Establishment of CRISPR-Cas9 system in Bifidobacteria animalis AR668

Jiao Li  
University of Shanghai for Science and Technology

Xin Song  
University of Shanghai for Science and Technology

Zhiqiang Xiong  
University of Shanghai for Science and Technology

Guangqiang Wang  
University of Shanghai for Science and Technology

Yongjun Xia  
University of Shanghai for Science and Technology

Yijin Yang  
University of Shanghai for Science and Technology

Lianzhong Ai (ailianzhong@126.com)  
University of Shanghai for Science and Technology

Research Article

Keywords: Bifidobacteria, CRISPR, gene knockout, inducible plasmid curing

Posted Date: October 20th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2172911/v1

License: ☑️ ☐ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Microbial Cell Factories on June 12th, 2023. See the published version at https://doi.org/10.1186/s12934-023-02094-2.
Abstract

_Bifidobacteria_ are representative intestinal probiotics that have extremely high application value in the food and medical fields. However, the lack of molecular biology tools limits the research on functional genes and mechanisms of _bifidobacteria_. The application of accurate and efficient CRISPR system to genome engineering can fill the gap in efficient genetic tools for _bifidobacteria_. In this study, CRISPR system of _B. animalis_ AR668 was established, which successfully knocked out gene 0348 and gene 0208. The influence of different homology arms and fragments on the knockout effect of the system was explored. In addition, the inducible plasmid curing system of _bifidobacteria_ was innovatively established. This study contributes to the genetic modification and functional mechanism analysis of _bifidobacteria_.

Introduction

In recent years, gut commensal microorganisms have received extensive attention due to their close relationship with human health and diseases. As a type of symbiotic bacteria in the intestines of humans and mammals, _bifidobacteria_ has been shown to have a variety of probiotic effects, including regulating the balance of intestinal flora, improving lactose intolerance, improving immune activity and inhibiting pathogens (1–3). These positive effects have prompted it to be used commercially as an active ingredient in probiotic foods which promote health (4). Although the functional activity of _bifidobacteria_ as probiotics has been extensively studied, it is little known about its exact molecular mechanism of action, which is the key to scientifically explaining its health benefits.

Since the whole genome sequence of _b. longum_ was reported in 2002, more and more _bifidobacterium_ genome information have been reported, which greatly improved the understanding of physiology, genetics and evolution of them with medical and commercial significance, and therefore the study of _bifidobacteria_ moved from initial morphology to molecular level. The research on the whole genome could be conducive to fully reveal the physiological and metabolic characteristics of _bifidobacteria_, accelerate the further excavation of important functional genes, and lay the foundation for the screening and application of _bifidobacteria_. As of 2022, there were 98 _bifidobacteria_ strains that had completed whole-genome sequencing and submitted to the National Center for Biotechnology Information (NCBI, National Center for Biotechnology Information). However, to make use of these abundant genomic data and prove the precise function of genes, it is essential to exploit effective genetic tools and technologies for _bifidobacteria_. Due to the high nutritional requirements, high sensitivity to oxygen, complex restrictive modification systems, the development of genetic tools for _bifidobacteria_ was hindered (5, 6). Due to the present, relatively few _bifidobacteria_ molecular tools have been developed, which explains why the genetics of these microorganisms is poorly understood compared to other bacteria with industrial importance.

