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Improving *Salmonella* vector with *rec* mutation to stabilize the DNA cargoes

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Abstract

Background: *Salmonella* has been employed to deliver therapeutic molecules against cancer and infectious diseases. As the carrier for target gene(s), the cargo plasmid should be stable in the bacterial vector. Plasmid recombination has been reduced in *E. coli* by mutating several genes including the *recA*, *recE*, *recF* and *recJ*. However, to our knowledge, there have been no published studies of the effect of these or any other genes that play a role in plasmid recombination in *Salmonella enterica*.

Results: The effect of *recA*, *recF* and *recJ* deletions on DNA recombination was examined in three serotypes of *Salmonella enterica*. We found that (1) intraplasmid recombination between direct duplications was RecF-independent in Typhimurium and Paratyphi A, but could be significantly reduced in Typhi by a $\Delta recA$ or $\Delta recF$ mutation; (2) in all three *Salmonella* serotypes, both $\Delta recA$ and $\Delta recF$ mutations reduced intraplasmid recombination when a 1041 bp intervening sequence was present between the duplications; (3) $\Delta recA$ and $\Delta recF$ mutations resulted in lower frequencies of interplasmid recombination in Typhimurium and Paratyphi A, but not in Typhi; (4) in some cases, a $\Delta recJ$ mutation could reduce plasmid recombination but was less effective than $\Delta recA$ and $\Delta recF$ mutations. We also examined chromosome-related recombination. The frequencies of intrachromosomal recombination and plasmid integration into the chromosome were 2 and 3 logs lower than plasmid recombination frequencies in Rec⁺ strains. A $\Delta recA$ mutation reduced both intrachromosomal recombination and plasmid integration frequencies.

Conclusions: The $\Delta recA$ and $\Delta recF$ mutations can reduce plasmid recombination frequencies in *Salmonella enterica*, but the effect can vary between serovars. This information will be useful for developing *Salmonella* delivery vectors able to stably maintain plasmid cargoes for vaccine development and gene therapy.

Background

Attenuated *Salmonella* are being developed as vaccines to protect against typhoid fever [1-3]. There are also endeavors employing *Salmonella* as delivery vectors for therapeutic molecules. One strategy utilizes attenuated *Salmonella*, which expresses a gene or gene fragment encoding a protective antigen as vaccine against bacterial pathogens [4-6]. The heterologous genes can be expressed from the *Salmonella* chromosome, or, more often, from a multi-copy plasmid [7,8]. Another strategy exploits *Salmonella* as a delivery vector of DNA vaccine against viral pathogens [4,5,9]. The later strategy is also used to deliver DNA encoding tumor antigen or

cytokine for therapeutic applications in oncology [10,11]. In addition, *Salmonella* is used to deliver small interfering RNAs (siRNA) [12], ribozymes [13] and large DNA molecules encoding a viral genome [14]. For instance, *in vivo* delivery of an artificial bacterial chromosome (BAC) carrying the viral genome of the murine cytomegalovirus (MCMV) by *Salmonella* Typhimurium led to a productive virus infection in mice and resulted in elevated titers of specific antibodies against lethal MCMV challenge [14].

Most vaccine designs utilize *Salmonella* delivery vectors carrying a single plasmid for expression of a single antigen or of a fusion protein carrying epitopes from more than one antigen [15]. To induce broader immunity against a particular pathogen or various pathogens, one might need to express multiple antigens from a

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single plasmid carrying different antigen cassettes or from multiple plasmids in a single cell, each expressing one or more relevant antigens. Co-delivery of plasmids encoding tumor antigens and cytokines by *Salmonella* has been successfully demonstrated to improve protective immunity against cancer [16]. In the case where multiple plasmids are carried in the same *Salmonella* vector strain, there are most likely regions of homology between the plasmids, since the widely used pUC- and pBR-based plasmids have origins of replication that are nearly identical and both share regions of homology with the p15A *ori*. Additionally, commonly used promoter sequences, transcriptional terminators and other expression plasmid components may also be present on plasmids coexisting in the same bacterial cell. The presence of these similar or identical DNA sequences would serve to facilitate undesirable interplasmid recombination. In some cases the bacterial vector may intentionally harbor multiple copies of the same DNA sequence, which may lead to plasmid instability. Recently, we encountered such a situation during the development of a bacterial based influenza vaccine. We constructed a single plasmid carrying eight head-to-tail connected influenza cDNA cassettes [17]. The plasmid was intended for delivery into host cells by an attenuated *Salmonella* strain. The multiple repetitive sequences residing in the plasmid make its stability within the attenuated *Salmonella* an important concern because any intraplasmid recombination event results in deletion of one or more influenza gene cassettes.

Recent work in our laboratory has focused on developing new strategies for attenuated *Salmonella* vaccine strains, with features including regulated delayed *in vivo* attenuation [18,19], regulated delayed *in vivo* antigen synthesis [18,20-22], and programmed delayed *in vivo* cell lysis [23,24]. For all of these systems, one or more chromosomal and/or plasmid genes are placed under the control of the *araC* P_{BAD} promoter. Eventually, our goal is to combine all of these features into a single *Salmonella* vaccine vector strain. Such a strain will therefore carry multiple chromosomal and plasmid copies of *araC* P_{BAD}, providing sites for potential recombination, which could lead to unwanted chromosomal or plasmid rearrangements.

However, to our knowledge, there have been no published studies specifically designed to evaluate plasmid recombination in *Salmonella enterica*. Deletions of several *Escherichia coli* genes are known to reduce the frequency of plasmid recombination, including the *recA*, *recE*, *recF* and *recJ* genes [25-30]. The *recA* gene encodes the general recombinase RecA, involved in nearly all forms of recombination in the cell [31]. The RecE, RecF and RecJ proteins play a role in plasmid

recombination and recombination repair [32,33]. The RecA, RecF and RecJ proteins are highly homologous between *E. coli* and *S. enterica*, therefore they may play similar roles in DNA recombination. Despite these possible similarities, the recombination systems in the two organisms differ somewhat, as *S. enterica* does not encode *recE* [34]. Based on these concerns, we decided to determine the effect of *rec* gene deletions on intraplasmid recombination, interplasmid recombination, intrachromosomal recombination and plasmid integration in *S. enterica*.

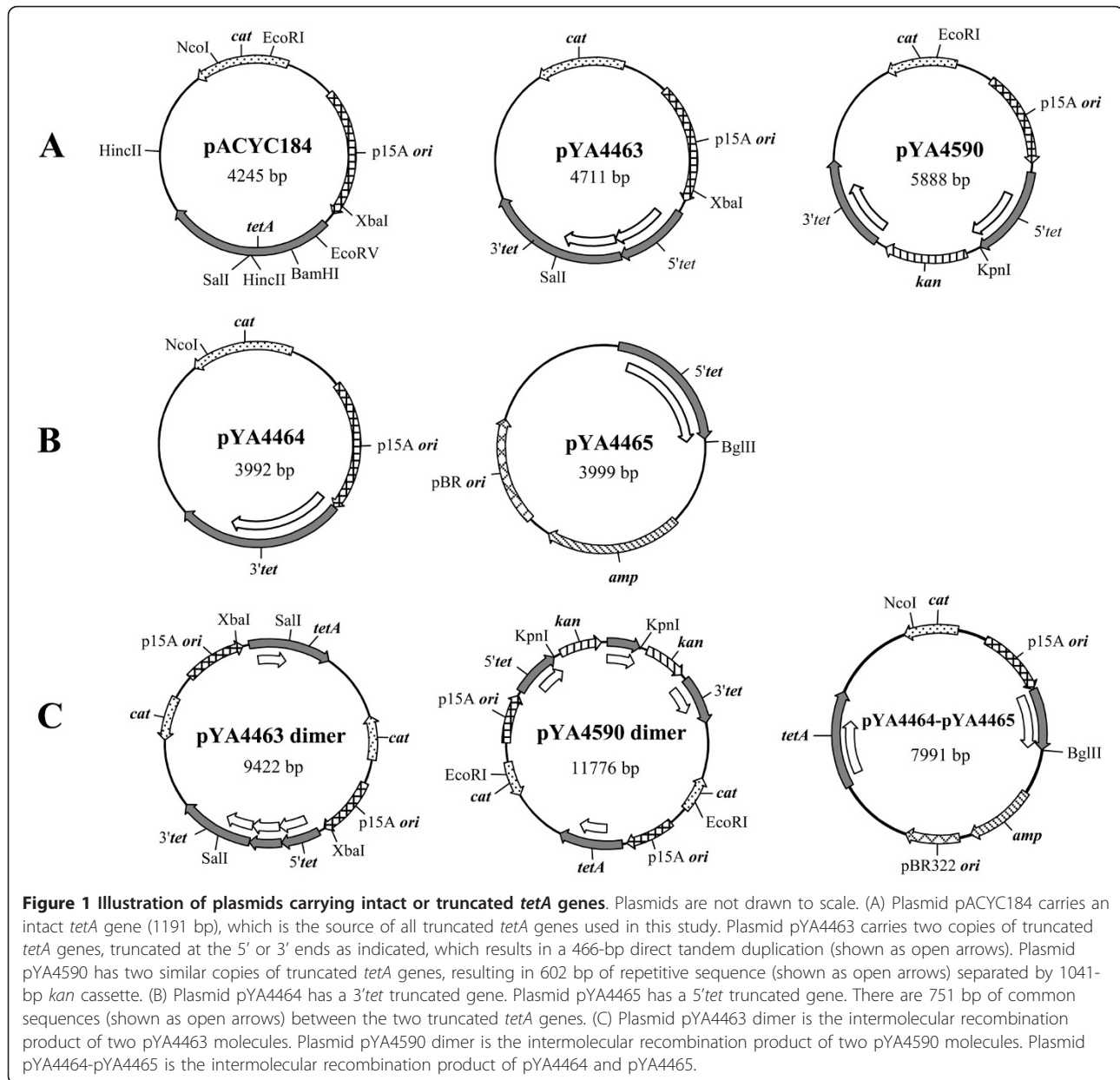
In this work, we examine the effect of $\Delta recA$, $\Delta recF$ and $\Delta recJ$ mutations on DNA recombination frequencies in three serovars of *Salmonella enterica* currently relevant to vaccine development. Our results show that the effect of these mutations on recombination can vary among *Salmonella* serovars and with previously published results in *E. coli*.

