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Methodology Report

An Improved Method to Knock Out the *asd* Gene of *Salmonella enterica* Serovar Pullorum

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An asd-deleted (Δ*asd*) mutant of *Salmonella enterica* serovar Pullorum (*SP*) was constructed using an improved method of gene knockout by combining the π-suicide plasmid system with the Red Disruption system. The *asd* gene was efficiently knocked out by the recombinant suicide vector, which replaced the *asd* gene with the Cm^R gene. Based on the balanced lethal host-vector system, the phenotype of the Δ*asd* mutant was further defined. The improved method was simpler and more effective than previously reported conventional methods.

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1. Introduction

Salmonella enterica serovar Pullorum (*SP*) is highly adapted to species of fowl, although *SP* infections in primates have been reported [1]. As a fowl-specific pathogen, *SP* has a considerable worldwide economic impact, especially in developing countries. The growing problems of antibiotic resistance and the lengthy persistence of the bacteria in chickens after infection [2] necessitate the development of novel and efficient measures to control this pathogen.

A balanced lethal host-vector system, based on the Δ*asd* mutant of *Salmonella typhimurium,* has previously been used in a vaccine [3]. However, because of the high specificity of *SP* for fowl, *SP* is a better live vaccine vector for mucosal immunization of fowl than other *Salmonella* spp. The balanced lethal host-vector system can be used to produce a live vaccine, can be used as a vaccine vector [4–7], and is also a tool with which to study the genetics and pathogenesis of *SP* infection. This requires construction of an *SP* Δ*asd* mutant and the development of an *SP* balanced lethal host-vector system.

Previously, multiple attempts have failed to produce a mutant when either the π-suicide plasmid system or the Red Disruption system was used to knock-out the *asd* gene [8] of *SP*. However, we describe here the successful ablation of the *asd* gene of *SP* using a combination of these two systems above. The basic strategy was to replace the chromosomal *asd* sequence with a selectable antibiotic resistance gene (*Cm*) using a suicide vector based on the π-suicide plasmid system, and *E. coli* χ7213 as a donor strain. After selection with the appropriate antibiotic, the *Cm* resistance gene can be eliminated using the helper plasmid pCP20.

2. Materials and Methods

2.1. Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are given in Table 1. Bacteria were grown in rich liquid or solid (12 g/L agar) Luria broth (LB) medium. The media were supplemented with ampicillin (*Amp,*100 μg/mL), kanamycin (*Km*, 50 μg/mL), chloramphenicol (*Cm*, 30 μg/mL), streptomycin (*Sm*, 25 μg/mL), or nalidixic acid (*Nal*, 30 μg/mL) as required. NA (solid LB medium without NaCl) and NB (liquid LB medium without NaCl) with 10% sucrose were used during the gene allelic exchange experiments to select plasmids that had been excised from the chromosome.

2.2. Construction of Suicide Plasmid pGMB151-asdp1234 (Cm). The PCR product *asd*p12, using primers *asd*p1/*asd*p2,

Table 1: Strains and plasmids used in this study.

	Name	Description	Source	Reference
Strains	*S. Pullorum* S06004 (Nal^R)	Recipient	Lab collection	
	χ7213 (pGMB151- $\Delta asd/Cm$)	Donor	This work	
	χ7213 (Km^R Δasd)	*E. coli* for cloning pGMB151	Dr. Roy Curtiss III	Kang et al. [5]
	Spy372 (λ-pir)	*E. coli* for cloning pGMB151	Lab collection	Huang et al. [9]
	E. coli. DH5α	*E. coli* for cloning pMD18	Lab collection	
	pKD3	Plasmid containing Cm^R gene	Dr. Cristina Marolda	Datsenko and Wanner [10]
Plasmids	pCP20	Plasmid of expressing FLP	Dr. Cristina Marolda	Datsenko and Wanner [10]
	pGMB151 (Amp^RSm^R)	Suicide plasmid	Lab collection	Huang et al. [9]
	pYA3334 (with *asd* gene)	Plasmid of expressing DAP	Dr. Roy Curtiss III	Kang et al. [8]
	pYA3334-dsRED	Plasmid of expressing dsRED	This work	
	pMD18	Plasmid for cloning	Takara	
	pMD-*asd*p12	*asd*p12 cloned into pMD18	This work	
	pMD-*asd*p34	*asd*p34 cloned into pMD18	This work	
	pMD- Cm^R	Cm^R gene cloned into pMD18	This work	
	pMD-*asd*p1234	*asd*p1234 cloned into pMD18	This work	
	pMD-*asd*p1234(*Cm*)	*asd*p1234(*Cm*) cloned into pMD18	This work	

Table 2: The primer sequences used for PCR amplification.

