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Removal of Antibiotic Resistance of Live Vaccine Strain *Escherichia coli* MM-3 and Evaluation of the Immunogenicity of the New Strain

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Abstract MM-3 was a live vaccine strain candidate for protecting neonatal piglets from diarrhea. Designed in the 1980s, a high degree of protection from colibacillosis was afforded to piglets in a challenge study and field trials. However MM-3 had a drawback of carrying the antibiotic resistance gene (chloramphenicol acetyltransferase gene, *cat*). The introduction of a host-plasmid balanced lethal system into the vaccine was a good idea to solve the problem. The λ-Red recombination system was adopted in this study to realize the replacement of *cat* by aspartate-semialdehyde dehydrogenase gene (*asd*) in the plasmid pMM085. The new plasmid named pMMASD was introduced into an *Escherichia coli* strain χ6097 and *Salmonella typhimurium* χ4072 where the *asd* gene had been knocked out in their chromosomes. Cultured in an Erlenmeyer flask, expression levels of two antigens K88ac fimbriae and heat-labile enterotoxin B subunit (LTB) in cell lysate were similar among MM-3, χ4072(pMMASD) and χ6097(pMMASD). However, χ4072(pMMASD) possessed the more effective secretion mechanism to transport LTB enterotoxin into culture liquid. The relatively higher stability of pMMASD in *Salmonella typhimurium* χ4072 than that of pMM085 in MM-3 was determined both *in vitro* in the absence of selective pressure, and *in vivo* following oral inoculation. Oral immunization of BALB/c mice with χ4072(pMMASD) or χ6097(pMMASD) was sufficient to elicit IgA responses in mucosal tissues as well as systemic IgG antibody responses to the K88 fimbriae, while MM-3 failed to elicit specific antibody responses to K88 fimbriae in mucosal tissues. Among three live strains, only χ4072(pMMASD) could develop strong humoral responses against LTB enterotoxin. The results suggest that χ4072(pMMASD) is expected to be a promising live vaccine strain.

Key words λ-Red recombination system; host-plasmid balanced lethal system; K88ac fimbriae; LTB enterotoxin; ETEC; live vaccine strain; *Salmonella*

Diarrheal disease caused by enterotoxigenic strains of *Escherichia coli* (ETEC) expressing the K88 (F4) fimbrial adhesin was a significant source of mortality and morbidity among newborn and weaned piglets. Recent vaccination strategies include the oral immunization of pregnant sows with a vaccine containing purified K88, K99 and 987P fimbriae, and B subunit of LT enterotoxin (LTB) [1], the utilization of killed recombinant strain expressing enterotoxigenic *E. coli* K88ac-ST_1-LTB fusion protein [2], using *Salmonella typhimurium* as a vector to express the ETEC ST_1-LTB and having a non-antibiotic selection marker (asd^+) [3], or oral immunization of new born piglets with live avirulent *E. coli* vaccine strains expressing K88ac fimbriae and LTB subunit [4].

The use of live attenuated bacteria to deliver recombinant protein vaccine antigens was a promising approach to overcoming the difficulties and costs of vaccine antigen purification, and it enhanced presentation to the immune system [5]. Bacterial vectors may mimic natural infection and therefore interact with the mucosal, humoral and cellular compartments of the immune system [6]. Live bacteria vaccine might be superior to an orally administered

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bacterin if the attenuated strain colonized the intestine, thus stimulating the immune system over a longer period of time than that would be possible with killed bacteria [4]. When this approach was used, genes encoding vaccine antigens were expressed from multicopy plasmids in the live attenuated bacterial vectors.

LTB was heat-labile toxin B subunit and was able to bind to the ganglioside GM1 and other related receptors presented on enterocyte [7]. K88 fimbrial adhesins were filamentous surface appendages whose lectin (carbohydrate binding) activity allowed K88 ETEC to attach to specific glycoconjugates (receptors) on porcine intestinal epithelial cells, which was an essential step in the colonization of the small intestine by ETEC [8]. Live bacterial vector MM-3 was designed by Chen and colleagues [9] and played a function of protecting neonatal piglets from diarrhea. The strain MM-3 carried pMM085, which possessed ETEC immunogens of both LTB and fimbriae antigen K88ac. In a challenge study and field trials, a high degree of protection from colibacillosis was afforded to piglets [9,10].

MM-3 always lost pMM085 *in vivo* because the plasmid brought the chloramphenicol resistance gene (*cat*). Antibiotic pressure worked well for plasmid maintenance *in vitro*, however, it was not presented *in vivo*. As a result, under the circumstance of lacking selective pressure, plasmid would be quickly lost during cell division. Furthermore, the antibiotic resistance gene was no longer recommended in live bacterial vaccines [11]. Removal of *cat* gene in MM-3 seemed necessary. β-galactosidase as a tracking marker was also attempted in the work of Chen *et al.* [9]. Although this tracking marker was safer than the antibiotic resistance gene, the common problem of instability remained.

Stability of protective antigen expression *in vivo* was essential for recombinant vaccine efficacy. The use of a balanced-lethal host-vector system wherein the plasmid possessed a gene complementing to a chromosomal deletion mutation of a vital gene, such as a gene for cell wall synthesis or DNA stability and replication, ensured that the plasmid was maintained [5]. The other approaches to ensure retention of the plasmid included the "Hok-Sok" strategy [12] and "Operator-Repressor Titration" [13] which also resulted in bacterial cell death if the plasmid was lost.

A classic example of the balanced lethal host-vector system was the *asd* system. A deletion of the chromosomal aspartate-semialdehyde dehydrogenase gene (*asd*) was made in the bacterial vector, resulting in an absolute requirement for diaminopimelic acid, an essential component of the peptidoglycan of the cell wall of gram-negative bacteria that was not found in mammalian hosts. The *asd* gene was supplied on a plasmid and complemented the mutation. In the original work describing this system in serovar *typhimurium*, the plasmid was stable in the absence of any exogenous selective pressure *in vitro* or *in vivo* [14]. In this study, the host-plasmid balanced lethal system was adopted and the work included the replacement of the *cat* gene by the *asd* gene and the transformation of the new plasmid into the strain which the *asd* gene was knocked out.

Considering that the pMM085 was constructed in 1980s and it was difficult to find its accurate physical map, the λ-Red recombination system was introduced to mediate the *cat* gene removal and the *asd* gene substitution. Recent advances in homologous recombination-based DNA engineering technology, termed "recombinogenic engineering" or "recombineering", have led to the development of highly efficient procedures with which chromosomal or plasmid DNA could be modified in *E. coli* by the introduction of mutations, such as single base substitutions, deletions and insertions. These methods were based on the rac-encoded RecET system or the bacteriophage λ-Red recombination system, eliminating the need for *in vitro* manipulations using restriction enzymes or DNA ligases. The three proteins of the Red system, Exo, Beta and Gam, mediated recombination between a linear double-stranded DNA donor and its homologous target sequence by promoting double-strand break repair [15–17]. The results of this study also indicated that the λ-Red recombination system was convenient and efficient to reconstruct plasmid.

