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Okshevsky, Mira; Xu, Yali; Masson, Luke; Arbour, Melanie

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Note

Efficiency of CRISPR-Cas9 genetic engineering in *Escherichia coli* BL21 is impaired by lack of Lon protease

Mira Okshevsky, Yali Xu, Luke Masson, Melanie Arbour *

Human Health Therapeutics Research Center, National Research Council of Canada, 6100 Royalmount Ave, Montreal, Quebec, Canada

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ABSTRACT

The efficiency with which *E.coli* BL21 can be modified using CRISPR-Cas9 genetic engineering is several orders of magnitude lower than that of *E. coli* W3110. We show that the lack of Lon protease is responsible, and demonstrate that restoration of the Lon protease or knock-out of *sulA* improves CRISPR-Cas9 engineering efficiency of BL21 to levels comparable to *E. coli* W3110.

The CRISPR-Cas9 system has become a useful tool for genetic engineering in many organisms (Sander and Joung, 2014), including *Escherichia coli* (*E. coli*). This genetic engineering process involves targeted DNA restriction, accomplished by the *Streptococcus pyogenes* Cas9 nuclease enzyme, followed by DNA repair. Molecules of guide RNA bind both Cas9 and the target sequence, resulting in a blunt double-stranded break (DSB) at a specific target locus (Loureiro and da Silva, 2019). An exogenous DNA template with homology to flanking sequences of the DSB is introduced to the cell. If the cell uses this template to repair the DSB, the intended mutation will be incorporated into the genome, and the cell will no longer be targeted by the CRISPR-Cas9 system. Cells that do not incorporate the change are subject to DNA restriction and death.

The efficiency of homologous recombination required for CRISPR-Cas9 editing has been greatly improved in *E. coli* through the simultaneous use of phage-derived recombinases RecET (Zhang et al., 1998) or λ-Red proteins Exo, Beta, and Gam (Murphy, 1998; Li et al., 2015; Pyne et al., 2015). CRISPR-Cas9 with phage recombineering allows for the efficient introduction of point mutations (Wang et al., 2018), integration of large genomic fragments (Abdelaal et al., 2019), elimination of plasmids (Wang et al., 2019), and facilitation of gene deletions, insertions, and replacements in *E. coli* (Li et al., 2015; Pyne et al., 2015).

E. coli strains W3110 (Wu et al., 2018; Li et al., 2019; Su et al., 2020) and BL21 (Seo et al., 2017; Wang et al., 2018, 2019) are commonly used for CRISPR-Cas9 genetic engineering. *E. coli* BL21 is a popular choice for the expression of recombinant proteins due to its lack of proteinases OmpT and Lon. It is therefore desirable to have efficient and high-quality tools for genetic engineering of this strain. Unfortunately, BL21 is orders of magnitude less efficiently modified than W3110, making creating and screening for a correct mutant time-consuming and inefficient. This phenomenon has been previously reported (Chung et al., 2017), but no genetic mechanism has been determined. To this end, we here identify the genetic mechanism of the reduced CRISPR-Cas9 engineering efficiency of *E. coli* BL21 and present solutions to this problem.

The CRISPR-Cas9 engineering system used here consists of the constitutively expressed Cas9 cassette and IPTG-inducible λRED recombineering system (pREDcas9), a gRNA expression plasmid (pGRB-lacZ), and a single stranded template DNA (ssDNA_lacZ) as described in Li et al. (2015). To allow successful mutations to be determined by color selection on X-gal, the *lacZ* gene encoding beta-galactosidase was targeted. Nine nucleotides encoding two stop codons were added to the beginning of the lacZ gene. Colonies carrying the intended mutation appear white due to inactivation of *lacZ*, while false positive colonies retain the blue color of the starting strain. In theory, only cells with the desired genetic modification should survive to form a white colony. However, cells might survive to form a blue colony if mutations alter the gRNA target site or inactivate the Cas9 cassette.

The number of colonies that appeared following CRISPR-Cas9 engineering in *E. coli* BL21 was several orders of magnitude lower than *E. coli* W3110 (Fig. 2a). Also, visible colonies of W3110 appeared after 24 h,

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; DSB, double-strand break; IPTG, Isopropyl β-d-1-thiogalactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside.

