTR of mRNA, might offer a solution to identify species-specific riboswitches (49, 50).

2. CHALLENGES TO THE PARADIGMS FOR sRNAs AND RIBOSWITCHES

While there are general principles for designing sRNAs or riboswitches for synthetic biology applications (Table 1), as more sRNAs and riboswitches are discovered in diverse organisms, an increasing number remain functionally uncharacterized, while some of those that are studied have proven to be exceptions to the general rules. In this section, we focus on specific challenges to the paradigms that have arisen from fundamental studies on sRNAs and riboswitches that should be instructive for those interested in engineering regulatory RNAs.

2.1. Challenges to binding target identification

Identification of target genes for sRNAs and target small molecule ligands for riboswitches remains an enormous challenge, often lagging far behind discovery of the sRNA gene or prediction of the conserved riboswitch structure in a UTR (Figure 1). For sRNAs, the difficulty is in part due to the sRNA acting in trans to target multiple mRNAs. Moreover, sRNAs are known to have multiple targets that can be differentially regulated (or not) based on specific cellular conditions. Furthermore, combinations of different regulatory mechanisms are also possible, as one sRNA can regulate multiple mRNA targets with different mechanisms. For example, DsrA activates translation of the alternative sigma factor *rpoS* upon binding by relieving a hairpin that blocks the RBS, but represses translation of its other target, *hns*, by blocking the RBS (51, 52). Additionally, DsrA prevents Rho-dependent transcription termination of *rpoS* by sharing the same binding site with the Rho terminator (25). Several new sequencing-based methods have been developed to aid in the rapid, high-throughput identification of sRNA targets (34, 53–55) to complement computational tools (56, 57) and validation of hypothesized sRNA-mRNA interactions by additional fluorescence (58), genetics (deletion and overexpression), and biochemical assays (59). Determination of the target network aids in engineering sRNAs to regulate multiple targets under specific conditions without off-target effects.

For riboswitches, the difficulty in ligand identification is in part due to incomplete knowledge of gene function outside of major metabolic pathways. Furthermore, gene annotations for most bacterial genomes are based on homology, and may not be accurate or supported by experimental evidence. In fact, the riboswitches for cyclic di-AMP, fluoride, and guanidine were discovered as conserved regulatory structured RNA elements several years before their ligands were known to be physiologically relevant metabolites (44). The identification of the riboswitch ligand provided insight into signaling pathways for cyclic di-AMP and cyclic AMP-GMP (60–63), and the function of transporters for fluoride, preQ₁ and guanidine (64–67).

Like gene annotations, the target ligand for most riboswitches annotated in bacterial genomes are based on homology to a known riboswitch. However, there is a growing

realization that hidden diversity of riboswitch-ligand pairs within structural classes is more prevalent than previously assumed. This is exemplified by riboswitches for adenine (68), 2'-dG (69), and cyclic AMP-GMP (61, 62). These variant classes appear to have diverged from the parent riboswitch classes to evolve an altered ligand specificity. Two complementary strategies have been demonstrated for revealing such hidden diversity. A "bottom-up" strategy biochemically examines the structure-activity relationship to identify key nucleotide positions, which guides the refinement of the consensus sequence and covariance model to search for rare variants among the parent riboswitch class (62). A more generalizable "top-down" strategy utilizes the x-ray crystal structures of riboswitches to identify key positions and their associated gene annotations to predict novel riboswitch functions. The resulting refined bioinformatics search led to discovery of riboswitch subclasses that await biochemical validation (70). These two strategies have led to the discovery of variants among glycine, FMN, and the two cyclic di-GMP riboswitch classes. These many examples of natural riboswitch reprogramming showcase the potential for engineering riboswitch scaffolds to specifically target new metabolite ligands.

