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Regulated delayed synthesis of lipopolysaccharide and enterobacterial common antigen of *Salmonella* Typhimurium enhances immunogenicity and cross-protective efficacy against heterologous *Salmonella* challenge

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Abstract

Lipopolysaccharide (LPS) O-antigen and enterobacterial common antigen (ECA) are two major polysaccharide structures on the surface of *Salmonella enterica* serovar Typhimurium. Previous studies have demonstrated that regulated truncation of LPS enhances the cross-reaction against conserved outer membrane proteins (OMPs) from enteric bacteria. We speculate that the regulation of both O-antigen and ECA may enhance the induction of immune responses against conserved OMPs from enteric bacteria. In this work we targeted *rfbB* and *rffG* genes which encode dTDP-glucose 4,6-dehydratases and share the same function in regulating O-antigen and ECA synthesis. We constructed a mutant, S496 (*rfbB6 rffG7 pagL73::TT araCP_{BAD} rfbB-3*), in which *rfbB* gene expression was dependent on exogenously supplied arabinose during *in vitro* growth and achieved the simultaneous tight regulation of both LPS and ECA synthesis, as demonstrated by the LPS profile and Western blotting using antisera against LPS and ECA. When administered orally, *S. Typhimurium* S496 was completely attenuated for virulence but still retained the capacity to colonize and disseminate in mice. In addition, we found that oral immunization with S496 resulted in increased immune responses against OMPs from enteric bacteria and enhanced survival compared with immunization with S492 possessing *rfbB6 rffG8* mutations when challenged with lethal doses of *Salmonella* Choleraesuis or *Salmonella* Enteritidis. These results indicate that *S. Typhimurium* arabinose-regulated *rfbB* strain S496 is a good vaccine candidate, conferring cross-protection against lethal challenge with heterologous *Salmonella*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2016.07.010>.

Keywords

Salmonella Typhimurium; *rfbB* and *rffG* genes; Arabinose-regulated expression; Cross-protection

1. Introduction

Lipopolysaccharide (LPS) is one of the key virulence factors and a major surface component of *Salmonella enterica* serovar Typhimurium [1]. LPS is critical for successful *Salmonella* infection and survival in the host as the lack of LPS or modification of LPS will compromise many biological characteristics, including swimming and swarming motility [2], intestinal colonization [3], serum resistance [4], invasion/intracellular replication [5], and resistance to killing by macrophages [6]. Full-length LPS consists of three domains: lipid A, core oligosaccharide and O-antigen polysaccharide [1]. The O-antigen polysaccharide, which is composed of a polymer of repeating sugar units and is the primary surface antigen of *Salmonella*, contributes to defense against serum complement activation [7].

The enterobacterial common antigen (ECA) is the second major immunogenic antigen on the surfaces of *Salmonella* and other enteric bacteria [8]. ECA is composed of linear polysaccharide chains, consisting of a repetitive tri-saccharide composed of 4-acetamide-4,6-dideoxy-D-galactose (Fuc4NAc), N-acetyl-D-mannosaminuronic acid (ManNAcA) and N-acetyl-D-glucosamine (GlcNAc) in *Salmonella* [9]. Previous studies have demonstrated that ECA plays an important role in bacterial virulence [10] and that an ECA-deficient mutant was able to induce a protective immune response against lethal challenge of homologous and heterologous *Salmonella* when used as a live-attenuated vaccine in the mice model [11]. We and others have demonstrated that regulated O-antigen synthesis contributes to the induction of cross-reactive immune responses to conserved outer membrane proteins (OMPs), thereby enhancing cross protection against infection by multiple pathogens [12–14]. Regulated O-antigen synthesis can be achieved by the deletion of particular genes essential for O-antigen synthesis, such as *pmi* (*manA*) and *galE*, or by the replacement of the promoter of a gene of interest with a regulated promoter, such as the *araC* P_{BAD} promoter [12,13]. The *pmi* and *galE* gene deletions resulted in mutants with a reversible rough LPS, in which the smooth LPS phenotype was restored when grown in the presence of mannose and galactose, respectively [15–17].