* Corresponding author at: Human Health Therapeutics Research Center, National Research Council of Canada, 6100 Royalmount Ave, Montreal, Quebec H4P 2R2, Canada.

E-mail address: Melanie.arbour@nrc-cnrc.gc.ca (M. Arbour).

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while BL21 required an additional 24 h of incubation, which is not typical growth behavior for BL21.

To determine the genetic basis of these observations, the whole genome of one BL21 white colony was sequenced using Ion Torrent technology, with the amplicon library prepared according to the Ion Xpress Plus™ gDNA and Amplicon Library Preparation protocol (Life technologies), and read assembly and mutation analysis performed using Geneious Prime 10.2.2. Compared to the BL21 type strain, the CRISPR-Cas9 engineered strain had mutations in six places; the intended 9-nucleotide mutation in *lacZ* was confirmed, as well as a base deletion 29 bases downstream in *lacZ*, a single base deletion in gene *sulA*, a two-base deletion in *ydiF*, and about 2000 kb deletion including the 5′ end of *mcrB*, and all of *symE*. These genome regions were investigated in seven other white clones using PCR amplification of target regions and Sanger sequencing (performed at *Centre d'expertise et de service* Genome Quebec), but the only region harbouring a mutation in all seven clones was around *sulA*.

All sequenced BL21 clones mutated using CRISPR-Cas9 engineering contained spontaneous mutations in or upstream of gene *sulA* (Table 1). To confirm that the inactivation of *sulA* is necessary for CRISPR-Cas9 engineering in *E. coli* BL21, we used CRISPR-Cas9 engineering (Li et al., 2015) to generate a site-directed knock-out of the *sulA* gene in BL21 (BL21ΔsulA) by introducing two premature stop codons following the first 30 amino acids (Fig. 1c). The number of successfully mutated cells (white colonies) divided by the total number of starting cells was calculated for each strain to compare CRISPR-Cas9 efficiency. The proportion of white colonies generated using *E. coli* BL21ΔsulA was 171 times higher than for BL21 ($1.6 \times 10^{-4} \pm 1.5 \times 10^{-5}$ vs. $9.4 \times 10^{-7} \pm 1.2 \times 10^{-6}$ for BL21) (Fig. 2), and colonies appeared within the 24 h typical of the BL21 growth rate. Strain BL21ΔsulA also showed a much lower proportion of blue colonies (5.4 ± 4.4% blue colonies vs. 47.8 ± 33.3% blue for BL21) (Fig. 2).

Table 1
Mutations in or upstream of *sulA* in CRISPR-Cas9 engineered BL21 clones.

clone	mutation
21	T → C substitution 42 base pairs upstream of start codon
22	A deletion at position 80, causing frameshift
23	T → C substitution 42 base pairs upstream of start codon
24	A deletion at position 80, causing frameshift
27	G → A substitution at position 198, changing Trp to stop codon
31	G → A substitution at position 198, changing Trp to stop codon
34	A → G substitution 18 base pairs upstream of start codon

SulA is a component of the coordinated cellular response to DNA damage known as the SOS system (Baharoglu and Mazel, 2014). The SulA protein (Uniprot accession A7ZK62) binds to and inhibits FtsZ polymerization, thereby stopping cell division and preventing the premature segregation of damaged DNA to daughter cells. When the DNA damage is repaired, SulA is degraded by the Lon protease, and cell division can resume.

The Lon protease is an ATP-dependent cytoplasmic serine protease. Studies in *E. coli* have shown that Lon is involved in unfolding and