Recombinant attenuated *Salmonella* strains were receiving much attention regarding their potential as an antigen delivery system for mucosal immunization. They were particularly useful as oral vaccines since these organisms colonized a major mucosal inductive site, i.e., the gut-associated lymphoid tissue (GALT). Specifically, the *Salmonella* were taken-up by the specialized M cells interspersed throughout the luminal epithelium of the Peyer's patches in the GALT and transported to underlying lymphoid cells [18,19]. *S. typhimurium* Δ*cya*Δ*crp*Δ*asd* mutants, which lacking adenylate cyclase and the cyclic AMP (cAMP) receptor protein had been shown to be stable and safe for *in vivo* use [11,20].

In this study, the new plasmid possessing *asd* gene was introduced into attenuated Δ*cya*Δ*crp*Δ*asd S. typhimurium* χ4072. BALB/c mice orally immunized with the *S. typhimurium* χ4072(pMMASD), which expressed heat-labile enterotoxin LTB and fimbriae antigen K88ac, produced stronger humoral and mucosal antibody

responses against the two antigens than the former live vaccine strain MM-3.

Materials and Methods

Bacterial strains, media, plasmids and antisera

Escherichia coli MM-3, containing pMM085, expressing K88ac fimbriae and LTA$^-$B$^+$, with *cm*^{R} antibiotic resistance gene was kindly provided by Prof. T. M. CHEN (Beijing Institute of Biotechnology, Beijing, China) [9]. *E. coli* χ6097, a Δ*asd* mutant strain, and *S. typhimurium* χ4072, a Δ*cya*Δ*crp*Δ*asd* mutant strain, were kindly provided by Curtiss *et al*. [11,20]. *S. typhimurium* χ3730, a Δ*galE*Δ*hsd*Δ*asd* mutant strain which was a restriction-negative, modification-positive strain used as an intermediate for the transfer of plasmids from *E. coli* to *Salmonella*, was also provided by Roy Curtiss III. The plasmid pKD46 was kindly provided by Dr. B. L. WANNER [16], expressing Red proteins-Exo, Beta and Gam when induced by arabinose and containing *amp*^{R} gene. The plasmid pZL21, containing *asd* gene was preserved in our lab. MM-3 and *E. coli* containing pKD46 were cultured in LB media with corresponding antibiotics. *E. coli* χ6097, *S. typhimurium* χ3730 and *S. typhimurium* χ4072 were grown in LB media supplemented with diaminopimelic acid (50 μg/ml). All strains and plasmids used in this study are provided in **Table 1**. The mouse anti-K88ac fimbriae antiserum and mouse anti-CTB enterotoxin antiserum were prepared in our laboratory.

Preparation of DNA fragments for recombineering

Polymerase chain reaction (PCR) was used for the amplification of DNA fragments with Thermocycler (Biometra, Gottingen, UK). The high-fidelity *Pfu* DNA polymerase (Tiangen, Beijing, China) was used in all of the reactions, prepared as described by the supplier. DNA fragments were generated by touchdown PCR amplification and the appropriate primers are shown in **Table 2**. Primers Forcatasd and Revcatasd were used to amplify a 1.7-kb DNA fragment containing the full-length *asd* gene using plasmid pZL21 as a template. The PCR conditions included an initial denaturation at 94 ºC for 5 min, followed by touchdown procedure of 16 cycles of denaturation at 94 ºC for 40 s, annealing at 58 ºC for 40 s (–0.5 ºC increment of every cycle) and a final extension at 72 ºC for 2 min, followed by general conditioning including 20 cycles of denaturation at 94 ºC for 40 s, annealing at 50 ºC for 40 s and extension at 72 ºC for 2 min. PCR products were used as templates in the second round of PCR with the same primers and the same reaction conditions to decrease the concentration of pZL21 [21]. The final PCR products were gel-purified and suspended in elution buffer

Table 1 ***Salmonella typhimurium*** **and** ***Escherichia coli*** **constructs used for construction of new live vaccines**

Bacterial strain	Parent strain	Characteristics	Plasmid	K88ac and LTA$^-$B$^+$	Select marker	References
S. typhimurium						
χ3730	LT2-Z	*leu hsdLT galE trpD2 rpsL120 ΔasdA1Δ[zhf-4::Tn10] metE551 metA22 hsdSA hasSB ilv*	None	None	asd$^-$	[11,20]
χ4072	SR-11	pStSR100$^-$ *gyrA1816 Δcya-1 Δcrp-1ΔasdA1Δ[zhf-4::Tn10]*	None	None	asd$^-$	[11,20]
χ4072(pMMASD)			pMMASD	K88ac, LTA$^-$B$^+$	asd$^+$	This study
E. coli						
MM-3	C600	*supE*44 *hsdR thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21	pMM085	K88ac, LTA$^-$B$^+$	*cm*^{R}	[9]
χ6097	K-12	F-*Δ[lac-pro] rpsL ΔasdA4 Δ[zhf-2::Tn10] thi 80dlacZ ΔM15*	None	None	asd$^-$	[11,20]
χ6097(pMMASD)			pMMASD	K88ac, LTA$^-$B$^+$	asd$^+$	This study
Plasmid						
pKD46		araBp-*gam-bet-exo*$^+$	None	None	*amp*^{R}	[16]
pZL21		*asd*$^+$	None	None	asd$^+$	This lab

Table 2 Primers used in polymerase chain reaction

Primer	Primer sequence (5′→3′)	Position	Orientation
Forcatasd	**GAGGCATTTCAGTCAGTTGCTCAATGTACC-TATAACCA***GAAGCGGTTAGCTCCTTCGGT*	+67 to +105::–28 to –9	Forward
Revcatasd	**CCGACATGGAAGCCATCACAAACGGCATGA-TGAACCTGA***CCAACTTACTTCTGACAACG*	+567 to +605::+1696 to +1677	Reverse
Forcat	**ATATCCCAATGGCATCGTAA**	+37 to +56	Forward
Revcat	**TGGAAGCCATCACAAACG**	+599 to +582	Reverse

The positions of the primers are relative to the start codon of the chloramphenicol acetyltransferase gene (*cat*) and *asd* gene. c*at* sequences are in bold letters and *asd* sequences are in italics.

(10 mM Tris, pH 8.0). Primers Forcat and Revcat were used to identify the correct mutants.