In fact, gene knockout is a direct procedure to clearly understand the function of specific coding sequences. There have been only a few attempts to genome editing of _bifidobacteria_. Some studies have proved that the standard knockout method based on traditional homologous recombination is feasible in _bifidobacteria_, but this requires a sufficiently high transformation efficiency(7, 8). For example, strict anaerobic conditions are used in the preparation and electro-transformation of competent cells to overcome the oxygen limitation of _bifidobacteria_. Nevertheless, due to the lack of RecBCD protein in most _bifidobacteria_ genomes responsible for the main homologous recombination pathway of prokaryotes, the homologous recombination method is time-consuming and inefficient which is not suitable for multiple genetic modifications of the _bifidobacteria_ genome. As a widely used conditional knockout system, the Cre-loxP system is of great significance for exploring the phenotype and function of specific genes in the genome. However, when Cre recombinase is integrated into the genome, its insertion position has certain limitations and may affect the expression of endogenous genes. In conditional knockout, the Cre promoter must be effective and specific which requires pre-screening of Cre promoter genes with high expression levels (9). Also, scars will stay on the genome after genetic modification. The CRISPR-Cas system has been widely used in the genetic modification of various organisms in recent years because of its precise, fast, and traceless operation (10). The classic CRISPR-Cas9 technology generates a DNA double-strand break (DSB) at the target site, inducing intracellular homologous recombination (Homology-directed repair, HDR) and non-homologous end joining (NHEJ) repair pathway to achieve genetic modification such as site-specific knockout, replacement, and insertion of genomic DNA(11). In lactic acid bacteria (LAB), CRISPR-Cas9 gene editing technology can be used to clarify and enhance beneficial properties. Jee-Hwan combined CRISPR-Cas9 with recombination technology in the genetic engineering of _Lactobacillus reuteri_ (12). This is the first time that CRISPR technology has been applied to the genetic engineering of LAB. Research by Ryan et al.
showed that CRISPR system could be used for genetic modification of \textit{L. plantarum} WCFS1 and successfully site-directed mutation from D480Y to rpoB \cite{13}. Subsequently, Huang et al. established a RecE/T-assisted CRISPR genome editing toolbox that could be used in \textit{L. plantarum} WCFS1 and \textit{L. brevis} ATCC367, which easily achieved gene knockout in \textit{Lactobacillus} \cite{14}. Song X et al. established a gene editing plasmid pLCNICK for \textit{Lactobacillus casei} based on CRISPR-Cas9\textsuperscript{D10A}. The independent gene knockout and green fluorescent protein (eGFP) gene insertion efficiency of this system was 25–62\%, which proved that the pLCNICK gene editing system could be used as a genome editing tool for \textit{L. casei} \cite{15}. In recent years, CRISPR technology has also been applied to anaerobic microorganisms. Cobb et al. applied the CRISPR-Cas9 system to \textit{Streptomyces} for the first time \cite{16}. Huang et al. established a CRISPR system for rapid genome editing of \textit{Clostridium ljungdahlii}, with a gene knockout efficiency of 50–100\%, which overcame the lack of genetic tools available for anaerobic \textit{Clostridium} \cite{17}. The successful application of CRISPR technology in LAB and anaerobic microorganisms prompted us to apply it to \textit{bidobacteria}.

A strain of \textit{B. animalis} called AR668 was screened from baby feces in our laboratory, and its most suitable electro-transformation method through preliminary experiments was established. Based on this, it is planned to establish CRISPR system for \textit{B. animalis} AR668, which will provide a tool platform for better elucidation of its probiotic mechanism and help to systematically elucidate the physiological and metabolic mechanisms at the molecular level that speed up the transformation and breeding of excellent strains.

**Materials And Methods**

**Strains**

\textit{Escherichia coli} TOP10 was used as a cloning host. All \textit{E. coli} carrying pAM1 series plasmids were cultured on LB plates containing ampicillin at 37°C. \textit{Bifidobacteria} AR668 was cultured anaerobically in BS medium at 37°C. If necessary, erythromycin was added at a concentration of 5\(\mu\)g/ml. All recombinant plasmids enter the competent cells of \textit{bifidobacterium} by electroporation.

**Enzyme and biotechnology toolkit**

Phanta Max Super-Fidelity DNA Polymerase and 2× Taq Master Mix (Novozymes Kunming, China) are used for high-fidelity DNA amplification and PCR screening of the required genotypes. The oligonucleotide primers used were synthesized by BGI (Shenzhen, China). Use conventional restriction enzymes and ligases purchased from Takara, Dalian, China to construct plasmids, or use ClonExpress MultiS one-step cloning kit (Vazyme Biotech, Nanjing, China) to assemble plasmids. Plasmid isolation and DNA purification using kits purchased from Axygen (Hangzhou, China).

**Plasmid construction**

The plasmids and primers used are shown in Table 1 and 2. The CRISPR-Cas9 editing plasmid consists of the following parts: the linearized vector is obtained by double digestion of pAM1-ldh2 with \textit{Pst} and \textit{Spe}; the Cas9 gene is obtained by PCR amplification with Cas9-\textit{Spe}-F and Cas9-\textit{Spe}-R using plcp\textsubscript{0537} as the template; the whole genome of AR668 is used as the template, PCR amplification of 0348-sgRNA-F and 0348-sgRNA-R was used to obtain 20 nt of the target \textit{gene} 0348; the AR668 whole genome was used as a template, and primers of 0348-up and 0348-down were used for PCR amplification to obtain the homology arms on both sides of the target gene. Note that all 20 nt need to be aligned with the AR668 genome in advance to avoid off-target due to the presence of highly similar sequences.