Results

Plasmid construction

We constructed a series of plasmids (Figure 1 and Table 1) encoding various truncated *tetA* genes to assay plasmid recombination frequencies using the strategies similar to those described previously [28,35]. Restoration of a functional *tetA* gene via intra- or intermolecular recombination resulted in a change of the bacterial phenotype from tetracycline sensitive to tetracycline resistant, and served as a marker allowing us to measure the frequency of recombination events (Figure 2).

Plasmids pYA4463 and pYA4590 were constructed to test intraplasmid recombination (Figure 1 panel A). Plasmid pYA4463 carries two truncated *tetA* genes (5' end and 3' end), which have 466-bp of tandemly repeated sequence. An intramolecular recombination event can delete one of the repeats resulting in an intact *tetA* gene, thereby recreating the structure of plasmid pACYC184 (Figure 1 panel A). Theoretically, intermolecular recombination may occur between two pYA4463 molecules to form a plasmid dimer with a functional *tetA* gene (Figure 1 panel C). Plasmid pYA4590 contains a 602-bp *tetA* sequence duplication separated by a 1041-bp *kan* cassette. The intramolecular recombination product is equivalent to pACYC184. The intermolecular recombination product is a dimer plasmid containing an intact *tetA* gene (Figure 1 panel C). Plasmids pYA4464 and pYA4465 carry the 3'*tet* gene and 5'*tet* gene, respectively (Figure 1). The Rec⁺ *Salmonella* strain χ 3761 carrying either plasmid individually was sensitive to tetracycline. There is 751-bp of *tetA* DNA in common between the two truncated *tetA* genes. Recombination between the two plasmids creates a hybrid plasmid containing an intact *tetA* gene (Figure 1 panel C).



Intraplasmid recombination products

To verify the recombination products, plasmid DNA was prepared from tetracycline resistant (Tc^R) single colonies derived from χ 3761(pYA4463), χ 3761(pYA4590) and χ 3761(pYA4464, pYA4465). Plasmids extracted from Tc^R clones of χ 3761(pYA4463) were digested with *XbaI* and *Sall*. Theoretically, *XbaI/Sall* digestion of pYA4463 will yield two fragments (3524 bp and 1187 bp), pACYC184 will yield two fragments (3524 bp and 721 bp) and pYA4463 dimer will yield four fragments (3524 bp, 3524 bp, 1653 bp and 721 bp). The results (Figure 3A) showed that digestion of all 16 Tc^R clones yielded a 721-bp band, indicating either a

pYA4463 dimer or a plasmid equivalent to pACYC184. Three clones (lane 1, 5 and 10) yielded the pYA4463 dimer-specific 1653-bp band. Therefore, we conclude that the other 13 clones recombined to form the pACYC184-like structure. Of note, several clones (2, 13-16) also yielded the 1187-bp pYA4463-specific band, suggesting that the original plasmid (pYA4463) and its recombination product (pACYC184-like) could coexist in the same bacterial cell.

Plasmids extracted from Tc^R clones of χ 3761(pYA4590) were digested with *KpnI* and *EcoRI*. Theoretically, plasmid pYA4590 will be digested into two fragments (3414 bp and 2474 bp), plasmid pACYC184 will

Table 1 Plasmids used in this study

Plasmid	Relevant characteristic(s)*	Reference or source
pACYC184	<i>cat</i> , <i>tetA</i> , p15A <i>ori</i>	[59]
pBAD-HisA	<i>amp</i> , pBR <i>ori</i>	Invitrogen
pKD46	λ Red recombinase expression plasmid	[60]
p15A-PB2-kan	<i>cat</i> , <i>kan</i> , p15A <i>ori</i>	This study
pYA4463	pACYC184, adjacent 5'tet and 3'tet	This study
pYA4464	pACYC184, 3'tet	This study
pYA4465	pBAD-HisA; 5'tet	This study
pYA4590	pACYC184, 5'tet-kan-3'tet	This study
pYA4373	<i>cat-sacB</i>	[54]
pRE112	<i>oriT</i> , <i>oriV</i> , <i>sacB</i> , <i>cat</i>	[61]
pYA3886	pRE112, Δ <i>recF126</i>	This study
pYA4783	pYA3886, Δ <i>recF1074</i>	This study
pYA3887	pRE112, Δ <i>recJ1315</i>	This study
pYA4680	pRE112, Δ <i>recA62</i>	This study
pYA4518	pYA4464, <i>cat</i> , p15A <i>ori</i> , GFP gene	This study
pYA4518-cysG	Two <i>cysG</i> fragments	This study
pYA4689	pYA4518-cysG, 5'tet-kan-3'tet	This study
pYA4690	pYA4518-cysG, 5'tet-kan	This study
pYA5001	<i>aacC1</i> , pSC101 <i>ori</i> , T vector	This study
pYA5002	pYA5001, <i>recA</i> cassette from Typhimurium χ 3761	This study
pYA5004	pYA5001, <i>recA</i> cassette from Typhi Ty2 χ 3769	This study
pYA5005	pYA5001, <i>recF</i> gene from Typhimurium χ 3761	This study
pYA5006	pYA5001, <i>recF</i> gene from Typhi Ty2 χ 3769	This study

**cat*: chloramphenicol resistance gene; *tetA*: tetracycline resistance gene; *amp*: ampicillin resistance gene; *kan*: kanamycin resistance gene; 3'tet: 3' portion of the *tetA* gene; 5'tet: 5' portion of the *tetA* gene together with its promoter; *aacC1*: 3-N-aminoglycoside acetyltransferase.

be linearized (4245 bp) and the pYA4590 plasmid dimer will be digested into four fragments (4245 bp, 3414 bp, 2474 bp and 1643 bp). Examination of the restricted DNA (Figure 3B) showed that only one clone (lane 12) had the pYA4590 dimer-specific 1643-bp band. The most prominent band in the other lanes was a 4245-bp band expected for pACYC184-like recombination products. Nine clones contained a mixture of pACYC184 and pYA4590 (lane 1, 3-5, 8, 9, 14-16).

Interplasmid recombination products

Plasmids extracted from Tc^R clones of χ 3761(pYA4464, pYA4465) were digested with *NcoI* and *BglIII*. Both pYA4464 and pYA4465 are linearized into a DNA fragment about 4 kb. Therefore, in cells containing each or both monomeric plasmids, the digested product will be a single band. The pYA4464-pYA4465 hybrid will be cut into two fragments (5510 bp and 2481 bp). All four of the Tc^R clones we isolated and examined showed