Genetic manipulation of *Escherichia coli* χ6097

The plasmid pMM085 and pKD46 were transformed into *E. coli* χ6097 and the transformants were selected from an LB agar plate supplemented with 100 μg/ml ampicillin, 75 μg/ml chloramphenicol and 50 μg/ml diaminopimelic acid (DAP). Transformants containing a Red helper plasmid were grown in 50 ml LB cultures with appropriate selective pressure and *L*-arabinose at 30 ºC to allow the expression of Exo, Beta and Gam when A_{600} reached 0.5, then made electrocompetent by concentrating 100 folds and washing three times with ice-cold 10% glycerol [17]. A total of 25 μl of competent cells were mixed with 20–25 ng of the PCR amplified DNA in a pre-cooled 0.1 cm Gene Pulser cuvette (Bio-Rad, Hercules, USA) and electroporated using the Bio-Rad Gene Pulser with conditions as 1.8 kV, 25 μF and 200 Ω. Following electroporation, the cells were grown in 1 ml of LB at 37 ºC for 1 h, plated 200 μl of cells on every LB agar plate without supplementation, and incubated at 37 ºC for 20 h. The correct mutants were identified by whole cell PCR using Primers Forcat and Revcat.

Expression of K88ac and LTA$^-$B$^+$ (LTB) in different live vaccine strains

The verified pMMASD obtained from *E. coli* χ6097 was transformed into *S. typhimurium* χ3730, which had a characteristic of Δ*galE*Δ*hsd*Δ*asd*. Plasmid pMMASD was then extracted from χ3730 and transformed into *S. typhimurium* χ4072 by the $CaCl_2$ transformation method. The strains were cultured at 37 ºC in 75 ml of LB medium (250 ml Erlenmeyer flask) for 12 h, and when A_{600} reached 4–5, 30 ml of culture was removed for centrifugation. The precipitants were resuspended in 10 ml of 1×phosphate-buffered saline (PBS) buffer and lysed by ultrasonication, centrifuged at 12,000 *g* for 20 min at 4 ºC. The lysate supernatant and culture supernatant were preserved at –20 ºC for checking the expression level of both K88ac fimbriae and LTB enterotoxin as described as follows in the enzyme-linked immunosorbent assay (ELISA).

In vitro plasmid maintenance

A single colony of *S. typhimurium* χ4072(pMMASD) was inoculated into 5 ml of nonselective medium (LB supplemented with 50 μg/ml DAP), grown at 37 ºC in an orbital shaker at 220 rpm for 12 h, then successively subcultured with 1:100 into flesh nonselective media every 12 h until the total time was 48 h. The culture of the first 12 h and the fourth 12 h was diluted 1:160,000 and 40 μl was plated on the nonselective agar plates respectively. After incubation at 37 ºC for 20 h, 100 colonies on the plate were selected randomly and inoculated on a selective medium (LB agar plate), and viable colonies were enumerated. The cells grew approximately three generations per hour, so there were 36 generations and 144 generations after 12 h growth and 48 h growth respectively.

The procedure of determining *in vitro* plasmid maintenance of *E. coli* MM-3 was almost the same as that of *S. typhimurium* χ4072(pMMASD), except that the non-selective and selective media were changed respectively to LB media and LB media plus chloramphenicol.

In vivo plasmid maintenance

Bacteria were cultured in an Erlenmeyer flask for 12 h, and centrifuged at 4000 *g* for 10 min. The density of the bacteria was adjusted by optical density measurement at 600 nm and confirmed by serial dilutions on LB agar plates. Female BALB/c mice were obtained from the Central Animal House of Beijing Institute of Biotechnology (Beijing, China) and were 8 weeks old at the commencement of experiments. Mice were fasted for 4 h before feeding the

bacteria. After treatment with 300 μl of 50% saturated sodium bicarbonate solution, each mouse received a single oral dose of 1.0×10^9 colony forming units (200 μl) of *S. typhimurium* χ4072(pMMASD) vaccine or 1.0×10^9 CFU of *E. coli* MM-3 vaccine and fasted for an additional 2 h.

On day 10 postinoculation, 10 mice were killed by cervical dislocation, and the spleen and 6–7 Peyer's patches of each mouse were removed. The spleen or the Peyer's patches were homogenized in 1.8 ml of sterile PBS using 50 μm pore size cell strainers, and 50 μl of the mixture was plated onto suitable agar for enumeration. Specifically, the samples were plated onto LB agar and LB agar containing chloramphenicol respectively for checking *E. coli* MM-3, and were plated onto Hektoen enteric (HE) agar and HE agar containing diaminopimelic acid for checking *S. typhimurium* χ4072(pMMASD).

Plasmid maintenance in the alimentary canal was also detected. The fecal pellets of each group were collected every day and resuspended in 60 ml of enrichment medium. Enrichment medium of selenite broth was employed for detection of *S. typhimurium* χ4072(pMMASD) in fecal specimens since the vaccine strains represented only a small percentage of the intestinal flora [22]. After incubation at 37 ºC for 10–12 h, 50 μl of broth was plated on HE agar plates, and the suspicious colonies on HE agar plates were green or transparent without a black center (lactose negative, no production of H_2S), and were further identified by whole cell PCR using primers Forcat and Revcat. For detection of MM-3 in fecal specimens, LB plus chloramphenicol was used as an enrichment medium and selective plate medium. The suspicious colonies were further identified by whole cell PCR.

Enzyme-linked immunosorbent assay

For detecting the expression level of K88ac fimbriae, the lysate supernatants were diluted to 1:10 and the culture supernatants were diluted to 1:3. The diluted antigens were then used to coat 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) at 100 μl per well. The plates were incubated overnight at 4 ºC, washed with PBST (PBS containing 0.05% Tween 20) three times, then blocked by 0.1% bovine serum albumin (BSA), and followed by incubation with mouse anti-K88ac fimbriae antiserum (1:2000 dilution). The bound Ag-Ab complex was detected using goat anti-mouse IgG conjugated to horseradish peroxidase (1:10,000 dilution) and color was developed using o-phenylene dimine (OPD) as a chromogen and H_2O_2 as substrate and the absorbance was read at 492 nm. For detecting the expression level of LTB, ganglioside GM1 at 2 μg/ml was used to coat 96-well microtiter plates, blocked by 0.1% BSA, and incubated with diluted lysate supernatant dilution or culture supernatant dilution. After being washed with PBST three times, mouse anti-CTB enterotoxin antiserum (1:2000) was added and the following steps were the same as those used when detecting the expression level of K88ac.

Oral immunization of mice

The preparation of bacteria was in the same way as in the method of "*in vivo* plasmid maintenance". Before immunization, mice were fasted for 4 h, and then pretreated with 300 μl of 50% saturated sodium bicarbonate solution. Finally, the mice were fed with feeding needles for intragastrical delivery of 200 μl of bacterial suspensions (2.0×10^9 CFU for *E. coli* strains or 1.4×10^8 CFU for *Salmonella* strains) and fasted for an additional 30 min. The mice were immunized at day 0, 7 and 21.