Gene amplified	Primers	Primer sequences (5′-3′)	Amplicon size (bp)	Note
Upstream of *asd*	*asd*p1	tt*ggatcc*ccgttgaatgatgatgaccg	1959	*Bam*H I
	*asd*p2	tt*ctcgag*tgcgttaggaagggaatc		*Xho* I
Downstream of *asd*	*asd*p3	tt*ctcgag*gtagcttaatcccgcgggta	2079	*Xho* I
	*asd*p4	tt*ggatcc*gagcgttcattgtcatcgac		*Bam*H I
asd	*asd*p5	ttgcttccaactgctgagc	1796(wt)	
	*asd*p6	tcctatctgcgtcgtcctac	1360($\Delta asd + Cm$)	
Cm^R	Cm^F	a*ctcgag*gtgtaggctggagctgcttc	1032	*Xho* I
	Cm^R	a*ctcgag*atgggaattagccatggtcc		*Xho* I
*rfb*S	*rfb*SF	tgcctatcagagtattagtgt	400	*S.Pullorum*
	*rfb*SR	tattcacgaattgatatatcc		
hto	*hto*F	actggcgttatccctttctctgctg	495	*genus Salmonella*
	*hto*R	atgttgtcctgcccctggtaagaga		

and the PCR product *asd*p34, using primers *asd*p3/*asd*p4, were amplified from genomic DNA of *SP* S06004. The PCR product Cm^R, which spans the Cm^R cassettes and includes the flanking FRT sites of the Cm^F/Cm^R primers, was amplified from the pKD3 plasmid. These fragments were purified and cloned into the pMD18 vector and the resulting plasmids were named pMD-*asd*p12, pMD-*asd*p34 and pMD-Cm^R, respectively. The *asd*p12 and *asd*p34 fragments acted as two arms for homologous recombination. Subsequently, *asd*p12 and *asd*p34 were ligated via an *Xho*I site to produce *asd*p1234. A fragment of the Cm^R gene was then cloned into the *Xho*I site of *asd*p1234 to produce pMD-*asd*p1234 (*Cm*) (Figure 1). pMD-*asd*p1234 (*Cm*) and the pGMB151 suicide plasmid were digested with *Bam*HI and religated to produce pGMB151-*asd*p1234 (*Cm*), which was then transferred to *E. coli* Spy372 [9]. pGMB151-*asd*p1234 (*Cm*) was then further transferred to *E. coli* χ7213, and was termed the donor strain *E. coli* χ7213 (pGMB151-$\Delta asd/Cm$). The sequences of all primers used are given in Table 2.

2.3. Generation of the SP Δasd (Cm) Mutant by Conventional Allelic Exchange. The donor strain, *E. coli* χ7213 (pGMB151-$\Delta asd/Cm$), and the recipient strain, *SP* S06004, were grown in LB at 37°C with shaking to OD 0.6-0.7 at 600 nm. The recipient strain was heat-treated at 50°C for 30 minutes immediately before conjugative mating to temporarily inactivate the host-restriction systems. Samples of the donor strain and the heat-treated recipient strain were mixed in 10 mM $MgSO_4$ solution, the mixture was immobilized on a 0.45 μm membrane filter, and the filter was incubated on LB agar at 28°C for 18 hours. The transconjugants were recovered in LB for 1 hour at 37°C, and spread onto LB agar containing *Nal*, *Amp*, and *Cm* [11]. The Colonies were also streaked onto NA with *Nal*, *Amp*, and *Cm* and NA with *Nal*, *Amp*, *Cm* and sucrose to select bacteria which are sensitive to sucrose [11]. Single bacterial colonies, of single-crossover plasmid insertions (S06004::pGMB151-$\Delta asd/Cm$), which are sensitive to sucrose, were subcultured 8–10 times on NB containing 10 mM $MgSO_4$, 10% sucrose

Table 3: Antibiotics resistance of bacteria during the selection of Δ*asd* mutant.

Bacteria	Antibiotics				
	Nal	*Km*	*Cm*	*Sm*	*Amp*
Salmonella Pullorum (S06004)	+	−	−	−	−
χ7213(pGMB151-Δ*asd*/*Cm*)	−	+	+	+	+
First crossover (S06004::pGMB151- Δ*asd*/*Cm*)	+	−	+	+	+
Second crossover (Δ*asd* (*Cm*) mutant)	+	−	+	−	−
SP Δ*asd* mutant	+	−	−	−	−

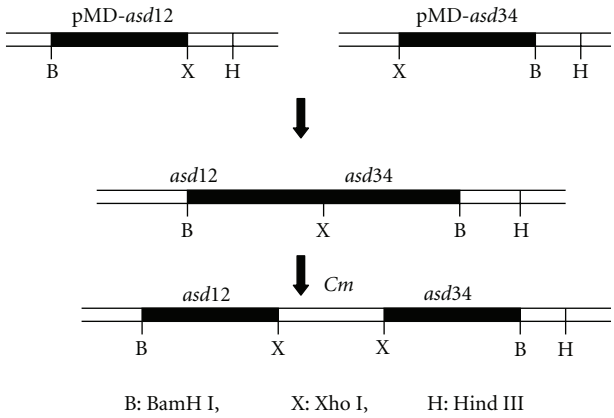


Figure 1: The construction of pMD-*asd*p1234 (*Cm*).

