Advances in the application of gene knockout technology in bacterial drug resistance research

Chunyu Tong  
Heilongjiang Bayi Agricultural University  https://orcid.org/0000-0002-2164-5524

Yimin Liang ( liangyimin1314@foxmail.com )  
Heilongjiang Bayi Agricultural University  https://orcid.org/0000-0003-0392-6039

Zhelin Zhang  
Heilongjiang Bayi Agricultural University

Sen Wang  
Heilongjiang Bayi Agricultural University

Xiaohui Zheng  
Heilongjiang Bayi Agricultural University

Qi Liu  
Heilongjiang Bayi Agricultural University

Bocui Song  
Heilongjiang Bayi Agricultural University

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Abstract

Gene knockdown has been confirmed as a common problem-solving method in biology. It is effective in investigating bacterial drug resistance. The technologies of gene knockdown comprise zinc-finger nuclease technology (ZFN technology), suicide plasmid vector systems, transcription activator-like effector protein nuclease technology (TALEN technology), Red homologous recombination technology, CRISPR/Cas, and so forth. To be specific, Red homologous recombination technology, CRISPR/Cas9 technology, and suicide plasmid vector systems have been the most extensively used technologies for the knockdown of bacterial drug resistance genes. The three above-described technologies have been employed in a considerable number of studies to obtain significant results in the research on bacterial gene functions. This study aims to provide an overview of effective gene knockout methods that are currently used for genetic drug resistance testing in bacteria and to provide a reference for the selection of gene knockout techniques.

Introduction

Microbial drug resistance has become a major challenge in the field of microbial infection prevention and control over the past few years\cite{1}. The overexpression of genes related to active drug efflux pumps has been reported as one of the major mechanisms of bacterial multidrug resistance\cite{2}. Bacteria are capable of regulating the stability of their internal environment and protecting themselves from damage by actively pumping out toxic substances (e.g., antibacterial drugs, and metabolites) through the efflux pump\cite{3}. The discovery of novel drug resistance genes and the research on the function of known drug resistance genes have taken on a great significance. Knockout can eliminate the function of a drug resistance gene, gene overexpression can positively verify the gene's function, and gene back-complementation has the capability of theoretically reverting the changes of knockout. The function of the target gene can be precisely explored by constructing knockout, back-complementation, and overexpression strains and through the comparison and assessment of the differences between strains. Knockout has been widely adopted to address problems in biology, and it serves as a vital method to study microbial drug resistance efflux pumps.

1. Common Bacterial Gene Knockout Techniques

Red homologous recombination refers to a physiological phenomenon that is prevalent in living organisms; it serves as an intrinsic mechanism where organisms correct their or external factors-induced DNA mutations\cite{4}, i.e., the molecular biological basis for gene knockout. On that basis, the first three types of knockout technologies developed are elucidated as follows. The first is the Red recombination system, which is performed for prokaryotic cells using the Red region of the $\lambda$ phage gene to encode a gene that is capable of initiating homologous recombination of bacterial chromosomes with exogenous DNA\cite{5}. The second type is the Cre-loxP and Flp-FRT systems\cite{6}, which are primarily performed for eukaryotic cells,
both comprising a specific DNA sequence and a recombinase to knock out a specific gene. The third type refers to large-scale random insertion mutations, theoretically achieving the genome-wide knockout of any gene; these insertion mutations have become the more effective methods currently used in plants, mainly comprising transposons and T-DNA insertion mutations.

An efficient suicide vector system refers to a chromosome modification technology developed in the 1980s. Its basic principle is to construct recombinant suicide plasmids containing homologous DNA fragments of a certain length; the host's recombination system is adopted for the exchange between homologous recombination sequences; the suicide vector can lose the above recombinant plasmids during two recombination and complete recombination. The above-mentioned system exhibits a wide host range and transferability, and it has been extensively employed to study bacterial physiology, virulence, and drug-resistance genes.

Rudin et al. and Rouet et al. discovered that double-strand break (DSB) DNA introduced at the target site dramatically increases the target gene's integration efficiency. Researchers first introduced specific double-strand break DNA into the genome by homing nucleases (i.e., extensive nucleic acid endonucleases), mostly through non-homologous end joining (NHEJ) repair. On that basis, the TALEN, ZFN, and CRISPR/Cas Systems are considered the three major technologies for genome editing.

