Fish and Shellfish Immunology 92 (2019) 377–383

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Multiplex genome editing by natural transformation in *Vibrio mimicus* with potential application in attenuated vaccine development

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ARTICLE INFO

Keywords:
Vibrio mimicus
Natural transformation
FLP-Recombination
Targeted mutagenesis

ABSTRACT

Vibrio mimicus (*V. mimicus*) is a significant pathogen in freshwater catfish, though knowledge of virulence determinants and effective vaccine is lacking. Multiplex genome editing by natural transformation (MuGENT) is an easy knockout method, which has successfully used in various bacteria except for *V. mimicus*. Here, we found *V. mimicus* strain SCCF01 can uptake exogenous DNA and insert it into genome by natural transformation assay. Subsequently, we exploited this property to make five mutants (△Hem, △TS1, △TS2, △TS1△TS2, and △II), and removed the antibiotic resistance marker by Flp-recombination. Finally, all of the mutants were identified by PCR and RT-PCR. The results showed that combination of natural transformation and FLP-recombination can be applied successfully to generate targeted gene disruptions without the antibiotic resistance marker in *V.* mimicus. In addition, the five mutants showed mutant could be inherited after several subcultures and a 668-fold decrease in the virulence to yellow catfish (*Pelteobagrus fulvidraco*). This study provides a convenient method for the genetic manipulation of *V. mimicus*. It will facilitate the identification and characterization of *V. mimicus* virulence factors and eventually contribute to a better understanding of *V. mimicus* pathogenicity and development of attenuated vaccine.

1. Introduction

Vibrio mimicus, a Gram-negative bacterium distributed widely in natural aquatic environments [1], causes gastrointestinal diseases and food poisoning in humans around the world [2–4]. Meanwhile, it is one of the most prevalent pathogen in aquaculture and can infect the shrimp, crab and fish [5–10]. Recently, *V. mimicus* has been identified as a freshwater catfish pathogen leading to consequent massive economic burden to the freshwater catfish industry [8,9]. Editing bacterial genomes is an essential tool in research of infection molecular mechanisms and development of attenuated vaccine. However, there is no easy and rapid method reported to knock out genes in this bacterium.

Natural transformation is the ability of bacterium to take up foreign DNA and integrate the them into the genome via homologous recombination [11], this process is exploited to make mutant strains in various bacteria. Meibom first reported that chitin can induce natural competence in *Vibrio cholerae* under certain conditions [12], competent *V. cholerae* can take up foreign DNA by natural transformation. Subsequently, Melanie Blokesch used natural transformation as a tool to genetically manipulate *V. cholera* [13]. Recently, other members of the *Vibrionaceae* family have been shown to be naturally competent and secure it on gene knockout tool [11,14,15]. There are no studies reported that natural transformation existed in *V. mimicus*, in this study, we investigated the natural transformation of *V. mimicus* and demonstrated whether it was possible for natural transformation to be used as an effective method to knock out genes in *V. mimicus*.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture media

The strains and plasmids used in this study are listed in Table S1 in the supplemental material. The *V. mimicus* strains were cultured in liquid cultures in LB (Luria-Bertani) medium supplemented with 5% NaCl at 30 °C with agitation. Antibiotics were used at the following concentrations: 50 mg/mL kanamycin (Kan) and 25 mg/mL

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https://doi.org/10.1016/j.fsi.2019.06.025
Received 19 December 2018; Received in revised form 9 June 2019; Accepted 12 June 2019

