



Visualized and precise design of artificial small RNAs for regulating T7 RNA polymerase and enhancing recombinant protein folding in *Escherichia coli*

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ARTICLE INFO

Article history:

Received 5 July 2016

Received in revised form

30 August 2016

Accepted 30 August 2016

Keywords:

Artificial small RNAs

Visualized and precise design

Prokaryotic T7 expression system

Inclusion body

ABSTRACT

Small non-coding RNAs (sRNAs) have received much attention in recent years due to their unique biological properties, which can efficiently and specifically tune target gene expressions in bacteria. Inspired by natural sRNAs, recent works have proposed the use of artificial sRNAs (asRNAs) as genetic tools to regulate desired gene that has been applied in several fields, such as metabolic engineering and bacterial physiology studies. However, the rational design of asRNAs is still a challenge. In this study, we proposed structure and length as two criteria to implement rational visualized and precise design of asRNAs. T7 expression system was one of the most useful recombinant protein expression systems. However, it was deeply limited by the formation of inclusion body. To settle this problem, we designed a series of asRNAs to inhibit the T7 RNA polymerase (Gene1) expression to balance the rate between transcription and folding of recombinant protein. Based on the heterologous expression of *Aspergillus oryzae* Li-3 glucuronidase in *E. coli*, the asRNA-antigene1-17bp can effectively decrease the inclusion body and increase the enzyme activity by 169.9%.

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1. Introduction

Recently, many sRNAs have been identified and characterized in diverse organisms, particularly in *Escherichia coli* [1,2]. They have been divided into cis-acting sRNA (csRNAs) and trans-acting sRNAs (tsRNAs) [3]. The tsRNAs usually interact with the 5' un-translated region (5'UTR) or the translation initiation region (TIR) of target mRNA by base pairing to regulate gene expression [4,5]. The tsRNAs have been used as versatile and flexible genetic tools in many fields, such as building logic gate in synthetic biology [6], knocking down shunt pathway in metabolic engineering [7] and studying bacterial physiology [8]. Since they are such a powerful genetic tool that many groups have tried to develop asRNA to regulate gene expression.

Yokobayashi et al. [9] developed an efficient throughout screening method for asRNAs by designing antisense domain as a series of N bases, and fusing randomized antisense domain with

natural sRNA scaffolds. However, the operation was complicated and time consuming. To simplify the operation, Lee [10] rationally designed the antisense domain of asRNA by introducing complementary base pairing with the front of target 24bp sequence. This can deeply predigest the screening process, but the inhibition efficiency of designed asRNAs was dramatically different. Apart from the antisense domain, Lee [11] and Ikebukuro [12] found that the replacement of sRNA scaffolds could also improve the efficiency of asRNAs. But sRNA scaffolds still need to screen efficient antisense domain to gain effect.

Based on previous study, the key step to implement reasonable design of asRNAs was to design efficient antisense domain, so we focus on designing efficient antisense domain.

T7 expression system was one of the most popular protein expression systems [13] in which T7 RNA polymerase specifically recognized T7 promoter [14] and elongated target mRNA 5-fold faster than *Escherichia coli* RNA polymerases [15]. It includes two parts: a plasmid containing T7 promoter and a factitial DE3 lysogenic host [16]. The DE3 lysogenic host has one copy of T7 RNA polymerase gene (Gene1), which was pre-integrated into genome to produce a very limited amount of T7 RNA Polymerase [17]. The

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Peer review under responsibility of KeAi Communications Co., Ltd.

pET expression system from Novagen Inc. was one representative of such approach and has been widely used for protein expression in *E. coli* [18]. Although the amount of T7 RNA polymerase was very limited, the speed of target gene transcription was still too fast. The high-level expression of recombinant proteins in *Escherichia coli* often accumulate as insoluble aggregate in vivo as the inclusion body [19] that severely limit its application.

We proposed that the inclusion body formation was due to the high efficiency of T7 RNA polymerase. The inclusion body is very likely to form due to the imbalance between the speed of target gene transcription and protein folding. In this study, we developed a series of asRNAs with different inhibition efficiency to fine tune Gene1 expression, which dramatically decreased the inclusion body formation of T7 expression system.

