Methodology Report

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

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Received 9 February 2009; Revised 28 April 2009; Accepted 26 May 2009

Recommended by Han De Winde

An asd-deleted (Δasd) 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 CmR gene. Based on the balanced lethal host-vector system, the phenotype of the Δasd 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 Δasd 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 Δasd 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 asdp12, using primers asdp1/asdp2,
PCR product CmR and SP were amplified from genomic DNA of strain E. coli all primers used are given in Table 2.

and the PCR product asdp34, using primers asdp3/asdp4, were amplified from genomic DNA of SP S06004. The PCR product CmR, which spans the CmR cassettes and includes the flanking FRT sites of the CmF/CmR primers, was amplified from the pKD3 plasmid. These fragments were purified and cloned into the pMD18 vector and the resulting plasmids were named pMD-asdp12, pMD-asdp34 and pMD-CmR, respectively. The asdp12 and asdp34 fragments acted as two arms for homologous recombination. Subsequently, asdp12 and asdp34 were ligated via an XhoI site to produce asdp1234. A fragment of the CmR gene was then cloned into the XhoI site of asdp1234 to produce pMD-asdp1234 (Cm) (Figure 1). pMD-asdp1234 (Cm) and the pGMB151 suicide plasmid were digested with BamHI and religated to produce pGMB151-asdp1234 (Cm), which was then transferred to E. coli Spy372 [9]. pGMB151-asdp1234 (Cm) was then further transferred to E. coli χ7213, and was termed the donor strain E. coli χ7213 (pGMB151-Δasdp/Cm). The sequences of all primers used are given in Table 2.

### Table 1: Strains and plasmids used in this study.

| Strains               | Description                           | Source                     | Reference                  |
|-----------------------|---------------------------------------|----------------------------|----------------------------|
| S. Pullorum S06004   | Recipient                             | Lab collection             | Datsenko and Wanner [10]   |
| χ7213 (pGMB151-Δasdp/Cm) | Donor                                 | 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 | asdp1   | ttggatcctgaagaattgtagtc  | 1959               | BamH1                 |
|                | asdp2   | tcctgagctgtgagaaggaatc   |                    |                       |
| Downstream of asd | asdp3   | ttctccgtagctataactcgcgcgtta | 2079               |                       |
|                | asdp4   | ttgacgccagctctgctgctgctgctg|                    |                       |
| asd            | asdp5   | ttgcctcataacctgctagc     | 1796(wt)           |                       |
|                | asdp6   | ttctactgctgtgctactac     | 1360(Δasdp + Cm)   |                       |
| CmR            | CmR     | actcgcagttgcttgcagcttc   | 1032               |                       |
|                | CmF     | actcgcagtagtagggttagggtg  |                    |                       |
| rfbS           | rfbSF   | tttatctgtaagttttagttag   | 400                | S. Pullorum           |
|                | rfbSR   | tattcagttgtgcttataactcc  |                    |                       |
| hto            | htoF    | actgttggcttcccctttctgtccgtg  | 495                | genus Salmonella      |
|                | htoR    | atgccgctttccttgcttgcttgctgctg|                    |                       |
3. Results

3.1. Antibiotic Resistance of the SP Δasd Mutant. During the selection process, the antibiotic resistance of the SP Δasd mutant, the SP Δasd (Cm) mutant, and other bacterial strains was determined (Table 3). As expected, the SP Δasd mutant showed the profile NalR AmpR SmR, 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 Δasd mutant and the SP Δasd (Cm) mutant. The IMViC of the mutant was “−−−−” which was consistent with those of wt SP S06004. However, the growth velocity of the Δasd mutant and the Δasd (Cm) mutant in LB media

2.6. Elimination of the CmR Gene from of the SPΔasd (Cm) Mutant. Plasmid pCP20 is an AmpR and CmR plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis [18]. The SP Δasd (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 Δasd mutant population.

2.7. Construction of the SP Balanced Lethal Host-Vector System. The SP Δasd mutant can be complemented with a foreign asd gene from the plasmid that forms the SP balanced lethal host-vector system. The SP Δasd 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 Δasd 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 Δasd mutant to express the red fluorescent protein. This was used to further demonstrate stability of the plasmid in different passages of the SP Δasd mutant (pYA3334-dsRED), using flow cytometry (FACS) analysis.