Assessment of immune responses

Mice of each group were killed on day 35. The blood was collected and centrifuged at 10,000 *g* for 5 min and the serum was stored at –20 ºC. Whole small intestines, from the duodenum to the ileocecal junction, were excised, snipped into pieces, and resuspended with 800 μl of PBS with 50 mM EDTA (pH 8.0). Recovered intestinal contents were vortexed vigorously for 5 min. After centrifugation at 10,000 *g* for 10 min at 4 ºC, supernatants were collected and stored at –20 ºC. For each immunized group of mice, fecal pellets were collected on day 34. Approximately 100 mg of feces were added to tubes containing 400 μl of PBS with 50 mM EDTA (pH 8.0) and incubated ice for 15 min. And then, tubes were agitated vigorously for 10 min on a vortex mixer at maximum speed. The suspensions were centrifuged at 10,000 *g* for 10 min at 4 ºC, and the supernatants were stored at –20 ºC.

Purification of K88ac fimbriae was as follows: *S. typhimurium* χ4072(pMMASD) was cultured in 150 ml of LB medium for 12 h at 37 ºC and shaken at 200 rpm. The cell suspensions were collected by centrifugation at 4000 *g* for 10 min, and the pellets were resuspended in 30 ml of 50 mM Tris buffer (pH 7.45) containing 1.0 M NaCl. The bacteria were subjected to heat shock at 63 ºC for 20 min to obtain K88ac fimbriae. The bacteria were pelleted at 4000 *g* for 10 min, and the K88ac fimbriae were precipitated from the supernatant by adding ammonium sulfate (30% saturation) and stirring for 1 h at 4 ºC. The precipitate was collected by centrifugation at 10,000 *g* for 10 min, resuspended in 2 ml of 50 mM Tris buffer (pH 7.45), and dialyzed against the same buffer for 16 h at 4

ºC. The purity of the isolated K88ac fimbriae was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by the Lowry method. The purified K88ac fimbriae were used to coat the 96-well ELISA plates.

Antibody responses were assayed by ELISA. Individual mouse serum, intestinal washes and fecal pellet extract were tested for IgG and IgA antibodies against K88ac fimbriae by ELISA. The 96-well plates were coated with purified K88ac fimbriae (0. 5 μg in 100 μl of 0.1 M carbonate buffer, pH 9.6, per well) overnight at 4 ºC. The plates were blocked with 0.1% BSA in PBST at 37 ºC for 2 h, washed three times with PBST, and incubated respectively with dilutions of serum, intestinal washes and fecal pellet extract samples in PBST-0.1% BSA for 1 h at 37 ºC. For detecting IgG and IgA antibodies against enterotoxin LTB, ganglioside GM1 (0.2 μg in 100 μl of 0.1 M carbonate buffer, pH 9.6, per well) was used to coat 96-well microtiter plates, blocked by 0.1% BSA, and followed by incubation with diluted cell lysate supernatants as described in "Materials and Methods". After being washed with PBST three times, diluted serum, intestinal washes, or fecal pellet extract samples in PBST-0.1% BSA were added, respectively. The bound Ag-Ab complexes were detected using goat anti-mouse IgG conjugated to horseradish peroxidase (1:10,000 dilution). The color was developed using OPD as a chromogen and H_2O_2 as substrate. The absorbance was read at 492 nm.

Statistical analysis

The absorbance at 492 nm (A_{492}) in ELISA was plotted using GraphPad Prism. Probability values were determined by the two-tailed, unpaired *t* test with Welch's correction (95% confidence interval) for comparisons of two sets of data.

Results

Construction of plasmid pMMASD

Using the λ-Red recombination system presented in pKD46, the *cat* gene was knocked out and the *asd* gene was knocked in through homologous recombination between pMM085 and a PCR-amplified *asd* gene fragment. The homologous recombination was between a plasmid pMM085 containing the *cat* gene sequence and the PCR-generated DNA fragment containing *asd* sequence flanked by 38 to 39 bp of *cat* sequence, corresponding to the 5′ and 3′ regions of the intended site of mutagenesis, respectively. When the λ-Red recombination system was activated, the flanking sequences of homology to normal *cat* sequence were sufficient to allow homologous recombination in *E. coli* χ6097 to promote the incorporation of the *asd* gene into the *cat* sequence, with an efficiency of 2.2×10^2 recombinants/μg DNA when growth was selected in conditional medium lacking DAP. Among the 21 colonies selected, 20 colonies showed a varying mixture of parentally and correctly recombined plasmids by PCR amplification of the region corresponding to the site of *asd* gene integration (**Fig. 1**). Colonies containing only the recombinant plasmid pMMASD were obtained by retransformation in *E. coli* χ6097 and selected for *asd* function. The correct recombination event was also confirmed by restriction enzyme and sequence analysis.

Expression of LTB and K88ac in *Salmonella typhimurium* χ4072 and in *Escherichia coli* χ6097

Salmonella typhimurium χ3730 in which the *asd* gene was knocked out was the Δ*galE* mutant strain of *S. typhimurium* LT2-Z and lost the effect of restriction but had the effect of modification. Therefore, the plasmid transformed into *S. typhimurium* χ3730 could obtain the methylated model of *S. typhimurium* and thus existed in this strain stably. The plasmid with the methylated model extracted from χ3730 was then transformed into the final host strain *S. typhimurium* χ4072 without any restriction. Five vaccine strains, *E. coli* MM-3, *E. coli* c6097, *E. coli* χ6097(pMMASD), *S. typhimurium* χ4072 and *S. typhimurium* χ4072(pMMASD), were cultured in LB medium at 37 ºC for 12 h. When A_{600} reached 4.0 to 5.0, 30 ml of mixture was obtained for analyzing the expression levels of K88ac fimbriae and LTB enterotoxin, respectively by ELISA.

Salmonella typhimurium χ4072(pMMASD) and *E. coli* χ6097(pMMASD) were found to stably express K88ac fimbriae and LTB enterotoxin without antibiotics. As shown in **Fig. 2**, *E. coli* MM-3, *E. coli* χ6097(pMMASD) and *S. typhimurium* χ4072(pMMASD) expressed similar levels of LTB in the cell lysate. However, the secretion level of LTB enterotoxin in χ4072(pMMASD) was highest, indicating that *S. typhimurium* had an advantage of secreting antigen over *E. coli*. Meanwhile, the negative contrasts χ6097 and χ4072 had a very low background, suggesting that the "GM1 sandwich" method was suitable for checking the LTB enterotoxin. As to the expression of K88ac fimbriae, although the background of negative contrasts χ6097 and χ4072 were relatively high in culture supernatant, the conclusion that K88ac fimbriae were displayed on the surface of the bacteria and dropped into