The first-generation ZFN gene editing technology and the second-generation TALEN gene editing technology have been extensively employed in genome editing of eukaryotic organisms (e.g., animals and plants), and they can achieve fine manipulation at the genome level (e.g., insertion, deletion or replacement). Compared with the conventional methods, ZFN and TALEN technologies are more efficient, and the main disadvantages of ZFN and TALEN technologies comprise their complexity, long cycle time and high cost. Moreover, the application of ZFN and TALEN technologies in bacterial gene editing has not been rarely reported. Compared with first- and second-generation technologies, third-generation CRISPR/Cas9 gene editing is easier, faster, and more efficient. It outperforms other gene editing techniques. The main problem is off-target effects. It has been widely used for gene editing in a wide variety of eukaryotes and prokaryotes (e.g., Escherichia coli).

Red homologous recombination, CRISPR/Cas9, and suicide plasmid techniques have been the more commonly used knockout techniques in the laboratory, whereas all three can induce DNA double-strand breaks to replace the knockout gene via non-homologous end or homologous end repair mechanisms. The main strategies are elucidated as follows: (1) One-step screening, with a screening marker in the middle and linear DNA fragments with homologous sequences at both ends of the gene, where the marker gene displaces the target gene with the help of recombination between the homologous sequence and the target vector. (2) Screening + reverse screening, in the first round of recombination, the linear DNA fragment comprises two genes of screening marker and reverse screening marker, and the recombinant molecule is selected out by screening the marker gene. The homologous fragment in the linear DNA fragment displaces the two screening marker genes on the recombinant molecule in the second round of recombination, while the recombinant molecule is selected by the reverse screening marker gene. (3)
Screening + site-specific recombination, the two sides of the screening marker genes contain special sites recognized by Cre or FLP site-specific recombinase. The screening marker genes can be deleted through site-specific recombinase after the first screening, whereas a special sequence of more than ten bp is left on the recombinant molecule. (4) Screening + restriction enzyme cleavage reaction, in which restriction enzyme cleaves the recombinant molecule on both sides of the screening marker gene and then ligates it with a restriction endonuclease. The technical means of gene manipulation has significantly enriched through the combination of Red homologous recombination technology with additional DNA experimental techniques. Researchers are free to use the above-described techniques to achieve different experimental objectives.

2. Common Knockout Techniques In Bacterial Drug Resistance Research

2.1 Red homologous recombination technologies

Knockouts in certain Gram-negative bacteria (e.g., Escherichia coli (E. coli), Salmonella, and Klebsiella) are often performed using a two-step homologous recombination approach. The development of this method has already been explored over a long period, and the conventional method mainly uses the RecA and RecBCD proteins encoded by the strains themselves as mediators\(^{[15]}\). However, this system has obvious shortcomings (e.g., serious problems with the operation steps, very low recombination rates, and the need for a long homologous arm of the target gene). As a result, it is difficult obtaining the desired recombinant, the knockout efficiency is greatly limited, and the development of this technique is affected.

In 1998, Zhang et al.\(^{[16]}\) have suggested that the RedE/RecT system presented in E. coli exhibits the recombination function. In 2000, it was also found that the Exo and Beta proteins of \(\lambda\)-phage exhibit the same function\(^{[17]}\), and the E. coli can be specifically modified using a small homologous arm. It was later termed ET Recombination, which can significantly improve the shortcomings of the conventional method, reduce homologous arms required in recombination, and increase the recombination rate. Thus, it has been widely used in the genetic modification of E. coli.

Recombination of double-stranded DNA (dsDNA) requires three bacteriophage \(\lambda\)-Red proteins (i.e., Gam, Exo, and Beta)\(^{[18]}\). Exons encode exonucleases that degrade dsDNA from the 5’ end, while \(\beta\) proteins bind the single-stranded regions derived from exons and facilitate recombination by facilitating pairing with cognate genomic targets\(^{[19]}\). It prevents the degradation of linear dsDNA by Gam E. coli RecBCD protein and SbcCD nuclease\(^{[20]}\).

Murers et al.\(^{[21]}\) initially constructed a plasmid expressing phage recombinase in 1999. Since then, numerous researchers have modified the plasmid on that basis. The most extensively used one is pKD46 constructed by Datsenko et al.\(^{[22]}\), which can express the complete Red homologous recombination system in the auxotrophic plasmid pKD46, make an innovation in the two-step homologous recombination method, and successfully perform gene knockout in E. coli K-12. In its established Red
recombinant technology, the primer requires only 36 nucleotides for the homologous arm, and pKD46 serves as the homologous recombinant plasmid with benzyl-resistant low copy temperature-sensitive type. The $\text{exo}$, $\text{bet}$, and $\text{gam}$ genes can be integrated under the control of the phage arabinose promoter. The templates for targeting DNA comprise pKD3 and pKD4 plasmids with chloramphenicol and kanamycin resistance genes, respectively, with FRT sites (flip-flop binding sites)\cite{23}. Depending on the removal of the gene of interest, the resistance gene can be removed with a helper plasmid expressing FLP recombinase that recognizes direct repeat FRT (FLP Recognition Target) sites flanking the resistance gene. Red and FLP helper plasmids contain temperature-sensitive replicons that can be eliminated through incubation at 37°C (Fig. 1).