The sgRNA and artificially synthesized promoter P23 are assembled by overlap PCR to obtain the sgRNA expression cassette. The above fragments are assembled by two rounds of overlap PCR, and the vector and fragments are assembled by a seamless cloning kit. When preparing a knockout plasmid targeting \textit{gene} 0208, use pLJ2 as a template, and replace the corresponding homology arms on both sides and 20 nt.

Similarly, knockout plasmids with different homology arms are also constructed based on pLJ2, and the homology arms of the corresponding size are replaced with the other originals unchanged. When constructing knockout plasmids with different knockout fragments, only the sequences of the homology arms on both sides of pLJ2 need to be changed.

Construction of the inducible reporter plasmid pLJ9: The linearized vector was obtained by double digestion of pAM1-ldh2 with \textit{Nde1} and \textit{Spe1}. The promoter sequence of \(\beta\)-galactosidase in the AR668 genome was amplified by pAM1- \textit{lacZ-F} and pAM1- \textit{lacZ-}
R, and amplified by pAM1-gfp-F and pAM1-gfp-R. The eGFP sequence of pAM1-ldh2-eGFP was increased, the above fragments were connected by overlap PCR, and the vector and fragments were connected by the seamless cloning kit to obtain the recombinant plasmid pLJ9.

The inducible plasmid elimination system consists of the corresponding knockout plasmid and the inducible sgRNA_Em expression cassette. The linearized vector is obtained by single-enzyme digestion of pLJ2 with Xho1, and the lacZ sequence of pAM1-lacZ-eGFP is amplified by sgRNA-Em-lacZF and sgRNA-Em-lacZ-R, designed to target Em SgRNA_Em is amplified by sgRNA-Em-F and sgRNA-Em-R, and the two ends are connected to form an inducible sgRNA expression cassette by overlap PCR. The vector and fragments are assembled by seamless cloning kit to construct recombinant plasmid pLJ10.

**Competent cell preparation and electroporation**

The constructed plasmids were delivered into AR668 by electroporation. The competent cells were prepared as follows. One milliliter of overnight cultures in BS broth were diluted into 50 ml of fresh BS broth supplemented 0.5 mol/L sucrose. The inoculated 50 ml BS broth with the additives were anaerobically incubated at 37 °C until the OD$_{600nm}$ reached at 0.3. The cells were chilled on ice for 30 min and harvested by centrifugation at 5000 rpm and 4°C for 10 min. Cells were washed twice with 0.5 mol/L sucrose and 0.1mmol/L ammonium citrate buffer. The final cell pellet was resuspended in 1ml of ice-cold buffer with 10% glycerol. One hundred-microliter aliquot of the competent cells were stored at -80°C.

The competent cells were mixed with 1000 ng of plasmid DNA and kept on ice for 30 min. Then the mixture was transferred to a pre-cooled Gene Pulser cuvette (Bio-Rad, Hercules, CA, USA). The cuvette was pulsed at various field strengths and parallel resistances using the Gene Pulser Xcell Microbial Electroporation System (Bio-Rad). Following the electroporation, 0.9 ml of BS broth (supplemented with 0.4 mol/L sorbitol, 2 mmol/L calcium chloride and 20 mmol/L magnesium chloride) was added to bacteria and incubated at 37 °C for 4 hours under anaerobic conditions. The bacteria were then plated onto BS agar containing 5 μg/ml of Em. The plates were incubated for more than 48 hours under anaerobic conditions.

**Screening and identification of edited genes**

The mutants were screened by PCR, and the primers on both sides of the upper and lower homology arms were designed as verification primers. The PCR-correct strains were further identified by sequencing.

**Evaluation of homology arms and knockout fragments in the crispr system**

In this study, plasmid pLJ2 was used as an example. On this basis, knockout plasmids with upper and lower homology arms of 1000 bp, 500 bp, 250 bp, and 150 bp were constructed, electrot transformed into AR668, and incubated at 37°C for more than 48 hours. The knockout result of the plasmid was verified, and the result was further confirmed by sequencing.

In this study, the plasmid pLJ2 was taken as an example. On this basis, knockout plasmids with knockout fragments of 1000 bp, 300 bp, and 5000 bp were constructed, electrot transformed into AR668, and incubated at 37°C for more than 48 hours. In addition to the results for verification, and further confirm the results by sequencing.

