Short Paper

Gene disruption in *Salmonella typhimurim* by modified λ Red disruption system

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Summary

There are many techniques to knock out directed genes in bacteria, some of which have been described in *Salmonella* species. In this study, a combination of SOEing PCR method and the λ Red disruption system were used to disrupt *phoP* gene in wild type and standard strains of *Salmonella typhimurium*. Three standards PCR and one fusion PCR reactions were performed to construct a linear DNA including upstream and downstream of *phoP* gene and Kanamycin cassette. As a template plasmid, we used pKD4 which carries kanamycin gene flanked by FRT (FLP recognition target) sites. The resulting construct was electroporated into prepared competent cells of *S. typhimurium*. The transformants colonies related to the standard strain appeared on the LB-Km-agar plates after incubation, but there was no colony on LB-Km-agar plates corresponding to the wild type strain. The failure in transformation of the wild type strain may be because of inflexibility of the λ Red disruption system in this strain or its unique restriction-modification system. However, by this construct we are able to generate *phoP* mutant in many of the *Salmonella* species due to high homology of the *phoP* gene which exists in different species.

Key words: Gene disruption, Kanamycin cassette, λ Red disruption system, *Salmonella typhimurium*, SOEing PCR method

Introduction

Knocking out directed genes has been used by researchers for many purposes such as demonstration of gene function, vaccine production and advance understanding of structure or expression of proteins (Braman, 2010). There are different methods to disrupt genes in bacteria, from which some have been described in *Salmonella* spp. including use of the R6K-suicide plasmid, the λ Red disruption system, the suicide plasmid combined with the Red system or the plasmid temperature-sensitive with *SacB* gene (pKO3) (Geng *et al*., 2011).

In this study, SOEing PCR method combined with the λ Red disruption system was used to delete *phoP* gene in a wild type *Salmonella typhimurium*. The *phoP* gene is a transcriptional regulator and a member of two-component regulatory system (PhoPPhoQ) (Rychlik and Barrow, 1991) that plays a key role in adaptation of the organism to intracellular environments and survival within macrophages (Miller, 1991). It also controls the expression of more than 40 genes required for virulence of the *S. typhimurium*, and its resistance to adverse environments encountered in host such as low pH of stomach, bile salts, low oxygen in the small intestine and cationic antimicrobial peptides on epithelial cells (Gahan and Hill, 1999; Navarre *et al*., 2005). Thus disruption of *phoP* gene in *S. typhimurium* leads to its inability to survive within macrophages and increased susceptibility to stress factors in the host (Vescovi *et al*., 1994).

Materials and Methods

Bacterial strains and plasmids

The bacterial strains used in this study were *S. typhimurium* 14028 as a positive control and a wild type strain of *S. typhimurium* isolated from a Caspian horse. The plasmids were pKD46, a Red helper plasmid containing a temperature sensitive origin of replication and ampicillin resistant marker which expresses recombinase enzyme in the presence of L-arabinose and pKD4, a template plasmid which carries kanamycin gene flanked by FRT (FLP recognition target) sites (Datsenko and Wanner, 2000).

Media and chemicals

In this study, various media including Luria-Bertani (LB) agar and broth, SOC and SOB were employed. If required, they were supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml) and 1 mM L-arabinose. In all PCR assays, two restriction enzymes: Taq DNA polymerase enzyme and Pfu DNA polymerase were accordingly used in the conventional PCR assay and/or for cloning and mutagenesis.

PCR reaction

Using PCR, the *phoP* gene of the wild type and standard strains were amplified with designed primers based on known *phoP* sequences data for *S. typhimurium*. The pair of primers used for PCR amplification successfully primed the synthesis of anticipated DNA fragments of *phoP* with 631 bp length (Fig. 1).

