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Evolution of genes on the *Salmonella* Virulence plasmid phylogeny revealed from sequencing of the virulence plasmids of *S. enterica* serotype Dublin and comparative analysis

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ABSTRACT

Salmonella enterica serotype Dublin harbors an approximately 80-kb virulence plasmid (pSDV), which mediates systemic infection in cattle. There are two types of pSDV: one is pSDVu (pOU1113) in strain OU7025 and the other pSDVr (pOU1115) in OU7409 (SD Lane) and many clinical isolates. Sequence analysis showed that pSDVr was a recombinant plasmid (co-integrate) of pSDVu and a plasmid similar to a 35-kb indigenous plasmid (pOU1114) of *S.* Dublin. Most of the F-transfer region in pSDVu was replaced by a DNA segment from the pOU1114-like plasmid containing an extra replicon and a *pilX* operon encoding for a type IV secretion system to form pSDVr. We reconstructed the particular evolutionary history of the seven virulence plasmids of *Salmonella* by comparative sequence analysis. The whole evolutionary process might begin with two different F-like plasmids (IncFI and IncFII), which then incorporated the *spv* operon and fimbriae operon from the chromosome to form the primitive virulence plasmids. Subsequently, these plasmids descended by deletion from a relatively large plasmid to smaller ones, with some recombination events occurring over time. Our results suggest that the phylogeny of virulence plasmids as a result of frequent recombination provides the opportunity for rapid evolution of *Salmonella* in response to the environmental cues.

Introduction

Salmonella infection of human beings and animals continues to be a distressing health problem worldwide. Taxonomically, *Salmonella* organisms are considered as a single species, known as *Salmonella enterica*, which include more than 2,500 different serotypes, or serovars. Some serotypes, such as *S.* Typhi and *S.* Paratyphi, are highly adapted to humans without other known natural hosts, while others, such as *S.* Typhimurium, have a broad host range and infect a wide variety of animal hosts. A few serotypes, such as *S.* Dublin and *S.* Choleraesuis, are most adapted to single animal species and only occasionally infect humans. In cattle, two main clinical patterns of salmonellosis are evident [1]: in young animals, *S.* Typhimurium is the predominant etiological agent of acute enteritis, which results in severe dehydration and high mortality if left untreated, while *S.* Dublin is predominant in older animals to cause both enteric and systemic infections, including septicemia and abortion.

Among *Salmonella* serotypes, some, i.e., serotypes Abortusequi, Abortusovis, Choleraesuis, Dublin, Enteritidis, Gallinarum-Pullorum, Sendai, and Typhimurium, are known to harbor a *Salmonella* virulence plasmid involved in the virulence of the host serotype. Nearly all the clinical isolates of *S.* Dublin from veterinary or human sources harbor a virulence plasmid (pSDV) [2–5]. The pSDV carries an *spv* operon, which is universally present in the virulence plasmids of *Salmonella* [6,7] and in the chromosome of *S. enterica* subspecies I, II, IIIa, IV, and VII [8]. The *spv* operon consists of five genes, *spvRABCD*, which are regulated by environmental and bacterial growth conditions [9–11]. For *S.* Dublin, this operon is a prerequisite for causing severe enteritis and systemic infection in cattle [12].

Previous studies have shown that there are two types of pSDV, according to physical and genetic mapping: one is pSDVr (pOU1115 or pSDL2) of *S.* Dublin strain Lane [13] and the other pSDVu (pOU1113) of strains OU7025 and OU7052 [6]. Incompatibility analysis also revealed that the two plasmids of *S.* Dublin were different from each other. Previously we have sequenced pSDVu (Accession No. AY517905). In this study, we further sequenced pSDVr and a 35-kb indigenous non-pSDV plasmid, pOU1114, of *S.* Dublin. We carried out comparative analysis of the DNA sequences of the two pSDVs and other *Salmonella* virulence plasmids, aiming to reconstruct the particular evolutionary

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history of the genetically and medically important virulence plasmids of *Salmonella*.

