Journal of Biotechnology 230 (2016) 40–43

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Short communication

Stable and enhanced gene expression in *Clostridium acetobutylicum* using synthetic untranslated regions with a stem-loop

Joungmin Lee[a,b,1], Yu-Sin Jang[a,b,2], Eleftherios T. Papoutsakis[c], Sang Yup Lee[a,b,*]

[a] *Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical and Biomolecular Engineering (BK21 Program), KAIST, 291 Daehakro, Yuseong-gu, Daejeon 34141, Republic of Korea*
[b] *BioProcess Engineering Research Center, and Center for Systems and Synthetic Biotechnology, Institute for the BioCentury, KAIST, 291 Daehakro, Yuseong-gu, Daejeon 34141, Republic of Korea*
[c] *Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, DE, USA*

ARTICLE INFO

Article history:
Received 4 February 2016
Received in revised form 2 April 2016
Accepted 13 May 2016
Available online 14 May 2016

Keywords:
Clostridium acetobutylicum
Gene overexpression
5′ untranslated region

ABSTRACT

Gene overexpression is one of the most basic strategies in metabolic engineering, but the factors determining gene expression levels have been poorly studied in *Clostridium* species. In this study, we found that a short single-stranded 5′ untranslated region (UTR) sequence led to decreased gene expression in *Clostridium acetobutylicum*. Using an in vitro enzyme assay and reverse transcription-quantitative PCR, we found that addition of a small stem-loop at the 5′ end of mRNA increased mRNA levels and thereby protein expression levels up to 4.6-fold, possibly protecting mRNA from exonuclease attack. Gene-expression levels were apparently independent of the stability of the added stem-loop; the existence of a stem-loop itself appears to be more important. Our results indicate that efficient expression cassettes can be designed by taking the 5′ UTR into consideration, as the expression levels can vary even though the same promoter and RBS are used. These findings will be useful for developing a more reliable gene expression system for metabolic engineering of *Clostridium* strains.

Clostridium is a genus containing Gram-positive, strictly anaerobic bacterial species and has been attracting great interest in biotechnology. This huge genus consists of industrially important species as well as pathogenic species concerned with human health (Durre, 2014). Several clostridial species are able to produce industrially important solvents including acetone, 1-butanol, and ethanol (ABE), among which 1-butanol is of interest to industries since this chemical is not only an important industrial solvent, but also is a better biofuel than ethanol (Durre, 2008; Lee et al., 2008).

Gene overexpression is one of the most basic but important tools of metabolic engineering and synthetic biology. It has been shown that overexpression of several key enzymes leads to enhanced 1-butanol yield and selectivity in *Clostridium* species (Hou et al., 2013; Sillers et al., 2008; Yu et al., 2012). Sillers et al. (2008) reported that overexpression of *adhE1* in the non-solventogenic *Clostridium acetobutylicum* M5 strain under the control of the phosphotransbutyrylase (*ptb*) promoter resulted in higher butanol production compared to that using the native *adhE1* promoter (11.1 *vs.* 6.2 g L^{-1}). A study using *C. tyrobutyricum* demonstrated that strong expression of *C. acetobutylicum adhE2* using the most stable plasmid harboring the pBP1 replicon led to production of higher titer of butanol (20 g L^{-1}) from mannitol without deleting any competing pathways (Yu et al., 2012). Even though gene overexpression in *C. acetobutylicum* has been demonstrated since the early 1990s (Lee et al., 1992; Mermelstein et al., 1992), only a few promoters have been used for metabolic engineering (Jang et al., 2012; Sillers et al., 2008; Tummala et al., 1999). Furthermore, the impact of the structure of recombinant mRNAs on protein expression has not yet been examined in gene overexpression studies in *Clostridium* species.

This study was motivated by an observation during a study on the effects of promoter and terminator on gene expression in *C. acetobutylicum*. Plasmid pLJM72 was constructed from pSOS95-Cm by replacing the *adc* terminator with a synthetic terminator BBa_B1010 (Table 1). Then, plasmid pLJM76 was constructed by replacing the *C. acetobutylicum thl* promoter (P_{thl}) in pLJM72 with the *C. acetobutylicum ptb* promoter (P_{ptb}). Plasmids were introduced

* Corresponding author at: Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical and Biomolecular Engineering (BK21 Program), KAIST, 291 Daehakro, Yuseong-gu, Daejeon 34141, Republic of Korea.
E-mail address: leesy@kaist.ac.kr (S.Y. Lee).
[1] CJ CheilJedang R&D Institute, Suwon, Gyeonggi-do 16495, Republic of Korea.
[2] Institute of Agriculture & Life Science (IALS), Department of Agricultural Chemistry and Food Science, Gyeongsang National University, Jinju, Gyeongsangnam-do 52828, Republic of Korea.

http://dx.doi.org/10.1016/j.jbiotec.2016.05.020
0168-1656/

Table 1
Activities of chloramphenicol acetyltransferase (CAT) when using different promoters and terminators.