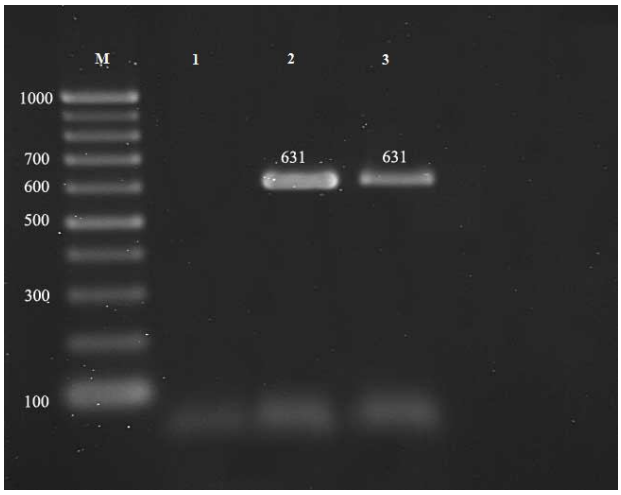

Fig. 1: Amplification of *phoP* gene. Lane M: 100 bp DNA ladder as a size standard, Lane 1: Negative control (no DNA), Lane 2: *phoP* gene of the *Salmonella typhimurium* 14028, and Lane 3: *phoP* gene of the wild type strain

Electroporation of pKD46 into *S. typhimurium*

We prepared electro-competent cells from the bacteria according to the Maloy protocol. Then, we electroporated pKD46 plasmid into the competent cells using a Micropulser (Bio Rad). After electroporation, 1 ml SOC was immediately added to the shocked bacteria and incubated at 30°C for 1 h, and shaken at 200 rpm. Then one-tenth portion of the mixture was plated on LB agar supplemented with ampicillin to select ampicillin resistant transformants.

Amplification of fragments for SOEing PCR method

Using PCR, upstream and downstream segments of *phoP* gene and Kanamycin cassette were amplified by phoP-F (1) and phoP-R (2), phoP-F (5) and phoP-R (6), Kan-F (3) and Kan-R (4) primers in respect. The primers phoP-R (2) and phop-F (5) were designed so that they have a 5 tail complementary to the primers kan-F (3) and Kan-F (4) in order. The primers used in this study are summarized in Table 1. To amplify the Kanamycin cassette, the pKD4 plasmid was used as a template. A 75 µL reaction mixture was prepared including 7.5 µL of 10 X Pfu buffer, 3 µL of 25 mM $MgSO_4$, 1.8 µL of 10 Mm dNTP Mix, 1.8 µL of each primers (10 pmol), 4.2 µL of Pfu DNA polymerase (5 U/µL) and 3 µL of DNA template. The PCR amplification was performed with 35 cycles of denaturation at 94°C for 35 s, annealing at 50°C for 35 s, and extension at 72°C for 45 s. The initial denaturation and final extension were 94°C for 5 min and 72°C for 5 min, respectively. The PCR amplification was performed successfully and anticipated fragments with 694 bp, 1530 bp and 871 bp lengths were observed on 1% agarose gel (Fig. 2). Then, the PCR products were purified from the agarose gel using the Gel extraction kit and suspended in double-distilled water (DDW).

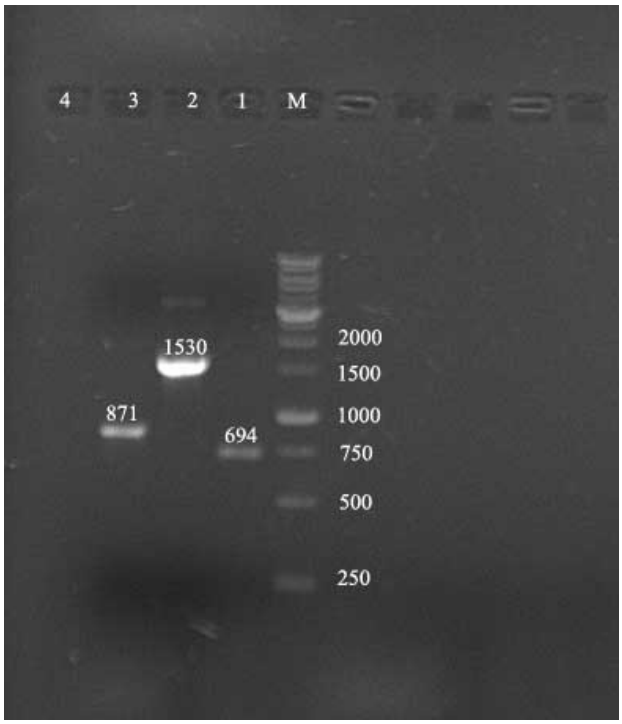

Fig. 2: Amplification of fragments for SOEing PCR method. Lane M: 1 Kb DNA ladder as a size standard. Lane 1: Upstream segment of the *phoP* gene, Lane 2: Kanamycin cassette, and Lane 3: Downstream segment of the *phoP* gene