**Establishment of eGFP-based inducible reporting system**

A fluorescent reporter plasmid containing eGFP was constructed. Based on plasmid pAM1-ldh2, a vector was prepared with Hind and Nde as double restriction sites, lacZ in the AR668 genome was used as a promoter, and eGFP was used as a fluorescent reporter gene to construct a plasmid. The plasmid was transformed into AR668 by electroporation, and a single colony was picked for PCR verification. The single colony containing the correct plasmid was grown in fresh BS broth for lactose induction. The fluorescence is detected by a fluorescence microscope and the relative fluorescence intensity is measured with a microplate reader. The best induction conditions were obtained by optimizing the OD$_{600nm}$ value before induction, the concentration of lactose and the induction time.

**Mutant purification and plasmid elimination for continuous genome engineering**
Cultivate the mutant strains verified by PCR and sequencing in a resistant medium. For double-banded strains, that is, when the wild type and the mutant type are present at the same time, they should be streaked and separated in the solid medium first, and then the single-banded strains will be verified by colony PCR. The knock-out strains were cultured in non-antibiotic medium. When it is raised to a proper OD$_{600nm}$, a certain amount of lactose is added to induce. Under the guidance of the sgRNA targeting the erythromycin resistance gene in the plasmid, Cas9 cuts the erythromycin gene to complete the elimination of the plasmid.

**Results**

**CRISPR-Cas9-mediated bifidobacteria genome editing**

In the working process of CRISPR-Cas9 gene editing system, DSB is caused by sgRNA-guided double-stranded cleavage of Cas9 protein at the target site. Next, HDR pathway is needed to repair the broken genome and finally gene knockout is achieved. Cas9 protein has a certain toxic effect on cells, so we first constructed a shuttle plasmid pLJ1 containing the Cas9 expression box and transformed it into *bifidobacteria* by electroporation. We observed more than $10^2$ transformants were produced, which were verified by PCR. This proved that Cas9 protein could be expressed in *bifidobacteria* and had less toxic effect. In order to prove the guided cleavage activity of sgRNA in pLJ3, we constructed a recombinant plasmid pLJ4 containing only Cas9 expression box and sgRNA expression box, lacking homology arms. After transformation, only a few colonies appeared, indicating that the sgRNA we designed had a high cleavage activity. It guided the cleavage of Cas9 protein to cause DSB, and the lack of homology arms destroyed the genome and caused cell death. We constructed a recombinant plasmid pLJ2 targeted *gene 0348*, containing homology arms and sgRNA expression box. After it transformed into *bifidobacteria*, colony PCR verification and sequencing proved that almost all the obtained colonies were mutants. The result had shown that the system successfully knocked out *gene 0348* (figure 1).

In addition, the recombinant plasmid pLJ3 targeting *gene 0208* was also constructed which encoded *upp*. Similarly, when the recombinant plasmid introduced into *bifidobacteria*, the knockout efficiency from obtained transformants was 80 %. In general, we have constructed a CRISPR plasmid that can be used for *bifidobacteria* gene knockout.

**Exploring the smallest homology arm of the CRISPR system**

Next, we explored the effect of different homology arms in recombinant plasmids on the knockout efficiency. Based on pLJ2, recombinant plasmids pLJ4, pLJ5, and pLJ6 with homology arms of only 500 bp, 250 bp, and 150 bp were designed, and the other parts unchanged. After these plasmids electroporation into *bifidobacteria* respectively, the knockout effect was observed in the figure. When the homology arm was 1000 bp, the system had the highest ability for *gene 0348*, and when the homology arm was 500 bp, the knockout efficiency was 58 %. In a word, the system had a quite good knockout ability, even if the homology arm was 150 bp with the knockout efficiency of more than 50 % (table 3).

**Exploration of the largest knockout fragment of the CRISPR system**

Similarly, the largest knockout fragment of the system was explored. Based on pLJ2, we designed recombinant plasmids pLJ7 and pLJ8 with knockout fragments of 3000 bp and 5000 bp, and other elements remained. The above recombinant plasmids were introduced into competent cells respectively, and the result was shown in the figure. When the knockout fragment was 3000 bp, its knockout effect was only 20%, but pLJ8 didn't knocked out yet when the target fragment was 5000 bp (table 3).