Results and discussion

Plasmid analysis of S. Dublin

The sizes of the two virulence plasmids of *S.* Dublin were both nearly 80 kb (Table 1). The *Hin*dIII-digested RFLP pattern differed between the two pSDVs, and when pSTV was used as a probe, four *Hin*dIII-digested DNA fragments with sizes of 14.2 kb (H3), 9.3 kb (H4), 5.6 kb (H6), and 1.5 kb (H9) in pSDVr and the pSDV of all clinical isolates were not hybridized; in contrast, these fragments hybridized to the probe derived from the 35-kb pOU1114 of OU7029 (Fig. 1). This result indicates that all clinical isolates harbored pSDVr, thereby suggesting an evolutionary advantage of pSDVr over pSDVu. Previously, we have shown that when OU7025 was introduced with pSDVr::Tn*5*, the original pSDVu as well as the 35-kb pOU1114 was expelled [6]. In this study, we further tagged Tn*5* onto pSDVu and pOU1114, and tested if the pSDVu::Tn*5*, the pSDVr::Tn*5*, and the pOU1114::Tn*5* were conjugative. In accordance with previous reports [14,15], we found that pSDVu was nonconjugative. However, pOU1114 was highly conjugative, with an efficiency of $4.3\,10^{-2}$; in contrast, pSDVr was barely conjugative, with an extremely low efficiency of $<10^{-8}$.

Sequence comparison among pSDVu, pSDVr, and pOU1114

Physical and genetic maps showed that pSDVr was seemingly a recombinant plasmid of pSDVu and the non-pSDV pOU1114 (Fig. 1). Consistent with this, the pSDVr was incompatible with pSDVu and pOU1114 [6]. Earlier, pSDVu has been sequenced with a size of 80,156 bp [16]. In this study, we further determined the sizes of pSDVr and pOU1114 to be 74,598 and 34,595 bp, respectively. The G+C content and number of *orf*s differed among pSDVu, pSDVr, and pOU1114 (Table 2). The highest G+C content was observed in pSDVu with 53.8% and the lowest in pOU1114 with 41.45%. Sequence annotation and comparison showed that the recombination event occurred between *traI* and *tlpA*. It appears that a 28-kb fragment of pOU1114 replaced the original F-transfer region from *traI* to *tlpA* in pSDVu to form pSDVr. This region consists of 37 *orf*s, which include a *pilX* operon encoding a Type IV secretion system, plasmid stability genes, *stbD* and *stbE*, and a plasmid replication gene, *stpA* (Fig. 2).

Nevertheless, pOU1114 still differed from its counterpart in pSDVr in the segment encoding the plasmid partition system. Between 30K and 32K in pOU1114, there lie *parA* and two hypothetical genes (no *parB*); but in the corresponding region (from 62K to 63K), pSDVr has *parG* and *parF* instead. The *parG* and *parF* are homologs of *parA* and *parB*; they might share the same function, but their sequences differed greatly [17].

Taken together, it is likely that a relative plasmid of pOU1114 recombined with pSDVu to form pSDVr. Another possibility is that after the recombination of pSDVu and pOU1114, some other homologous recombination events occurred and therefore changed the gene composition of pSDVr. The former explanation gains more

Table 1
*Hin*dIII restriction fragment profiles of seven virulence plasmids of *Salmonella*[a]

Fragment	pSAV	pSCV	pSDVu	pSDVr	pSEV	pSPV	pSTV
H1	37.1	15.9	23.4	17.4	16.5	21.0	37.1
H2	16.5	12.2	16.5	16.5	12.7	16.5	16.5
H3	12.7	11.8	15.8	13.5	12.3	15.5	12.7
H4	10.0	3.8	9.0	9.0	10.1	15.5	9.4
H5	6.0	3.7	8.5	7.1	3.7	8.5	7.4
H6	5.0	2.6	3.7	5.5	2.8	3.7	3.7
H7	3.7		2.6	3.7	2.0	2.6	3.3
H8				2.6		2.6	2.6
H9				1.5			
Total size	91.6	50.0	79.5	76.8	60.1	85.9	92.7

[a] pSAV, virulence plasmid of *S.* Abortusovis OU7058; pSCV, virulence plasmid of *S.* Choleraesuis OU7085; pSDVu, virulence plasmid of *S.* Dublin OU7025; pSDVr, virulence plasmid of *S.* Dublin strain Lane (OU7409); pSEV, virulence plasmid of *S.* Enteritidis; pSPV, virulence plasmid of *S.* Gallinarum-Pullorum OU7115; pSTV, virulence plasmid of *S.* Typhimurium C5.