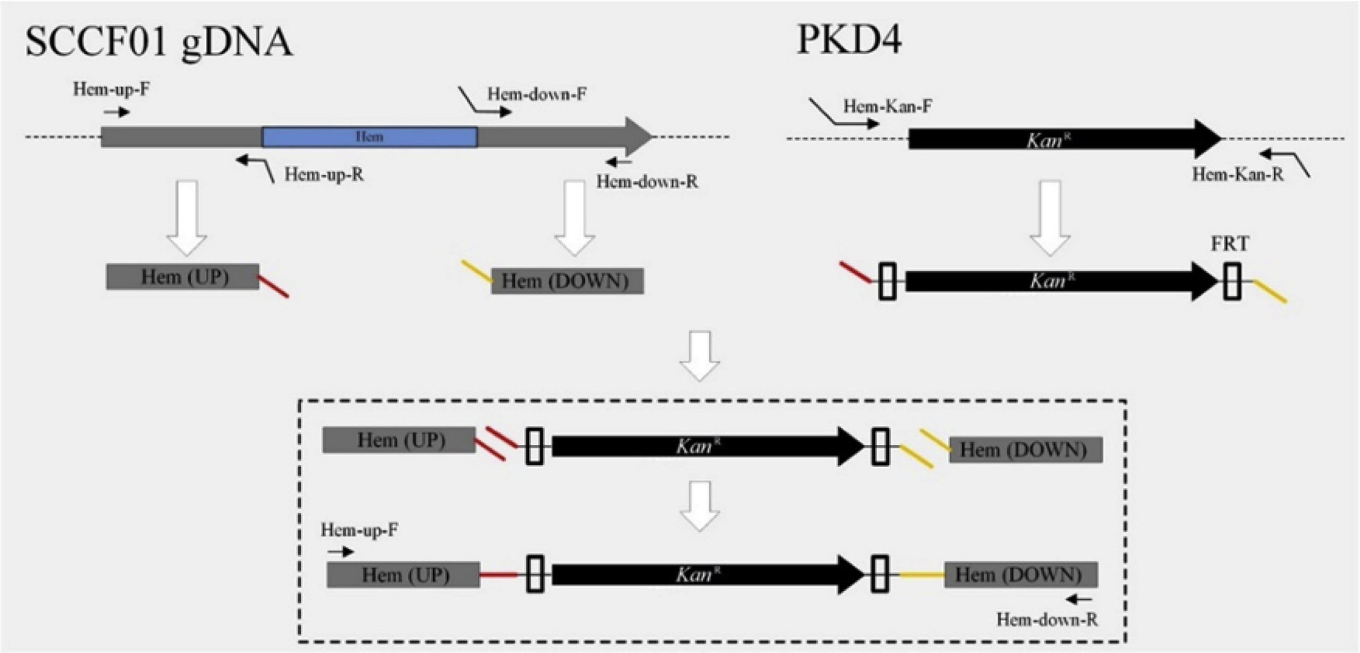

Fig. 1. Schematic diagram of SOE-PCR.

chloramphenicol (Cm).

2.2. Generation of mutagenic PCR fragments

DNA manipulation: Genomic DNA of *V. mimicus* strain SCCF01 was extracted using the Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). Plasmids (listed in Table S1) were purified from *Escherichia coli* DH5 using the Bacterial Plasmid Rapid Preps Kit. **Primers Design:** All the primers used in generation of PCR fragments were designed based on *V. mimicus* SCCF01 genome sequence (GenBank under accession numbers: CP016383 and CP016384) [16]. The information of target genes and primers are listed in Table S2 and Table S3 in the supplemental material. **Splicing-by-overlap extension PCR (SOE-PCR)** [13]: PCR fragments as transforming DNA were created by joining three independent PCR reactions corresponding to the regions flanking the sequence to be deleted and FRT-flanked antibiotic resistance cassette. Here, we used the hemolysin gene as an example to describe the process (Fig. 1). Approximately 2 kb upstream and downstream DNA of the hemolysin gene were amplified with Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech, Nanjing, China) using the primer pairs (Hem-up-F, Hem-up-R and Hem-down-F, Hem-down-R) with genomic DNA as template. Concomitantly, FRT-flanked kanamycin resistance cassette was amplified with Phanta Polymerase using the primer pairs (Hem-kan-F and Hem-kan-R) with PKD4 as template (if amplify chloramphenicol resistance cassette, PKD3 is served as template). Three PCR fragments (upstream DNA, downstream DNA and kanamycin cassette) were purified using DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China). All three fragments obtained in the first round were used as templates and mixed in equal ratios in a SOE-PCR reaction with the primers (Hem-up-F and Hem-down-R) to generate the mutagenic PCR fragments (ΔHem:Kan). The SOE-PCR product (Hemolysin genes upstream - kanamycin cassette - Hemolysin genes downstream) served as transforming DNA in the natural transformation experiment.

2.3. Natural transformation assay

Natural transformation was performed as described previously for *V. cholera* [17] with a little modification. The steps were as follows (Fig. 2): (1) the *V. mimicus* strain was grown in liquid LB medium with shaking at 180 rpm at 30 °C up to an optical density at 600 nm (OD600) of 0.4–0.5. Then, the bacteria were washed three times in 2 vol of M9 medium containing 5 mM $CaCl_2$ and 32 mM $MgSO_4$. (2) 1 ml of cell suspension to approximately 80 mg chitin flakes (weighted and autoclaved in standard Eppendorf tubes) and incubate for 24 h under static conditions at 30 °C. (3) Carefully exchanging the medium above the settled chitin flakes with fresh medium and adding ≥ 200 ng of the prepared PCR fragment. (4) Plating 50 µl of the cell suspension on antibiotic-containing LB-agar plates to select transformants.

2.4. Removal of antibiotic cassette

Firstly, transferring the PCP20 plasmid into transformants by electro-transformation [13]. Inoculating transformants contained the PCP20 plasmid LB with 1/100 volume of a fresh overnight culture at 30 °C. Note: the Flp recombinase from plasmid will express in transformants, the flp recombinase can mediate recombination at the FRT sites (gaagttcctatactttctagagaataggaacttc) of flanked antibiotic cassette [18], then antibiotic cassette was removed (Fig. S1). Secondly, growing the bacteria on LB agar plates at 42 °C for 2 h without antibiotics and then changing the temperature to 30 °C for overnight culture (Note: the bacterium will loss PCP20 plasmid at 42 °C as the PCP20 was a temperature-sensitive replication) [18]. Thirdly, re-streak the bacteria in parallel on antibiotic agar plates. If the bacteria were susceptible to the antibiotic, it indicated that antibiotic cassette was removed.

