Effect of Deletion of Genes Involved in Lipopolysaccharide Core and O-Antigen Synthesis on Virulence and Immunogenicity of *Salmonella enterica* Serovar Typhimurium

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Lipopolysaccharide (LPS) is a major virulence factor of *Salmonella enterica* serovar Typhimurium and is composed of lipid A, core oligosaccharide (C-OS), and O-antigen polysaccharide (O-PS). While the functions of the gene products involved in synthesis of core and O-antigen have been elucidated, the effect of removing O-antigen and core sugars on the virulence and immunogenicity of *Salmonella enterica* serovar Typhimurium has not been systematically studied. We introduced nonpolar, defined deletion mutations in *waaG* (rfaG), *waaI* (rfaI), *rfab*/*waaI* (rfaI), *rfaH*, *waaL* (rfal), *wbaP* (rfBP), *waaL* (rfal), or *wzy* (rfc) into wild-type *S*. Typhimurium. The LPS structure was confirmed, and a number of *in vitro* and *in vivo* properties of each mutant were analyzed. All mutants were significantly attenuated compared to the wild-type parent when administered orally to BALB/c mice and were less invasive in host tissues. Strains with ΔwaaG and ΔwaaL mutations, in particular, were deficient in colonization of Peyer’s patches and liver. This deficiency could be partially overcome in the ΔwaaL mutant when it was administered intranasally. In the context of an attenuated vaccine strain delivering the pneumococcal antigen PspA, all of the mutations tested resulted in reduced immune responses against PspA and *Salmonella* antigens. Our results indicate that nonreversible truncation of the outer core is not a viable option for developing a live oral *Salmonella* vaccine, while a *wzy* mutant that retains one O-antigen unit is adequate for stimulating the optimal protective immunity to homologous or heterologous antigens by oral, intranasal, or intraperitoneal routes of administration.

Live attenuated *Salmonella* vaccines can colonize the gut-associated lymphoid tissue (e.g., Peyer’s patches [PP]) and the secondary lymphatic tissues to elicit immune responses against both *Salmonella* and the heterologous antigens delivered by the vector during colonization of the host (9, 10, 12). Attenuation of *Salmonella* virulence can be achieved either by deleting the genes involved in central metabolic pathways such as *aroA*, *aroC*, *purA*, *pabA*, and *pabB* or by deletion or regulated expression of global regulators such as *phpQ*, *fur*, and *crp* and other genes important for establishing an infection (reviewed in references 8, 10, 12, and 14).

Lipopolysaccharide (LPS) of *Salmonella* is a recognized virulence determinant and is essential for functions, including swarming motility (60), intestinal colonization (47), serum resistance (57), invasion/intracellular replication (43), and resistance to killing by macrophages, all of which are critical for successful infection by the pathogen. LPS consists of three different regions: a conserved lipid A, a short core oligosaccharide (C-OS), and the O-antigen polysaccharide (O-PS) assembled in a variable number of oligosaccharide repeating units (51). Lipid A is a ligand that stimulates the TLR4-MD2-CD14 pathway to activate the antimicrobial host defense during bacterial infection (41, 50). The O-PS is *Salmonella*’s primary defense against serum complement activation (42). Truncating the LPS O-antigen and ceasing its synthesis *in vivo* are strategies for attenuating *Salmonella* for use as live vaccines.

Galactose is a component of both core and O-antigen, and mannose is a component of O-antigen. The *galE* and *pmi* genes encode enzymes that are critical for UDP-galactose and GDP-mannose synthesis, respectively. Thus, *galE* (19, 29) and *pmi* (13, 37, 58) mutants of *Salmonella enterica* serovar Typhimurium are conditionally rough (lacking O-antigen), depending on the availability of galactose or mannose, respectively, resulting in strains that are attenuated and immunogenic. For use as vaccines, these strains are grown with their respective sugars prior to immunization and produce complete O-antigen. RfaH, a transcriptional antiterminator, is required for the synthesis of secreted and surface-associated cell components of *S*. Typhimurium, including O-PS and core sugar components of LPS (2). An rfaH deletion mutant is described as “gently rough,” and exhibits deep-rough characteristics and stimulates protective immune response against *Salmonella* challenge in BALB/c mice (13, 28). The *wzy* (rfc) gene encodes the O-antigen ligase, required for polymerization of multianti O-antigen molecules. A *wzy* (rfc) mutant produces semifroag LPS, consisting of a complete core and a single O-antigen subunit. We have previously shown that a ∆pabB ∆pabA vaccine strain carrying a Δwzy mutation is immunogenic and can effectively deliver the heterologous *Streptococcus pneumoniae* antigen PspA, resulting in protective immunity against *Streptococcus*
pneumoniae challenge (34). The immunogenicity of rfaH and wzy mutant strains can be further enhanced by programmed downregulation of rfaH or wzy expression in a recombinant attenuated Salmonella vaccine (RASY) without compromising their ability to colonize lymphoid tissues (34, 35). These previous studies led us to consider manipulation of other genes involved in the synthesis of LPS, including the genes for core oligosaccharide (C-OS) that have not been explored previously in the context of vaccine construction.

