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RESEARCH PAPER

Engineering *Escherichia coli* BL21 Genome to Improve the Heptanoic Acid Tolerance by using CRISPR-Cas9 System

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Abstract Acid tolerance is one of the critical factors to determine the quality of the industrial production strains. Therefore, we have investigated the introduction of the acid tolerance genes into the genome of *Escherichia coli* BL21 by using CRISPR-Cas9 system. The *dsrA* and *rcsB* genes of *E. coli* K-12, which are involved in the heptanoic acid tolerance, were inserted into the genome of *E. coli* BL21 without scar. The native transcription unit (TU) of *dsrA* and the synthetic TU of *rcsB* were integrated in *E. coli* BL21 genome. We found that the position of genomic coordinate of 1,300,270 was more efficient to integrate *dsrA* and *rcsB* than genomic coordinate of 3,876,428. Furthermore, the *rcsB* was successfully expressed in the resulting engineered strains (*i.e.,* $rcsB^+$ or $dsrA^+$ $rcsB^+$ strains). The engineered strains expressing *dsrA* and/or *rcsB* showed the higher survival rate and specific growth rate under *n*-heptanoic acid stress than wild-type *E. coli* BL21. These results indicate that the newly introduced acid-tolerance systems were active in the *E. coli* BL21 strain.

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1. Introduction

The production of carboxylic acids (*e.g.*, acetic acid, butyric acid, hexanoic acid, octanoic acid) in microbial cells may lead to a decrease of intracellular pH and the damage to cell membranes, thus lowering the product yields and titers [1,2]. If the acid tolerance is achieved in production host, there is additional advantage in downstream process cost in addition to the increase in yields and/or titers. For example, the production of carboxylic acids around the neutral pH additionally increases the purification cost [3]. Therefore, regardless of the workhorses (*i.e. Saccharomyces cerevisiae*, *Corynebacterium glutamicum* or *Escherichia coli*), the acid tolerance is desirable for fatty acids production.

Among the usual candidates for the development of recombinant strain, *E. coli* BL21 is one of the most utilized bacterial strains. However, unlike the *E. coli* K-12, *E. coli* BL21 lost a few genes including the ones involved in the acid or stress tolerance. For example, *E. coli* BL21 does not have *dsrA* (noncoding small RNA (sRNA)), which inhibits H-NS and activates *rpoS*, thereby affecting acid tolerance [4]. *E. coli* BL21 does not have *rcsB* either, which is related to the activation of *gadE* [5,6]. Without *rcsB*, GadE of glutamate-dependent acid resistance (GDAR) system does not form heterodimer with RcsB and cannot bind to GAD box [7]. Therefore, GDAR system does not work without RcsB. Regarding the contribution of RcsB to acid tolerance, Woo *et al.* showed that complementation of *rcsB* in *E. coli* BL21 can increase the acid tolerance by activating GDAR [6]. In addition, they also complemented *dsrA* [8], to enhance the acid tolerance of *E. coli* BL21.

Although they showed the enhanced acid tolerance, industrial strain having plasmids needs additional cost of the use of expensive antibiotics to maintain plasmids. Therefore, if we can integrate recombinant genes into genome, we may additionally reduce the operation cost for antibiotics. In addition, if genes of interest are integrated into genomes, we can make additional room for the use of plasmids encoding the enzymes of desired synthetic pathway. These days, genome engineering has become a routine work due to the rapid development of genome engineering tools such as CRISPR-Cas system. In spite of several concerns about CRISPR-Cas system such as off-target effect [9], CRISPR-Cas system has enabled the scar-free and reliable genome engineering.

In our previous study, genes for enhancing acid tolerance, *i.e. dsrA* and *rscB*, were recruited using recombinant plasmids and employment of two genes showed improved acid tolerance [6]. To make the recombinant host of our previous study more industrially friendly and to investigate CRISPR-Cas system in *E. coli* BL21, we investigated the integration of *dsrA* and *rcsB* into *E. coli* BL21 genome using CRISPR-Cas9 system.

2. Materials and Methods

2.1. Enzymes, plasmids, and chemicals

Luria-Bertani (LB) medium was purchased from Becton Dickinson Korea (Seoul, Republic of Korea). Glucose was purchased from Duksan Pure Chamicals (Ansan, Gyeonggi-do, Republic of Korea). Chemicals for Riesenberg medium [10], antibiotics, L-arabinose, isopropyl β-D-1-thiogalactopyranoside (IPTG), *n*-heptanoic acid, glycerol, and M9 minimal salt were purchased from Sigma-Aldrich Korea (Seoul, Republic of Korea). pCas (Addgene plasmid number 62225) and pTargetF (Addgene plasmid number 62226) were purchased from Addgene (Cambridge, MA, USA).