The dTDP-glucose 4,6-dehydratase, encoded by *rfbB* (*rmIB*) in the O-antigen gene cluster, is the second enzyme necessary for the synthesis of dTDP-rhamnose, which is the rhamnose donor for the *S. Typhimurium* O-antigen structure [18]. The *rffG* gene, which is located in the ECA gene cluster, is a functional homolog of *rfbB* and is responsible for synthesis of dTDP-Fuc4NAc, the precursor of Fuc4NAc in the ECA structure [9]. Previous results have demonstrated that these two genes are exchangeable [8,19]. We are planning to manipulate one gene to achieve the regulated synthesis of two major surface antigens, with the goal of maximal exposure of the conserved OMPs to the host immune system, thus inducing enhanced protective immunity.

In this study, we achieved regulated LPS and ECA synthesis via *rfbB* gene regulation by the arabinose-regulated *araC* P_{BAD} promoter. In this system, LPS and ECA were synthesized *in*

vitro, where arabinose was available, but not *in vivo*, where arabinose was absent. This vaccine strain displayed the wild-type phenotype at the time of immunization and became attenuated after colonization in the host tissue [13]. The resultant strain was evaluated for its capacity to induce immunity and cross-protection against *S. Enteritidis* and *S. Choleraesuis*.

2. Materials and methods

2.1. Bacterial strains, plasmids, media, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *S. Typhimurium* vaccine strains were derived from the highly virulent strain S100. *S. Typhimurium* cultures were grown at 37 °C in Luria–Bertani (LB) broth or on LB agar with or without 0.1% arabinose. Diaminopimelic acid (DAP) (50 µg/ml) was added for the growth of the *asd* strains [20]. LB agar containing 5% sucrose was used for *sacB* gene-based counterselection in the allelic exchange experiments [21].

2.2. Plasmids and mutant strain construction

The primers used in this study are listed in Supplementary Table 1. DNA manipulations were performed as described elsewhere [22], and detailed procedures could be found in the supplementary materials and methods. Transformation of *Escherichia coli* and *S. Typhimurium* was accomplished by electroporation (2.5 kV, 5 ms). The transformants were selected on LB agar plates containing chloramphenicol (25 µg/ml). The construction of the *rfbB rffG* mutant and the arabinose-regulated mutant is described in the Supplementary Materials and Methods.

2.3. LPS and ECA analysis

LPS was analyzed as previously described by Hitchcock and Brown [23]. The ECA phenotypes were characterized by immunoblotting. ECA was prepared as previously described [24]. The isolated ECA samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the resulting bands were transferred to nitrocellulose membranes. The membranes were probed with a 1:1000 dilution of the murine anti-ECA monoclonal antibody mAb898 [24].

2.4. Growth kinetics and motility and sensitivity assays

For determining the growth kinetics of the wild-type strain S100 and the mutant strains, bacterial density was monitored by optical density (OD) at 600 nm every hour. LB plates containing 0.3% agar with or without arabinose were inoculated with each strain for the motility assays. For the polymyxin B and sodium deoxycholate (DOC) sensitivity assays, 100 µl of 100-fold diluted bacterial cultures that had been grown to 0.8 of OD₆₀₀ were inoculated with polymyxin B at a final concentration of 0.1 µg/ml or DOC at a final concentration of 10 mg/ml for 1 h at 37 °C. The bacteria were diluted to the appropriate concentration and plated on LB plates, and bacterial colony counts were used to calculate survival rates. The assays were repeated three times.

2.5. Attachment and invasion assays

The human intestinal cell line INT407 was obtained from the American Type Culture Collection (ATCC strain CCL-6). The attachment and invasion tests were performed three times as described previously [25].

2.6. Determinations of virulence and colonization in mice

All animal research was conducted in compliance with the Animal Welfare Act and regulations related to experiments involving animals following the principles stated in the Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering during the experiments.

Six-week-old female BALB/c mice were obtained from Dashuo Experimental Animal Ltd. (Chengdu; Sichuan, China). Determination of the 50% lethal dose (LD₅₀) ($n = 5/\text{group}$) and colonization evaluation ($n = 3/\text{group}$) were conducted as previously described [12]. Peyer's patches (PP), spleen and liver tissues were harvested on day 6 post-infection to evaluate colonization.

2.7. Immunization and lethal *Salmonella* challenge

The bacterial cultures were prepared as previously described [12]. Each mouse was orally inoculated with 20 μl of buffered saline gelatin (BSG) containing 1.0×10^9 colony-forming units (CFU) of each strain on day 0 and was boosted on day 28 with the same dose of the same strain. Blood was obtained from mice by eye venous plexus bleeding at biweekly intervals after the first immunization.