2.2. Noncoding RNAs that still code and riboswitches that are not "switches"

Although sRNAs are traditionally described as being noncoding RNAs, in multiple systems, it is now evident that sRNAs can encode for small peptides as dual function sRNAs (71, 72). The first bacterial dual function sRNA described is RNAIII from *Staphylococcus aureus* that regulates several virulence factors. The 5' region of RNAIII contains an ORF (*hld*) that encodes a secreted 26 amino acid peptide, δ -hemolysin, that causes lysis of host cell membranes (71). Regulation of the *hld* ORF occurs by the partial overlap by one of RNAIII mRNA targets, *map* (a surface adhesion protein) which could serve to regulate expression of δ -hemolysin (10). Other dual function sRNAs appear to have virulence-related functions (73) however, not all dual function sRNAs appear to regulate virulence factors (71). In fact, dual function sRNAs are capable of regulating one system through two distinct mechanisms as demonstrated by *SgrS*; this sRNA negatively regulates the glucose transporter *ptsG* mRNA under glucose-phosphate stress, contains an ORF, *sgrT*, translated in *E. coli* under glucose-phosphate stress and interferes with PtsG transport activity (74, 75). The regulatory ability of the cell to regulate translation of the ORF within a sRNA is still to be determined for some dual function sRNAs, however some dual function sRNAs appear to be translationally dependent on σ^S (76). The presence of dual function sRNAs indicates the need to verify ORFs contained in an sRNA as part of the discovery and validation process as the small peptide encoded could aid sRNA function, or be separately regulated by the mRNA targets. Additionally, dual function sRNAs suggest an opportunity to engineer multi-function sRNAs to obtain both the regulation of an sRNA and the enzymatic activity of a small peptide in a promising new research frontier.

Similar to the inaccuracy of naming sRNAs as noncoding, the term riboswitch propagates the classic "induced fit" model that riboswitch conformation consists of two main states, "ON" and "OFF", and ligand binding switches the RNA from one conformation to the other. However, this classic view has been challenged by biophysical studies tracking single molecule trajectories for RNA folding (77–79) and using advanced NMR spectroscopy techniques to detect highly transient states (80). In the "conformational selection" model, a

riboswitch can fold into and dynamically sample different conformational states without the ligand. Presence of ligand drives the equilibrium toward a favored conformational state (81). For example, a recent single molecule FRET study of the full SAM-I riboswitch revealed that there are four discrete conformational states that are populated even in the absence of SAM (82). Note that such dynamics is revealed *in vitro* under thermal equilibrium conditions for full-length riboswitches. It is expected that riboswitch functional dynamics *in vivo* would be even more complicated due to co-transcriptional folding. For example, co-transcriptional folding of a fluoride riboswitch appeared highly dynamic, such that addition of a newly transcribed single nucleotide might greatly alter the folding landscape of the nascent riboswitch (83). Undoubtedly, rational riboswitch engineering would greatly benefit from a better understanding of such riboswitch dynamics.

2.3. Not all sRNAs depend on Hfq chaperoning but all RNA regulators depend on intracellular protein interactions

Many sRNAs (but not all) require an RNA chaperone protein, Hfq, for proper function (including sRNAs OxyS and DsrA). Hfq acts as a “meeting platform” to mediate a number of steps in sRNA-mRNA interaction including exposing the seed region for sRNA-mRNA hybridization, neutralizing negative charges of the RNAs, protecting RNAs from degradation and assisting annealing of the RNA strands (84). Crystal structures of Hfq suggest that each of the four faces of the homohexameric toroid protein has a different binding preference and Hfq-dependent sRNAs have been classified into two groups depending on the binding preference to Hfq (85). Moreover different sides of Hfq have also been attributed to aiding release of the sRNA-mRNA pairs to allow rapid Hfq cycling to other sRNA-mRNA pairs (86) and to interact with bacterial membranes (87). However, it is now known that Hfq is not present in all bacteria, even in those that contain sRNA-based regulation. While some bacteria contain Hfq homologs, these are often not required for proper sRNA function (although Hfq can aid function) and are functionally distinct between organisms (88). Hfq is largely not required in gram positive bacteria, with only a few exceptions (88, 89). Recent efforts have identified FinO-domain proteins, like ProQ, as another form of specific sRNA chaperone (90). However, the FinO-domain proteins do not appear to be alternatives to Hfq, as almost all bacterial families that lack Hfq homologs also lack FinO-domain proteins (90). Although there appear to be some general trends of Hfq-based interactions with sRNAs (such as binding U or A rich sequences of RNA) (84), the understanding of what makes an sRNA Hfq-dependent is still lacking, and there is even less understanding about the importance of FinO-domain RNA chaperones.