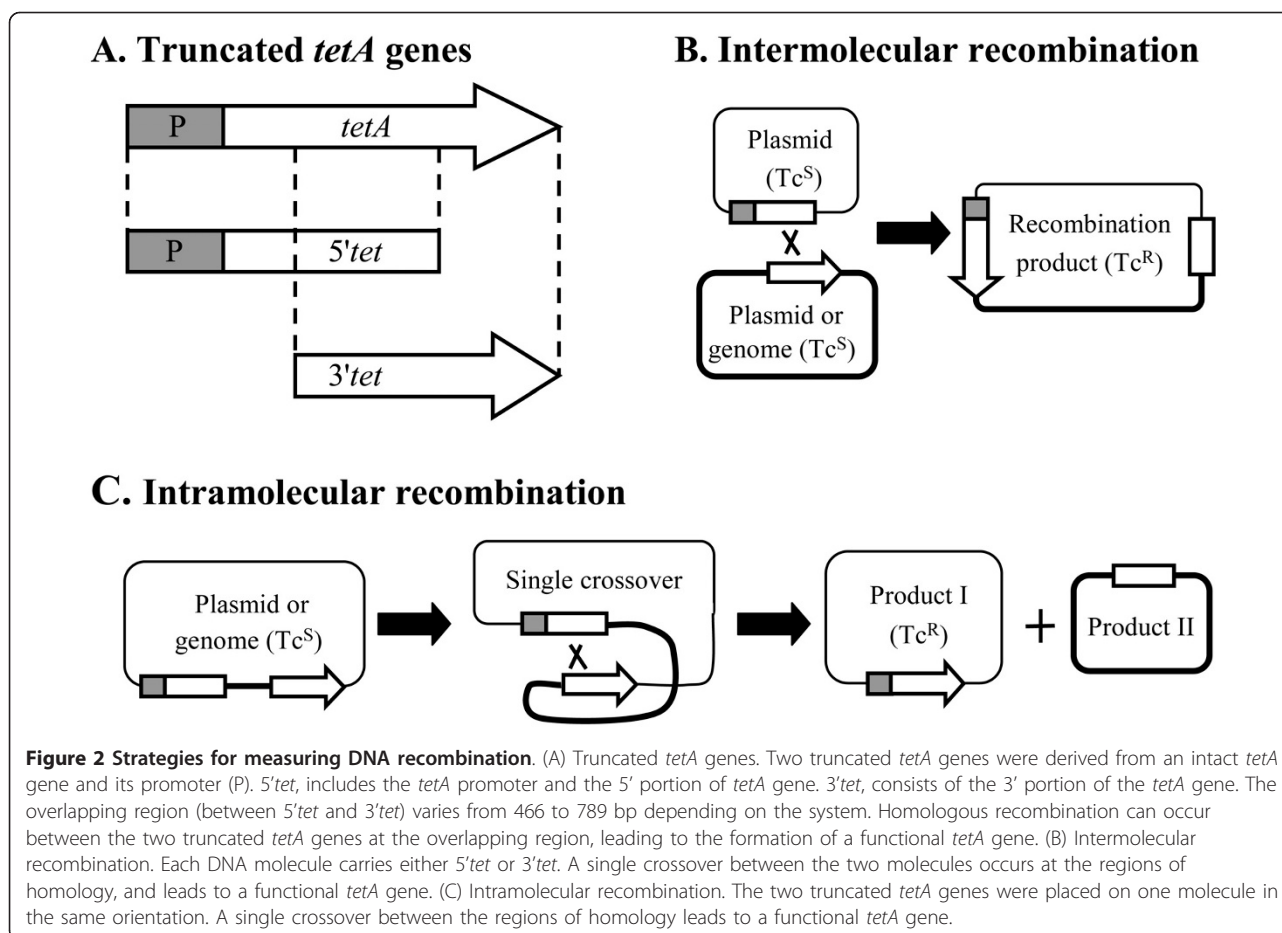
recombination product specific bands and the 4-kb band expected when each plasmid exists separately in the cell. Four tetracycline sensitive (Tc^S) isolates were examined and only a single band was observed, as expected (Figure 3C). These results suggest that interplasmid recombination occurred in the Tc^R cells and that both dimer and individual monomers corresponding to at least one of the two starting plasmids can coexist in the same bacterial cell. We performed a similar experiment in *S. Typhi* strain Ty2(pYA4464, pYA4465) and obtained identical results (data not shown).

Construction of *rec* deletion strains

We constructed a series of strains for these studies carrying deletions in either *recA*, *recF* or *recJ* in *S. Typhimurium* UK-1, *S. Typhi* Ty2 and *S. Paratyphi* A (Table 2). We also constructed Δ *recA* Δ *recF* and Δ *recJ* Δ *recF* double mutants in *S. Typhimurium*. Deletion of *recA*, *recF* and *recJ* results in an increase in sensitivity to UV irradiation [36,37]. To verify the presence of these deletions phenotypically in our strains, the UV sensitivity of the *S. Typhimurium* mutant strains was measured. The Δ *recF* and Δ *recJ* mutants showed significantly lower surviving fractions than the wild type strain after the same exposure dose (Figure 4). By contrast, after five seconds of UV exposure (16 J/m²) to 2.2×10^9 CFU of the Δ *recA62* mutant (χ 9833), we were unable to recover any surviving cells (not shown). UV resistance similar to the wild-type strain χ 3761 was restored to *S. Typhimurium* Δ *recA* and Δ *recF* mutants strains after introduction of *recA* plasmid (pYA5002) or either *recF* plasmid (pYA5005/pYA5006), respectively. Transformation of either mutant strain with vector plasmid pYA5001 did not restore UV resistance (Figure 4 and data not shown for *recA* mutant).

Effect of *rec* deletions on intraplasmid recombination

To examine the influence of Δ *recA*, Δ *recF* and Δ *recJ* mutations on intraplasmid recombination frequencies, plasmid pYA4463 (tandem duplication) or pYA4590 (tandem duplication with intervening sequence) were introduced into *Salmonella rec* mutants and their parental strains and analyzed as described in the Methods section. The recombination frequency of plasmid pYA4463 was approximately $1.5-5.0 \times 10^{-3}$ in Rec⁺ Typhimurium, Typhi and Paratyphi A (Table 3). In *S. Typhimurium* and Paratyphi A, most of the *rec* deletions had no effect on the intraplasmid recombination frequency of plasmid pYA4463 except that a small, but statistically significant decrease in recombination was observed in the Δ *recA* mutant of Paratyphi A. However, in both *S. Typhi* strains, both Δ *recF* mutations resulted in approximately 10-fold decrease in recombination frequency ($P < 0.01$), while the Δ *recA* and Δ *recJ* mutations resulted in a 2-3-fold reduction ($P < 0.01$). In the



complementation test, the recombination frequency of plasmid pYA4463 in *S. Typhi* χ 11053 was restored to $2.52 \pm 0.18 \times 10^{-3}$ and $1.71 \pm 0.68 \times 10^{-3}$ by introduction of plasmid pYA5005 encoding *S. Typhimurium* *recF* gene and pYA5006 encoding the *S. Typhi* *recF* gene, respectively (Table 3).

The results with plasmid pYA4590 were also variable among strains. The recombination frequency in *Rec*⁺ *S. Typhimurium* and *S. Paratyphi* A strains was approximately $2\text{-}3 \times 10^{-3}$ and in both *S. Typhi* strains, the frequency was 3-fold higher, at 1.16×10^{-2} (Ty2) and 1.31×10^{-2} (ISP1820). In *S. Typhimurium* and *S. Typhi* Ty2, the Δ *recA* and Δ *recF* mutations reduced the recombination frequency of plasmid pYA4590 by 5-20-fold ($P < 0.01$; Table 3). The results were similar for *S. Paratyphi* A, though the Δ *recF* mutation only led to 3-fold lower plasmid pYA4590 recombination ($P < 0.01$). The Δ *recJ* mutation had no effect in *S. Typhimurium* and resulted in a 2-3-fold decrease in recombination in both *S. Typhi* Ty2 and *S. Paratyphi* A. Combining the Δ *recA* Δ *recF* mutations in *S. Typhimurium* led to a recombination frequency similar to the frequencies observed for both mutations individually, indicating no additive effect. In the complementation test, plasmid

pYA5002, which encodes *S. Typhimurium* *recA*, was transformed into *S. Typhimurium* Δ *recA* mutant χ 9833 (pYA4590) and *S. Typhi* Δ *recA* mutant χ 11159 (pYA4590). Their respective recombination frequencies were $2.50 \pm 0.42 \times 10^{-3}$ and $14.35 \pm 2.44 \times 10^{-3}$, which were comparable to the corresponding wild type strains ($P > 0.05$) (Table 3). The *recF*-encoding plasmids pYA5005 and pYA5006 were transformed into *recF* mutant strains χ 9070 (pYA4590) and χ 11053 (pYA4590), respectively. The respective recombination frequencies were increased to $2.00 \pm 0.24 \times 10^{-3}$ and $2.86 \pm 0.59 \times 10^{-3}$.

Effect of *rec* deletions on interplasmid recombination

To evaluate interplasmid recombination, plasmids pYA4464 and pYA4465 were co-electroporated into the wild-type and *rec* deletion strains. Electroporants from each test strain were grown in LB broth containing both ampicillin and chloramphenicol to maintain selection for both plasmids. The frequency of recombination was determined as described in the Methods section. The interplasmid recombination frequency was $1\text{-}4 \times 10^{-3}$ for *Rec*⁺ *S. Typhimurium*, *S. Typhi* and *S. Paratyphi* A strains (Table 3). For *Typhimurium* and *Paratyphi* A,

