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Deletion of *Salmonella enterica* serovar typhimurium *sipC* gene

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ABSTRACT

Objective: To construct a novel plasmid as *Salmonella enterica* serovar typhimurium (*S.* typhimurium) *sipC* gene knockouts candidate.
Methods: In this research, 5′ upstream and 3′ downstream regions of *S.* typhimurium *sipC* gene and kanamycin gene were PCR amplified. Each of these DNA fragment was cloned into pGEM T-easy vector. The construct was confirmed by PCR and restriction digest.
Results: PCR amplified 320, 206 and 835 bp DNA fragments were subcloned into pET-32 vector resulting with a plasmid called pET-32-*sipC* up-*kan- sip C* down.
Conclusions: The new plasmid (pET-32-*sipC* up-*kan- sip C* down) is useful for genetic engineering and for future manipulation of *S.* typhimurium *sipC* gene.

1. Introduction

Salmonella is a member of the Enterobacteriaceae family, a large group of Gram-negative, non-spore-forming bacilli and facultative anaerobes [1]. *Salmonella enterica* (*S. enterica*) causes nearly 99% of *Salmonella* infections in animals and humans [2]. The *S. enterica* includes about 2600 diverse serotypes which consist of two species, *Salmonella bongori* and *S. enterica* and are divided into six subspecies: *salamae*, *enterica*, *diarizonae*, *arizonae*, *indica* and *houtenae* [1]. *S. enterica* serovar typhimurium (*S.* typhimurium) is a widely distributed food-borne pathogen and one of the most popular causes of bacterial food-borne disease in humans and animals and deaths globally [3,4]. Salmonellosis is considered one of the most significant food-borne zoonoses that is viewed in two kinds of different diseases, enteric fever which can be typhoid or paratyphoid and gastroenteritis which is non-typhoidal [5,6]. Bacterial pathogen in human causes 21 million cases of typhoid fever and 200000 deaths per year, mainly in Southern Asia, Africa and South America [7]. The discovery of a new vaccine for salmonellosis is a challenge for the scientists. Several vaccines have undergone clinical tests. The need of an efficacious vaccine against salmonellosis providing strong humoral as well as cellular immunity still persists [8]. *S. enterica* serovars have been classified based on reactivity of antigen to somatic lipopolysaccharide, flagellar and capsular antigens. From a clinical aspect, these may be broadly grouped on the basis of host range and disease representation [9]. The *Salmonella* pathogenicity islands (SPIs) 1 and 2 are two important virulence determinants of *S. enterica*. They encode type III secretion systems (T3SS), which carry the effector proteins and enable the injection of effector proteins directly into the cytosol of eukaryotic cells. These effectors finally manipulate the cellular functions of the infected host and comfort the development of the infection [10]. At present, 21 SPIs have been recognized in *Salmonella*. Many of the identified SPI-encoded genes have only putative functions with no obvious role in *Salmonella* pathogenesis.

The SPI-1 locus is a 40 kb chromosomal island, which carries among all the others genes needed for the biosynthesis of a functional T3SS, a number of some effector and regulatory proteins and their chaperones [1]. The T3SS allows the secreted proteins to pass through the bacterial outer and inner membranes and a translocon creates a pore in the host cell membrane [11]. Location and function of these proteins in this system are shown below. Inner membrane proteins: InvA, SpaP, Q, R, S;

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associated inner membrane proteins: InvA, E; outer membrane proteins: InvG, PrgK, PrgH; chaperone: SicA; putative chaperone: InvI; secreted proteins involved in secretion: InvJ, SpaO; secreted proteins with an effector function (target proteins): SipA, SipB, *Salmonella* invasion protein C (SipC), SipD, SptP [12].

Invasion is initiated by pathogen binding to the host cell surface [13]. After invasion and entering the lumen of the small intestine, environmental conditions enable T3SS-1 genes to be expressed and subsequently the secretion system to be assembled at the bacterial membrane [14]. Cell entry is determined by rearrangements of the actin cytoskeleton, which is partly interceded by SipC, a component of the bacterial translocon, at the place of bacteria–host-cell contact [1]. SipC includes two membrane-spanning areas with C- and N-terminal domains front of the host-cell cytoplasm. This topological assembly of this effector protein is a reason to the actin nucleation and the translocation processes. Remarkably, both of these processes are primarily dependent on the C-terminus of SipC. SipC can localize actin polymerization at bacterial attachment site and employ actin directly [15]. Kanamycin is inactivated with the aminophosphotransferases by transferring the γ-phosphate to the OH group in the 3′ position of the pseudosaccharide. The *kan* gene was cloned and transformed in *Escherichia coli* (*E. coli*) cells [16].