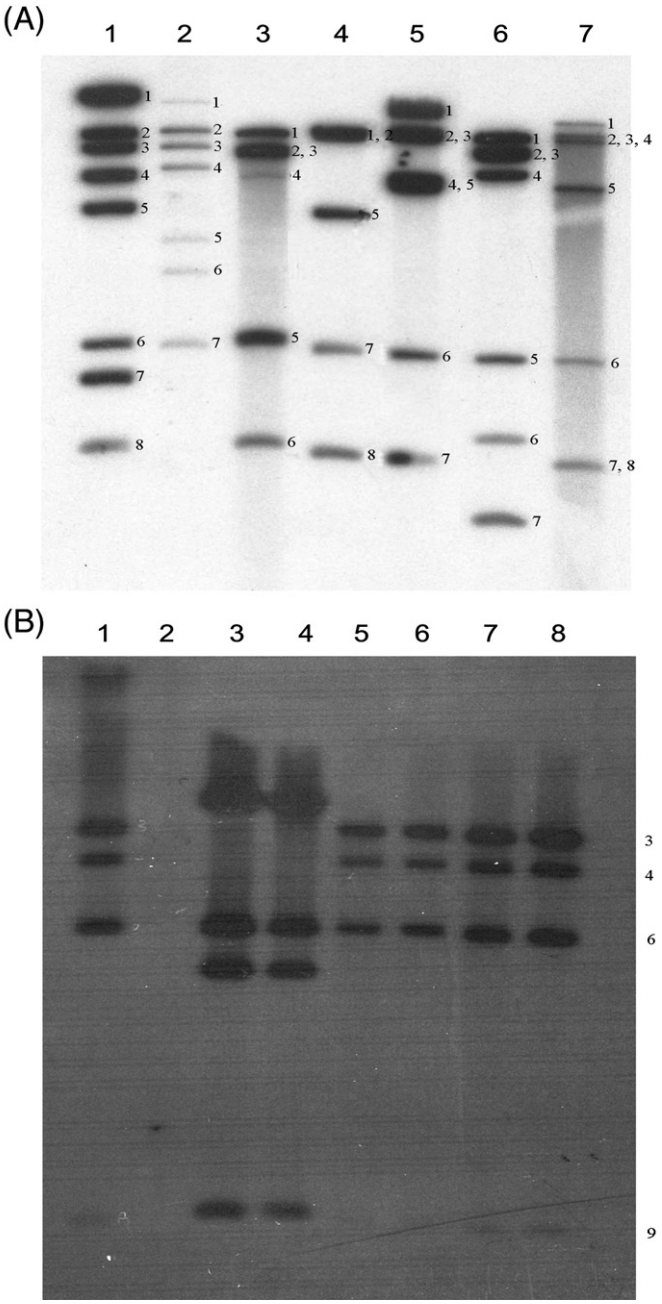

Fig. 1. Comparative analysis of the seven *Salmonella* virulence plasmids. (A) Restriction fragment length polymorphism (RFLP) analysis of the seven virulence plasmids by *Hin*dIII digestion, followed by DNA-DNA hybridization using the 92.7-kb pSTV of OU5045 as the probe. Lanes: 1, pSTV of *S.* Typhimurium; 2, pSAV of *S.* Abortusovis; 3, pSCV of *S.* Choleraesuis; 4 and 5, pSDVr and pSDVu of *S.* Dublin; 6, pSEV of *S.* Enteritidis; 7, pSPV of *S.* Gallinarum-Pullorum. (B) DNA-DNA hybridization of the virulence plasmids of clinical and laboratory strains of *S.* Dublin using the 35-kb pOU1114 as the probe. Lanes: 1, pSDVu from OU7052; 2, STV from S. Typhimurium OU5045; 3, pSDVu from OU7025; 4, pOU1114 from OU7029 (4), OU7432 (5), OU7444 (6), OU7501 (7 and 8). The numbers indicated on the right side of each band correspond to the *Hin*dIII-digested DNA fragments shown in Table 1.

Table 2
General features of pOU1114, pSDVu, and pSDVr[a]

Characteristics	pOU1114	pSDVr	pSDVu
Plasmid size (bp)	34595	74589	80156
G+C content (%)	41.5	48.6	53.8
Number of *orf*s	41	85	82

[a] GenBank accession numbers: pOU1114, DQ115387; pSDVr (pOU1115), DQ115388; pSDVu (pOU1113), AY517905. The number of *orf* is estimated based on the deduced amino acid length not smaller than 50.

support from the analysis of nucleotide divergence: divergence (mismatch divided by comparable bases) calculated from alignment between pSDVu and pSDVr is 0.002, while the value is 0.012 between pOU1114 and pSDVr.