[12], 1% diaminopimelic acid (DAP) and *Cm*. The mutants, ie the *SP* Δ*asd* (*Cm*) mutant, without *Amp* resistance were screened on LB plates that contained DAP and *Cm* [13, 14] (Figure 2). At the same time, The presence of the Δ*asd* allele in the *SP* Δ*asd* (*Cm*) mutant was confirmed by *asd*p5/*asd*p6 primers which PCR product,1360 bp, was smaller than 1796 bp amplified from wt *SP*S06004 (Figures 3 and 5(b)).

2.4. Antibiotic Resistance, Growth and Biochemical Characteristics of the SP Δasd (Cm) Mutant. During the selection process, the *SP* Δ*asd* (*Cm*) mutant was cultured in medium supplemented with several antibiotics, including *Nal*, *Amp*, *Cm*, and/or *Km*. When the *asd* gene was replaced by the Cm^R gene, the mutant became resistant to *Cm* and depended on exogenous DAP for growth. The *SP* Δ*asd* (*Cm*) mutant was cultured in medium containing DAP, and also in medium without DAP, as a control, to determine if its growth was dependent on the presence of DAP. The basic biochemical characteristics of the *SP* Δ*asd* (*Cm*) mutant were evaluated using IMViC tests.

2.5. PCR Verification of the SP Δasd (Cm) Mutant. In addition to the primers *asd*p1/*asd*p2, *asd*p3/*asd*p4 and Cm^F/Cm^R, primers *asd*p5/*asd*p6 (*asd*p5 is in the *asd*p12 sequence and *asd*p6 is in the *asd*p34 sequence), and primers *asd*p5/Cm^F and *asd*p6/Cm^R (Table 2) were used to further characterize the *SP* Δ*asd* (*Cm*) mutant. The PCR products obtained were compared with those of wt *SP* S06004. At the same time, the genus *Salmonella* was identified by PCR amplification of the *hto* gene [12, 15] with the primers *hto*F/*hto*R (Table 2). The *rfbS* gene, which specifically identifies *SP*[16, 17], was amplified with the primers *rfb*SF/*rfb*SR (Table 2).

2.6. Elimination of the Cm^R Gene from of the SPΔasd (Cm) Mutant. Plasmid pCP20 is an Amp^R and Cm^R plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis [18]. The *SP* Δ*asd* (*Cm*) mutants were transformed with plasmid pCP20, and transformants resistant to ampicillin were selected at 30°C.

Subsequently, a few colonies were purified once, nonselectively, at 43°C, and were then tested for loss of all antibiotic resistance. The majority lost the *Cm* resistance gene and the FLP helper plasmid simultaneously, and comprised the *SP* Δ*asd* mutant population.

2.7. Construction of the SP Balanced Lethal Host-Vector System. The *SP* Δ*asd* mutant can be complemented with a foreign *asd* gene from the plasmid that forms the *SP* balanced lethal host-vector system. The *SP* Δ*asd* mutant, with the plasmid containing the foreign *asd* gene, can grow without DAP. The pYA3334 plasmid [5, 19], which contains the *asd* gene, was transformed into the *SP* Δ*asd* mutant to verify its growth without DAP. The pYA3334-dsRED plasmid, which contains the*asd* gene and the dsRED gene, was transformed into the *SP* Δ*asd* mutant to express the red fluorescent protein. This was used to further demonstrate stability of the plasmid in different passages of the *SP* Δ*asd* mutant (pYA3334-dsRED), using flow cytometry (FACS) analysis.

3. Results

3.1. Antibiotic Resistance of the SP Δasd Mutant. During the selection process, the antibiotic resistance of the *SP* Δ*asd* mutant, the *SP* Δ*asd* (*Cm*) mutant, and other bacterial strains was determined (Table 3). As expected, the *SP* Δ*asd* mutant showed the profile $Nal^R Amp^S Cm^S Sm^S$, which was the same as that of the wt *SP* S06004.

3.2. Growth and Biochemical Characteristics of the SP **Δasd** *Mutant.* DAP was an absolute requirement for growth of the *SP* Δ*asd* mutant and the *SP* Δ*asd* (*Cm*) mutant. The IMViC of the mutant was "−+−−" which was consistent with those of wt *SP* S06004. However, the growth velocity of the Δ*asd* mutant and the Δ*asd* (*Cm*) mutant in LB media

