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Immunological Responses Induced by *asd* and *wzy*/*asd* Mutant Strains of *Salmonella enterica* serovar Typhimurium in BALB/c Mice

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Attenuated bacteria have long been developed as vaccine candidates but can have some disadvantages, such as the potential for damage to immune organs due to insufficient clearance. To minimize these disadvantages, we generated *Salmonella enterica* serovar Typhimurium mutants SHJ2104 (*asd::cm*) and HTSaYA (*wzy::km, asd::cm*). The *wzy* gene codes for the O-antigen polymerase, which is involved in lipopolysaccharide (LPS) biosynthesis, and *asd* codes for aspartate ß-semialdehyde dehydrogenase, which participates in cell wall formation. The strains synthesized LPS with a short-chain length, and showed lower cytotoxicity and reduced intracellular proliferation in animal cells compared to wild-type bacteria. After oral infection, the mutants were cleared in immune tissues, including the Peyer's patch, mesenteric lymph node, and spleen, within 5 days. The LD_{50} of the mutants in Balb/c mice was estimated to be 10^6 higher than wild-type bacteria when administered either via an oral or *i.p.* route, indicating that the two strains are highly attenuated. To compare the immune response to and protective effects of the mutants against wild-type bacterial infection, we inoculated the mutants into mice via an oral (1×10^{10} CFU) or *i.p.* (1×10^7 CFU) route once or twice at a two week interval. All immune responses, such as serum IgG and secretory IgA levels, cytokine production, and delayed hypersensitivity, were highly induced by two rounds of immunization. HTSaYA and SHJ2104 induced similar immune responses, and mice immunized with HTSaYA or SHJ2104 via an *i.p.* route were protected against wild-type *Salmonella* infection even at 100-fold of the LD_{50} (5×10^6 CFU). Taken together, these data indicate that HTSaYA and SHJ2104 could be developed as live attenuated *Salmonella* vaccine candidates.

***Keywords*:** *Salmonella enterica* serovar Typhimurium, live vaccine, immune responses, *wzy* and *asd*

Salmonella enterica contains 2,300 serogroups, and typhoid fever caused by *Salmonella* infection is highly prevalent in developing countries with an annual global incidence of approximately 16 million cases and 600,000 deaths (Pang *et al.*, 1998). Among the various serogroups of *Salmonella*, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is most commonly associated with human and animal infections due to the consumption of contaminated food and water. To protect against diseases caused by *Salmonella* spp., various types of vaccines have been developed and studied (Karem *et al.*, 1995). The use of soluble protein antigens as oral vaccines often induces oral tolerance unless the antigens are administered together with an appropriate adjuvant, such as cholera toxin or heat-labile toxin (Douce *et al.*, 1997). Killed vaccines are safe and stable, but antibody responses to these vaccines are not very effective owing to the weak generation of memory B cells. These vaccines thus require the administration of several subsequent boosts. Live attenuated vaccines have been shown to confer greater and longer-lasting immunity than killed vaccines (O'Callaghan *et al.*, 1988). Although the current inactivated whole-cell typhoid vaccine, *S.* Typhi Ty21a, a *galE* mutant of *S.* Typhi Ty2, has been used as a live oral typhoid vaccine in human field trials, it caused serious side effects in some recipients (Stuhl *et al.*, 1964; Levine *et al.*, 1987). Various highly attenuated *S.* Typhimurium strains also have been developed as live oral vaccines and display strong immunogenicity in mice. These vaccine strains include amino acid auxotrophic mutants (O'Callaghan *et al*., 1988) and strains defective in purine biosynthesis (McFarland and Stocker, 1987).

The *asd* gene encodes the aspartate β-semialdehyde dehydrogenase, which generates an essential component of the peptidoglycan in the cell wall of Gram-negative bacteria (Schleifer and Kandler, 1972). *S.* Typhimurium containing an *asd* mutation indispensably requires diaminopimelic acid (DAP) for growth, and in its absence the bacteria undergo lysis (Li *et al.*, 2008). Since DAP is not present in mammalian tissues, the *asd*-disrupted *Salmonella* fails to proliferate in infected animals. Moreover, the balanced-lethal phenotype of this mutant allows the asd^+ plasmid to be stably maintained (Galan *et al.*, 1990; Kang *et al.*, 2002; Kang and Curtiss, 2003). Lipopolysaccharide (LPS) is the major virulence determinant of Gram-negative bacteria, including *S.* Typhimurium, and is composed of three domains: lipid A (endotoxin), an oligosaccharide core, and O-antigen. O-antigen is a polymer of a tetrasaccharide repeat unit (RU) that determines the O serotype (Raetz and Whitfield, 2002). A mutation in the *wzy*

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(also called *rfc*) gene results in the ligation of only one O-antigen RU to the lipid A-core, a phenotype known as semi-rough (SR) LPS (Collins *et al.*, 1991). When the *wzy* mutant was inoculated orally into BALB/c mice, it showed 100,000-fold attenuation relative to the wild-type strain (Collins *et al.*, 1991). The *wzy* mutant colonized Peyer's patches to a level apparently similar to that seen with the *galE* mutant, but, unlike this mutant, was still capable of progressing from the Peyer's patches to the spleen (Collins *et al.*, 1991). Early reports demonstrated that colonization of the mouse intestine is impaired in LPS-defective *S.* Typhimurium mutants (Nevola *et al.*, 1985) and that mutants lacking O-antigen are attenuated in mice (Miller *et al.*, 1989). Not only the presence of O-antigen but also the proper distribution of O-antigen chain lengths is required for full *S.* Typhimurium virulence (Murray *et al.*, 2003). However, O-antigen is not required for invasion of epithelial cells *in vitro* (Kihlstrom and Edebo, 1976). Thus, the *wzy* mutant strain is not sufficiently attenuated for use as a *Salmonella* vaccine in humans or animals. In addition, one of the fears of using strains attenuated by single-locus mutations as a live vaccine is the possibility that the strain could undergo phenotypic reversion and become fully virulent. Double-gene disruptions that attenuate bacteria virulence greatly reduce the odds of reversion (Hone *et al.*, 1991; Karem *et al.*, 1995), and it is thus possible that the *wzy* mutation may be of use in conjunction with other attenuating mutations (Collins *et al.*, 1991). In this work, we assessed the immune responses to and protective effects of *Salmonella asd* and *asd*/*wzy* mutants in mice and demonstrate that these strains may have utility as attenuated live vaccine candidates.