Feng et al.\cite{24} constructed a gene deletion strain of the E. coli efflux protein YddA, AcrB using the Red recombinant system and examined the drug susceptibility of the deletion strain. Their result has suggested that YddA mainly effluxes norfloxacin, and it is an ATP-dependent efflux protein. Ogawa et al. \cite{25} investigate the mechanism of multidrug and toxic compound extrusion (MATE) resistance of Klebsiella using the above-mentioned recombinant plasmid, and their results indicated that the MIC values of antibacterial drugs (e.g., kanamycin) do not change after knocking down the $\text{ketM}$ gene of the exocytosis pump, thus confirming that the MATE exocytosis pump is not the direct cause of drug resistance in Klebsiella.

2.2 CRISPR/Cas9 technologies

CRISPR technology was originally discovered in 1987\cite{26}, whereas it was not until 2012 that Professor Jennifer Doudna and Professor Emmanuel Charpentier confirmed in vitro experiments that the CRISPR-cas9 system can "localize" DNA breaks\cite{27}. CRISPR knockout screens can be employed in functional genomics studies to detect genomic loci of cellular drug resistance\cite{28,29,30}, elucidate how cells induce host immune responses, and determine how certain viruses cause cell death\cite{31}. The above-mentioned technique has been extensively applied in prokaryotes and eukaryotes.

CRISPR/Cas systems currently fall into two categories. Types I, III, and IV pertain to the first category of CRISPR/Cas systems, and types II, V and VI belong to the second category of CRISPR/Cas systems\cite{32}. Type 1 systems share some common features. Precursor crRNAs are processed using specialized Cas endonucleases, and after the maturation, the respective crRNA is assembled into a large multi-Cas protein complex that is capable of secreting nucleic acids complementary to the crRNA and split. In Class 2 systems, a single multinomial large protein serves as the effector. However, in the CRISPR Type II (CRISPR/Cas9) system found in Streptococcus, CRISPR-RNA (tracrRNA) is linked to a small crRNA region complementary and lays a basis for the formation of partial dsRNA. This dsRNA can be attached to Cas9 and target the prototypical spacer sequence, which is subsequently degraded by the nucleic acid endonuclease Cas9\cite{33} (Fig. 2).

Normally, artificial double-strand breaks in E. coli must be repaired by RecA-mediated homologous recombination using homologous sequences as editing templates; however, natural homologous
recombination pathways are generally considered to be difficult repairing double-strand breaks caused by CRISPR-Cas9 cutting of chromosomes. The introduction of phage-derived λ-Red recombinase into the CRISPR system improves the probability of obtaining mutant strains, and precise gene modification can be obtained when editing templates are supplemented by the target plasmids [34,35,36]. The processing efficiency is further increased by introducing an exogenous DNA repair system (e.g., when editing templates and λ-Red systems were introduced simultaneously) [37].

Liu et al. [38] targeted and disrupted plasmids encoding kanamycin resistance genes in Escherichia coli using the CRISPR-Cas9 system, thus restoring kanamycin susceptibility in over 99% of bacteria for 32 h, demonstrating that the CRISPR-Cas9 system is effective against resistant plasmids. Wu et al. [39] used the CRISPR/Cas9 genome editing function for modification and removal. They introduced a repair template for homologous recombination, such that the gene editing efficiency was significantly increased. Qiu et al. [40] used the CRISPR-Cas9 system to create a gyrA gene mutation, which altered the susceptibility of the strain to quinolones, thus demonstrating a causal relationship between the E. coli gyrA mutation and its resistance to quinolone antimicrobials.

2.3 Suicide Plasmid Vector System

The suicide plasmid vector system refers to a chromosome modification technique developed in the 1980s, which has a wide host range and is transferable [41]. Conventional suicide plasmids contain the R6K replication initiator that replicates only in recipient bacteria with the pir gene, which encodes a protein required for R6K initiation function and is eliminated once the pir gene is missing [42,43]. Accordingly, it cannot be replicated in general bacteria and should be incorporated into the bacterial chromosome and co-replicated with the bacterial chromosome, which is the characteristic condition and special feature of suicide plasmids.