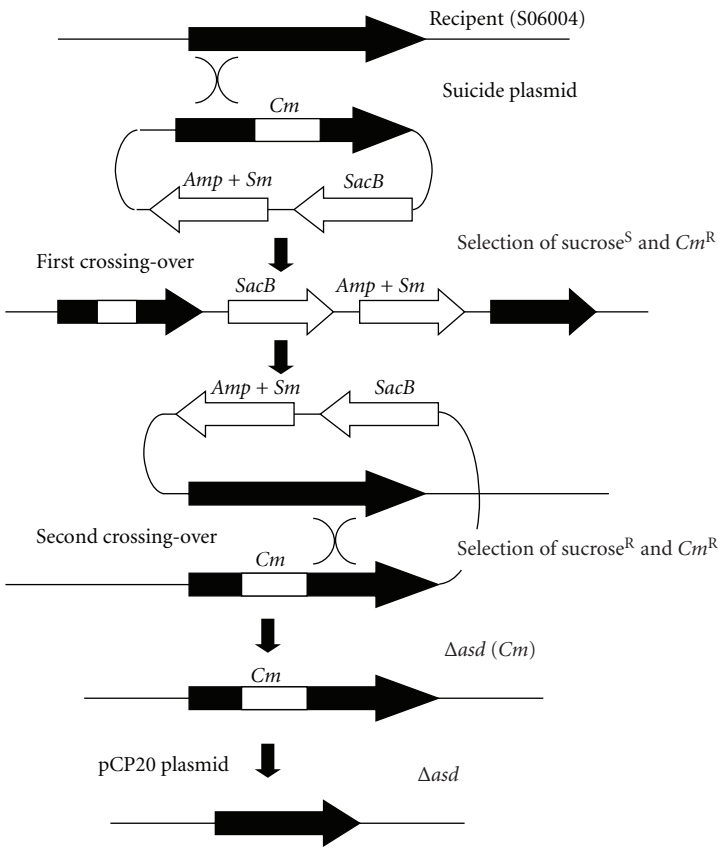


Figure 2: The screening strategy for the *SP* Δ*asd* mutant.

containing DAP was slow compared with that of the wt S06004 (Figure 4).

3.3. PCR Verification of the SP Δ*asd* (*Cm*) *and* Δ*asd Mutants.* PCR was used to identify the *SP* Δ*asd* (*Cm*) and *SP* Δ*asd* mutants. PCR amplification of the *hto* and the *rfbS* genes showed that the Δ*asd* (*Cm*) mutant was *SP*. The PCR products were amplified using primers *asd*p1/*asd*p2, *asd*p3/*asd*p4, Cm^F/Cm^R, *asd*p5/*asd*p6, *asd*p5/Cm^F, and *asd*p6/Cm^R (Figure 5(a)), which demonstrated that the *asd* gene had been replaced by the *Cm* gene. PCR amplification using the primers *asd*p5/*asd*p6 showed that bacteria of the first crossover possessed two copies of an upstream fragment and a downstream fragment of the *asd* gene. After the second crossover, the *asd* gene was replaced by the *Cm* gene, and the Cm^R gene was eliminated from the *SP* Δ*asd* (*Cm*) mutant by plasmid pCP20 (Figures 3 and 5(b)). These results indicated that an *SP* Δ*asd* mutant had been developed whose genomic DNA lacked the *asd* gene.

3.4. Construction of the SP Balanced Lethal Host-Vector System. When the *SP* Δ*asd* mutant was transformed with plasmid pYA3334, which contains the *asd* gene, the recombinant *SP* Δ*asd* mutant (pYA3334) could grow without DAP. This showed that the *asd* gene in the plasmid could functionally complement the Δ*asd* mutant. After transformation with the plasmid pYA3334-dsRED, the *SP* Δ*asd* mutant (pYA3334-dsRED) expressed red fluorescent protein (Figure 6); in contrast, there was no red fluorescence from the *SP* Δ*asd* mutant (pYA3334). The FACS analysis of dsRED expression in the different passages of the *SP* Δ*asd* mutant (pYA3334-dsRED) showed that pYA3334-dsRED was stable. In the 2nd and 20th passages of the *SP* Δ*asd* mutant (pYA3334-dsRED), 96.3% and 95% of bacteria, respectively, showed strong red fluorescence (Figure 7).

4. Discussion

Allelic exchange experiments [20] allow investigation of the functions of many unknown genes identified during the sequencing of entire genomes. A number of allele replacement methods can be used to inactivate bacterial chromosomal genes. These all require the engineering of gene disruption on a suitable plasmid. Amberg et al. [21] reported the successful knock-out of a gene by homologous recombination in yeast using fusion PCR technology. Kuwayama et al. [22] showed that genes can be directly disrupted in *Saccharomyces cerevisiae* by transformation with PCR fragments encoding a selectable marker and having only 35nt of flanking homologous DNA. Most bacteria, however, are not readily transformable with linear DNA, in