2. Materials and methods

2.1. Strains, plasmids and culture condition

Escherichia coli Trans1-T1, TOP10 were selected as the clone hosts and BL21 (DE3) as the expression host. Plasmids pEASY-Blunt (TRANSGEN BIOTECH), pSB1A3, pSB1C3 and pSB1K3 (parts from iGEM) were used to express asRNAs. To verify the function of asRNAs in optimizing the T7 expression system, plasmid pET-28a-pGUS which expressed *Aspergillus oryzae* strain Li-3 glucuronidase (GenBank: EU095019.1) was used as a model system. Since the recombinant *Aspergillus oryzae* strain Li-3 glucuronidase (pGUS) was expressed as the inclusion body that severely limit our study. The strains were cultured in Luria-Bertani (LB) media at 37 °C, 30 °C and 16 °C with 200 rpm. Restriction enzymes and other modifying enzymes were purchased from Thermo Fisher Scientific and used according to the manufacturer's recommendations.

2.2. Plasmids and strains construction

Plasmid pSB1A3-system was constructed as a platform to express asRNA and to test its inhibition efficiency. This vector contains

both asRNA expression cassette and verification cassette that was developed by BioBrick method [20] (Fig. 1). Besides, pSB1C3-asRNA cassette was constructed to express asRNAs. And the pSB1K3-verification cassette was constructed to verify the efficiency of asRNAs.

By one step mutation, 24bp of ipgC, sicA* and exsC 5'leader mRNA sequence were fused to the reporter gene *LacZ* to get pSB1A3-system-ipgC, pSB1A3-system-sicA* and pSB1A3-system-exsC. The complementary base pairing sequence of ipgC, sicA* and exsC 5'leader mRNA were fused to sRNA scaffold MicC in the same way to get pSB1A3-system-ipgC-anti-ipgC-24bp, pSB1A3-system-sicA*-anti-sicA*-24bp and pSB1A3-system-exsC-anti-exsC-24bp.

Besides, pSB1A3-system-ipgC-anti-ipgC-20bp and pSB1A3-system-exsC-anti-exsC-20bp have been constructed to verify whether the exposure of the antisense domain is necessary for asRNA to show function. Furthermore, pSB1A3-system-ipgC-anti-ipgC-15bp, pSB1A3-system-ipgC-anti-ipgC-10bp and pSB1A3-system-ipgC-anti-ipgC-5bp were developed to test the efficiency of the length of antisense domain.

To optimize the T7 expression system, asRNA-anti-gene1-17bp, asRNA-anti-gene1-13bp and sRNA-anti-gene1-10bp were designed to inhibit Gene1 expression independently to see whether these asRNAs can decrease the inclusion body of pGUS and improve its activity.

These asRNAs were developed by fusing 17bp, 13bp and 10bp with complementary base pairing sequence of Gene1 5'mRNA respectively to MicC scaffold. Using plasmid pSB1C3-sRNA cassette as template the plasmids pSB1C3-asRNA cassette-anti-gene1-17bp, pSB1C3-asRNA cassette-anti-gene1-13bp and pSB1C3-asRNA cassette-anti-gene1-10bp were constructed. After amplified by primer pair Primer SphI-fw & Primer BglII-rv, the PCR fragments were digested with SphI and BglII, gel-purified, and then ligated into plasmid pET-pGUSE, which was digested with the same enzyme pair. The final plasmids were named pET-28a-pGUSE-asRNA-anti-gene1-17bp, pET-28a-pGUSE-asRNA-anti-gene1-13bp and pET-28a-pGUSE-asRNA-anti-gene1-10bp respectively.