The purpose of this study was to generate a plasmid that carries kanamycin resistance gene replacement of *S.* typhimurium native *sipC* gene with Up-Kan-Down fragment, using homologous recombination technique.

2. Materials and methods

2.1. Bacterial complex, plasmids construction and media

In this study, the protocol and informed consent forms were approved by the Islamic Azad University, Shahrekord Branch, Shahrekord, Iran with 17621105 grant number. Virulent *S.* Typhimurium (ATCC-13311) collected from Microbiology Laboratory of Islamic Azad University of Shahrekord Branch was preserved as frozen glycerol stocks and cultured into Luria-Bertani (LB) broth until the log growth phase (optical density 600 = 0.9) was reached. The *kan* gene was isolated from the pET-28 vector. For cloning, preservation of DNA fragment and subcloning, pGEM T-easy vector using TA cloning kit (Promega, U.S) and pET-32 vector with *E. coli* strain TOP10F′ were used, respectively. Bacterial cultures were grown at 37 °C in LB broth and LB agar plates.

2.2. Extraction of genomic DNA from S. typhimurium

DNA was isolated from colonies of bacteria using DNA extraction kit (DNP™ Kit, CinnaGen, Iran) according to the manufacturer's protocol. The quality of DNA was checked on 1% agarose gel electrophoresis and quantified by spectrophotometric mensuration at 260 nm, according to Sambrook and Russell [17].

2.3. Amplification of flanking regions of sipC gene and kan gene

Oligonucleotide primers were designed for amplification of flanking regions of *sipC* gene of *S.* typhimurium. The sequences of these primers were up-*sipC*-F: 5′-ATGTCTAGA CCCTAAATAAAGTGGCG-3′ and up-*sipC*-R: 5′ ATTAG ATCTCTCCCTTTATTTGGCAG-3′ (accession number: CP008928.1) containing *Xba* I and *Bgl* II restriction sites and down-*sipC*-F: 5′-ATTGAGCTCTGACCACTGAAAGCCAC-3′ and down-*sipC*-R: 5′-ACACTCGAGTAATACCCAGACTT TCCG-3′ (accession number: CP007360.1) containing *Sac* I and *Xho* I restriction sites. Primers were used for amplification of up and down region of *sipC* gene, respectively. Moreover, *kan*-F: 5′-ATAAGATCTATGAGCCATATTCAGCGTG-3′ and *kan*-R: 5′-ATAGAGCTCTTAGAAAAATTCATCCAG-3′ primers containing *Bgl* II and *Sac* I restriction sites were designed for amplification of *kan* gene and pET-28 vector was used as template. Underlined sequences indicate restriction sites.

Three collections of PCR programs were performed singly in high volume for amplification of *kan* gene and up and down regions of *sipC* gene. PCR amplification programs were carried out in a total volumes of 25 μL in 0.2 μL tubes containing 1 μL of template DNA, 1 μmol/L of each primer, 2.5 μmol/L of 10× PCR buffer, 2 μmol/L $MgCl_2$, 200 μmol/L deoxy-ribonucleoside triphosphate and 1 unit of *Taq* DNA polymerase (Fermentas, Germany). For the optimal amplification of flanking regions of *sipC* gene and *kan* gene, an initial denaturation step was performed at 95 °C for 5 min, then amplified for 32 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 1 min. Lastly, a final extension phase was programmed for 5 min at 72 °C and amplified samples were held at 10 °C.

2.4. Analysis of PCR products

The amplified products (10 μL) were analysed by electrophoresis in 1% agarose gel in tetrabromoethane [tris-base 10.8 g 89 mmol/L, boric acid 5.5 g 2 mmol/L, ethylene diamine tetraacetic acid 4 mL of 0.5 mol/L ethylene diamine tetraacetic acid (pH 8.0) buffer]. Constant voltage of 80 V was used for products differentiation. The 100 bp DNA ladder (Fermentas, Germany) was used as a molecular weight marker. After electrophoresis, the gel was stained with ethidium bromide and images were taken in UVIdoc gel documentation systems (UK).

