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Review Article

Regulatory RNAs in *Bacillus subtilis:* A review on regulatory mechanism and applications in synthetic biology

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ABSTRACT

Bacteria exhibit a rich repertoire of RNA molecules that intricately regulate gene expression at multiple hierarchical levels, including small RNAs (sRNAs), riboswitches, and antisense RNAs. Notably, the majority of these regulatory RNAs lack or have limited protein-coding capacity but play pivotal roles in orchestrating gene expression by modulating transcription, post-transcription or translation processes. Leveraging and redesigning these regulatory RNA elements have emerged as pivotal strategies in the domains of metabolic engineering and synthetic biology. While previous investigations predominantly focused on delineating the roles of regulatory RNA in Gram-negative bacterial models such as *Escherichia coli* and *Salmonella enterica*, this review aims to summarize the mechanisms and functionalities of endogenous regulatory RNAs inherent to typical Gram-positive bacteria, notably *Bacillus subtilis*. Furthermore, we explore the engineering and practical applications of these regulatory RNA elements in the arena of synthetic biology, employing *B. subtilis* as a foundational chassis.

1. Introduction

Regulatory RNAs are recognized as ubiquitous and functionally diversified post-transcriptional regulator of gene expression in both prokaryotes and eukaryotes [1]. They participate in many cellular physiological processes, such as biofilm formation, ion homeostasis, metabolism regulation, anti-toxification, pathogenesis [1,2]. Regulatory RNAs found in prokaryotes are categorized as riboswitches, small non-coding RNAs (sRNAs), antisense sRNAs etc [2]. The regulatory RNAs vary in length and function through distinct mechanisms.

Riboswitches are typical regulatory RNAs in 5′ or 3′ untranslated region (UTR) of mRNA (messenger RNA). It could bind to specific small molecules (ligands) and regulate gene expression through changes in secondary structure of the mRNAs and thus the binding of the ribosomes [3,4]. sRNAs are typically *trans*-encoded regulatory RNAs with an average length of 50~300 nt [5] and interact with multiple target mRNAs by imperfect base pairing, causing mRNA degradation or translation blocking. Antisense RNAs engage in extensive base-pairing interactions with the complementary mRNA, as they are transcribed from the DNA strand opposite to that encoding the mRNA and can span from ten to thousands of nucleotides in length [6].

In addition to regulating their natural targets, regulatory RNAs such as riboswitches and sRNAs have been structurally redesigned and developed as efficient and independent regulatory tools in prokaryotes to regulate non-natural target gene expression via the canonical or noncanonical regulation mechanism [7–16]. CRISPR RNAs (clustered regularly interspaced short palindromic repeat RNAs), essential components of the bacterial innate immune system against bacteriophage, guide Cas proteins (CRISPR-associated proteins) to targeted DNA or RNA sequences. They were endowed with regulatory function in the CRISPRi (CRISPR interference) scenario [17–20]. The interference of CRISPRi is mediated by both deactivated Cas protein and non-coding RNA, controlling the mRNA generation of the target genes [21]. Considering only a short transcript is required, the sRNA regulation systems could be quickly constructed. Comparing to CRISPRi, some engineered or *de novo* designed sRNA have less polar effect on regulating polycistronic mRNA expressions [8,22]. Besides, the expression of the deactivated Cas protein may impose a larger metabolic burden than

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sRNA.

Till now most research on regulatory RNA primarily focused on Gram-negative bacteria [23], such as *Escherichia coli* and *Salmonella enterica*. Compared to their counterparts in Gram-negative bacteria, regulatory RNAs in *B. subtilis*, which is an important chassis in synthetic biology, are less explored. Here we focus on riboswitches and sRNAs in *B. subtilis*, summarizing endogenous RNA regulatory mechanism, analyzing design methods of artificial RNA devices and predicting future development of artificial RNA tools for application in the field of synthetic biology and metabolic engineering. Although CRISPR RNAs/gRNAs are involved in the regulation of gene expression when applied to CRISPR interference, they do not fall into the category of classic regulatory RNAs and thus are not within the scope of discussion for this review.

2. Riboswitch in *B. subtilis*

2.1. Regulation mechanism of riboswitches

Riboswitches are predominantly located in 5′ UTR of mRNA and act in *cis*, consisting of an aptamer domain to sense and bind target ligands and an expression platform that modulates the activation or repression of downstream genes [24]. Generally, riboswitches primarily regulate transcription in Gram-positive bacteria but translation in Gram-negative microorganisms [25,26]. Some riboswitches in Gram-negative bacteria lack intrinsic terminators; their termination is assisted by Rho [25]. Transcriptionally, riboswitches could mediate transcription anti-termination or termination after ligand binding. Transcription termination is categorized into Rho-dependent termination and Rho-independent termination. Rho-dependent termination requires binding of Rho (recognizes C-rich residue and unstructured RNA) to rut site (Rho utilization site) on mRNA [26]. Rho moves along mRNA until it meets and interacts with RNA polymerase (RNAP), leading to the dissociation of the transcription elongation complex and subsequent termination of transcription. In certain situations, riboswitch-mediated translational control and transcriptional control are coupled (Fig. 1A). When ligands bind to riboswitches, rut site would be exposed and RBS (ribosome binding site) is simultaneously sequestered. This dual action results in Rho binding and ribosome detachment [27]. Rho-independent termination requires an intrinsic transcription terminator consisting of a strong hairpin structure followed by poly-uridine residues [28]. Riboswitch-mediated transcription termination leads to formation of terminator and release of RNAP from DNA template and RNA transcript (Fig. 1B). Conversely, in Rho-independent transcription anti-termination, ligand-bound riboswitch sequesters the terminator, allowing RNAP to continue elongating through the DNA-RNA complex (Fig. 1C) [29]. Translationally, RBS could be obscured or exposed by secondary structure change of riboswitches in 5′UTR, dynamically controlling ribosome binding or detachment (Fig. 1D and E). For instance, the ribozyme-riboswitch *glmS* in *B. subtilis*, located in the 5′UTR, activates its self-cleavage activity upon binding to GlcN6P, leading to the degradation of mRNA [30] (Fig. 1F). In *B. subtilis*, all identified riboswitches regulate through Rho-independent transcriptional termination or anti-termination, translation inhibition and self-cleavage. Riboswitches could also exert control via Rho-dependent transcription termination or translation initiation in other prokaryotes [31–33].

