Journal of Animal and Veterinary Advances 10 (16): 2090-2094, 2011
ISSN: 1680-5593
© Medwell Journals, 2011

A Simpler Method for the Efficient and Precise Deletion of Genes in *Salmonella* sp.

Shi-Zhong Geng, Xin-An Jiao, Zhi-Ming Pan, Qiang Fang,
Zhi-Fa Wen and Xiang Chen
Jiangsu Key Laboratory of Zoonosis, Yangzhou University,
Yangzhou, 225009 Jiangsu, P.R. China

Abstract: A simpler method for the efficient and precise deletion of genes in *Salmonella* sp. was developed. To demonstrate this approach, the *prgH* gene of *Salmonella typhimurium* SL7207 was deleted by homologous recombination with a temperature-sensitive plasmid containing a cassette that two DNA fragments as homologous arms flanking chloramphenicol resistance gene (Cm^R) which replaced the *prgH* gene. During screening mutant at 44°C, the temperature-sensitive plasmid was lost easily only the mutant which *prgH* gene was replaced by Cm^R gene could grow in the LB media with chloramphenicol. The results showed that the method was simpler, more effective to delete target gene in genomic DNA of *Salmonell* sp. than those conventional methods.

Key words: Salmonella, deletion of gene, *prgH* gene, homologous recombination, temperature, China

INTRODUCTION

Allelic exchange experiments allow investigation of the functions of many unknown genes identified during the sequencing of entire genomes (Zhou *et al*., 2010).

Deleting directed gene is a central technique used to demonstrate gene function in many studies (Zhou *et al*., 2010). Several main different methods of gene deletions have been reported in *Salmonella* sp. including use of the R6K-suicide plasmid (Miller and Mekalanos, 1988; Philippe *et al*., 2004), the λ-Red disruption system (Kuwayama *et al*., 2002; Datsenko and Wanner, 2000; Doublet *et al*., 2008), the suicide plasmid combined with the Red system (Geng *et al*., 2009) or the plasmid with temperature-sensitive with *SacB* gene (pKO3) (Link *et al*., 1997; Hamann *et al*., 2005; Hamilton *et al*., 1989).

Although, these methods were reported many times few of them were applied successfully when we attempted to delete prgH (Kubori *et al*., 1998; Kimbrough and Miller, 2000) gene of *Salmonella* sp. In this study, researchers reported a simpler and precise method to knock out *prgH* gene of *Salmonella* sp.

MATERIALS AND METHODS

Bacteria and plasmids: The strains and plasmids used in this study were shown in Table 1. Strains were grown in rich liquid or solid (12 g L^{-1} agar) Luria Broth (LB) medium. The media were supplemented with ampicillin (Amp, 100 μg mL^{-1}), chloramphenicol (Cm, 50 μg mL^{-1}), streptomycin (Sm, 25 μg mL^{-1}).

Table 1: Strains and plasmids used in this study

Parameters	Name	Description	Source	References
Strains	S. *typhimurium* SL7207 (Sm^R)	Parent	Lab collection	-
	E. coli. DH5α	*E. coli* for cloning pMD18	Lab collection	-
	E. coli. BW25113	*E. coli* for cloning pKD46	CGSC, USA	-
Plasmids	pKD4	Plasmid containing Cm^R gene	Dr. Cristina Marolda	Datsenko and Wanner (2000)
	pCP20	Plasmid of expressing FLP	Dr. Cristina Marolda	Datsenko and Wanner (2000)
	pKD46	Plasmid with repA101(ts)	CGSC,USA	Datsenko and Wanner (2000)
	pMD18	Plasmid for cloning	Takara, JP	-
	pMD-prgH-u	prgH-u cloned into pMD18	This work	-
	pMD-prgH-d	prgH-d cloned into pMD18	This work	-
	pMD-Cm^R	Cm^R gene cloned into pMD18	This work	-
	pMD-prgH-u+d (Cm^R)	prgH-u+d(Cm^R) cloned into pMD18	This work	-
	pKD46-prgH-u+d (Cm^R)	prgH-u+d (Cm^R) cloned into pKD46	This work	-

Corresponding Author: Xin-An Jiao, Jiangsu Key Laboratory of Zoonosis, Yangzhou University, 12 East Wenhui Road, Yangzhou, 225009 Jiangsu, P.R. China

Construction of plasmid pKD46-prgH-u+d (Cm^R) for knocking out of *prgH* gene: The plasmid pKD46-prgH-u+d (Cm^R) was constructed following the process shown in Fig. 1. All of the primers used in this study were shown in Table 2.

