Strain engineering via regulatory noncoding RNAs: not a one-blueprint-fits-all

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Abstract

Increasing demands for efficient, sustainable chemical production continue to motivate engineering of microbial strains. Noncoding RNAs (ncRNAs) represent a class of powerful regulators of cellular processes and are emerging as significant tools for strain engineering, particularly for complex phenotypes. Current strategies include adjusting expression levels of natural ncRNAs and developing synthetic ncRNAs to target specific genes. In this review, we summarize and analyze the effectiveness of the design blueprints for a variety of natural and synthetic ncRNA systems recently reported in the literature to offer a concise and easy to read engineering guide to exploiting trans-regulatory ncRNAs for strain engineering.

Introduction

With rising demands of efficiency, environmental care, and sustainability, the use of microbes as chemical factories is increasingly attractive. As regulatory noncoding RNAs (ncRNAs) continue to be discovered in organisms of traditionally high relevance to biotechnology, understanding of and exploitation of natural cellular regulation to help achieve production and efficiency goals have expanded. These ncRNAs are ~50-300 nt transcripts that act as regulators of mRNA and protein expression, typically by blocking translation or changing stability [1]. In bacteria, small ncRNAs have

been highlighted as powerful tools due to their regulatory roles in cellular pathways [2–5]. Cis-regulatory ncRNAs act on regions within the same RNA transcript while trans-regulatory ncRNAs regulate separate RNA transcripts or proteins [5]. Riboswitches are a prominent class of cis-regulatory ncRNAs typically in 5'- or 3'-untranslated regions (UTRs) of RNA transcripts with great potential for local control of gene expression in metabolic engineering efforts; these have recently been reviewed [6] and are not discussed here. So far, engineering strategies involving trans-regulatory ncRNAs fall into two general approaches as shown in Figure 1: (A) altering expression of well-characterized natural ncRNAs to induce enhanced regulatory effects on protein expression levels (potentially in multiple pathways) and (B) designing synthetic ncRNAs to knockdown expression of individual proteins.

In this review, we offer a concise guide to exploiting trans-regulatory ncRNAs for strain engineering by considering the effectiveness of the design blueprints that have been recently proposed for a variety of natural and synthetic ncRNA systems (Table 1). More historical accounts of ncRNAs and their applications are available [4,5,7]. Due to the absence of RNA interference in bacterial systems, the use of synthetic ncRNAs shows significant promise as an effective gene-silencing tool. Most engineering efforts have focused so far on the exploitation of ncRNA translational repression mechanisms, preventing translation by blocking the ribosome-binding site (RBS). The limited exploitation of ncRNAs stems from the challenge that much remains unknown about the networks of mRNAs, proteins, and transcriptional factors that are regulated in response to environmental changes. As a growing number of ncRNA mechanisms continue to be uncovered, applications for strain engineering will likewise continue to expand. For example, target activating ncRNAs are now known to bind at 5'- or 3'-UTRs of their targets to allow accessibility of a previously blocked RBS or to increase the stability of the target transcript [8,9]. These activating mechanisms may provide unique protein overexpression possibilities for strain engineering.

Exploitation of naturally discovered ncRNAs: construction of overexpression and deletion strains

Hundreds of natural ncRNAs have been discovered throughout bacteria, largely enabled by high-throughput sequencing technologies [4,10,11]. In *E. coli* alone, over a hundred are now confirmed. Many ncRNAs are known to impact expression of multiple mRNA targets, some of which have been biochemically and genetically identified and others yet to be uncovered. Importantly, a significant number of these ncRNAs (particularly in Gram-negative bacteria) depend on the RNA-binding protein Hfq as a cofactor that enables mRNA targeting [12,13]. Hfq homologs are widespread across bacterial species, although still undetected in many species [14].

Recently, natural ncRNAs have been exploited to increase acid tolerance in *E. coli* [15]. These efforts were largely enabled by the fact that multiple Acid Resistance (AR) systems in *E. coli* are well-characterized and considered global stress response systems, encompassing a wide variety of genes. For instance, AR1 depends on the RpoS sigma factor that directly or indirectly regulates about 500 genes [16,17]. Given this dependence on RpoS, engineering efforts on the AR1 system in *E. coli* have focused on manipulating RpoS levels. Interestingly, simple overexpression of *rpoS* by removal of its natural 5'-UTR does not significantly improve acid tolerance and is not sufficient to fully induce the genes it regulates [18]. The inability to use conventional overexpression strategies to increase levels of RpoS is not surprising given that expression of this protein is tightly regulated at multiple levels by a variety of ncRNAs (e.g. DsrA, RprA, and ArcZ) that stimulate *rpoS* translation through its 5'-UTR [18]. This is an

interesting case in which the cis-regulatory ncRNA (5'-UTR) blocks the RBS until the trans-regulatory ncRNAs (DsrA, RprA, ArcZ) bind to complementary nucleotide sequences in the cis-regulator and free the RBS to allow translation.