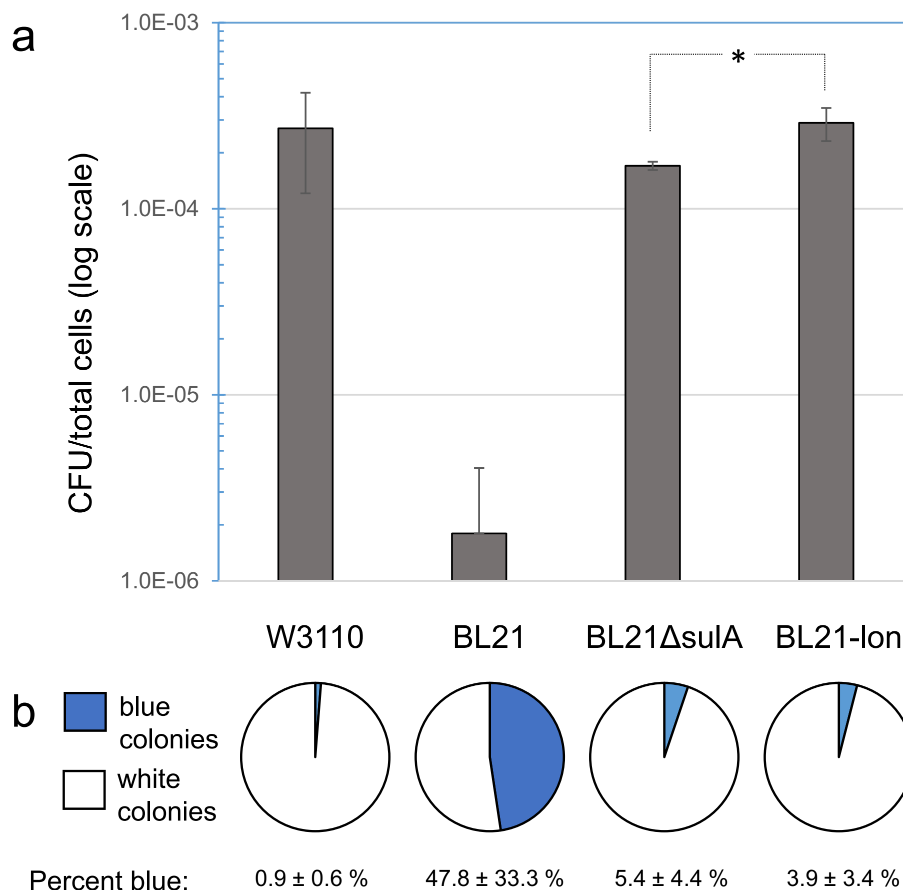

Fig. 2. Comparison of CRISPR-Cas9 engineering efficiency of strains W3110, BL21, BL21ΔsulA and BL21-lon

a) Number of colonies (CFU) following CRISPR-Cas9 engineering/total cells, shown on a log 10 scale. Data show means +/− standard deviation from three independent experiments. Star indicates a significant difference between strains as determined by Student's t-test ($p < 0.05$).
b) Proportion of colonies with a blue color, indicating intended mutation did not occur (false positives). Percentage shown is the average of three independent experiments ± standard deviation.

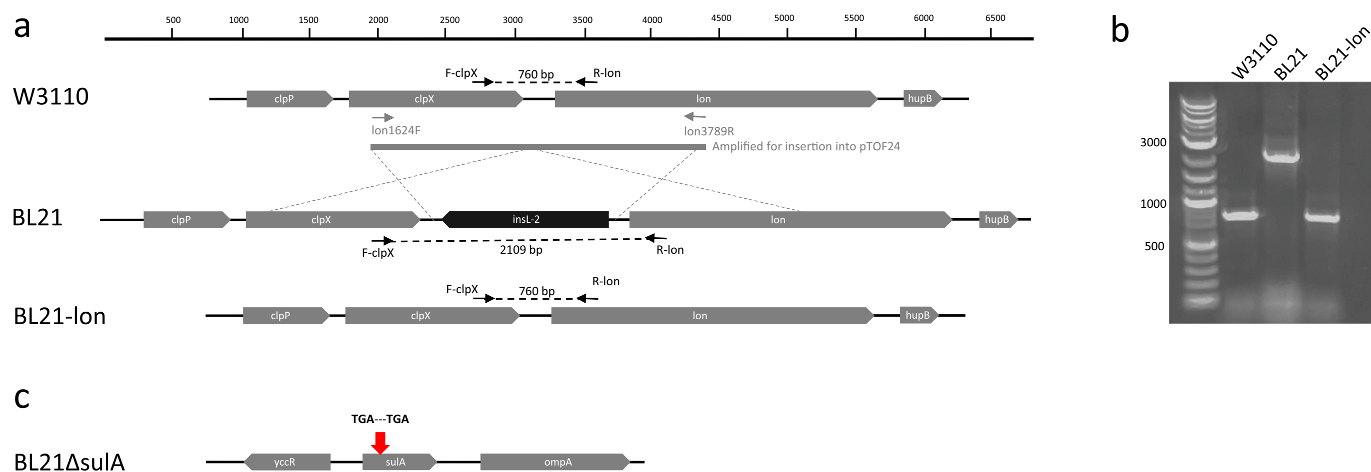

Fig. 1. Genotypes of *E. coli* W3110, BL21, BL21-lon and BL21ΔsulA

a) The *E. coli* BL21 genome harbours an insertion in the promoter region of the *lon* gene (shown in black). This insertion was removed and the lon promoter region of BL21-lon modified to be identical to the *lon* promotor region or W3110.