For the lethal challenge studies, the immunized mice were challenged by oral inoculation with 6.5×10^8 CFU, 2.4×10^7 CFU and 2.4×10^7 CFU of the wild-type *S. Typhimurium*, *S. Choleraesuis* and *S. Enteritidis*, respectively. The LD₅₀ values of the wild-type *S. Typhimurium*, *S. Choleraesuis* and *S. Enteritidis* in BALB/c mice were 5.0×10^5 , 1.0×10^5 and 1.0×10^5 by oral administration, respectively (data not shown). The challenged mice were monitored and deaths were recorded daily for 30 days.

2.8. Quantitative enzyme-linked immunosorbent assay (ELISA)

S. Typhimurium LPS was purchased from Sigma (St. Louis, MO, USA). *Salmonella* outer membrane proteins (SOMPs) were prepared as previously described in [26]. A quantitative ELISA was used for the analysis of antibodies in serum to *S. Typhimurium* LPS and SOMPs. The wells of polystyrene 96-well flat-bottom microtiter plates were coated with 100 ng/well LPS or SOMPs suspended in 100- μl volumes of sodium carbonate/bicarbonate coating buffer (50 mM Na₂CO₃, 50 mM NaHCO₃, 0.1% sodium azide, pH 9.6). Goat anti-mouse Ig (H + L) (BD Pharmingen; San Diego, CA) in PBS was added to extra wells without coating with OMPs or LPS in triplicate to determine the standard curve. The coated plates were incubated overnight at 4 °C, followed by blocking with PBS containing 10% fetal bovine serum (FBS) for 1 h at room temperature. A 100- μl volume of 200-fold diluted sample was added to each corresponding well in triplicate, and 100- μl volume of the mouse IgG (BD Pharmingen; San Diego, CA, USA) with twofold dilutions in PBS from 0.5 mg/ml was successively added to the well coated by goat anti-mouse Ig (H + L) for the standard

curve. The plates were incubated for 1 h at 37 °C and were then treated with biotinylated goat anti-mouse IgG (Southern Biotechnology Associates; Birmingham, AL, USA). The wells were developed with a streptavidin-alkaline phosphatase conjugate (Southern Biotechnology Associates; Birmingham, AL, USA), followed by *p*-nitrophenylphosphate substrate (Sigma-Aldrich; St. Louis, MO, USA) in diethanolamine buffer (pH 9.8). Color development (absorbance) was recorded at 405 nm using an iMark™ Microplate Reader (Bio-Rad; Hercules, CA, USA). The standard curve was drawn using Curve Expert (Hyams DG; Starkville, MS, USA), and the concentration of serum antibodies was calculated using the standard curve.

2.9. Statistical analysis

Antibody titers were expressed as means $\pm$ SEM. Statistical analyses were performed using the GraphPad Prism 5 software package (Graph Software; San Diego, CA, USA) [27]. One- or two-way ANOVA were performed to determine the statistical significance of the differences between or among mean values for the various experimental and control groups. The log-rank test was used for survival curves. $P < 0.05$ was considered a significant difference.

3. Results

3.1. Mutant construction

The single mutants S490 (*rffB6*) and S491 (*rffG8*) had the same profiles of LPS and ECA as the parent wild-type strain, and double deletion of *rffB* and *rffG* resulted in a mutant S492 with deficient LPS and ECA profiles (Fig. 1A and B), indicating that the proteins encoded by *rffB* and *rffG* have the same function as dTDP-glucose 4,6-dehydratase and that the amount of dTDP-glucose 4,6-dehydratase encoded by either *rffB* or *rffG* gene is sufficient for synthesizing the full LPS and ECA structures in LB media. To further elucidate this phenomenon, the two complement plasmids were constructed and transformed into the mutant S492 (*rffB6 rffG8*). The LPS and ECA profiles indicated that each complement plasmid has capacity to restore truncated LPS and ECA to the wild-type state (Fig. 1A and B), which further demonstrated that the *rffB* and *rffG* genes performed the same function in the LPS and ECA biosynthetic pathways [8,19].