Screening the ΔprgH/Cm^R mutant of *S. typhimurium* SL7207: Plasmid pKD46-prgH-u+d (Cm^R) was transformed to *S. typhimurium* SL7207 by electroporation instrument and the recombinants (SL7207::pKD46-prgH-u+d (Cm^R)) with Amp and Cm resistance were cultured in 28°C, one colony was cultured in the LB media with Cm at 28°C and subcultured (100 μL of the culture was used to inoculate 4 mL of fresh media) for 12 h at 44°C and repeat 8-10 times (Fig. 2). At last, serial dilutions of the culture were prepared and plated 250 μL of the 10^{-4}, 10^{-5} and 10^{-6} dilutions onto LB (Cm) agar plates and incubated for ~16 h at 44°C. Each colony screened should be patched on an LB (Cm) agar plate and an LB (Amp) plate. Only those colonies that grew on LB (Cm) plate and did not grow in LB (Amp) plate would be correct. On average 50-100 positive colonies were screened.

The colonies of the ΔprgH/Cm^R mutant that could only grow on the LB (Cm) plates were furtheridentified by PCR and its characteristics related to *prgH* gene was verified. The SL7207 ΔprgH mutant was received from the ΔprgH/Cm^R mutant by pCP20 plasmid (Fig. 2) (Datsenko and Wanner, 2000; Doublet *et al*., 2008).

Verification of the SL7207 ΔprgH/Cm^R mutant

PCR identification of SL7207ΔprgH/Cm^R mutant: Primers asdp5/asdp6 (asdp5 is in the asdp12 sequence and asdp6 is in the asdp34 sequence) were used to further identify the ΔprgH/Cm^R mutant. The *stn* gene (Geng *et al*., 2009) was amplified with the primer set stnF/stnR (Table 2) which specifically identified *Salmonella* sp.

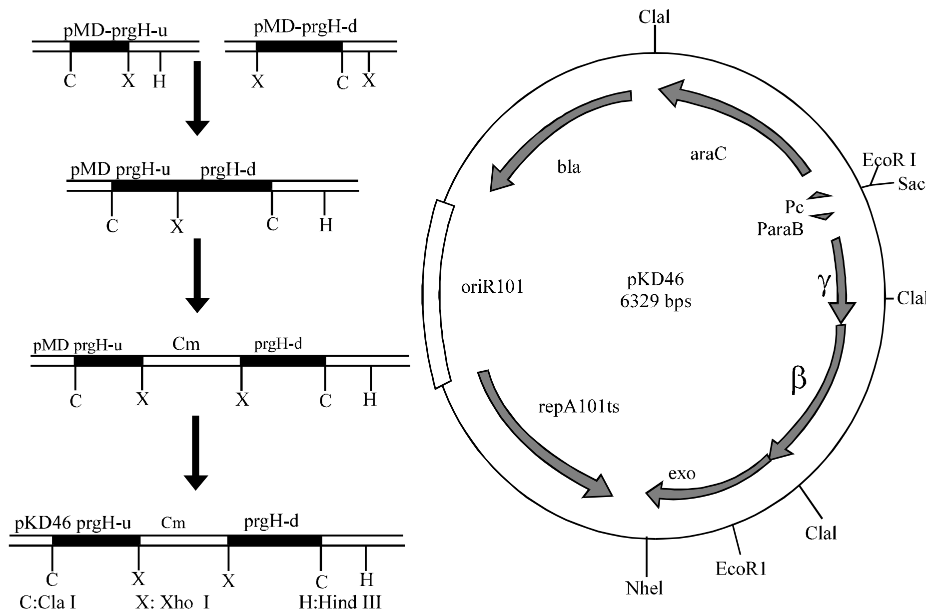

Fig. 1: The construction of plasmid pKD46-prgH-μ+d (Cm^R)