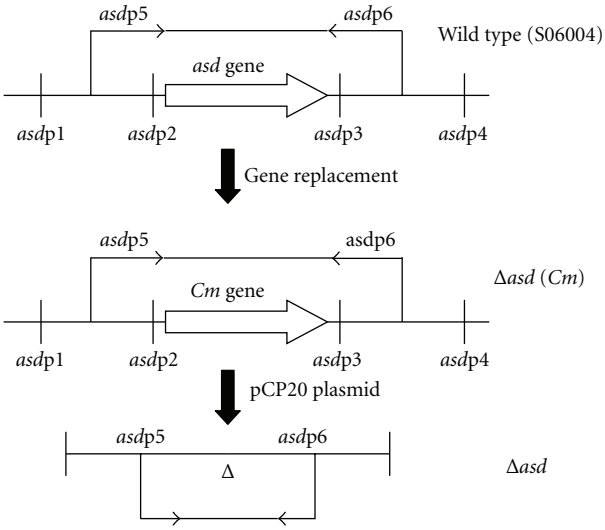


Figure 3: Genetic organization of a recombinant construct containing a defined deletion. The map shows the recombinant Δ*asd* (1488 bp) region deleted from the *SP* genome. The open arrow indicates the coding region of the *asd* gene (Cm^R gene), and dotted lines represent the limits of the deleted region. The deletion is shown as an open triangle, and the sizes of the flanking regions adjacent to the deletion on the suicide vector are indicated. The position and orientation of PCR primers used in this study are indicated by filled arrows on the map of the wt DNA. The sizes of the PCR amplified products from the wt, the *SP* Δ*asd* (*Cm*) mutant, and the *SP* Δ*asd* mutant are 1796 bp, 1360 bp, and 328 bp, respectively.

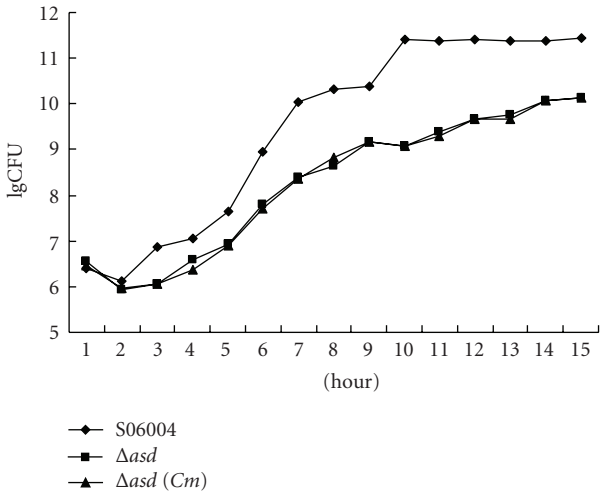
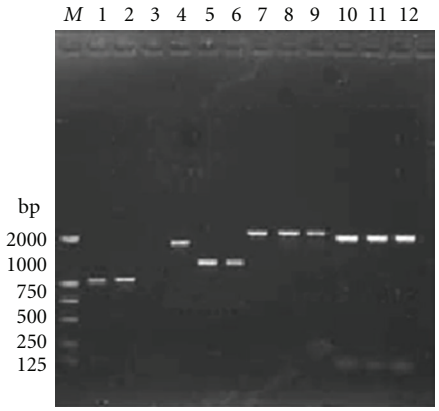
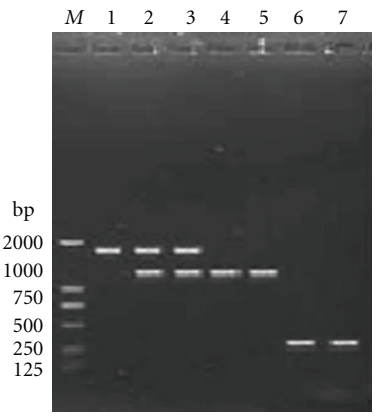


Figure 4: The growth curves of the Δ*asd* mutant, the Δ*asd* mutant (*Cm*) and the parental strain S06004 in LB media with DAP.



(a) Overall of PCR identification of the *SP* Δ*asd* (*Cm*) mutant Lanes 1, 2, 5, 6, 7, 8, 10, 11: the *SP* Δ*asd* (*Cm*) mutant; Lanes 3, 4, 9, 12:wt, Lanes 1, 2,3: Cm^F/Cm^R; 4, 5, 6: *asd*P5/*asd*P; 6, 7, 8, 9: *asd*P1/*asd*P2; 10, 11, 12: *asd*P3/*asd*P4



(b) PCR with primers *asd*P5/*asd*P6 identified wt, *SP* Δ*asd* (*Cm*) and *SP* Δ*asd* mutant, Lane 1: wt; Lanes 2, 3: First crossover of conjugants; Lanes 4, 5: Second crossover of conjugants (the *SP* Δ*asd* (*Cm*) mutant); Lanes 6, 7: the *SP* Δ*asd* mutant.

Figure 5: PCR identification of the *SP* Δ*asd* (*Cm*) mutants.

part, because of intracellular exonucleases that degrade linear DNA. Datsenko and Wanner [10] developed the simple and highly efficient Red Disruption system to directly inactivate chromosomal genes in *E. coli* K-12 using PCR products based on the phage λ-Red recombinase, which is synthesized under the control of an inducible promoter on an easily curable, low copy number plasmid, such as pKD46 (or pKD20). To adapt it to more distantly related bacteria, it may be necessary to express the Red system under different control or from another low copy number vector.