To achieve regulated LPS and ECA synthesis, we applied the same strategy as we previously used for the regulation of *wzy* and *rfaH* expression in *Salmonella* to the *rffB* gene [12,13]. We used the arabinose-regulated *araC*P_{BAD} promoter to regulate *rffB* expression in turn regulate LPS and ECA synthesis. Four mutants with arabinose-regulated *araC*P_{BAD} *rffB* carrying variable Shine-Dalgarno (SD) sequences, ATG/GTG start codons or both were constructed (Table 1). The effects of arabinose-regulated *rffB* on LPS and ECA synthesis were evaluated by silver staining and Western blotting. The mutants S496 (P_{BAD} *rffB*-3) and S497 (P_{BAD} *rffB*-4) produced an LPS and ECA pattern similar to that of the wild-type *Salmonella* when they were grown with arabinose and displayed the phenotypes of rough LPS and ECA-deficiency similar to that of the mutant S492 (*rffB6 rffG8*) in the absence of arabinose, whereas the other two constructs did not show tight regulation by arabinose (Fig. 1C and E). Thus, S496 and S497 were selected for further phenotypic evaluation.

3.2. Phenotypic evaluation of the mutant strains

To investigate whether regulation of LPS and ECA synthesis affects *Salmonella* phenotypes, we performed several assays, including growth curve assays and assays of swarming capacity and susceptibility to environmental factors. The growth rates of the mutant strains were determined in LB broth with or without 0.1% arabinose. No differences were observed for growth rates of the mutants and the parent strain (Supplementary Fig. 1). As expected, the mutant S492 (*rfbB6* *rffG8*) showed less ability to swarm in the absence of arabinose than the wild-type strain, and the arabinose-regulated mutants also had reduced motility capacity compared with the wild-type strain when grown in the absence of arabinose. The motility of the arabinose-regulated mutants was partially restored to wild-type levels when arabinose was available (Table 2). In assays examining susceptibility to environmental factors, the mutant S492 (*rfbB6* *rffG8*) and the arabinose-regulated mutants also showed more sensitivity to polymyxin B and DOC than the wild-type strain in the absence of arabinose ($P < 0.001$) (Fig. 2B). When arabinose was supplied, the arabinose-regulated mutant S496 was restored to the wild-type level of resistance to polymyxin B but S497 still showed significant sensitivity to polymyxin B compared with the wild-type strain ($P < 0.05$) (Fig. 2A). Whereas both S496 and S497 exhibited significant sensitivity to DOC compared with the wild-type strain under arabinose availability ($P < 0.05$), S496 showed more resistance to DOC than S497.

3.3. Attachment and invasion assays

To evaluate the effects of the *rfbB* and *rffG* gene mutations on attachment and invasion, we examined the capacity of the mutant strains to attach to and invade INT407 human epithelial cells. The mutant S492 (*rfbB6* *rffG8*) and the arabinose-regulated mutant strains in the absence of arabinose showed significant reductions in attachment (approximately 5-fold) compared with the parent strain ($P < 0.05$). No significant differences in attachment were observed between the arabinose-regulated mutant strains and the parent strain S100 in the presence of arabinose; similar results were observed for the invasion assays (Fig. 2C and D). The mutant S496 (P_{BAD} *rfbB-3*) showed significantly higher attachment to the INT407 cells than the S497 mutant (P_{BAD} *rfbB-4*) (89% vs 42%) ($P < 0.01$) (Fig. 2C), but no difference between these two mutants was observed for INT407 invasion (Fig. 2D). The mutant S496 (P_{BAD} *rfbB-3*) possessed a more similar phenotype than S497 (P_{BAD} *rfbB-4*) to the wild-type strain. S496 (P_{BAD} *rfbB-3*) was selected for further evaluation in animal experiments.

3.4. Virulence and colonization of mutant strains in BALB/c mice

To assess the virulence of the mutants, the oral LD₅₀ values for each strain were determined in BALB/c mice. Groups of female BALB/c mice received gradual doses of each strain orally and were monitored for 30 d after inoculation. The results showed that the LD₅₀ of the wild-type *S. Typhimurium* S100 was 5.0×10^5 CFU, displaying the most virulence among all of the tested strains. The LD₅₀ of S491 (*rffG8*) of 3.2×10^7 CFU indicated that it was 64-fold less virulent than the wild-type S100. The mutant strains S490 (*rfbB6*), S492 (*rfbB6* *rffG8*) and S496 (P_{BAD} *rfbB-3*) were avirulent while grown with arabinose prior to oral administration to mice (LD₅₀ $> 10^9$ CFU) (Table 2).