In contrast to sRNAs, a hallmark of riboswitches is their ability to directly bind a metabolite ligand without assistance from protein factors. However, in order for gene regulation to occur, the riboswitch must interact with or recruit protein/RNP factors required for transcription termination, translation initiation, or mRNA degradation, e.g. RNA polymerase, Rho, ribosome, or RNase. Thus, understanding riboswitch-protein interactions is important. For example, riboswitches fold while being transcribed by RNA polymerase. Thus, the kinetics of co-transcriptional RNA folding and the transcription speed of RNA polymerase need to match well for riboswitch function, and in fact natural riboswitches have been shown to harbor transcriptional “pause” sequences (91). Furthermore, binding of

ribosomes to the RBS also contributes to the overall thermodynamic energy equilibrium, along with the intramolecular RNA-RNA and intermolecular ligand-RNA interactions (92). Similar considerations of sRNA-protein interactions (e.g. interactions with the cellular machinery) are important for fully understanding sRNA function in vivo (e.g. transcription, degradation, target binding, ribosome binding). Ignoring these riboswitch-protein and sRNA-protein interactions would result in misunderstanding of native biological functions of these regulators and in failure of engineering them.

2.4. Complex network regulation involves crosstalk, competition, and coordination

RNA networks involve multiple levels of regulation and recent studies have demonstrated that levels of individual mRNA targets, or shared resources (like Hfq) can affect the regulatory ability of an sRNA across entire regulatory networks. These competing endogenous RNA (ceRNA) (which can be another sRNA or mRNA) interfere with sRNA target binding, either through competition/sequestration of sRNA binding sites through mimicry or alternative binding, or competition for Hfq, such that the level of sRNA based regulation is dependent on the level of the ceRNA (93, 94). In this manner, communication occurs between the numerous regulatory pathways that sRNAs can mediate in an organism. Based on this hypothesis of crosstalk between sRNA and their RNA targets and protein mediators, it is important to consider the effect across a network when engineering changes to a system. This competition and crosstalk can result in unexpected results when modulating a desired system. For example, when engineering acid tolerance in *E. coli*, researchers overexpressed three sRNAs that all translationally activate target *rpoS* (DsrA, RprA and ArcZ) but found the benefit of overexpression to be supra-additive instead of the expected linear trend (95).

In contrast to the competition of sRNA-based networks, natural riboswitches arranged cooperatively in tandem have been discovered, which reveals the great potential of riboswitches to form complex bio-computational logic circuits. In nature, some riboswitches contain tandem aptamer domains for the same ligand, such as a glycine riboswitch in both *Vibrio cholerae* and *B. subtilis* (96), or tandem complete riboswitches from the same class, such as a TPP riboswitch in *B. anthracis* (97) and a triple-tandem cyclic di-GMP riboswitch in *B. thuringiensis* (98). The multivalent organization yields a more digital response to the cognate ligand due to a steeper ligand-dependent binding curve. Tandem arrangements with riboswitches for distinct ligands result in a logic gate, which is exemplified by the *metE* UTR in *B. clausii* (99). This tandem arrangement contains a SAM riboswitch and an adenosyl cobalamin (AdoCbl) riboswitch and functions as a two-input Boolean NOR logic gate. Only when both SAM and AdoCbl concentrations are low in the cells will expression of the downstream gene *metE*, which encodes an AdoCbl-independent SAM synthase, be turned on. These natural examples are inspiring for constructing artificial RNA-based logic gates.

Furthermore, several natural riboswitch-ribozyme mechanisms have been discovered to date. Ligand binding to the *glmS* riboswitch catalyzes site-specific cleavage that triggers mRNA degradation. The *glmS* sequence acts as both the riboswitch and the ribozyme by placing the ligand's nucleophilic amine group in position to attack a specific phosphodiester bond (100–

102). In contrast, a c-di-GMP-II riboswitch in *Clostridium difficile* is found adjacent to a group I self-splicing intron that acts as its expression platform. The ligand-bound riboswitch induces rearrangement of the group I intron conformation that allows GTP to attack and activates the splicing event, resulting in a translatable mRNA (29). The eukaryotic TPP riboswitches found in plants and filamentous fungi also regulate gene expression by splicing mechanisms, albeit through the spliceosome rather than a self-splicing element (103). These natural examples reveal quite different mechanisms from artificial aptazymes that previously have been engineered by fusing an aptamer to a ribozyme to effect ligand-induced self-cleavage (12, 13).

