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Enhancements in existing genomic editing methods

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ABOUT THE STUDY

As a class of versatile living systems, bacteria are useful in many fields of synthetic biology. In bacteria, genetic information contained on the single-copy chromosome determines the characteristics of a specific strain. To understand bacterial characteristics and utilize them to explore the world and serve human life, researchers frequently conduct chromosomal engineering to reprogram the genetic information of bacteria. Through DNA editing, researchers can add desired exogenous genetic information to or delete unwanted endogenous genetic information from the bacterial chromosome. The long fragment editing technique is of great importance in accelerating bacterial chromosome engineering to obtain genetically stable strains.

To accelerate the process of chromosomal engineering, researchers have reported many methods for generating insertions and deletions in bacterial chromosome. Homologous recombination with Polymerase Chain Reaction (PCR) fragments forms the basis of these methods. However, since RecA-mediated homologous recombination with linear DNA is of low efficiency, researchers created the desired mutagenesis on a suitable plasmid before recombining it into the genome. To enhance the efficiency of homologous recombination, the bacteriophage-derived λ-Red system was introduced into bacteria on either the genome or plasmids. Genomic editing based on λ-Red recombinases is referred to as recombineering. In recombineering, an antibiotic resistance gene is required as a selectable marker. To remove the selectable marker after genomic editing, researchers introduced counter-selection systems or site-specific recombination systems, including FLP/FRT and Cre/loxP.

Moreover, eliminating selectable markers and plasmids is complicated and time-consuming, and the residual FRT or loxP site may influence a new round of genomic editing. Generating a Double-Strand Break (DSB) in the target DNA is an effective strategy for improving the efficiency of long-fragment manipulations. Though the homing endonuclease I-SceI is efficient for cleaving double-stranded DNA (dsDNA), researchers had to integrate an 18-bp recognition site into the target DNA before inducing DNA cleavage. Engineered endonucleases, such as Zinc-Finger Nucleases (ZFNs) and Transcription Activator-like Effector Nucleases (TALENs), can be programmed to recognize and cleave the genome at a specific locus. However, these approaches require engineering new enzymes for each target sequence.

Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology was developed based on research into the adaptive immune system of *Streptococcus pneumoniae*. Cas9 endonuclease complexed with a designed single-guide RNA (sgRNA) can generate DSB in a specific protospacer sequence where a proper Protospacer-Adjacent Motif (PAM) exists. The technique relies on sgRNAdirected cleavage at the target site to kill wild-type cells, thus circumventing the need for selectable markers or counter-selection systems. Changing the 20-bp spacer sequence can reprogram the specificity of the Cas9-sgRNA complex, making CRISPR/Cas9 technology much more convenient than ZFNs and TALENs. Many methods based on the CRISPR/Cas9 technology are efficient for short-sequence editing in *Escherichia coli*.

As a powerful chromosome engineering tool, CARS has great application potential. In this study, to demonstrate its potential, we have applied CARS in genome simplification and metabolic engineering. *E. coli* has been the prominent prokaryotic organism in research laboratories since the origin of molecular biology, and is arguably the most completely characterized single-cell life form. According to previous studies, different *E. coli* strains possess different genome sizes. For example, MG1655, an *E. coli* K-12 strain, has a 4.6-Mb genome

that harbors 4497 genes, including 4296 protein-encoding genes and 201 RNA-encoding genes. Functional analyses have shown that *E. coli* cells grown under given conditions use only a fraction of their genes.