2.2. Ligands of B. subtilis riboswitches

According to types of ligands, riboswitches are classified into metabolite riboswitches, tRNA riboswitches and protein riboswitches. Details of endogenous riboswitches from *B. subtilis* are shown in Table 1. Metabolites are the main source of riboswitch ligands. Elucidated metabolite riboswitch ligands in *B. subtilis* include Flavin mononucleotide (FMN) [34], S-adenosylmethionine (SAM) [35–37], adenine [38–40], ATP [41], c-di-AMP [42–46], lysine [47] and glucosamine-6-phosphate (G6P) [30,48]. T-box or tRNA riboswitches that selectively bind to a cognate tRNA regulate gene expression through transcription antitermination by binding uncharged tRNA (to which its

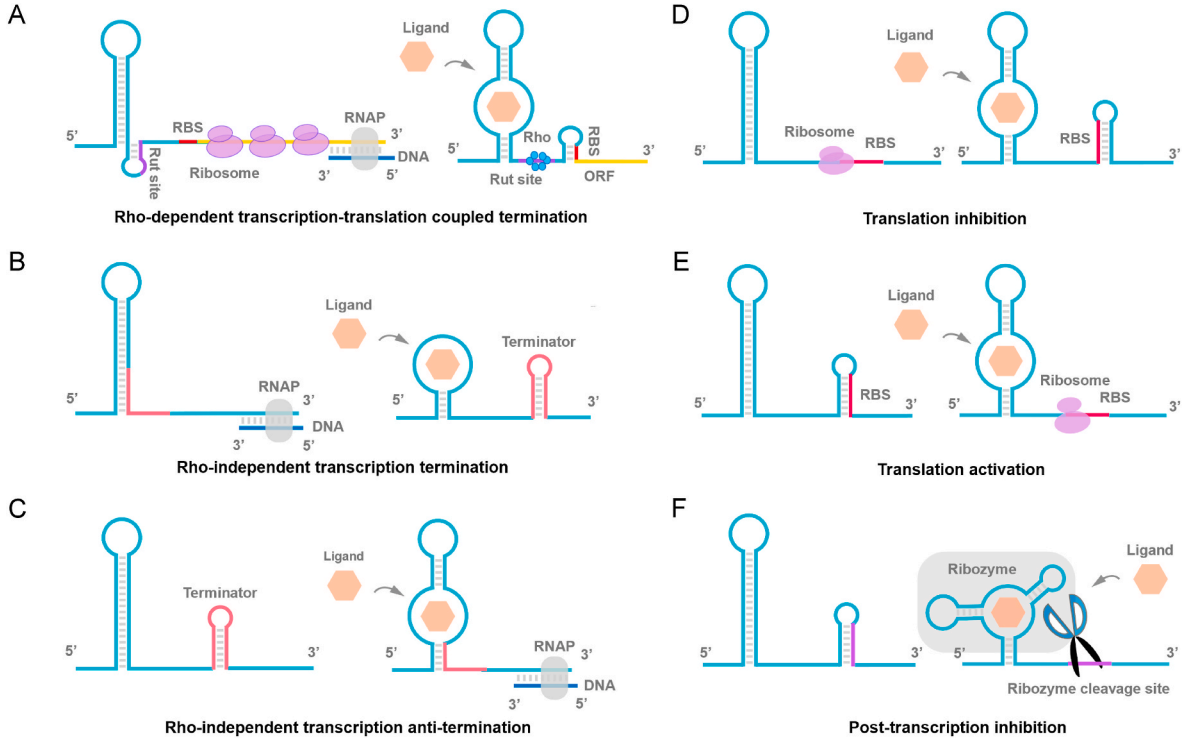

Fig. 1. Regulatory mechanism of riboswitch in prokaryotes. A. Rho-dependent transcription-translation coupled termination. **B.** Rho-independent transcription termination. **C.** Rho-independent transcription anti-termination. **D.** Translation inhibition. **E.** Translation activation. **F.** Post-transcription inhibition.

Table 1
Endogenous riboswitches in *B. subtilis*.

Type	Name	Ligand	Regulatory mechanism	Target	Binding position	Gene products
Metabolite riboswitch	FMN riboswitch	FMN	Rho-independent transcription termination	*ribD*	5′ UTR	5-Amino-6-(5-phosphoribosylamino) uracil reductase
	SAM riboswitch	SAM	Rho-independent transcription termination	*yitJ*	5′ UTR	Bifunctional homocysteine S-methyltransferase/5,10-methylenetetrahydrofolate reductase
	pbuE riboswitch	Adenine	Rho-independent transcription termination	*pbuE*	5′ UTR	Hypoxanthine efflux transporter
	xpt riboswitch	Guanine	Rho-independent transcription Termination	*xpt*	5′ UTR	Xanthine phosphoribosyltransferase
	ydaO riboswitch	ATP and c-di-AMP	Rho-independent transcription termination	*ktrA* and *ydaO*	3′ UTRs	Potassium uptake protein and putative amino acid transporter
	lysC riboswitch	lysine	Rho-independent transcription termination	*lysC*	5′ UTR	Aspartokinase II (alpha and beta subunits)
	glmS riboswitch	Glucosamine -6-phosphate	Self-cleavage	*glmS*	5′ UTR	Glutamine-fructose-6-phosphate transaminase
T-box riboswitch	*glyQS* riboswitch	Uncharged tRNA*Gly*	Rho-independent transcription antitermination	*glyQS*	5′ UTR	Glycyl-tRNA synthetase
	tyrS riboswitch	Uncharged tRNA*Tyr*	Rho-independent transcription antitermination	*tyrS*	5′ UTR	Tyrosyl-tRNA synthetase
Protein riboswitch	L13-S9 riboswitch	S13/S9	Translation inhibition	*rplM, rpsI*	5′ UTR	Ribosomal protein L13–S9
	L19 riboswitch	L19 protein	Translation inhibition	*rplS*	5′ UTR	Ribosomal protein L19
	L10(L12)4 riboswitch	L10(L12)4 protein	Transcription attenuation and antitermination	*rplJ, rplL*	5′ UTR	Ribosomal L10(L12) 4 complex
	L20 riboswitch	L20 protein	Rho-independent Transcription antitermination	*infC-rpmI-rplT*	5′ UTR	Translation initiation factor IF3 and the r-proteins L35 and L20
	S6–S18 riboswitch	S6–S18	Translation inhibition	*rpsF-ssbA-rpsR*	5′ UTR	Ribosomal proteins S6 and S18
	S4 riboswitch	S4	Rho-independent transcription termination	*rpsD*	5′ UTR	Ribosomal protein S4
	trp riboswitch	TRAP	Rho-independent transcription termination and translation inhibition	*trpEDCFBA* operon, *trpE, pabA, trpP* and *ycbK*	5′ UTR	Tryptophan biosynthetic enzymes
	glcT riboswitch	GlcT	Transcription antitermination	*ptsG*	5′ UTR	Glucose permease
	sacT riboswitch	SacT	Transcription antitermination	*sacPA*	5′ UTR	Phosphosucrase and sucrose-specific PTS permease
	SacY riboswitch	SacY	Transcription antitermination	*sacB*	5′ UTR	Levansucrase
	licT riboswitch	LicT	Transcription antitermination	*licS*	5′ UTR	β-1,3-1,4-endoglucanase
	pyrR riboswitch	PyrR-UMP complex	Transcription attenuation	*pyr*	5′ UTR	Transcriptional attenuator and uracil phosphoribosyltransferase
	glpP riboswitch	GlpP	Transcription antitermination	*glpP*	5′ UTR	Glycerol-3-phosphate dehydrogenase
	hutP riboswitch	Mg^{2+} ion and l-histidine	Transcription antitermination	*hutp*	5′ UTR	Transcriptional anti-terminator