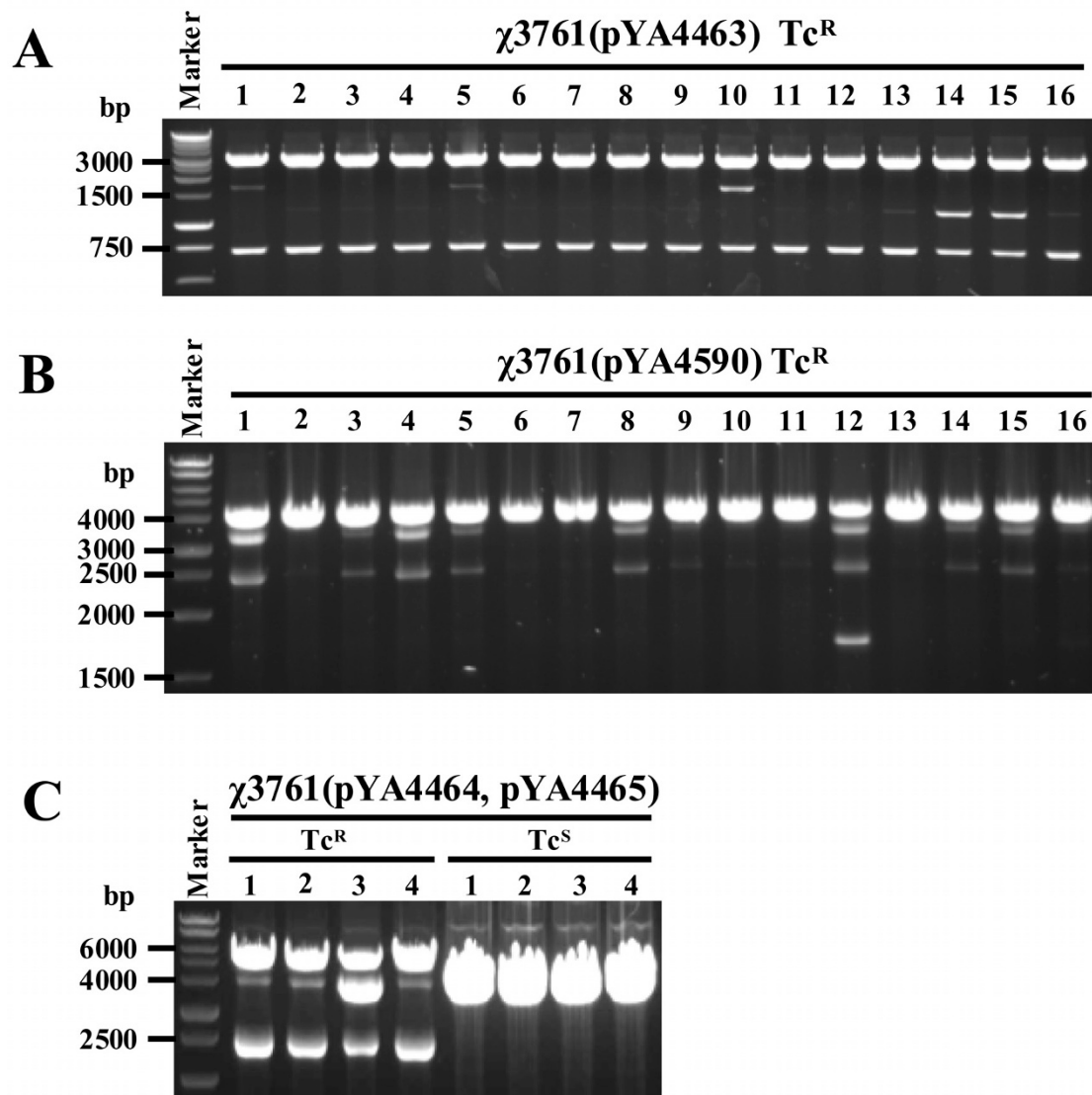


Figure 3 Verification of plasmid recombination product by agarose gel separation. (A) Plasmid DNA was isolated from Tc^{R} clones derived from $\chi 3761(\text{pYA4463})$ and digested by *XbaI* and *SalI*. (B) Plasmid DNA was isolated from Tc^{R} clones of $\chi 3761(\text{pYA4590})$ and digested by *KpnI* and *EcoRI*. (C) Plasmid DNA was isolated from Tc^{R} or Tc^{S} clones of $\chi 3761(\text{pYA4464, pYA4465})$. The purified plasmids were digested with *NcoI* and *BglII*.

the $\Delta recA$ and each $\Delta recF$ mutation reduced the interplasmid recombination frequency by about 3-10-fold ($P < 0.01$). In contrast, the $\Delta recA$ mutation had no effect on interplasmid recombination in *S. Typhi* Ty2. The $\Delta recF$ mutations did not reduce interplasmid recombination in either of the Typhi strains. Surprisingly, introduction of the $\Delta recF1074$ mutation into *S. Typhi* Ty2 resulted in significantly higher interplasmid recombination ($P < 0.01$). Note that we performed this analysis in eight independent experiments and observed a higher recombination frequency of interplasmid recombination each time. The $\Delta recJ$ mutation had no significant effect in *S. Typhi*, and a small (< 3 -fold) but significant effect

in *S. Typhimurium* and *S. Paratyphi A*. The recombination frequencies were also determined in *S. Typhimurium* strains $\Delta recA \Delta recF$ and $\Delta recF \Delta recJ$ double deletions. No additive effect between the two mutations was observed with respect to each single mutation.

Effect of *rec* deletions on chromosome related recombination

To measure intrachromosomal recombination frequencies, we introduced the pYA4590-derived DNA sequence containing two truncated *tetA* genes ($5'tet\text{-}kan\text{-}3'tet$) into the *S. Typhimurium* chromosome at *cysG*. The two truncated *tetA* genes had 602 bp of overlapping sequence.

Table 2 The bacterial strains used in this study

Strain	Genotype* [parental strain]	Reference or source
S. Typhimurium UK-1		
χ3761	wild type	[62]
χ9833	Δ <i>recA</i> 62 [χ3761]	This study
χ9070	Δ <i>recF</i> 126 [χ3761]	This study
χ9072	Δ <i>recJ</i> 1315 [χ3761]	This study
χ9081	Δ <i>recJ</i> 1315 Δ <i>recF</i> 126 [χ9072]	This study
χ9931	<i>cysG</i> 494::(5'tet-kan-3'tet) [χ3761]	This study
χ9932	Δ <i>recF</i> 126 <i>cysG</i> 494::(5'tet-kan-3'tet) [χ9070]	This study
χ9933	Δ <i>recJ</i> 1315 <i>cysG</i> 494::(5'tet-kan-3'tet) [χ9072]	This study
χ9934	Δ <i>recA</i> 62 <i>cysG</i> 494::(5'tet-kan-3'tet) [χ9833]	This study
χ9935	<i>cysG</i> 493::(5'tet-kan) [χ3761]	This study
χ9936	Δ <i>recF</i> 126 <i>cysG</i> 493::(5'tet-kan) [χ9070]	This study
χ9937	Δ <i>recJ</i> 1315 <i>cysG</i> 493::(5'tet-kan) [χ9072]	This study
χ9938	Δ <i>recA</i> 62 <i>cysG</i> 493::(5'tet-kan) [χ9833]	This study
χ9939	Δ <i>recF</i> 126 Δ <i>recA</i> 62 [χ9070]	This study
S. Typhi Ty2		
χ3769	wild type	[63]
χ11053	Δ <i>recF</i> 126 [χ3769]	This study
χ11134	Δ <i>recF</i> 1074 [χ3769]	This study
χ11159	Δ <i>recA</i> 62 [χ3769]	This study
χ11194	Δ <i>recJ</i> 1315 [χ3769]	This study
S. Typhi ISP1820		
χ3744	wild type	D.M. Hone
χ11133	Δ <i>recF</i> 1074 [χ3744]	This study
S. Paratyphi A		
χ8387	Plasmid pSPA1 was cured from wt isolate ATCC 9281	This study
χ11243	Δ <i>recA</i> 62 [χ8387]	This study
χ11244	Δ <i>recF</i> 126 [χ8387]	This study
χ11245	Δ <i>recJ</i> 1315 [χ8387]	This study
<i>E. coli</i> K-12		
EPI300	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ80 <i>dIacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ ⁻ <i>rpsL</i> <i>nupG</i> <i>trfA</i> <i>dhfr</i>	Epicentre
χ7213 (MGN-617)	<i>thi-1</i> <i>thr-1</i> <i>leuB6</i> <i>glnV44</i> <i>fhuA21</i> <i>lacY1</i> <i>recA1</i> <i>RP4-2-Tc::Muλpir</i> Δ <i>asdA4</i> Δ <i>zhf-2::Tn10</i>	[55]

* *kan*: kanamycin resistance gene; 5'tet: 5' portion of the *tetA* gene together with its promoter; 3'tet: 3' portion of the *tetA* gene.

Intrachromosomal recombination deletes the kanamycin resistance cassette and restores one intact copy of the *tetA* gene (Figure 2C). Deletion of *recA* resulted in a 5-fold reduced recombination frequency compared to the Rec⁺ strain χ9931 ($P < 0.01$), while the *recF* or *recJ* deletions had no effect, indicating that RecF and RecJ are not involved in this process (Table 4).

To examine plasmid integration, the 5'tet gene was introduced into the *S. Typhimurium* chromosome at *cysG*. The resulting strains were transformed with plasmid pYA4464 (3'tet) (Figure 1B). The 789 bp of

overlapping sequence between 5'tet on the chromosome and the 3'tet on the plasmid could result in plasmid integration into the chromosome, generating an intact *tetA* gene (Figure 2B). Deletion of *recA* had a profound effect, reducing the integration frequency to less than 7×10^{-10} , which was below the limits of detection in this assay ($P < 0.01$), indicating a strict requirement for RecA in this process. Introduction of plasmid pKD46, which encodes the λ Red recombinase, into χ9938 (Δ*recA*) carrying pYA4464 restored the integration frequency to the level of the Rec⁺ strain χ9935. Deletion of *recF* reduced the frequency of integration less than 3-fold ($P < 0.01$; Table 4) and the Δ*recJ* deletion had no effect.