Materials and Methods

Bacterial strains and mutagenesis

The bacterial strains used in this study are listed in Table 1. *S.* Typhimurium sch2005 (renamed from ATCC 14028s) was used as wild-type strain. The *asd*, *wzy*, and *wzy*/*asd* mutant strains were constructed using the λ-Red recombinase method as described (Datsenko and Wanner, 2000). A *wzy*-disrupted gene fragment carrying the kanamycin resistance gene (*km*) in the place of the open reading frame was generated by PCR amplification using pKD4 as a template. The primers used were 5′-TGCCTGATGGTAATATTTTTAATACTAAGC ATTTTTTCTAAAGGCTCTAT (forward), and 5′-ATTTTTACGCTT CAGAGCCAAATAAAACGGCGGCATTGCCGCCGTATAACATG GGAATTAGCCATGGTCC (reverse). The 1.2-kb PCR products were purified and transformed into sch2005 carrying a λ-Red helper plasmid (pKD46) by electroporation. The *wzy*-disrupted mutant was termed HTSaY (*wzy::km*). We also used the same method to disrupt the *asd* gene using pKD3 as a template. The primers used were 5′-AGGATACTGGCGCGCATACACAGCACATCTCTTTGCAGGAA AAAAACGCTGTGTAGGCTGGAGCTGCTTC (forward), and 5′-TATCCGGCCTACAGAACCACACGCAGGCCCGATAAGCGCTG CAATAGCCAATGGGAATTAGCCATGGTCC (reverse). The 1.1-kb PCR products were purified and transformed into sch2005 or HTSaY carrying a λ-Red helper plasmid (pKD46) by electroporation, producing SHJ2104 (*asd::cm*) and HTSaYA (*wzy::km*, *asd::cm*), respectively. The transformed cells were then grown in LB broth with ampicillin (50 μg/ml) and L-arabinose (1 mM) at 30°C to an optical density at 600 nm (OD_{600}) of 0.5. Antibiotic resistant transformants were then cultured in the absence of antibiotic selection at 42°C and assayed for ampicillin sensitivity to confirm the loss of pKD46. The gene deletion in mutant bacteria was confirmed with PCR analysis. To make *wzy* expression vector (pGEM-T/wzy), 1.6-kb fragment was amplified with Sawzy-F (5′-TGCTATCGGAGGCGCAGTAG) and Sawzy-R (5′-GGATTAGGGCGGAGTAGCGG) primers and cloned in pGEM-T Easy vector (Invitrogen, USA) against bacterial genome. The expression vector of *asd* gene (pGA) was described (Collins *et al.*, 1991).

Table 1. *S.* Typhimurium strains used in this study

Strains	Relevant properties	Source
sch2005 (ATCC 14028s)	Wild type	ATCC
SHJ2104	Δ*asd::km*	This study
HTSaY	Δ*wzy::km*	This study
HTSaYA	Δ*wzy::km*, Δ*asd::cm*	This study

Bacterial growth conditions

Wild-type *S.* Typhimurium and the mutant strains were grown in Luria-Bertani (LB) medium (Difco Laboratories, USA) containing 1% NaCl with vigorous aeration at 37°C. For the solid support medium, 1.5%-bacto agar was included. Nutrient Broth and Brain Heart Infusion (BHI) media were purchased from Difco Laboratories (Song *et al.*, 2004). The concentrations of antibiotics used were 50 μg/ml ampicillin, 50 μg/ml kanamycin, and 15 μg/ml chloramphenicol. The *asd* disruption mutants were cultured in the additional presence of 100 μg/ml DAP.

Eukaryotic cell lines and culture conditions

The RAW 264.7 murine macrophage and Caco-2 murine intestinal epithelial cell lines were used in this study. The cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

LPS analysis

LPS purification was performed as previously described (al-Hendy *et al.*, 1991). LPS was separated on 12% acrylamide gels using a Tricine-SDS buffer system (Lesse *et al.*, 1990) and visualized using a Power Stain Silver Staining kit (ELPIS-biotech, Korea).

Lactate dehydrogenase (LDH) assay

An LDH Cytotoxicity Detection kit (Promega, USA) was used to determine the cytotoxic effects of wild-type and mutant *S.* Typhimurium strains in RAW 264.7 and Caco-2 cell lines. This kit uses a colorimetric assay to quantify cell death based on the measurement of LDH activity released from the cytosol of damaged cells (Zychlinsky *et al.*, 1994). Cells (1×10^5/well) were seeded into 24-well plates in 0.5 ml DMEM (without phenol red, GIBCO, USA) containing 2.5% FBS. Cells were inoculated with viable *S.* Typhimurium wild-type and mutant bacteria at a multiplicity of infection (MOI) of 100 in 0.5 ml DMEM without phenol red. After infection for 30 min, fresh media containing 10 μg/ml of gentamicin (Sigma-Aldrich, UK) was added after simple washing. The cells were incubated for the indicated times at 37°C in 5% CO_2. The supernatants were then removed, centrifuged at 13,000 rpm at 4°C for 10 min, and then 50 μl was transferred to a 96-well ELISA plate. The cytotoxicity assay was performed according to the manufacturer's instructions, and plates were read at OD_{490}. All measurements were performed in triplicate, and each experiment was carried out three times.

Bacterial invasion and proliferation assay in RAW 264.7 cells

Invasion and intracellular survival tests were performed principally as

previously reported (Gahring *et al.*, 1990). RAW 264.7 cells (1×10^5 cell/well) were cultured in 24-well plates as above for 16 h prior to infection. Before infection, wild-type *S.* Typhimurium and the mutant strains were cultured overnight and then diluted 40-fold in LB broth and grown for another 4 h at 37°C with vigorous shaking as described (Song *et al.*, 2004; Kim *et al.*, 2006). Bacterial inoculums were prepared by harvesting the cells at 4,000 rpm for 5 min and then directly resuspending the bacteria in pre-warmed DMEM containing 10% FBS. The inoculums were diluted and added to the mammalian cells at an MOI 100, and the cells were incubated for 30 min at 37°C and 5% CO_2. After infection, extracellular bacteria were removed by extensive washing with phosphate buffered saline (PBS). Fresh DMEM containing 10% FBS and 10 μg/ml gentamicin was then added to kill extracellular bacteria, and the RAW 264.7 cells were incubated at 37°C in 5% CO_2 for 4, 8, and 24 h. To calculate the bacterial number, the infected cells were washed three times with PBS and lysed with 0.05% Triton X-100 (Usb, USA) for 10 min. The lysates were then serially diluted and plated onto BHI agar plates supplemented with antibiotics and DAP. The colony forming units (CFU) were determined after overnight incubation at 37°C.

Determination of the 50% lethality dose (LD_{50})

Female BALB/c mice (8-week old) were obtained commercially (Orient, Korea) and fasted overnight prior to inoculation. To determine the LD_{50}, groups of five animals were infected with bacteria. The inoculums were administered orally (10^6 to 10^{10} CFU in 300 μl PBS) or intraperitoneally (*i.p.*) (10^5 to 10^9 CFU in 100 μl PBS). Animal deaths were recorded for 30 days, and LD_{50} values were estimated as described (Reed and Muench, 1938).