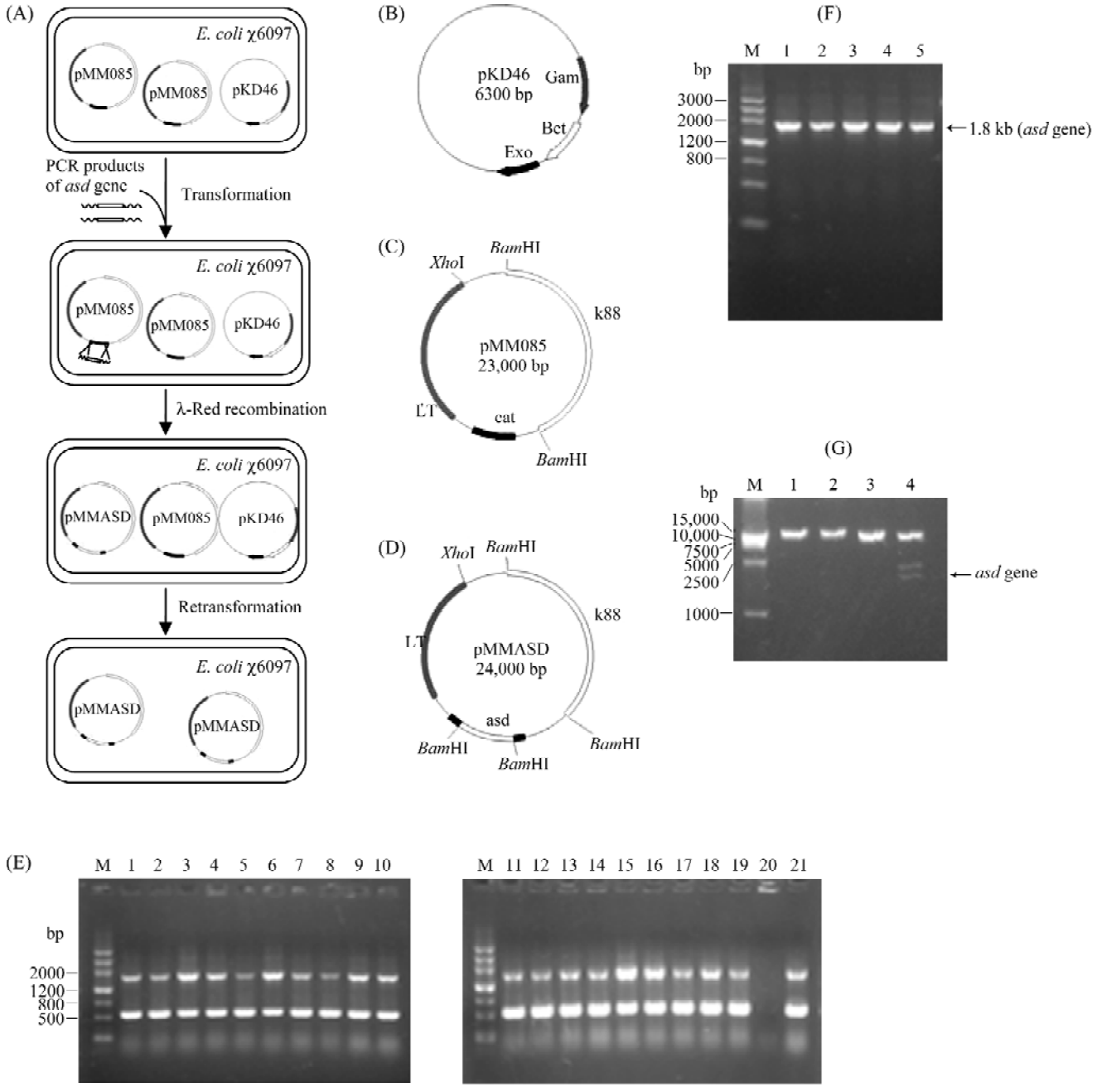

Fig. 1 Construction and identification of plasmid pMMASD

(A) Schematic representation of the strategy for replacement of the *cat* gene by the *asd* gene. The *cat* gene was partly deleted through homologous recombination between the plasmid gene and a DNA fragment *asd* gene generated by polymerase chain reaction (PCR) described in "Materials and Methods". This process was assisted by the λ-Red recombination system presented in the plasmid pKD46. (B–D) Map of plasmid pKD46, pMM085 and pMMASD respectively. (E) Confirmation of the resultant colonies grown on the selective LB agar plates. The DNA products after PCR amplification of plasmid DNA with primers Forcat and Revcat would be only a 1.8 kb fragment (*asd* gene containing the flanking sequence homologous to the *cat* gene) when the *cat* gene was replaced by the *asd* gene in every plasmid, otherwise, the DNA products would be the mixture fragments of 1.8 kb and 0.5 kb for the mixture of parental plasmids and correctly recombined plasmids in one host cell. Lanes 1–21 are the whole cell PCR products of colonies 1–21 respectively; M, DNA marker. (F) Confirmation of resultant colonies grown on selective LB agar plates after retransformation of mixture plasmids in *Escherichia coli* χ6097. The DNA products after PCR amplification of plasmid DNA with primers Forcat and Revcat were analyzed by 1% (*W*/*V*) agarose gel electrophoresis. Lanes 1–5, the whole cell PCR products of five random selected colonies; M, DNA marker. (G) Confirmation of the *cat* gene removal and introduction of the *asd* gene substitution by restriction enzyme. The substitution of the *asd* gene introduced two new *Bam*HI restriction enzyme sites in the recombinant plasmid pMMASD, and would result in two additional fragments of 1.7 kb and 2.1 kb following digestion. Lanes 1 and 2 are the plasmid pMM085 and pMMASD digested with *Xho*I respectively; Lanes 3 and 4 are pMM085 and pMMASD digested with *Bam*HI respectively; M, DNA marker.

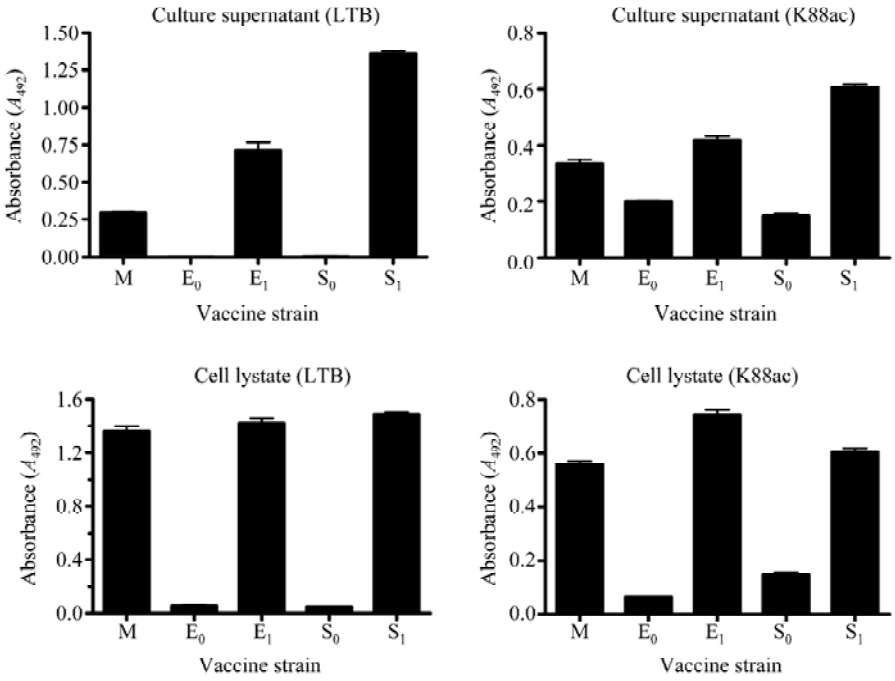

Fig. 2 Enzyme-linked immunosorbent assay (ELISA)analysis of expression of both K88ac fimbriae and LTB enterotoxin in different live vaccine strains