Finally, riboswitches and sRNAs can work together. One example is the ethanolamine (EA) utilization (eut) locus of *Enterococcus faecalis* in which the EutX sRNA contains an adenosyl cobalamine (AdoCbl) riboswitch that, in the absence of AdoCbl (a cofactor required for EA catabolism), sequesters a binding site for the response regulator, EutV. EutV prevents transcription termination upon binding EutX; however, EA is required for EutV to be functionally activated (104). In this manner, both the presence of AdoCbl and EA are required to (i) release the EutV binding site and (ii) activate EutV to prevent transcriptional termination (104). In another example, two SAM riboswitches, SreA and SreB in *Listeria monocytogenes*, regulate gene expression not only in cis (via binding of SAM ligand and termination of transcription) but also independently in trans as sRNAs by binding to the 5'UTR of the mRNA target, *prfA*, to down-regulate expression of the virulence regulator (105). Such dual-acting regulatory RNA elements should find applications in constructing more complex regulation networks in synthetic biology.

3. COMPARISON OF SYNTHETIC BIOLOGY APPLICATIONS FOR sRNAS AND RIBOSWITCHES

Even as regulatory RNA functions in native systems continue to be elucidated, a plethora of applications of ncRNA have already been demonstrated, although it is not possible to provide a comprehensive list here. Earlier sRNA applications focused on metabolic engineering and earlier riboswitch applications focused on orthogonal gene regulation. However, recent approaches have expanded the use of ncRNAs for novel *in vivo* applications such as characterization of cellular RNA-RNA interactions, cellular metabolism, and signaling, and construction of synthetic logic gates (Figure 3).

sRNAs are primarily known to interact through antisense interactions with a target mRNA, so a native or synthetic sRNA can be designed to bind and regulate desired mRNA targets. Although native sRNAs are frequently useful (especially in an overexpressed or deleted manner), these efforts are primarily used to alter an sRNA's native pathway in its native organism. To modulate other targets, or use an sRNA in another organism, synthetic sRNAs are frequently designed. While some sRNAs (e.g. DsrA, MicF) are uniquely portable in their ability to be utilized in other organisms (106, 107), many native sRNAs cannot easily be transported between different organisms. Portability of sRNAs can be enhanced by an alternative approach that incorporates specific seed regions of sRNAs into an unrelated sRNA and maintain the original targeting, despite the new RNA context (108, 109). As

many sRNAs are associated with cellular factors (e.g. Hfq), design of sRNAs should account for the effect (aid or hindrance) of these factors in the system (93, 107). Additionally, interaction of the sRNA with other mRNAs or sRNAs in a competitive manner should also be considered and decoy sequences should be avoided (94). Often the accessibility (ability of RNA to interact with another RNA) of the sRNA and RNA target should be considered in design to ensure that the sRNA and target will be able to functionally interact (2, 3, 33). High-throughput screening of a large set of synthetic sRNAs has also proved useful at designing sRNAs for target regulation (110–112). Factors to consider are summarized in Table 1, but for detailed discussion on synthetic sRNA engineering strategies, please see ref. (2, 3, 107, 113, 114).

Thus far, synthetic sRNAs have been derived *de novo* from artificial libraries (110, 111, 114), by modularizing and re-designing natural sRNA scaffolds (106, 107, 112, 115), and by rationally engineering the affinity with its targets (116). Two specific examples are the rational engineering of altered affinity of CsrB to CsrA (and thus the downstream gene expression of CsrA targets) (116) and the modification of the DsrA scaffold to switch its targeting regions (for *rpoS* and *hns* in *E. coli*) to a heterologous target from γ -butanol synthesis in *Clostridium acetobutylicum* (106). However, it is important to note that while these methods may have the advantage of a direct target choice, synthetic sRNA systems might not be as robust as native regulatory systems.