Several different methods of gene knock-out have been reported in *Salmonella*, including the π-suicide plasmid containing R6K ori, the λ-red system, the Red Disruption system, and a plasmid with temperature-sensitive replication. Among these methods, π-suicide plasmids and the Red Disruption system have been preferred in *Salmonella* and *E. coli*, because they possess many advantages. However, the performance of the Red Disruption system in different bacteria can be variable due to intrinsic differences, such as Recombinase expression. Similarly, the major problem of the π-suicide plasmid system is that its efficiency is very poor. Most bacteria subjected to homologous recombination, even under negative selection for the *sacB* gene [23, 24], are wild type (wt),

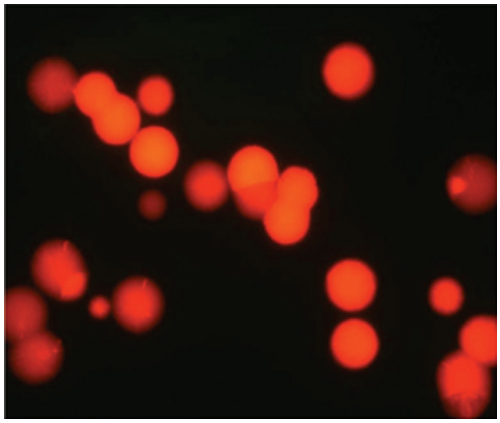


Figure 6: dsRED expression in the *SP* Δ*asd* mutant (pYA3334-dsRED). *SP* Δ*asd* mutant (pYA3334-dsRED) colonies showed red fluorescence on LB plates without DAP.

and only a few are mutant, therefore, it is difficult to directly isolate the desired mutant. An increase in the efficiency for screening recombinants is needed. Application of the π-suicide plasmid system requires two problems to be solved: (1), the requirement of antibiotic resistance in the engineered bacteria and (2), the efficiency of selection for mutants.

Previously we have made multiple attempts, to obtain mutants using the Red Disruption system, but without success (unpublished data). This is possibly because phage λ-Red recombinase was not expressed in S06004 from the pKD46 plasmid. It is probable that this system is not adaptable to some "recalcitrant" strains, such as *SP* S06004, as we showed here.

The π-suicide plasmid containing R6K ori is universally used for gene ablation. The *E. coli* host strains SM10 [25] and S17 for this plasmid are resistant to *Km*, but the recipient *Salmonella* strain used in this study had no special antibiotic resistance. When screening bacteria of the first crossover, it is difficult to separate donor bacteria (SM10 or S17) from recipient bacteria (*SP* S06004). The efficiency of screening recombinants requires improvement.

In an attempt to solve these difficulties, a new approach that combined the π-suicide plasmid system with the Red Disruption system was developed. First, we used *E. coli* strain χ7213 [26] instead of strain SM10 or S17, because *E. coli* χ7213 is a Δ*asd* mutant that depends on exogenous DAP for its growth. It was easy, therefore, to isolate donor and recipient bacteria after conjunction on an LB plate without DAP. The donor bacterium, *E. coli* χ7213, could not grow on medium without DAP, but the first-cross bacteria could grow. Second, the FRT-flanked resistance gene (Cm^R or Km^R) of the pKD3 plasmid of the Red Disruption system was used to replace the gene of interest. Plasmid pCP20, which is Amp^R and Cm^R, shows temperature-sensitive replication and thermal induction of FLP synthesis was used to knock-out the FRT-flanked resistance gene. This improved method made the knock-out of a gene simple in comparison with the π-suicide plasmid system or the Red Disruption system alone.