Since affecting a complex phenotype like acid tolerance is well-suited for trans-regulatory ncRNA engineering, a new strategy overexpressed the three ncRNAs known to up-regulate RpoS levels (instead of overexpressing RpoS directly) [15]. All three overexpression strains (RprA, ArcZ, and DsrA under their native promoters) showed increased survival percentage after 1 h growth in acidified media (pH 2.5) with 3-, 5-, and 106-fold improvements over the empty plasmid control strain, respectively. Furthermore, a triple overexpression strain (combination of all three ncRNAs) showed an impressive 8500-fold improvement in cell survival after acid stress, demonstrating a supra-additive effect of combinatorial overexpression that cannot be solely attributed to simple increase in RpoS protein expression levels.

Importantly, the engineering of ncRNAs to improve survival under acid stress set a precedent for using natural ncRNA regulators to tune expression of entire sets of pathways. However, unlike this demonstration, the vast majority of ncRNAs have not been characterized, leaving their mRNA targets and mechanisms of action unknown, particularly beyond *E. coli*. For example, in *Streptomyces coelicolor*, no natural ncRNAs had been functionally characterized and no Hfq homologs were known, but a natural cis-encoded antisense RNA was overexpressed and phenotypic impacts on antibiotic production were observed [19]. An additional challenge with this overexpression approach is the risk of expressing ncRNAs at non-native levels given the variety of pathways that can be negatively impacted.

As a demonstration that effective engineering of ncRNA overexpression could be achieved successfully under a non-native promoter, recent work showed that constitutive overexpression of ncRNA RyhB in *E. coli* DALA under the RNA polymerase binding region of the P_{BAD} promoter enabled production of 16% more 5-aminolevulinic acid (ALA), as listed in Table 1 [20]. This ncRNA approach was motivated by the lack of success with traditional methods involving gene expression of selected enzymes given the complex nature of this pathway and the synergistic interdependence of the enzymes involved. RyhB had been shown to enable cell survival in iron-deficient environments by down-regulating iron-containing proteins to free iron for other essential cellular functions [21]. RyhB was also known to down-regulate four enzymes in the TCA-heme pathway with two additional computationally predicted targets (hemH and hemB).

To date, knockouts (or knockdowns) of natural ncRNAs are not widely used as engineering strategies, but have been performed with the motivation of characterizing the functions of ncRNAs as they are discovered [22–24]. This approach is particularly common for bacterial pathogens, for which some ncRNA knockout strains can have differential ability to infect their hosts [25,26]. The variety of bacterial ncRNA roles in virulence have been well reviewed in recent years [27–29], including within the particular context of *Listeria* [30], *Mycobacterium* [31], *Pseudomonas* [32], *Staphylococcus* [33], *Streptococcus* [34], and *Vibrio* [35]. The lack of ncRNA knockouts for strain engineering is not surprising as the nature of ncRNA regulation is dynamic with respect to cellular stresses and growth phases; as such, simple knockouts may not be beneficial in all conditions throughout cellular growth. Additionally, the necessary genetic tools for ncRNA deletions are more complex than plasmid overexpression approaches, limiting the screening of combinatorial effects involving multiple ncRNAs.

Design of synthetic ncRNAs: modular blueprints inspired by natural mechanisms

Soon after antisense RNAs were discovered to bind complementary to mRNAs and regulate their translation [36], ncRNAs were constructed to accomplish this regulation artificially in *E. coli* [37,38] and in a variety of organisms including *Mycobacterium* [39,40], *Clostridium* [41], *Staphylococcus* [42], *Lactobacillus* [43], and *Bacillus* [44]. Although the efficiencies were initially low and highly gene-dependent, the potential of using antisense RNAs to knockdown (but not completely knockout) essential genes has motivated a variety of new designs to express these antisense binding sequences [45]. As shown in Figure 2, a basic modular design that surfaced for engineering efficient expression of trans-regulatory ncRNAs includes: (i) a promoter, (ii) an antisense binding domain to a target mRNA, (iii) a scaffold for stability and/or Hfq binding, and (iv) a terminator [46–49]. As we highlight below and in Table 1, a variety of synthetic antisense ncRNA strategies has surfaced within this basic architectural blueprint.