By comparison, riboswitches are known to confer small molecule-dependent control of gene expression, so a natural or synthetic riboswitch can be placed downstream of a native promoter to regulate the target transgene in cis. For metabolic engineering or metabolite reporter/biosensor applications, typically an endogenous metabolite is the target of interest, so synthetic riboswitch designs in these cases have focused on expression platform engineering to convert from a turn-off to a turn-on system (and vice versa), to implement a different mechanism of gene regulation or biosensor output, or to adapt a riboswitch to function between different organisms. For orthogonal gene regulation, the goal is usually to utilize a chemical-inducible riboswitch in place of a chemical-inducible promoter to control gene expression, since the latter requires introduction of an orthogonal transcription factor. Thus, the synthetic designs have focused on riboswitch aptamer engineering to reprogram the ligand binding pocket to bind nonnative, cell permeable small molecules. Alternatively, the *in vitro* selected aptamer for theophylline (117) has demonstrated high portability for orthogonal gene regulation in many different bacteria, although in almost every case, optimization of the expression platform through screening has been necessary (118, 119). Thus far, synthetic riboswitches have been derived via rational or structure-based design (120, 121), medium-throughput selection or screening strategies (118, 122), and computational modeling (92, 123). Factors to consider are summarized in Table 1, but for detailed discussion on riboswitch engineering strategies, please see ref. (1, 5).

3.1. Applications to metabolic engineering

3.1.1. sRNAs provide network-level manipulation of complex phenotypes—A

unique characteristic of sRNAs is their ability to regulate multiple mRNA targets in response to changes in environmental conditions. This provides an excellent building point for

metabolic/network engineering by being able to control entire genetic circuits with one sRNA. Additionally, sRNAs have been determined to be useful regulators in less conventional organisms (such as many industrially relevant strains) where genetic manipulation and understanding of regulatory mechanisms are less available. Moreover, examples in this section involve engineering further robustness for stress tolerance of a specific environmental threat (i.e. acid, ethanol, butanol, overproduction of a compound) that arise in biotechnology applications.

For example, *Clostridium acetobutylicum* or *Zymomonas mobilis*, two model organisms for acetone-butanol-ethanol (ABE) fermentation and ethanol and/or farnesene production, respectively, do not have many genetic tools available for metabolic engineering efforts or traditional transcriptional modifications. However, deletion and overexpression of sRNAs (both native and heterologous) have proven to be useful tools to improve product yields in these organisms (124–126). Likewise, manipulation of well characterized sRNAs (DsrA, ArcZ and RprA) has led to improved acid stress resistance in *E. coli* (95). For many of these systems, discovery of important sRNAs for the desired phenotype is often in conjunction with development of new strains. Typically, analysis of transcriptome changes under desired stress or production conditions reveals native sRNAs that could be manipulated (by deletion or overexpression) to enhance stress resistance or production of desired product. Recent examples of this method of sRNA-based metabolic engineering by overexpression of native sRNAs include increasing butanol tolerance in *Clostridium acetobutylicum* (127), ethanol and butanol tolerance in cyanobacterium *Synechocystis* sp. PCC 6803 (128) and *Zymomonas mobilis* (126), and steroid intermediate production in *Mycobacterium neoaurum* (129). Deletion of sRNAs that interfere with the desired pathway, or produce undesired phenotypes or growth rates, has also become a useful engineering method to ensure high production of final products (such as subtilisin in *Bacillus licheniformis* (130)).

Although many sRNA-based systems utilize native sRNAs, synthetic sRNAs can be designed to interact and regulate a specific target; alternatively, a conserved sRNA can be adapted for use in another organism. For example, the *E. coli* sRNA system IS10 RNA-IN/OUT regulator was adapted for use in *Synechococcus* sp strain PCC 7002 to achieve 70% knockdown of the desired mRNA target (131). Another example utilized an inducible *E. coli* sRNA and Hfq construct to knockdown UDP-glucose pyrophosphorylase (UDPPase) which is involved in cellulose synthesis to permit fine-tuning of cellulose production (132). The addition of Hfq with the heterologous sRNA is capable of improving efficiency of targeted gene knockouts. This has been demonstrated by the use of an *E. coli* MicC scaffold in combination with expression of *E. coli* Hfq in *C. acetobutylicum* PJC4BK to increase butanol production (124). This system in *C. acetobutylicum* was found to be more efficient than other asRNA knockdown systems lacking Hfq (124). In a similar way, synthetic sRNAs based on known scaffolds have been designed to improve tyrosine production in *E. coli* (115).