2.5. Detection of deletion mutants

To investigate whether deletion mutants were successfully constructed. Detection of deletion mutants was performed by PCR method. The primers used for detection of deletion mutants were shown in Table S4.

2.6. RT-PCR detection of upstream and downstream genes

To investigate whether the deletion influence the expression of upstream and downstream regions of target gene. Total RNA was extracted from deletion mutants and the synthesized cDNA was used as

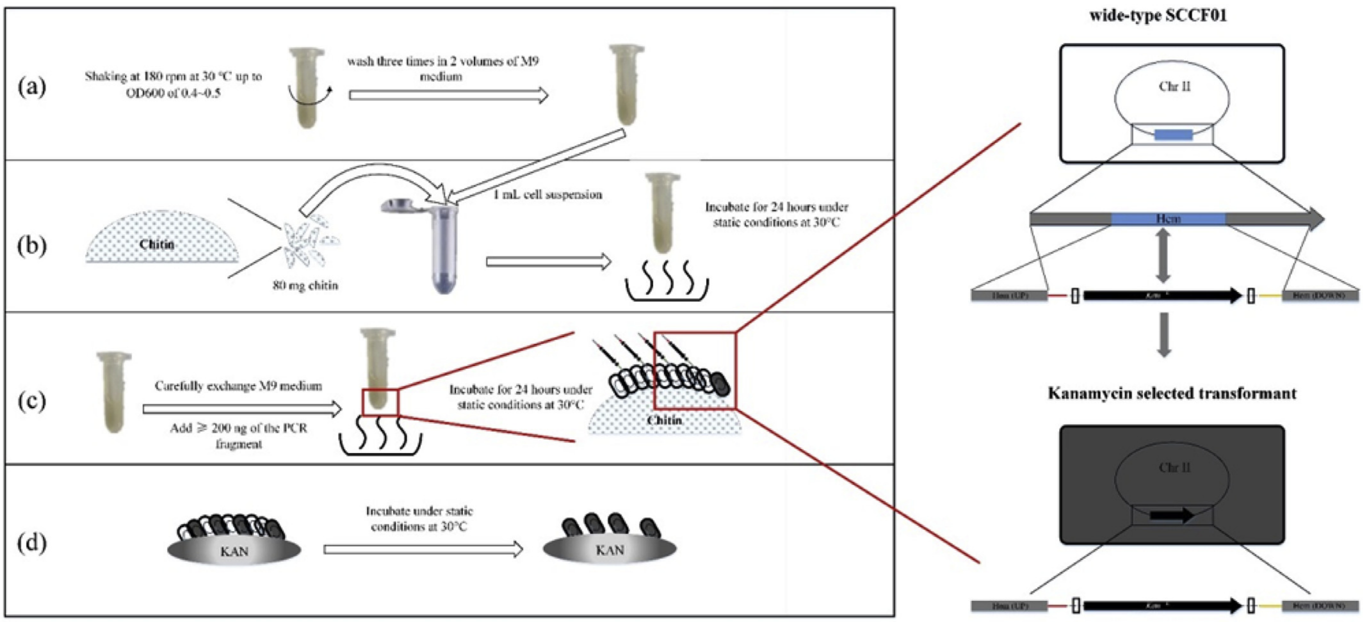

Fig. 2. Flow chart of natural transformation assay.

the PrimerScript RT reagent Kit. RT-PCR detection was performed with cDNA as template, and the primers used for RT-PCR detection were shown in Table S5.

2.7. Virulence determination in yellow catfish

Five mutants (△Hem, △TS1, △TS2, △TS1△TS2, and △II) were constructed successfully by natural transformation and Flp-recombination. The mutants as starter, multiple genes deletion mutants (△TS1△TS2△Hem and △TS1△TS2△II) were constructed using our previous work.

Subsequently, yellow catfish were used to evaluate the 50% lethal doses (LD^{50}) of the wild-type and mutants. Groups of 30 yellow catfish (14.58 ± 0.27 cm and 31.07 ± 2.04 g) were kept in 50 L tanks supplied with flow-through water. Immersion challenge was conducted by adding bacterial cultures directly to the tanks with 10 L water (5 × 10^4–10^7 CFU ml^{-1}). After 1 h of incubation, water flow was resumed. Mortalities were monitored for 4 weeks and the LD$_{50}$ values were calculated according to the method described by Karber's method.

3. Results

3.1. Generation of mutagenic PCR fragments

The three independent fragments (upstream homologous arm, resistance gene and downstream homologous arm) were amplified using high-fidelity DNA polymerase. All of amplification products were consistent with the anticipated sizes (Fig. 3, Lanes 1–3). Then three PCR fragments were fused by SOE-PCR and obtained the mutagenic PCR fragments (Fig. 3, Lanes 4).