cognate amino acid is not chemically bonded). In *B. subtilis, glyQS* [49–51] and *tyrS* [52,53] are regulated by tRNA. For some riboswitches their ligands are the proteins encoded by the downstream coding regions. For instance, the expression of some ribosomal protein genes are autoregulated through protein riboswitches [54], including L10(L12)4 [55], L13-S9 [56], L19 [56], L20 [57,58], S4 [58], S10 [59], and S15 [58]. These regulatory elements primarily serve to limit the accumulation of excessive unbound ribosomal proteins by suppressing the transcription or translation of downstream genes [55,58]. Tryptophan synthesis is also regulated by riboswitches responding to *trp* mRNA binding attenuation protein (TRAP, synthesis of TRAP is controlled by tryptophan) [60]. Utilization of alternative sugars is also regulated by riboswitches [61], the ligands of which belong to BglG family, including GlcT [62], SacT [63], SacY [64], and LicT [65]. There are other three riboswitches, PyrR [66], GlpP [67], and HutP [68] responsible for biosynthesis and uptake of nucleotides, glycerol-3-phosphate, and histidine utilization respectively.

3. Engineering of riboswitches in *B. subtilis* and their applications

3.1. Monitoring DNA mutation

Under harsh environmental pressure, *B. subtilis* sporulate to aid their survival. In biological production, sporulated cells have the potential to serve as time-delayed chassis for expression at specific time. Recently, sporulated cells have also been applied as vessels for DNA storage [69]. However, after DNA replication during late growth phase before sporulation or after continuous subculture, DNA mutation would happen (Fig. 2A). Denis Tamiev et al. created a DNA mutation monitor on plasmid based on theophylline riboswitch [70]. By monitoring fluorescence of riboswitch-controlled RFP (red fluorescence protein), DNA point mutation could be indirectly monitored (Fig. 2A). The length of riboswitch is always much shorter compared to coding gene, thereby exerting little metabolic pressure. This approach is significant for monitoring mutations of critical genes in industrial production, since mutation is a major issue in industrial culture.

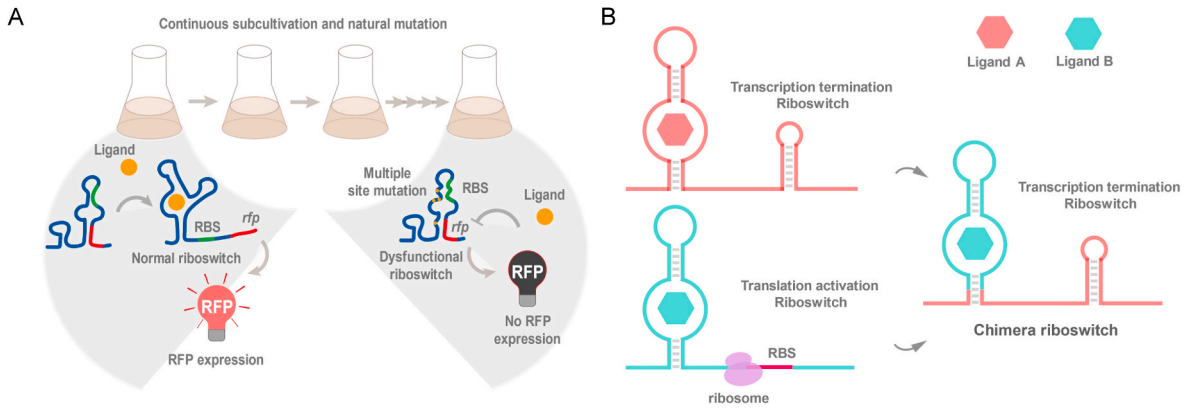

Fig. 2. Application of riboswitches in *B. subtilis*. A. Riboswitch DNA mutation monitor. **B.** Chimera riboswitch.

3.2. Transforming constitutive gene expression into inducible gene expression

Inducible systems, capable of turning genes on and off, are essential for biochemical expression in microbial production. But in *B. subtilis*, inducible systems are widely used, while the high price of inducers like IPTG or xylose limit their application in industry [71,72]. In that sense, alternative induction systems with low basal level, high induction rate and low-cost inducer need to be constructed. By inserting riboswitches in 5′UTR, constitutive systems could be reformed into inducible systems. Phan et al. discovered *gcv* operon controlled by glycine riboswitch in *B. subtilis* can be converted into an inducible expression-secretion system [72]. Furthermore, riboswitches could also strengthen inducible systems. For instance, inserting lysine or theophylline riboswitches downstream of an inducible promoter can increase the induction fold change up to a hundredfold [73].

3.3. Orthogonal regulation

Orthogonal regulation in synthetic biology refers to independent regulation of genes without interference with each other or the biological environment. This approach enables the design of more complex metabolic networks. There are two main strategies for achieving orthogonal regulation based on riboswitch.

The first strategy is designing analogues of riboswitch ligands. Regulation by analogues of natural ligands could mitigate influences on other metabolic reaction *in vivo*. Artificial analogues, synthesized through chemical reactions [74], could be designed to bind more tightly to aptamers [75,76]. While synthesizing new ligands is challenging, altering the nucleotide sequence of aptamers is comparably less complicated. Additionally, the inherent modularity of riboswitches lays the foundation for riboswitch engineering, allowing the exchange of aptamer domains and expression platforms between different riboswitches [77].