Enumeration of mutant *Salmonella* in mouse lymphoid organs

Groups of three mice were orally inoculated with wild-type or mutant *S.* Typhimurium (1×10^{10} CFU in 300 μl PBS) and sacrificed on the indicated days. Peyer's patches, mesenteric lymph nodes, and spleens were collected from the inoculated mice, and tissues were homogenized in PBS containing 0.05% Triton X-100 with a homogenizer (Ika-Works, Germany). After homogenization, the bacteria were enumerated in serial $\log_{10}$ dilutions. All mutant bacteria strains were cultured on BHI agar plate supplemented with antibiotics and DAP.

Mouse immunization and sample collection

SHJ2104 and HTSaYA were grown overnight and then diluted 40-fold to generate fresh cultures. After 4 h of growth (OD_{600} approximately equal to 4.0), the cultures were centrifuged at 6,000×g for 5 min. Cell pellets were maintained on ice and resuspended in cold, sterile PBS. For oral immunization, mice were fasted overnight and inoculated with 300 μl bacteria (1×10^{10}) by insertion of a feeding needle into the stomach. In other mice, 100 μl bacteria (1×10^7) was injected *i.p*. All immunization procedures were performed once or twice after a 2-week interval. After oral or *i.p*. immunization with SHJ2104 and HTSaYA, mice (n=4 per group) were orally challenged with wild-type sch2005. Challenged mice were monitored daily for 2 weeks.

Samples were taken from each group of immunized mice as described (Hvalbye *et al.*, 1999). To collect saliva, the mice were *i.p*. injected with 100 μl of 0.1% pilocarpine-HCl (Sigma, USA). Mice were then anaesthetized with an *i.p*. injection of 100 μl of a Rompun (BAYER, Korea) and Zoletil 50 solution (VIRBAC, France). After 1-2 min, the saliva was collected from the mouth by a plastic transfer pipette into a 1.5-ml tube. Blood samples were collected from the eyes using heparinized capillary tubes (Chase, USA). Samples were kept at 4°C for 6 h and then centrifuged at 3,000 rpm at 4°C for 30 min to separate the serum. The serum samples were then incubated at 56°C for 30 min to inactivate complement. Fecal samples were mixed with PBS (2-fold v/w), stored overnight at 4°C, vigorously vortexed for 15 min, and centrifuged at 13,000 rpm at 4°C for 10 min. The supernatants were then removed and used to analyze antigen-specific antibody titers. Saliva, serum, and fecal samples were stored at -20°C until antibody analysis.

Antigen preparation

Wild-type *S*. Typhimurium was grown in 50 ml LB broth until it reached log-phase. The bacteria were then collected by centrifugation, resuspended in PBS, and sonicated in the presence of 10 mM PMSF (Boehringer, Germany). After centrifugation at 13,000 rpm at 4°C for 10 min, the supernatant was collected and analyzed using the BCA protein analysis method (Intron Biotechnology Inc., Korea). *S.* Typhimurium-specific LPS was purchased from Sigma (USA).

Enzyme-linked immunosorbent assay (ELISA)

To measure the antibody titer against *Salmonella* whole cell lysate and LPS, ELISAs were performed as described (Li *et al.*, 2008). 100 μl of whole cell lysate (150 ng) or LPS (10 μg/μl) suspended in sodium carbonate-bicarbonate coating buffer (pH 9.6) was applied to each well of 96-well EIA/RIA plates (Corning, USA). The plates were incubated overnight at 4°C to allow antigen binding. Free binding sites were blocked with a blocking buffer (PBS; pH 7.4, 0.1% Tween 20, and 1% bovine serum albumin). Plates were brought to room temperature and rinsed three times with a PBS-Tween 20 (PBS-T) solution. Serially diluted samples were then added to the wells, and plates were incubated for 2 h and then rinsed three times with PBS-T. Goat anti-mouse IgG-, IgG1-, IgG2a-, and IgA-horseradish peroxidase conjugated secondary antibodies (1:4,000, Southern Biotechnology, USA) were then added and incubated at room temperature for 2 h. Wells were developed with *o*-phenylenediamine dihydrochloride (OPD) (Sigma) substrate in 0.05 M phosphate citrate buffer. The reaction was stopped by the addition of 3 N H_2SO_4, and the absorbance was measured at 490 nm using an ELISA reader (Molecular Devices, USA).

Determination of delayed type hypersensitivity (DTH) responses

Mice were immunized as described above. After 4 weeks, the delayed type hypersensitivity reaction was assessed by subcutaneous administration of 50 μg of sonicated *Salmonella* whole cell lysate diluted in 50 μl PBS into one hind foot pad. As a negative control, 50 μl PBS was injected into a different foot pad. 24 h after administration, footpad swelling was measured using a spring loaded caliper (Mitutoyo, Japan). The results are expressed as the percent increase in swelling of the inoculated footpad as described previously (Na *et al.*, 2006).

Cytokine assays

Splenocytes were isolated from immunized and unimmunized BALB/c mice. Single cell suspensions were generated in RPMI medium contain 10% of FBS using 1-ml syringe needles. Splenocytes were then passed through a cell strainer into a polystyrene round-bottom tube (BD Falcon, USA). The red blood cells (RBC) were lysed at room temperature for 5 min using RBC lysis buffer (Sigma-Aldrich). Subsequently, 1×10^6 splenocytes were seeded into 12-well dishes (NUNC, Denmark) together with *Salmonella* whole cell lysates (10 μg/μl) for stimulation. After 24 h of stimulation, the culture supernatants were collected to assess the concentrations of IL-2, IL-4, IL-5, IL-6, IL-17, IFN-γ, and TNF-α. Cytokine concentrations were

determined via ELISA with immunoassay kits from BIOSOURCE (Invitrogen).

Statistical analysis

Data are expressed as the Mean±standard deviation (SD). The means were evaluated using one-way ANOVA analysis of variance and the least-significant difference was determined using the Wilcoxon-Mann-Whitney rank test. A p value of <0.05 was considered statistically significant. Mouse mortality was analyzed using Kaplan-Meier survival curves.

Results

Phenotype characterization of *wzy* and *asd* mutants

The *wzy* gene encodes the O-antigen polymerase, which is involved in LPS synthesis (Murray *et al.*, 2003; Hoare *et al.*, 2006). Mutations in *wzy* should result in a SR phenotype that by SDS-PAGE analysis appears as a very prominent band representing the LPS core with one O-antigen unit. After disrupting *wzy* using the recombination method (Datsenko and Wanner, 2000), we analyzed LPS purified from the mutants (Fig. 1). Unlike the wild-type (sch2005) and SHJ2104 (*Δasd::km*) strains (lanes 1 and 2), which had both long-chain (L-LPS) and SR-type LPS, the *wzy* mutants (HTSaY and HTSaYA) expressed only SR-type LPS (lanes 3 and 4). After transformation with plasmid expressing *wzy* gene, such mutants recovered wild-type LPS phenotype (lanes 5 and 6). After transformation with plasmid expressing *asd* gene, SHJ2104 and HTSaYA, recovered their growth even in the absence of DAP as patterns similar to wild-type bacteria (data not shown).