Due to the significant attenuation of these mutant strains, we also determined whether these mutants retained the capacity to efficiently colonize and disseminate in mouse tissues. The bacterial numbers in tissues from mice infected with the wild-type *Salmonella* strain recovered from PP (Fig. 3A), spleen (Fig. 3B), and liver (Fig. 3C) were approximately 10^6 , 10^4 , and 10^4 , respectively. The colonization levels of the *rfbB6* and *rffG8* single mutants and the arabinose-regulated mutant S496 (P_{BAD} *rfbB-3*) in the spleen and liver were similar to those of the parent strain S100. However, in all three different organs from infected mice including spleen, liver and PP, the mutant S492 (*rfbB6* *rffG8*) showed significantly lower colonization levels than the wild-type S100 ($P < 0.05$), but no significant differences in the bacterial loads in the PP, spleen and liver were observed between the wild-type S100 and arabinose-regulated mutant S496 (P_{BAD} *rfbB-3*) ($P > 0.05$) (Fig. 3).

3.5. Evaluation of immunogenicity and protective efficacy

As the mutant S496 (P_{BAD} *rfbB-3*) with the arabinose-regulated synthesis of LPS and ECA was significantly attenuated but nonetheless efficiently colonized and disseminated to mouse tissues (Table 2 and Fig. 3), we then determined whether immunization with this mutant induces protective immunity and enhances protection efficacy. The mutant S496 (P_{BAD} *rfbB-3*) strain induced the highest titer of anti-LPS IgG, which was significantly higher than that of the mutant S492 (*rfbB6* *rffG8*) at week 8 ($P < 0.001$) (Fig. 4A). The anti-OMP IgG level from mice immunized with the mutant S496 (P_{BAD} *rfbB-3*) was higher than that from mice immunized with S492 (*rfbB6* *rffG8*) with no significant difference (Fig. 4B). The immunized mice were challenged orally with 6.5×10^8 CFU of the wild-type *S. Typhimurium* 4 wks after the second immunization. The mice immunized with either S492 (*rfbB6* *rffG8*) or S496 (*rfbB-3*) were fully protected against a subsequent lethal challenge of 6.5×10^8 CFU of the wild-type *Salmonella* S100, and the mice in the control group succumbed after a challenge of 15 days.

3.6. Evaluation of cross-reactive immunity and cross-protection against heterologous *Salmonella* challenge

The arabinose-regulated mutant S496 (P_{BAD} *rfbB-3*) induced a strong immune response to OMPs from *S. Typhimurium* (Fig. 4B). Therefore, it was worthwhile to investigate whether S496 (P_{BAD} *rfbB-3*) elicits cross-reactive immunity and confers cross-protection against other *Salmonella* infections. The serum IgG responses to OMPs from other enteric bacteria were determined in the blood of mice immunized with S496 (P_{BAD} *rfbB-3*) or S492 (*rfbB6* *rffG8*) at week 8. Although S496 (P_{BAD} *rfbB-3*) was able to induce a higher IgG immune response to OMPs than S492 (*rfbB6* *rffG8*) from all of the tested bacteria, no significant differences were observed in the immune responses (Fig. 4B).

To evaluate the cross protective efficacy conferred by S496 (P_{BAD} *rfbB-3*), the clinically relevant serogroups *S. Choleraesuis* and *S. Enteritidis* were selected to challenge the immunized mice with doses of 2.4×10^7 ($\sim 200 \times LD_{50}$) and 2.6×10^7 CFU ($\sim 200 \times LD_{50}$) by oral administration 4 wks following the second immunization, respectively. For the challenge with *S. Choleraesuis*, the survival rate of the mice immunized with S496 (P_{BAD} *rfbB-3*) was 60%, which was higher than the protection level afforded by S492 (*rfbB6* *rffG8*) (33.3%) and the mock-immunized controls (0%) (Fig. 4C). There were significant

increases in survival after S492 (*rfbB6* *rffG8*) or S496 (P_{BAD} *rfbB*-3) immunization following *S. Enteritidis* challenge compared with the oral mock-immunized animals (Fig. 4D) ($P < 0.01$). Moreover, S496 (P_{BAD} *rfbB*-3) afforded better cross-protection than S492 (*rfbB6* *rffG8*) (Fig. 4C and D).