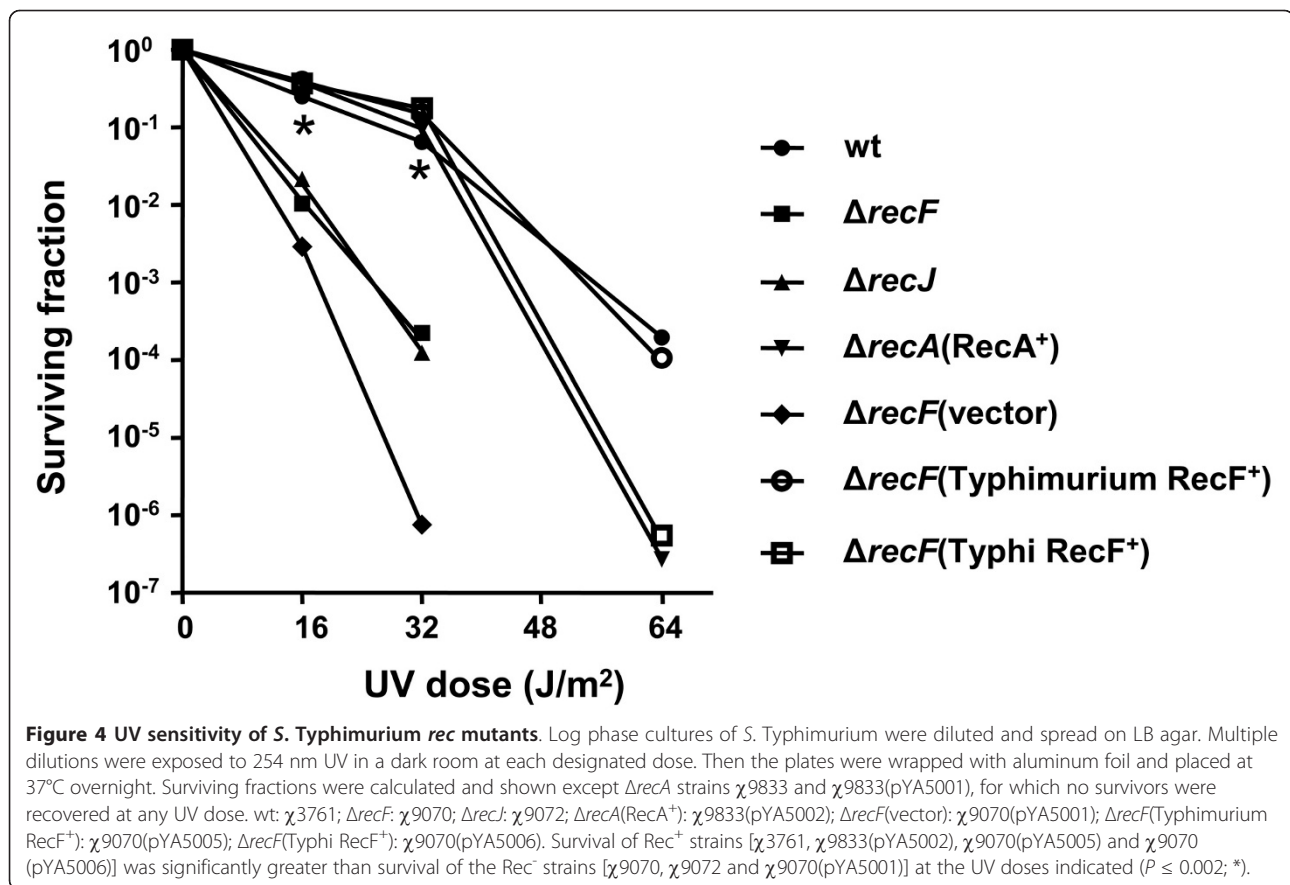
Effect of rec deletions on the virulence of S. Typhimurium

BALB/c mice were orally inoculated with the highly virulent *S. Typhimurium* strain χ3761 and its *rec* mutant derivatives. The LD₅₀s of χ3761, χ9070 (Δ*recF*) and χ9072 (Δ*recJ*) were similar, 3.2×10^4 , 6.8×10^4 and 1.5×10^5 CFU, respectively (Table 5). The LD₅₀ of the Δ*recF* Δ*recJ* double mutant was approximately 100-fold higher than χ3761, at 2.2×10^6 CFU. All mice inoculated with 1.3×10^9 CFU of the Δ*recA* mutant survived, indicating that the LD₅₀ was $> 1.3 \times 10^9$ CFU. Two months following the initial inoculation with the Δ*recA* mutant strain, surviving mice were challenged with either 1.5×10^8 or 1.5×10^9 CFU of wild-type strain χ3761. All mice survived the challenge, indicating that Δ*recA* mutant strain χ9833 was both attenuated and immunogenic.

Discussion

We began our studies using information gathered in *E. coli* as a reference point. In *E. coli*, *recA*-dependent homologous recombination relies on the RecBCD pathway, the RecFOR pathway (originally designated the RecF pathway) and the RecE pathway [38]. The RecBCD pathway is important in conjugational and transductional recombination [39], and may also be involved in the recombination of plasmids containing one or more Chi sites [40]. Recombination in small plasmids lacking a Chi sequence is primarily catalyzed by the RecFOR pathway [41]. RecF, RecO, and RecR bind to gaps of ssDNA and displace the single-strand DNA binding proteins to allow RecA to bind [42,43]. The RecJ ssDNA exonuclease acts in concert with RecFOR to enlarge the ssDNA region when needed. Strand exchange is then catalyzed by RecA [44]. Because of their prominent role in plasmid recombination in *E. coli*, we analyzed the effect of mutations in *recF*, *recJ* and *recA* on plasmid recombination in *Salmonella*.

Attenuated *S. Typhi* strains have been developed as antigen delivery vectors for human vaccine use. Due to the host restriction phenotype of *S. Typhi*, preliminary work is typically done in *S. Typhimurium* using mice as



the model system to work out attenuation and antigen expression strategies. Recently, we have also been investigating attenuated derivatives of the host-restricted strain *S. Paratyphi* A as a human vaccine vector. Therefore, it was of interest to evaluate and compare the effects of *rec* mutations in these three *Salmonella* serovars. We selected *S. Typhi* strain Ty2 as exemplary of this serovar because most of the vaccines tested in clinical trials to date have been derived from this strain [45]. *S. Typhi* strain ISP1820 has also been evaluated in clinical trials [46,47] and we therefore included it in some of our analyses. We found that, for some DNA substrates, the effects of *ΔrecA* and *ΔrecF* deletion mutations differed among *Salmonella enterica* serotypes. In particular, we found that deleting *recA*, *recF* or *recJ* in *S. Typhi* Ty2 and deleting *recF* in strain ISP1820 had significant effects (3-10 fold) on the recombination frequency of our direct repeat substrate, pYA4463 (Table 3). No or very limited effect (< 2 fold) was observed for our *S. Typhimurium* and *S. Paratyphi* A strains, consistent with results reported for *E. coli* indicating that recombination of this type of substrate is *recA*-independent [35]. In contrast, the *ΔrecA* and *ΔrecF* mutations resulted in lower interplasmid recombination in Typhimurium and

Paratyphi A but not in Typhi strains. Deletion of *recJ* led to a reduction in intraplasmid recombination frequencies in *S. Typhi*, while no effect was seen in *S. Typhimurium*. The *ΔrecJ* mutation also affected plasmid recombination frequencies for two of the three substrates tested in *S. Paratyphi* A. Taken together, these results suggest that the recombination system in *S. Typhi*, or at least in strains Ty2 and ISP1820, is not identical to the recombination system in *S. Typhimurium* and *S. Paratyphi* A.

To investigate the mechanism responsible for the observed differences, we analyzed the genome sequences of *S. Typhimurium* UK-1 (Luo, Kong, Golden and Curtiss, unpublished whole genome sequence), *S. Paratyphi* A (NC_006511) [48] and *S. Typhi* Ty2 (NC_004631) [49]. No paralogs of the *recA*, *recF* and *recJ* genes were found in the three strains. The *S. Typhimurium* UK-1 has RecA, RecO and RecR protein sequences identical to Typhi Ty2, and RecF and RecJ protein sequences with over 99% identity. Plasmids expressing Typhimurium *recF* or Typhi *recF* complemented the *ΔrecF126* mutation in Typhi, as evidenced by the UV sensitivity profile (Figure 4) and intraplasmid recombination of pYA4463 (Table 3). Therefore, the basis for these

Table 3 Plasmid recombination frequency (Mean ± STD, × 10⁻³)