C-OS is assembled by the sequential transfer of sugars from a lipid acceptor by specific glycosyltransferases in the inner membrane (17, 63). The genes encoding these proteins are organized in the waa operon (rfa operon) (53). The potential of Salmonella mutants with truncated core and O-antigen as attenuated vaccines has not been systematically analyzed. In this work, we constructed strains with an ordered series of S. Typhimurium nonpolar deletion mutations causing stepwise truncations of core sugars and evaluated these mutant strains for virulence and ability to colonize BALB/c mice. The effect of each mutation in a well-characterized attenuated vaccine strain on its ability to deliver the Streptococcus pneumoniae antigen PspA and to affect an antigen-specific protective immune response was also determined.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media, and growth conditions.** The strains and plasmids used in this study are listed in Table 1. S. Typhimurium cultures were grown at 37°C in LB broth (4) or nutrient broth (Difco) or on LB agar with or without 0.1% arabinose. Selenite broth, with or without supplements, was used for enrichment of Salmonella from mouse tissues. Diaminopimelic acid (DAP) (50 μg/ml) was added for the growth of ΔwaaD strains (45). LB agar containing 5% sucrose was used for ssaC gene-based counterselection in allelic exchange experiments. MOPS (morpholinepropanesulfonic acid) minimal medium (46) with/ without 10 μg/ml p-aminobenzoic acid (pABA) was used to confirm the phenotype of ΔpabA ΔpabB mutations.

### TABLE 1. Bacterial strains and plasmids used in this study

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<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
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<td>Recombinant plasmids</td>
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<td>Plasmid Asd&lt;sup&gt;+&lt;/sup&gt;; pBR ori β-lactamase signal sequence-based periplasmic secretion plasmid (blac&lt;sub&gt;SS&lt;/sub&gt;)</td>
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<td>pYA4088</td>
<td>852-bp DNA encoding the α-helical region of PspA (aa 3-285) into pYA3493; blac&lt;sub&gt;SS&lt;/sub&gt;-pasp</td>
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<td>χ7232</td>
<td>endA1 hsdR17 (K&lt;sup&gt;−&lt;/sup&gt; m&lt;sup&gt;−&lt;/sup&gt;) glnV44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 &lt;sup&gt;hir&lt;/sup&gt; deoR (Δ80llac ΔlacZΔM15)</td>
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<td>S. pneumoniae WU2</td>
<td>Wild-type virulent, encapsulated type 3</td>
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*a* The mutations introduced into χ9241 background are indicated in bold.
Plasmids and mutant strain construction. The primers used in this study are listed in Table 2. DNA manipulations were carried out as described elsewhere (55). Transformation of Escherichia coli and S. Typhimurium was done by electroporation. Transformsants were selected on LB agar plates containing appropriate antibiotics. Selection for Aac′ plasmids was done on LB agar plates.

For the waaGΔ2 deletion, two pairs of primers, DrfαG-1F/DrfαG-1R and DrfαG-2F/DrfαG-2R, were used to amplify approximately 350-bp fragments upstream and downstream of gene rfaG, respectively. The two fragments were then joined by PCR using primers RfaG-1F and RfaG-2R.

