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Research article

Improving the expression of recombinant pullulanase by increasing mRNA stability in *Escherichia coli*

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ABSTRACT

Background: Pullulanase production in both wild-type strains and recombinantly engineered strains remains low. The Shine-Dalgarno (SD) sequence and stem-loop structure in the 5′ or 3′ untranslated region (UTR) are well-known determinants of mRNA stability. This study investigated the effect of mRNA stability on pullulanase heterologous expression.

Results: We constructed four DNA fragments, pulA, SD-pulA, pulA-3t, and SD-pulA-3t, which were cloned into the expression vector pHT43 to generate four pullulanase expression plasmids. The DNA fragment pulA was the coding sequence (CDS) of pulA in *Klebsiella variicola* Z-13. SD-pulA was constructed by the addition of the 5′ SD sequence at the 5′ UTR of pulA. pulA-3t was constructed by the addition of a 3′ stem-loop structure at the 3′ UTR of pulA. SD-pulA-3t was constructed by the addition of the 5′ SD sequence at the 5′ UTR and a 3′ stem-loop structure at the 3′ UTR of pulA. The four vectors were transformed into *Escherichia coli* BL21(DE3). The pulA mRNA transcription of the transformant harboring pHT43-SD-pulA-3t was 338.6%, 34.9%, and 79.9% higher than that of the other three transformants, whereas the fermentation enzyme activities in culture broth and intracellularly were 107.0 and 584.1 times, 1.2 and 2.0 times, and 62.0 and 531.5 times the amount of the other three transformants (pulA, SD-pulA, and pulA-3 t), respectively.

Conclusion: The addition of the 5′ SD sequence at the 5′ UTR and a 3′ stem-loop structure at the 3′ UTR of the pulA gene is an effective approach to increase pulA gene expression and fermentation enzyme activity.

1. Introduction

Pullulanase (EC 3.2.1.41) has been the most extensively studied and industrially applied carbohydrate debranching enzyme. There are two types of pullanases, type I and type II. Type I preferentially and efficiently hydrolyzes the α-(1,6) glucosidic bonds in pullulan and branched polysaccharides, giving maltotriose as the final product [1,2]. Type II, or amylopullulanase, can hydrolyze either α-(1,6) or α-(1,4) glucosidic bonds in pullulan and branched polysaccharides. The final product is maltotriose when hydrolyzing pullulan, whereas the final products are glucose, maltose, and maltotriose when hydrolyzing poly- and oligosaccharides (starch) [3]. Therefore, pullulanase is usually used in combination with α-amylase and glucoamylase to enhance the hydrolysis rate and saccharification rate [4] or with β-amylase to increase the maltose yield [5] in the starch conversion process. However, pullulanase production is low in wild-type strains, and thus, industrially applied pullulanase is solely derived from recombinantly engineered strains.

Heterologous gene expression in engineered bacteria is affected by many factors, such as host strain selection, plasmid copy number, promoter selection, mRNA stability, and codon usage [6]. Many host strains have been used for the heterologous expression of pullulanase, including *Escherichia coli* [7,8,9], *Bacillus flavothermus* [10], *Bacillus licheniformis* [11], *Bacillus subtilis* [12,13], *Brevibacillus choshinensis* [14], *Klebsiella aerogenes* [15], *Pichia pastoris* [16], *Raoultella planticola* [17], and *Saccharomyces cerevisiae* [18], with various plasmids, promoters, or signal peptides. However, improvement in the expression of recombinant pullulanase by increasing mRNA stability has not been reported.