To express a synthetic ncRNA *in vivo*, they are typically encoded on a plasmid with a promoter region upstream of the ncRNA to initiate transcription. Selection of appropriate (i.e. tightly regulated promoters) promoters is a key for the effective inhibition of target mRNA expression [46,50,51]. Regulatory promoters (Module 1 in Figure 2) are especially useful as the expression can be turned on and off with ligand inputs or with temperature [47]. As shown in Table 1, recent studies employ a variety of promoters. Importantly, the transcription start site (TSS) of the selected promoter should be known. In at least one study, different types of promoters (P_{Tet} , P_{T7} and P_{Trc}) were tested and selected for the efficient gene silencing according to the sensitivity of the target mRNA [50]. An alternative to plasmid expression has been to deliver the ncRNAs via M13 phage infection [52], as shown in *E. coli* for engineering antibiotic sensitivity (Table 1). An optimization challenge that arose from this study is the need for appropriate copy numbers with phage infection.

Downstream of the promoter, a stretch of nucleotides is designed for antisense base pairing with a target mRNA near its start codon (Module 2 in Figure 2). This antisense binding region is central to the mechanism of ncRNA translational repression of the mRNA. The most efficient length and location of binding to the target mRNA varies from target to target. One approach to find the optimal binding sequence is by randomly screening a library of synthesized ncRNAs using a GFP reporter system [53,54]. As illustrated in Module 3 (Figure 2A), this approach was used to identify binding sequences with the highest repression efficiencies for OmpF, FliC, and RecA in *E. coli*. In the case of RecA, this screening process was used to uncover key regions within the target recA mRNA that could be most affected by mutations to disrupt gene expression. Indeed, after using IntaRNA [55] to predict interactions between the synthetic ncRNA and recA, targeted mutations to the Shine-Dalgarno (SD) sequence of recA showed 86% of repression for RecA (improved from 78% from the random screen).

Recently, a systematic rational design process of the antisense binding region has been reported [56,57]. In this work, instead of a random library of sequences, various target-binding sequences from the transcription initiation region (TIR) (including RBS and SD sequences to outside of TIR regions) were screened for efficient repression. In addition, binding energies were evaluated to confirm the correlation with repression capability. Here, target-binding regions containing the TIR were selected due to their highest repression capabilities. This result suggested that prediction of binding energy (less than -20kcal/mol) can be used one of the parameters for selecting target binding sequence. Using this strategy, repression of the *tyrR* and *csrA* genes involved in tyrosine biosynthesis pathway resulted in the highest tyrosine production (2g/L) achieved to date in *E. coli*. In addition, use of these synthetic ncRNAs enabled identification of new gene targets for engineering cadaverine production (Table 1). These

authors applied the same design process to develop *E. coli* strains for short-chain alkanes and phenol production in combination with traditional metabolic engineering strategies [58,59].

Antisense binding regions may be attached to scaffolds derived from natural ncRNAs (Module 3 in Figure 2) for structural stability and for recruitment of the Hfq protein to mediate binding to the mRNA target [48,53]. Natural ncRNA scaffolds (DsrA, GcvB, MicF, and Spot42) have been fused to the target binding sequence screened for repression efficiency and engineered for Hfq binding through mutational analysis increasing predicted ncRNA secondary structure stability [60]. In another study, 101 known ncRNAs were analyzed, leading to the selection of MicC, a natural ncRNA involved in OmpC outer membrane protein translation, as a synthetic ncRNA scaffold [56]. The MicC scaffold was used in several following studies as shown in Table 1 including tyrosine, cadaverine, phenol and N-acetylglucosamine production. This scaffold is particularly attractive as its targeting and Hfq binding sequences are well known and it is thought to bind only one target. Although computational target prediction programs for ncRNA-mRNA pairs can effectively predict real targets, experimental confirmation of these predictions can be slow, limiting the use of ncRNAs that are suspected to regulate multiple targets [61].