2.5. Cloning of sipC gene and plasmid construction

The PCR products were purified using gel extraction kit (Bioneer, Korea) according to manufacture's protocol. All PCR products were cloned into pGEM T-easy vector and the recombinant vectors were transformed by heat shock at 42 °C and calcium chloride method into *E. coli* TOP10F′ competent cells in LB culture media (Merck, Germany). Kanamycin resistance was used for the selection. The presence of flanking regions of *sipC* gene and *kan* gene was confirmed by restriction endonucleases analysis.

2.6. Subcloning of the sipC and kan genes

The up-*sipC* fragment was removed from the pGEM T-easy vector by *Xba* I-*Bgl* II double digestion and subcloned in *Xba* I-*Bgl* II linearized pET-32 to get pET-32-*sipC* up. Then, *kan* fragment was released from the pGEM T-easy vector by

Bgl II-*Sac* I double digestion and subcloned into *Bgl* II-*Sac* I linearized pET-32-*sipC* up producing pET-32-*sipC* up-*kan*. Finally, pGEM-*sipC* down double digested with *Sac* I-*Xho* I and down fragment of *sipC* was subcloned into *Sac* I-*Xho* I linearized pET-32-*sipC* up-*kan* to produce pET-32-*sipC* up-*kan*-*sipC* down recombinant vector. Competent cells of *E. coli* TOP10F′ strain were transformed with pET-32-*sipC* up-*kan*-*sipC* down recombinant vector. Flanking regions of *sipC* gene and *kan* gene containing restriction point of *Xba* I, *Bgl* II, *Sac* I and *Xho* I were inserted in polyclonal site in pET-32. *E. coli* TOP10F′ strain competent cells were used for transformation and cultured in LB agar media containing ampicillin. The final construct was confirmed by double digestion by *Xba* I-*Xho* I and by PCR using up-*sipC*-F and down-*sipC*-R primers.

3. Results

DNA was successfully extracted and PCR amplified products for flanking regions of *sipC* and *kan* genes on 1% agarose gel revealed 320 bp, 206 bp and 835 bp fragments, respectively (Figure 1).

In the next step, the up and down regions of *sipC* gene of *S.* typhimurium and *kan* gene were cloned with TA cloning technique in pGEM T-easy vector successfully, separately (Figure 2).

Plasmid purification and *Xba* I, *Bgl* II, *Sac* I and *Xho* I restriction endonuclease digestion of pET-32-*sipC* up-*kan*-*sipC* down recombinant plasmid confirmed the correction of up and down regions of *sipC* gene of *S.* typhimurium and *kan* gene cloning (Figure 3). A 7261 bp large fragment was related to pET-32 vector (5900 bp) and 320, 206 and 835 fragments were up and down regions of *sipC* gene and *kan* gene bands, respectively (Figure 3).

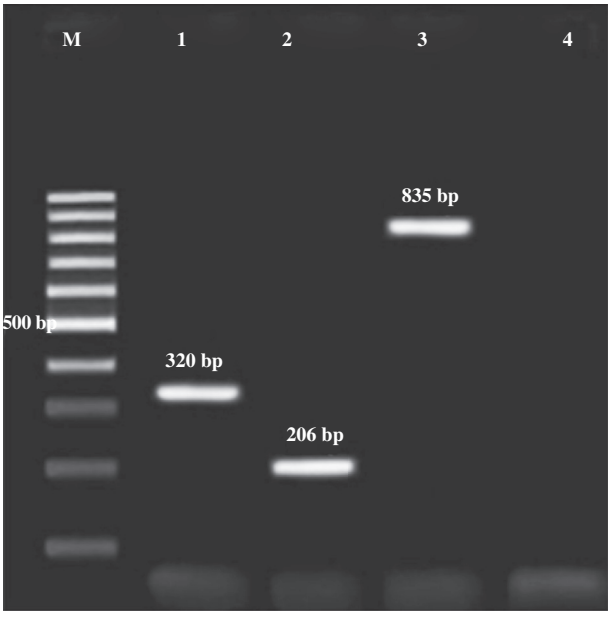

Figure 1. Agarose gel of PCR amplified products of flanking regions of *sipC* gene and *kan* gene.
Line M: 100 bp DNA marker (Fermentas, Germany); Lines 1 and 2: Up and down regions of *sipC* gene, respectively; Line 3: *kan* gene; Line 4: PCR negative control sample without bacterial DNA.