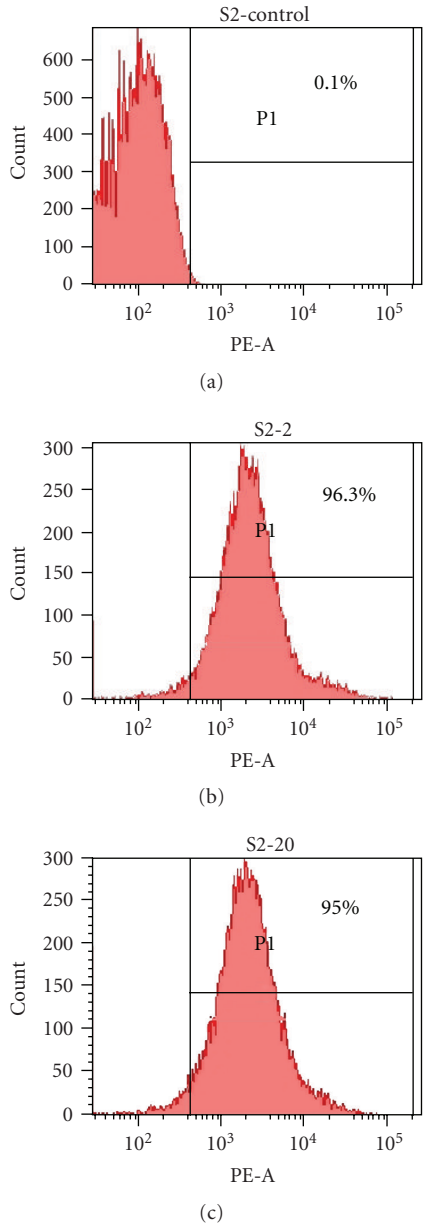


Figure 7: FACS analysis of dsRED expression in the different passages of the *SP* Δ*asd* mutant (pYA3334-dsRED). S2-control: control for the *SP* Δ*asd* mutant (pYA3334); S2-2: The 2nd passage of the *SP* Δ*asd* mutant (pYA3334-dsRED); S2-20: The 20th passage of the *SP* Δ*asd* mutant (pYA3334-dsRED).

This improved method has been successfully applied in our lab to knock-out many bacterial genes. we anticipate that it will be widely applied for gene targeting in the future.

5. Conclusions

In this paper, we have described a new improved approach for gene targeting in *Salmonella enteric* serovar Pullorum to knock-out gene(s), replaceing target gene (*asd* gene) with *Cm* (or *Km*) gene from the Red Disruption system

based on π-suicide plasmid system, which is simpler in the procedures and more effective for screening recombinants than previously reported conventional methods.

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References

[1] R. A. Ocholi, L. U. Enurah, and P. S. Odeyemi, "Fatal case of salmonellosis (*Salmonella pullorum*) in a chimpanzee (Pan troglodytes) in the Jos Zoo," *Journal of Wildlife Diseases*, vol. 23, no. 4, pp. 669–670, 1987.

[2] P. Wigley, A. Berchieri Jr., K. L. Page, A. L. Smith, and P. A. Barrow, "*Salmonella enterica* serovar pullorum persists in splenic macrophages and in the reproductive tract during persistent, disease-free carriage in chickens," *Infection and Immunity*, vol. 69, no. 12, pp. 7873–7879, 2001.

[3] K. L. Karem, S. Chatfield, N. Kuklin, and B. T. Rouse, "Differential induction of carrier antigen-specific immunity by *Salmonella typhimurium* live-vaccine strains after single mucosal or intravenous immunization of BALB/c mice," *Infection and Immunity*, vol. 63, no. 12, pp. 4557–4563, 1995.

[4] J. E. Galán, K. Nakayama, and R. Curtiss III, "Cloning and characterization of the asd gene of *Salmonella typhimurium*: use in stable maintenance of recombinant plasmids in *Salmonella* vaccine strains," *Gene*, vol. 94, no. 1, pp. 29–35, 1990.

[5] H. Y. Kang, J. Srinivasan, and R. Curtiss III, "Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica serovar typhimurium* vaccine," *Infection and Immunity*, vol. 70, no. 4, pp. 1739–1749, 2002.

[6] C. O. Tacket, S. M. Kelly, F. Schodel, et al., "Safety and immunogenicity in humans of an attenuated *Salmonella typhi* vaccine vector strain expressing plasmid-encoded hepatitis B antigens stabilized by the Asd-balanced lethal vector system," *Infection and Immunity*, vol. 65, no. 8, pp. 3381–3385, 1997.

[7] S.-L. Yuan, P. Wang, H.-X. Tao, et al., "Removal of antibiotic resistance of live vaccine strain *Escherichia coli* MM-3 and evaluation of the immunogenicity of the new strain," *Acta Biochimica et Biophysica Sinica*, vol. 38, no. 12, pp. 844–856, 2006.

[8] H. Y. Kang, C. M. Dozois, S. A. Tinge, T. H. Lee, and R. Curtiss III, "Transduction-mediated transfer of unmarked deletion and point mutations through use of counterselectable suicide vectors," *Journal of Bacteriology*, vol. 184, no. 1, pp. 307–312, 2002.

[9] X. X Huang, L. V. Phung, S. Dejsirilert, et al., "Cloning and characterization of the gene encoding the z66 antigen of *Salmonella enterica* serovar Typhi," *FEMS Microbiology Letters*, vol. 234, no. 2, pp. 239–246, 2006.

[10] K. A. Datsenko and B. L. Wanner, "One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 12, pp. 6640–6645, 2000.

[11] M. R. Sarker and G. R. Cornelis, "An improved version of suicide vector pKNG101 for gene replacement in gram-negative bacteria," *Molecular Microbiology*, vol. 23, no. 2, pp. 410–411, 1997.

[12] P. Gay, D. Le Coq, M. Steinmetz, T. Berkelman, and C. I. Kado, "Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria," *Journal of Bacteriology*, vol. 164, no. 2, pp. 918–921, 1985.

[13] P. Gay, D. Le Coq, M. Steinmetz, E. Ferrari, and J. A. Hoch, "Cloning structural gene sacB, which codes for exoenzyme levansucrase of Bacillus subtilis: expression of the gene in *Escherichia coli*," *Journal of Bacteriology*, vol. 153, no. 3, pp. 1424–1431, 1983.