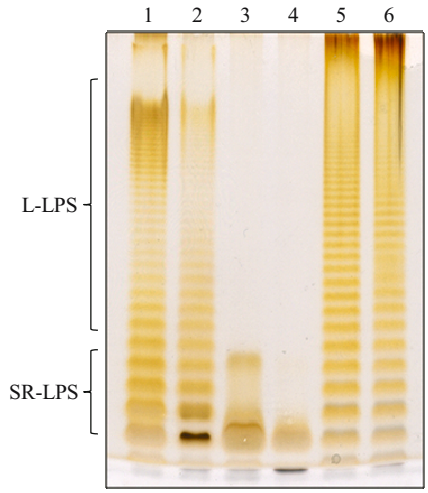

Fig. 1. Analysis of LPS from wild-type and mutant strains of *S.* Typhimurium. LPS was purified from bacterial cells (2×10^7 CFU) by the hot-phenol method, separated on a 12% SDS-PAGE gel, and visualized by silver staining. 1, sch2005; 2, SHJ2104; 3, HTSaY; 4, HTSaYA; 5, HTSaY transformed with pGEM-T/wzy; 6, HTSaYA transformed with pGEM-T/wzy.

In vitro cytotoxicity and intracellular proliferation

To examine the cytotoxic effects of the mutant *Salmonella* in mammalian cells, we incubated bacteria (MOI 100) with RAW 264.7 and Caco-2 mouse cells for the indicated times and assayed LDH activity (Fig. 2A). Wild-type bacteria and the HTSaY mutant (*Δwzy::km*) showed much higher levels of cytotoxicity than either SHJ2104 (*Δasd::cm*) or HTSaYA (*Δwzy::km*, *Δasd::cm*) in both mammalian cell types. Subsequently, we examined the ability of these mutants to invade animal cells and proliferate intracellularly (Fig. 2B). The number of intracellular bacteria was similar in all cases 30 min after infection of RAW 264.7 cells. These results indicate that the *wzy* and *asd* genes do not play a direct role in bacterial invasion of host cells. However, the number of mutant bacteria rapidly decreased after 4 h, while the number of wild type bacteria increased ~100 fold in 24 h. The decrease in the number of intracellular bacteria was more severe for the

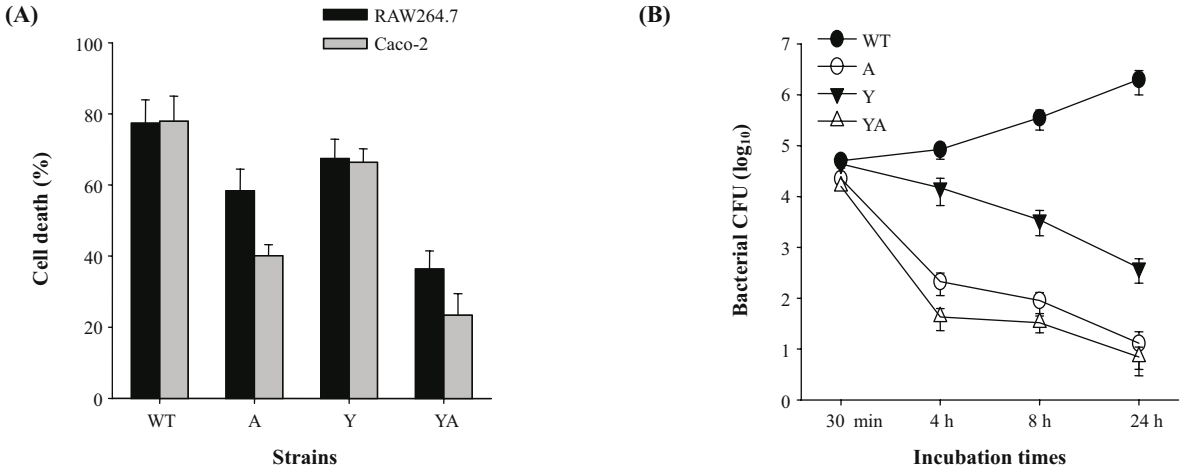

Fig. 2. The cytotoxicity and proliferation of *S.* Typhimurium mutants in host cells. (A) Bacteria-mediated cell death. RAW 264.7 and Caco-2 cells were infected with wild-type and mutant *S.* Typhimurium (MOI 100). After infection, the LDH activity in the supernatant was measured at 4 h and 24 h. (B) Invasion and proliferation of bacterial mutants in RAW 264.7 cells. 30-min after infection with *Salmonella* (MOI 100), the cell culture media was replaced with media supplemented with gentamicin (10 μg/ml) to remove extracellular bacteria. The number of intracellular bacteria was determined after the indicated times by a colony counting method. WT, sch2005; A, SHJ2104; Y, HTSaY; YA, HTSaYA.