M, *Escherichia coli* MM-3; E_0, *E. coli* χ6097; E_1, *E. coli* χ6097(pMMASD); S_0, *Salmonella typhimurium* χ4072; S_1, *S. typhimurium* χ4072(pMMASD). The cell lysate were diluted to 1:10, and the culture supernatant were dilute to 1:3 by 0.1 M carbonate buffer, pH 9.6.

culture fluid was easy to make. In the cell lysate of MM-3, χ6097(pMMASD) as well as χ4072(pMMASD), the expression levels of K88ac fimbriae were almost the same as others, with χ6097(pMMASD) possessing the relatively highest ability to display the K88ac fimbriae.

The *asd* balanced-lethal system was used to stabilize plasmid maintenance for *in vivo* experiments. Data showed in this study that the changes in the reconstructed plasmid pMMASD had no negative effect on the expression of K88ac fimbriae and LTB enterotoxin; on the other hand, the new host cell *S. typhimurium* χ4072 possessed the more effective secretion mechanism to transport LTB enterotoxin into culture liquid, indicating that *S. typhimurium* χ4072(pMMASD) might be the better vaccine strain to elicit the humoral and mucosal antibody responses against the LTB enterotoxin.

In vitro plasmid maintenance

To demonstrate whether pMMASD would be stably maintained in a host-plasmid balanced lethal system, *in vitro* plasmid maintenance of *S. typhimurium* χ4072 (pMMASD) was investigated compared with that of *E. coli* MM-3. Serial subcultures in nonselective broth showed that more than 70% of equivalent colony numbers were obtained on selective agar plates for total viable cells after the 144 generations. In contrast, there were less than a quarter of equivalent colony numbers obtained in MM-3, which maintained its plasmid with antibiotic selective pressure, indicating that the plasmids were more stable in the host-plasmid balanced lethal system than in the antibiotic selective system (**Fig. 3**).

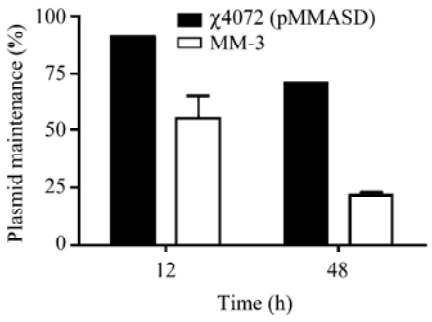

Fig. 3 Plasmid maintenance *in vitro*

Salmonella typhimurium χ4072(pMMASD) and *Escherichia coli* MM-3 were grown in nonselective broth before being plated on the nonselective plates. One hundred colonies were selected randomly and inoculated on the selective plates. Viable colonies were counted.

In vivo plasmid maintenance

Maintenance of plasmid pMMASD in *S. typhimurium* χ4072, as well as pMM085 in MM-3 *in vivo* was evaluated. *In vivo*, recombinant bacteria could be detected neither in the spleens and in Peyer's patches of BALB/c mice orally inoculated with 1.0×10^9 of *E. coli* MM-3, nor in the spleens of BALB/c mice orally inoculated with 1.0×10^9 of χ4072 (pMMASD). Only three of ten mice inoculated with χ4072 (pMMASD) were detected having the invasion of the vaccine strain in Peyer's patches (**Table 3**).

S. typhimurium χ4072 was a Δ*cya*Δ*crp* mutant strain in which the adenylate cyclase and the cAMP receptor protein genes were deleted. These proteins were necessary for the transcription of many genes and operons concerning with the transportation and breakdown of catabolites [23]. It was said that the Δ*cya*Δ*crp* mutant abolishes the synthesis of at least two adhesins that *S. typhimurium* uses for attachment to, and thus invasion of enterocytes [24]. Curtiss *et al.* also proved that the Δ*cya*Δ*crp* mutations do not significantly impair the ability of *S. typhimurium* to attach to, invade and persist in Peyer's patches, but do impair the ability to reach or survive in the spleen [25]. Although the invasion ability of *S. typhimurium* χ4072 in spleens and Peyer's patches was relatively poor, the conclusion that the plasmid in the vaccine strain was stably maintained could be made according to the percentages of retaining plasmid in Peyer's patches that were from 60% to 115% in three of ten susceptible mice. Furthermore, when the total of 63 colonies on the nonselective agar plates (HE plus DAP) were selected and inoculated on a selective medium, 61 colonies grew well, indicating the stability of plasmid pMMASD *in vivo*.

Maintenance of plasmid in vaccine strains in the alimentary canal was also evaluated (**Table 4**). If plasmid pMMASD or pMM085 were lost in the host cell, there was no vaccine strain in fecal pellets. The results suggested that the pMM085 had a greater chance of being lost from the host strains comparing with pMMASD, and the reason was perhaps that the environment *in vivo* did not provide an antibiotic pressure for the stable maintenance of pMM085. On the contrary, this environment provided a selective pressure (the lack of DAP) for the survival of pMMASD in the host-plasmid balanced lethal system. Perhaps the other reason for the relatively longer persistence in alimentary canal for χ4072(pMMASD) was the ability of invasion of intestinal enterocytes.

Table 3 Plasmid maintenance of χ4072(pMMASD) in Peyer's patches following intragastric inoculation of mice

Mice	HE agar plate	HE+DAP agar plate	Bacteria retaining plasmid (%)
n_1	45	39	115
n_2	11	14	79
n_3	6	10	60

Groups of BALB/c mice (n=10) were orally inoculated with *Salmonella typhimurium* χ4072(pMMASD) or *Escherichia coli* MM-3 at day 0. Spleens and six Peyer's patches per mouse were removed at day 10 after inoculation and homogenized in 1.8 ml phosphate-buffered saline (PBS) before 50 μl of preparations were plated onto selective or nonselective agar for enumeration of the bacteria retaining plasmids. In Peyer's patches only three of ten mice inoculated with *S. typhimurium* χ4072(pMMASD) were detected the invasion of the vaccine strain . The values are the percentages of organisms that retained plasmids to the nearest 1%. HE agar, hektoen enteric agar; DAP, diaminopimelic acid.