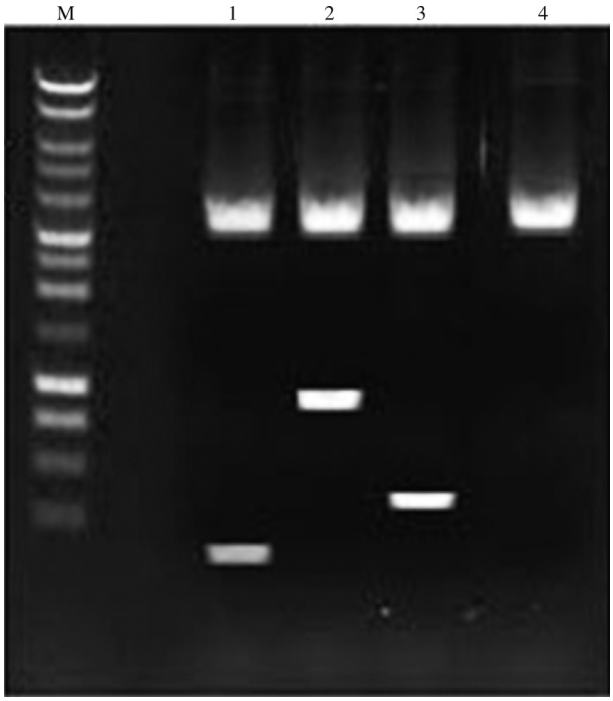

Figure 2. Digested pGEM recombinant plasmid.
Analysis of digested pGEM recombinant plasmid was performed using *Xba* I, *Bgl* II, *Sac* I and *Xho* I restriction endonuclease enzymes. Line M: 1 kb DNA ladder (Fermentas, Germany); Line 1: pGEM vector with down regions; Line 2: pGEM vector with *kan* gene; Lines 3: pGEM vector with up regions; Line 4: pGEM vector without inserted sequence.

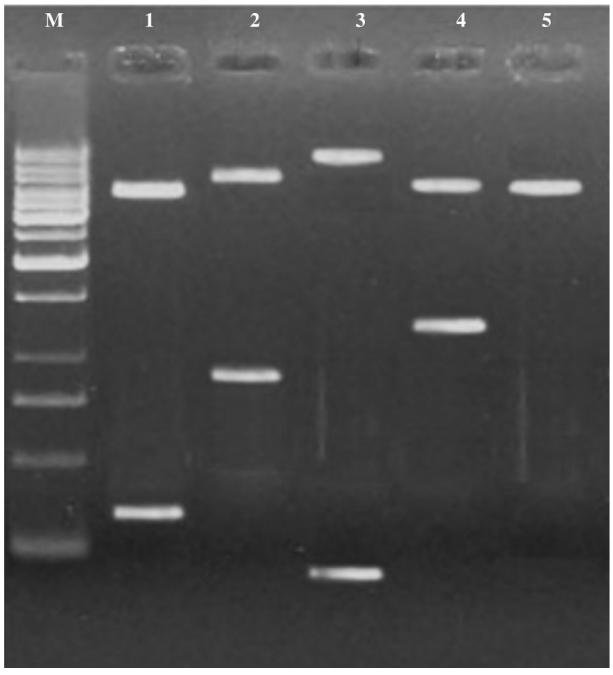

Figure 3. Digested pET-32 recombinant plasmid.
Analysis of digested pET-32-*sipC* up-*kan*-*sipC*-down recombinant plasmid was performed using *Xba* I, *Bgl* II, *Sac* I and *Xho* I restriction endonuclease enzymes. Line M: 1 kb DNA ladder (Fermentas, Germany); Lines 1, 2 and 3: Digested pET-32-*sipC* up-*kan*-*sipC*-down recombinant vector to fragments of up, *kan* and down regions respectively; Line 4: pET-32-*sipC* up-*kan*-*sipC*-down recombinant vector using *Xba* I and *Xho* I restriction enzymes to *sipC* up-*kan*-*sipC*-down recombinant fragment; Line 5: pET-32 vector without inserted sequence.