The second strategy is reforming riboswitches to recognizing new ligands, encompassing three kinds of approaches: 1) introducing mutation on aptamers to generate derivatives of riboswitches [78,79]; 2) utilizing exogenous riboswitches from other strains [79,80]; 3) designing chimera riboswitches hybridized by more than two different riboswitches [77,81,82], which could also alter regulation mechanism (Fig. 2B). These approaches facilitate interactions between different riboswitches and ligands. Such strategies are instrumental in creating complex gene circuits, enabling multiple simultaneous regulations or multistep cascading regulations.

4. sRNA in *B. subtilis*

4.1. Regulation mechanism of sRNAs

Most of the sRNAs discovered act post-transcriptionally or translationally by base-pairing with target mRNA. With the burgeoning discovery of the sRNA mechanisms in prokaryotes, sRNAs are found to work almost in all levels of gene expression.

Transcriptionally, sRNAs could mediate gene expression through transcription read-through (transcription could not stop normally at terminators). In bacteria, there are two kinds of transcription read-through. The first type of transcription read-through involves abnormal transcription termination of sRNA itself. This phenomenon, observed in both SR6 and SR7, is speculated as the result of inefficient transcription terminator [83,84]. The correlation between environmental stress and sRNA read-through has not been fully explained. After sRNA read-through, the range of target genes may be expanded. Another form of sRNA-mediated transcription read-through involves competition between sRNA and Rho of Rho utilization (rut) site (Fig. 3A). DsrA, ArcZ, and RprA in *E. coli* [85] and SraL in *S. enterica* [86] have been demonstrated to compete with Rho for a specific mRNA rut site. Rho-dependent termination was most discovered in enteric Gram-negative bacteria and relatively less known in Gram-positive bacteria [87]. But recent research have increasingly shown importance of Rho-dependent termination in Gram-positive bacteria [88]. However, competition between sRNA and Rho of rut site has not been clarified in *B. subtilis* yet.

Post-transcription regulation mediated by sRNAs is widespread in bacteria. Most of the post-transcription regulation are mediated by ribonuclease. However, due to the different ribonuclease repertoire among species, the mechanism of post-transcription differs [89–91]. In *B. subtilis*, sRNAs from type I TA systems (toxin-antitoxin systems) commonly regulate through post-transcriptional degradation. The degradation is mainly mediated by RNase III and assisted by RNase Y and RNase J1 (Fig. 3B). Until now, only four type I TA systems have been fully investigated in *B. subtilis*: *txp*/RatA [92], *bsrG*/SR4 [93,94], *bsrE*/SR5 [95,96], and *yonT-yoyJ*/SR6 [84] (Fig. 4). Those sRNAs from TA systems act as small antitoxin molecules and base-pair with toxin mRNA. With RNase III recognizing base pair region, the toxin-antitoxin complex would be cleaved by RNase III and further digested by RNase Y and RNase J1 [97] (Fig. 3B). Exceptionally, SR6 regulates *yoyJ* through translation inhibition [84]. Post-transcription protection is another way of regulation opposite to degradation. In *B. subtilis*, for example, when sRNA RoxS binds to 5' end of *yflS* mRNA, it prevents RNase J1 from degrading the mRNA (Fig. 3B) [98].

Translationally, sRNAs could also mediate translational inhibition by binding RBS on mRNA or by binding ribosome proteins (Fig. 3C). Base-pairing of sRNA and RBS is widely seen in bacteria, *yoyJ*/SR6 in *B. subtilis* [84], for example. Even though sRNAs are not found in

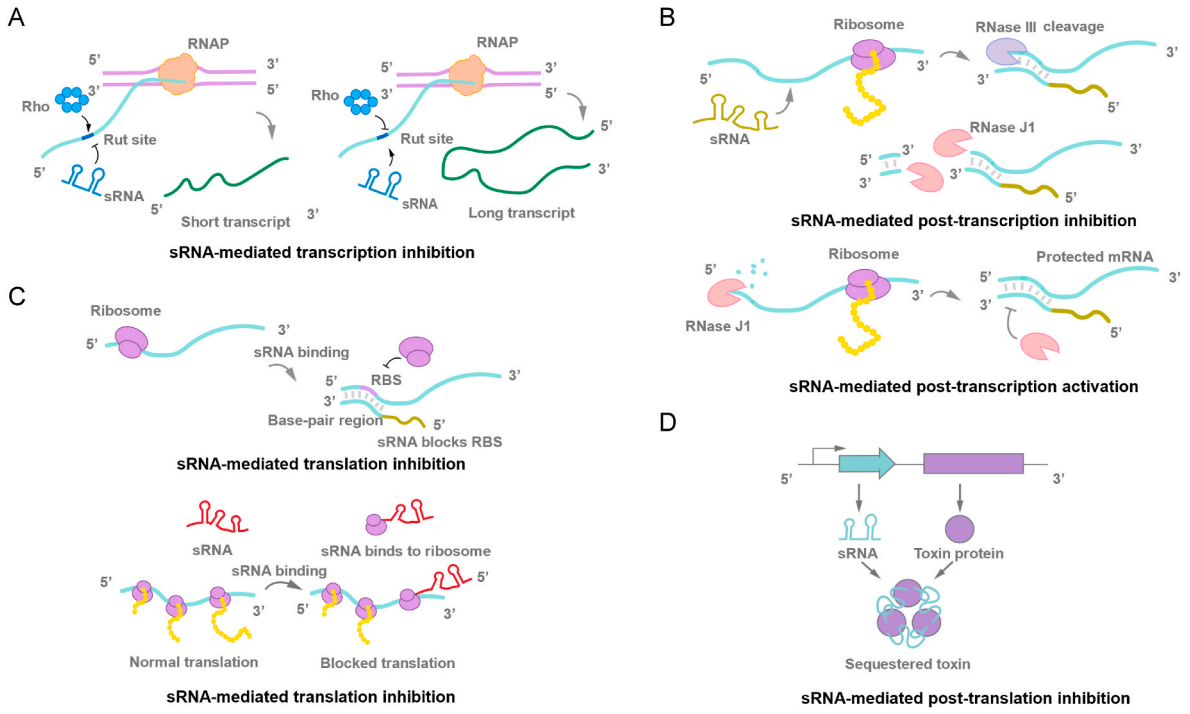

Fig. 3. Regulatory mechanism of sRNA in prokaryotes. A. sRNA-mediated transcription inhibition. **B.** sRNA-mediated translation inhibition. **C.** sRNA-mediated post-transcription inhibition. **D.** sRNA-mediated post-translation inhibition.