DrfaG-2R ..........................TTCTATCATATAGCCGATCTG
DrfaG-2F ......................TGGAGAAAAGTAAAAACGCGCTGAT

For the rfaLΔ2 deletion, two pairs of primers, DrfβL-1F/DrfβL-1R and DrfβL-2F/DrfβL-2R, were used to amplify approximately 600-bp fragments around the gene rfaL. The two fragments were then joined by PCR using primers DrfβL-1F and DrfβL-2R.

DrfβL-2F ..................TTCTATCATATAGCCGATCTG
DrfβL-2R ..................TGGAGAAAAGTAAAAACGCGCTGAT

The mutations were also introduced into wild-type (pYA4896, pYA4897, pYA4898, pYA4899, and pYA4900) to generate ΔwaaG, ΔwaaL, ΔwbaP, ΔwbbJ, and ΔwbbK. The presence of both the mutations was done on LB agar plates.

The experiments were repeated three times.

Attachment and invasion assays. Attachment and invasion tests were performed as described previously (18). The human intestinal cell line INT407 was cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 100 μg/ml penicillin and streptomycin. Cells were seeded 16 h prior to infection at a density of 5 × 10^5 cell/well (24-well plates). Bacteria were grown overnight statically in 3 ml LB. One ml of the overnight culture was added to 50 ml LB and grown at 37°C to an OD600 of 0.95. The culture was diluted to 2 × 10^7 CFU/ml in DMEM without antibiotics. The tissue culture medium was replaced by DMEM supplemented with 10% fetal calf serum and 2 mM l-glutamine immediately before 100 μl of the bacterial suspension was added to each well to achieve a multiplicity of infection (MOI) of 5:1. The plates were centrifuged at 200 × g for 5 min to synchronize the interaction between bacteria and monolayer cells and incubated at 37°C in a 5% CO2 incubator to allow attachment for 1 h. Wells were washed 3 times with PBS, followed by the addition of 1% Triton X-100 to lyse the INT407 cells. Aliquots of serial dilutions from each well were spread onto LB plates and incubated overnight to determine the number of attached bacteria. Attachment was calculated as follows: percent attachment = 100 × (number of cell-associated bacteria/initial number of bacteria added). The same procedure was used to evaluate invasion with the following modifications. Prior to the lysis step, 1 ml of serial dilutions made from each well were spread onto LB plates and incubated overnight to determine the number of attached bacteria. Attachment was calculated as follows: percent invasion = 100 × (number of bacteria resistant to gentamicin/initial number of bacteria added). Both adherence and invasion assays were performed three times.

Serum bactericidal assay. Pooled human serum (PHS) was purchased from Equitech-Bio and stored at −70°C until used. Heat-inactivated serum (HIS) was generated by incubating PHS for 2 h at 56°C. Bacteria grew statically in LB media overnight, and the next day they were grown to an OD600 of 0.9 to 0.95 under 37°C at 180 rpm. Bacteria (1 ml) were suspended in Dulbecco’s phosphate-buffered saline (DPBS) to a final concentration of 2 × 10^6 to 3 × 10^5 CFU/ml; 50-μl portions of the bacterial suspensions were added to 50 μl PHS or HIS and incubated for 30 min at 37°C. After incubation, the samples were plated on ice to determine the bacterial cell counts (CFU/ml) of the HB101 cells and on LB agar plates and incubated overnight at 37°C. Bacterial colonies were counted to determine survival.

Determinations of LD₅₀ and colonization in mice. All animal procedures were approved by the Arizona State University Animal Care and Use Committee. Seven-week-old, female BALB/c mice were obtained from the Charles River Laboratories (Wilmington, MA). Mice were acclimated for 7 days before starting the experiments. Determination of the oral 50% lethal dose (LD₅₀) followed our standard procedures (35).

To evaluate the effect of the administration route on colonization, mice were inoculated orally with 20 μl of buffered saline with gelatin (BSS) containing 1 × 10^7 CFU or intranasally (i.n.) with 10 μl of BSS containing 1 × 10^5 CFU. Six days after oral inoculation, or 6 days for mutants and 3 days for wild-type after i.n. inoculation, three animals per group were euthanized. Peyer’s patches (PP) were collected from all sites of the small intestinal surface, and bacterial colonization in PP represented the mean bacterial burden in pooled PP from each mouse. Spleen and liver samples were collected, and their individual weights were measured. Each sample was homogenized in a total volume of 1 ml BSS, and appropriate dilutions were plated onto MacConkey agar and/or LB agar to determine the number of viable bacteria. The remaining tissue homogenate was enriched for Salmonella by incubation in selenite cystine broth overnight at 37°C. Samples that were negative by direct plating and positive by enrichment in selenite cystine broth were recorded as 1 CFU/orange.

