

Original Article

CRISPR-Cas9 Mediated Capsule Gene Silencing in *Escherichia coli*

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Abstract

Background: Genomic engineering of *Escherichia coli* is applied to design and produce recombinant proteins as the new drugs. The aim of this study was to CRISPR-Cas9 mediated capsule gene silencing in *E. coli*.

Materials and Methods: We suppressed genes involved in capsule expression of *E. coli* by CRISPR cas9 process. The constructed *E. coli* was confirmed by microscopic smear, transmission electron microscopy and T7 phage influence assay.

Results: The results were shown that the inhibition of capsule production was carried out successfully and there was not any capsule layer around the bacteria.

Conclusion: *E. coli* without any capsule around may proper for replacement of it with other molecules in future.

Keywords: Capsule, CEISPR, *Escherichia coli*, TEM

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Introduction

Metabolic engineering of *Escherichia coli* is widely practiced to obtain recombinant protein factories. To perform manipulations of genome and targets editing are required efficient protocols. Wide varieties of techniques for targeted gene editing, which can be classified into homologous recombination, are available for *E. coli*¹. There are few tools for genome modification such as multiplex automated genome engineering (MAGE) is an only single stranded DNA (ssDNA) based gene modification mediated by λ -Red which was further developed as a multiplex genome editing tool². Another one the clustered regularly interspaced short palindromic repeats-CRISPR-associated system (CRISPR-Cas9 system) has been

used as an efficient technology for genome engineering in prokaryotes and eukaryotes. The type II CRISPR-Cas9 system from *Streptococcus pyogenes* contains a maturation CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) guiding the nuclease Cas protein9 (Cas9) to the target of any DNA sequence, known as a protospacer, with a protospacer-adjacent motif (PAM) present at the 3'end (NGG in the case of *S. pyogenes*, where N represents any nucleotide³.

According to other research⁴ on *E. coli*, the CRISPR-Cas9 system has been predicted to apply allelic modification with efficiency as high as 65%. Therefore, in the present study we have decided to inhibit the capsule construction in *E. coli*. Therefore, we developed a CRISPR-Cas9 system-based genome

editing strategy, including gene knockouts of multiple targets in several operons coding a cluster for capsule layer production in *E. coli*.

Methods

Construction of a plasmid with multi-sgRNAs:

The cluster contains capsule coding genes of *E. coli* in NCBI GenBank was initially considered. We used Database of prokaryotic operons (DOOR) to find operons that have similar composition and structure to a query operon⁴. PubMLST server has a part named bacterial isolate genome sequence database (BIGSdb) for gene-by-gene package annotation and analysis that was applied in this research. In BIGS database, large numbers of loci is defined with alleles assigned by reference to sequence definition databases. The capsule cluster for *Kps EFMT* sequences (accession numbers L19929, U59301, AF037588, X53819) were considered for finding the gRNA fragments and arranged with loop RNAs for inhibition of capsule synthesis which were designed according to CRISPR/Cas9 system protocol (addgene, USA). The tRNA fragments specific for *Escherichia coli* joined the guide RNA sequences to each other. The designed construct was synthesis into the pBR322 vector under the *Tet* promoter by gene Ray Company (Fig.1).

Transformation of *E. coli* strain BL21 with designed plasmid: The designed plasmid with Ampicillin resistant gene was transferred into the *E. coli* strain BL21 competent cells together with 10ng Cas9 plasmid with kanamycin resistant gene, which coded endonuclease (addgene, USA). The transformed bacteria were then cultured on an agar plate contained both antibiotics then incubated at 37°C (Fig. 2).

Study of recombinant bacteria by smear Fuchsin staining and transmission electron microscopy (TEM): In Fuchsin staining a colony of bacteria in each phase of examination was suspended in a drop of water on the slide and allowed to dry. After fixing and staining with Fuchsin (Merck, Germany) the slides were studied under the light microscopy with 100X lens. Following the overnight incubation, the bacteria transferred to Eppendorf tubes and washed with sterile water. Finally, the bacteria were resuspended in 1 mL sterile nanopure water. The

bacterial suspension was fixed at 4°C for 90 min with 2% glutaraldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.4. Cells were fixed in 1% OsO₄ for 90 min at 4°C and stained with 0.25% uranyl acetate at 4°C for 1 h. Finally, the cells were embedded into Spurr resin and left to polymerize in an oven at 60°C for 24 h. Resins were sectioned by cutting an 80-nm film at 25°C using an ultra-microtome Sorvall MT 5000 (Dupont, Boston, MA) equipped with a diamond knife. Thin section was mounted on copper grids covered with carbon film and observed using a TEM JEOL+JEM 1200 EX II (Jeol Ltd, Tokyo, Japan) at an accelerating voltage of 80 kV. Cutting films were deposited on the grid. Aliquots of 20 EL of the liquid cultures were deposited on TEM copper grids covered with carbon film. Bacterial cells were then fixed with 20 EL of 5% formaldehyde without staining. The samples were promptly observed under the TEM.