The first report of ncRNAs for strain engineering in *Bacillus subtilis* also used this blueprint (Figure 2A) with the *E. coli* MicC scaffold [62]; in this case, Hfq was expressed from a plasmid to overcome the lack of Hfq in *Bacillus*. The success of this strategy was reflected in the 134% improvement in N-acetylglucosamine production shown by the ncRNA-engineered strain over the *B. subtilis* control (Table 1). Still, the lower repression efficiency and lower overall productivity in *B. subtilis* compared to *E. coli* demonstrates the need to further optimize this blueprint for organisms that naturally lack Hfq. Limited information about ncRNAs in non-model organisms hinders wider exploitation of these powerful tools.

An alternative scaffold architecture has been pursued with the development of paired-terminus antisense RNAs (PTasRNAs) [50], which have complementary 38 nt flanking regions on each side of the antisense targeting region to form a stable hairpin as illustrated in Figure 2B. PTasRNAs have been successful in optimizing pyruvate production, fatty acid synthesis, and antibacterial sensitivity in *E. coli* [46,49,63–65] and inhibition of actinorhodin biosynthesis in *Streptomyces coelicolor* [66]. As noted in the *Streptomyces* study, natural ncRNA mechanisms are largely unknown, making further optimization of the ncRNA design challenging [66]. For example, because there are no Hfq homologs known in this organism [19], it is unclear whether a scaffold mimicking natural ncRNA recruitment of Hfq would increase efficiency or if the stability provided by the PTasRNA structure (Figure 2B) is sufficient. Importantly, the PTasRNA strategy has also been shown effective in silencing multiple genes in combination [49].

Most recently, a new class of synthetic RNAs, small transcription activating RNAs (STARs) [67], has been engineered as antisense to a synthetically constructed terminator-like hairpin that is placed upstream of a target gene. In this design, the hairpin prevents gene transcription unless it is disrupted by the STAR (various lengths, 17-60 nt); in this case, the hairpin is opened to allow transcription. A key design parameter of STARs is the selection of the hairpin sequence and length coupled to the STAR length. This system parallels translational control schemes that have been reported in the context of SD-antiSD hairpins that act as riboregulators at the 5' of a target gene [68,69]. STARs have been designed to successfully target natural terminators in *E. coli*, but given that these are at the end of genes, they have undetectable effects on expression of the surrounding genes. Although they do not have a clear role in metabolic engineering at this point, STARs show promise for developing RNA-only logic networks [70].

Conclusion

As indicated in Table 1, current engineering efforts have mostly focused on model organisms in well-characterized pathways. Limited information about ncRNAs in non-model organisms has hindered wider exploitation of these powerful tools. However, there is great potential to adapt these modular ncRNA designs to target the same pathways in a variety of industrially relevant organisms. Even in *E. coli*, mechanisms of ncRNA action on their targets are not fully characterized, preventing rational ncRNA design strategies. Lack of understanding of ncRNA mechanisms in non-model organisms has also limited the design of synthetic ncRNAs to include scaffolds for cofactor binding as the chaperoning role of Hfq in mediating ncRNA-target binding is not clear in other organisms (particularly in Gram-positive bacteria) [71]. As such, current blueprints that involve Hfq are not as effective in all bacteria.

Another major hurdle is the characterization of precise regions where ncRNAs bind their targets *in vivo*. Studies of native or synthetic antisense ncRNAs have demonstrated that target-binding region is crucial for efficient regulation of target mRNAs. Although computational target prediction programs for ncRNA-mRNA pairs can effectively predict real targets, experimental confirmation of these predictions can be slow, leaving false positive rates of these programs unknown [61]. In this capacity, thermodynamics analysis has uncovered strong positive correlations between ncRNA-target binding energies and gene repression [57]. Likewise, information regarding binding energy calculations has been valuable for further enhance knockdown efficiency by mutagenesis approaches that fine-tune target binding regions on the ncRNA [56]. We suspect that recent *in vivo* structural analysis methods will be of high benefit to this problem [69,72,73].

Finally, methods to more efficiently use “omics” datasets would allow genome-wide insights into the ncRNA strain engineering process. As more natural ncRNAs are discovered to be relevant to industrial phenotype goals, combinations of ncRNA overexpressions and deletions in a single strain could be a powerful strain development approach. Combined with traditional metabolic engineering approaches, these strains may produce currently unachievable phenotypes.