Immune responses of BALB/c mice after oral immunization with the live vaccine strains

The immunogenicity of the K88ac fimbriae and LTB enterotoxin expressed in *S. typhimurium* χ4072 (pMMASD), *E. coli* χ6097(pMMASD) and MM-3 were tested by oral administration of the strains to its corresponding group of BALB/c mice. Thirty mice were divided into six groups with five mice in each group. After three immunizations, each mouse was killed and the blood, small intestines and fecal pellets were collected separately for analyzing the effectiveness of different live vaccine strains delivering K88ac fimbriae and LTB enterotoxin to the

Table 4 Plasmid maintenance in alimentary canal following intragastric inoculation of mice

Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
MM-3	+	+	–	–	ND	ND	ND	ND
χ4072(pMMASD)	+	+	+	+	+	+	–	–

Groups of BALB/c mice (n=12) were orally inoculated with *Salmonella typhimurium* χ4072(pMMASD) or *Escherichia coli* MM-3 at day 0. Fecal pellets were collected every day and cultured in enrichment broth for 12 h, then plated on selective agar plates. The suspicious colonies were identified by whole cell polymerase chain reaction (PCR) to identify the plasmid maintenance. +, vaccine strains were detected in the sample; –, vaccine strains could not be detected in the sample; ND, the results were not detected.

immune system.

S. typhimurium χ4072(pMMASD), *E. coli* χ6097 (pMMASD) and *E. coli* MM-3 developed significantly high levels of K88ac fimbriae-specific serum IgG [**Fig. 4(D)**]. However, K88ac fimbriae-specific mucosal secretory IgA levels determined in intestinal washes and fecal pellet washes for the mice immunized with χ4072(pMMASD) and χ6097(pMMASD) were significantly higher than secretory IgA levels for the mice immunized with *E. coli* MM-3 [**Fig. 4(E,F)**]. Meanwhile, serum IgG against the LTB enterotoxin were only developed in the mice immunized with *S. typhimurium* χ4072(pMMASD), as visualized in absorbance of A_{492} by ELISA [**Fig. 4(A)**]. The results indicated that the more effective secretion system of *S. typhimurium* enhanced the immune response to the LTB enterotoxin. Comparison of the immune response with *S. typhimurium* χ4072(pMMASD) versus *E. coli* MM-3 resulted in significant differences in both K88ac fimbriae-specific mucosal secretory IgA and LTB enterotoxin-specific serum IgG, suggesting that the use of *S. typhimurium* as a delivery system improved the immunogenicity of both K88ac fimbriae and LTB enterotoxin.

Although *S. typhimurium* χ4072(pMMASD) elicited mucosal secretory IgA specific for LTB enterotoxin, the antibody levels were generally low [**Fig. 4(B,C)**]. There were no statistically significant differences in LTB-specific mucosal secretory IgA levels between the mice immunized with χ4072 and the ones immunized with χ4072 (pMMASD), or between the control mice and ones immunized with χ4072(pMMASD).

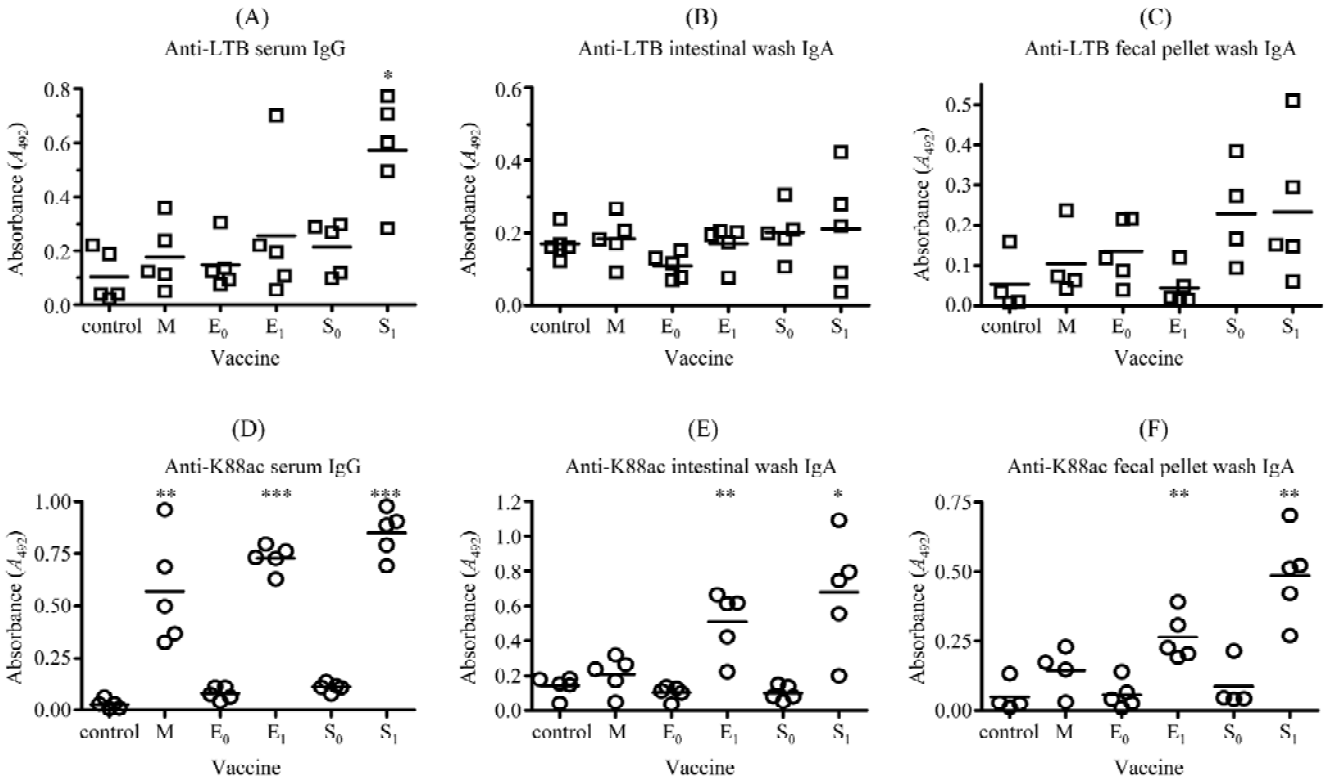

Fig. 4 K88ac fimbriae and LTB enterotoxin specific IgG and IgA responses after oral immunization of BALB/c mice with different live vaccine strains

Mice were immunized on day 0, 7, and 21 with 2.0×10^9 CFU *E. coli* strains or 1.4×10^8 CFU (colony forming units) *Salmonella* strains. Control, the group of mice that was immunized with physiological saline solution instead of vaccine strains; M, mice immunized with *Escherichia coli* MM-3; E_0, mice immunized with *E. coli* χ6097; E_1, mice immunized with *E. coli* χ6097(pMMASD); S_0, mice immunized with *Salmonella typhimurium* χ4072; S_1, mice immunized with *S. typhimurium* χ4072(pMMASD). Individual serum, intestinal washes and fecal pellet washes of anti-LTB enterotoxin and anti-K88ac fimbriae responses from live vaccine strains are shown as scatter plots. The lines among the scatter plots are the means of the absorbance of A_{492} of each group. The samples of serum, intestinal washes and fecal pellet washes were diluted to 1:250, 1:10 and 1:10 respectively before determining the antibody level by ELISA. Compared data sets were between control and M, E_0 and E_1, as well as S_0 and S1 when the probability values were analyzed. *P<0.05; **P<0.01; ***P ≤0.001.