Construction of vaccine strain derivatives. Each of the mutations under study was introduced into the previously described attenuated Salmonella strain χ9241.
Expression plasmid pYA4088, which specifies a Bla-SS-PspA secreted fusion protein, was introduced into each of the H9273 derivatives. The level of Bla-SS-PspA fusion protein produced by each strain was examined by using Western blots probed with anti-PspA antibody.

Immunogenicity of vaccine strains in mice. Strains were grown statically overnight in LB broth with 0.1% arabinose at 37°C. Arabinose was added to reduce antigen expression during in vitro growth (61). The following day, 2 ml of the overnight culture was inoculated into 100 ml of LB broth with 0.1% arabinose and grown with aeration at 37°C to an OD600 of 0.8 to 0.9. Cells were harvested by room temperature centrifugation at 4,000 rpm for 15 min, and the pellet was resuspended in 1 ml of BSG. Groups of mice (6 or 7 mice per group) were orally inoculated with 20 μl of BSG containing 1 × 109 CFU of each strain, or mice were i.n. or intraperitoneally (i.p.) inoculated with 1 × 107 or 1 × 106 CFU of each strain, respectively, on day 0 and boosted on day 35 with the same dose of the same strain. Blood was obtained every 4 weeks by mandibular vein puncture. Blood was allowed to coagulate at 37°C for 2 h. Following centrifugation, the serum was removed and stored at −80°C.

Antigen preparation and ELISA. Recombinant PspA (rPspA) was purified as described previously (32). The rPspA clone, which encodes the α-helical region of PspA (amino acids [aa] 1 to 302) in pET20b, was a kind gift from Susan Hollingshead at the University of Alabama at Birmingham. S. Typhimurium LPS was purchased from Sigma. Outer membrane proteins were prepared as described previously (32). An enzyme-linked immunosorbent assay (ELISA) was used to assay serum antibodies against S. Typhimurium LPS, rPspA, and bacterial outer membrane proteins, including those from Salmonella (SOMPs), as previously described (38). Color development (absorbance) was recorded at 405 nm using a SpectraMax M2e automated ELISA plate reader (Molecular Devices, Menlo Park, CA). A405 readings 0.1 higher than PBS control values were considered positive (serum dilution start at 1:50).

Statistical analyses. Statistical analyses were performed by using the GraphPad Prism 5 software package (Graph Software, San Diego, CA). Antibody titers were expressed as means ± standard deviations. The means were evaluated with one or two-way analysis of variance (ANOVA) and Bonferroni’s multiple comparison test for comparisons among groups of antibody titers.

RESULTS

Mutant construction and LPS phenotypes. We constructed an isogenic set of S. Typhimurium strains with nonpolar gene deletions that resulted in progressively shorter LPS, beginning with a Δwzy mutant whose LPS includes a single O-antigen unit, through a ΔwaaG mutant with a core truncated to the heptose residues (Fig. 1A). Each deletion mutation resulted in a strain with the expected length of LPS (Fig. 1B). The wild-type parent strain, UK-1, produced a complete or smooth LPS.

FIG. 1. Schematic representation of S. Typhimurium lipopolysaccharide molecule (inner core, outer core, and O-antigen) and LPS phenotypes of mutant and parent strains. (A) Dotted lines indicate the level of LPS truncation resulting from each mutation (Kdo, 3-deoxy-D-manno-octulosonic acid; PPEtN, pyrophosphorylethanolamine; Hep, Heptose; GlcNAc, N-acetylglicosamine; Glc, glucose; Gal, Galactose; P, phosphate). (B) Cells from indicated strains were grown and processed for LPS analysis as described in Materials and Methods. LPS was visualized by silver staining polyacrylamide gel electrophoresis (PAGE) gels. The mutant strains (from left to right): χ11308 (ΔwaaG42), χ11309 (ΔwaaI43), χ9945 (ΔfaI49), χ11310 (ΔwaaL45), χ11311 (ΔwbaP45), and χ9944 (Δwzy-48). The expected location of O-antigen regions and core is indicated on the right of the gel.
(lane UK-1 in Fig. 1B). The LPS produced by the ΔwaaG42 mutant included only lipid A and the inner core (Fig. 1A) and migrated the fastest in the gel as expected (Fig. 1B). The LPS produced by the other mutants, as ordered from left to right in Fig. 1B, included more core and/or O-antigen sugars, resulting in the expected reduced mobility in the gel. The ΔfaH mutant produced a truncated core with a molecular mass similar to that of the ΔwaaI mutant, as previously reported (35, 39).