Table 2: The primer sequences of PCR amplification

Genes	Primers	Primer sequences (5'-3')	Amplicon size (bp)	Note
prgH-U	prgH p1	aaactcgagCGCCATGGTCCAGCGGGATA	1.164	Xho I
	prgH p2	aaagtcgacatcgatTGCCGGCCTTAATCCACAGGG	-	Sal I, Cla I
prgH-D	prgH p3	aaagtcgacatcgat CTGATGCGCAAGCGGCGAAC	1.749	Sal I, Cla I
	prgH p4	aaactcgagACTGGCTCAAGGGGCGCTCA	-	Xho I
prgH	prgH p5	AGTAGCGCCGGATCGGAGGG	1.697 (parent)	-
	prgH p6	TGCGGCAGGACGCTGATGAC	1.841 (Δ prgH+Cm^R)	-
prgH-(RT)	prgH-(RT)-F	ACATCATCGAGCGCCCCCTGA	446	-
	prgH-(RT)-R	GTGTTGCCAGGCCGGGACAAA	-	-
Cm^R	CmF	actcgagGTGTAGGCTGGAGCTGCTTC	1.032	Xho I
	CmR	*actcgag*ATGGGAATTAGCCATGGTCC	-	Xho I
Stn	stnF	CTTTGGTCGTAAAATAAGGCG	260	*Salmonella* sp.
	stnR	TGCCCAAAGCAGAGAGATTC	-	-

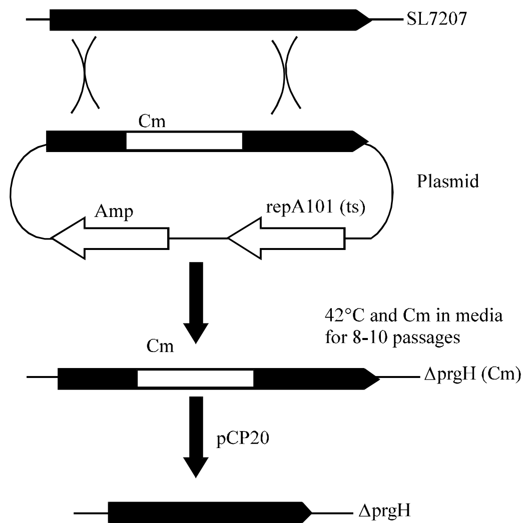

Fig. 2: The screening strategy for the ΔprgH mutant

Detecting of mRNA of *prgH* gene by Reverse Transcription-PCR (RT-PCR): The SL7207ΔprgH/Cm^R mutant and parent SL7207 were cultured and their mRNA were extracted by mRNA extraction Kit of RNAiso Plus respectively, *prgH* gene was detected of by RT-PCR to determine whether *prgH* gene was expression the primers were shown in Table 2.

Biochemical characteristics: The basic biochemical characteristics of the ΔprgH/Cm^R mutant were evaluated by biochemical tube test.

RESULTS AND DISCUSSION

PCR identification of SL7207ΔprgH/Cm^R mutant: The PCR products of primer set asdp5/asdp6 showed that bacteria with Plasmid pKD46-prgH-u+d (Cm^R) possessed two copies of an upstream fragment and a downstream fragment of the *prgH* gene, the SL7207 had only one copy. After mutant being screened, the *prgH* gene was replaced by the *Cm*^{R} gene, the amplicons were 1,697 bp (parent) and 1,697bp and 1,841 bp (the recombinant with Plasmid pKD46-prgH-u+d (Cm^R), 1,841 bp (ΔprgH/Cm^R mutant) (Fig. 3). These results indicated that an ΔprgH/Cm^R mutant had been constructed whose genomic DNA lacked the *prgH* gene. The *stn* gene was amplified with the primer set stnF/stnR, the amplicon size was 260 bp (Fig. 4), the result showed that the mutant was *Salmonella* sp.

Detecting of mRNA of *prgH* gene by reverse transcription-PCR (RT-PCR): The result of RT-PCR showed that SL7207ΔprgH/Cm^R mutant did not express mRNA of *prgH* gene as a control, the parent SL7207 could express mRNA of *prgH* gene (Fig. 5). This illustrated further that the SL7207ΔprgH/Cm^R mutant did not have the *prgH* gene which had been knocked out from genomic DNA of the parent SL7207.