3.1.2. Riboswitches control metabolic flux to favor metabolic production—

Riboswitches also have been applied to control metabolic flux to maximize the yield of target products. For example, an industrially relevant lysine-fermenting bacterium *Corynebacterium glutamicum* was engineered for improved lysine yield. In this organism,

oxaloacetate (OAA) can either be converted into the desired product lysine by a multi-step biosynthesis pathway or into citrate by citrate synthase in the TCA cycle. To redirect the citrate synthase-mediated metabolic flux to favor lysine production, a natural lysine-OFF riboswitch from *E. coli* was inserted into the genome to regulate expression of the citrate synthase gene. The net effect of this engineering is to titrate expression of citrate synthase in response to lysine levels, which led to an increase of lysine yield by 63% (133). A further 21% improvement in yield was achieved by engineering the *E. coli* lysine-OFF riboswitch into a synthetic lysine-ON riboswitch and inserting it into the *C. glutamicum* genome to regulate expression of a natural lysine transporter (134). This engineering released the feedback inhibition that lowers lysine yield.

3.1.3. Riboswitches as sensors for screening enzymes or strains to favor metabolic production—

Another application of riboswitches in metabolic engineering is to act as sensors to screen for enzymes or strains that give higher yield of a target product. For instance, to improve lysine fermentation efficiency, a natural *E. coli* lysine riboswitch controlling a tetracycline resistance gene was used to select for a chimeric aspartate kinase that was not feedback inhibited by the end-product, lysine (135). For strain engineering, a similar riboswitch-regulated selection cassette was used to select for pathway-optimized *E. coli* strains from a library of 10^9 - 10^{10} variants with different expression levels of phosphoenolpyruvate carboxylase, an enzyme involved in OAA biosynthesis and thus lysine production (136). Another example of strain engineering used an engineered *E. coli* strain as a cell-based reporter to screen a *Bacillus subtilis* production strain library for riboflavin production. The *E. coli* reporter strain carries an engineered riboswitch-ribozyme based on the endogenous *E. coli* FMN riboswitch to turn on GFP expression in response to FMN. By co-encapsulating *E. coli* reporter cells with individual *B. subtilis* cells in nanoliter reactors, the entire production strain library could be sorted using fluorescence to correlate to levels of released riboflavin. The winner *Bacillus* strain displayed up to 150% improvement in riboflavin production compared to the industrially optimized parent strain (137).

3.2. Applications of riboswitches to chemically-inducible expression systems

Protein-based chemically-inducible expression systems, such as the IPTG-inducible *lac* operon or the arabinose-inducible *araC* promoter, have proven to be important tools for conditional gene expression in bacteria. However, such protein-based systems are cumbersome to implement in new species due to the requirement of multiple protein factors. Furthermore, protein-based systems also respond to environmental changes and/or cellular stress, which make them not entirely bio-orthogonal. By contrast, riboswitches do not require extra biomolecular factors and can be encoded within a few hundred nucleotides. To engineer riboswitch-based inducible expression systems, one strategy is to reprogram the ligand specificity of the aptamer domain of natural riboswitches. For example, screening structure-based mutants of purine riboswitches with a small library of purine analogues resulted in orthogonal riboswitches that respond to PPDA and PPAO, respectively (138, 139). The PPDA riboswitch called M6'' was used to control *cheZ* expression and caused dose-dependent change in cell motility in *E. coli*. A similar strategy applied to PreQ₁ riboswitches identified a C17U riboswitch mutant that selectively responds to DPQ₀, a

diamino analog of PreQ₀. The DPQ₀ riboswitch was used for chemically-inducible regulation of a gene involved in cell morphology in *B. subtilis*(140).

An alternative strategy involves engineering *in vitro* selected RNA aptamers into functional riboswitches in live cells. One of the most successful examples is the theophylline aptamer, due to the stability, cell permeability and bio-orthogonality of theophylline and the specificity of the aptamer (118, 119, 141). In particular, synthetic theophylline riboswitches with engineered expression platforms to control ribosome binding have enabled research studies in a number of bacterial species that otherwise lacked inducible expression systems, including *Streptococcus pyogenes*, *Francisella*, *Mycobacteria tuberculosis*, *Streptomyces coelicolor*, *Cyanobacteria*, and *Bdellovibrio bacteriovorus* (142–149). However, more orthogonal riboswitches are still needed as they can have broad applications in the study and engineering of non-model organisms in particular.