2.2. Bacterial strains and growth condition

E. coli BL21 (DE3), mutant strains, and *E. coli* K-12 strains were cultivated at 37°C in LB medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) supplemented with appropriate antibiotics for plasmid DNA preparation and seed cultivation. The Riesenberg medium with 10 g/L glucose and appropriate antibiotics was used for the main cultivation. The Riesenberg medium consisted of 4 g/L $(NH_4)_2HPO_4$, 13.5 g/L, KH_2PO_4, 1.7 g/L citric acid, 1.4 g/L $MgSO_4$, and 10 mL/L trace metal solution. The trace metal solution was composed of 10 g/L $FeSO_4$, 2.25 g/L $ZnSO_4$, 1.0 g/L $CuSO_4$, 0.5 g/L $MnSO_4$, 0.23 g/L $Na_2B_4O_7$, 2.0 g/L $CaCl_2$, and 0.1 g/L$(NH_4)_6Mo_7O_{24}$. The minimal M9 medium was used for colony forming unit (CFU) calculation. The M9 minimal medium consisted of 33.9 g/L Na_2HPO_4, 15 g/L KH_2PO_4, 5 g/L NH_4Cl, and 2.5 g/L NaCl.

2.3. Construction of pgRNA and editing template

Genome sequence of *E. coli* K-12 MG1655 (accession number NC_000913.3) and *E. coli* BL21 (accession number CP001509.3) were retrieved from GenBank database and used to design guide RNA and editing template. To construct plasmid encoding guide RNA for Cas9 endonuclease (pgRNA, hereafter), following primers were used; sense primer: 5'-ATATATACTAGT-N(20)-GTTTTA GAGCTAGAAATAGC-3' (N(20) means the genomic sequence of 20 nucleotides upstream of PAM (protospacer adjacent motif) of target site), antisense primer: 5'-ATATATACTAGTATTATACCTAGGACTGAGCTAG-3'. The PCR product was ligated using T4 DNA Ligase (Elpis Biotech, Daejeon, Republic of Korea) after the consecutive enzymatic digestion with DpnI and SpeI. Genomic DNA of *E. coli* BL21 (DE3), *E. coli* K-12 MG1655 were prepared from the 3 ml scale overnight culture in LB medium using DOKDO-Prep bacterial genomic DNA purification extraction kit (Elpis Biotech, Daejeon, Republic of Korea). *dsrA* and *rcsB* genes were amplified from *E. coli* K-12 MG1655 genomic DNA and 400 bp-length homologous arms were amplified from *E. coli* BL21 genomic DNA. Following the previous report [8], the complete transcription unit of *dsrA* including native promoter and terminator was amplified. For *rcsB*, open reading frame was amplified to have C-terminal His-tag using the primers having synthetic constitutive promoter (BBa_J23100, 5'-TTGACGGCT AGCTCAGTCCTAGGTACAGTGCTAGC-3'), synthetic ribosome binding site (RBS) (BBa_B0034, 5'-AAAGAG GAGAAA-3'), and *E. coli rrnB* terminator (BBA_B0010, 5'-CCAGGCATCAAATAAAACGAAAGGCTCAGTCGA AAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGG TGAACGCTCTC-3'). For target site 1 (TS1, genomic coordinate of *E. coli* BL21 genome = 3,876,409), left arm and right arm sequences were chosen to have genomic coordinates of 3,876,020 – 3,876,419 and 3,876,430 – 3,876,829, respectively. For target site 2 (TS2, genomic coordinate of *E. coli* BL21 genome = 1,300,270), left arm and right arm were chosen to have genomic coordinates of 1,299,881 – 1,300,280 and 1,300,291 – 1,300,690, respectively. Genomic coordinate numbers were based on the *E. coli* BL21 genome sequence. Left arm, right arm, *dsrA* and/or *rcsB* transcription unit were fused by extension PCR. Gel purification of the PCR products was performed prior to electroporation. All primers were purchased from Cosmo genetech (Seoul, Republic of Korea).

2.4. Transformation and plasmid curing

E. coli BL21 (DE3) competent cells containing pCas were prepared. L-Arabinose (final concentration of 10 mM) and kanamycin (50 mg/L) were added to the culture for the induction of λ-red recombinase [11]. The electrocompetent cells was grown at 30°C to OD_{600} = 0.5 and harvested at 3,500 rpm and 4°C by centrifugation for 5 min. The cells were then washed third times with ice-cold 10% glycerol solution. After the removal of supernatant, cells were suspended with residual 10% glycerol solution (~ 500 μL). For electroporation, 100 μL of competent cells was mixed with 100 ng of pgRNA and 400 ng of editing template. Electroporation was done in a 2-mm Gene Pulser cuvette (Bio-Rad Korea, Seoul, Republic of Korea) at 2.5 kV, and the product was suspended immediately in 700 μL of ice-cold LB medium. Cells were recovered at 30°C for 1 h before being spread onto LB agar containing kanamycin (50 mg/L) and ampicillin (50 mg/L) and incubated overnight at 30°C. Positive clones were identified by colony PCR.