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Figures

Figure 1. General engineering approaches of noncoding RNAs. (A) Altering levels of selected mRNA targets by overexpression or deletion of natural ncRNAs (green). Some mRNA targets may be known (red) while others may be undiscovered and uncharacterized (black). Overexpression of ncRNAs from plasmids with non-natural promoters (black arrow) and of cofactors known to mediate ncRNA-target interactions (black triangle) have been used to observe the effect of ncRNAs on target proteins and enzymes in a diversity of pathways. Deletion of ncRNAs from genomic DNA has been used to confirm the regulatory effects on the target genes. Both overexpression and deletion strategies can alter protein expression levels, potentially in multiple cellular pathways. (B) Designing synthetic ncRNAs to knockdown expression of individual proteins by blocking translation of mRNA targets (red). Basic strategies include an antisense-binding region (green) that binds to the RBS (black square) or another

target region and a scaffold (yellow) for stability and/or to facilitate cofactor binding. This strategy is effective for knocking down known mRNA targets.

Figure 2. Design strategies of synthetic noncoding RNAs for effective gene regulation. Modular design of synthetic ncRNAs: Module 1 is the selection of a known promoter. Module 2 comprises of target-binding sequence that is antisense to a ribosomal binding sequence. Potential target binding sequences are tested for the effective gene knockdown. Selected binding sequences can be further mutagenized to achieve higher effectiveness. Module 3 represents a scaffold for the stabilization of ncRNAs. A scaffold can be chosen from well-known natural ncRNAs (A) such as Spot42, DsrA, MicC, and MicF to recruit additional cofactors (Hfq). Alternatively, (B) PTasRNAs have been designed with complementary 38 nt flanking regions, on each side of the antisense targeting region to form a stable hairpin. Module 4 involves natural terminators. Each module can be engineered to fine-tune the action of synthetic ncRNAs for effective target gene regulation.

Tables and boxes

Table 1. Examples of natural and synthetic ncRNAs used for engineering metabolic pathways.

phenotype objective	organism	ncRNA type	ncRNA targets (mRNA)	promoter	binding sequence on the target	scaffold(s)	terminator	Hfq-dependent?	strain performance	Ref
acid resistance	<i>E. coli</i>	natural DsrA, RprA, ArcZ	rpoS ¹	natural	natural	natural	natural	yes	8502-fold increase in acid stress survival (after triple sRNA overexpression)	[15]
5-aminolevulinic acid production	<i>E. coli</i>	natural RyhB	hemBH, acnAB, sdhAB, fumA, cydAB ¹	P _{BAD}	natural	natural	natural	yes	16% increase in 5-aminolevulinic acid production	[20]
RNaseE reduction	<i>E. coli</i>	traditional asRNA	rne	P _{tet}	RBS to the 51bp (-32bp to +51) of rne transcript	N/A	t ₀	no	35% reduction in Rnase level	[74]
RpoS repression	<i>E. coli</i>	traditional asRNA	rpoS	P _{T7}	randome library screening	N/A	T7	no	RpoS repression	[75]
Cobalamine (Vitamin B12) production	<i>B. mega-terium</i>	traditional asRNA	hemZ	P _{xyIA}	82 nt upstream of start codon to the first 47nt of hemZ	N/A	N/A	no	20% increase in vitamin B12 production	[44]
butyrate formation	<i>C. Aceto-butylicum</i>	traditional asRNA	bk, ptb	P _{ptb}	102-nucleotide asRNA with 87% complementarity /698-nucleotide asRNA with 96% complementarity	N/A	adc terminator	no	50% increase in acetone and 35% in butanol/300% increase in lactate dehydrogenase activity (no change in butyrate formation)	[41]
S-adenosylmethionine production	<i>E. coli</i>	synthetic multi-domain scaffold ²	aroE, proA, ilvC, proC, argC, rfp uidA	P _r	TIR of target mRNA	MicC	T7	yes	70% increase in S-adenosylmethyl production	[76]
uidA gene silencing	<i>E. coli</i>	synthetic multi-domain scaffold ²	uidA	P _{tac}	5' UTR containing region	RyhB, Spot42, DsrA and OxyS	natural from scaffold	yes	4- to 7-fold repression of target genes	Man 201 1
decreased cell motility	<i>E. coli</i>	synthetic multi-domain scaffold ²	ompF, fliC	P _{BAD}	20-30 nt random library screen	DsrA, GcvB, MicF, Spot42	natural from scaffold	yes	decreased motility (qualitative assay)	[53]
decreased cell motility, increased fluroquinone antibiotic sensitivity	<i>E. coli</i>	synthetic multi-domain scaffold ²	recA	P _{LtetO1}	20-30 nt random library screen	Spot42	natural from scaffold	yes	decreased motility, increase ciprofloxacin sensitivity (qualitative assays)	[54]
short-chain alkane production	<i>E. coli</i>	synthetic multi-domain scaffold ²	fabH	P _{tac}	TIR region	MicC	T1/TE	yes	increase in short-chain fatty acids	Choi 201 3
tyrosine production	<i>E. coli</i>	synthetic multi-	tyrR, csrA, pgI,	P _R	24 nt near TIR	MicC	T1/TE	yes	2 g/L tyrosine produced	[56]

