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An efficient system for deletion of large DNA fragments in *Escherichia coli* via introduction of both Cas9 and the non-homologous end joining system from *Mycobacterium smegmatis*

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ABSTRACT

Accompanied with the internal non-homologous end joining (NHEJ) system, Cas9 can be used to easily inactivate a gene or delete a fragment through introduction of DNA double-stranded breaks (DSBs) in eukaryotic cells. While in most prokaryotes (*e.g. Escherichia coli*), due to the lack of NHEJ, homologous recombination (HR) is required for repair of DSBs, which is less convenient. Here, a markerless system was developed for rapid gene inactivation or fragment deletion in *E. coli via* introduction of both Cas9 and a bacterial NHEJ system. Three bacterial NHEJ systems, *i.e. Mycobacterium smegmatis* (*Msm*), *Mycobacterium tuberculosis* (*Mtb*) and *Bacillus subtilis* (*Bs*), were tested in *E. coli*, and the *Msm*NHEJ system showed the best efficiency. With the employment of Cas9 and *Msm*NHEJ, we efficiently mutated *lacZ* gene, deleted *glnALG* operon and two large DNA fragments (67 kb and 123 kb) in *E. coli*, respectively. Moreover, the system was further designed to allow for continuous inactivation of genes or deletion of DNA fragments in *E. coli*. We envision this system can be extended to other bacteria, especially those with low HR efficiency.

1. Introduction

To study the biological function of a gene, gene knockout experiment is usually performed prior to the phenotypic exploration. In eukaryotic cells, a knockout mutant can be obtained through the introduction of a DNA double-stranded break (DSB) within the gene, followed by subsequent error-prone non-homologous DNA end-joining (NHEJ) repair [1]. Besides, through introduction of two DSBs at the end of a designated DNA sequence, the outside ends of the chromosome can be linked *via* NHEJ, which meanwhile deletes the DNA fragment [2–4]. In the past decades, many successful methods have been developed for introduction of DSBs in eukaryotic chromosome, employing meganucleases, zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) [5–7]. Recently, with the discovery and development of the CRISPR system [8,9], the work becomes much easier [1]. On the contrary, most prokaryotic cells lack the NHEJ system [10]. Therefore, to perform gene inactivation or DNA deletion in prokaryotes, traditional techniques based on homologous recombination (HR), group II intron retrohoming and site-specific recombination have been developed [11–15]. Although antibiotic resistance markers can be used to improve the editing efficiency of the above techniques, subsequent removal of the selection marker is also a workload. Recently, Cas9-assisted genome editing systems have been developed in several bacterial species, allowing for markerless genome editing [16]. Because HR is usually required for repair of the Cas9-introduced DSBs, DNA fragments or plasmids for HR must be constructed, which is time-consuming and less convenient.

Eukaryotic NHEJ systems are complicated and consist of a series of proteins [17]. In some bacteria (*e.g. Mycobacterium smegmatis* (*Msm*), *Mycobacterium tuberculosis* (*Mtb*) and *Bacillus subtilis* (*Bs*)),

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there exists a much simpler NHEJ system that needs only two key proteins, *i.e.* Ku and ligase D (LigD) [10,18–20]. More importantly, the NHEJ system from *Mtb* has been shown to be functional in some bacteria [21]. For example, introduction of the *Mtb*NHEJ system into *E. coli* enabled the circularization of linear plasmids or led to small deletion at a very low efficiency at the Cas9-introduced DSB sites [22,23]. In this study, above three bacterial NHEJ systems were tested in *E. coli*, and the *Msm*NHEJ system was found to have the highest activity. With the employment of the *Msm*NHEJ system and Cas9, the *glnALG* pathway (4.2 Kb) and two large fragments (67 and 123 Kb) were successfully deleted in *E. coli*.

2. Materials and methods

2.1. Bacterial strains and media

E. coli strain DH10B was used for plasmid cloning and strain MG1655 was used for testing of the NHEJ system. *E. coli* strains were grown in Luria-Bertani (LB) medium and incubated at 37 °C. If needed, appropriate concentration of antibiotics (100 μg/mL ampicillin, 50 μg/mL kanamycin and 34 μg/mL chloramphenicol) was added to the medium. L-arabinose (10 mM or as mentioned in the article) was added to the liquid medium to induce the expression of arabinose-inducible genes. IPTG (isopropyl-β-D-thiogalactopyranoside, 100 μM) and X-gal (40 μg/mL) were used for blue/white screening of the phenotypes of *lacZ* mutants. M9 medium (12.8 g $Na_2PO_4{\cdot}7H_2O$, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl and 10 g glucose per liter) was used to characterize the phenotypes of *glnALG* mutant, while M9 with glutamine (2 mM) added was used as a positive control for culture of *E. coli* mutants. IPTG (0.5 mM) was added to the liquid medium to induce the expression of IPTG-inducible genes. Sucrose (10 g per liter) was added to NaCl-free LB plates to select cells that lost the *sacB*-containing plasmid pZX21.