4. Discussion

S. enterica is a facultative intracellular pathogen of universal significance and Gram-negative enteric bacterium [18]. While some have a restricted host range, for example the serovars typhi and pullorum are restricted to humans and chickens and most of the *S. enterica* serovars can infect a broad range of cold and warm-blooded animals and humans. *S. enterica* infects its hosts by the oral route and causes two types of disease: a gastroenteritis determined by the extension of bacteria in the intestinal tract and typhoid fever that determined by the invasion of the systemic compartment [19,20]. SipC is a *Salmonella* actin-binding protein that nucleates actin filament formation [12]. This protein was found to enhance the entry of wild-type *S.* Typhimurium into cultured cells, by interacting with phospholipid membranes and oligomerizing in solution. Research to date on *sipC* genes and SipC proteins that have been obtained, further study of the molecular properties and performance are discussed. Nuclear actin-related activity displacement factor SipC was placed under study by Chang *et al.* who reported that the central region of the protein SipC for nuclear actin and the C-terminal amino acid region for transfer factors are required [21,22]. Gene cloning allows scientists to find exclusive genes, cut them out and insert them into the genome of another organism and is the act of making copies of a single gene. Genetic engineering is the process of cloning genes into new organisms to change the protein product. Bacterial plasmids used in gene cloning naturally contain antibiotic resistance genes [23]. He CH *et al.* [24] constructed a recombinant plasmid based on *AAV* gene. This gene carried human endothelial nitric oxide synthase. The study by He CH *et al.* showed that pSNAV-eNOS was successfully constructed with the ability to express human endothelial nitric oxide synthase mRNA in cultured animals and humans cells, which is similar to the results of our study, but the plasmid and restriction enzyme of that research differed from those of our study [24]. The study of cloning of the encoding enterohemorrhagic *E. coli* Shiga-like toxin subunits to pGEM T-easy vector showed that cloning of this gene was successful and their findings were similar to this study [25]. Another study of Peerayeh SN *et al.* [26] in Iran, constructed a recombinant vector based on *UreB122* gene that carried the urease of *Helicobacter pylori*. Prokaryote expression vector pET-32a was inserted with *UreB122* gene. The recombinant vector was used to transform the competent *E. coli* DH5α. This study showed that pET-32a-*UreB122* was successfully constructed and the expression of recombinant protein was induced by isopropythio-β-D-galctoside at different concentrations [26]. Haghi *et al.* generated a gene construct based on *porA* gene [27]. This protein (PorA) is a major member of the outer membrane of *Neisseria meningitidis* and functions as a cationic protein. *porA* was cloned into prokaryotic expression vector pET-32a and recombinant vector was transformed into competent Origami B (DE3) cells. Haghi *et al.* study showed that pET-32a-*porA* was successfully constructed and recombinant protein was overexpressed, which is similar to our study [27]. A gene construct to *vacB* gene deletion of *Brucella melitensis* (*B. melitensis*) *vacB* gene that was generated by Iranian researchers in 2012 is an important gene of *B. melitensis* that encoded a RNase R. This construct carries a kanamycin resistance gene replacement in downstream and upstream region of *vacB* gene of *B. melitensis*. Results showed that cloning of this gene was successful and their findings are similar to our research [16]. In another study in China, a recombinant vector for deletion of *yncD* gene in *S. enterica* was constructed. The *yncD* gene encodes a putative TonB-dependent transporters and was identified recently as an *in vivo* induced antigen [28]. In this study, *yncD* deletion mutant was successfully constructed in pYG4 vector, which is similar to the results of the present research [28]. The recombinant bacteria have become a useful instrument in various aspects. In this research, we have constructed the new recombinant plasmid carrying a kanamycin resistance gene replacement in up and down regions of *sipC* gene of *S.* typhimurium by improving the plasmid of *E. coli*.

In conclusion our results showed that *sipC* gene was cloned and subcloned in *E. coli* successfully. The recombinant gene construct has became a useful tool in various aspects on basic knowledge and applied science. In this research, we have constructed a novel recombinant gene construct that carries a kanamycin resistance gene replacement of *S.* typhimurium native *sipC* gene with *sipC* up-*kan*-*sipC* down fragment using homologous recombination technique. The new recombinant construct (pET-32-*sipC* up-*kan*-*sipC* dwon) in this study can be useful for genetics engineering. According to the results of the present study, gene construct that was produced can be used for *sipC* gene deletion and then manipulated strain can be used for engineering attenuated vaccine against *S.* typhimurium in future researches.

Conflict of interest statement

We declare that we have no conflict of interest.

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