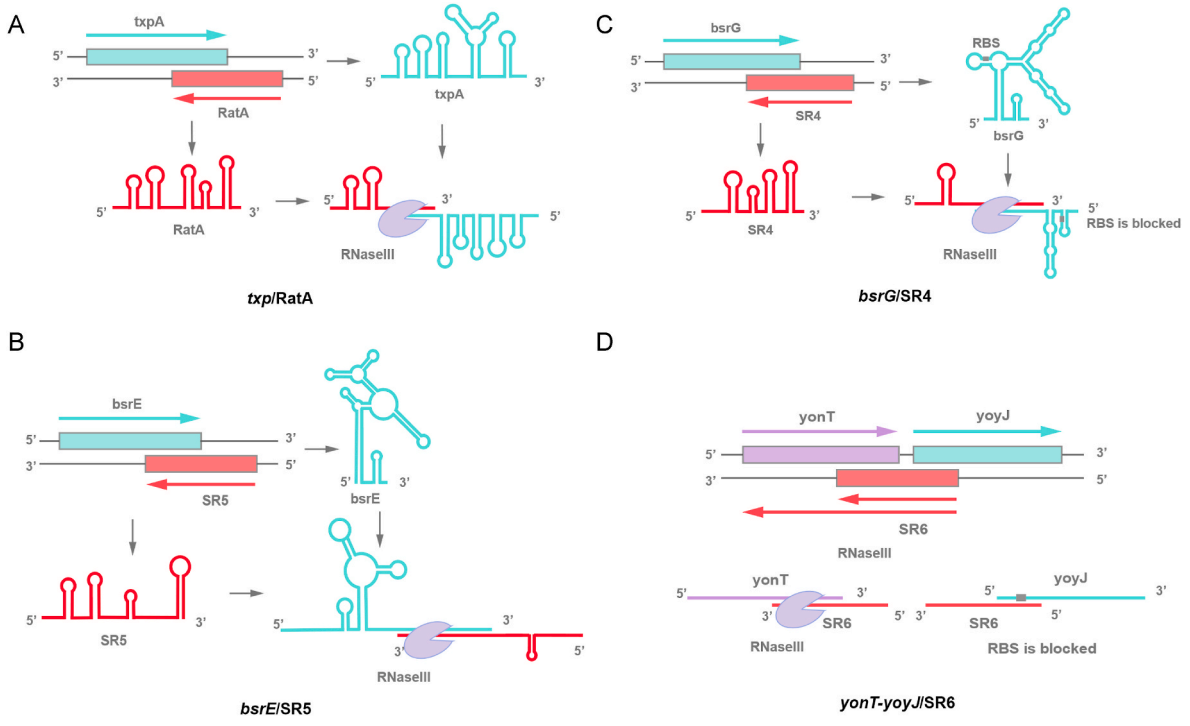

Fig. 4. sRNAs play an important role in toxin-antitoxin systems in *B. subtilis*. A. *txp*/RatA. **B.** *bsrG*/SR4. **C.** *bsrE*/SR5. **D.** *yonT-yoyJ*/SR6.

B. subtilis to bind ribosomes, SprF1, a ribosome-binding sRNA, is recently found in *Staphylococcus aureus* to block binding from ribosome to RBS, thus inhibiting translation [99] (Fig. 3C). Translational activation usually acts through opening up secondary structure near RBS. In *B. subtilis*, RosX binds to 5′UTR of *yflS* mRNA, protecting it from RNase J1 and stimulating 30S (ribosomal subunit) binding to RBS [98].

Post-translationally, sRNAs in type III TA system act as antitoxins by binding to toxin proteins and sequestering them by forming protein-RNA complexes [100] (Fig. 3D).

4.2. Classification of sRNAs in B. subtilis

The types and mechanisms of plenty endogenous sRNAs in *B. subtilis* are thoroughly characterized [101]. Here we provide a new perspective

based on the role of sRNAs in metabolism and physiological process (Fig. 5). Some characterized sRNAs in *B. subtilis* directly participate in physiological processes like transportation and sporulation. Others are actively involved in metabolic pathways regulations including arginine metabolism, iron metabolism and control of NAD^+/NADH balance. Additionally, some sRNAs are part of the immune system of *B. subtilis*, TA system, for example.

FsrA plays an important part in the tricarboxylic acid cycle (TCA cycle), down-regulating aconitase (*citB*) and succinate dehydrogenase (*sdhCAB*) post-transcriptionally [102]. FsrA also mediates repression of glutamate synthase, which serves as a vital link between central carbon metabolism and nitrogen metabolism. FsrA also represses dicarboxylate transporter (DctP), important for increasing TCA cycle intermediates. RoxS [98] is another important sRNA in TCA cycle. RoxS activates *yflS* by binding to the 5' end of the *yflS* mRNA with the C-rich region CRR3, protecting *yflS* (encoding a malate transporter) from RNase J1 and stimulating 30S binding to the RBS. RoxS also down-regulates several genes post-transcriptionally, including *ppnkB* (encoding NAD-kinase) and TCA components *sucC* (encoding succinate dehydrogenase) and *citZ* (encoding citrate synthase). Another sRNA, corroborated by electrophoretic mobility shift assays (EMSA) to interact with RoxS and FsrA, is called RosA [103,104]. The length of RosA varies in different species (225, 193, 128, or 92 nt), demonstrated to be the result of activity of endo- and exo-ribonucleases [103].

Arginine metabolism is regulated by at least two transcriptional regulators RocR [105,106] and AhrC. SR1 down regulates *ahrC* post-transcriptionally by targeting *ahrC* mRNA, which encodes a transcriptional activator of rocABC and rocDEF operon in arginine metabolism. Toeprinting studies and secondary structure probing of the *ahrC*/SR1 complex indicated that SR1 inhibits translation initiation by inducing structural change downstream from the RBS of *ahrC*. The interaction between SR1 and *ahrC* mRNA, facilitated by Hfq, has more than 7 base-pairing regions as predicted by computational analysis.

The iron-sparing response is regulated by sRNA FsrA in conjunction with three small basic proteins, FbpABC. FsrA, working alongside FbpABC, represses many "low-priority" iron-containing enzymes. The lactate-inducible lutABC operon encodes iron sulfur-containing enzymes required for growth on lactate. FsrA, together with FbpB, also represses the synthesis of the LutABC lactate oxidase enzymes [107].