Suicide plasmids have two options when transferred into the host cell since they do not contain replicon structures that can replicate in the host cell. The first option is automatically removed without replication, and the other is to be incorporated into the chromosome and replicates as it replicates. Using the above function, the homologous arm is cloned into the suicide plasmid vector through enzymatic cleavage and DNA ligation. Moreover, after splicing transfer, the homologous fragment on the suicide plasmid will integrate with the homologous fragment on the bacterial genome. After the introduction of suicide plasmid into bacteria, the bacteria can rely on their recombination function (i.e., RecA recombination system), and genes can be precisely deleted [15].

Gene deletion using suicide plasmids is based on a two-step homologous recombination process involving a counter-selection system that substitutes the homology arms of the suicide plasmid with the bacterial target gene [44]. Thus, the first exchange integration refers to the replacement of the target gene with the homologous arm in the suicide plasmid, which integrates the suicide plasmid into the genome in its entirety, and the second exchange integration is the removal of the plasmid backbone from the bacterial genome to obtain a trace-free gene knockout. After successful replacement, the suicide plasmid
is removed by rejection for its inability to replicate in an environment lacking relevant replication conditions, thus causing a trace-free knockout deletion strain\cite{45} (Fig. 3).

Oh et al.\cite{46} developed a method for the knockout of drug resistance genes in *Acinetobacter baumannii* (*A. baumannii*) using suicide plasmids by amplifying fragments with the upstream and downstream fragments of the target gene and antibiotic resistance genes by overlapping PCR. Subsequently, the recombinant suicide plasmids are ligated into *A. baumannii* for homologous recombination via flat ends to obtain mutants. TSAI Y K et al.\cite{47} deleted and recovered copies of the *ompK35* and/or *ompK36* genes directly from the chromosome of *Klebsiella* using suicide plasmid allele exchange method. Compared with the parental strain, only the strain lacking *OmpK36* is resistant to cefazolin, cephalothin, and cefoxitin. Deletion of *OmpK35* further increased MICs, suggesting that strains with double deletions are significantly resistant to the above-described drugs.

### 3. Advantages And Disadvantages Analysis

The two-step homologous recombination method has been the most commonly used method, where the plasmid encoding the recombinase is first transferred into the bacteria, and then the exogenous DNA is transferred into the recombinase-expressing bacteria by electroporation for homologous recombination. The defects are elucidated as follows: (1) it requires a high concentration of DNA fragments, especially for some strains with dense cell membranes, due to low uptake efficiency, the amount of DNA entering the cells often fails to meet the minimum standard for homologous recombination, resulting in gene knockout failure. Moreover, the electro-transformation will cause the death of numerous cells, and the remaining cells will not reach the required concentration, thus resulting in knockout failure. (2) The Red system is required to eliminate the recombinase expression vector from the bacteria to allow for the subsequent introduction of another vector expressing the FLP recombinase, which should be eliminated again to eventually produce a markerless mutant. In brief, this λ-Red recombination-based approach refers to a time-consuming process, and knockout by Red homologous recombination leaves a residual FRT locus on the bacterial genome, and the probability of securing a recombinant strain in practice is often extremely low.

Herring et al.\cite{48} first proposed the Genegorging method in 2003 to tackle down the shortcomings of conventional methods. The targeting fragment is ligated on a plasmid to achieve mass replication in the host without exogenous transformation, and cleavage by homing endonuclease leads to the production of targeting DNA in each cell, raising the base number of cells that may undergo homologous recombination. The single plasmid knockout method was first proposed by Yu in 2008\cite{49}, in which the λ-Red recombination system and the homing endonuclease are joined on a plasmid under the control of arabinose and rhamnose promoters. Moreover, the target fragment is homologously recombined into the chromosome and then introduced into the homing endonuclease for cleavage through double-swap homologous recombination, similar to the suicide plasmid vector system, to achieve a trace-free knockout.
without repeated transformation of plasmids. It outperforms previous methods, and it applies to a range of gene modification work (e.g., point mutation of genes in *E. coli*, gene-targeted insertion).