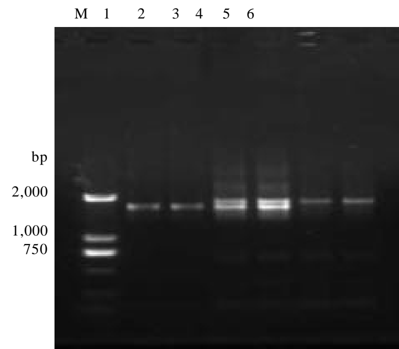

Fig. 3: PCR identification by prgHp5/p6 of SL7207 ΔprgH/Cm^R M DNA marker DL20001; 2: SL7207; 3-4: the recombinants; 5: SL7207 ΔprgH/Cm^R

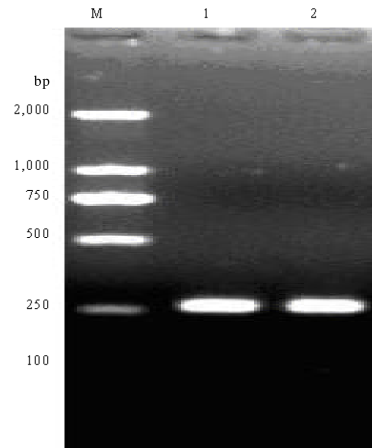

Fig. 4: PCR identification based on *stn* gene of *Salmonella* sp. M DNA Marker DL2000; 1: SL7207 ΔprgH/Cm^R; 2: SL7207

Table 3: Biochemical identification of the SL7207 (ΔprgH/Cm^R) and parent strain SL7207

Parameters	SL7207 (ΔprgH/Cm^R)	SL7207
Glucose	+	+
Maltose	+	+
Mannose	+	+
Sorbitol	-	-
Sucrose	-	-
Lactose	-	-
H_2S	+	+
Urea	-	-
Lysine decarboxylase	+	+
KCN	-	-
Gelatin	-	-
TSI	-	-
IMViC	---+	---+

Biochemical characteristics: The results of biochemical tube test of the mutant were consistent with those of parent SL7207 (Table 3). The SL7207 (ΔprgH/Cm^R) mutant

Table 4: The difference of different methods of gene deletions in *Salmonella* sp.

	The mutant being screened			
Methods	Vector	*Cm*^{R} gene	The recombinants of first crossing	Vector lost
λ-Red system	pKD46, phage λ-Red recombinase inducted by arabinose (difficult)	Mutant with *Cm*^{R} gene	The recombinants with *Cm*^{R}gene	-
R6K-Suicide plasmid	pGMB151, λ-pir protein	No	Diffcult to get sucrose-sensitive recombinants and differentiate the donor and the recipient	Sucrose (difficult)
Cm^R replacement based on R6K-Suicide plasmid	pGMB151, λ-pir protein	Mutant with *Cm*^{R} gene	Diffcult to get sucrose-sensitive recombinants and easy to differentiate the donor and the recipient	Sucrose (difficult)
Cm^R replacement based on temperature-sensitive plasmid	pKD46 at 28°C	Mutant with *Cm*^{R} gene	the recombinants with *Cm*^{R} gene	44°C (easy)

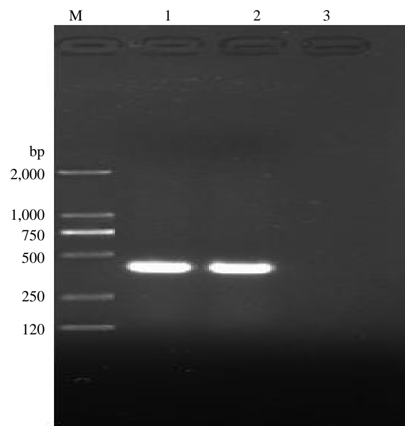

Fig. 5: RT-PCR identification by prgH-(RT) primers M:DNA Marker DL2000; 1: SL7207; 2: SL7207; 3: SL7207 (△prgH/Cm^R)

was constructed successfully during 3 weeks using this method based on temperature-sensitive plasmid with RepA101 (ts). Because target gene was replaced by *Cm*^{R} gene in the temperature-sensitive plasmid. At 44°C, this plasmid was lost easily from the recombinants at the same time, the parent SL7207 had no Cm restance, SL7207 without pKD46-prgH-u+d (Cm^R) was dead in the LB (Cm) media. Only when target gene (*prgH*) was replaced by *Cm*^{R} gene via allele replacement and the recombinants [SL7207::pKD46-prgH-u+d (Cm)] lost plasmid, the mutant with *Cm*^{R} gene and without *prgH* gene could grow in the Cm and 44° and could not grow on LB (Amp) plates. And so, by subculturing 8-10 times, the mutant could be retrieved. Comparing with the Red system (Table 4), the method need not what pKD46 plasmid was transformated into target bacteria and induced by arabinose to express phage λRed recombinase which was unsure in the try (Datsenko and Wanner, 2000). Although, λ-Red Disruption system looked simple and was applied in *E. coli* and other G-bacteria, researchers tried many times with Red system in *Salmonella* sp. but at last failed. As a control, researchers cloned the cassette into the R6K-suicide plasmid with *SacB* gene (Geng *et al*., 2009) and the temperature-sensitive plasmid. In theory, the recombinant bacteria with the R6K-suicide plasmid was very sensitive to sucrose because levansucrase was expressed by the *SacB* gene which was lethal to bacteria in the presence of 10% sucrose and utilized as a positive and counter-selectable markers in the template plasmid (Gay *et al*., 1983) but during screening mutant, it is difficult to receive a clony with suicide plasmid which is sensitive to sucrose in spite that we success in knocking out this gene.