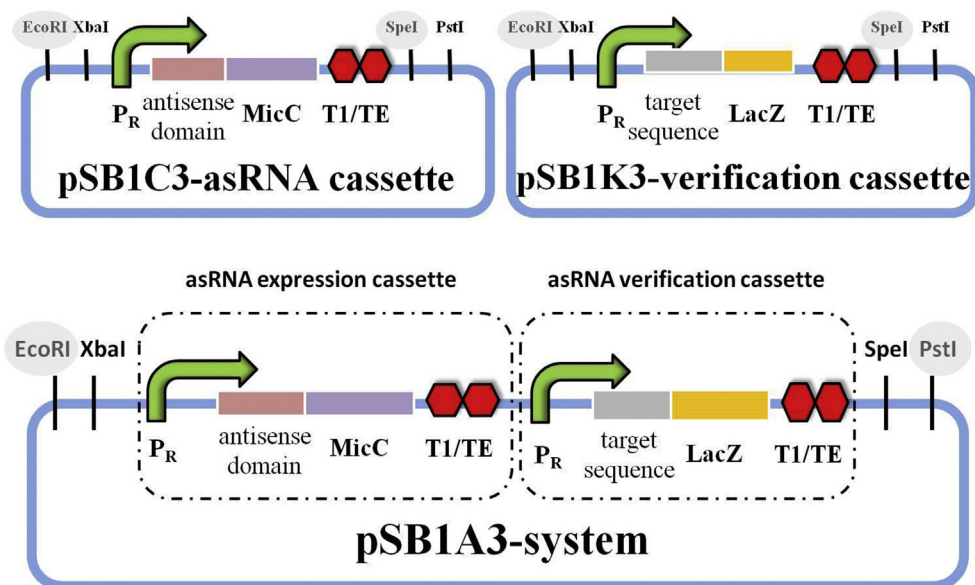


Fig. 1. The schematic of the design and construction of asRNA expression and verification system. The plasmid pSB1C3-asRNA cassette was consisted of promoter P_R , antisense domain of the asRNA which was reversely complement base pairing with the target inhibition sequence, MicC scaffold of asRNA and the bi-directional terminator T1/TE. The plasmid pSB1K3-verification cassette was consisted of promoter P_R , initial 24bp sequence from the target inhibition gene to fuse with the reporter gene *LacZ* which has deleted the ATG. The plasmid pSB1A3 was consisted of both asRNA expression cassette and asRNA verification cassette. It was constructed by BioBrick method.

All the primers used in this experiment were listed in [supplementary materials Table 1](#) and the plasmids were listed in [supplementary materials Table 2](#).

2.3. Assay of enzyme activity

2.3.1. β -galactosidase

One milliliter actively growing culture of *Escherichia coli* was taken in Eppendorf tube and centrifuged at 1000 rpm for 10 min. The cell pellet was washed twice with phosphate buffer and re-suspended in 500 μ L of phosphate buffer (pH 7.0). The cells were permeabilized by vortexing for 10 s with a solution containing 25 μ L of toluene. To 100 μ L of permeabilized cells, 10 μ L ONPG was added. The reaction mixture was incubated at 37 °C in water bath till the development of yellow. Reaction was stopped by adding 2 mL of 0.4 M Na₂CO₃ solution. Yellow intensity was determined at 420 nm using a spectrophotometer against a blank containing all ingredients except the cell culture. β -galactosidase activity is expressed as Millers unit calculated as formula given below:

$$\text{Millers unit} = \frac{\text{Absorbance}_{420}}{\text{Absorbance}_{420} \times T \times V} \times 1000$$

T = time of reaction (minute)

V = volume of the cell culture used (mL)

2.3.2. *Aspergillus oryzae* strain Li-3 glucuronidase

The activity of pGUS was determined by the hydrolysis of *p*-nitrophenyl (pNPG). The assay was consisted of 20 μ L crude enzyme and 30 μ L 1 mM pNPG. After incubation at 40 °C for 10 min, the reaction was stopped by mixing with 200 μ L 0.4 mM Na₂CO₃. The production of *p*-nitrophenol was assayed by using an ultraviolet spectrophotometer (Hitachi) at 405 nm through the standard curve.

2.4. Assay of protein production

The total crude protein concentration was determined using a BCA Protein Assay Kit (Applygen, China). Semi-quantitative determination of recombinant protein concentration was analyzed by SDS-PAGE and western-blot.

3. Results and discussion

3.1. Design and construction of plasmid system for characterizing and verifying asRNAs

Small RNAs were composed of two functional parts: anti-sense domain and scaffold domain [21]. The antisense domain binds to target mRNA by base pairing to inhibit gene translation. A majority of tsRNAs interact with 5'UTR or TIR of target mRNA. The scaffold domain recruits Hfq protein to avoid sRNA degradation by RNaseE [22] and scaffold MicC [23–25] was frequently used in natural sRNAs.