Functionally, pOU1114 was conjugative, whereas pSDVr showed a low efficiency of conjugation. Transfer regions including *taxB*, *pilX* operon, *actX*, and *tax* from the two plasmids involved in conjugation have been carefully examined. Only the sequence of *pilX11* was different between the two plasmids. In pSDVr, the 1,032-bp *pilX11* had an internal frameshift mutation and therefore it might be a pseudogene. However, because the frameshift occurred at the 740th bp (GGGGG to GGGGGG), *pilX11* in pSDVr still encoded a ca. 250-amino acid peptide, whose sequence was the same as its counterpart in pOU1114. It might be a genetic degeneration and the *pilX11* in pSDVr could still possess its function. This is a possible reason for pSDVr still being conjugative but showing lower conjugation efficiency than pOU1114. Furthermore, because pSDVr is much larger than pOU1114, its conjugation capability is expected to be lower than that of the smaller pOU1114.

Phylogenetic analysis of Salmonella virulence plasmids and reconstruction of the evolutionary route

Previously, we have compared the physical and genetic maps of four virulence plasmids in *S.* Typhimurium, *S.* Enteritidis, *S.* Choleraesuis, and *S.* Dublin and reported two types of virulence plasmids in *S.* Dublin [6,14]. *S.* Abortusovis and *S.* Gallinarum-Pullorum also contain a virulence plasmid. Using pSTV of *S.* Typhimurium OU5045 and 14 PCR products as probes, we were able to identify differences among the seven known virulence plasmids from six serotypes by *Hin*dIII restriction analysis (Table 1). DNA fragments of 16.5 and 3.7 kb are conserved in all virulence plasmids, except for a minor difference (15.9 kb) of the H1 fragment in pSCV (Table 1). Using pSTV as a standard, DNA variation was found in fragments H1, H5, and H6 of pSAV, H1 of pSDVu, and H1, H3, H4, H6, and H9 of pSDVr (Fig. 1). The virulence plasmids of *Salmonella* can therefore be separated into two distinct lineages based on their physical and genetic maps: the first group includes pSCV, pSEV, pSTV, and probably pSAV and the other pSPV and the two pSDVs. The major difference between the two lineages was located between *repA* of RepFIIA and the *ccd* operon. The change in gene order was as follows: *repA* (RepFIIA)-*rck-srgA-srgB-pefI-pefD-pefC-pefA-pefB-repA* (RepFIB)-*ccdA* for the first group and *repA* (RepFIIA)-*fedE-faeH-faeI-faeJ-faeA-srgB-vagD-vagC-ccdA* for the second. Apparently, the major difference between the two lineages of virulence plasmids is the plasmid-associated fimbrial genes. All seven virulence plasmids shared a conserved core structure, suggesting that the plasmids are likely to descend from a common ancestor, but the question is how these virulence plasmids with different sizes, which are unique to the host serotype, are formed. A hypothesis is that virulence plasmids are sequentially or independently formed by recombination. This possibility was supported by earlier studies showing that there were pSCV with a size larger than 50 kb, the commonest size, and these large pSCVs were mostly co-integrates formed with a resistant plasmid [18]. A co-integrate (pWR33) between a pSTV and F′::Tn*5lac*$^+$ was also shown to form in vitro [19]. We have found in this study that pSDVr is a recombinant plasmid (co-integrate) of pSDVu and a relative plasmid of pOU1114. In this event, pSDVr obtained an additional replicon highly homologous to that of *E. coli* IncX plasmid R6K (Accession No. AJ006342) and a *pilX* operon of pOU1114. The genes of the *pilX* operon encode a type IV secretion system similar to VirB in T-DNA of *Agrobacterium tumefaciens*, responsible for DNA transfer [20]. In the case of pSCV, the recombination event gives *Salmonella* a survival advantage in an unfavorable drug environment [18]. The integration of additional replicons from another indigenous plasmid to pSDVu constitutes a new evidence for the evolution of virulence plasmid in *Salmonella* via recombination. The result supports the hypothesis that the phylogeny of virulence plasmids as a result of frequent recombination provides the opportunity for rapid evolution of *Salmonella* in response to environmental cues. As shown in this study, the majority of the clinical isolates of *S.* Dublin that circulated in the community to cause infections harbored pSDVr, rather than pSDVu.