The ΔwaaL46 and ΔwbaP45 mutants synthesize complete core with no O-antigen attached. The ΔwaaL46 mutant produces an undecaprenyl-phosphate-O-unit (Und-PP-O-unit) through the action of WbaP (49), but it cannot be added to the lipid A core due to the absence of O-antigen ligase (WaaL) (5, 33). Alternatively, the ΔwbaP mutant synthesizes O-antigen ligase (WaaL) but lacks WbaP, the Und-P galactose phosphotransferase that produces an O-antigen ligase substrate by catalyzing the transfer of galactosyl-1-phosphate from UDP-galactose to Und-P. The Δwzy-48 mutant displayed the expected semirough phenotype with the addition of one O-antigen unit (Fig. 1B) (34).

To confirm the observed alterations in LPS profiles, we performed infection studies with the O-antigen-specific phase P22. Strains grown in LB broth were used as recipients for transduction assays. As expected, all mutants except the ΔfaH49 and wild-type UK-1 strains were resistant to P22 infection (Table 3). The ΔfaH49 strain was sensitive to P22 and was transducible; however, the number of transductants obtained was much lower than that obtained with the wild-type strain, as we observed previously (35), indicating that, although undetectable on our silver-stained gel (Fig. 1B), the ΔfaH49 mutant still produced minute amounts of the full-length O-antigen.

**Phenotypic evaluation of mutant strains.** The mutants’ growth rates were evaluated in LB broth. All strains were similar to the UK-1 parent except strains χ11308 (ΔwaaG42) and χ11309 (ΔwaaH43), which had a slightly lower rate of growth. Under similar conditions, it took these mutants 5 h to reach an OD_{600} of 1.3, compared to 4.5 h for the parent strain UK-1 (data not shown). We also noted that these two mutants tended to settle out of suspension more quickly than the other strains when held statically for 30 min.

The effect of LPS alterations on the resistance of these mutants to detergents, such as deoxycholate (DOC) and ox bile, and to the antimicrobial peptide polymyxin B was determined by measuring the MIC in each case (Table 3). All mutants were more sensitive to DOC and polymyxin B than wild-type UK-1, and the sensitivity varied based on the degree of truncation of the outer core. The ΔwaaG42 mutant, lacking the entire outer core, was the most sensitive to DOC, with an MIC approximately 6-fold lower than that of the wild-type parent strain. These results are in agreement with our previous report that a ΔfaH mutant is more sensitive to DOC than the parent strain (35). However, the strain with the ΔwaaG42 mutation exhibited at least 2-fold greater resistance to polymyxin B than the ΔwaaH43, ΔfaH49 and ΔwaaH44 mutants, each of which contains more of its outer core. Strains carrying the ΔwaaL46, ΔwbaP45, and Δwzy-48 mutations contain all the core sugars and displayed intermediate sensitivity between the smooth parent and the other mutants (Table 3). All strains were resistant to the highest concentrations of ox bile tested.

Initial rough mutants are unable to swarm because of insufficient moisture on the agar surface but can still swim in 0.3% soft agar (60). Therefore, we determined the relative ability of each mutant to swim on soft agar (Table 3). All mutants were able to swim to various degrees on soft agar. Three mutants, ΔwaaG42, ΔwbaP45, and ΔwaaH43, each had substantially reduced bacterial motility compared to other mutants (Table 3), whereas ΔfaH49, ΔwaaH44, and ΔwaaL46 showed 2-fold reductions in motility as determined by the motility diameter on soft agar. Deletion of wzy-48 had no effect on swimming motility, which was similar to that measured for wild-type UK-1 (34).