CRISPR/Cas9 technology is capable of targeting almost any gene to achieve a trace-free knockout, with the advantage of simple operation and high recombination efficiency, simply by designing a single guide RNA. Under its targeting action, the Cas9 protein tracks close to the PAM sequence to recognize and shear the exogenous dsDNA. However, for most existing industrial microorganisms, DNA repair generally selects non-homologous recombination. Accordingly, Jiang et al.\textsuperscript{[50]} coupled CRISPR/Cas9 technology with Red recombinase, which significantly increased the chance of homologous recombination compared with λ-Red recombination technology. The downside is the case that the application of CRISPR technology in bacteria becomes significantly less prevalent than in eukaryotes, and most CRISPR/Cas9 gene editing systems are employed on microorganisms mainly to obtain gene deletion strains to lay a basis for further studies. Besides, CRISPR/Cas9 systems have several defects (e.g., off-target effects, unstable tool plasmids, and toxic effects of Cas9 proteins). Furthermore, the targeting efficiency of Cas9 protein is unique in different strains, and switching to a tissue-specific promoter to drive the targeted expression of Cas9 nucleic acid endonuclease should be considered in the design of vector plasmids\textsuperscript{[51]}. In addition, gene editing using suicide plasmid homologous recombination technology can be performed without trace knockout by simply constructing consecutive homologous fragments upstream and downstream of the target gene. The homologous fragments are linked to the suicide plasmid, and the bacteria have the capability of integrating the suicide plasmid into the chromosome in accordance with its RecA recombination function. Subsequently, the suicide plasmid backbone is eliminated through a second homologous recombination. The defect of the above method is that the suicide plasmid should be transferred to the host bacterium after splice transfer, and more uncertainties exist during the process. However, since the suicide plasmid cannot be replicated in the host bacterium, it is not required to eliminate the tool plasmid. It is noteworthy that suicide plasmid homologous recombination technology is still a relatively safe and stable gene editing technology to avoid the subsequent normal regulation from being affected.

4. **Summary And Outlook**

Drug resistance has emerged in a wide variety of common diseases due to antibiotic abuse over the past few years, such that drug resistance genes can be investigated from a functional genetic perspective. Bacteria reduce the concentration of drugs in bacteria by mainly relying on active efflux mechanisms, and energy-dependent efflux systems (e.g., quinolones, macrolides, and chloramphenicol) have been discovered successively, i.e., a "single" gene encoding a "single" antibiotic or a class of antibiotics. A "single" drug system goes against one or more classes of antibiotics. The foundation can be laid for the development of modern drugs by knocking these genes out or modifying them specifically to explore changes in the resistance phenotype of knockout and proto-bacteria.
Considerable drug transporter genes have been identified through the complete sequencing of bacterial genomes. In Escherichia coli, based on sequence similarity, 37 open reading frames (orf) have been identified as drug transport genes, which are the earliest identified drug resistance genes, whereas some drug resistance genes remain undiscovered. Currently, bioinformatics prediction, gene expression profiling, gene knockout, gene knockin, gene silencing, and gene editing techniques have served as common techniques for the research on microbial gene functions. In existing research, specific research plans can be formulated, and research methods suitable for microbial gene functions can be selected based on the prerequisites for functional research on microbial target genes (groups). Sometimes two or more methods should be adopted in parallel, and these methods should be combined and confirm each other.

Our laboratory has developed a method for knocking out bacterial-related genes using the λ-Red recombinant system and has successfully knocked out drug resistance genes in *E. coli* through multiple knockouts and tedious elimination of resistance. Bacterial multidrug resistance is widely present in various bacteria in nature. Moreover, *E. coli*, a model strain among bacteria, should not be anti to any antibiotics. The above three knockout methods all employ antibiotic resistance screening for knockout strains and have been extensively utilized. For some multi-drug resistant strains, however, replacement of the screening resistance gene is obliged. Moreover, the plasmids utilized in the above three methods are not stable in all strains, and the knockout plasmids should be designed rationally in accordance with the characteristics of the corresponding strains.

Knockout technologies have evolved through three generations and continue to be evolving since they generate irreplaceable benefits in gene deletion. Although all existing knockout methods exhibit several defects, they can be optimized through unique applications and technological developments.

**Declarations**

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yimin Liang. The evaluation of the final manuscript was done by Chunyu
Tong and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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References


Figures
Figure 1

(a) The targeting fragment contains homologous arms (H1, H2), FRT sites and resistance genes for screening; (b) The homologous arms (H1, H2) on both sides of the target gene are used to undergo homologous recombination; (c) The targeting fragment successfully displaces the target gene under the mediation of Red homologous recombination; d. The pcp20 plasmid expresses FLP recombinase, recognizes the FRT sites and eliminates the resistance genes.
Figure 2

crRNA binds to tracrRNA through base pairing to form Guide RNA, which forms a complex with the nuclease Cas9 and guides Cas9 to shear ds DNA at the sequence target site paired with crRNA. after shearing down the target gene, the cell can use its own non-homologous end-joining repair to repair the broken chromosome, or it can use Red homologous recombination, which artificially introduces homologous fragments for homologous end-joining repair.
Suicide vectors were integrated into the bacterial genome after undergoing the first homologous recombination, after which they underwent the second homologous recombination to obtain two results, strains with the same sequence as the wild type (a), and strains without trace knockout (b).