b) Gel electrophoresis of PCR fragments generated with primers F-clpX and R-lon, showing successful modification of lon promoter region in BL21-lon.

c) Strategy for inactivation of *sulA*. Two stop codons added after first 90 base pairs of *sulA*.

degrading misfolded proteins during heat shock, as well as degrading SulA (Mizusawa and Gottesman, 1983). The *lon* gene is present in the *E. coli* BL21 genome (locus tag ECD_00391, NCBI accession NC_012971), but a mobile genetic element just upstream (ECD_00390) prevents its expression (Fig. 1a). This suggests that in *E. coli* BL21, when the DSB created by Cas9 triggers the SOS response, cell division cannot resume following DNA repair unless a spontaneous mutation arises to inactivate *sulA*. The expression of DNA polymerases of low fidelity during the SOS response (Baharoglu and Mazel, 2014) can be expected to facilitate such random mutations. To confirm that the lack of Lon is responsible for the poor CRISPR-Cas9 engineering efficiency of BL21, we recovered the Lon protease in BL21 by replacing the disrupted promoter region with the *lon* promoter from W3110, creating BL21-lon (Fig. 1). To achieve this mutation, the indicated region in Fig. 1a was ligated into pTOF24 (Merlin et al., 2002), and allelic exchange was performed using sacB counter-selection.

The proportion of successfully mutated cells of *E. coli* BL21-lon was 295 times greater than for BL21 ($2.8 \times 10^{-4} \pm 5.7 \times 10^{-5}$ vs. $9.4 \times 10^{-7} \pm 1.2 \times 10^{-6}$ for BL21) (Fig. 2), and as observed for BL21ΔsulA, colonies appeared within the typical 24-h window. Strain BL21-lon also showed a much lower proportion of blue colonies (3.9 ± 3.5% blue colonies vs. 47.8 ± 33.3% blue for BL21), indicating a higher proportion of cells harbouring the intended mutation (Fig. 2). Here, correct mutations are indicated by color and false positives can be easily determined. Most mutations, however, will require labor-intensive molecular investigations to verify, making it highly desirable to minimize false positives.

Although BL21ΔsulA and BL21-lon were both within the range of mutation efficiency observed for W3110, BL21-lon produced significantly more colonies than BL21ΔsulA. This may be because the usual role of SulA is to ensure that the cell does not expend energy on replicating non-functional copies of itself. In the absence of a functional SulA to inhibit cell division, more cells will perish making non-functional copies, leaving fewer cells to survive to form colonies.

From these results it can be concluded that the poor CRISPR-Cas9 engineering efficiency of *E. coli* BL21 is due to the absence of a functional Lon protease in this strain, and the mutations observed in *sulA* are one mechanism by which the cell can bypass its inability to degrade SulA following DNA damage. Researchers should be aware that such unintended mutations will arise when performing CRISPR-Cas9 engineering in *E. coli* strain BL21.

In summary, the efficiency of CRISPR-Cas9 engineering in *E. coli* BL21 can be improved to levels similar to *E. coli* W3110 through the recovery of Lon or knock-out of *sulA*. Targeted recovery of the Lon protease removes the need for spontaneous *sulA* mutations and greatly increases the efficiency of CRISPR mutagenesis in BL21. For cases in which restoring a protease such as Lon is undesirable (production of recombinant proteins, for example), a *sulA* mutant is the more desirable option to increase CRISPR-Cas9 efficiency. Alternatively, CRISPR-Cas9 should be avoided in *E. coli* BL21 in favour of a genetic engineering method that does not create a DSB.

Declaration of Competing Interest

The authors declare no conflict of interest associated with the manuscript "Efficiency of CRISPR-Cas9 genetic engineering in *Escherichia coli* BL21 is impaired by lack of Lon protease."

Data availability

Data will be made available on request.

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