As mentioned earlier, there were at least two lineages of virulence plasmids discerned by sequence comparison [14]. After the discovery of the *spv* operon in the chromosome of *S. enterica*, it was proposed that the formation of the virulence plasmids might be through integration and excision events of F-like plasmids near the *spv* operon in the chromosome [8]. A recent study suggested a two-stage theory of incorporation of RepFIIA and RepFIB independently to give rise to pSPV from pSCV, pSEV, and pSTV [21]. According to the sequence analysis of known virulence plasmids, especially the regions from *oriT* to *traA*, we further proposed that the genesis of the *Salmonella* virulence plasmids might begin with two different F-like plasmids. One IncFI-like plasmid is the ancestor of pSTV, pSEV, and pSCV, and the other IncFII-like plasmid is that of pSPV and pSDV. All the current virulence plasmids of different sizes and with more resistance genes and replicons evolved from recent recombination and deletion events. Furthermore, although the virulence plasmids could be divided into two groups based on sequence comparison, all of them carried the same signature sequences for transposition flanking at both ends of the *spv* operon, suggesting that other evolutionary processes such as

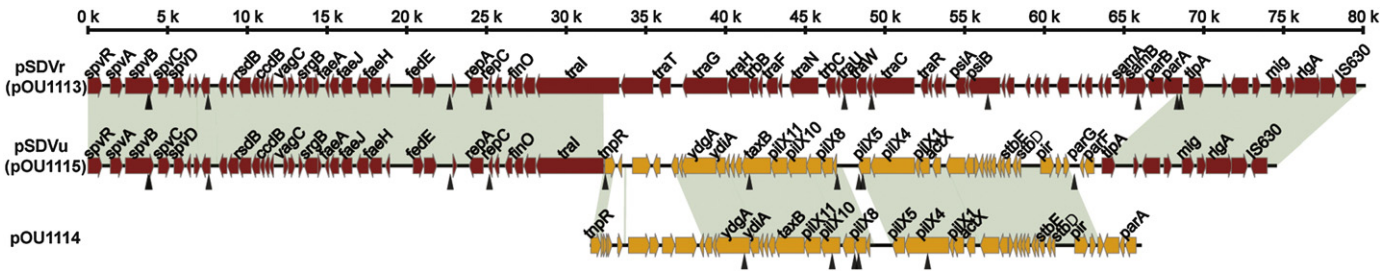

Fig. 2. Linear representation of pOU1114, pSDVu, and pSDVr. Red arrows on pSDVr axis and yellow arrows on pOU1114 axis represent their genes and direction of transcription, respectively. The same genes are represented with the same color in pSDVu, reflecting the recombinant nature of pSDVu. Light green blocks represent homologous region between plasmids (nucleotide similarity > 90%). Black triangles under axes indicate *Hin*dIII restriction sites.

Table 3
Bacterial strains used in this study

Strain name	Species or serotype	Virulence plasmid (kb)	Other plasmid or source of the strain
Hu735	*E. coli*	-	F'*lac*$^+$Tn*5ts*
OU5045	*S.* Typhimurium C5	pSTV (92.7)	Laboratory strain
OU7025	*S.* Dublin	pSDVu (79.5)	pOU1114 (35-kb non-pSDV plasmid)
OU7029	*S.* Dublin	-	A derivative of OU7025 lacking pSDV but containing pOU1114
OU7052	*S.* Dublin	pSDVu (79.5)	Laboratory strain
OU7058	*S.* Abortusovis	pSAV (~90)	Laboratory strain
OU7085	*S.* Choleraesuis	pSCV (50)	Laboratory strain
OU7130	*S.* Enteritidis	pSEV (60)	Laboratory strain
OU7115	*S.* Gallinarum-Pullorum	pSPV (86)	Laboratory strain
OU7409	*S.* Dublin Lane	pSDVr (76.8)	Laboratory strain
OU7499	*S.* Dublin Lane	PSDVr::Tn*5* or pSDL2::Tn*5* (82.6)	Laboratory strain
OU7432	*S.* Dublin	pSDVr (76.8)	Clinical isolate
OU7444	*S.* Dublin	pSDVr (76.8)	Clinical isolate
OU7501	*S.* Dublin	pSDVr (76.8)	Clinical isolate

transposition have occurred during the formation of various virulence plasmids. This is also true for the resistance genes, most of which are carried by transposons or integrons in *Salmonella* [18,22,23].