2.2. Plasmids construction

Oligonucleotides and plasmids used in this study were listed in Tables S1 and S2, respectively. Detail information about plasmids construction was showed in Supplementary method.

2.3. Measurement of the NHEJ efficiency in E. coli

Three NHEJ expression vectors (pZX08, pZX09 and pZX10) were individually transformed into *E. coli* MG1655 together with pZCas9. Then, electrocompetent cells of the transformants were prepared and L-arabinose was initially added into the culture to induce the expression of the NHEJ systems till the OD600 reached 1.0. Plasmid pGRNA-dlacZ (200 ng) was electroporated into the above competent cells (200 μL) and the cells were then plated on corresponding selective LB plates supplemented with IPTG (100 μM) and X-gal (40 μg/mL), followed by colony forming units (CFU) counting after 17 h incubation. To facilitate the counting of white colonies, another 24 h culture was needed. All transformation experiments were repeated at least three times.

2.4. Cas9 and NHEJ-assisted gene or DNA fragment deletion in E. coli

The *Msm*NHEJ expression vector (pZX09) was transformed into *E. coli* MG1655 together with pZCas9. Subsequent procedures for preparation of electrocompetent cells, electroporation and colony formation on selective plates were the same procedures as described in the "Measurement of the NHEJ efficiency in *E. coli*", except that distinct plasmids expressing different sgRNAs were used in the result described.

2.5. Phenotypic validation of glnALG mutant

After transformation of plasmid plasmid pGRNAD-dgln, colonies were innoculated into liquid LB medium individually. After 7 h culture, 2 μL bacterial suspension from each tube was individually dripped on M9 plate for phenotypic test, and M9 plate with glutamine was employed for positive controls. After 17 h incubation, phenotypes were checked based on the growth of colonies, and colonies that failed to grow probably contained *glnALG* mutations.

2.6. Analysis of mutation or deletion

To confirm the mutation of *lacZ* gene, we designed primers lacZ-F-F/lacZ-R-R on the outsides of the Cas9 cleavage site. Primer lacZ-F-F was 722 bps upstream of the Cas9 cleavage site while primer lacZ-R-R was 567 bps downstream of the cleavage site. Single colonies were directly used for colony PCR, and the PCR products were subsequently analyzed by agarose gel electrophoresis and Sanger DNA sequencing.

Similarly, primers of gln-F/gln-R or glnot-F/glnot-R were used for verification of the deleted region in operon *glnALG*, primers of d67k-yan-F/d67k-yan-R were used for verification of the deletion of LF1, and d123k-yan-F/d123k-yan-R were designed for verification of LF2 deletion. Positions of the above primers can be found in Figs. S2 and S3, respectively.

2.7. Continuous inactivation of genes or deletion of DNA fragments in E. coli

Plasmid pZX21 was transformed into *E. coli* MG1655, and subsequent preparation of the electrocompetent cells and electroporation of sgRNA plasmid were same as mentioned above. Colonies grown on the plates were verified by PCR amplification and candidates were subjected for further sequencing. A positive colony was innoculated into liquid LB medium, which was supplemented with kanamycin to maintain pZX21 and 0.5 mM IPTG was added to eliminate pGRNA. After being cultured by shaking at 37 °C for at least 8 h, cells were streaked on LB plates with kanamycin to form single colonies, and the ampicillin-sensitive colonies can be used for a new round of fragment deletion. Finally, pZX21 could be removed *via* counter-selection under the condition of sucrose (10 g per liter).

3. Results

3.1. The NHEJ system from Msm had the highest activities in E. coli

We first tested the efficiency of Cas9 coupling with sgRNA in *E. coli* (MG1655). Plasmid pZCas9 which contained the *Streptococcus pyogenes cas9* gene driven under a strong constitutive promoter (BBa_J23101) was constructed and transformed into *E. coli*. Then, pGRNA-dlacZ (expressing the sgRNA targeting the *lacZ* gene, Table S2) was transformed into *E. coli* which contained pZCas9, and nearly all transformants were killed (Fig. S1a), which demonstrated that sgRNA-lacZ was effective and could be used for subsequent testing of the NHEJ efficiency. For those surviving transformants, the Cas9-introduced DSBs might either be repaired by HR with the sister chromosomes [23] or by alternative end-joining (A-NHEJ) [24].

Three NHEJ systems from *Mtb, Msm and Bs*, which contained both Ku and LigD proteins, were individually co-transformed with pZCas9 into MG1655. Then, pGRNA-dlacZ was further transformed

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