**Screening and optimization of inducible promoter based on enhanced green fluorescent protein (eGFP)**

The complete genetic operating system included plasmid curing procedure for subsequent operations. However, the traditional plasmid elimination method by continuous passage with no-antibiotic medium was used, the plasmids still existed, which was time-consuming and inefficient. Therefore, this method was not applicable in our system. In this study, the *bifidobacteria* self-inducible promoter was selected to eliminate the recombinant plasmid. The *ldh2* promoter in pAM1-ldh2 was replaced with the β-galactosidase promoter *PlacZ* in AR668, and eGFP was inserted to form the recombinant plasmid pLJ9. The plasmid was electroporated into AR668. After the PCR program was verified, it was cultured in liquid medium. After induction of lactose, the
The development of whole genome sequences and genetic tools provides opportunities for improving the analysis of physiology, genetics and gastrointestinal metabolism in bidobacteria. Through appropriate genetic modification, the survival rate in vivo of bidobacteria can be enhanced, which is essential for the effective manipulation of these probiotic microorganisms.
probiotics can be improved, and the ingested probiotic strains can be molecularly tracked in human or animal disease models. Future research needs to optimize the system to expand the scope of application such as exploring the practicability of this system in other bifidobacteria, genetically modifying AR668, and selecting mutant strains with excellent traits for industrial large-scale production.

**Conclusion**

Taken together, the present study established CRISPR system of *B. animalis* AR668 for genetic modification and functional mechanism analysis.

**Declarations**

**Author contributions**

Jiao Li performed the experiments. Jiao Li, Xin Song and Zhiqiang Xiong conceived this project. Guangqiang Wang, Yongjun Xia and Yijin Yang analyzed the data. Lianzhong Ai guided the experiment and edited the manuscript. All authors read and approved the final manuscript.

**Funding**

This work was supported by grants from the National Natural Science Foundation of China (31972101), National Science Foundation for Distinguished Young Scholars (32025029), Shanghai Education committee scientific research innovation projects of China (2101070007800120), Natural Science Foundation of Shanghai (22ZR1444000), National Natural Science Foundation of China (32101928).

**Availability of data and materials**

All data have been stored on dedicated computers at the University of Shanghai for science and technology. All data and strains are available upon request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

All authors have approved the manuscript.

**Acknowledgements**

Not applicable.

**Competing interests**

The authors declare no competing interests.

**Author details**

1 Shanghai Engineering Research Center of Food Microbiology, School of Health Science and Engineering, University of Shanghai for Science and Technology, Shanghai 200093, China.