3.3. Applications to studying biological systems

3.3.1. Utilizing sRNA-based antisense interactions for in vivo molecular studies—Recent techniques have utilized mimicking the natural mechanism of sRNA-mRNA binding for fundamental molecular characterization studies in cells. In these systems, antisense RNA probes that bind a specific region within a target RNA of interest are used to understand RNA-RNA interactions *in vivo*. Using a similar logic as split-protein complementation (150), researchers developed a Split-Broccoli system, adapted from the dye-binding aptamer Broccoli and a three-way junction RNA. RNA-RNA interactions can be detected *in vivo* by co-expression of the predicted RNA interacting partners as fusions with the two parts (top and bottom) of the Split-Broccoli system (151). Validation of this system with a RNA Toehold Switch demonstrated that both binding of the RNAs (visualized by the Broccoli fusion) and translational regulation (by an mCherry fusion with the toehold portion of RNA) could be observed from the single construct with relatively low background fluorescence (151).

Another method has been demonstrated to characterize the interactions of an entire RNA molecule. The *in vivo* RNA Structural Sensing System (iRS³) utilizes a fluorescent reporter based system to determine tertiary interactions of a RNA molecule *in vivo* based on *in vivo* hybridization profiles (152). To determine RNA-RNA interactions, a set of antisense probes are designed that cover the RNA sequence space. These probes are then attached to a fluorescence reporter such that upon binding of the probe the RBS of a fluorescent reporter is released and thereby can be measured by an increase in fluorescence. Through a set of probes the entire accessibility of an RNA molecule can be determined and linked to its structure and predicted RNA-RNA interactions (152). Currently, this method has been demonstrated to contribute to further understanding the *Tetrahymena* group I intron *in vivo* (152) and the accessibility of CsrB regions to CsrA (33). Utilization of this method has also provided a biophysical model to predict sRNA-mRNA binding interactions by considering the availability of RNA interactions using the suboptimal structures (33).

3.3.2. Using riboswitches as in vivo reporters and biosensors for target small molecules—Riboswitches can function as reporters or be engineered as fluorescent

endogenous genes (165). A similar approach of using antisense interactions to regulate circuits involves designing synthetic RNAs to disrupt transcription terminators placed upstream of reporter genes (166). These small transcription activating RNAs (STARs) are capable of creating diverse RNA-only logic gates (166).

Natural riboswitches function as basic computational units for a complex bio-computation network in the living organism. The input of ligand concentration is converted by the riboswitch into gene expression (ON or OFF) as an output signal. By combining these basic units, higher-level architectures such as Boolean logic gates can be constructed. Some natural examples of such riboswitch-based logic circuits have been presented in section 2.4. Inspired by natural tandems, theophylline riboswitches engineered to regulate transcription anti-termination were placed in tandem to decrease leaky expression. The resulting expression was less leaky, but maximum expression level was compromised (167). In another study, previously engineered theophylline and tetracycline riboswitches were arranged in tandem to construct an AND logic gate that should activate gene expression only when both ligands are present. Interestingly, it was found that the sequence order of the two riboswitches affected the performance of the logic gate (168). AND and NAND Boolean logic gates were also realized via a cell-based selection using a theophylline aptamer followed by a TPP riboswitch (169). For the AND logic gate, ligand-dependent conformational change of the theophylline riboswitch rearranged the TPP riboswitch to allow for TPP binding, which reorganizes the downstream expression platform to initiate translation. Therefore, an 18-fold turn-on of gene expression requires high concentrations of both theophylline and thiamin. These exciting examples suggest that riboswitches can be powerful building blocks for constructing complex logic networks. However, it should also be noted that the current way to design a riboswitch logic gate is not yet fully rational. Semi-rational cell-based screen or selection methods still play an important role in developing such logic circuits. It is expected that growing understanding of natural systems and experience gained from engineering studies will help provide general rules for designing such complex RNA-based computational networks.

4. CONCLUSIONS AND FUTURE HORIZONS OF RNA ENGINEERING

In this chapter we have discussed the history of regulatory RNA discovery and innovations, giving insight into current discoveries and recent applications. Regulatory RNAs are expected to expand as useful tools for synthetic biology applications (Figure 3), even beyond the applications demonstrated so far. For example, we envision novel synthetic systems being developed for medicine and nanotechnology. While not discussed here in detail, the inspiration of sRNA-based regulation in bacteria has already been utilized in many fields, including CRISPR systems (reviewed in (170, 171)). Regulatory RNAs have also been described as key points for antibiotic targeting in bacterial infections as several elements of virulence are controlled by sRNAs (reviewed in (172); (173)) and compounds targeting riboswitches have demonstrated antibiotic activity (174). Recently, insights from RNA folding have led to an entire new field of RNA nanotechnology for biological applications (175). While many of the applications are in eukaryotes, the basis of RNA-based interactions and folding remains the same as in bacteria and can serve as inspiration for a wide variety of applications. These studies have produced examples of RNA structures such as lattices and