In the process of sporulation, it has been demonstrated that SR1 targets *kinA* (a sporulation-specific ATP-dependent histidine kinase) mRNA. The deletion of *sr1* accelerates sporulation but results in lower spore quality [108]. SR1 inhibits the translation of *kinA* mRNA *in vivo,* but does not affect its stability. Research by Mars RA et al. has predicted that sRNAs S25, S31, S37, S526, S547, S623, S661, S1009, S1083, S1279, S1388, S1445 and S1559 could also be related to sporulation [54]. According to PhD thesis of Holly Hall, promoters of S357, S547, S612 and S849 are active during the early stages of the sporulation process [109]. These findings suggest that sporulation in *B. subtilis* could be significantly influenced by the activity of many sRNAs. The overexpression or knock-out of sporulation related sRNAs could also influence sporulation efficiency [109]. This understanding could be crucial for scaling-up production in industrial settings, potentially reducing the negative impact on the dormancy of spores.

In *B. subtilis*, sRNAs play a role in type I toxin-antitoxin (TA) systems as part of its immune mechanisms. The *txpA*/RatA system consists of the antitoxin sRNA RatA and toxin TxpA (59 aa). TxpA could lead to cell lysis in the absence of RatA [92]. The *bsrG*/SR4 system includes the

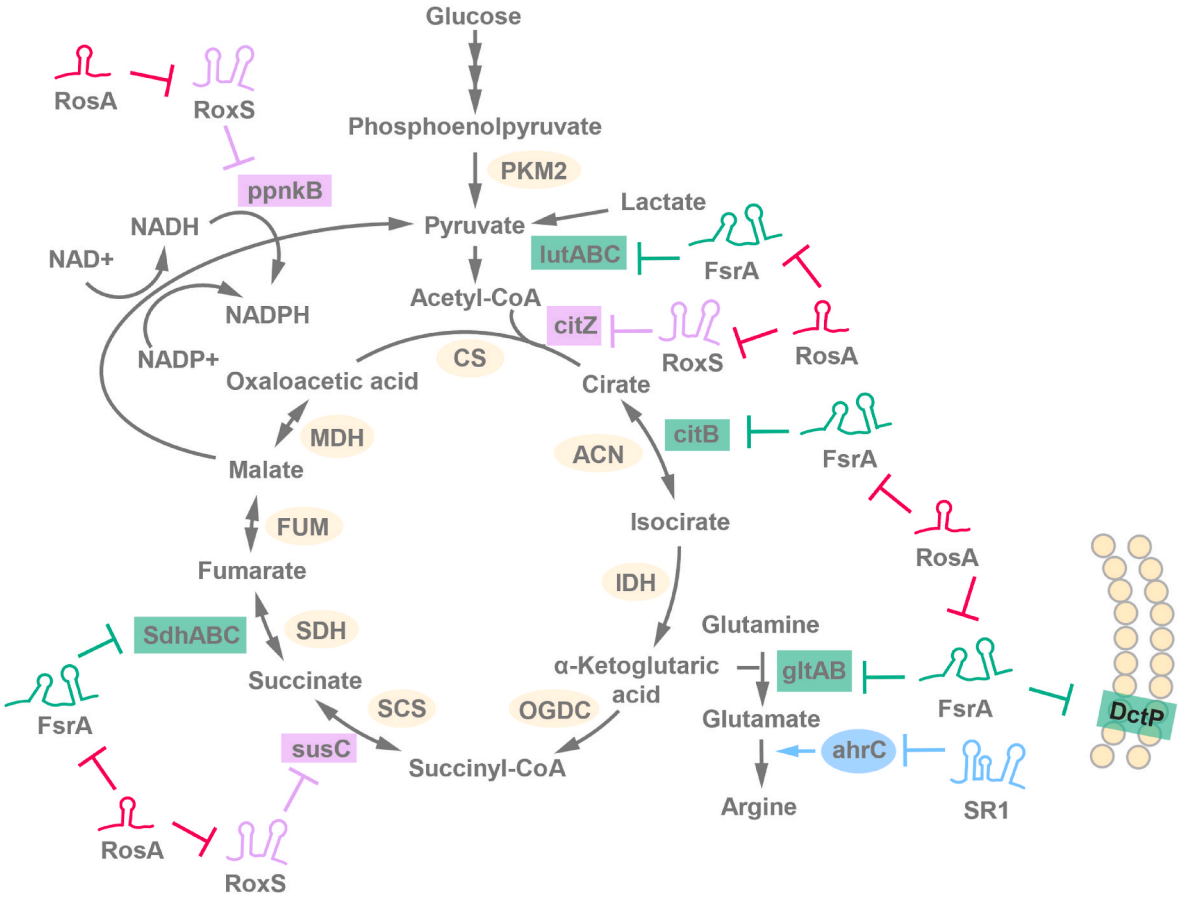

Fig. 5. sRNAs involved in the regulation of central metabolism in *B. subtilis*. GltAB: iron-sulfur-containing enzyme glutamate synthase; DctP: dicarboxylate transporter; CS: citrate (Si)-syn-thase; MDH: malatedehydrogenase; FUM: fumarase; SDH: succinate dehydrogenase; SCS: succinyl-CoA synthetase; OGDC: 2-oxoglutarate dehydrogenase complex; IDH: isocitrate dehydrogenase; ACN: Aconitase.

antitoxin sRNA SR4 and toxin BsrG (38 aa). BsrG causes cell wall defects, membrane invaginations, and altered cells shape in the absence of SR4 [93,94]. The *bsrE*/SR5 system includes the antitoxin sRNA SR5 and toxin BsrE (30 aa), where BsrE is less toxic than the other type I TA system toxins [95,96]. The *yonT-yoyJ*/SR6 system encodes antitoxin sRNA SR6 and two toxins YonT and YoyJ. YonT causes cell lysis, while YoyJ is weaker than YonT but is still detrimental in the absence of SR6 [84].

From the perspective of endogenous sRNAs' function in the physiological process, sRNAs play important roles in transportation, metabolism, sporulation, and the immune system to bacteriophage. According to function of base-pairing genes, predicted sRNAs in *B. subtilis* may also be involved in the replication of genes, cell wall biogenesis, ribosome synthesis, tRNA synthesis, two-component systems and so on [54]. Given the diverse regulatory mechanisms of sRNAs and the comprehensive understanding of their regulatory pathways. sRNAs are being engineered and utilized as efficient toolboxes in synthetic biology, enabling the fine-tuning of specific gene expression.