For the curing of pCas and pgRNA, the mutant strains harboring both pCas and pgRNA plasmid was inoculated into 3 mL of LB medium containing kanamycin (50 mg/L) and 0.1 mM IPTG. The culture was incubated for 8 h, diluted, and spread onto LB plates containing ampicillin (50 mg/L). The colonies were confirmed as cured by determining their sensitivity to ampicillin (50 mg/L). pCas was cured by growing the colonies overnight at 42°C. The colonies were confirmed as cured by determining their sensitivity to kanamycin (50 mg/L). Otherwise, many subculture in LB medium could cured two plasmids. Plasmid-cured mutants were identified by PCR amplification of transcription unit of *dsrA* and/or *rcsB* and DNA sequencing.

2.5. Real time PCR and western blot

Real time PCR samples were harvested at OD_{600} = 1 in stationary phase of *E. coli* cultivated in Riesenberg medium. Real time PCR was performed using Labopass TMSYBR Green Q Master (Cosmo genetech, Seoul, Republic of Korea). Expression levels were calculated using the Bio-Rad CFX manager software, using the *E. coli* K-12 sample as the reference and the 16S rRNA as the internal control.

To investigate the expression of RcsB (theoretical size 24.49 kD), western blotting was performed with Anti-6X His tag antibody. *E. coli* BL21 (DE3) $rscB^+$ and *E. coli* BL21 (DE3) $dsrA^+rcsB^+$ strain were used for western blotting. Lysate was prepared by sonication in 200 mL of 50 mM Tris buffer (pH 8.0, protease inhibitor cocktail (Sigma-Aldrich Korea, Seoul, Republic of Korea) included) after harvest at the stationary growth phase. 2.5, 5, and 10 mL of the lysate was loaded, respectively. For western blot membrane detection, exposure time was changed to 1, 5, and 10 min.

2.6. Acid tolerance assay

Tolerance assay for *n*-heptanoic acid was carried out as described previously [6,8]. Firstly, *E. coli* BL21 (DE3), *E. coli* K-12, *E. coli* BL21 (DE3) $dsrA^+$, *E. coli* BL21 (DE3) $rcsB^+$, and *E. coli* BL21 (DE3) $dsrA^+rcsB^+$ were inoculated into 3 mL of LB medium and cultured overnight. After overnight culture, cells were inoculated into 25 mL of LB medium and cultivated until OD_{600} reaches 1. Then, the cultures were diluted to 1/10 with M9 minimal medium and cultivated for 1 h after the stressor was added. After the cells were incubated in M9 medium containing 3 or 5 mM of *n*-heptanoic acid, cells were serially diluted and spread evenly on LB agar plates. Cells without *n*-heptanoic acid stress were also spread on LB agar plate. Survival rate based on colony forming unit was calculated as follows; survival (%) = colony forming unit (CFU) (post stress)/ CFU (prior stress) × 100.

3. Results And Discussion

3.1. Integration of *dsrA* and/or *rcsB*

There have been studies to report the genome editing in *E. coli* K-12 strains using CRISPR-Cas system [11,12]. For instance, Bassalro *et al.* reported the integration and expression efficiency of several integration positions of *E. coli* K-12 genome when genes are integrated using CRISPR-Cas system [13]. Based on the report by Bassalro *et al.,* we have selected the two intergenic regions as target sites for gene integration (Fig. 1). Firstly, we searched for the integration site, corresponding to the safe site 9 (SS9) of *E. coli* K-12 MG1655, because the SS9 site showed integration efficiency of 100% [13]. In addition, the GFP gene integrated at SS9 was relatively well-expressed (fluorescent intensity of about 70% compared to the strongest fluorescent intensity). However, the target sequence of SS9 (*i.e.,* TCTGGCGCAGTTGATATGTA) was missing in *E. coli* BL21 genome. Therefore, we selected one site in the intergenic region between *aslA* and *glmZ*. The target site (TS) has the sequence of TTCAATCCTACCTCTGGCGC and PAM sequence of AGG (Fig. 1A). In spite of several trial, we could not integrate *dsrA* and/or *rcsB*. The reason for the different integration efficiency was not clear. When considering the previous reports [14,15], we guess that lots of gene deletion in *E. coli* BL21 genome have made the local genome structure of TS1 of *E. coli* BL21 different from that of SS9 of *E. coli* K-12 MG1655. Another reason might include the different protein occupancy [16] of TS1 from SS9, which might result in the inhibition of Cas9's approach to TS1.