tubular structures (176), triangles from a tetra-uracil motif (177), and catalytic triangles and squares based on the Tetrahymena group I intron (178). Construction of these elaborate RNA origami structures has been largely aided by computational methods that model the structures formed by RNA interactions (123), and it is anticipated that next steps in this field would incorporate functional regulatory elements like sRNAs and riboswitches. Additional applications include biocontainment (179) and use of logic gates for diagnostics. An example of the latter has already been reported using toehold switches in the context of paper-based diagnostics to detect viral RNAs (180). It is therefore expected that elucidation of the mechanistic diversity of sRNAs and riboswitches would lead to improved applications in these areas spanning biotechnology and medicine.

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Figure 1.

Timeline of sRNA and riboswitch discovery including relevant technological advances that aided identification and verification of regulatory RNAs. The explosion of sRNA and riboswitch discovery in *E. coli* and nonmodel organisms was frequently at points of technological advancement such as around 2001 with the development of deep-sequencing techniques. However, although identification of sRNAs and riboswitches has rapidly expanded, verification of function still lags behind.

Figure 2.

General function of sRNAs (A-D) and riboswitches (a-f). sRNAs regulate gene expression in trans through several functions enacted by antisense interactions, including transcription attenuation/enhancement through interactions with the RNA polymerase (A), inhibition of protein or ribosome binding either indirectly (B) or directly (C), and sequestration of protein factors (such as CsrA) (D). Riboswitches regulate gene expression in cis through a ligand-induced conformational change in the expression platform. The resulted gene expression consequences include rho-dependent/independent transcription termination (a, b),

transcription antitermination (c), translation activation (d)/inhibition (e) and mRNA degradation (f).

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Figure 3. Examples of applications of sRNAs and riboswitches. Applications of these regulatory RNAs stem from the base characteristics of the RNA function (antisense interactions for sRNA and ligand binding for riboswitches). Recent applications of these systems have begun to interweave these mechanisms to provide more complex engineering strategies.

Table 1

General Considerations for Synthetic Design.

Factors	sRNAs	Riboswitches
Protein Factors	Several, but not all, require protein chaperone Hfq ¹ ; Hfq scaffolds can be added to synthetic sRNAs	No chaperone required, but interacts with transcriptional or translational machinery ^A
Length	50-500 nt with ~12 nt “seed” region for binding mRNA target	~30-120 nt for aptamer domain, variable for expression platform
Sequence complementarity to target/Structural considerations	Minimal mismatches in sRNA seed binding region	Accurate secondary structure model is critical; tertiary structure is most helpful ^B
Target/ligand recognition	Specificity for desired mRNA target(s) to minimize off-target effects	Specificity for desired ligand to minimize off-target binding to other cellular metabolites
Cellular stability	Designs that avoid recognition by intracellular RNases	Designs that fold stably in cells
Kinetics of binding	Short or long-term interaction with target RNA depending on application ²	Ligand on-rate vs thermodynamic binding stability depending on regulatory mechanism ^C
Design methods to alter targets/ligands	Modify the sRNA binding region and location on mRNA target; by modification of sRNA scaffolds (through redesign/transport of sRNA seed regions) or high-throughput screening of synthetic sRNAs	Structure-guided mutagenesis, computational modeling, or high throughput screening/selection

Comparison of general factors to be considered in synthetic applications of small regulatory RNAs and riboswitches.

¹The “requirement” for Hfq chaperone is typically species and RNA dependent and both presence and absence of Hfq should be tested in application.

²Additional design aspects include utilizing different promoter strengths to express the sRNA of interest at varying levels, and inducible promoters to alter the timing of expression.

^ARiboswitch-based fluorescent biosensors do not function via gene regulation.

^BWhile computational modeling of 3D RNA structure is advancing rapidly, *de novo* prediction of small molecule ligands has not yet been achieved.

^CKinetics of ligand binding is important for transcriptional control, whereas thermodynamic stability is important for translational control.