Shine-Dalgarno (SD) sequence and stem-loop structure in the 5′ or 3′ untranslated region (UTR) are well-known determinants of mRNA stability. The SD sequence in the 5′ UTR increases mRNA stability in *Bacillus thuringiensis* [19] and *B. subtilis* [20]. The SD sequence complements the 3′-OH end of the 16S ribosomal RNA and increases the affinity between the ribosomal subunits and the mRNA [19]. A ribosomal subunit bound to the 5′ UTR functions as an mRNA stability

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inducer [21] to increase the stability of the gene transcripts and improve protein production in *B. subtilis* [22]. The 5′ stem-loop structure at the 5′ UTR or 3′ stem-loop at the 3′ UTR also increases RNA stability in *E. coli* [23], *B. subtilis* [20], and *B. thuringiensis* [24]. A 5′ stem-loop at the 5′ UTR prevents the degradation of the downstream mRNA by RNase E, PNPase, and RNase J [25,26,27], whereas a 3′ stem-loop at the 3′ UTR blocks the processive activities of the two 3′–5′ exoribonucleases PNPase and RNase II and stabilizes the upstream mRNA [28].

We previously isolated a pullulanase-producing bacterial strain of *Klebsiella variicola* [29] and identified the pullulanase expressed by *E. coli* as type I, encoded by the gene *pulA* (GenBank accession number: KJ146839). The optimal pH and temperature for the enzyme reaction were 5.6 and 45°C, respectively [30]. In the present study, we aimed to increase the levels of pullulanase production by adding a 5′ SD sequence at the 5′ UTR and a 3′ stem-loop structure at the 3′ UTR of *pulA* in *E. coli*.

2. Materials and methods

2.1. Bacterial strains, DNA, and plasmids

K. variicola Z-13 is a wild-type pullulanase-producing strain isolated by the Key Laboratory of Enzyme Engineering of Agricultural Microbiology, Ministry of Agriculture, Zhengzhou, stored at the China General Microbiological Culture Collection Center (CGMCC), Beijing, and numbered as CGMCC NO.10357. DNA fragment 3t is a stem-loop structure of the *B. thuringiensis* crystal protein gene (GenBank accession number: M37207.1) located at 2504–2982 bp (5′–3′) with a length of 479 bp and was chemically synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The plasmid used to transform and express the *pulA* constructs in *E. coli* BL21(DE3) was the *E. coli–B. subtilis* shuttle vector pHT43 (MoBiTec, Goettingen, Germany).

2.2. Expression vector construction

To construct the *pulA* expression vectors and determine the effects of the SD sequence and stem-loop structure on pullulanase expression, four DNA fragments were cloned by PCR (Fig. 1). The pulA fragment was the coding sequence of *pulA* in *K. variicola* Z-13. The PCR template was the genomic DNA of Z-13, and the primers were pul-F and pul-R (Table 1). The SD-pulA fragment was cloned using the genomic DNA of Z-13 as the template and SD-pul-F and SD-pul-R (Table 1) as the primers. Fragments pulA-3t and SD-pulA-3t were cloned by PCR and enzyme ligation. First, the fragments pulAa and SD-pulAa were cloned by PCR using the genomic DNA of Z-13 as the template and the primer pairs pul-F and pul-3t-R and SD-pul-F and pul-3t-R, respectively. Second, the fragment 3tb was cloned by PCR using the DNA fragment 3t as the template and 3t-F and 3t-R (Table 1) as primers. Third, fragments pulAa, SD-pulAa, and 3tb were digested by the restriction enzyme *Spe I* and then ligated by T4 DNA ligase to generate the fragments pulA-3t and SD-pulA-3t.

The four DNA fragments pulA, SD-pulA, pulA-3t, and SD-pulA-3t were cloned into the expression vector pHT43 by enzyme digestion and ligation to construct four pullulanase expression vectors: pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t, respectively.