Next, we have tried the integration of *dsrA* and/or *rcsB* into another site of *E. coli* BL21 genome (Fig. 1B). We

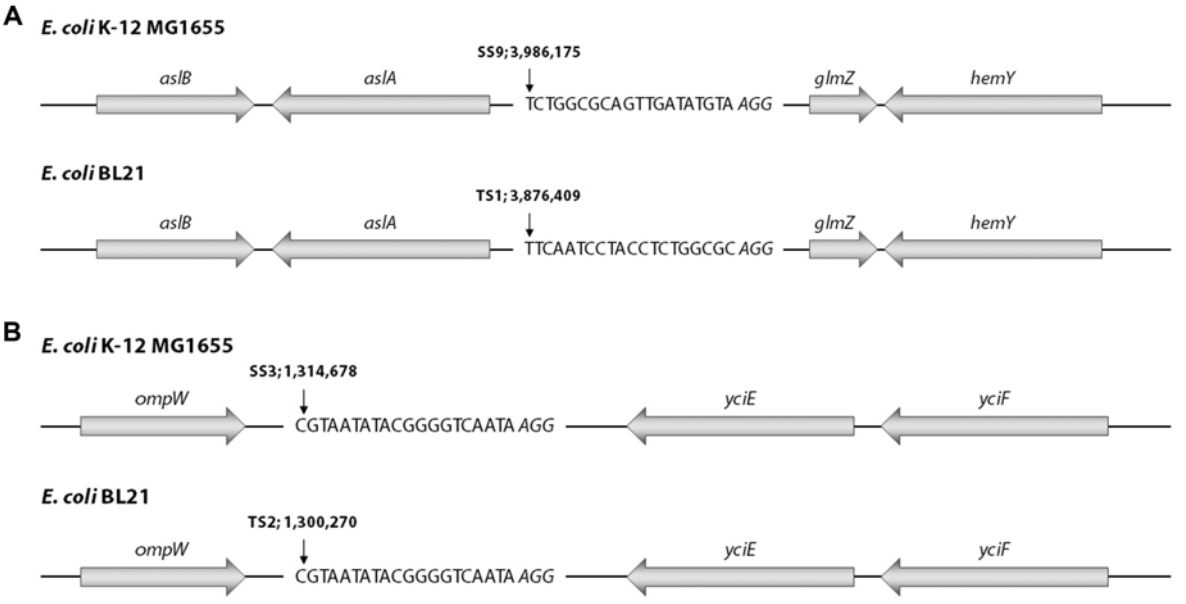

Fig. 1. Genome coordinates of target sites. (A) Genomic environment and genome coordinate of safe site 9 (SS9) and target site 1 (TS1). The target site 1 in the *E. coli* BL21 genome, which is comparable to the SS9 of *E. coli* K-12 MG1655 [13], was designated as TS1. (B) Genomic environment and genome coordinate of SS3 and TS2. The target site 2 in the *E. coli* BL21 genome, which is comparable to the SS3 of *E. coli* K-12 MG1655 [13], was designated as TS2. The nucleotide sequences in normal style (20 nt) are target sequence of guide RNA. The nucleotide sequences in italic are PAM sequences.

found the same target sequence as that of SS3 (safe site 3 [13]) at genomic coordinate of 1,300,270. We designated this site as TS2. When the integration using 400 ng of editing template having *dsrA*, *rcsB*, or *dsrA*/*rcsB* transcription unit was tried, it showed 32, 73, and 41% of integration efficiency, respectively. Compared to the previous report [13], integration efficiency at TS2 was lower than that of SS3 although two integration sites have similar genomic environment and genomic coordinate.

3.2. Expression of integrated *dsrA* and *rcsB*

The native *dsrA* transcription unit was used to ensure the native transcription start and stop of *dsrA* and complete construction of sRNA DsrA. Therefore, transcription unit of *dsrA* in *E. coli* BL21 *dsrA*$^+$ and *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$ was designed to be identical to that in *E. coli* K-12. With this native transcription unit, we investigated the expression of *dsrA* in *E. coli* K-12, *E. coli* BL21 *dsrA*$^+$, and *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$. Transcript level of *dsrA* was measured for *E. coli* K-12, *E. coli* BL21 *dsrA*$^+$ and *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$ using real time PCR. When there was no *n*-heptanoic acid stress, the *dsrA* expression level was lower than that in *E. coli* K-12 (Figs. 2A and 2B). However, when 3 or 5 mM *n*-heptanoic acid was present in the culture medium, the *dsrA* transcription level of *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$ was much higher or similar compared to *E. coli* K-12 (Fig. 2B). In particular, under 3 mM of *n*-heptanoic acid stress, the *dsrA* transcription level of *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$ was 2.3-fold higher than *E. coli* K-12. The reason for the different transcription level among wild type and mutant *E. coli* strains is not clear. One possible reason for this different transcription level could be a different transcription rate depending on the position on the genome, as previously reported [17].