Plasmid pSB1A3-system was established as a platform to express asRNA and to detect the inhibition efficiency. It contained two cassettes: expression cassette and verification cassette. The expression cassette was consisted of P_R promoter, MicC scaffold and T1/TE terminator. The antisense domain was base paired with the TIR of target gene and then inserted into the sequence between P_R promoter and MicC scaffold by one-step mutation. The verification cassette was consisted of P_R promoter, *LacZ* gene which was fused

to target 24bp mRNA leader sequence and T1/TE terminator. The schematic design of pSB1A3-system was shown in [Fig. 1](#). The inhibition efficiency of asRNAs was characterized by β -galactosidase activity.

3.2. Visualized and precise design for artificial sRNAs

Based on previous work, designing antisense domain was the key step to develop asRNAs and the inhibition efficiency was directly related to the antisense domain of asRNAs.

We developed asRNA-anti-*ipgC*-24bp, asRNA-anti-*sicA**-24bp and asRNA-anti-*exsC*-24bp. However, only asRNA-anti-*sicA**-24bp decreased the expression level by 38%. The inhibition efficiency of other asRNAs was indistinctive ([Fig. 2-a](#)). By analyzing the secondary structure of these three asRNAs through web open software RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>), it was found that only asRNA-anti-*sicA**-24bp showed exposed antisense

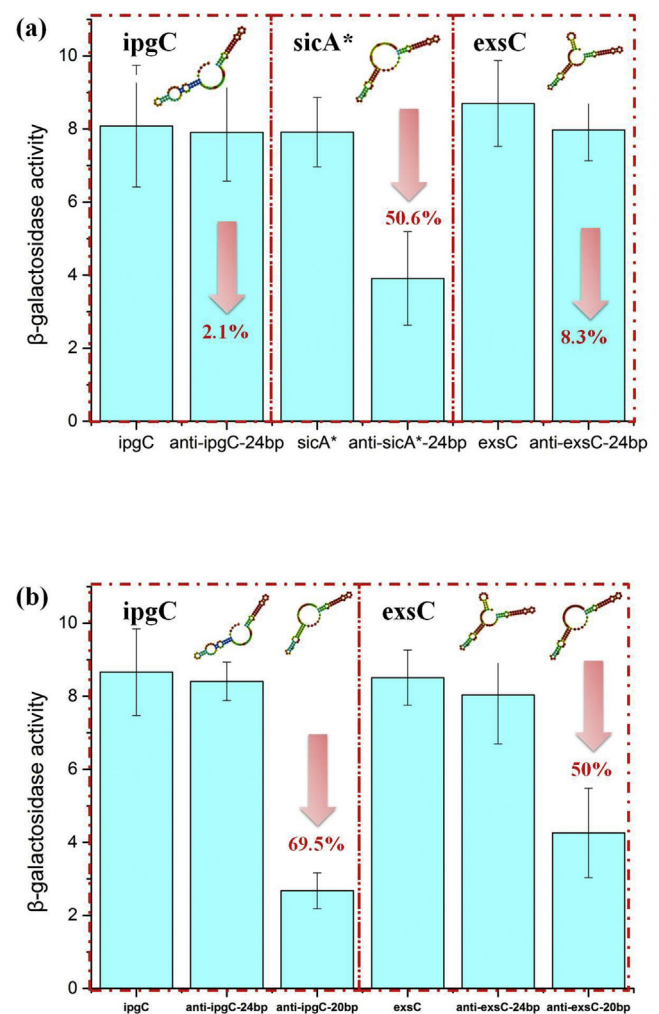


Fig. 2. The secondary structure of designed asRNAs predicted by software RNA fold, the inhibition efficiency of β -galactosidase activity and the binding free energy predicted by software RNAsubopt. (a) The secondary structure of asRNA anti-*ipgC*-24bp, asRNA anti-*sicA**-24bp, asRNA anti-*exsC*-24bp and the inhibition efficiency of these asRNAs. (b) The secondary structure of asRNA anti-*ipgC*-20bp, asRNA anti-*exsC*-20bp and the inhibition efficiency of these asRNAs. (c) The secondary structure of asRNA anti-*ipgC*-20bp, asRNA anti-*ipgC*-15bp, asRNA anti-*ipgC*-10bp, asRNA anti-*ipgC*-5bp and the inhibition efficiency of these asRNAs. (d) The binding free energy between asRNA anti-*ipgC*-20bp, asRNA anti-*ipgC*-15bp, asRNA anti-*ipgC*-10bp and asRNA anti-*ipgC*-5bp with target *ipgC* mRNA, respectively, that was predicted by software RNAsubopt.