Table 1: Primers used in this study

Primer designation	Sequence (5→3)	Template
phoP-F	GCGTACTGGTTGTAGAGGATAATG	Genomic DNA
phoP-R	GTGGTAATGACATCGTGCGGAT	-
Kan-F (3)	TCTTGAGCGATTGTGTAGGCTGGAGCTGCTTCGAAG	pKD4
Kan-R (4)	GTGACACAGGAACACTTAACGGCTGACATGGGAATTAG	-
phoP-F (1)	AGAAGGCAACCTCGGTCTGTCTAACGCAGTGTTG	Genomic DNA
phoP-R (2)	**CTTCGAAGCAGCTCCAGCCTACACAATCGCTCAAGA**TTCATTAAGGTAGTAATCAGCTTC	-
phoP-F (5)	**CTAATTCCCATGTCAGCCGTTAAGTGTTCCTGTGTCAC**ACGATGTCATTACCACCGTAC	Genomic DNA
phoP-R (6)	TTGCGCACAAGGCTGGTCAGCTCACGCGTC	-
E1	CAAGCTGGAAGTAAACCG	Genomic DNA
E2	GCGCTGCGTCCATAATAA	-

The regions marked in bold are complementary to the pKD4 plasmid template

Fusion PCR reaction

Three purified PCR products related to upstream and downstream segments of the *phoP* gene and Kanamycin resistance cassette were applied in fusion PCR as primers. A 75 µL reaction mixture was prepared including 2.5 µL of 10 X Pfu buffer, 1.25 µL of 25 mM $MgSO_4$, 0.63 µL of 10 Mm dNTP Mix, 1.4 µL of Pfu DNA polymerase (5 U/µL), 6 µL, 4.8 µL and 1 µL of purified PCR products of the upstream and downstream segments of the *phoP* gene and Kanamycin resistance cassette respectively as a primer. The PCR amplification was performed with 20 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 20 s, and extension at 72°C for 1 min. The initial denaturation and final extension were 94°C for 3 min and 72°C for 1 min, respectively. The band related to construct with 3095 bp length was observed on a 1% agarose gel (Fig. 3) and was purified with Gel Extraction kit. Thereafter, the construct was amplified and purified.

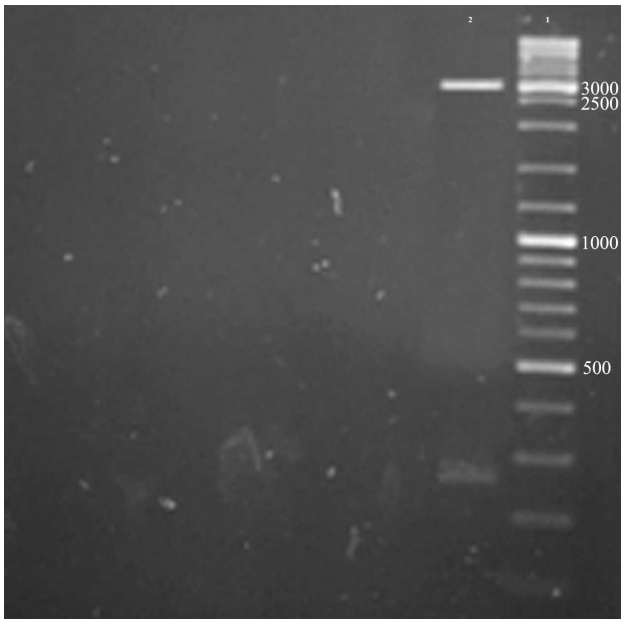

Fig. 3: Fusion PCR product. Lane 1: DNA ladder Mix as a size standard, and Lane 2: Construct