Strain	rec deletion	pYA4463 ^a	pYA4590 ^b	pYA4464+pYA4465 ^c
<i>S. Typhimurium</i>				
χ3761	None	1.55 ± 0.31	2.40 ± 0.54	2.88 ± 0.85
χ9833	ΔrecA62	1.07 ± 0.24	0.22 ± 0.07**	0.27 ± 0.07**
χ9070	ΔrecF126	1.14 ± 0.15	0.52 ± 0.07**	0.33 ± 0.09**
χ9072	ΔrecJ1315	1.87 ± 0.44	2.37 ± 0.21	1.10 ± 0.20**
χ9081	ΔrecJ1315 ΔrecF126	NA ^d	NA	0.35 ± 0.08**
χ9939	ΔrecF126 ΔrecA62	NA	0.41 ± 0.09**	0.35 ± 0.08**
χ9833(pYA5002)	ΔrecA62 (RecA ⁺)	NA	2.50 ± 0.42	NA
χ9070(pYA5005)	ΔrecF126 (RecF ⁺)	NA	2.00 ± 0.24	NA
<i>S. Typhi</i> Ty2				
χ3769	None	4.69 ± 0.26	11.59 ± 2.61	4.20 ± 1.44
χ11159	ΔrecA62	1.32 ± 0.27**	0.60 ± 0.19**	3.37 ± 0.96
χ11053	ΔrecF126	0.51 ± 0.06**	0.57 ± 0.09**	6.19 ± 2.71
χ11134	ΔrecF1074	0.45 ± 0.05**	0.52 ± 0.17**	16.28 ± 2.64**
χ11194	ΔrecJ1315	1.69 ± 0.26**	4.88 ± 1.56**	2.31 ± 0.90
χ11053(pYA5005)	ΔrecF126 (RecF ⁺)	2.52 ± 0.18	NA	NA
χ11053(pYA5006)	ΔrecF126 (RecF ⁺)	1.71 ± 0.68	NA	NA
χ11159(pYA5002)	ΔrecA62 (RecA ⁺)	NA	14.35 ± 2.44	NA
χ11053(pYA5006)	ΔrecF126 (RecF ⁺)	NA	2.86 ± 0.59	NA
<i>S. Typhi</i> ISP1820				
χ3744	None	4.93 ± 0.67	13.10 ± 1.23	4.22 ± 0.25
χ11133	ΔrecF1074	0.65 ± 0.26**	0.71 ± 0.06**	5.38 ± 0.58
<i>S. Paratyphi</i> A				
χ8387	None	2.70 ± 0.39	3.32 ± 0.61	1.03 ± 0.15
χ11243	ΔrecA62	1.91 ± 0.69**	0.55 ± 0.20**	0.13 ± 0.03**
χ11244	ΔrecF126	5.00 ± 0.70	1.16 ± 0.21**	0.34 ± 0.04**
χ11245	ΔrecJ1315	2.56 ± 0.41	1.83 ± 0.99**	0.64 ± 0.15**

^aIntraplasmid recombination without intervening sequence (5'tet-3'tet).

^bIntraplasmid recombination with a 1041-bp intervening sequence (5'tet-kan-3'tet).

^cInterplasmid recombination.

^dNot assayed.

**P < 0.01, relative to the parental rec⁺ strain.

differences are not clear and indicates that there may be other genes or gene products involved. A more detailed analysis of this phenomenon is under investigation.

Plasmid recombination frequencies were higher in our *Salmonella* strains than those reported in *E. coli*. We

Table 4 Chromosome related recombination in *S. Typhimurium*^a

rec deletion	Intrachromosomal recombination		Plasmid integration	
	Strain	Frequency (10 ⁻⁵)	Strain	Frequency (10 ⁻⁶)
None	χ9931	6.02 ± 0.38	χ9935	5.59 ± 0.94
ΔrecF126	χ9932	7.05 ± 1.40	χ9936	2.13 ± 0.60**
ΔrecJ1315	χ9933	9.18 ± 2.18	χ9937	4.89 ± 0.41
ΔrecA62	χ9934	1.29 ± 0.51**	χ9938 ^b	<0.00071**

^aMean ± STD from 3-5 assays were shown in the table.

^bUpon introduction of pKD46 (30°C, 0.2% arabinose), the frequency was 6.41 ± 0.85 × 10⁻⁶ (P = 0.425).

** P < 0.01, relative to the parental rec⁺ strain.

observed intra- and interplasmid recombination frequencies on the order of 1 × 10⁻³ in Rec⁺ *Salmonella*, whereas measurements made in *E. coli* strain AB1157 using a similar plasmid system (equivalent to our substrates pYA4590 and pYA4464 + pYA4465) revealed a basal frequency around 10-fold lower, approximately 1 × 10⁻⁴ for both types of substrates [26]. Interestingly, the effect of a recF mutation in *E. coli* was to reduce the recombination frequency of intra- and interplasmid recombination approximately 30-fold, to roughly the same frequencies we observed for *S. Typhimurium* (Table 3). However, consistent with the results in *E. coli*, the effects of recA, recF, and recA recF mutations were similar, indicating that the mutations are epistatic.

RecF has been shown previously to play a role in recombinational repair of chromosomal DNA in response to DNA damaging agents [50], including a major role in homologous recombination between direct repeats in the chromosome of *S. Typhimurium*. In our

Table 5 Virulence of *S. Typhimurium* *rec* mutants in BALB/c mice (oral inoculation)

Strain	<i>rec</i> deletion	Dose (CFU)	Survivor/total	LD ₅₀ (CFU)
χ3761	None	1.5 × 10 ⁶	0/4	3.2 × 10 ⁴
		1.5 × 10 ⁵	1/4	
		1.5 × 10 ⁴	3/4	
		1.5 × 10 ³	4/4	
χ9070	Δ <i>recF126</i>	1.0 × 10 ⁷	0/4	6.8 × 10 ⁴
		1.0 × 10 ⁶	1/4	
		1.0 × 10 ⁵	1/4	
		1.0 × 10 ⁴	4/4	
χ9072	Δ <i>recJ1315</i>	1.0 × 10 ⁷	0/4	1.5 × 10 ⁵
		1.0 × 10 ⁶	0/4	
		1.0 × 10 ⁵	3/4	
		1.0 × 10 ⁴	3/4	
χ9081	Δ <i>recJ1315</i> Δ <i>recF126</i>	1.0 × 10 ⁷	1/4	2.2 × 10 ⁶
		1.0 × 10 ⁶	3/4	
		1.0 × 10 ⁵	4/4	
		1.0 × 10 ⁴	3/4	
χ9833	Δ <i>recA62</i>	1.3 × 10 ⁹	10/10	>1.3 × 10 ⁹

study, we did not observe any effect of *recF* on intrachromosomal recombination, although it did have an effect on the frequency of plasmid integration (Table 4). This discrepancy can be explained by the fact that we did not use DNA damaging agents in our study. These agents lead to single stranded stretches of DNA that represent substrates for *recF* (and *recA*). Our observation that *recF* did affect plasmid integration may reflect the presence of stretches of ssDNA in the plasmid, presumably due to supercoiling effects.

To induce strong primary and memory immune responses, *Salmonella* delivery vectors should be sufficiently invasive and persistent to allow antigen expression in targeting organs, while maintaining a high degree of safety. This requires the use of mutations that attenuate the *Salmonella* vector without impairing its antigen delivery ability. Many attenuating mutations impair invasion and colonization ability. In our study, we confirmed a previous report that *recF* is not required for *S. Typhimurium* virulence in mice [51], indicating that the *recF* mutant remains invasive and replicates well in colonized organs. Therefore, including a Δ*recF* mutation in a *Salmonella* vaccine strain is unlikely to affect its immunogenicity. Our results with the *S. Typhimurium* Δ*recA* strain are consistent with two previous, independent studies showing that *recA* mutations reduce *Salmonella* virulence [51,52]. To evaluate the potential effect of Δ*recA* mutation on immunogenicity, mice inoculated with the *recA* mutant were challenged with a lethal dose of virulent wild-type *S. Typhimurium*. All the challenged mice survived, indicating that a Δ*recA* mutant retains immunogenicity and therefore may be

suitable for use in a vaccine. However, since it does not affect virulence, inclusion of a Δ*recF* mutation into a *Salmonella* vector that has been attenuated by other means to reduce the frequency of intra- and interplasmid recombination, may be more desirable than a Δ*recA* mutation. Studies are currently underway to investigate these possibilities.

Our data show that Δ*recA* and Δ*recF* mutations resulted in reduced frequencies of intraplasmid recombination in all *Salmonella* strains tested, which included three serovars, when there was an intervening sequence between the direct duplications (Table 3). Our results also show that it is likely that deletions in *recA*, *recF* or *recJ* will not be useful for reducing interplasmid recombination in *S. Typhi* vaccine strains, since we did not observe any reduction in interplasmid recombination frequency. This result was disappointing, since the majority of human trials with live *Salmonella* vaccines have focused on *S. Typhi*. In the case of *S. Typhi*, it appears that the best approach to preventing interplasmid recombination will be in the careful design of each plasmid, avoiding any stretches of homology. However, for vaccines based on *S. Typhimurium* or *S. Paratyphi* A, introduction of a Δ*recF* mutation into attenuated *Salmonella* vaccine strains carrying multiple plasmids is a useful approach to reduce unwanted plasmid/plasmid or plasmid/chromosome recombination without further attenuating the strain or negatively influencing its immunogenicity. The Δ*recA* mutation had a similar or more pronounced effect on reducing various classes of recombination and it clearly had an effect on virulence. We did not examine the effect of a Δ*recA* mutation on the immunogenicity of a vectored antigen. Based on its effect on virulence, it may affect the immunogenicity of the vectored antigen in some attenuation backgrounds and therefore may not be applicable for all attenuation strategies.