5. Artificial sRNA design and application

5.1. Transcription regulation with riboswitch-targeting sRNAs

Synthetic sRNAs could also play the role as transcription activator. Small transcription activating RNAs (STARs) have been previously designed to target transcription attenuators and riboswitches in *E. coli* [9,110]. Those sRNAs could pair with premature terminator and activate gene expression. This work achieved artificial sRNA-mediated transcription activation *in vivo* first time [9]. Lins et al. firstly implemented STARs in *B. subtilis*, called riboswitch-targeting sRNAs (rtRNA) [111]. By targeting at terminator stem-loop, rtRNAs activate gene expression by turning riboswitches into ON state (Fig. 6A). rtRNAs could both work *in vitro* and *in vivo*, increasing gene expression up to 103-fold [111]. This work effectively engineered a natural RNA transcriptional repressor as well as the ability to convert intrinsic terminators into transcription-on regulators. This also achieved sRNA-based metabolic regulation and RNA-only genetic networks *in vivo*. The simplicity of rtRNAs suggests that sRNA based transcription activation may be a natural mechanism of gene regulation waiting to be discovered in *B. subtilis* [9].

5.2. Post-transcription regulation with redesigned bsrG/SR4

According to the way of inhibition mechanism of base-pair, TA systems could be constructed into gene regulation tools without manipulating any protein. Post-transcriptionally, we have modified the TA system *bsrG*/SR4 in *B. subtilis* to a useful genetic tool, named as modulation via the small RNA-dependent operation system (MS-DOS) [112]. Operation region, a part of the toxin *bsrG* coding region, is required to insert after the stop codon of target genes. Base-pairing between operation region and SR4 triggers RNase III degradation of a complex of the target gene and SR4 (Fig. 6B) , achieving post-transcription inhibition over targeted genes. MS-DOS was verified by inhibiting *ftsZ* in *B. subtilis*, the cell of which was lengthened greatly because of abnormal cell division [112]. MS-DOS was also applied to regulate crucial genes in hyaluronan biosynthesis. Down-regulation of *pfkA* resulted in the highest hyaluronan titer (1.52 g/L) which was 1.6-fold of the parental strain [112]. Inhibition by MS-DOS could be more stable than sRNA regulation translationally, because MS-DOS introduce RNase III cleavage site. So orthogonal regulation of multiple genes could be easier using MS-DOS.

5.3. Translation regulation with redesigned yonT-yoyJ/SR6

Endogenous sRNAs could be modified and designed as useful metabolic regulation tool, requiring no genome-editing process. But not all endogenous sRNAs have the potential to be designed as robust tools to regulate target genes without destroying core scaffold [113]. There were a lot of examples of artificial sRNA systems in *E. coli*, finding out more native scaffold [114,115] or designing novel scaffold [116,117]. The sRNA system MicC-Hfq from *E. coli* was also successfully transplanted into *C. glutamicum* [118], but such translation regulation sRNA system was not established in *B. subtilis*. We have modified TA system *yoyJ*/SR6 in *B. subtilis* into regulatory tool acting translationally by base-pairing 24 nucleotides with mRNA starting from N-terminal coding sequence AUG (Fig. 6C). With minimized structure, SR6 was proven to maintain a strong repression activity of 83%. This artificial sRNA system was also

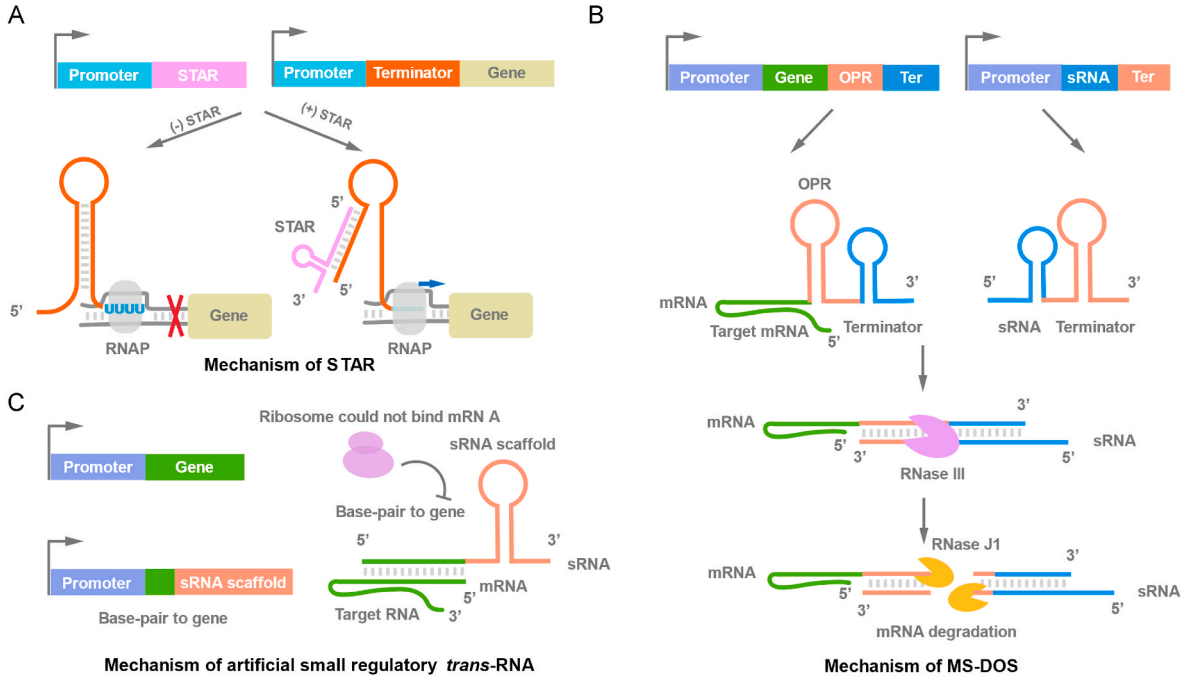

Fig. 6. Development and application of artificial sRNA in *B. subtilis*. **A.** Mechanism of small transcription activating RNAs. **B.** Mechanism of artificial small regulatory *trans*-RNA. **C.** Mechanism of Modulation via the small RNA (sRNA)-dependent operation system (MS-DOS). OPR: operation region.

applied in *E. coli*, demonstrated to have repression efficiency above 80%, which could function without Hfq, causing lower metabolic burden [119]. sRNAs with arbitrary sequences and fixed secondary structures were also designed by a *de novo* sRNA design program to match any gene of interest, which was demonstrated to be pretty efficient in down-regulating expression of *comER* and *ftsZ* and functioned well in acetoin biosynthesis regulation [119]. Arbitrary sequence gets rid of traditional fixed sRNA scaffold, making gene regulation by sRNA more customized. To regulate different genes under different situations, sRNAs with different inhibition efficiency could be chosen. Because of its convenience to construct, this method could also be applied to screen genes of interest through high-throughput screening [119].