Plasmid	CAT activity[a] (mU/mg protein)
Wild-type	ND
pSOS95-Cm	3.76 ± 0.84
pLJM72[b]	5.12 ± 1.14
pLJM76[c]	1.88 ± 0.64

[a] Values shown are (mean) ± (SD) obtained from triplicate experiments; ND, not detected.
[b] This plasmid was constructed by ligation of *Nar*I/*Eco*RI double-digested pSOS95-Cm and an annealed product of two oligonucleotides 5′-CGCCCGCCGCAAACCCCGCC-CCTGACAGGGCGGGGTTTCGCCGCG-3′ and 5′- AATTCGCGGCGAAACCCCGCCCT-GTCAGGGGCGGGGTTTGCGGC-GGG-3′.
[c] This plasmid was constructed by ligation of *Pst*I/*Bam*HI double-digested pLJM72 and a *Pst*I/*Bam*HI double-digested PCR product containing the *C. acetobutylicum ptb* promoter, amplified using two oligonucleotides 5′-ATATCTGCAGGTGGATGGAGTTAAG-3′ and 5′-ATATGGATCCGCTCATTTATATTTTAACA-3′.

into *C. acetobutylicum* ATCC 824 through electroporation following a published protocol (Mermelstein et al., 1992) using an ECM 630 electroporator (Harvard Apparatus Inc., Holliston, MA). Prior to transformation into *C. acetobutylicum*, all plasmids were methylated *in vivo* in *E. coli* harboring a suitable methylation plasmid (Mermelstein and Papoutsakis, 1993). Transformed colonies were selected on the 2x YTG (Bacto™ tryptone, 16 g L^{-1}; Bacto™ yeast extract, 10 g L^{-1}; NaCl 4 g L^{-1}; glucose, 5 g L^{-1}) agar plates supplemented with the 40 μg mL^{-1} of erythromycin.

To examine gene expression levels, the wild-type and recombinant *C. acetobutylicum* strains were cultivated in the flasks containing 100 mL of clostridial growth medium (Jang et al., 2012; Tummala et al., 1999) at 37 °C in an anaerobic glove box (Coy, Grass Lake, MI). For wild-type *C. acetobutylicum*, the spore stock was inoculated and germinated by heating at 70–80 °C for 10 min after inoculation. For recombinant strains, a frozen glycerol stock of vegetative cells was inoculated. When the OD_{600} reached between 1.0 and 1.5, cells were harvested for chloramphenicol acetyltransferase (CAT) assay as reported (Scotcher et al., 2003).

When comparing of pLJM72 with pSOS95-Cm with respect to gene expression level, it was found that use of a strong terminator increased CAT activity by about 36% (Table 1). However, replacement of P_{thl} with the P_{ptb} (plasmid pLJM76) in pLJM72, decreased the CAT activity by 63% compared to that obtained with pLJM72. This result was unexpected because the strength of P_{ptb} is known to be greater than (Girbal et al., 2003), or comparable with that of P_{thl} (Tummala et al., 1999) during the acidogenic phase. The 5′ untranslated region (UTR) of the expression cassette in pLJM76 is only 37-nt long (Fig. S1 of Supplementary data). Mfold (Zuker, 2003) predicts that the 5′ end of the mRNA remains single-stranded. Many previous studies, in both eukaryotic and prokaryotic organisms, suggest that the 5′ UTR of mRNA affects the strength of gene expression (Curran et al., 2013; Emory et al., 1992; Sharp and Bechhofer, 2005; Smolke and Keasling, 2002). Hence, we decided to test the effects of 5′-terminal stem-loop of mRNA on gene expression in *C. acetobutylicum*. For this, 8 variants of the 5′ UTR were constructed from pLJM76, resulting in pLJM76v0 to pLJM76v7 (Table 2). From pLJM76v1 to pLJM76v7, the 5′ UTR sequence was modified to form a short stem-loop with various strengths at the 5′ end of mRNA (Table 2). Plasmid pLJM76v0 is a control that has the same 5′ UTR length as UTRv1 but does not form a strong stem-loop at the 5′ end. After transformation of the plasmids, each *C. acetobutylicum* strain was cultured in duplicates in New Brunswick BioFlo 110 bioreactors (Eppendorf) containing 0.5 L of CGM. When the OD_{600} reached 1.0–1.5 (exponential phase) and 8–10 (solventogenic phase), cells were harvested to perform CAT assays and to isolate total RNA. For total RNA isolation, cells were immediately treated

Table 2
Sequences of 5′ UTR and its stability.