[14] N. D. Cohen, E. D. McGruder, H. L. Neibergs, R. W. Behle, D. E. Wallis, and B. M. Hargis, "Detection of *Salmonella enteritidis* in feces from poultry using booster polymerase chain reaction and oligonucleotide primers specific for all members of the *genus Salmonella*," *Poultry Science*, vol. 73, no. 2, pp. 354–357, 1994.

[15] N. D. Cohen, D. E. Wallis, H. L. Neibergs, et al., "Comparison of the polymerase chain reaction using genus-specific oligonucleotide primers and microbiologic culture for the detection of *Salmonella* in drag-swabs from poultry houses," *Poultry Science*, vol. 73, no. 8, pp. 1276–1281, 1994.

[16] A. R. Desai, D. H. Shah, S. Shringi, et al., "An allele-specific PCR assay for the rapid and serotype-specific detection of *Salmonella pullorum*," *Avian Diseases*, vol. 49, no. 4, pp. 558–561, 2005.

[17] D. H. Shah, J.-H. Park, M.-R. Cho, M.-C. Kim, and J.-S. Chae, "Allele-specific PCR method based on *rfbS* sequence for distinguishing *Salmonella gallinarum* from *Salmonella pullorum*: serotype-specific *rfbS* sequence polymorphism," *Journal of Microbiological Methods*, vol. 60, no. 2, pp. 169–177, 2005.

[18] P. P. Cherepanov and W. Wackernagel, "Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant," *Gene*, vol. 158, no. 1, pp. 9–14, 1995.

[19] K. Nakayama, S. M. Kelly, and R. Curtiss III, "Construction of an ASD+ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain," *Bio/Technology*, vol. 6, no. 6, pp. 693–697, 1988.

[20] N. Philippe, J.-P. Alcaraz, E. Coursange, J. Geiselmann, and D. Schneider, "Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria," *Plasmid*, vol. 51, no. 3, pp. 246–255, 2004.

[21] D. C. Amberg, D. Botstein, and E. M. Beasley, "Precise gene disruption in Saccharomyces cerevisiae by double fusion polymerase chain reaction," *Yeast*, vol. 11, no. 13, pp. 1275–1280, 1995.

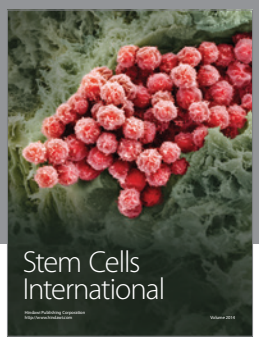
[22] H. Kuwayama, S. Obara, T. Morio, M. Katoh, H. Urushihara, and Y. Tanaka, "PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors," *Nucleic Acids Research*, vol. 30, no. 2, p. E2, 2002.

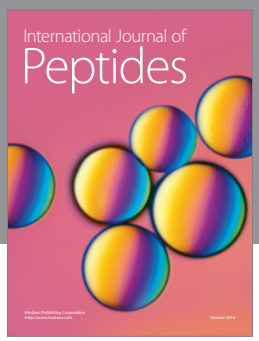
[23] R. A. Edwards, L. H. Keller, and D. M. Schifferli, "Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression," *Gene*, vol. 207, no. 2, pp. 149–157, 1998.

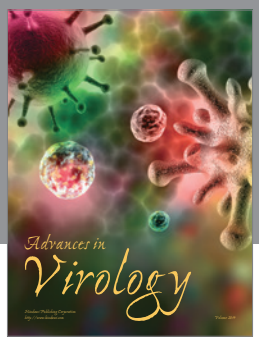
[24] J. L. Ried and A. Collmer, "An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-eviction mutagenesis," *Gene*, vol. 57, no. 2-3, pp. 239–246, 1987.

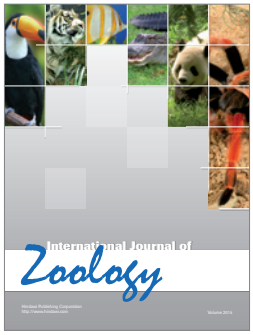
[25] H. Schmidt, M. Bielaszewska, and H. Karch, "Transduction of enteric *Escherichia coli* isolates with a derivative of Shiga toxin 2-encoding *Bacteriophage* Φ3538 isolated from *Escherichia coli O157:H7*," *Applied and Environmental Microbiology*, vol. 65, no. 9, pp. 3855–3861, 1999.

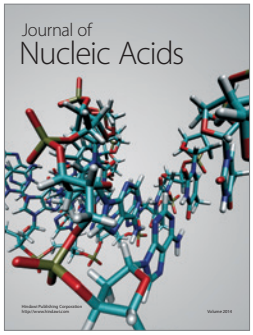
[26] F. Daigle, J. E. Graham, and R. Curtiss III, "Identification of Salmonella typhi genes expressed within macrophages by selective capture of transcribed sequences (SCOTS)," *Molecular Microbiology*, vol. 41, no. 5, pp. 1211–1222, 2001.











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