Confirmation of capsule synthesis inhibition by T7 phage effect:

The colony harboring both pCas9 and pgRNAs was inoculated into 1ml of BL medium containing Ampicillin (100mg/liter) and Kanamycin (50mg/1liter). The culture was incubated at 37°C with shaking at 200rpm. After 8h when the optical density at 600nm of a culture reached 0.2, 100 cfu T7 phages (at a multiplicity of infection of 0.01) was added to the bacterial suspension. To isolate the bacteria without the capsule, in this step the suspension was centrifuged at 8000 rpm for 10 minutes. By removing the above phase containing *T7 phages*, the pellet was spread onto LB agar plates containing both antibiotics. The Bacteria grown onto plates in compared with control colonies with capsule were lysed.

Results

Construct design contains Capsule cluster gRNAs:

gRNAs (lowercase letters) complementary to capsule cluster genes (KpsE,F,T,M) of *E. coli* were designed as below. They were separated with RNA loops (dots) and tRNA sequences (capital letters). The complete sequence was flanked with *BamHI* and *SaLI* restriction enzymes sites.

```
ggatccactaggaataaagacggtcgcc.....ACTTTTA
ATCAATTGGtCGCAGGTTTCGAATCCTGCACGA
CCCACCAtcagcggtaatttgcgagcc.....ACTTTT
AATCAATTGGtCGCAGGTTTCGAATCCTGCACG
```

ACCCACCAcgcgatggacttctctaacc.....ACTT
 TTAATCAATTGGtCGCAGGTTCGAATCCTGCA
 CGACCCACCAcagcttcttaaacggcttgagcc
gtcgac

Microscopic demonstration of the capsule layer of *E. coli*: In smear of *E. coli* stained by fuchsin we have observed coccobacilli shaped bacteria without any capsule around it under the light microscope with 100X lens (Fig. 3).

Isolation of Bacteria with T7 phage effectiveness after capsule gene silencing: *Escherichia coli* K1's capsule is a barrier to bacteriophage T7⁷. Therefore, the T7 phage tail components are sequentially assembled into the capsid during the virus morphogenesis after DNA packaging is completed. Here after adding T7 phage the cultivated *E. coli* colonies are bacteria with capsule and killed bacteria by T7 phage are *E. coli* without capsule. Therefore decreased the number of colonies after treatment compared to control (without treatment) showed inhibition of *E. coli* capsule synthesis.

Discussion

In the present study, we induced the application of the CRISPR/Cas9 system for inhibition of *E. coli*.

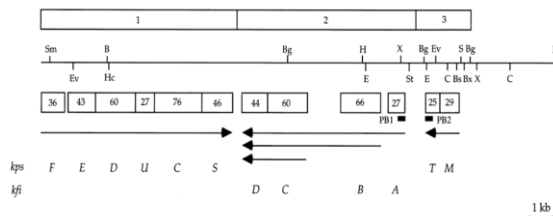


Figure 1. *E. coli* Capsule gene cluster, *kps* locus of the *E. coli* K5⁵.

Capsule synthesis based on guide RNAs for *kpsE*, *F*, *M*, *T* genes of capsule cluster of *E. coli*. The *Enterobacteriaceae* family includes a variety of intestinal symbionts as well as pathogens. Among the *Enterobacteriaceae* family, *Escherichia coli* is the well-known bacterial species on Earth, *E. coli* is a cadre of different strains that have adapted to diverse environmental conditions and lifestyles. While the typical *E. coli* genome contains exactly 4800 genes, only approximately 1700 genes are shared by every *E. coli* strain⁷.