Conclusions

In this study we showed that Δ*recA* and Δ*recF* mutations reduce intraplasmid recombination in *S. Typhimurium*, *S. Typhi* and *S. Paratyphi* while there is an intervening sequence between the duplicated sequences. The Δ*recA* and Δ*recF* mutations reduce interplasmid recombination in *S. Typhimurium* and *S. Paratyphi* but not in *S. Typhi*. The Δ*recF* mutations also sharply reduce intraplasmid recombination between direct duplications in *S. Typhi*. Since Δ*recA* mutation results in an avirulent *Salmonella* strain, the Δ*recF* mutation is ideal for reducing plasmid recombination in *Salmonella* delivery vectors without impairing the virulence. The intrachromosomal recombination and plasmid integration are 2-3 orders lower than plasmid recombination, therefore are less concerned. These information help

develop *Salmonella* delivery vectors able to stably maintain plasmid cargoes for vaccine development and gene therapy.

Methods

Bacterial strains and media

E. coli K-12 strain EPI300™ was used for cloning and stable maintenance of plasmids. All *Salmonella* strains used in this work were derived from *Salmonella enterica* serovar Typhimurium wild-type (wt) strain χ 3761 (UK-1), serovar Typhi strains Ty2 and ISP1820 or serovar Paratyphi A strain χ 8387. Their origin and relevant genotypes are presented in Table 2. Bacteria were grown in LB broth [53].

Plasmid construction

All plasmids used in this study and their relevant characteristics are presented in Table 1. Primers used for plasmid construction are shown in Table 6. All enzymes were obtained from New England Biolabs or Promega.

To construct plasmid pYA4463 (Figure 1 panel A), a *Xba*I-*Hinc*II fragment containing the *tetA* promoter and 568 bp of the 5' end of *tetA*, was excised from pACYC184 and ligated into *Xba*I-*Eco*RV digested pACYC184.

To generate plasmid pYA4590 (Figure 1 panel A), the 5' end of *tetA* gene together with its promoter was amplified from pACYC184 with primers P1 and P2, which contain engineered *Xba*I and *Kpn*I restriction sites, respectively. The resulting PCR fragment was digested with *Xba*I and *Kpn*I. The *kan* gene was amplified from plasmid p15A-PB2-*kan*, a pACYC184 derivative carrying a influenza virus PB2 gene and a *kan* cassette, with primers P3 and P4, which were engineered to contain *Kpn*I and *Bam*HI sites, respectively. The resulting PCR fragment was digested with *Kpn*I and *Bam*HI. The two digested PCR fragments were ligated into pACYC184 digested with *Xba*I and *Bam*HI. The resulting plasmid, pYA4590, contains the *tetA* promoter and 891 bp of the 5' end of *tetA*, a 1041-bp fragment encoding *kan* and its promoter followed by 902 bp of the 3' end of *tetA*.

To construct plasmid pYA4464 (Figure 1 panel B), plasmid pACYC184 was digested with *Xba*I and *Eco*RV to remove the 5' 102 bp of the *tetA* gene and the *tetA* promoter. The cohesive ends were filled using the Klenow large fragment of DNA polymerase and the linear plasmid was self-ligated to yield plasmid pYA4464.

To construct plasmid pYA4465 (Figure 1 panel B), the 5' 853 bp of *tetA* together with its promoter was amplified from pACYC184 using primers P5 and P6, which were engineered with *Sma*I and *Bgl*II sites, respectively. The resulting PCR fragment was digested with *Sma*I and *Bgl*II, and ligated to *Eco*RV and *Bgl*II digested pBAD-HisA.

Creation of *rec* deletions

The *recA62* deletion, which deletes 1062 bp, encompassing the entire *recA* open reading frame, introduced into the bacterial chromosome using either λ Red recombinase-mediated recombination [54], or conjugation with *E. coli* strain χ 7213(pYA4680) followed by selection/counterselection with chloramphenicol and sucrose, respectively [55]. The *cat-sacB* cassette was amplified from plasmid pYA4373 by PCR with primers P7 and P8 to add flanking sequence. The PCR product was further amplified with primer P9 and P10 to extend the flanking sequence. Those two steps of amplification resulted in the *cat-sacB* cassette flanked by 100 bp of *recA* flanking sequences at both ends. The PCR product was purified with QIAquick Gel Extraction Kit (QIAGEN) and electroporated into *Salmonella* strains carrying plasmid pKD46 to facilitate replacement of the *recA* gene with the *cat-sacB* cassette. Electroporants containing the *cat-sacB* cassette were selected on LB plates containing 12.5 μ g chloramphenicol ml⁻¹. From *S. Typhimurium* chromosome, a 500-bp sequence upstream *recA* gene was amplified with primers P11 and primer P12 and a 500-bp sequence downstream *recA* gene was amplified with primers P13 and P14. Primers P12 and P13 were engineered with a *Kpn*I site. The two PCR fragments were digested with *Kpn*I, ligated and amplified with primers P11 and P14. The resulting PCR product was digested with isocaudarner *Spe*I and *Xba*I and ligated into *Xba*I-digested pRE112 to yield plasmid pYA4680. In addition, undigested, agarose-gel purified PCR product was electroporated into the *cat-sacB Salmonella* strains carrying plasmid pKD46 and spread onto LB plates containing 5% sucrose to select for deletion of the *cat-sacB* cassette. Chloramphenicol-sensitive isolates were verified as Δ *recA62* by PCR using primers P15 and P16 (Δ *recA62*: 1360 bp; wt: 2412 bp). *S. Typhimurium* strains χ 9833 and χ 9939 were constructed by this method (Table 2). For construction of a Δ *recA62* mutant of *S. Typhi*, wild-type strain Ty2 was mated with *E. coli* strain χ 7213 (pYA4680). Transconjugants were selected on LB plates containing chloramphenicol, followed by counterselection on sucrose plates as described above. The resulting Δ *recA62* strain was designated χ 11159. The *S. Paratyphi* A strain χ 11243 was generated from wild-type strain χ 8387 using the same strategy.

The Δ *recF* deletion strains were constructed using suicide vectors pYA3886 and pYA4783. From the *S. Typhimurium* chromosome, a 397-bp sequence upstream of the *recF* gene was amplified with primers P17 and P18, which were engineered with *Xba*I and *Kpn*I sites, respectively. The downstream 296-bp sequence (including 78 bp from the 3' ORF of *recF*) was amplified with primers P19 and P20 containing *Kpn*I and *Sph*I sites, respectively. The two fragments were digested and

Table 6 Primers used in this study

Primer	Sequence ^a	Direction ^b
P1	tatttctagattcagtgcaat	F
P2	ttaggtaccgccaacgccaagacg	R
P3	taaggtaccggaattgccagctggg	F
P4	ttaggatcctccgcgacattccccg	R
P5	taaccgggaattctcatgtttgac	F
P6	ttaagatctccatgccggcgataat	R
P7	tgcttcaacagtacgaattcactatccggttaaccaagtgcgatgacgcctgcagggcg	F
P8	gttttgctgaatggcggcttctgtttgccgccccaccatcacctgatgattttgtaactgtaattg	R
P9	ggcaacaattctacaaaacttgatactgatgacatacagataattgcttcaacagtacgaa	F
P10	gagaaatgccaaaaggcgccataaatgcagcccttgatgtaattaacgttttgctgaatggcggc	R
P11	taaactagtagcagcagagagtcctgtaccg	F
P12	ttaggtaccggaagcttgcacactggattgaac	R
P13	taaggtaccgcatcctcatcaggtgatggggcg	F
P14	ttatctagatttgcgaacggcctgttcacgt	R
P15	gatagcacgtgctatctgtgc	F
P16	tcgtcgacagcgtgttcg	R
P17	ctagtctagcgtcagtgagaatcagctcaaa	F
P18	caaggtaccatattagtagcattcgtccagg	R
P19	cgcggtaccagcgtgaacacgttatagacat	F
P20	acatgcatgcaatagtcacgacgatacttt	R
P21	ctagtctagcgtcagtgagaatcagctcaaaatc	F
P22	cggggtaccatcaactcataaccagggcgttatc	R
P23	cgactttatcttacctgaagctgtggat	F
P24	gttacggacacggagttatcggcgtgaata	R
P25	ctagtctagaagattataacgcgctggg	F
P26	cggggtaccgctattattaccactggtc	R
P27	cgcggtacctaatcggggcatttaacaac	F
P28	acatgcatgcttcgagcgtgaacgctct	R
P29	gtctataaagcggcgatgagaaacatg	F
P30	tcgacgatcgttcgagcgtgaacgctct	R
P31	taaaagcttgaccgagctgtctgacgt	F
P32	tcaagatctctcggcgcgaggtgcccg	R
P33	taaagatcttgactgagtgaaaaagcagttgccacgat	F
P34	ttagagctcagaaaggaataccggcatgaca	R
P35	taaagatctcgatataagttgtaattctc	F
P36	ttactgcaggcaggtgcccggccttc	R
P37	ttactgcagtcgacacattccccg	R
P38	gggtaatgctgtggaccatttgc	F
P39	ccgggtaatccccggcactaccg	R
P40	gcgctacaaacctgtggcaacaat	F
P41	gctgtgatcggcagcagaatac	R
P42	ttctcaacataaaaaagttgtgtaatactgaggatgcccgcacag	F
P43	gttacggacacggagttatcggcgtgaata	R

^aThe underlined sequences are enzyme sites mentioned in the text.