Electroporation of the construct into *S. typhimuruim*

The purified PCR product was electroporated into *S. typhimuruim* carrying pKD46. This transformant was grown at 30°C in 50 ml SOB supplemented with ampicillin 50 µg/ml and L-arabinose 1 mM to an OD_{600} of ~0.6. Then it was made electrocompetent by concentrating and washing with ice-cold 10% glycerol. Electroporation was performed according to the manufacturer's instructions, using 100 µL of competent bacteria and 350 ng of PCR product. After adding 1 ml SOC broth to the shocked bacteria, they were incubated at 37°C for 1 h. Then one-half was spread onto LB-Km-agar plates and incubated at 37°C for 24 h to select Km transformants (Dominguez-Bernal *et al*., 2008).

PCR confirmation

To verify that the Kanamycin resistance cassette has been replaced with *phoP* gene correctly, two PCR_s were carried out. The first PCR was performed by Kan-F (3) and Kan-R (4) primers to confirm presence of the Kanamycin cassette in the appeared colonies. The second one was done with external primers (E1 and E2), the flanking locus-specific primers to confirm the correct position of the construct in the transformants chromosome. Then, the PCR products were observed on 1% agarose gel (Fig. 4).

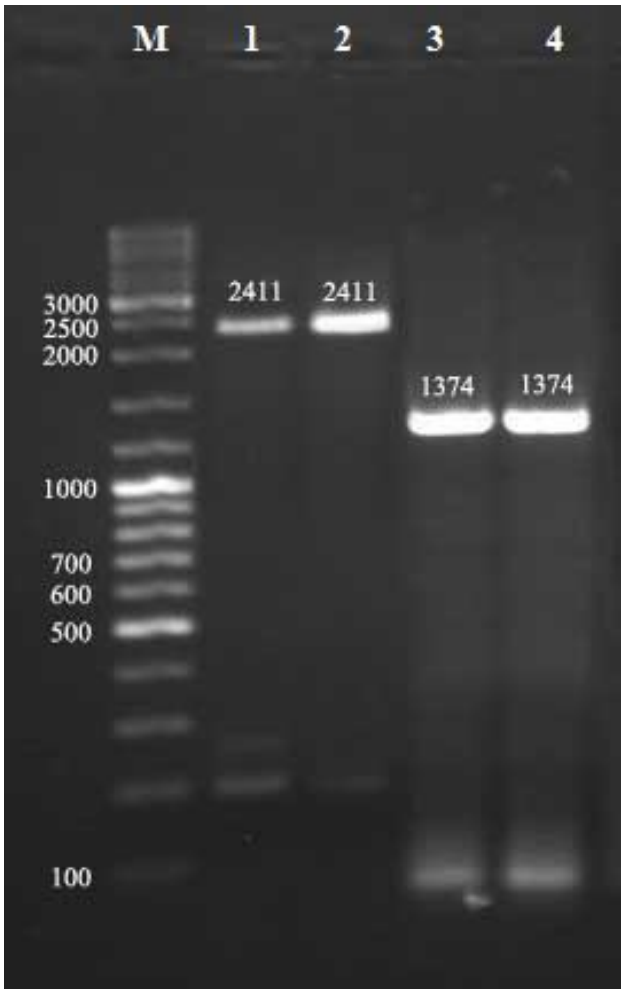

Fig. 4: PCR confirmation. Lane M: DNA ladder Mix as a size standard, Lanes 1 and 2: PCR result on the on the genomic DNA of transformants with E1 and E2 primers, Lanes 3 and 4: PCR result on the genomic DNA of parent strain with E1 and E2 primers