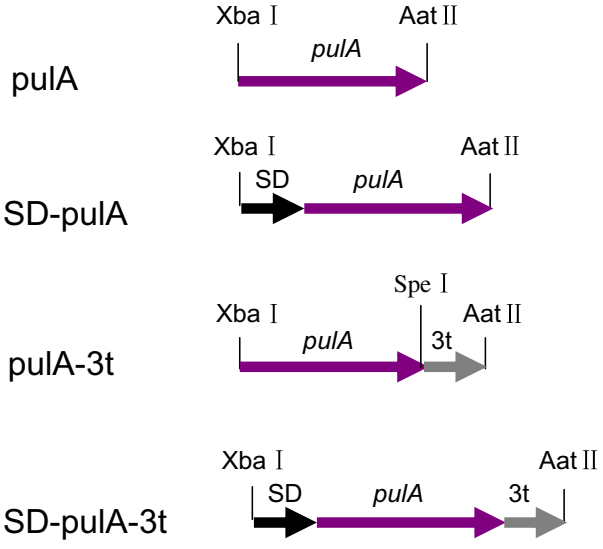

Fig. 1. The four DNA fragments cloned into plasmid pHT43 to construct the pullulanase gene *pulA* expression vectors. pulA, coding sequence of the pullulanase gene in *Klebsiella variicola* Z-13; SD, Shine-Dalgarno (SD) sequence; 3t, stem-loop structure of *Bacillus thuringiensis* crystal protein gene.

2.3. Transformation and transformant identification

The four pullulanase expression vectors were used to chemically transform competent *E. coli* BL21(DE3) for expression. To screen for the transformants, single colonies growing on LB agar containing 100 mg ampicillin l^{-1} were picked and subcultured on LB agar for five passages. The transformants were identified by colony PCR using the primer pairs pul-F and pul-R (Table 1).

2.4. Pullulanase fermentation and enzyme assay

Transformants were inoculated into a 50-ml LB shake flask and cultivated at 200 rpm, 37°C for 2–3 h until OD_{600} reached 0.6; IPTG was added to a final concentration of 0.5 mM to induce the expression of the recombinant pullulanase. Incubation was continued for another 16 h at 20°C, and then the cells and supernatants were separated by centrifugation at 6500 *g* for 15 min at 4°C. The supernatants were stored at 4°C. The cell pellet was suspended in distilled water and sonicated (pulsed on for 3 s, off for 4 s) for 30 min in an ice bath. The crushed cell suspension was centrifuged at 6500 *g* for 30 min at 4°C, and then the supernatants were collected and adjusted to a final volume of 50 ml with distilled water. The protein contents of the crude enzyme extracts were measured by the Bradford method [31]. Pullulanase activity was assayed depending on the concentration of reducing sugars liberated after incubating the pullulanase solution with 0.5% pullulan at 45°C for 30 min. Reducing sugars were quantified by the 3,5-dinitrosalicylate method [32]. One unit of pullulanase activity was defined as the amount of enzyme required to release 1 μM reducing sugars per minute from pullulan under the specified assay conditions [33].

Table 1
Primers used in this study.

Primers	Nucleotide sequence (5′–3′)[a]
pul-F	GCTCTAGA**ATG**CTCAGATATACCTGTCATGCCC
pul-R	GCCGACGTCAATTATTTACTGCTCACCGGCAGG
SD-pul-F	GCTCTAGA**GGAGG**ACAGCT**ATG**CTCAGATATACCTGTCATGCCC
SD-pul-R	GCCGACGTCAATTATTTACTGCTCACCGGCAGG
pul-3t-R	GCCGACGTCAGGACTAGTCCTTATTTACTGCTCACCGGCAGG
SD-pul-3-F	GCTCTAGA**GGAGG**ACAGCT**ATG**CTCAGATATACCTGTCATGCCC
SD-pul-3-R	GCCGACGTCAGGACTAGTCCTTATTTACTGCTCACCGGCAGG
3t-F	GGACTAGTCCATTAACTAGAAAGTAAAGAAGTAGTGACC
3t-R	GCCGACGTCAAGCTTACAGAGAAATACACGAGGGC
gapA-F	ATGACTATCAAAGTAGGTATCA
gapA-R	TTATTTGGAGATGTGAGCGATCAG

[a] The SD mRNA stabilizing sequence and start codon are indicated in bold, and the *Xba* I, *Aat* II, and *Spe* I restriction enzyme sites are underlined.