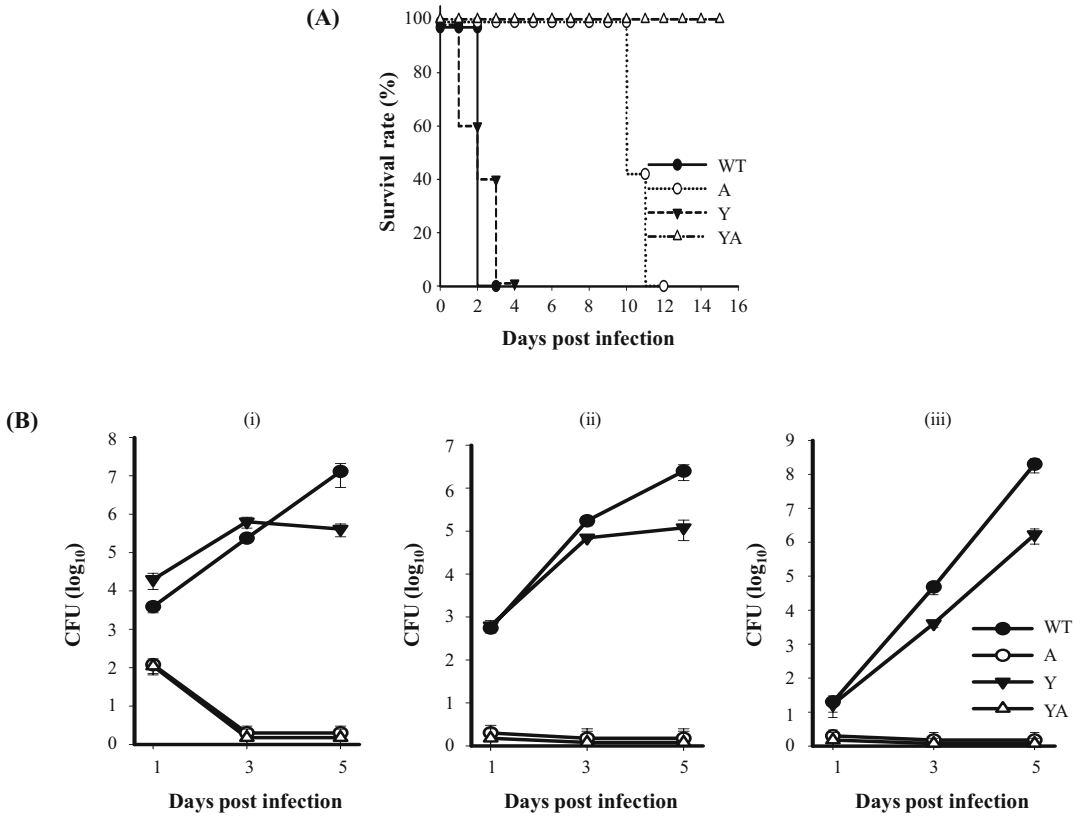

Fig. 3. Survival of mice orally infected with *S.* Typhimurium. (A) Survival of mice infected with wild-type and mutant bacteria. Mice (five per group) were orally infected with bacteria (5×10^{10} CFU) and the number of surviving mice was counted for the indicated days. (B) Enumeration of bacteria in immune organs after oral infection. Mice were orally infected with bacteria (1×10^{10} CFU). At the indicated days after infection, the number of bacteria in the Peyer's patches (i), mesenteric lymph nodes (ii), and the spleen (iii) was determined. WT, sch2005; A, SHJ2104; Y, HTSaY; YA, HTSaYA.

strains carrying the *asd* mutation (SHJ2104 and HTSaYA) than for the *wzy* mutant strain (HTSaY). These data indicate that the *asd*, and not the *wzy* mutation, is primarily responsible for the observed decrease in cytotoxicity, presumably owing to a failure to proliferate within animal cells.

In vivo cytotoxicity and persistence within host organs

To assess the virulence of the *Salmonella* mutants, we first determined their LD_{50} values in mice. The LD_{50} of HTSaYA was $\sim10^{10}$ CFU for oral administration and 5.5×10^{7} CFU for *i.p*. administration (data not shown). Moreover, the surviving mice infected with this mutant did not show any signs of illness and remained healthy for the entire 30 day observation period after inoculation. In a previous study, we reported that the LD_{50} values for wild-type *S*. Typhimurium were 4×10^{4} CFU and less than 10 CFU for oral and *i.p*. administration, respectively (Na *et al.*, 2006). Therefore, the LD_{50} of HTSaYA is approximately $\sim10^{6}$ higher than that of the wild-type strain irrespective of the route of inoculation.

In addition, we compared the survival rate of mice after oral injection with 5×10^{10} CFU of wild-type bacteria and the three other mutants (Fig. 3A). All of the mice injected with wild-type or HTSaY expired within 3 days after inoculation. Mice injected with SHJ2104 expired more slowly than those infected with wild type bacteria, but all mice eventually succumbed by day 11 post infection. Only the HTSaYA-injected mice survived and showed no sign of illness. These results confirmed that HTSaYA was the most highly attenuated strain of those tested.

Next, we examined the ability of these mutants to persist in the immune organs of infected mice. Mice infected with 1×10^{10} mutant bacteria through the oral route were periodically sacrificed to enumerate the number of bacteria in various organs (Fig. 3B). Wild-type *S.* Typhimurium colonized and proliferated in Peyer's patches, mesenteric lymph nodes, and the spleen up to day 5 until the mice expired. HTSaY, which contained a single mutation in *wzy,* were detected in the immune organs, but at >10-fold lower levels than wild-type bacteria (Fig. 3B). In contrast, SHJ2104, containing a single mutation in *asd,* and HTSaYA, with a double mutation in *wzy* and *asd,* were detected in Peyer's patches, but not in mesenteric lymph nodes or the spleen 1 day after infection, and infection was cleared completely within 3 days (Fig. 3B).

Induction of humoral and mucosal immune responses

The above results indicated that SHJ2104 and HTSaYA mutants could potentially be developed as live attenuated vaccines. To test their potential as vaccine candidates, we injected mice with these mutants via an oral (1×10^{10}) or *i.p*. (1×10^{7}) route once or twice at a 2-week interval and analyzed