A constitutive promoter was used to prevent any possible regulation of gene expression of *rcsB* in *E. coli* BL21. Therefore, the *rcsB* transcription unit of *E. coli* BL21 *rcsB*$^+$ and *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$ was different from that in *E. coli* K-12. It turned out that the *rcsB* transcription level of *E. coli* BL21 *rcsB*$^+$ was >3-fold of that of *E. coli* K-12 (Fig. 3A). Surprisingly, the *rcsB* transcription level of *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$ was 38-fold and 23-fold of that of *E. coli* K-12 in the presence of 3 and 5 mM of *n*-heptanoic acid, respectively (Fig. 3B). The *rcsB* transcription level of *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$ was > 6-fold of that in *E. coli* K-12 even without *n*-heptanoic acid stress. This much higher transcription level of *rcsB* may be due to the different transcription rate depending on the position of genome [17]. Another reason for this higher transcript level may be the accumulation of mRNA under stress condition. Since we used constitutive promoter, it was hard to attribute the higher transcript level to an elevated transcription rate through transcriptional regulation. Rather, the accumulation of mRNA can be more reasonable. According to Benard,

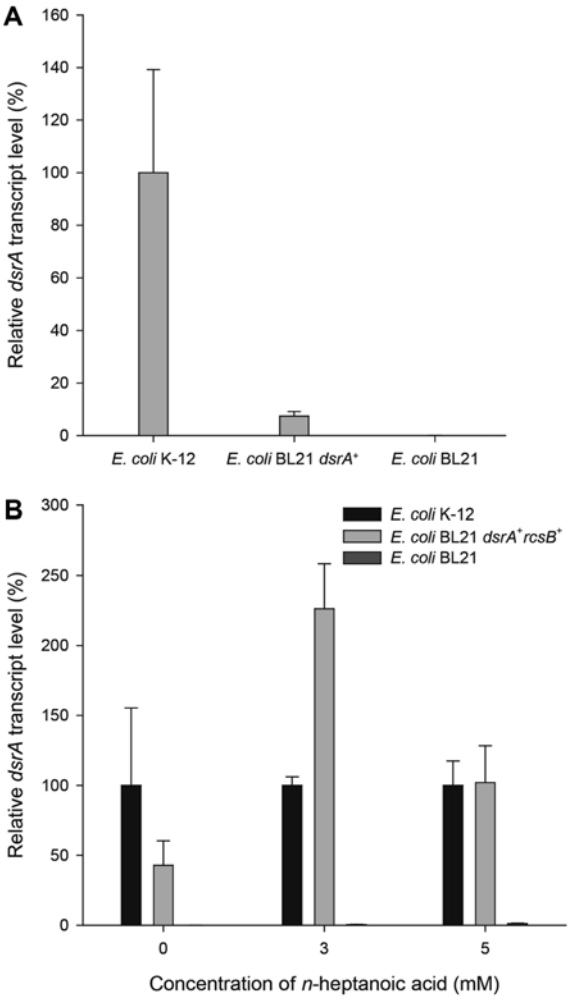

Fig. 2. Comparison of *dsrA* transcript level in *E. coli* wild type with mutant strains. (A) Relative *dsrA* transcript level of *E. coli* K-12, *E. coli* BL21 $dsrA^+$, and *E. coli* BL21. When *dsrA* transcript level of *E. coli* K-12 was set to 100, that of *E. coli* BL21 $dsrA^+$ and *E. coli* BL21 were 7.38 and 0, respectively. (B) Relative *dsrA* transcript level of *E. coli* K-12, *E. coli* BL21 $dsrA^+rcsB^+$, and *E. coli* BL21 was measured in the presence of 0, 3, or 5 mM of *n*-heptanoic acid in the cultivation medium. The *dsrA* transcript level of *E. coli* K-12 was set to 100. Compared to the *dsrA* transcript level of *E. coli* K-12, that of *E. coli* BL21 $dsrA^+rcsB^+$ in 0, 3, or 5 mM *n*-heptanoic acid were 43, 230, and 100%, respectively. The *dsrA* transcript was not detected in *E. coli* BL21 because *E. coli* BL21 does not possess *dsrA* gene. Experiments were run in triplicate. Error bars mean the standard deviations.