2.5. SDS-PAGE

Samples of 10 μl were loaded onto a SDS–10% polyacrylamide gel, and the proteins were separated by electrophoresis.

2.6. Semi-quantitative RT-PCR

The *pulA* expression levels in *E. coli* harboring the *pulA* expression plasmids were determined by semi-quantitative RT-PCR as described elsewhere [34] with slight modifications after the induction of expression in the cells by IPTG for 16 h. The primers gapA-F and gapA-R were used to amplify the housekeeping gene glycerol 3-phosphate dehydrogenase gene (*gapA*). The PCR conditions were 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 29 cycles. The primer pairs pul-F and pul-R, SD-pul-F and SD-pul-R, pul-F and pul-3t-R, and SD-pul-F and pul-3t-R were used to amplify *pulA* from pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t, respectively. The PCR conditions were 94°C for 30 s, 58°C for 30 s, and 72°C for 320 s for 28 cycles. A 5-μL aliquot of the resultant PCR solution was analyzed on 1% agarose gel and photographed; the density of each band was quantified using an image analysis software, UVI band V. 97 (UVI Tech, Cambridge, UK).

3. Results

3.1. PCR identification of transformants

To identify the transformants harboring pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t, the *pulA* genes were amplified by PCR. The sizes of the PCR products (3328 bp) matched the expected sizes (Fig. 2), indicating that the transformants were positive clones.

3.2. Pullulanase expression by the transformants

A putative 130-kDa band corresponding to pullulanase was observed in the protein profiles of the transformants harboring pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t by SDS–PAGE. SDS–PAGE analysis revealed that a high level of expression was achieved by the transformants harboring pHT43-SD-pulA and pHT43-SD-pulA-3t. In these cultures, pullulanase was found in both the culture broth and intracellularly (Fig. 3). The pullulanase activities of the transformant harboring pHT43-SD-pulA-3t in culture broth and intracellularly were 2.68 and 5.21 U mg^{-1} protein, 1.2 and 2.0 times that of the transformant harboring pHT43-SD-pulA, respectively (Fig. 4). However, the pullulanase expression was very low in both the culture broth and intracellularly for the transformants harboring pHT43-pulA and pHT43-pulA-3t. The pullulanase activities of the transformant harboring pHT43-SD-pulA-3t in culture broth and intracellularly were 107.0 and 584.1 times that of the transformant harboring pHT43-pulA and were 62.0 and 531.5 times that of the transformant harboring pHT43-pulA-3t, respectively. The pullulanase activities of the transformant harboring pHT43-pulA-3t in culture broth and intracellular were 72.4% and 9.9% higher than that of the transformant harboring pHT43-pulA, respectively (Fig. 4).

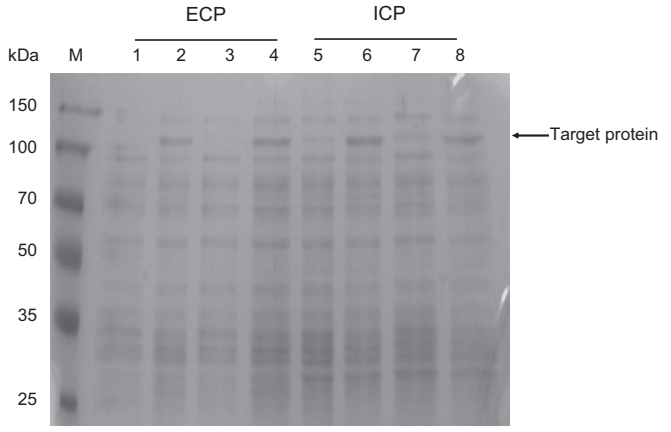

Fig. 3. SDS–PAGE analysis of extracellular and intracellular pullulanase from transformants harboring pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t. ECP, extracellular pullulanase; ICP, intracellular pullulanase; M, protein molecular mass markers; 1–4 (extracellular) and 5–8 (intracellular), pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t. pulA, pullulanase gene (*pulA*) of *Klebsiella variicola* Z-13; SD, 5′ Shine-Dalgarno sequence; 3t, the stem-loop structure of the *Bacillus thuringiensis* crystal protein gene.