3.2. Natural transformation of exogenous DNA

To qualitatively judge whether natural transformation occurs in *V. mimicus* strain SCCF01. Natural transformation assay was performed with fusion PCR fragments (Table S3 Hemolysin deletion) as exogenous DNA. The results showed that transformants can grow on the LB-Kan agar plates. In addition, transformants DNA were extracted as templates and PCR dection of antibiotic-resistant genes (Kan) and target genes (Hem) were performed. The results showed that kanamycin gene can detected in transformants, while hemolysin gene was not detected in transformants (Data not shown), which indicated that the fusion PCR fragments were successfully transferred into *V. mimicus* strain SCCF01 via natural transformation method and integrated into the genome by homologous recombination.

3.3. Application of natural transformation

Through an initial natural transformation assay, we discovered that natural transformation can be used to transfer exogenous DNA into *V. mimicus*. To further search natural transformation can be used for targeted gene deletions in different situations. We tested four types of genes/gene cluster disruption constructs (△Hemolysin genes:Kan^R, △TonB1 system genes:Kan^R, △TonB2 system genes:Cm^R and △II secretory system genes:Cm^R; abbreviate as △Hem:Kan^R, △TonB1:Kan^R, △TonB2:Cm^R and △II:Cm^R, respectively) by natural transformation. Then, the PCP20 plasmid was transferred into transformants to remove the antibiotic-resistance genes, resulting in deleted mutants without antibiotic resistance (△Hem, △TonB1, △TonB2 and △II). Finally, we used PCR and RT-PCR to identify all mutants.

3.4. PCR detection of antibiotic-resistance genes

The results of Fig. 4 showed that antibiotic-resistance genes (kanamycin gene/chloramphenicol gene) can be detected in all of transformants (△Hem:Kan^R, △TonB1:Kan^R, △TonB2: Cm^R and △II: Cm^R), and indicated that antibiotics resistance gene can be transferred into *V. mimicus* strain SCCF01 and integrated into the genome by natural transformation and homologous recombination. In addition, all of the final mutants (△Hem, △TonB1, △TonB2 and △II) with removing antibiotic-resistance genes were detected no antibiotic-resistance genes, which indicated that antibiotic-resistance genes in transformants have been removed using PCP20 plasmid.

3.5. PCR detection of target genes

The results of Fig. 5 showed that target genes can be detected in wild-type strain SCCF01 (wt-SCCF01), while all transformants (△Hem:Kan^R, △TonB1:Kan^R, △TonB2:Cm^R and △II:Cm^R) were detected no corresponding target genes. This result indicated that the