^bForward (F) or reverse (R) primers.

inserted into *XbaI-SphI* digested pRE112, resulting in plasmid pYA3886. The corresponding deletion was designated $\Delta recF126$. Strains $\chi 9070$, $\chi 9081$ and $\chi 11244$ were generated by conjugation using *E. coli* strain $\chi 7213$ (pYA3886). Phage P22HT^{int} mediated transduction was

used to construct Typhi strain $\chi 11053$ [56]. The $\Delta recF126$ deleted 996 bp from the 5' end of *recF* in serovars Typhimurium and Paratyphi. The upstream flanking sequence of *S. Typhi* is different with the other serotypes. To construct a serovar Typhi-specific $\Delta recF$

mutation, we constructed a new suicide vector. The *recF* upstream flanking sequence in plasmid pYA3886 was replaced with the corresponding DNA sequence (447 bp) from *S. Typhi* Ty2. Primers P21 and P22 were used for this modification. The resulting plasmid was designated as pYA4783. The Typhi-specific $\Delta recF1074$ mutation was introduced into *S. Typhi* strains ISP1820 and Ty2 by conjugation with *E. coli* strain $\chi 7213$ (pYA4783) to yield strains $\chi 11133$ and $\chi 11134$, respectively. Primers P23 and P24 were used to verify the *recF126* and *recF1074* deletions.

Similar strategies were used to construct the $\Delta recJ1315$ deletion with suicide vector pYA3887. From the *S. Typhimurium* chromosome, 330 bp upstream of the *recJ* gene was amplified with primers P25 and P26, which were engineered with *XbaI* and *KpnI* sites, respectively. The 299-bp downstream sequence was amplified with primers P27 and P28, engineered with *KpnI* and *SphI* sites, respectively. The two fragments were digested and ligated with *XbaI-SphI* digested pRE112. The resulting plasmid was designated pYA3887 and the corresponding deletion was named $\Delta recJ1315$. Strains $\chi 9072$ and $\chi 11245$ were generated by conjugating the parental strains with *E. coli* strain $\chi 7213$ (pYA3887). Strain $\chi 11194$ was constructed by phage P22HTint mediated transduction. The $\Delta recJ1315$ mutation is a deletion of the entire *recJ* gene (1734 bp). Primers P29 and P30 were used to verify the *recJ1315* deletion ($\Delta recJ1315$: 736 bp; wt: 2461 bp).

To test chromosome-related recombination, the 5'tet and 3'tet fragments were inserted into the *cysG* gene of each *S. Typhimurium* strain using the λ Red system. The 460-bp fragment of the *cysG* gene was amplified using primers P31 and P32 that were engineered with *HindIII* and *BglII* sites, respectively. The PCR product was digested with *HindIII* and *BglII*. A 480 bp adjoining fragment of *cysG* was amplified with primers P33 and P34. Primer P33 was engineered with *BglII* and *PstI* sites and primer P34 was engineered with a *SacI* site. The PCR product was digested with *BglII* and *SacI*. The two digested PCR fragments were ligated into *HindIII* and *SacI* digested pYA4518, deleting green fluorescent protein (GFP) gene. The resulting plasmid pYA4518-*cysG* has *BssHIII* and *PstI* sites between the two *cysG*-fragments. This plasmid was digested with *BssHIII*, followed by treatment with the Klenow large fragment. The linear plasmid was further digested with *PstI* for insertion of truncated *tetA* genes. The 5'tet-kan-3'tet cassette was amplified from pYA4590 with primers P35 and P36. Primer P36 was engineered with a *PstI* site. The PCR product was digested with *PstI* and inserted between the *cysG* fragments in pYA4518-*cysG* to yield plasmid pYA4689. The 5'tet-kan cassette was amplified from pYA4590 with primers P35 and P37. Primer P37

was engineered with a *PstI* site. The PCR product was digested with *PstI* and inserted into treated pYA4518-*cysG* to obtain plasmid pYA4690. The 5'tet-kan-3'tet cassette, together with *cysG* flanking sequences, was amplified from pYA4689 using primers P31 and P34. The PCR product was electroporated into strains $\chi 3761$ (pKD46), $\chi 9070$ (pKD46), $\chi 9072$ (pKD46) and $\chi 9833$ (pKD46) with selection on LB plates containing 25 μ g/ml chloramphenicol. After growth at 37°C to cure plasmid pKD46, the resulting strains containing chromosomal copies of the 5'tet-kan-3'tet cassette in *cysG* were designated $\chi 9931$ (Rec⁺), $\chi 9932$ ($\Delta recF$), $\chi 9933$ ($\Delta recJ$) and $\chi 9934$ ($\Delta recA$), respectively. Primers P38 and P39 were used to verify insertion in the *cysG* gene. The 5'tet-kan cassette together with *cysG* flanking sequences was amplified from pYA4690 with primers P31 and P34. Using the same strategy, the PCR product was electroporated into pKD46 transformants of strains $\chi 3761$, $\chi 9070$, $\chi 9072$ and $\chi 9833$ to yield strains $\chi 9935$ (Rec⁺), $\chi 9936$ ($\Delta recF$), $\chi 9937$ ($\Delta recJ$) and $\chi 9938$ ($\Delta recA$), respectively, each containing the 5'tet-kan cassette inserted into *cysG*. These strains were transformed with plasmid pYA4464 to test plasmid integration based on the 789-bp of *tetA* sequence common to both the plasmid and the bacterial chromosome.

Analysis of recombination frequency

To examine plasmid recombination and plasmid integration, plasmid(s) containing truncated *tetA* genes were introduced into *Salmonella* strains with or without *rec* mutations. The resulting strains were inoculated into 3 ml of LB broth supplemented with 100 μ g/ml ampicillin and/or 25 μ g/ml chloramphenicol, as needed. After 8 h growth at 37°C, bacteria were serially diluted in 10-fold steps. 100 μ l of the 10⁻², 10⁻³ or 10⁻⁴ dilution were spread onto LB-agar plates supplemented with 10 μ g tetracycline ml⁻¹ and 100 μ l of the 10⁻⁵, 10⁻⁶ or 10⁻⁷ dilutions were spread onto LB-agar plates with or without the addition of antibiotics, as needed. Plates were incubated overnight at 37°C. The ratio of tetracycline resistant colonies to total colonies was calculated as the recombination frequency. The average mean frequency was calculated using the frequencies obtained from 3-10 assays for each strain. Following one-way ANOVA, the Dunnett's test was used to compare multiple groups against the control. The Student's *t*-test was used to analyze two independent samples.

Complementation of *rec* mutation

Plasmid pYA5001 has a pSC101 *ori*, a gentamicin resistance marker and a prokaryotic green fluorescent protein (GFP) gene cassette flanked by two *AhdI* sites. A linearized T vector for cloning PCR products can be obtained by removing the GFP cassette by *AhdI*

digestion. The *recA* genes from *S. Typhimurium* and *S. Typhi* were amplified using their respective chromosomal DNAs as template with primers P40 and P41. The *recF* genes were amplified similarly using primers P42 and P43. The forward primer P42 was engineered to include the *S. Typhimurium lpp* promoter sequence ttctcaacataaaaaagtgtgtaataact (the -35 and -10 boxes are underlined). Amplified DNA fragment were treated with Taq DNA polymerase in the presence of dATP to add 3' A overhangs. Then the treated PCR products were cloned into pYA5001-derived T vector to yield *recA* plasmids pYA5002 (*Typhimurium*) and pYA5004 (*Typhi*), and *recF* plasmids pYA5005 (*Typhimurium*) and pYA5006 (*Typhi*). The *recA* plasmids, *recF* plasmids or empty vector plasmid pYA5001 were transformed into *S. Typhimurium recA* or *recF* mutants, respectively for complementation studies. The *recA* and *recF* plasmids were also introduced into *Salmonella* strains carrying pYA4590 or pYA4463 to complement the *rec* mutation and measure the plasmid recombination frequency.

UV sensitivity test

Quantitative UV killing curves were measured as described previously [57]. Briefly, cells were grown in 3 ml of LB broth at 37°C with vigorous shaking to mid-log phase. The cells were then 10 fold serially diluted in buffered saline with gelatin (BSG) and spread on LB agar plates. Multiple dilutions were exposed to 254 nm UV in a dark room at each designated dose. Then the plates were wrapped with aluminum foil and placed at 37°C overnight. The 10⁻⁶ dilutions were not exposed to UV to determine the total bacterial cell numbers present in the culture. Surviving fractions were calculated as the CFU remaining after UV exposure/total CFU present.

Virulence determination of the *rec* mutants

Eight-week old BALB/c female mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were held in quarantine for 1 week before use in experiments. Food and water were deprived 6 h before administration of bacteria. Each mouse was orally inoculated with 20 µl of *Salmonella* suspended in buffered saline with gelatin (BSG) by pipet feeding. Food and water were returned 30 min after inoculation. All mice were observed for a month to record mortality. The 50% lethal dose (LD₅₀) was determined via the Reed and Muench method [58]. Surviving mice were challenged orally with wild-type *Salmonella* χ 3761 two months after the first inoculation.

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Authors' contributions

RC, XMZ and WK conceived and designed the study. XMZ, SYW and KB constructed plasmids and *Salmonella* strains. XMZ performed all DNA recombination assays. XMZ, WK and XZ carried out the animal experiment. XMZ and KR performed UV killing experiment and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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