3.3. pulA gene mRNA transcription in the transformants

The mRNA expression level of the *pulA* gene in the transformants harboring pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t was determined by semi-quantitative RT-PCR. *pulA* gene expression was highest in the transformant harboring pHT43-SD-pulA-3t among the four transformants and was 339.0%, 34.9%, and 79.8% higher than that in the transformants harboring pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, respectively. *pulA* gene expression in the transformant harboring pHT43-SD-pulA was 225.4% and 33.3% higher than that in the transformants harboring pHT43-pulA and pHT43-pulA-3t, respectively. *pulA* gene expression in the transformant harboring pHT43-pulA-3t was 1.4 times higher than that in the transformant harboring pHT43-pulA (Fig. 5).

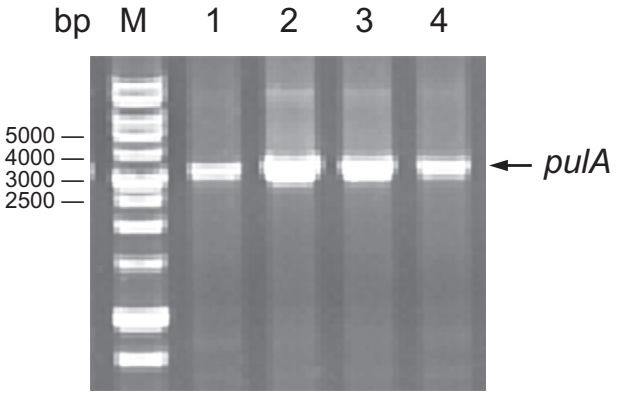

Fig. 2. PCR analysis of the recombinant *pulA* sequence in transformants harboring pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t. M, 1 kb plus DNA ladder; 1, pHT43-pulA; 2, pHT43-SD-pulA; 3, pHT43-pulA-3t; 4, pHT43-SD-pulA-3t.

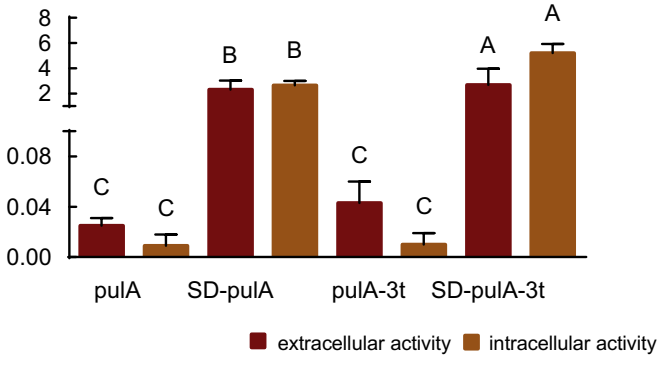

Fig. 4. Activities of extracellular and intracellular pullulanase from transformants harboring pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t. The results represent the mean ± SD of triplicate experiments. Different letters indicate significant differences among the activity of the transformants ($P < 0.01$).