		domain scaffold ²	ppc						(equivalent to traditionally engineered strains)	
cadaverine production	E. coli	synthetic multi-domain scaffold ²	130 targets	P _R	24 nt near TIR	MicC	T1/TE	yes	55% increase in cadaverine production over engineered strain XQ56	[56]
phenol production	E. coli	synthetic multi-domain scaffold ²	csrA, tyrR	P _R	24 nt near TIR	MicC	T1/TE	yes	3x greater phenol titer than previous best reported microbial fermentation	[59]
N-acetylglucosamine production	B. subtilis	synthetic multi-domain scaffold ²	pfk, glmM	P _{spac}	24 nt near TIR	MicC	T _{spoVG}	yes ³	134% increase over B. subtilis control	[62]
silencing antibiotic resistance (Cm, Kan)	E. coli	synthetic multi-domain scaffold ²	cam, nptII	P _R	24 nt near TIR	MicC	T1/TE	yes	>99% colonies sensitive to high Kan/Cm concentrations	[52]
sensitization to antibacterial essential oils eugenol and carvacrol	E. coli	PTasRNA	yidC, murA	P _{trc}	130-134 nt near TIR	paired-termini	T _{rrnB}	no	min inhibitory concentration reduced from 2.5 to 0.156 mg/mL	[65]
pyruvate accumulation	E. coli	PTasRNA	71 targets	P _{trc}	70-160 nt	paired-termini	T _{rrnB}	no	25 g/L pyruvate produced (equivalent to traditionally engineered strains)	[49]
(2S)-naringenin Production	E. coli	PTasRNA	fabBF	P _{trc}	148-245 nt near TIR	paired-termini	T _{rrnB}	no	431% increase of (2S)-naringenin production	[63]
acetate production	E. coli	PTasRNA	ackA	P _{trc} , P _{araBAD}	147bp near TSS	paired-termini	T _{rrnB}	no	48% reduction in ackA expression	[46]
essential gene screening	E. coli	PTasRNA	152 targets	P _{trc}	near Tss	paired-termini	T _{rrnB}	no	in case of fusA, 12-fold sensitization	[77]
actinorhodin production	S. coelicolor	PTasRNA	actI-ORF1	P _{trc}	155bp (-92 to +63) of actI-ORF1	paired-termini	T _{rrnB}	no	4-fold reduction in actinorhodin production	[66]
acetone production	C. acetobutylicum	asRNA based on secondary structure of target mRNA	ctfA, ctfB	P _{thl}	RBS and structural subunits	N/A	N/A	no	8-fold decrease in acetone production	[78]
scaffold engineering	E. coli	Natural sRNA-derived scaffolds	TaRNA, pT181sRNA	P _{araBAD}	RBS and 5'UTR	MicF, Spot42	T1/TE	yes	28-fold (TaRNA-MicF)/7.7 fold (pT181sRNA-spot42)	[60]
automated design of synthetic sRNA	E. coli	synthetic sRNA based on computational algorithm	GFP	P _{LacO}	5'UTR containing region	SokC, FinP, and DsrA structure	N/A	yes	1-4% repression	[79]
activation of suicide by targeting antitoxin	E. coli	PNA	hok mRNA:: sokRNA interaction	natural	5' region of sok RNA	N/A	N/A	no	inhibition of Sok mediated repression of hok mRNA	[9]

Notes ¹ E.coli Hfq overexpressed under P_{hbs}

² commonly used multi-domain scaffold (displayed in Figure 2A)

³ could be others (unknown)

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Figure1

A Alter Natural Expression of Known ncRNA Target

B Target Natural Expression of Desired mRNA Target

Figure2

A Synthetic multi-domain scaffold

B Paired-termini antisense RNA (PTasRNA)