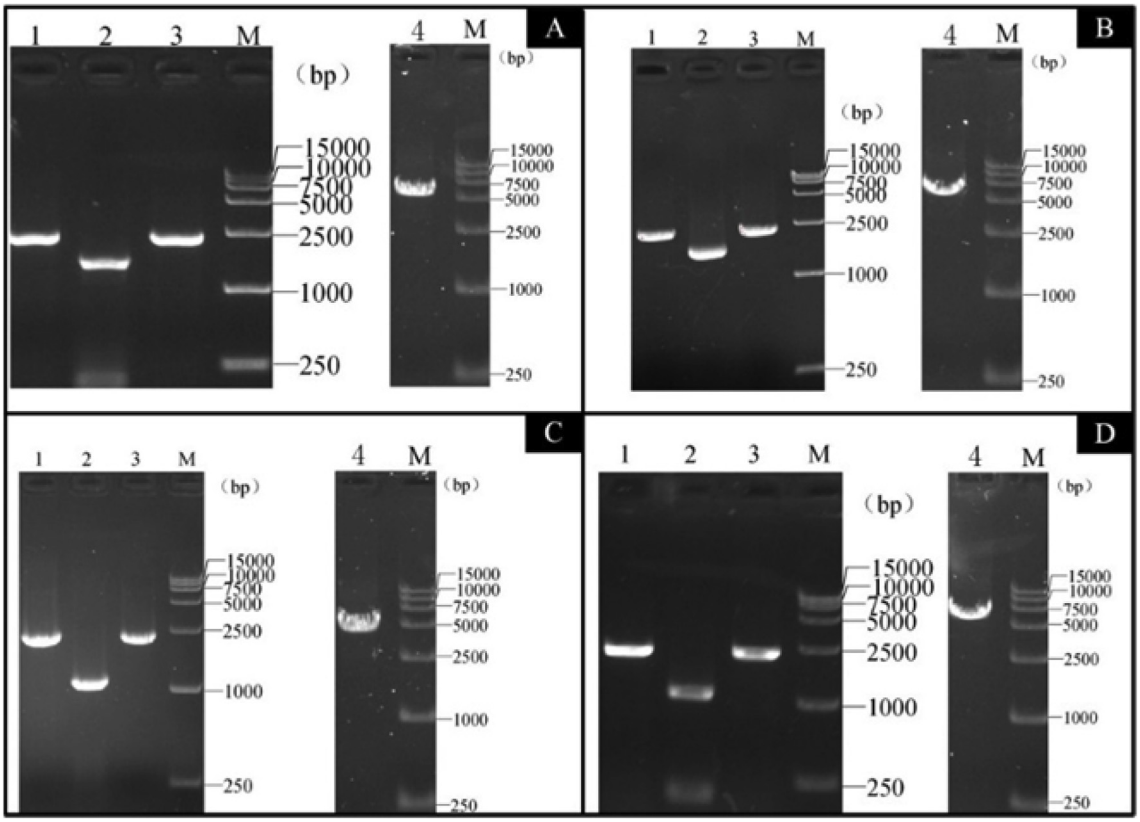

Fig. 3. Generation of mutagenic PCR fragments. A: Mutagenic PCR fragments of deleting hemolysin gene (fusion product: Hem-up-Kan-down), B: Mutagenic PCR fragments of deleting TonB1 system (fusion product: TS1-up-Kan-down); C: Mutagenic PCR fragments of deleting TonB2 system (fusion product: TS2-up-Cm-down); D: Mutagenic PCR fragments of deleting type II secretory system (fusion product: II-up-Cm-down). Lanes 1–3: upstream homologous arm, resistance gene, downstream homologous arm; lane 4: Fusion PCR product contained upstream homologous arm, resistance gene and downstream homologous arm.

antibiotic-resistance genes have replaced the antibiotic-resistance genes by homologous recombination after mutagenic PCR fragments transferred into *V. mimicus* strain SCCF01. Moreover, all of the final mutants (△Hem, △TonB1, △TonB2 and △II) were detected no corresponding target genes, indicating that target genes have been successfully deleted by homologous recombination and natural transformatio, and remove of antibiotic-resistance genes would not influence stably inherited of deletion.

3.6. PCR detection of PCP20 plasmid

To remove the antibiotic-resistance genes, we need transform the PCP20 plasmid into transformants (△Hem:Kan^R, △TonB1:Kan^R, △TonB2:Cm^R and △II:Cm^R), and obtained the final mutants (△Hem, △TonB1, △TonB2 and △II). However, PCP20 plasmid which exist in mutants would influence bacterial metabolism, consequently disturbed gene function analysis. Therefore, we raised culture temperature to 42 °C to remove the PCP20 plasmid. To investigate whether there is residual PCP20 exist in the final mutants, we performed PCR detection of residual PCP20 plasmid for all of the final mutants (△Hem, △TonB1, △TonB2 and △II). The results showed that only positive control can amplify a clear single target band (Fig. 6), indicating that the PCP20 plasmid in mutants had been completely remove with no residue.

3.7. RT-PCR (Reverse Transcription-PCR) detection of the transcription of upstream and downstream genes of target genes

If deletion of target genes would cause transcription termination of the upstream and downstream genes, it further influences functional verification of deletion genes. Therefore, we performed RT-PCR detection of the transcription of the upstream and downstream genes of target genes. The results showed that the upstream and downstream genes of target genes were detected in all of the transformants and the final mutants by RT-PCR. The results of RT-PCR also indicated that deletion of target genes by this method would not influence normal transcription of the upstream and downstream genes (Fig. 7).

3.8. Virulence determination in yellow catfish

As shown in Table 1, all of the mutants were lower mortality compared to the Wt-SCCF01 group. The mutants except for △Hem exhibited significantly attenuated virulence levels. In particular, the LD_{50} levels of multiple genes deletion mutants (△TS1△TS2△Hem and △TS1△TS2△II) were at least 300 times higher than that of the wild type, which suggested that the *V. mimicus* virulence were significantly decreased by the detection of Hemolysin, TonB1, TonB2 and Type II secretory system, and these mutant hold the potential used as the attenuated vaccines.

4. Discussion

V. mimicus is one of the most common bacterial pathogen in aquaculture, and consuming aquatic products contaminated with *V. mimicus* can cause food poisoning and gastrointestinal diseases. *V. mimicus* not only impedes healthy and sustainable development of aquaculture, but also threatens the human food safety. Gene knockout technology is an essential tool in research, multiplex genome editing by natural transformation (MuGENT) has been successfully used in various members of the *Vibrionaceae* family and other species [19,20]. To investigate the ability of *V. mimicus* to take up exogenous DNA, we performed natural transformation assay based on reference of the previous protocol