2.4. Antibiotic Resistance, Growth and Biochemical Characteristics of the SP Δasd (Cm) Mutant. During the selection process, the SP Δasd (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 CmR gene, the mutant became resistant to Cm and depended on exogenous DAP for growth. The SP Δasd (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 Δasd (Cm) mutant were evaluated using IMViC tests.

2.5. PCR Verification of the SP Δasd (Cm) Mutant. In addition to the primers asdp1/asdp2, asdp3/asdp4 and CmR/CmR, primers asdp5/asdp6 (asdp5 is in the asdp12 sequence and asdp6 is in the asdp34 sequence), and primers asdp5/CmRY and asdp6/CmRY (Table 2) were used to further characterize the SP Δasd (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 htoF/htoR (Table 2). The rfbS gene, which specifically identifies SP [16, 17], was amplified with the primers rfbSF/rtbSR (Table 2).

Table 3: Antibiotics resistance of bacteria during the selection of Δasd mutant.

| Bacteria                  | Nal | Km | Cm | Sm | Amp |
|---------------------------|-----|----|----|----|-----|
| Salmonella Pullorum (S06004) | +   | −  | −  | −  | −   |
| χ7213(pGMB151-Δasd/Cm)    | −   | +  | +  | +  | +   |
| First crossover (S06004:pGMB151-Δasd/Cm) | +  | −  | +  | +  | +   |
| Second crossover (Δasd (Cm) mutant) | +  | −  | +  | −  | −   |
| SP Δasd mutant           | +   | −  | −  | −  | −   |

[12], 1% diaminopimelic acid (DAP) and Cm. The mutants, i.e., the SP Δasd (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 Δasd allele in the SP Δasd (Cm) mutant was confirmed by asdp5/asdp6 primers which PCR product,1360 bp, was smaller than 1796 bp amplified from wt SP S06004 (Figures 3 and 5(b)).
containing DAP was slow compared with that of the wt S06004 (Figure 4).

3.3. PCR Verification of the SP Δasd (Cm) and Δasd Mutants. PCR was used to identify the SP Δasd (Cm) and SP Δasd mutants. PCR amplification of the hto and the rfb5 genes showed that the Δasd (Cm) mutant was SP. The PCR products were amplified using primers asdp1/asdp2, asdp3/asdp4, CmR/CmR, asdp5/asdp6, asdp5/CmR, and asdp6/CmR (Figure 5(a)), which demonstrated that the asd gene had been replaced by the Cm gene. PCR amplification using the primers asdp5/asdp6 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 CmR gene was eliminated from the SP Δasd (Cm) mutant by plasmid pCP20 (Figures 3 and 5(b)). These results indicated that an SP Δasd 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 Δasd mutant was transformed with plasmid pYA3334, which contains the asd gene, the recombinant SP Δasd mutant (pYA3334) could grow without DAP. This showed that the asd gene in the plasmid could functionally complement the Δasd mutant. After transformation with the plasmid pYA3334-dsRED, the SP Δasd mutant (pYA3334-dsRED) expressed red fluorescent protein (Figure 6); in contrast, there was no red fluorescence from the SP Δasd mutant (pYA3334). The FACS analysis of dsRED expression in the different passages of the SP Δasd mutant (pYA3334-dsRED) showed that pYA3334-dsRED was stable. In the 2nd and 20th passages of the SP Δasd 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 Δasd (1488 bp) region deleted from the SP genome. The open arrow indicates the coding region of the asd gene (CmR gene), and dotted lines represent the limits of the deleted region. 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 Δasd (Cm) mutant, and the SP Δasd mutant are 1796 bp, 1360 bp, and 328 bp, respectively.

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

The 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),
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-sucide 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-sucide 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-sucide 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 Δasd 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 (CmR or KmR) of the pKD3 plasmid of the Red Disruption system was used to replace the gene of interest. Plasmid pCP20, which is AmpR and CmR, 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-sucide plasmid system or the Red Disruption system alone.

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), replacing target gene (asd gene) with Cm (or Km) gene from the Red Disruption system.
based on \(\pi\)-suicide plasmid system, which is simpler in the procedures and more effective for screening recombinants than previously reported conventional methods.

**Acknowledgments**

The authors thank sincerely Dr. Roy Curtiss III and Dr. Cristina Marolda 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. The authors thank sincerely the editor and reviewers for their kind comments and suggestions. This research is supported by the National Natural Science Foundation of China (30425031) and National programs for Fundamental Research and Development of China (2006CB504404).

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