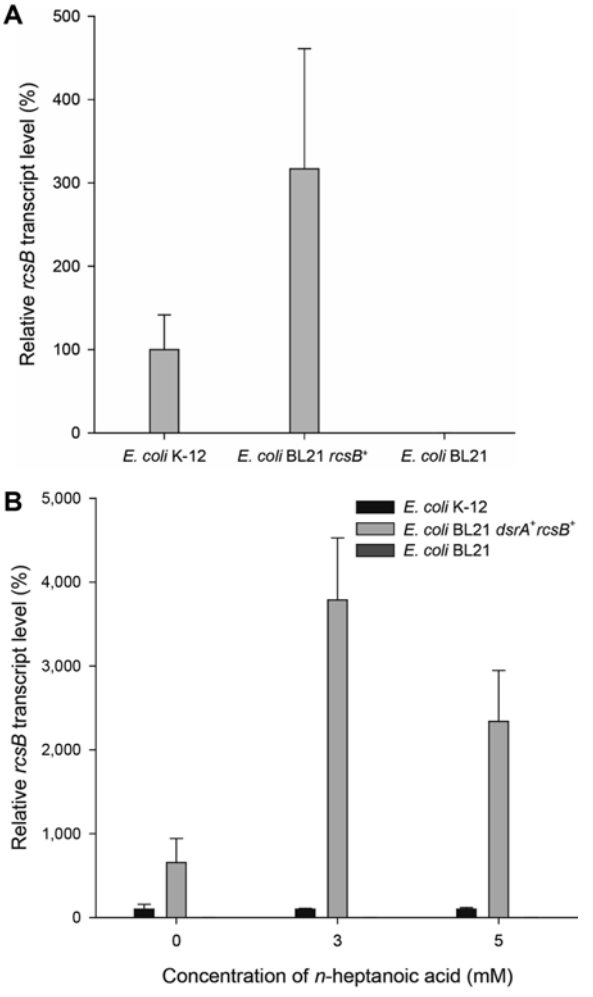

Fig. 3. Comparison of *rcsB* transcript level in *E. coli* wild type with mutant strains. (A) Relative *rcsB* transcript level of *E. coli* K-12, *E. coli* BL21 $rcsB^+$, and *E. coli* BL21. When the *rcsB* transcript level of *E. coli* K-12 was set to 100, that of *E. coli* BL21 $rcsB^+$ and *E. coli* BL21 was 320 and 0, respectively. (B) Relative *rcsB* transcript level of *E. coli* K-12, *E. coli* BL21 $dsrA^+rcsB^+$, and *E. coli* BL21 was measured in the presence of 0, 3, or 5 mM of *n*-heptanoic acid. The *rcsB* transcript level of *E. coli* K-12 was set to 100. Compared to the *rcsB* transcript level of *E. coli* K-12, that of *E. coli* BL21 $dsrA^+rcsB^+$ in 0, 3, or 5 mM *n*-heptanoic acid were 650, 3,800, and 2,300%, respectively. The *rcsB* transcript was not detected in *E. coli* BL21 because *E. coli* BL21 does not possess *rcsB* gene. Experiments were run in triplicate. Error bars mean the standard deviations.

5' to 3' mRNA degradation is inhibited in *Saccharomyces cerevisiae* under stress condition such as amino acid starvation [18]. Therefore, there may be a possibility that *E. coli* inhibits the mRNA degradation under acid stress.

We have next investigated whether the RcsB was translated or not. Western blotting was performed using anti-his-tag antibody because RcsB in this study has C-terminal his-tag. As shown in Fig. 4, the RcsB was successfully expressed in *E. coli* BL21 $rcsB^+$ and *E. coli* BL21 $dsrA^+rcsB^+$.

3.3. Acid tolerance of engineered *E. coli* BL21 strains

To estimate the acid tolerance of the newly engineered *E. coli* BL21 strains, specific growth rate and colony forming unit (CFU) were measured. Since one of the aims of this study was to develop a production strain for the production of medium chain fatty acids from long chain fatty acids [19-22], the specific growth rate was measured in the Riesenberg medium. *E. coli* K-12, *E. coli* BL21, *E. coli* BL21 $dsrA^+$, *E. coli* BL21 $rcsB^+$, and *E. coli* BL21