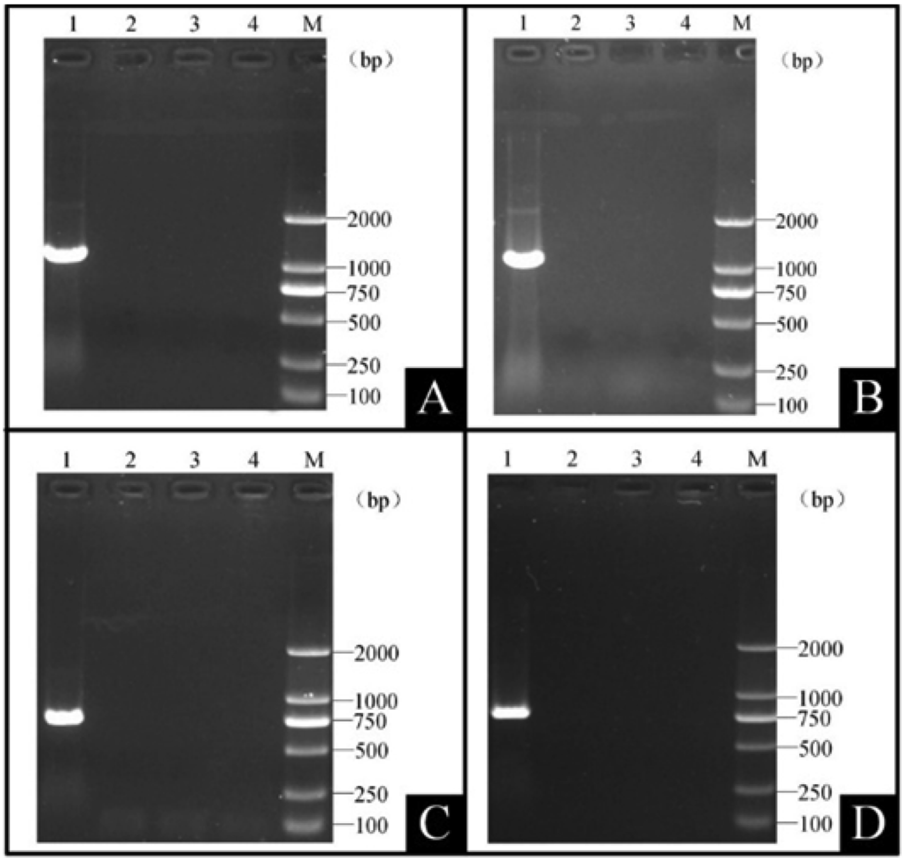

Fig. 4. PCR detection of antibiotic-resistance genes. A: Haemolysin; lane 1: △Hem:Kan^R, lane 2: △Hem, lane 3: wt-SCCF01, lane 4: negative control; B: TonB1 system; lane 1: △TS1:Kan^R, lane 2: △TS1, lane 3: wt-SCCF01, lane 4: negative control; C: TonB2 system, lane 1: △TS2:Cm^R, lane 2: △TS2, lane 3: wt-SCCF01, 4: negative control. D: Type II secretory system. Lane 1: △II:Cm^R, lane 2: △II; lane 3: wt-SCCF01, 4: negative control. M: DNA Marker 2000.