4. Discussion

The presence of multiple O-antigen serotypes in pathogenic *Salmonella* poses a major challenge for designing and developing a universal *Salmonella* vaccine to induce cross-protective immunity against numerous *Salmonella* serotypes, such as *S. Typhimurium*, *S. Choleraesuis* and *S. Enteritidis* [28]. O-antigen polysaccharide and ECA are two major immunogenic antigens on the surface of *Salmonella* [8,9]. Previous studies have indicated that O-antigen-deficient strains are typically less immunogenic and fail to effectively colonize in the host organ due to an inability to resist host pressures [6].

Truncation of LPS in *Salmonella* may result from blocking the synthesis of dNDP-sugars that are the precursors of core or O-antigen chains, from the inactivity of glycosyltransferases that are responsible for the formation of sugar linkages, or from the interruption of O-antigen processing genes [6,12,13,29]. Deletion of the glycosyltransferase genes or O-antigen processing genes leads to a nonreversible incomplete core or O-antigen of LPS [6]; however, deletion of genes such as *gale* or *pmi*, which are responsible for dNDP-sugar synthesis, results in mutants with reversible LPS [15,17,30]. It is obvious that an LPS- and ECA-negative strain would have a decreased capacity to withstand acidic, osmotic, bile and other stresses from the host, resulting in a decreased colonization capacity and impacting the induction of immune responses [6,11]. Thus, an optimal live oral *Salmonella* vaccine would retain its capacity to colonize and invade host lymphoid tissues while remaining completely avirulent [15,31–33]. In this study, we achieved construction of a mutant in which both O-antigen and ECA synthesis were simultaneously regulated by arabinose availability, resulting in enhanced cross-reactive immunity and cross-protection against challenge with multiple *Salmonella* serotypes compared with the double mutant.

Previous studies have demonstrated that the *rfbB* gene in the O-antigen gene cluster has functional homology with the *rffG* gene located in the ECA gene cluster [8,19]. Our results showed that the *rfbB* and *rffG* genes were exchangeable in terms of their impact on LPS and ECA synthesis (Fig. 1). Therefore, we targeted the *rfbB* gene to regulate O-antigen and ECA synthesis by arabinose availability via the $araC P_{BAD}$ promoter, which has been widely used to regulate expression of essential genes [12,13,34]. As the *rfbB* and *rffG* genes are located within operons, our strategy was to insert $araC P_{BAD}$ *rfbB* with a variable SD sequence and a start codon at a different position to maintain operon integrity. In this case, the $araC P_{BAD}$ *rfbB* cassette was inserted into the *pagL* position as we have demonstrated that deletion of *pagL* does not alter *Salmonella* virulence [35]. We obtained an ideal arabinose-regulated *rfbB* expression strain, S496, by assaying a series of biological characteristics and demonstrating wild-type characteristics when the strain was grown with arabinose (Figs. 1–3).

The animal experiments showed that the mutant S496 (P_{BAD} *rfbB*-3) was fully attenuated ($LD_{50} > 10^9$ CFU) but retained the capacity to disseminate in host organs (Fig. 3). Previous studies have shown that immunization with attenuated *Salmonella* strains with regulated LPS synthesis can elicit cross-reactive antibodies reacting with OMPs from different *Salmonella* serovars [6,29,36]. In addition, an ECA-deficient mutant induced a protective immune response against lethal challenge of homologous and heterologous *Salmonella* when used as a live-attenuated vaccine [11,37]. We observed that mutant S496 (P_{BAD} *rfbB*-3) induced higher levels of cross-reactive antibodies against OMPs from multiple enteric bacteria than the double mutant S492 (*rfbB6* *rffG8*) lacking O-antigen and ECA polysaccharides, indicating that regulation of both O-antigen and ECA would be beneficial for inducing cross-reactive immune responses against conserved OMPs from multiple *Salmonella* infections. The P_{BAD} *rfbB*-3 mutant strain was able to provide higher cross-protection levels against *S. Choleraesuis* and *S. Enteritidis* than the mutant S492 (*rfbB6* *rffG8*) (Fig. 4), but no significant difference was observed for the immune responses or protection efficacy ($P > 0.05$) (Fig. 4). We did not observe a significant advantage of S496 (P_{BAD} *rfbB*-3) over S492 (*rfbB6* *rffG8*) for inducing cross-reactive immune responses in this study, which may be due to the fact that the double mutant (*rfbB6* *rffG8*) still colonized and persisted well in the host organs (Fig. 3). As more than one means of attenuating *Salmonella* is necessary for live attenuated vaccine development [33,38], we will introduce other genetically unlinked mutations into this mutant to ensure the safety of the attenuated *Salmonella* strain and to evaluate cross-reactive immune responses and protection efficacy in future works.