Results

The PCR amplification of upstream and downstream segments of *phoP* gene and Kanamycin resistance cassette was performed successfully and anticipated fragments were observed on 1% agarose gel (Fig. 2). Then, the three purified PCR products related to upstream and downstream segments of the *phoP* gene and Kanamycin resistance cassette were applied in a fusion PCR as primers. The equal molar concentrations

of each purified segments were added to the reaction mixture in which the total concentration was nearly about 210 ng. The band related to the fusion PCR product (construct) with 3095 bp length was observed on a 1% agarose gel (Fig. 3). Then, the purified construct was electroporated into the competent *S. typhimurium* strains. The transformants colonies related to *S. typhimurium* 14028 appeared on the LB-Km-agar plates after incubation at 37°C for 24 h; meanwhile there was no colony on LB-Km-agar plates corresponding to the wild type strain. Using PCR with Kan-F (3) and Kan-R (4), E1 and E2 primers on the transformants colonies, the presence of the Kanamycin cassette in the transformants's chromosome and its replacement with *PhoP* gene was confirmed.

Discussion

The aim of this study was to make a site-directed mutagenesis in a wild type strain of *S. typhimurium* by combination of the SOEing PCR method and the λ Red disruption system. Using this combined method, we constructed a linear DNA with a long flanking homology to the phoP/Kanamycin resistance (Km) cassette. Because it has been revealed that the long flanking homology increases the frequency of recombination in some organisms such as *Salmonella*, in which it has been reported that 100 bp-1 kb of sequence homology is required for an efficient recombination in *Salmonella enteritidis* (Lu *et al*., 2003). However, in one research mutants of the *ropS* and *phoP* regulatory genes in *Salmonella enterica* serovar Choleraesuis (ATCC 13312) were generated by this system with primers that were designed with 56-nt extensions homologous to region adjacent to the target gene and template plasmid (Datsenko and Wanner, 2000; Dominguez-Bernal *et al*., 2008). In the present study we used a combination of the above mentioned methods to create amutation in the wild type strain of *S. typhimurium*. All steps were performed for the *S. typhimurium* 14028 in parallel with the wild type strain as a standard strain. After electroporation of the construct into the competent cells, we only obtained transformed strain from the *S. typhimurium* 14028 and no colony appeared on LB-Km-agar plates related to the wild type strain. Although the λ Red disruption system looks simple and has been applied in *E. coli* and other Gram-negative bacteria successfully but the performances of this system in different bacteria can be variable due to intrinsic differences, such as recombinase expression from the pKD46 plasmid. Particularly, most bacteria subjected to homologous recombination are wild types; it is probable this system is not adaptable to them (Geng *et al*., 2009). Another reason for the failure in transformation of the wild type strain may be due to its unique restriction-modification system that we had no knowledge about it. According to it has been reported that the *S. typhimurium* is untransformable by electroporation or transformable only at comparatively low levels (Callaghan and Charbit, 1990) in a research mutation in *smvA*, *acrB* and *tolC* genes of the *S. typhimuium* 14028 was performed by the λ Red disruption system but p22 was used to transduce *tolC* to *acrB* and *smvA* mutant strains. This method is not applicable to wild-type strains, because P22 is not able to infect some of these strains (Villagra *et al*., 2008). In some studies suicide plasmids have been used to obtain mutant strains of *Salmonella* such as mutation in *S. typhi* by the *sac*B-based recombinant suicide system or mutation in *Salmonella enterica* serovar Typhimurium F98 and serovar Enteritidis 147 by the suicide plasmid vector pDM4 (Methner *et al*., 2004; Lee *et al*., 2007). The suicide vectors have some disadvantages such as replication in a narrow host range and selection of bacterial strains containing the suicide plasmid vectors by environmental stresses, such as higher temperatures or antibiotics (Lee *et al*., 2007). The advantage of the construct in this study is presence of the FRT sites, flanked the Kanamycin resistance gene of the pKD4 plasmid of the Red disruption system, so the Kanamycin resistance gene can be removed by a pCP20 plasmid. As we know the pCP20 stimulates elimination of the FRT-flanked kanamycin resistance gene via expression of the FLP recombinase. This plasmid can also be cured from bacteria at 43°C because of having a temperature sensitive origin of replication (Cherepanov and Wackernagel, 1995; Griffith, 2001). Additionally, by this construct we are able to make phoP mutant in many *Salmonella* spp. Because the *phoP* gene has a high homology in different species of *Salmonella* and the construct can be replaced with their *phoP* gene through homologous recombination.

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