**References**


### Tables

**Table 1.** Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains and Plasmids</th>
<th>Characteristic</th>
<th>References and Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli TOP10</td>
<td><em>E. coli</em> K-12, F−, mcrAΔ (mrr-hsd RMS-mcrBC), φ80, lacZΔM15, Δlac 74, recA1, araΔ139Δ (ara-leu)7697, galU, galK, rps, (Strr) endA1, nupG.</td>
<td>Our laboratory</td>
</tr>
<tr>
<td>B. animalis AR668</td>
<td></td>
<td>Our laboratory</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAM1-ldh2</td>
<td>pMB1 ori, Amp, Em, repB, Pldh</td>
<td>Our laboratory</td>
</tr>
<tr>
<td>plcp_0537</td>
<td>Cas9, repE, repD, Em, Kan, repA101, sgRNA</td>
<td>Our laboratory</td>
</tr>
<tr>
<td>pLJ1</td>
<td>pMB1 ori, Amp, Em, repB, Pldh2, Cas9</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ2</td>
<td>pMB1 ori, Amp, Em, repB, Pldh2, Cas9, P23, sgRNA, <em>ldh</em> homologous arms(1kb)</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ3</td>
<td>pMB1 ori, Amp, Em, repB, Pldh2, Cas9, P23, sgRNA, <em>upp</em> homologous arms</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ4</td>
<td>pMB1 ori, Amp, Em, repB, Pldh2, Cas9, P23, sgRNA, <em>ldh</em> homologous arms(500bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ5</td>
<td>pMB1 ori, Amp, Em, repB, Pldh2, Cas9, P23, sgRNA, <em>ldh</em> homologous arms(250bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ6</td>
<td>pMB1 ori, Amp, Em, repB, Pldh-Cas9, P23-sgRNA, <em>ldh</em> homologous arms(150bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ7</td>
<td>pMB1 ori, Amp, Em, repB, Pldh2, Cas9, P23, sgRNA, 3kb homologous arms</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ8</td>
<td>pMB1 ori, Amp, Em, repB, Pldh2, Cas9, P23, sgRNA, 5kb homologous arms</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ9</td>
<td>pMB1 ori, Amp, Em, repB, <em>PlacZ</em>, eGFP</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ10</td>
<td>pMB1 ori, Amp, Em, repB, Pldh2, Cas9, P23, sgRNA, <em>ldh</em> homologous arms(1kb), <em>PlacZ</em>, sgRNA(Em)</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 2.** Oligonucleotides used in this study
<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence 5’-3’</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cas9-Spe-F</td>
<td>AATACTCGTCAAGACATGGGCACTAGTTTCGCTCACCTCAGGCTGACTC</td>
<td>For pLJ1 construction</td>
</tr>
<tr>
<td>Cas9-Pst-R</td>
<td>GCAAGCTTGCATGCCAGATGGGATAAGAATACTCAATAGGCTTAGATCG</td>
<td></td>
</tr>
<tr>
<td>0348-up-F</td>
<td>CGTCAGACATGGGCACTAGTCTCGAGGACCCGCGGCTGGATTCGCT</td>
<td>For pLJ2 construction</td>
</tr>
<tr>
<td>0348-up-R</td>
<td>AGAAGAGGAATCCGACTTCGTCGCGTC</td>
<td></td>
</tr>
<tr>
<td>0348-down-F</td>
<td>CGAAGTCGGATTCTTTCCTCTTCTCTGATCG</td>
<td></td>
</tr>
<tr>
<td>0348-down-R</td>
<td>GTGCTTTTTTTTCGCTCGAGAAGCGACC</td>
<td></td>
</tr>
<tr>
<td>0348-sgRNA-F</td>
<td>GCTTCGAGACCGAAAGACCCGACTCGGTCG</td>
<td></td>
</tr>
<tr>
<td>0348-sgRNA-R</td>
<td>AATGACAATGATGTTGCGGCCCCTGTCGCTCAAGAGGACGCGAGTTTGAAGCTGAATAAGGCTGGGGC</td>
<td></td>
</tr>
<tr>
<td>0348-P23-F</td>
<td>CGGCCTCTTGGACGACGGGCCCACATCATTTGTCTTTATATATTTGCTTGGG</td>
<td></td>
</tr>
<tr>
<td>0348-P23-R</td>
<td>TAGAGGTCGACTGAAACTAGTCGACAACCCTGACAAAACCCGGGTACCTTTTGGC</td>
<td></td>
</tr>
<tr>
<td>0208-up-F</td>
<td>ACATGGGCACTAGTCTCGAGGACCCGCGGTGTC</td>
<td>For pLJ3 construction</td>
</tr>
<tr>
<td>0208-up-R</td>
<td>ACTCTAGTAACACTAGCATATCGACAAACTGTACCC</td>
<td></td>
</tr>
<tr>
<td>0208-down-F</td>
<td>GATATGCTAGTATCTAGAGTAGAAGCCTTTGTTTG</td>