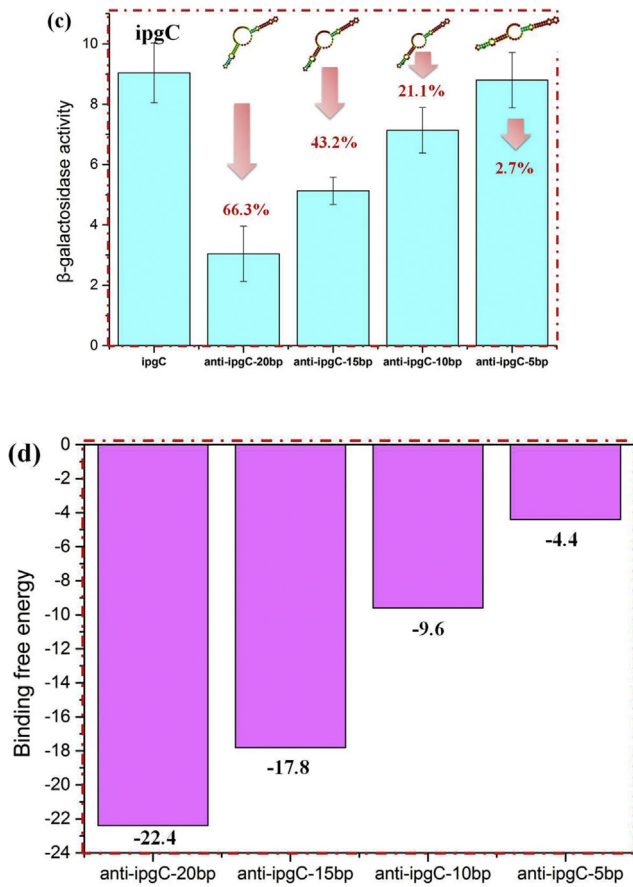


Fig. 2. (continued).

domain and maintained the natural secondary structure of MicC scaffold which was very important to recruit Hfq protein. So we proposed a hypothesis that the exposure of asRNA antisense domain

and the maintenance of asRNA scaffold nature structure were very important to asRNAs inhibition efficiency. This may be due to that the exposed antisense domain was easy to bind with target mRNA while the natural asRNA scaffold was important to recruit RNA charpon Hfq protein to avoid being degraded by RNaseE.

After redesigning asRNA-anti-ipgC-20bp and asRNA-anti-exsC-20bp to expose their antisense domain and maintain the natural MicC scaffold structure, these two asRNAs worked effectively (Fig. 2-b). Therefore, we proposed a criterion for asRNA design: the exposure of antisense domain out of scaffold and the maintenance of scaffold structure are necessary to implement reasonable design asRNAs, which is named as structure condition. The structure of antisense domain can be screened by software RNA fold and the virtual screening optimization is simple.

Furthermore, we designed asRNA-anti-ipgC-15bp, asRNA-anti-ipgC-10bp and asRNA-anti-ipgC-5bp which meet the structure requirement but in different length of antisense domain. We found that the longer antisense domain showed better performance. RNAsubopt analysis indicated that the longer antisense domain had lower binding free energy, thus resulting in the higher efficiency of asRNA (Fig. 2-c and d). We named it length condition.

Based on the proposed structure and length condition, we implemented visualized screening antisense domain structure and precise design of antisense domain length of asRNAs for regulating protein expression. Furthermore, we developed asRNAs which met these two conditions to optimize prokaryotic T7 expression system.