For sRNA knock-down tools in diverse bacteria, Cho et al. designed broad-host-range sRNA system (BHR-sRNA system) base-on sRNA scaffold of RoxS from *B. subtilis* [120]. Translationally, BHR-sRNA system achieved knockdown of reporter genes in 12 strains out of 16 strains, with slight modification in each strain. This demonstrated that sRNA system with same mechanism could be applied in multiple species, which is pretty meaningful in *trans*-bacteria regulation.

6. Conclusions and outlook

6.1. Create riboswitches for detection of small molecules

Due to its versality and designability, riboswitches have potential to bind various small molecules. To develop aptamers recognizing small molecules, SELEX (Systematic Evolution of Ligands by Exponential enrichment) is a prominent method, not only selecting for binding but also structure changes on binding in aptamers. SELEX could be divided into *in vitro* SELEX [121,122] and *in vivo* SELEX [123–125]. However, after being selected under *in vitro* conditions, aptamers may lose activity under *in vivo* conditions [124]. Another problem is immobilization of both aptamers and targets, inevitably changing function of target compound [123,126].

With the progress of bioinformatics, *in silico* design of riboswitches is developing with a high speed and applicable in various kinds of strains. Designing RNA aptamers recognizing versatile molecules have immense practical importance. Riboswitches could be applied as small molecule detectors monitoring metabolism (Fig. 7A), since *de novo* riboswitch responsive to specific ligand could be designed according to thermodynamic and kinetic analysis [76,127].

6.2. Rapid identification of metabolic targets with sRNA library

As gene regulation tools, sRNAs and their libraries could be constructed more easily, offering higher efficiency and non-polar regulatory advantages when compared to CRISPRi or CRISPRa [8]. For example, we have constructed single-stem loop small non-coding RNAs (ssl-sRNA) library with predictable and programmable activities and applied to screen out gene candidate in complex metabolic pathway in *E. coli* [8]. Knocking-down of some genes in a specific metabolic pathway, related transporter genes or related regulators could increase production [8]. This method could be improved for rapid identification of metabolic targets. After constructing a sRNA library with one-pot PCR using multiple primers and selecting out transformants with high production, next-generation DNA sequencing would be carried out to find out which sRNA plays the role and then the down-regulated gene would also be found out [8] (Fig. 7B). Rapid identification could quickly select out critical genes and hugely shorten the time of metabolic engineering. This method could be also applied in *B. subtilis* and other strains.

6.3. Designing of toehold switch based on regulatory RNA

Toehold switches are *de novo* RNA engineering elements, consisted of two strand, a triggering RNA strand and a toehold-hairpin strand with regulated gene (Fig. 7C) [128]. After base-paring with trigger RNA, toehold-hairpin would be opened and RBS would be exposed, enabling ribosome binding. Compared with other regulatory RNA devices, toehold switches are highly modular, orthogonal and programmable [128,129]. Toehold switches could not only regulate gene expression [13,128], but also detect mRNAs, such as virus RNAs [130]. To date, toehold switches have primarily been developed and applied in *E. coli*. There is a likelihood that they could also be adapted for use in *B. subtilis* synthetic biology and even in diagnostics [131]. Furthermore, enhancing existing regulatory RNA devices in *B. subtilis* might involve incorporating novel features inspired by the engineering of other

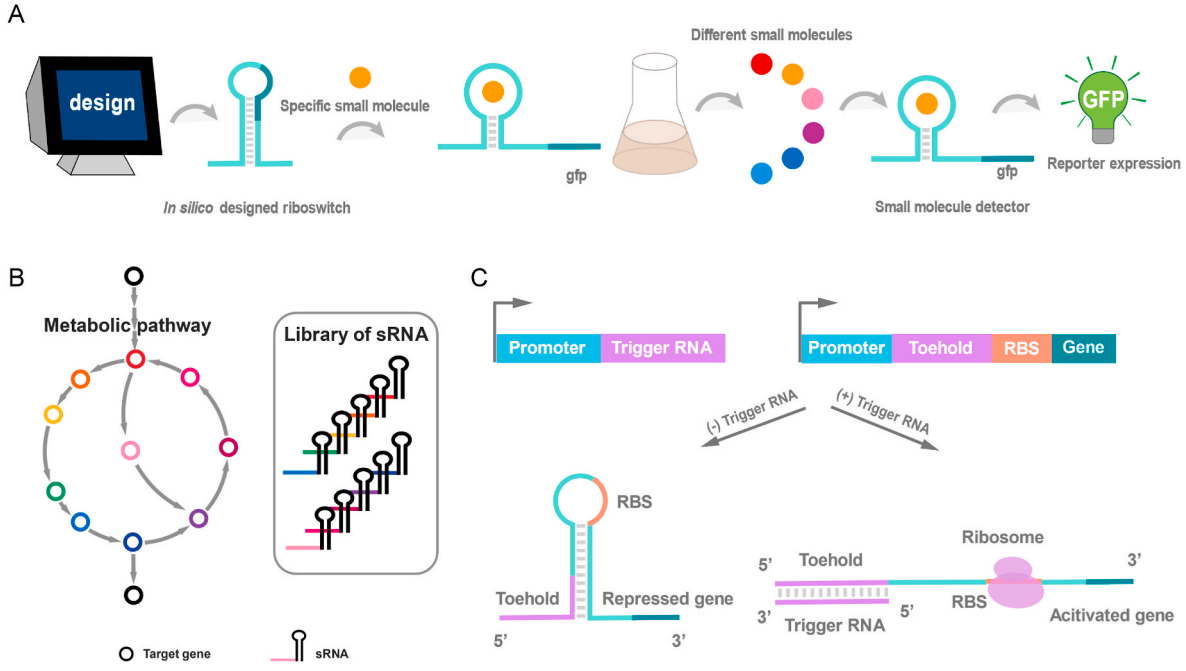

Fig. 7. Prospects for the future engineering and applications of *B. subtilis* regulatory RNA. A. Riboswitch small molecule detector. **B.** Rapid identification of metabolic targets with sRNA library. **C.** Designing of toehold switch.

regulatory RNAs.

Declaration of competing interest

There are no conflicts of interest to declare.

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