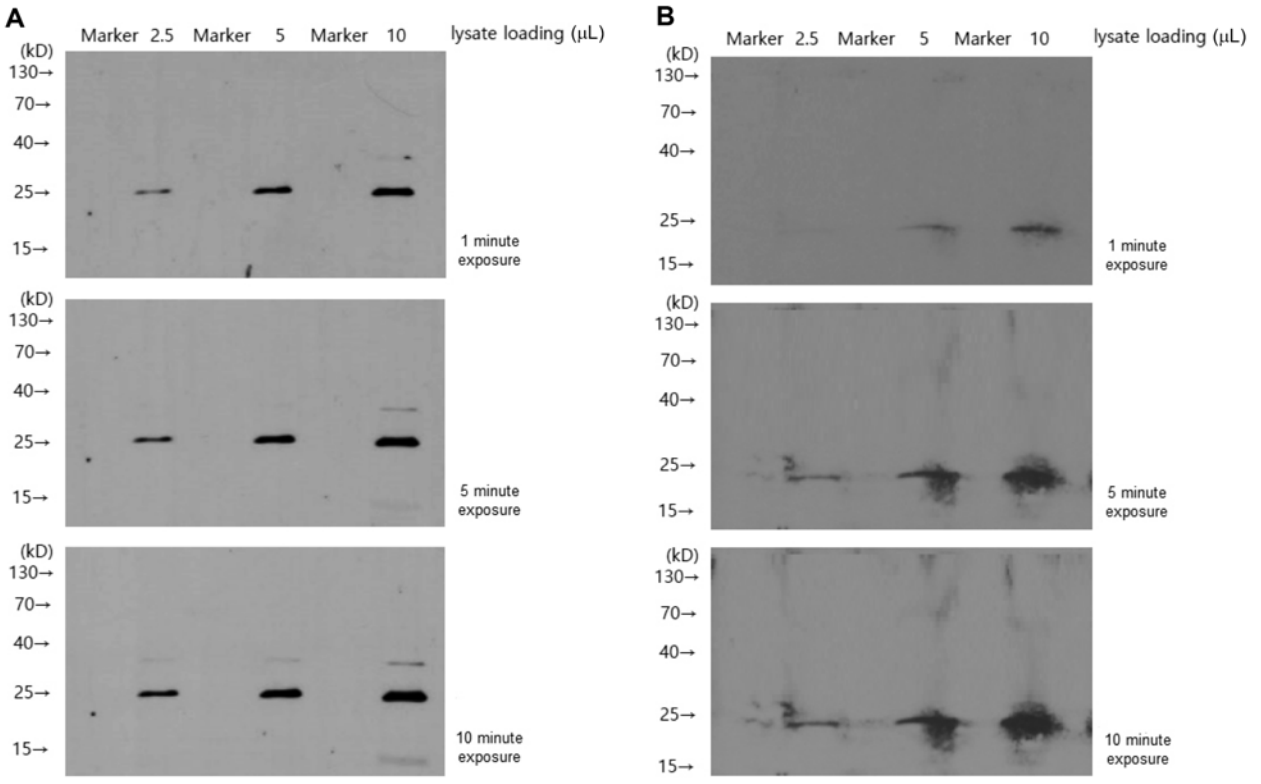

Fig. 4. Western blot analysis of RcsB translation in (A) *E. coli* BL21 *rcsB*$^+$ and (B) *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$. Loading amount of cell lysate was varied from 2.5 to 10 μL for each sample. Exposure times were 1, 5, and 10 min for each sample. Theoretical size of RcsB is 24.49 kD.

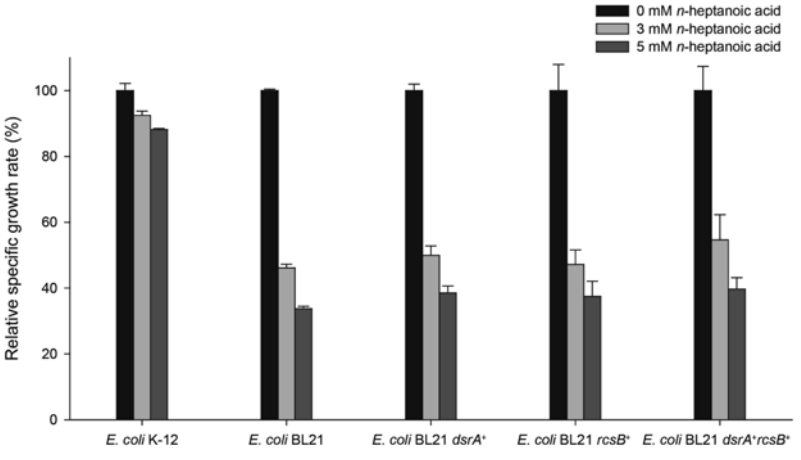

Fig. 5. Relative specific growth rates of *E. coli* K-12, *E. coli* BL21, *E. coli* BL21 *dsrA*$^+$, *E. coli* BL21 *rcsB*$^+$, and *E. coli* BL21 *dsrA*$^+$*rcsB*$^+$. Average growth rate of each strain without *n*-heptanoic acid stress was set to 100%. Experiments were run in triplicate. Error bars mean the standard deviations.

dsrA$^+$*rcsB*$^+$ were cultivated until OD_{600} reached 0.5 at 37°C under aerobic condition. Then, 3 or 5 mM of *n*-heptanoic acid was added into the Riesenberg medium and optical density was measured at every hour. The relative specific growth rate was compared in the absence and the presence of *n*-heptanoic acid. *E. coli* K-12 strain showed little decrease in the specific growth rate even when 3 or 5 mM of *n*-heptanoic acid was added (Fig. 5). In the case of *E. coli* BL21 strains, the relative specific growth rate was reduced to the half under 3 mM of *n*-heptanoic acid. However, small differences were found when comparing *E. coli* BL21 wild type and mutant strains to each other. *E. coli* BL21 showed a reduction of 54% under 3 mM *n*-heptanoic acid stress. On the other hand, the reduction of *E. coli*