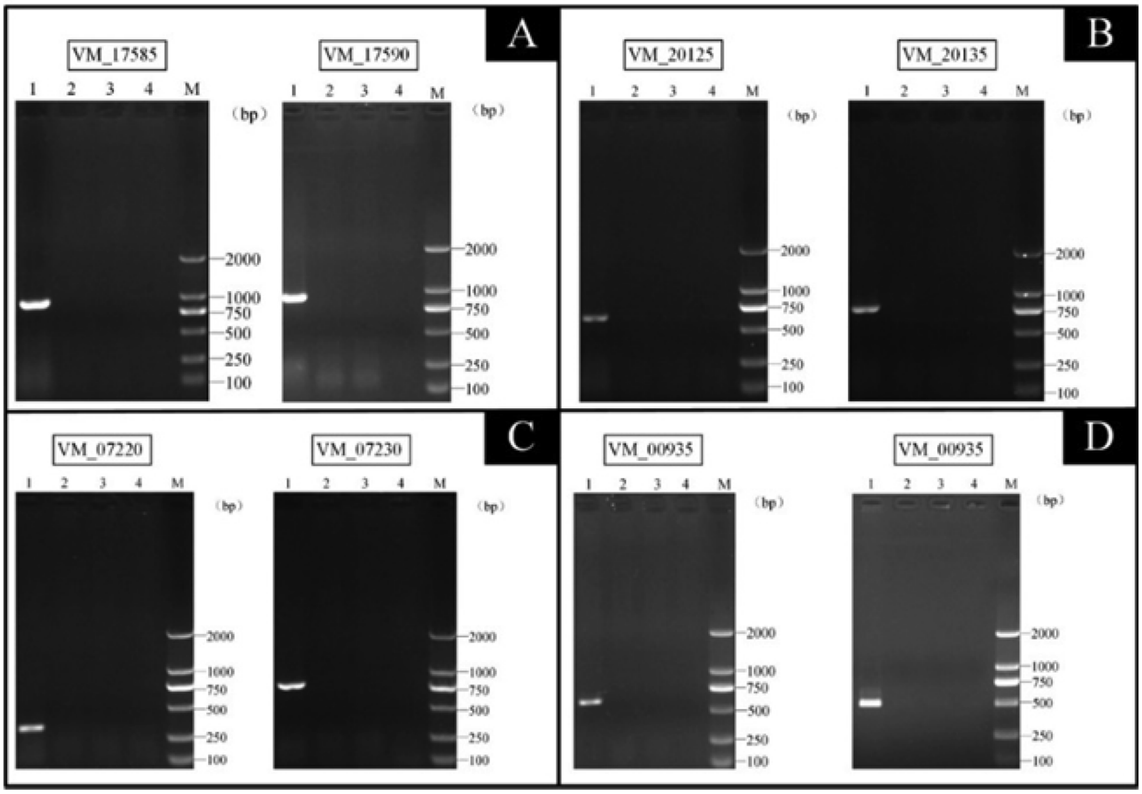

Fig. 5. PCR detection of target genes. A: Haemolysin; lane 1: wt-SCCF01, lane 2: △Hem:Kan^R, lane 3: △Hem, lane 4: negative control; B: TonB1 system; lane 1: wt-SCCF01, lane 2: △TS1:Kan^R, lane 3: △TS1, lane 4: negative control; C: TonB2 system, lane 1: wt-SCCF01, lane 2: △TS2:Cm^R, lane 3: △TS2, lane 4: negative control; D: Type II secretory system; lane 1: wt-SCCF01, lane 2: △II:Cm^R, lane 3: △II, lane 4: negative control. M: DNA Marker 2000.

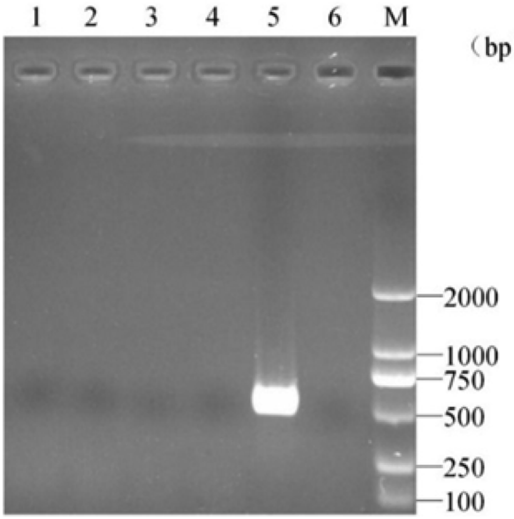

Fig. 6. PCR detection of PCP20 plasmid. Lanes 1–4: (△Hem, △TonB1, △TonB2 and △II; lane 5: PCP20 plasmid (positive control); lane 6: negative control.

[13,17]. The results showed that the *V. mimicus* strain SCCF01 can become competent by chitin-mediated induction, and take up exogenous kanamycin resistance cassette by natural transformation. Thus, we exploited the natural transformation phenomenon to create deletions of hemolysin genes, TonB1 system genes, TonB2 system genes and type II secretory system gene clusters. Then, to generate deletion mutants without antibiotic resistance marker, we used PCP20 plasmid to remove the antibiotic resistance marker [18]. Finally, all of the mutants were identified by PCR and RT-PCR. We found that all of the mutants were in accordance with our expection, suggesting that natural transformation can be successfully used in *V. mimicus* strain SCCF01 to delete target genes.

Target mutagenesis in *V. mimicus* was previously produced by suicide vector [21], which required SOE-PCR, cloning, enzymes restriction, adapter ligation and conjugation steps. Here, the targeting strategy based on natural transformation has the great advantages over the suicide vector mediated gene recombination. The method in this research was more simple and rapid than the traditional method. In addition, we successfully used natural transformation to knock out hemolysin genes, TonB1 system genes, TonB2 system genes and type II secretory system gene clusters. The total length of target deletion genes were 4204 bp (2 genes), 1761 bp (3 genes), 1563 bp (3 genes) and 11262 bp (12 genes). Therefore, this method has the advantage to delete multiplex genes and long genes (a study reported that the longest length of deleted genes is around 40 kb) [13]. This advantage contributes to a better understanding for the clusters of virulence factors, especially pathogenicity islands, secretion systems, pili and flagellar system.

In addition, this method is very advantageous to construct attenuated vaccine. Compared with non-replicating vaccines (DNA vaccines, inactivated vaccines and subunit vaccines etc), live attenuated vaccines generally are efficacious without the need for an adjuvant and can be administered orally or by immersion [22]. However, there are significant concerns over the safety of this vaccine because there is the risk of reversion to virulence (vaccine strain cause disease in the vaccinated animal) [22]. Fortunately, the safety of the attenuated vaccines can be increased by introducing more than one attenuating phenotype [23]. In this study, natural transformation was used to establish an easy multiple genes knockout method in *V. mimicus*. The type II secretory system gene clusters (12 genes, 11262 bp) of *V. mimicus* can be completely removed by natural transformation and it is conducive to eliminate the risk of reversion to virulence arising from recombination events. Besides, the detection of mutant virulence to yellow catfish suggested that *V. mimicus* virulence were significantly decreased using natural transformation and these mutant hold the potential used as the attenuated vaccines. Further research will be conducted to study the interaction of bacterial virulence and fish immunology responses.