In summary, we constructed a *Salmonella* mutant with arabinose-regulated O-antigen and ECA synthesis, and we demonstrated that immunization with this mutant induces cross-protection against heterologous *Salmonella* infection. Our results indicate that this mutation is a good choice to combine with other mutations to maximally unmask conserved immunogenic epitopes on the surface of *Salmonella* to develop a universal live vaccine for preventing infection by enteric bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Construction of *Salmonella* mutants. (A) LPS profiles. LPS from the wild-type *S. Typhimurium* S100 and the mutant strains S490 (*rfbB6*), S491 (*rffG8*), S492 (*rfbB6 rffG8*), S492 (*rfbB* complement plasmid) and S492 (*rffG* complement plasmid) were subjected to 12% SDS-PAGE and were silver-stained. (B) ECA profiles. ECA extracts from total membranes were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with the ECA-specific mAb898. (C) LPS profiles of the wild-type *S. Typhimurium* S100 and the arabinose-regulated mutants. The LPS molecules

from different mutant strains grown in nutrient broth with (+) or without (–) 0.1% arabinose were silver-stained after separation by 12% SDS-PAGE. (D) Western blots of LPS preparations from panel C. The blots were probed with anti-*Salmonella* group B antibodies. (E) ECA profiles of the wild-type *S. Typhimurium* S100 and the arabinose-regulated mutants. ECA from the wild-type *S. Typhimurium* S100 and the arabinose-regulated mutants grown in nutrient broth with (+) or without (–) 0.1% arabinose were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with the ECA-specific mAb898.

Fig. 2.

Polymyxin B and DOC sensitivity and attachment/invasion assays for the mutant strains. (A) Polymyxin B sensitivity assay. Approximately 1×10^7 CFU/ml bacteria with 0.1 μ g/ml polymyxin B were incubated for 60 min at 37 °C. The bacterial survival ratio was calculated after plating the bacteria on LB agar and counting colonies the following day. Values that are significantly different from those of the wild type as indicated by one-way ANOVA are indicated by asterisks (* $P < 0.05$). (B) DOC sensitivity assay. Approximately 1×10^7 CFU/ml bacteria with 10 mg/ml DOC were incubated for 60 min at 37 °C. The bacterial survival ratio was calculated after plating the bacteria on LB agar and counting colonies the

following day. Values that are significantly different from those of the wild type as indicated by one-way ANOVA are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$). (C) Attachment efficiency of the *Salmonella* mutants to INT407 cells. The mutant strains were added to wells with INT407 cells to achieve an MOI of 5:1. A 1% Triton X-100 solution was added to lyse the INT407 cells after incubation for 60 min at 37 °C. After plating on petri dishes and overnight incubation, the colony counts were determined. Attachment was calculated as follows: percent attachment = $100 \times (\text{number of cell-associated bacteria} / \text{initial number of bacteria added})$. Values that are significantly different as determined by one-way ANOVA are indicated by asterisks (** $P < 0.01$). (D) Invasion efficiency of *Salmonella* mutants to INT407 cells. Prior to the lysis step, 100 µg/ml gentamicin was added to kill the extracellular bacteria, and the plate was incubated for an additional 60 min at 37 °C. Invasion was calculated as follows: percent invasion = $100 \times (\text{number of bacteria resistant to gentamicin} / \text{initial number of bacteria added})$.

Fig. 3.

Colonization by the wild-type *Salmonella* and mutants in mice organs. Groups of mice ($n = 3/\text{group}$) were orally inoculated with approximately 1×10^9 CFU of the indicated strains, and the viable bacteria were recovered from the PP, spleen, and liver of BALB/c mice 6 days after oral inoculation. The bacterial counts were determined and are displayed as $\log_{10}$ CFU/g of PP (A), spleen (B), and liver (C) tissue. *Salmonella* S100 is the wild-type strain that was used as a positive control. The horizontal lines represent the means and the error bars represent the SEM. Values that are significantly different from those of the wild type as indicated by one-way ANOVA are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$).

Fig. 4.