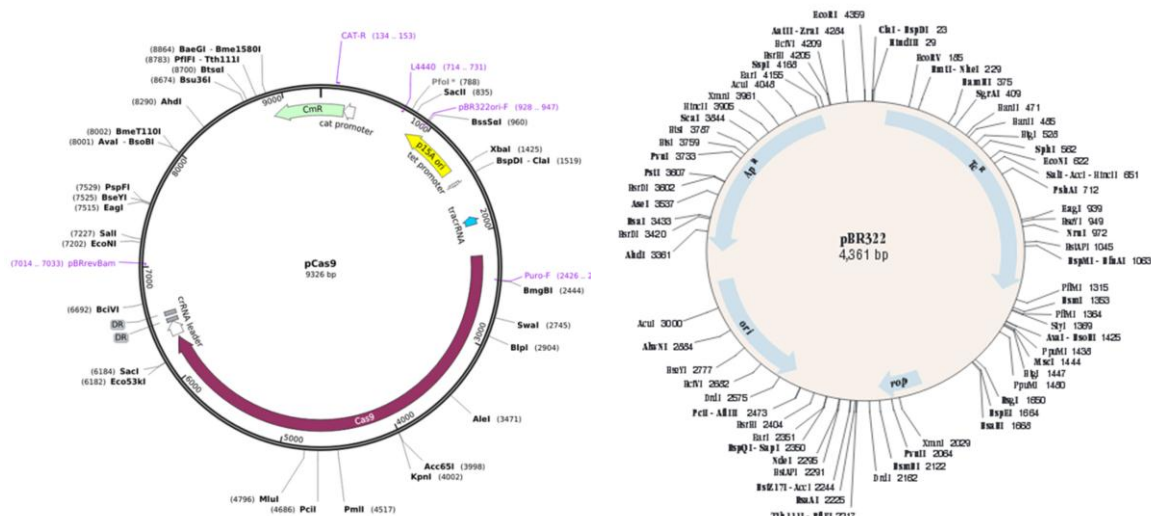


Figure 2. pCas9 plasmid contains bacterial Cas9 endonuclease coding gene (left), pBR322 vector contains capsule cluster gRNAs (right).

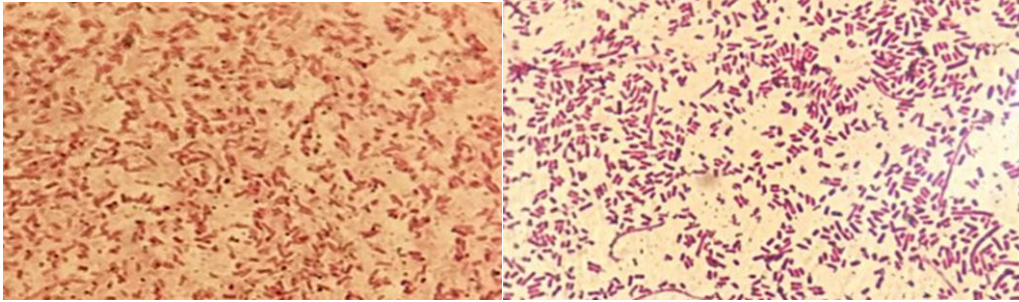


Figure 3. The light microscopic image was shown smear of *E. coli* stained with fuchsin, before inhibition (left) and after inhibition by CRISPR-Cas9 (right).



Figure 4. The TEM image of bacteria section after gene silencing.

Capsule in *E. coli* structure interferes with the action of complement and phagocytes. This effect is generally transient. In some cases, capsules are not or only poorly immunogenic, because of structural relationship or identity with the host material. Pathogen and non-pathogen strains with such capsules (e.g., K1 or K5) are disruptive. Bacterial capsules consist of acidic polysaccharides, which are made up from oligosaccharide repeating units. The capsules of *E. coli* are divided to two groups, which differ in chemistry, biochemistry, and genetic organization. All capsular polysaccharides are chromosomally determined: those of group I close to *his* gene and those of group II close to *serA* gene. The biosynthesis and surface expression have been studied. It could be shown that their biosynthesis is directed from a cluster of genes that determines the synthesis of the polysaccharide, its translocation across the bacterial cytoplasmic membrane, as well as its surface expression in a concerted process. The

mechanism(s) of their biosynthesis and expression is presented⁸.

Nowadays one of the gene expression regulations is programming of the Cas9 endonuclease with an RNA scaffold for sufficient breaks generation in a targeted DNA locus⁹. The CRISPR/Cas9 system can be designed to break multiple genes by co-expressing multiple sgRNAs carrying direct sequences. Several studies were developed to multiple genes interruptions like a method to clone a pair of sgRNAs to one plasmid by Vidigal et al¹⁰, sequentially use a multi-step strategy to apply sgRNA cassettes, Jiang et al developed to prepare a plasmid with several sgRNAs into separate specific vectors beforehand¹¹. Yu Jiang et al, has introduced a simultaneous multigene editing of up to three targets for construction of industrially useful microbes¹⁰.

In our study, we have inhibited the capsule construction by CRISPR-Cas9 system, which for determination and characterization of acquired *E. coli*; we used microscopic methods and T7 phage tropism. T7 phage is one the viruses that infect Gram-negative bacteria that have been described to use lipopolysaccharide (LPS) as a receptor, often assisted by porins or outer membrane proteins¹². The T7 tail components are sequentially assembled into the capsid during the virus morphogenesis after DNA packaging is completed. Therefore, this phage had been able to be applied for separating of capsule aborted *E. coli* from normal type.

The obtained *E. coli* K1 strain without any capsule layer is sensitive to T7 infection. T7 is able to form plaques on lawns of this strain, and phage growth is seen in liquid culture. According to Dean Scholl's paper⁷, T7 phage encodes a tail fiber protein that

specifically recognizes and binds to lipopolysaccharide and recognizes *E.coli* K1 produces a polysialic acid capsule. T7 phage encodes virion-associated endosialidases that can hydrolyze the K1 polysialic acid structure. So the conclude *E.coli* without capsule layer will be sufficient for applying in industrial production of recombinant drugs.

Conclusion

E. coli without capsule layer may be a proper cell for design a bacterial cell with other type of biological layers instead of it in future.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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