**Attachment/invasion and serum sensitivity assays.** To examine the effect of these LPS gene mutations on attachment and invasion, we evaluated the ability of the mutants to attach to and invade INT407 human epithelial cells. All strains were similar to UK-1 in their ability to attach to INT407 cells, indicating that the outer core and O-antigen are not essential for this process (data not shown). In contrast, the ΔwaaH43 and ΔwaaG42 mutations resulted in a significant reduction in invasion compared to the other strains, indicating a requirement for the inner glucose and the galactose residues of core for this activity (Fig. 2A). Deletion of wzy resulted in a small (2-fold) but significant increase in invasion compared to that of wild-

### Table 3. MICs of antimicrobials, swimming motility, transduction efficiency, and virulence of *S. Typhimurium* strain χ3761 and its isogenic mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>P22 phage sensitivity</th>
<th>MIC (DOCb bile) (mg/ml)</th>
<th>MIC (polymyxin B) (μg/ml)</th>
<th>MIC (ox bile) (mg/ml)</th>
<th>Motility (mm) on soft agar</th>
<th>LD_{50}e (LD50, median lethal dose)</th>
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<td>3.5***</td>
<td>&gt;10^9</td>
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<td>χ11309 (ΔwaaH43)</td>
<td>–</td>
<td>2.5</td>
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<td>4***</td>
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<td>0.08</td>
<td>&gt;20</td>
<td>17***</td>
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<td>χ11310 (ΔwaaH44)</td>
<td>–</td>
<td>2.5</td>
<td>0.08</td>
<td>&gt;20</td>
<td>15**</td>
<td>&gt;10^9</td>
</tr>
<tr>
<td>χ11312 (ΔwaaL46)</td>
<td>–</td>
<td>2.5</td>
<td>0.3</td>
<td>&gt;20</td>
<td>20**</td>
<td>&gt;10^9</td>
</tr>
<tr>
<td>χ11311 (ΔwbaP45)</td>
<td>–</td>
<td>2.5</td>
<td>0.3</td>
<td>&gt;20</td>
<td>7**</td>
<td>&gt;10^9</td>
</tr>
<tr>
<td>χ9944 (Δwzy-48)</td>
<td>–</td>
<td>2.5</td>
<td>0.3</td>
<td>&gt;20</td>
<td>45</td>
<td>&gt;10^9w</td>
</tr>
<tr>
<td>χ3761</td>
<td>++</td>
<td>10</td>
<td>0.6</td>
<td>&gt;20</td>
<td>45</td>
<td>1 x 10^8</td>
</tr>
</tbody>
</table>

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*α*, resistant; + and ++ +, degrees of sensitivity as determined by transduction efficiency.

*b* DOC, deoxycholate.

**P* < 0.01 compared with wild-type UK-1; ***P < 0.001 compared with wild-type UK-1.

*e* LD_{50}, median lethal dose.

* Some mice displayed symptoms (scruffy coat, lethargy) after inoculation with the Δwzy-48 strain but recovered after 14 days.
type UK-1 (P < 0.05), indicating that shortening the O-antigen contributes to bacterial invasion into epithelial cell. A similar phenomenon has been observed in *Shigella*, in which shortening the LPS by glucosylation promotes bacterial invasion and evasion of innate immunity by enhancing TTSS function without compromising the protective properties of LPS (62).

Complete LPS is an important factor in serum resistance (57). We examined the resistance of our mutants to the bacterial effects of human serum (Fig. 2B). All mutants were significantly more sensitive to human serum than the smooth parent strain. In general, the trend of sensitivity to the pooled human serum (PHS) followed the length of the LPS, with strains producing longer LPS being more resistant. These data are consistent with a previous study showing that a *wbaP* mutant was more sensitive to serum than a wild-type strain (30). However, one notable exception was that the Δ*waaL* mutant, producing the shortest core structure, was as resistant to serum killing as the Δ*waaL* mutant, which carries a complete core, and more resistant than mutants with intermediate core structures. There were no differences in the sensitivity of any of the strains to heat-inactivated serum.