<td></td>
</tr>
<tr>
<td>0208-down-R</td>
<td>GTGCTTTTTTTTACAGACCCTGCTGGGGG</td>
<td></td>
</tr>
<tr>
<td>0208-sgRNA-F</td>
<td>AATGACAATGATGTTGCGGCCCCTGTCGCTCAAGAGGACGCGAGTTTGAAGCTGAATAAGGCTGGGGC</td>
<td></td>
</tr>
<tr>
<td>0208-sgRNA-R</td>
<td>ATGCGAGGTCGCTCTTCCCTCCTCTCTCATCGG</td>
<td></td>
</tr>
<tr>
<td>0348-HR500-up-F</td>
<td>CGTCAGACATGGGCACTAGTCTCGAGGCCACTTCACGTCCTACGGCGGTGCT</td>
<td>For pLJ4 construction</td>
</tr>
<tr>
<td>0348-HR500-up-R</td>
<td>AGAAGAGGAAGAGGAATCCGACTTCGTCGCG</td>
<td></td>
</tr>
<tr>
<td>0348-HR500-down-F</td>
<td>CGCGGACGAAGTCGGATTTCTCTTCTCTCTCATCGG</td>
<td></td>
</tr>
<tr>
<td>0348-HR500-down-R</td>
<td>GTCGCTCTTTTTTTTCTAGAGACGACAGTCCCGACCG</td>
<td></td>
</tr>
<tr>
<td>0348-HR250-up-F</td>
<td>GGCACGTATCAGGCGGCTCGGCGGCTCGG</td>
<td>For pLJ5 construction</td>
</tr>
<tr>
<td>0348-HR250-up-R</td>
<td>AGAAGAGGAAGAGGAATCCGACTTCGTCGCGT</td>
<td></td>
</tr>
<tr>
<td>0348-HR250-down-F</td>
<td>GCGGACGAGTCGGATTTCTCTTCTCTCTCATCGG</td>
<td></td>
</tr>
<tr>
<td>0348-HR250-down-R</td>
<td>GTCGCTCTTTTTTTTCTAGACGCGGGCGGAACACG</td>
<td></td>
</tr>
<tr>
<td>0348-HR150-up-F</td>
<td>ACATGGGCACTAGTCTCGAGGGTTTCATCTGTTTCTCGTGTTTCGAG</td>
<td>For pLJ6 construction</td>
</tr>
<tr>
<td>0348-HR150-up-R</td>
<td>AGAAGAGGAATCCGACTTCGTCGCG</td>
<td></td>
</tr>
<tr>
<td>0348-HR150-down-F</td>
<td>CGAAGTCGGATTCTTCTCTCTCTCTCATCGG</td>
<td></td>
</tr>
<tr>
<td>0348-HR150-</td>
<td>TGTCCTTTTTCTAGAGTTTGCGCCACAGCTACG</td>
<td></td>
</tr>
<tr>
<td>down-R</td>
<td>3K-up-F</td>
<td>ACATGGGCACTAGTCTCGAGTGAAGGCGGCGTTGGC</td>
</tr>
<tr>
<td>3K-up-R</td>
<td>GGGCGAGCCCGTCACTCCCGCATGTCGAGCAC</td>
<td></td>
</tr>
<tr>
<td>3K-down-F</td>
<td>TCGACATGCGGGAGTGACGGCTCGCCCGG</td>
<td></td>
</tr>
<tr>
<td>3K-down-R</td>
<td>GGTGCTTTTTTTCTAGATCGGCGGAGAGCGT</td>
<td></td>
</tr>
<tr>
<td>5K-up-F</td>
<td>ACATGGGCACTAGTCTCGAGTGAAGGCGGCGTTGGC</td>
<td>For pLJ8 construction</td>
</tr>
<tr>
<td>5K-up-R</td>
<td>AGAGGAGAGAGGAAATCCGACTTCGTCGCGTC</td>
<td></td>
</tr>
<tr>
<td>5K-down-F</td>
<td>GCGGACAAAGTCGAGTTCCTTCTTCTCTCTCTCATGCGTCT</td>
<td></td>
</tr>
<tr>
<td>5K-down-R</td>
<td>GGTGCTTTTTTTCTAGATCGGCGGAGAGCGT</td>
<td></td>
</tr>
<tr>
<td>pAM1-gfp-F</td>
<td>CTGTTTGAGATCCTTTACTTTGACGTCGTTCCATGC</td>
<td>For pLJ9 construction</td>
</tr>
<tr>
<td>pAM1-gfp-R</td>
<td>GGCAAAGGAGACGGCAAGCTTGATGGTGAGCGCAGGAG</td>
<td></td>
</tr>
<tr>
<td>pAM1- lacZ-F</td>
<td>TCTCGCCCTTTGCTCCACATCAAGCTTGCCTCCTTTGCTCC</td>
<td></td>
</tr>
<tr>
<td>pAM1- lacZ-R</td>
<td>GACTGGAAGCGGCGATAGAATAACTTTACCTAGTATAGCGTGCGGG</td>
<td></td>
</tr>
<tr>
<td>sgRNA-Em-F</td>
<td>CGTCAGACATGGGCACTAGTCTCGAGAAAAAAAGCACCAGTCGTTGC</td>
<td>For pLJ10 construction</td>
</tr>
<tr>
<td>sgRNA-Em-R</td>
<td>GGCAAAGGAGACGGCAAGCTTGATGGTGAGCGCAGGAG</td>
<td></td>
</tr>
<tr>
<td>sgRNA-Em-lacZ-F</td>
<td>TATGCAAAACTACAGGCCGTCTCCTTTGCTCC</td>
<td></td>
</tr>
<tr>
<td>sgRNA-Em-lacZ-R</td>
<td>AATCCACGGCGGTCTCGAGCGCGCGCATATACTAGTATAGCGTGCGGG</td>
<td></td>
</tr>
<tr>
<td>0348-yz-F</td>
<td>GGTGGACGACACGGCCATG</td>
<td></td>
</tr>
<tr>
<td>0348-yz-R</td>
<td>GGAGCTCGGGCGCC</td>
<td></td>
</tr>
<tr>
<td>0208-yz-F</td>
<td>TAGGCTGCGCGCGTC</td>
<td></td>
</tr>
<tr>
<td>0208-yz-R</td>
<td>CCGCGCATCATCGACATACAG</td>
<td></td>
</tr>
<tr>
<td>3000yz-F</td>
<td>GGTTCGCGCCACACGG</td>
<td></td>
</tr>
<tr>
<td>3000yz-R</td>
<td>GGGGCACCGACGTCGATC</td>
<td></td>
</tr>
<tr>
<td>5000yz-F</td>
<td>CTCTGTGGAACATCGTCACCACA</td>
<td></td>
</tr>
<tr>
<td>5000yz-R</td>
<td>GTCGATGCGCTGGCCATC</td>
<td></td>
</tr>
</tbody>
</table>