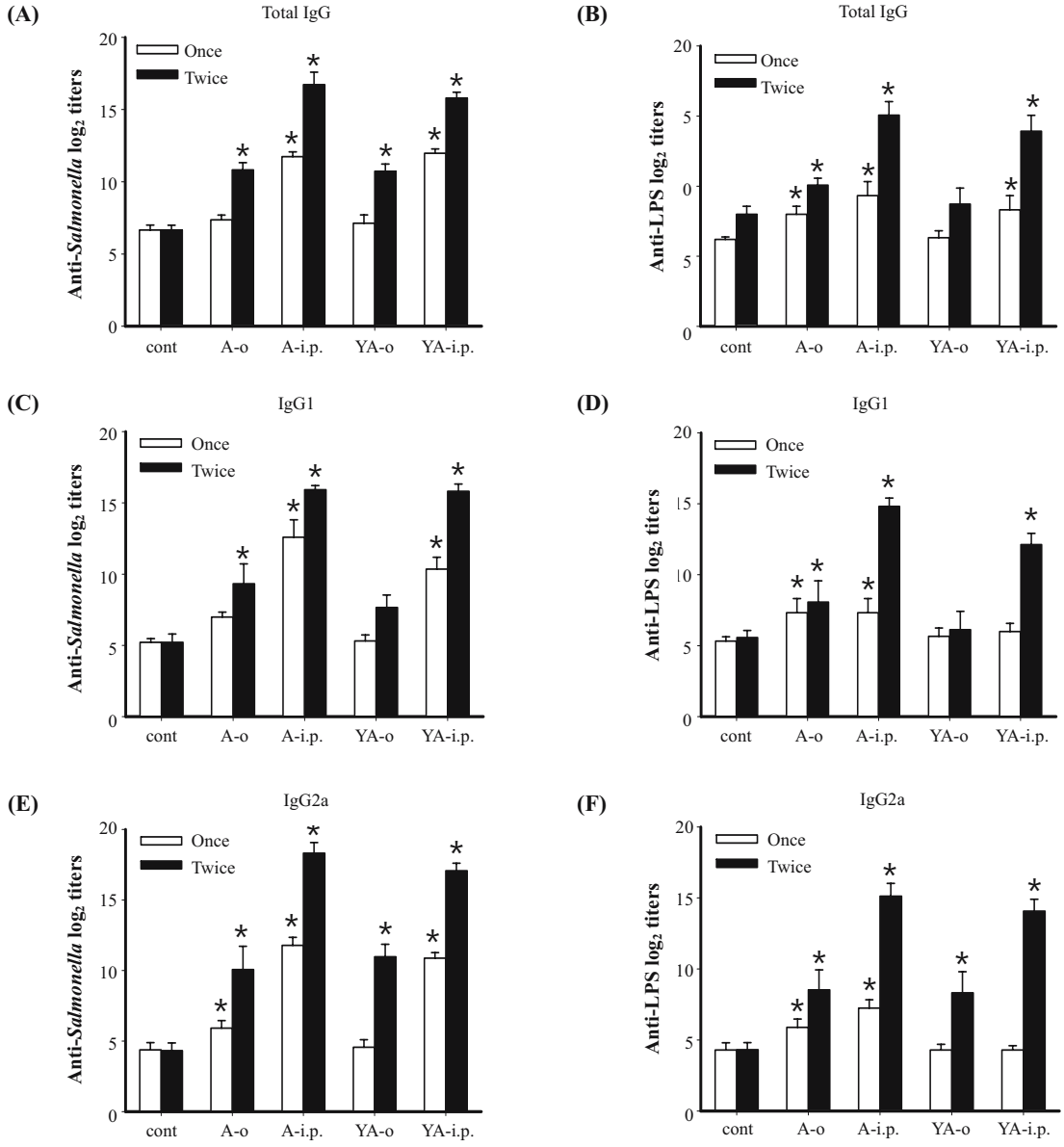

Fig. 4. Serum levels of *Salmonella*- and LPS-specific IgG. The level of IgG specific for *Salmonella* whole-cell lysates (A, C, and E) and *Salmonella* LPS (B, D, and E) in the sera of immunized mice was measured by ELISA. The mice were immunized once (white bars) or twice (black bars) with SHJ2104 (A) and HTSaYA (YA) via an oral (1×10^{10} CFU) or *i.p.* (1×10^{7} CFU) route. The sera were obtained 2 weeks after the final immunization, and control serum was harvested from non-immunized mice. Data are expressed as the geometric mean with the standard deviation. Asterisks (*) indicate that the *p*-value is <0.05.

the immune response. All the immunized mice survived and showed no signs of disease during the entire experimental period. The antibody response against *Salmonella* whole cell lysates and *Salmonella*-derived LPS in the sera, saliva, and stool of the immunized mice was measured by ELISA (Figs. 4 and 5). In our study, *i.p.* immunization with both mutants induced higher levels of total serum IgG, including both IgG1 and IgG2a, compared to oral immunization. However, orally immunizing twice with either mutant induced significantly higher serum antibody titers than a single oral immunization (Fig. 4). These data indicate that vaccination with SHJ2104 and HTSaYA mutants via an oral or *i.p.* route successfully induces an immune response against *Salmonella*.

In addition, we also determined the levels of IgA directed against *S.* Typhimurium whole-cell lysate and LPS in fecal fluid and saliva samples from the immunized mice (Fig. 5). Immunizing twice elicited significantly higher IgA titers than a single vaccination, and oral administration was more efficient in inducing mucosal immune responses than *i.p.* injection. IgA antibody titers against *Salmonella* whole-cell lysates in fecal and saliva samples increased similarly in mice immunized with either of the two mutants (Figs. 5A and C). Interestingly, the anti-LPS IgA levels in mice immunized with HTSaYA were significantly lower than those in mice immunized with

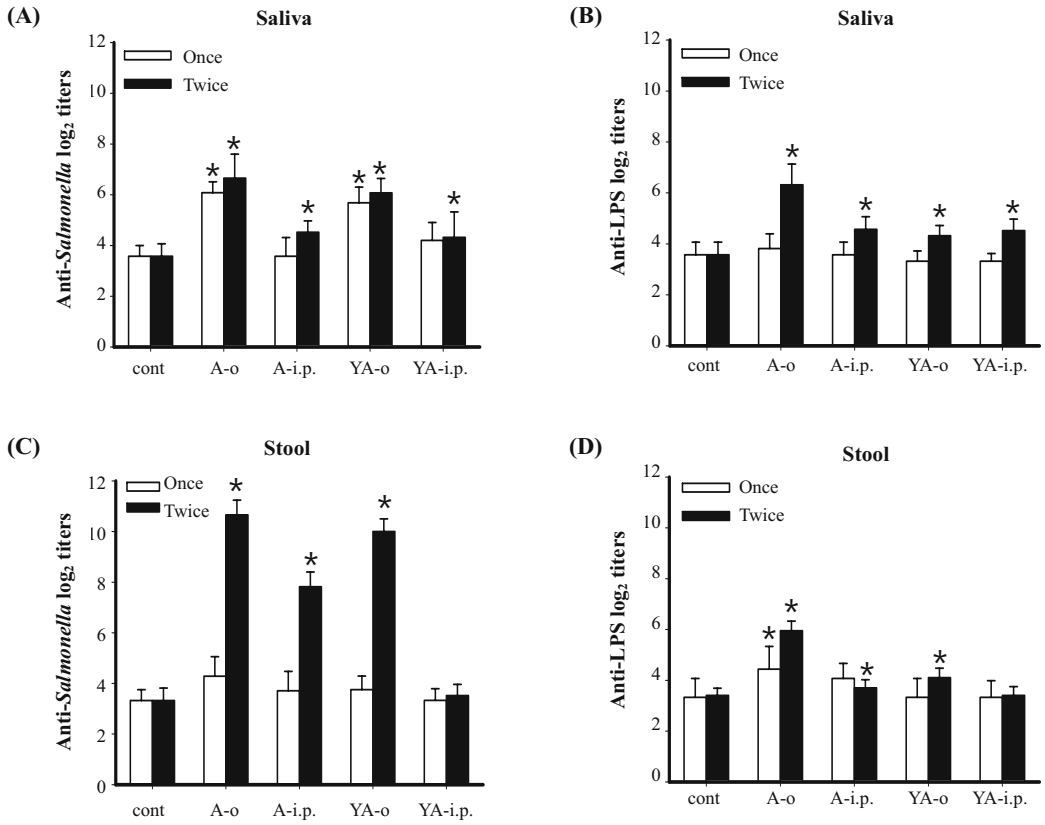

Fig. 5. Measurement of secretory IgA levels against *Salmonella* and its LPS. The levels of IgA against *Salmonella* whole-cell lysates (A and C) and *Salmonella* LPS (B and D) in the saliva and stool of mice immunized with SHJ2104 (A) and HTSaYA (YA) were measured by ELISA. Samples were obtained from the same mice analyzed in Fig. 4. White bars, one immunization; black bars, two immunizations. Control serum was harvested from non-immunized mice. Data are expressed as the geometric mean with the standard deviation. Asterisks (*) indicate a p-value of <0.05.

SHJ2104, irrespective of the route of immunization (Figs. 5B and D). This difference may be ascribed to the difference in the O-antigen chain length in HTSaYA.

Next, we measured cytokine production from splenocytes harvested from immunized mice (Fig. 6). Splenocytes were isolated and stimulated *in vitro* for 24 h with wild-type *S.* Typhimurium lysate. Cytokine production was dependent upon immunization frequency and route. Intraperitoneal immunization produced higher cytokines levels compared to oral immunization, and immunizing twice induced more cytokine production than a single immunization. Splenocytes from *i.p.* immunized mice produced a significant amount of proinflammatory cytokines and Th1-associated cytokines, including IL-6, TNF-α, IL-2, and IFN-γ. Interestingly, splenocytes from mice immunized via an oral route produced high levels of IL-17, but less IFN-γ, compared to mice immunized through an *i.p.* route, while production of other cytokines was only weakly induced. These data suggest that *i.p.* and oral immunization may differentially induce Th-1 and IL-17 mediated immune responses.