Concluding remarks

Plasmids, as part of the floating genome of bacterial organisms, appear to be a miniature of the bacterial pan-genome, which is composed of a core genome and a dispensable genome. The host-specificity of *Salmonella* indicates that different serotypes of *Salmonella* exploit different ecologies. It therefore can be expected that the host-specific virulence plasmids of *Salmonella* deal with different intracellular environments and extracellular selections. In this study, we found that the collection of genes shared by all seven virulence plasmids, i.e., virulence-related genes, is under negative selection. These genes, such as the *spv* operon, are conserved and stable, suggesting that they play an important role in the expression of virulence during the process of infection. On the other hand, the variable regions on these plasmids have been shaped by multiple forces that include deletion, gene loss (degeneration), recombination, and gene acquisition by lateral gene transfer. The evolution of the virulence plasmids is apparently linked to the adaptation of their *Salmonella* hosts to the environmental niche.

Materials and methods

Bacterial strains and plasmid analysis

The clinical isolates and laboratory strains used in this study are shown in Table 3. Clinical *S.* Dublin isolates were collected from Chang Gung Memorial Hospital, Taoyuan, Taiwan. Serotyping was performed by agglutination test using O and H antisera. Plasmid patterns of these strains were determined by a modified alkaline lysis method [24].

DNA preparation and DNA-DNA hybridization

The plasmid DNA of bacterial strains were extracted and purified by the CsCl gradient method described elsewhere [19]. Purified plasmid DNA was first digested with restriction endonuclease *Hin*dIII and then separated by 0.6% SeaKem GTG agarose. DNA was then transferred onto the Zeta-probe membrane (Bio-Rad). DNA-DNA hybridization was carried out according to the method of Southern [25]. The primers used to detect 14 gene fragments in the plasmids were described by Chu et al. [14]. The PCR products were purified from gel by a Wizard PCR Preps DNA Purification System (Promega, USA). The purified products and the virulence plasmid (pSTV) of *S.* Typhimurium strain C5 were labeled with ^{32}P-dCTP (specific activity 3,000 Ci/mmole, Amersham) by the random priming method described by the manufacturer (BRL) and used as probes. After the hybridization process, the sample was exposed to an X-ray film with intensifying screen.

DNA sequencing of pSDVr and pOU1114 and annotation

Purified pSDVr and pOU1114 were first fragmented by a Gene-Machine HydroShear (BST Scientific Pte, Ltd., Singapore). DNA approximately 2 kb in size was purified from gel by a Promega DNA purification kit (Promega, USA), modified by Klenow enzyme, and then cloned into *Sma*I-digested pBluescript II. Random clones were picked and the inserts sequenced by using ABI3730 autosequencer. Gap closure was carried out by PCR and sequencing. The total sequence of the reads approached approximately 6 coverage of the final size of each plasmid. Phred/Phrap/Consed software package was used for quality assessment and sequence assembly. The accuracy of the plasmid nucleotide sequence was verified by pulsed-field gel electrophoresis using *Bam*HI, *Hin*dIII, and *Xba*I for digestion. The nucleotide sequences of the two plasmids were analyzed and annotated according to the algorithm described earlier [26]. GenBank accession numbers of pOU1114 and pSDVr (pOU1115) are DQ115387 and DQ115388, respectively.

Bacterial conjugation

In conjugation, the donor and recipient strains were grown to the late exponential phase (4 10^8 bacteria/ml) and mixed 1:1. The bacterial mixture was incubated without shaking at 37C for 18 h and plated on MacConkey plates containing appropriate drugs (kanamycin, ampicillin, or both) for transconjugant selection. The efficiency of mating was calculated by dividing the number of transconjugants by the input number of the minority parent. To tag Tn*5* onto a *Salmonella* plasmid, *E. coli* HU735, an F' strain that harbored the F'*lac*$^+$ plasmid, was mated with a *Salmonella* recipient. *Salmonella* expressing lac$^+$ and Km^r on MacConkey plates were picked. The successful tagging was confirmed by plasmid extraction, followed by restriction fragment length polymorphism (RFLP) analysis.

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