Discussion

Live bacterial vector MM-3 played a function of protecting neonatal piglets from diarrhea. In a challenge study and field trials, a high degree of protection from colibacillosis was afforded to piglets when their dams were immunized orally or parenterally [9,10]. However, since the plasmid of MM-3 contained the *cat* gene, it had a large chance of being lost in the environment where antibiotic selective pressure was not presented. β-galactosidase as a tracking marker was also attempted in the work of Chen *et al.* [9], but the same problem remained. The target of this study was the introduction of the *asd* gene to replace the *cat* gene to result in a stable maintenance of recombinant plasmid in the *asd* balanced-lethal system. Moreover, finding a more suitable host cell to deliver the K88ac fimbriae and LTB enterotoxin was also expected.

Recently, the development of recombineering using homologous recombination in bacteria has allowed DNA manipulation without the need for restriction enzymes. The target gene could be introduced anywhere in chromosomes, bacterial artificial chromosomes or a plasmid DNA sequence specifically by homologous recombination. The replacement of the *cat* gene by the *asd* gene in pMM085 was achieved by a λ-Red recombination system. pMM085 was a multicopy plasmid, and this made the high frequency of recombination events *in vivo* for the greater chances of linear double-stranded DNA donor encountered its homologous target sequence. The recombined efficiency was only 2.2×10^2 recombinants/μg DNA (in the work of Wong *et al.* [15], the recombined efficiency was 1.32×10^4 recombinants/μg DNA), because the data were not gathered under the optimal conditions. Besides, this system showed a selection efficiency of more than 95% (20/21), requiring the screening of fewer colonies to ensure a successful outcome.

Recombinant plasmid pMMASD was transformed into two host cells: *S. typhimurium* χ4072 and *E. coli* χ6097. Cultured in LB medium, expression levels of K88ac fimbriae and LTB enterotoxin in cell lysate were similar among MM-3, χ4072(pMMASD) and χ6097(pMMASD), and χ6097(pMMASD) had a slightly higher expression level of K88ac fimbriae. It seemed that χ6097(pMMASD) was the candidate for taking the place of MM-3. However, analysis of the expression level of LTB enterotoxin in culture supernatant revealed that χ4072(pMMASD) possessed the more effective secretion mechanism to transport LTB enterotoxin into culture liquid. Work in mice with serovar *typhimurium* expressing the recombinant *Streptococcus pneumoniae* antigen PspA compared expression in the cytoplasm with secretion into the periplasm and culture supernatant [26]. There was a 10^4 increase in IgG titer in animals that received the strain bearing a secreted antigen. The same results in this study were reached when oral immunization of BALB/c mice with different live vaccine strains, only χ4072(pMMASD) developed a strong humoral response against LTB enterotoxin, while *E. coli* MM-3 and χ6097(pMMASD) did not elicit the corresponding response.

Although χ4072(pMMASD) failed to elicit strong IgA responses to LTB enterotoxin in mucosal tissues, expressed LTB might play an adjuvant effect on response to the K88 fimbriae. Consistent with this interpretation, only live *E. coli* strains carrying both K88ac and LTB antigens would afford litter pigs effective protection from the challenge inoculation, while strains carrying the K88ac antigen would afford pigs part protection to the challenge inoculation and the strain carrying LTB antigen afforded no protection to the pigs [4].

Enteroadhesive fimbriae played a critical role in the pathogenesis of ETEC. The binding of fimbriae to intestinal receptors ensures optimal mucosal colonization by the bacteria and efficient enterotoxin delivery to the enterocytes. Fimbriae can serve as an effective vaccine to induce an immune response against ETEC infections. The results of this study clearly demonstrate that oral immunization of BALB/c mice with the *Salmonella*-K88 construct χ4072(pMMASD), as well as *Escherichia*-K88 construct χ6097(pMMASD) were sufficient to elicit elevated IgA responses in mucosal tissues and increase the systemic IgG antibody responses to the K88 fimbriae. Although MM-3 could express ETEC K88 fimbriae and elicit good IgG responses, the ability of stimulating the mucosal inductive tissues was weaker than the other of two strains. Considering IgA was a major maternal antibody that passed to piglets through colostrum and milk and provided protection against infection, therefore, the higher level of IgA vaccine strains χ4072(pMMASD) and χ6097 (pMMASD) elicited, the higher protection effects might be gained.

S. typhimurium naturally infects Peyer's patches, a major IgA inductive site, and subsequently disseminates to systemic tissues. During the past several years, numerous studies have evaluated recombinant *Salmonella* vaccine strains for their ability to induce mucosal and systemic immune responses to heterologous protein antigens [27–29]. Although *S. typhimurium* Δ*cya*Δ*crp* mutant χ4072 had a weaker ability of invading and persisting in Peyer's patches and spleen than the Δ*aro*A mutants (defect in the

aromatic amino acid synthesis pathway) such as SL3261 which invaded both Peyer's patches and spleen [13,30], the fact that *S. typhimurium* χ4072(pMMASD) developed higher levels of specific IgA and IgG to K88ac fimbriae than that of *E. coli* χ6097(pMMASD) proved that *Salmonella* strains χ4072 as a living vector could enhance the mucosal and humoral immune response to the antigen.

Development of immunity to *Salmonella* infections relies on the cellular, humoral, and mucosal arms of the immune system. Although the role of humoral immunity has been long appreciated, recent work has shown the cellular responses stimulated by the *Salmonella* [31–33]. Furthermore, displaying of antigens or antigenic fragments by the autotransporter pathway on the surface of *Salmonella* vaccine strains would result in a pronounced T-cell response in mice [34,35]. The reason might be the efficient antigen presentation via the MHC class II pathway stimulating cellular immune response. Therefore, it would be speculated that the *Salmonella* strain χ4072 plus K88 fimbriae displaying on the surface of this cell might be involved in the cellular immune responses.

In conclusion, *S. typhimurium* χ4072(pMMASD) was identified in this work to have many advantages over the former live vaccine strain *E. coli* MM-3, not only in plasmid stability *in vitro* and *in vivo*, but also in developing immune response to both K88ac fimbriae and LTB enterotoxin, and was expected to be a safe and stable live vaccine strain. Therefore, this study gave an example of re-construction of a live vaccine strain carrying an antibiotic resistance gene.

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