Induction of cellular immune responses

To determine the ability of the SHJ2104 and HTSaYA mutants to induce cell-mediated immune responses, we examined the DTH response in mice 4 weeks after immunization with mutant bacteria via an oral (1×10^{10} CFU) or *i.p.* (1×10^{7} CFU) route (Fig. 7). Mice immunized once via either the oral or *i.p.* route showed increased foot-pad swelling, suggesting that a single immunezation was sufficient to induce cellular immune responses. Mice immunized twice via either route showed significantly higher levels of foot-pad swelling (more than four-fold), and *i.p.* immunization resulted in more consistent and stronger responses than oral immunezation. Together, these results indicate that the cell-mediated responses induced by SHJ2104 and HTSaYA are dependent on immunization frequency and route.

Protection of immunized mice challenged with virulent *S.* Typhimurium

Finally, we examined the survival of mice immunized with mutant SHJ2104 or HTSaYA following wild-type *S.* Typhimurium infection (Fig. 8). Mice were immunized twice with the mutant bacteria via an oral (1×10^{10} CFU) or *i.p.* (1×10^{7} CFU) route. The unimmunized mice succumbed to infection within 7 days after oral challenge with 5×10^{6} CFU (100-fold LD_{50}) wild-type bacteria. After two oral immunizations, 50% and 25% of the mice immunized with SHJ2104 and HTSaYA, respectively, survived for 15 days after oral challenge with wild-type bacteria (Fig. 8). However, all mice immunized twice through an *i.p.* route survived throughout the experiment. These data suggest that immunizing twice with SHJ2104 or HTSaYA via an *i.p.* route elicits significant levels of both

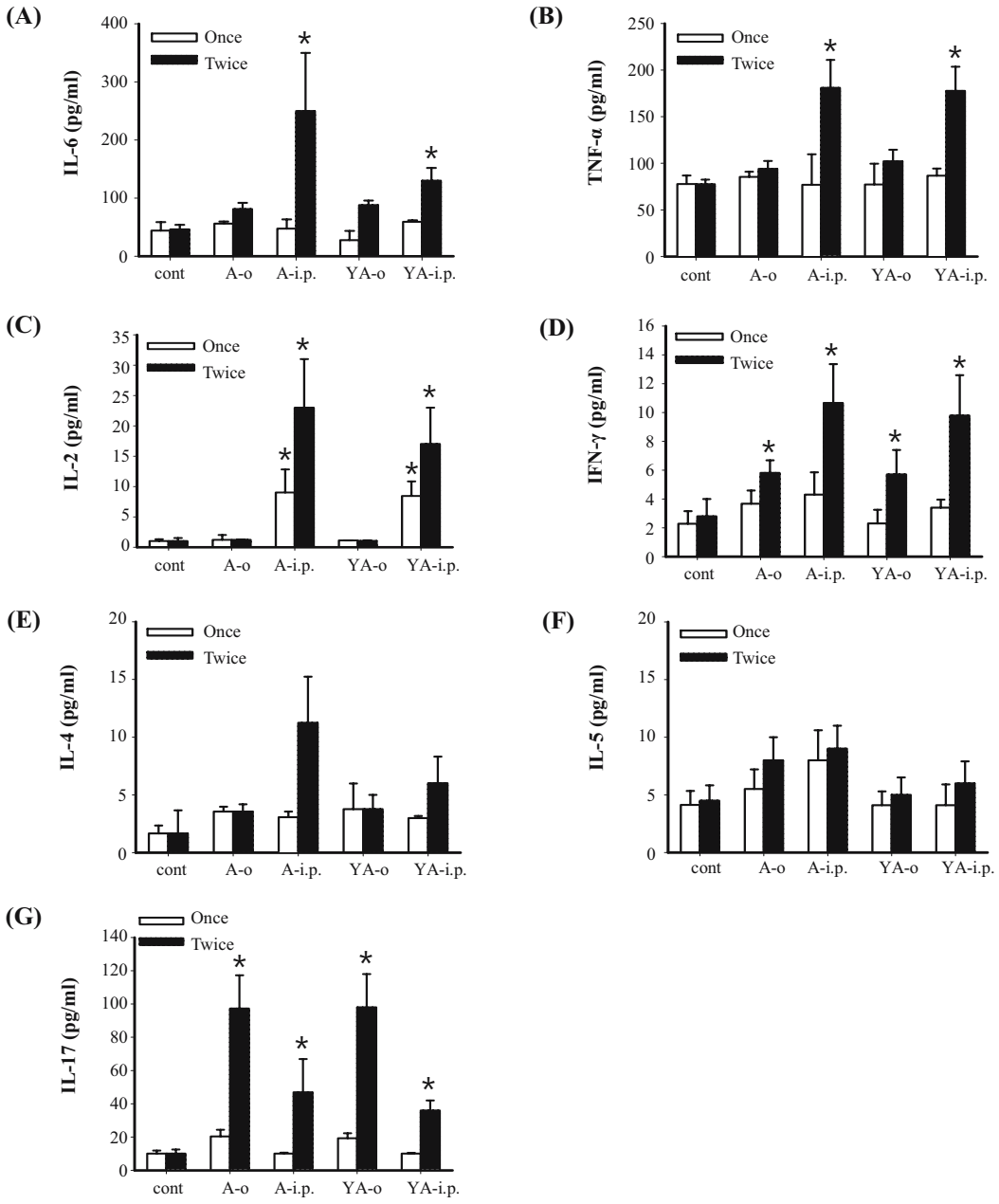

Fig. 6. Cytokine production from immunized spleen cells stimulated by *Salmonella* lysates. Splenocytes (1×10^6) were isolated from mice (n=3 per group) immunized with SHJ2104 (A) and HTSaYA (YA), and stimulated by *Salmonella* lysates for 24 h. The cytokine concentrations in the culture supernatants were measured by ELISA. Data are expressed as geometric mean with standard deviation. Asterisks (*) indicate a *p*-value of <0.05.

systemic and cell mediated immune responses, and consequently, perfectly protected animals after oral challenge with the virulent parent strain.