3.3. Regulating and balancing soluble protein expression by asRNAs in *E. coli*

The pET-20a and BL21 (DE3) from Novagen is a representative T7 expression system and has been widely used for protein expression in *E. coli*. However, it is easy to form the inclusion body and decrease enzyme activity that has limited its application severely. Although the inclusion body can be decreased by optimizing inducer concentration and induce condition, this cost a lot of time and energy on monotonous repetitive work. In this study,

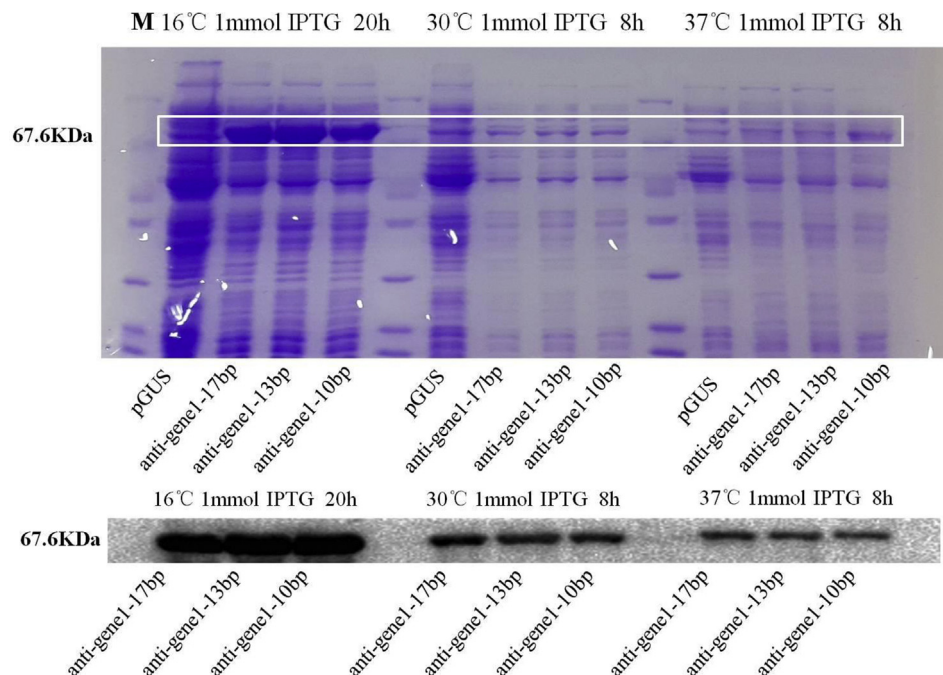


Fig. 3. The SDS-PAGE and western-blot results of the inducible expressed protein at 16 °C, 30 °C and 37 °C. The white square is pGUS with molecular weight of 67.6 kDa.