Plasmid[a]	UTR sequence[b]	ΔG[c] (kcal mol^{-1})
pLJM76	AGC	ND
pLJM76v0	AGTTGAATTAAGTTCT	0.3
pLJM76v1	**AGTAC**AATTAA**GTACT**	−3.9
pLJM76v2	**AGTGC**AATTAA**GCACT**	−5.9
pLJM76v3	**AGGCC**AATTAA**GGCCT**	−8.2
pLJM76v4	**AGTAC**AATATAA**GTACT**	−3.4
pLJM76v5	**AGTAC**AATAA**GTACT**	−3.7
pLJM76v6	**AGGCC**AATATAA**GGCCT**	−7.7
pLJM76v7	**AGTTGC**AATTAA**GCAACT**	−6.8

[a] Plasmids pLJM76v0 to v7 were constructed by inverse PCR of pLJM76 and self-ligation. The forward primer 5′-GGATCCACTTGAATTTAAAAGGG-3′ was used for construction of all plasmids. Reverse primers used are as follows: v0, 5′-AGAACTTAATTCAACTCATTTATAT-TTTAACAAACTTT-3′; v1, 5′-AGTACTTAATTGTACTCATTTATATTTTAACAAACTTT-3′; v2, 5′-AGTGCTTAATTGCACTCATTTATATTTTAACAAACTTT-3′; v3, 5′-AGGCCT-TAATTGGCCTCATTTATATTTTAACAAACTTT-3′; v4, 5′-AGTACTTATATTGTACTC-ATTTATATTTTAACAAACTTT-3′; v5, 5′-AGTACTTATTGTACTCATTTATATTTTAAC-AAACTTT-3′; v6, 5′-AGGCCTTATATTGGCCTCATTTATATTTTAACAAACTTT-3′, v7, 5′-AGTTGCTTAATTGCAACTCATTTATATTTTAACAAACTTT-3′.
[b] Only sequences at the upstream of the *Bam*HI recognition sequence are shown. Bold nucleotides indicate the regions that form a stem.
[c] Values were calculated using Mfold (Zuker, 2003).

with RNAProtect Bacterial Reagent (Qiagen). After collection of cells, cell pellets were resuspended in 50 μL lysozyme solution (30 mg mL^{-1}) and incubated for 15 min at room temperature. Total RNA was isolated using the Hybrid-R™ RNA extraction kit from the lysozyme-treated cells (Geneall, Seoul, Korea). One microgram of the total RNA was subjected to 20 μL of a RT reaction using RocketScript™ reverse transcriptase premix (Bioneer, Daejeon, Korea), using random nonadeoxynucleotides as the primer. Quantitative PCR (qPCR) was performed using 2x TOPreal™ SYBR master mix (Enzynomics, Daejeon, Korea) and LightCycler 96 (Roche Diagnostics). The *fabZ* gene (CAC3571) was chosen as an internal control (Jones et al., 2008). Primers 5′-TGCCAGGAGTTCTTA-TGATTGA-3′ and 5′-TTACTGCTCCAAGTATAGGCTT-3′ were used for the amplification of *fabZ*; 5′-TGAAAGTGATACGCAACGGTAT-3′ and 5′-TCAGATTAAAGCCATCGAAGGT-3′ were used for the amplification of the *cat* gene encoding CAT.

The addition of a small stem-loop to the 5′ end of mRNA successfully increased mRNA levels and consequently protein levels. Both in the acidogenic (Fig. 1A) and solventogenic (Fig. 1B) phases, the CAT activities were overall positively correlated with mRNA levels. Notably, mRNA and CAT levels of all plasmids tested rather increased in the solventogenic phase despite the use of P_{ptb}, which typically shows a decreased activity towards the later growth phase (Jones et al., 2008; Tummala et al., 1999). In pLJM76v0, in which the CAT mRNA does not possess a stem-loop at its 5′ end, the mRNA levels increased less than 2-fold in both phases; the increased length of the 5′ UTR seems to have partly impeded degradation of the 5′ end of mRNA. Addition of a stem-loop at the 5′ end of the mRNA further increased mRNA levels and therefore CAT activities up to 4.6-fold were measured compared to pLJM76. It was found, however, that the increase of mRNA levels depended on the existence of stem-loop itself; the gene expression level did not correlate with the strength of the stem-loop (see Table 2 for the predicted Gibbs free energies of the tested constructs).

Finally, to see whether a similar result would be observed with other genes, the *C. acetobutylicum adhE1* and *adhE2* genes were cloned into pLJM76 and pLJM76v1. For both *adhE1* and *adhE2*, the 5′ stem-loop clearly increased the protein expression levels in pLJM76v1 compared to those obtained with pLJM76, and the effect was much clearer in the solventogenic phase than the acidogenic phase (Fig. 2A). A similar result was obtained in *Clostridium beijer-*