CONCLUSION

In this improved method, some uncertain factors were avoid by temperature-sensitive plasmid replacing R6K-suicide plasmid such as phageλRed recombinase, levansucrase only temperature was used to make plasmid lose and speed process of screening mutant under the Cm antibiotic which was positive and counter-selectable markers in the mutant. In brief, this method was simpler than the others to generate precise gene deletions in *Salmonella* sp.

ACKNOWLEDGEMENTS

The researchers thank sincerely Dr. Cristina Marolda and CGSC (Coli Genetic Stock Center) in USA for their help and guide in gene knock-out and thank the cooperation of faculty members at Jiangsu Key Laboratory of Zoonosis in Yangzhou University. This research was supported by key projects in the National Science and Technology Pillar Program (2009BADB9B01), National Programs for Fundamental Research and Development of China (2006CB504404), National Nature Science Foundation of China (No. 30871860, 30425031) and the Government of Jiangsu Province (Nos. BK2008011 and BK2010039).

REFERENCES

Datsenko, K.A. and B.L. Wanner, 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA., 97: 6640-6645.

Doublet, B., G. Douard, H. Targant, D. Meunier, J.Y. Madec and A. Cloeckaert, 2008. Antibiotic marker modifications of [lambda] Red and FLP helper plasmids, pKD46 and pCP20, for inactivation of chromosomal genes using PCR products in multidrug-resistant strains. J. Microbiol. Meth., 75: 359-361.

Gay, P., D. Le Coq, M. Steinmetz, E. Ferrari and J.A. Hoch, 1983. Cloning structural gene SacB, which codes for exoenzyme levan-sucrase of *Bacillus subtilis*: Expression of the gene in *Escherichia coli*. J. Bacteriol., 153: 1424-1431.

Geng, S.Z., X.A. Jiao, Z.M. Pan, X.J. Chen, X.M. Zhang and X. Chen, 2009. An improved method to knock out the asd gene of *Salmonella enterica* Serovar Pullorum. J. Biomed. Biotechnol., 2009: 646380-646380.

Hamann, A., K. Krause, A. Werner and H.D. Osiewacz, 2005. A two-step protocol for efficient deletion of genes in the filamentous ascomycete *Podospora anserina*. Curr. Genet., 48: 270-275.

Hamilton, C.M., M. Aldea, B.K. Washburn, P. Babitzke and S.R. Kushner, 1989. New method for generating deletions and gene replacements in *Escherichia coli*. J. Bacteriol., 171: 4617-4622.

Kimbrough, T.G. and S.I. Miller, 2000. Contribution of *Salmonella typhimurium* type III secretion components to needle complex formation. Proc. Natl. Acad. Sci. USA., 97: 11008-11013.

Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil and M. Lara-Tejero *et al*., 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Sci., 280: 602-605.

Kuwayama, H., S. Obara, T. Mario, M. Katoh, H. Urushihara and Y. Tanaka, 2002. PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. Nucleic Acids Res., 30: E2-E2.

Link, A.J., D. Phillips and G.M. Church, 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: Application to open reading frame characterization. J. Bacteriol., 179: 6228-6237.

Miller, V.L. and J.J. Mekalanos, 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. J. Bacteriol., 170: 2575-2583.

Philippe, N., J.P. Alcaraz, E. Coursange, J. Geiselmann and D. Schneider, 2004. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. Plasmid., 51: 246-255.

Zhou, Q.M., D.J. Fan, J.B. Xie, C.P. Liu and J.M. Zhou, 2010. A method for generating precise gene deletions and insertions in *Escherichia coli*. World J. Microb. Biotech., 26: 1323-1329.