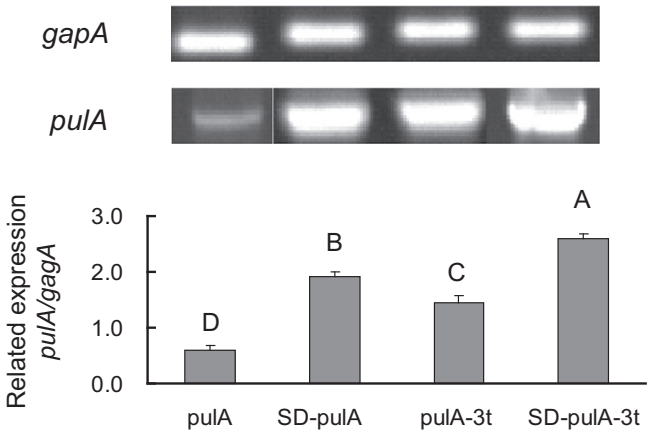

Fig. 5. Semi-quantitative RT-PCR analysis of *pulA* mRNA expression in transformants harboring pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t. *pulA*, pullulanase gene; *gapA*, glycerol 3-phosphate dehydrogenase gene. The results represent the mean ± SD of triple experiments. Bars with different uppercase letters indicate significant differences among the transformants ($P < 0.01$).

4. Discussion

Addition of the 5′ SD sequence at the 5′ UTR has been reported to increase heterologous gene expression and protein synthesis in bacteria [35,36,37]. For example, the addition of the 5′ SD sequence increased *cry1C-t* gene expression by two-fold and protein synthesis by one-fold. Furthermore, addition of both the 5′ SD sequence at the 5′ UTR and the 3′ stem-loop structure at the 3′ UTR of *cry1C-t* increased gene expression and protein synthesis by 24% and 40%, respectively, compared with the addition of the 5′ SD sequence only [37]. In this study, we confirmed that addition of the 5′ SD sequence at the 5′ UTR of *pulA* gene increased gene expression by more than two-fold and enzyme activity by approximately 260-fold. The addition of both the 5′ SD sequence at the 5′ UTR and the 3′ stem-loop structure at the 3′ UTR of the *pulA* gene increased the gene expression and enzyme activity by 34.9% and 1.2-fold, respectively, compared with the addition of the 5′ SD sequence only. These results confirmed that adding the 5′ SD sequence at the 5′ UTR and the 3′ stem-loop structure at the 3′ UTR of the *pulA* gene is an effective approach to increase the gene expression and fermentation enzyme activity.

Wong and Chang [24] reported that the addition of only the 3′ stem-loop structure at the 3′ UTR of interleukin-2 increased the half-life of the mRNA from approximately 2 to 6 min, and polypeptide synthesis increased 4.6- to 7-fold in both *E. coli* and *B. subtilis* [24]. However, in the present study, the addition of only the 3′ stem-loop structure at the 3′ UTR of *pulA* increased mRNA expression and fermentation enzyme activity by only 1.4-fold and 38.5%, respectively. The addition of only the 5′ SD sequence at the 5′ UTR of *pulA* increased mRNA expression and fermentation enzyme activity by more than two-fold and 2.6-fold, respectively. These results suggest that addition of the 5′ SD sequence at the 5′ UTR has a greater effect on increasing mRNA stability and corresponding protein production than the addition of the 3′ stem-loop structure at the 3′ UTR alone. Protein synthesis requires not only a stable mRNA but also ribosome and mRNA binding. The latter may be more important than the former for protein synthesis.

Wang et al. [13] used three promoters, P43, Papr, and Pamy, to increase pullulanase expression in *B. subtilis*. The pullulanase expression efficiency derived from the three promoters decreased in the order Papr > Pamy > P43, but the expression efficiency derived from Papr was only approximately 1.2-fold higher than that from the other two promoters [13]. P43, Papr, and Pamy are promoters of the cytidine deaminase gene in *B. subtilis*, alkali protease gene in *Bacillus amyloliquefaciens,* and α-amylase gene in *Bacillus alcalophilus*, respectively [38]. Addition of an osmolyte (betaine, glycine, or Na^+) or increasing the stability of pET expression system by using *lac* operator element also enhanced the pullulanase expression in *E. coli* manifold [7,8]. The strategy detailed in this study augmented the expression of the gene to an even greater extent by increasing the stability of mRNA. Therefore, increasing the mRNA stability is a more effective method to improve pullulanase expression.

Competing interests

The authors declare that they have no competing interests.

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