| Table 3. The effect of homology arms size and target fragment size on knockout. |

*gene 0348 knockout verification* |
*gene 0208 knockout verification* |
Knockout 3000 bp verification |
Knockout 5000 bp verification |
<table>
<thead>
<tr>
<th>Influencing factors</th>
<th>Size (bp)</th>
<th>(M/P/T)a</th>
<th>Efficiency (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homology arms</td>
<td>500</td>
<td>9/0/10</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>7/0/12</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>6/2/14</td>
<td>57</td>
</tr>
<tr>
<td>Target fragments</td>
<td>3000</td>
<td>3/0/15</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>0/0/8</td>
<td>0</td>
</tr>
</tbody>
</table>

a) M: number of colonies that harbored mixed wild-type and mutant cells; P: number of colonies that harbored pure mutant cells; T: total number of colonies used for PCR screening;
b) Efficiency: probability of deletion events occurring, calculated as (M+P)/T×100%.

**Figures**

(1) The map of plasmid pLJ2. (2) PCR verification and sequencing results of gene 0348 knockout. (3) AR668△0208 mutant and wild-type susceptibility test to 5-fluorouracil. (4) Summary of gene knockout in AR668.

**Figure 1**

CRISPR gene knockout process and results. (1) The map of plasmid pLJ2. (2) PCR verification and sequencing results of gene 0348 knockout. (3) AR668△0208 mutant and wild-type susceptibility test to 5-fluorouracil. (4) Summary of gene knockout in AR668.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Deletion size (bp)</th>
<th>Experimental rounds</th>
<th>(M/P/T)a</th>
<th>Efficiency (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>gene 0348</td>
<td>972</td>
<td>1</td>
<td>4/8/12</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>972</td>
<td>2</td>
<td>1/10/12</td>
<td>91</td>
</tr>
<tr>
<td>gene 0208</td>
<td>642</td>
<td>1</td>
<td>12/0/14</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>642</td>
<td>2</td>
<td>9/4/15</td>
<td>86</td>
</tr>
</tbody>
</table>

a) M: number of colonies that harbored mixed wild-type and mutant cells; P: number of colonies that harbored pure mutant cells; T: total number of colonies used for PCR screening;
b) Efficiency: probability of deletion events occurring, calculated as (M+P)/T×100%.
Figure 2

Construction of inducible plasmid curing system. (1) The map of plasmid pLJ9. (2) The optimization of lactose induction conditions. (3) Construction process of inducible plasmid pLJ10. (4) After the culture was induced according to the optimized conditions, it was cultured on a resistant plate and compared with the culture that was continuously passaged in a non-resistant medium.

Figure 3

The overview of CRISPR–Cas9 editing toolbox for AR668. The working plasmid contains three parts, the Cas9 expression box, the sgRNA1 constitutive expression box targeting knockout gene and the sgRNA2 inducible expression box targeting Em gene. The working plasmid is electro-transformed into bifidobacteria, then the target gene is cut by Cas9 protein under the guidance of sgRNA1, and homologous recombination repair can repair the broken genome through to complete gene editing. Next, the plasmid was induced and eliminated by lactose to obtain a pure mutant strain for the next round of genetic modification.