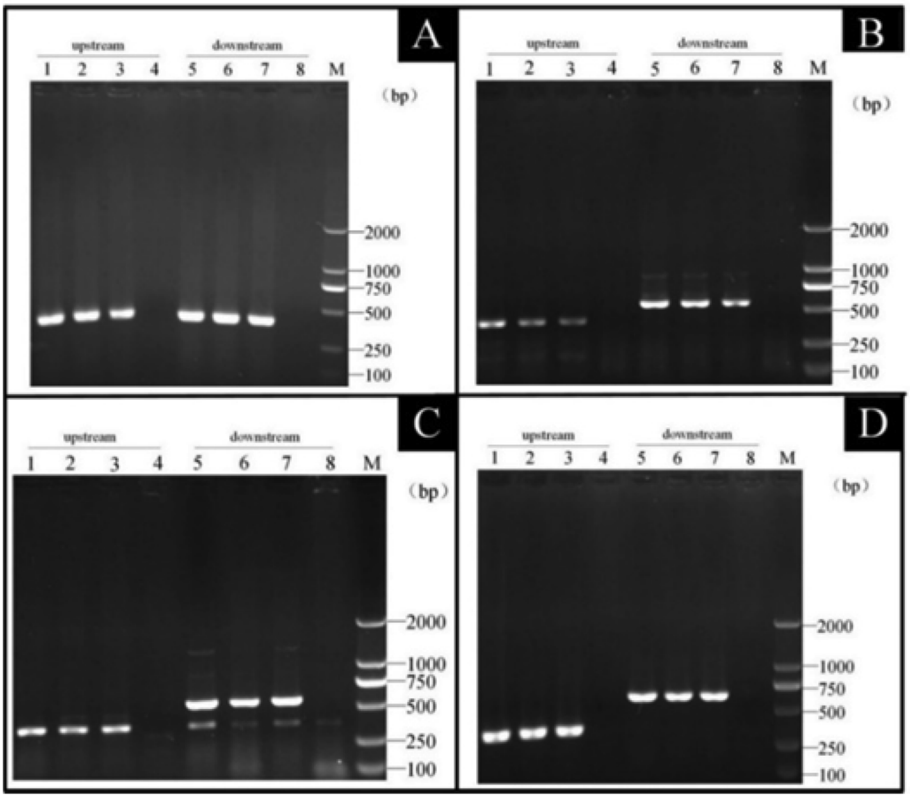

Fig. 7. RT-PCR detection of the transcription of the upstream and downstream genes of deletion target. A: Haemolysin; lanes 1 and 5: wt-SCCF01, lanes 2 and 6: △Hem:Kan^R, lanes 3 and 7: △Hem, lanes 4 and 8: negative control; B: TonB1 system; 1 and 5: wt-SCCF01, lanes 2 and 6: △TS1:Kan^R, lanes 3 and 7: △TS1, lanes 4 and 8: negative control; C: TonB2 system, 1 and 5: wt-SCCF01, lanes 2 and 6: △TS2:Cm^R, lanes 3 and 7: △TS2, lanes 4 and 8: negative control; D: Type II secretory system; 1 and 5: wt-SCCF01, lanes 2 and 6: △II:Cm^R, lanes 3 and 7: △II, lanes 4 and 8: negative control. M: DNA Marker 2000.

Table 1
LD_{50} values and attenuated-fold results for the mutants in yellow catfish.

	Cumulative mortality				LD_{50}	Attenuated-fold
	5.00×10^7	5.00×10^6	5.00×10^5	5.00×10^4		
Wt-SCCF01	100%	100%	75%	50%	6.67×10^4	1.00
△Hem	100%	60%	60%	35%	3.64×10^5	5.46
△TS1	70%	65%	30%	30%	1.77×10^6	26.61
△TS2	65%	50%	15%	20%	5.45×10^6	81.75
△TS1△TS2	50%	50%	10%	20%	9.42×10^6	141.25
△II	45%	25%	20%	10%	2.05×10^7	307.26
△TS1△TS2△Hem	40%	25%	20%	10%	2.37×10^7	354.81
△TS1△TS2△II	30%	10%	20%	10%	4.46×10^7	668.34

In summary, we successfully used natural transformation in *V. mimicus* strain SCCF01 to knock out genes. It can provide a new method for the construction of targeted mutants in this bacterium. And the method will facilitate the research of the pathogenic mechanism of *V. mimicus* and contribute to the development of attenuated vaccines.

Acknowledgments

This work was supported by Sichuan Innovation Team Project of Agricultural Industry Techology System (No.2017SICAD002), Sichuan Key Research and Development Project (2018NZ0007).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.025.

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