Serum immune responses and protection efficacy in orally immunized mice. Total serum IgG specific for *S. Typhimurium* LPS (A) and serum IgG against OMPs from *S. Typhimurium* and other enteric bacteria (B) were measured via quantitative ELISA. Each group consisted of 12 mice. The data represent antibody concentrations in pooled sera from orally immunized mice at the indicated number of weeks post-immunization (*** $P < 0.001$, S492 ($rfbB6 \ rffG8$) versus S496 ($P_{BAD} rfbB-3$)). The protection rates against *S. Choleraesuis* (C) ($n = 6/\text{group}$) and *S. Enteritidis* (D) ($n = 6/\text{group}$) challenge are shown. The immunized mice were challenged by oral inoculation with $200 \times \text{LD}_{50}$ *S. Choleraesuis* and $200 \times \text{LD}_{50}$ *S. Enteritidis*. The mice were monitored daily and deaths were recorded for 30 days to calculate the protection rates after challenge. Statistical analyses were performed using the log-rank test.

Table 1

Strains and plasmids used in this study.

Strains and plasmids	Description	Source or reference
<i>Plasmids</i>		
pYA4278	<i>SacB</i> mobRP4 R6K ori Cm ⁺ , pRE112-T-vector	[6]
pSS239	<i>rfbB6</i>	pYA4278
pSS240	<i>rffG8</i>	pYA4278
pYA4284	<i>pagL7</i>	[35]
pSS242	ctcgag AGGA gtcatt GTG	pSS241
pSS243	ctcgag AGGA gtcatt ATG	pSS241
pSS244	ctcgag GGAA gtcatt ATG	pSS241
pSS245	ctcgag GGAA gtcatt GTG	pSS241
pYA4518	GFP p15A ori Cm ⁺	[37]
pYA3700	TT <i>araC</i> P _{BAD} cassette plasmid; Ap ^r	[39]
pSS250	pYA4518- <i>rfbB</i>	
pSS251	pYA4518- <i>rffG</i>	
<i>Bacterial strains</i>		
S100	Wild-type <i>S. Typhimurium</i> , isolated from infected duck	[40]
S340	<i>S. Choleraesuis</i> , wild-type virulent	[40]
S246	<i>S. Enteritidis</i> , wild-type virulent	[40]
S490	<i>rfbB6</i>	S100
S491	<i>rffG8</i>	S100
S492	<i>rfbB6 rffG8</i>	S491
S493	<i>rfbB6 rffG7 pagL7</i>	S492
S494	<i>rfbB6 rffG7 pagL71:TT araC</i> P _{BAD} <i>rfbB-1</i>	S493
S495	<i>rfbB6 rffG7 pagL72:TT araC</i> P _{BAD} <i>rfbB-2</i>	S493
S496	<i>rfbB6 rffG7 pagL73:TT araC</i> P _{BAD} <i>rfbB-3</i>	S493
S497	<i>rfbB6 rffG7 pagL74:TT araC</i> P _{BAD} <i>rfbB-4</i>	S493
<i>E. coli</i>		
χ7232	<i>endA1 hsdR17</i> (⁺ _K ⁻ _m _K) <i>glnV44 thi-1 recA1 gyrA relA1</i> (<i>lacZYA-argF</i>) <i>U169</i> λ <i>pir deoR</i> (Φ80 <i>dlac</i> (<i>lacZ</i>)M15)	[41]
χ7213	<i>thi-1 thr-1 leuB6 glnV44 thuA21 lacY1 recA1</i> RP4-2-Tc::Mu[λ <i>pir</i>] <i>asdA4</i> (<i>zhf-2:Tn10</i>)	[41]

Table 2Motility and virulence of *S. Typhimurium* strain S100 and mutant derivatives.

Strains	0.1% arabinose	Motility (mm) on soft agar	LD ₅₀
S100	–	71 ± 2.00	5.0 × 10 ⁵
	+	75 ± 2.65	
S490 (<i>rfbB6</i>)	–	69 ± 2.60	>2.5 × 10 ⁹
	+	70 ± 3.46	
S491 (<i>rffG8</i>)	–	72 ± 2.64	3.2 × 10 ⁷
	+	73 ± 3.00	
S492 (<i>rfbB6</i> <i>rffG8</i>)	–	8 ± 1.00	>2.3 × 10 ⁹
	+	8 ± 2.00	
S496 (P _{BAD} <i>rfbB</i> -3)	–	8 ± 1.16	>2 × 10 ⁹
	+	37 ± 2.00	
S497 (P _{BAD} <i>rfbB</i> -4)	–	8 ± 0.00	ND
	+	39 ± 3.00	

ND: not determined.