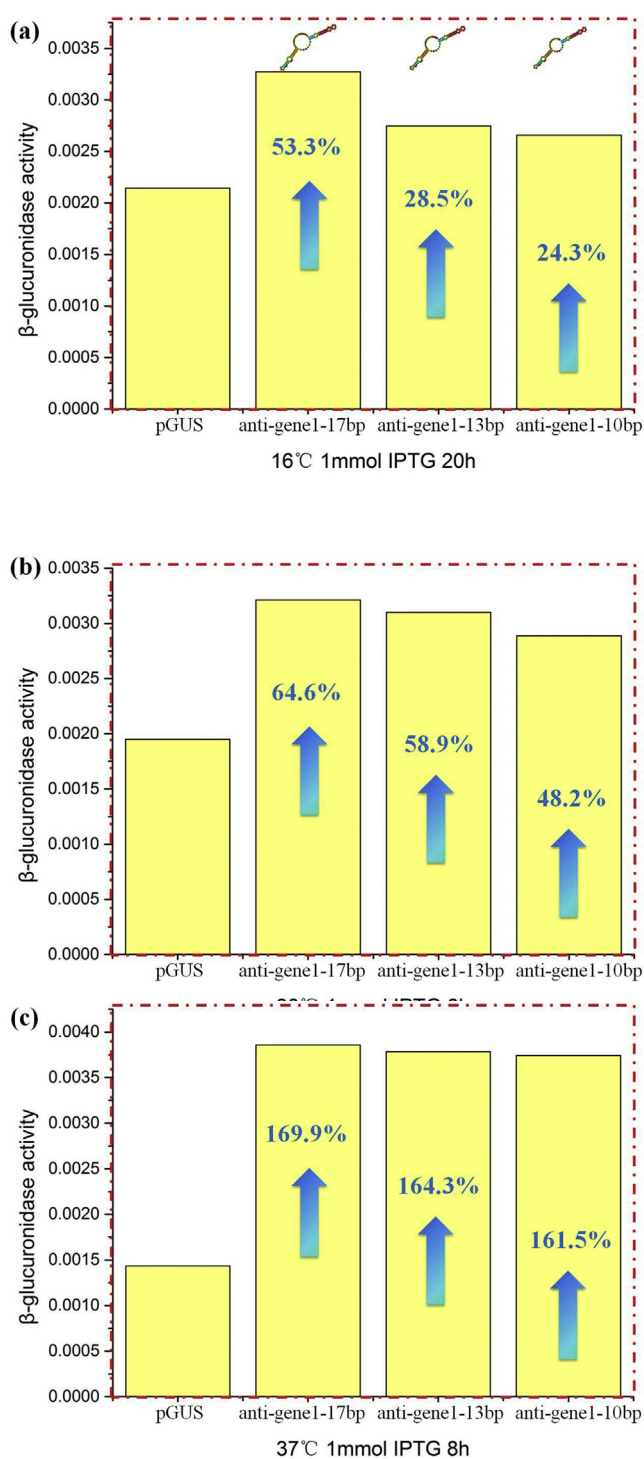


Fig. 4. The secondary structure of asRNA anti-gene1-17bp, asRNA anti-gene1-13bp and asRNA anti-gene1-10bp predicted by software RNA fold and the improved percentage of pGUS activity with 1 mM IPTG in 16 °C cultured 20 h, 30 °C cultured 8 h and 37 °C cultured 8 h condition respectively. (a) The secondary structure of asRNA anti-gene1-17bp, asRNA anti-gene1-13bp and asRNA anti-gene1-10bp predicted by software RNA fold. The improved percentage of pGUS activity with 1 mM IPTG at 16 °C, cultured 20 h. (b) The improved activity of pGUS by using the asRNAs with 1 mM IPTG in 30 °C, cultured 8 h. (c) The improved percentage of pGUS activity with 1 mM IPTG in 37 °C, cultured 8 h.

we aimed to decrease the inclusion body by reducing the amount of T7 RNA polymerase which is achieved by using asRNAs to inhibit Gene1 expression, which can balance the speed between target gene transcription and translation to allow the correct folding of target protein. We designed asRNA anti-gene1-17bp, asRNA anti-gene1-13bp and asRNA anti-gene1-10bp to inhibit Gene1 expression in three levels. The amount of soluble protein was detected via SDS-PAGE, western-blot and the activity of pGUS. The three asRNAs all worked effectively and asRNA anti-gene1-17bp had the best performance and the soluble protein and enzyme activity was increased by 169.9%, as shown in Figs. 3 and 4. In addition, it took only two days to design asRNAs and regulate protein expression, indicating that using asRNAs to regulate protein expression and decrease the inclusion body is a flexible and time-saving method.

4. Conclusions

In this paper, we have proposed two criteria for rational designing asRNAs: structure and length. The structure condition was to expose the antisense domain out of asRNA scaffold and maintain the natural structure of the asRNA scaffold. This operation can be visualized screening by software RNAfold. The structure condition was the length offer antisense domain was in positive correlation with the inhibition efficiency, which may be due to the longer antisense domain had the lower binding free energy thus resulting in the high efficiency of asRNA. But it was easy to form the stem-loop structure for a long antisense domain that may be hard to meet the structure condition of rational design asRNAs, so these two criteria should be met simultaneously to implement the visualized and precise design of asRNAs.

To expand the application of asRNAs, we developed three asRNAs in different inhibition level to inhibit T7 RNA Polymerase (Gene1) expression. By this way, we decreased the inclusion body on molecular level effectively in two days. That is a flexible and time-saving method to optimize T7 expression system in *Escherichia coli*.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

The author would like to acknowledge the National Science Fund for Distinguished Young Scholars (21425624) and the National Natural Science Foundation of China (21476026, 21376028).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.synbio.2016.08.005>.

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