Discussion

The LPS of Gram-negative bacteria is a major virulent determinant. The *Salmonella wzy* mutant is defective in O-antigen polymerization (Murray *et al.*, 2003). Although this mutation reduces bacterial cytotoxicity, the reduction is not sufficient enough to allow its use as a *Salmonella*-based vaccine. In a previous study, a *wzy S.* Typhimurium single mutant was reported to be ~10^5-fold attenuated compared to wild-type *Salmonella* in a mouse model (Collins *et al*., 1991). Therefore, this mutation has been used in conjunction with other mutations to create attenuated bacteria (Hone *et al.*, 1991; Karem *et al.*, 1995). Here, we examined the affects of combining a mutation in the *asd* gene with the *wzy* mutation. The *asd* gene encodes aspartate β-semialdehyde dehydrogenase, an enzyme in the DAP biosynthetic pathway, which is an essential component of peptidoglycan in the Gram-negative bacterial cell wall (Schleifer and Kandler, 1972). Although

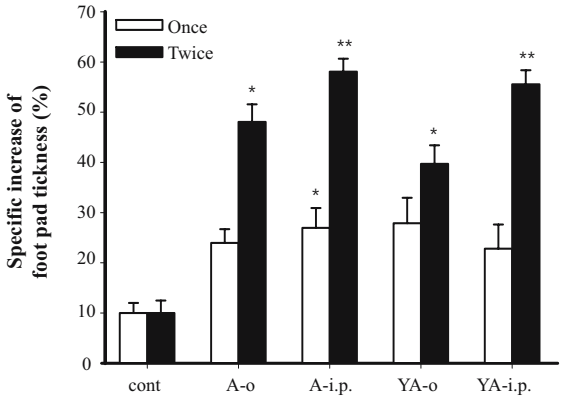

Fig. 7. Measurement of cellular immune responses after immunization. Mice (n=4 per group) were immunized once (white bars) or twice (black bars) with SHJ2104 (A) and HTSaYA (YA) via an oral (1×10^{10} CFU) or *i.p*. (1×10^{7} CFU) route. Delayed type hypersensitivity (DTH) reactions were initiated by injection of *Salmonella* whole cell lysate (50 μg) or PBS into the hind foot-pad. Foot-pad swelling was measured after 24 h. Data are expressed as the geometric mean with the standard deviations. The one or two asterisks (* or **) indicate a *p* value of <0.05 or <0.01, respectively.

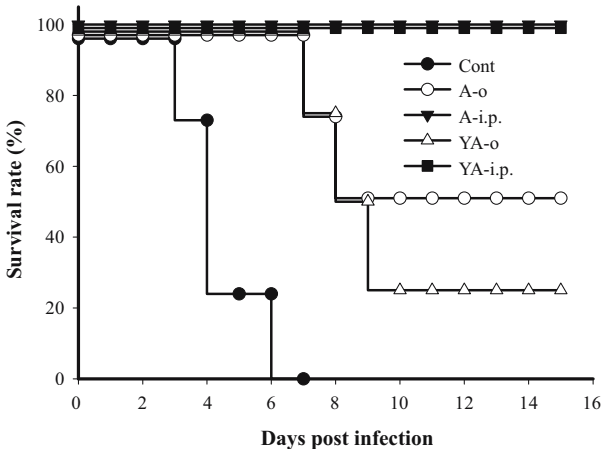

Fig. 8. Protection of immunized mice against oral challenge with wild-type bacteria. Mice (n=4 per group) were immunized twice with SHJ2104 (A) and HTSaYA (YA) via an oral (1×10^{10} CFU) or *i.p*. (1×10^{7} CFU) route. After 2 weeks, mice were orally challenged with virulent wild-type *S.* Typhimurium at a dose 100-fold times the LD_{50} (5×10^{6} CFU) and observed for the indicated days.

various antigen presenting systems using the balanced-lethal phenotype of the *asd* mutant have been developed (Galan *et al.*, 1990; Denich *et al.*, 1993; Ianaro *et al.*, 1995), attenuation by the *asd* mutation and the immune responses to this mutant had not been clearly defined. Thus, we constructed *S.* Typhimurium mutants lacking *wzy* and *asd* gene expression and examined their potential as vaccine strains using a murine model.

The *asd* mutants (SHJ2104 and HTSaYA) were much less cytotoxic in animal cells and mice than wild-type and *wzy* mutant strains (HTSaY). Host cell death was lower following *asd* mutant infection than following infection with wild-type *Salmonella* and the *wzy* strain. The *asd* mutants also failed to proliferate within both RAW 264.7 and Caco-2 cells (Fig. 2), indicating that the *asd* mutation significantly attenuates *Salmonella*. The addition of the *wzy* mutation further attenuated the *asd* mutant strain since all mice orally infected with a strain in which both *asd* and *wzy* were mutated (HTSaYA) survived at a bacterial dose (5×10^{10} CFU) that killed all mice infected with strains that contained only a mutation in *asd* (SHJ2104) (Fig. 3A).

After oral infection, *Salmonella* colonizes lymphoid organs, such as Peyer's patches and mesenteric lymph nodes specifically by invading the M cells, and then reaches deeper tissues like the liver and spleen (Carter and Collins, 1974). Bacterial colonization of Peyer's patches and internal organs, particularly mesenteric lymph nodes, can provide effective protective immunity even when low numbers of bacteria are present (McFarland and Stocker, 1987). As expected, wild-type *Salmonella* and HTSaY colonized and proliferated in Peyer's patches, mesenteric lymph nodes, and the spleen (Fig. 3B). However, the SHJ2104 and HTSaYA mutant strains were found only in Peyer's patches, were present at levels ~2 logs lower than wild type and HTSaY, and were rapidly cleared within three days. This rapid clearance might be responsible for the inefficient humoral immune response observed after oral immunization, since the serum IgG levels following oral SHJ2104 and HTSaYA administration were two-fold lower than those following *i.p*. immunization (Fig. 4). In contrast, secretary IgA responses were highly induced after oral immunization (Fig. 5). Consistent with these data, mice were not well protected against challenge with wild-type bacteria after two immunizations via an oral route. Some mice succumbed to infection with wild-type bacteria at doses at 100-fold of the LD_{50} (Fig. 8). However, *i.p*. immunization perfectly protected animals after an oral challenge with wild-type bacteria. Moreover, splenomegaly was observed after *i.p*. immunization with SHJ2104 and HTSaYA mutants, but not after oral immunization (data not shown). Splenomegaly is correlated with the degree of immunization protection afforded by mutant *Salmonella* (O'Callaghan *et al*., 1988; Zhang *et al.*, 1997). We also found that the immune responses induced by the two mutants depended upon the immunization frequency. Antibody titers and cytokine production were higher in mice immunized twice than in those immunized only once. These data correlate with the known susceptibility of *asd* mutants to lysis in the absence of DAP. Therefore, the *asd* mutants would be cleared before immune responses could be sufficiently induced, especially after only one injection.

Together, these data suggest that SHJ2104 and HTSaYA could be used as live attenuated vaccine candidates. These strains have several advantages: they are i) highly attenuated due to their low cytotoxicity and intracellular proliferation activity; ii) rapidly cleared from mouse organs; and iii) capable of eliciting a significant level of humoral, mucosal and cell-mediated immune responses especially after two *i.p*. immunizations. These features result in efficient protection against oral challenge with virulent wild-type *S.* Typhimurium.

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