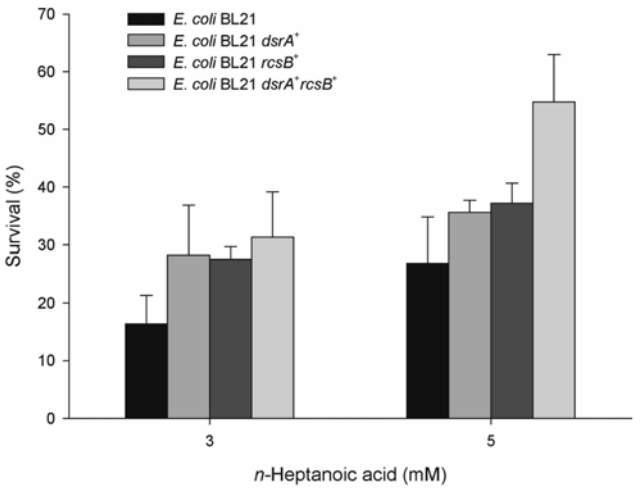

Fig. 6. Survival rate of the engineered strains. Calculation of survival rates was carried out based on colony forming unit (CFU). The survival rate was calculated by counting colony. The calculation formula of the survival rate (%) is as follows: survival (%) = CFU(post stress)/CFU (prior stress)×100. Experiments were run in triplicate. Error bars mean the standard deviations.

BL21 $dsrA^+$ and *E. coli* BL21 (DE3) $rcsB^+$ strains were 50 and 53%, respectively, which were slightly lower than *E. coli* BL21. The reduction of *E. coli* BL21 $dsrA^+rcsB^+$ strain was 45%, which is 9% lower than *E. coli* BL21. Similar results were obtained when 5 mM *n*-heptanoic acid was added. The reduction of *E. coli* BL21 $dsrA^+rcsB^+$ strain was reduced by 6%. The reduction of *E. coli* BL21 $dsrA^+rcsB^+$ was smaller than that of strains possessing *dsrA* or *rcsB* alone. This can be attributed to synergistic contribution of the expression of two genes *dsrA* and *rcsB* in *E. coli* BL21 $dsrA^+rcsB^+$.

In order to compare the colony forming activity of the engineered strains with the wild-type strain, the colony forming units (CFUs) and the survival rate were estimated. The survival rate was calculated based on the CFU before and after adding *n*-heptanoic acid. Each experiment was performed as previously reported [8]. As a result, the survival rate of the engineered *E. coli* BL21 strains were higher than that of *E. coli* BL21 (Fig. 6). Under 3 mM *n*-heptanoic acid stress, the survival rate of *E. coli* BL21 was 16%. *E. coli* BL21 $dsrA^+$ and *E. coli* BL21 $rcsB^+$ showed increased survival rate of 28 and 28%, respectively, which is 1.8-fold higher than *E. coli* BL21. *E. coli* BL21 $dsrA^+rcsB^+$ strains showed 31% of survival rate, which is 1.9-fold higher than *E. coli* BL21. Under 5 mM *n*-heptanoic acid stress, the survival rate of *E. coli* BL21 $dsrA^+rcsB^+$ was 55%, which is 2.0-fold higher than that of *E. coli* BL21 (27%). It was observed that the survival rate of mutant strains having both *dsrA* and *rcsB* genes were higher than mutant strains having *dsrA* or *rcsB* gene. Overall, it was assumed that the newly introduced *dsrA* and *rcsB* into the genome by CRISPR-Cas system contribute to tolerance against heptanoic acid-induced stress.

4. Conclusion

Although the integration efficiency of the target sites (*i.e.,* genomic coordinates of 3,876,409 and 1,300,270) in *E. coli* BL21 was much lower compared in *E. coli* K-12, the *dsrA* and/or *rcsB* of *E. coli* K-12 have been successfully introduced into the genome of *E. coli* BL21 by CRISPR-Cas9 system. For *dsrA*, in spite of the use of native transcription unit, the expression level was lower than in *E. coli* K-12. On the other hand, the *rcsB* with synthetic transcription unit showed much higher expression level. These results suggest that there would be difference in integration efficiency or expression level depending on the integration sites and the constituents of transcription unit. The future study will focus on the optimization of the expression level of *rcsB* using weak or medium-strength promoters to further increase the acid-tolerance of *E. coli* BL21. Besides the genetic optimization, metabolic optimization by metabolic flux analysis, like our previous work [23], would be helpful to further optimize the developed acid tolerant strains.

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