**Virulence and colonization of mutant strains in mice.** We evaluated our mutants for virulence by determining the oral LD_{50} of each strain in BALB/c mice. All mutants were highly attenuated, with no deaths at the highest dose tested, 1 × 10^{10} CFU (Table 3). To determine how well each strain could colonize mouse tissues, groups of mice were orally inoculated with 1 × 10^{9} CFU and the bacterial loads in Peyer’s patches (PP), spleen, and liver were determined 6 days after inoculation. The mean CFU counts of the wild-type UK-1 strain recovered from PP (Fig. 3A), spleen (Fig. 3B), and liver (Fig. 3C) were approximately 10^{3}, 10^{5}, and 10^{3}, respectively. Colonization levels of all mutants were significantly lower than the parent strain UK-1 (P < 0.05 to 0.001), and the relative differences observed among mutants were consistent for all organs examined. Notably, inactivation of *waaG* or *waaI* essentially eliminated the ability of *S. Typhimurium* to cause systemic infection. Both strains colonized PP poorly (<10^{5} CFU/g), and their presence in spleen and liver was below the limit of detection (Fig. 3B and C). The Δ*rrfH* mutant colonized both spleen and liver better than either the Δ*waaG* or Δ*waaI* mutants. Although the Δ*waaH*, Δ*waaL*, Δ*wbaP*, and Δ*wzy*-48 mutants were better able to colonize PP, spleen, and liver, the numbers of mutant bacteria isolated from each organ were significantly less than those of their UK-1 parent. Inflammation of the spleen, as determined by spleen weight, was also reduced significantly in mice inoculated with the mutants (P < 0.05 to 0.001) (Fig. 3D).

When *Salmonella* is administered orally, it must overcome the low-pH barrier of the stomach, followed by the harsh environment of the intestinal tract, including the presence of organic acids, bile salts such as DOC, and antimicrobial peptides similar to polymyxin B, before interacting with host cells. While we did not test the acid resistance of our mutants, previous reports have indicated that rough strains of *Salmonella* are more sensitive to the presence of organic acids than smooth strains (3). This, coupled with the observation that our mutants exhibit increased sensitivity to DOC and polymyxin B, led us to examine alternate routes of immunization to bypass the barriers imposed by the alimentary tract. Therefore, we evaluated the ability of the Δ*waaH*, Δ*waaL*, and Δ*wzy*-48 mutants to colonize PP, spleen, and liver after i.n. inoculation. Mice receiving the mutants were evaluated after 6 days, and mice receiving the virulent parent were evaluated after 3 days (Fig. 4). All three mutants achieved levels approximately 10-fold greater for each tissue than the levels reached when administered orally. The Δ*waaL* and Δ*wzy* mutants were able to colonize all tissues at levels comparable to those for UK-1, indicating that O-antigen is not required for colonization when *Salmonella* is administered intranasally. There does appear to be some benefit of core, since the Δ*waaI* mutant did not colonize as well as the other strains.

**Effects of the mutations on the immunogenicity of recombinant *S. Typhimurium* strains by oral administration.** We were also interested in determining the effect of core length on the ability of an attenuated vaccine to deliver a heterologous antigen. To address this, we introduced each of the mutations described above into attenuated *Salmonella* strain χ9241 (Table 1). Strain χ9241 is attenuated due to the presence of the Δ*pabA* Δ*pabB* mutations and produces smooth LPS. We introduced plasmid pYA4088 encoding pspA, a pneumococcal gene.
encoding the protective antigen PspA, into each strain. Immunoblot analyses of subcellular fractions from /H9273/9241(pYA4088) have shown that 51.5% of the Bla-ss-PspA fusion protein is secreted into the periplasm, 5.4% is secreted out of the cell, and the remaining 43.1% is retained in the cytoplasm (65). Nine groups of mice were immunized on day 1 to 2 with 1/H11003/109 of each /H9273/9241 derivative and boosted with the same dose of the same strain 5 weeks later. Mice immunized with strain

FIG. 3. Colonization of mice by /H9273/3761 and its isogenic mutants 6 days after oral inoculation. Groups of mice (3 mice/group) were orally inoculated with approximately 1 × 10^9 CFU of the indicated strains. Log_{10} CFU/g tissue determined on day 6 postinoculation in Peyer’s patches (PP) (A), spleen (B), and liver (C) of inoculated BALB/c mice. (D) Day 6 spleen weights from inoculated mice. The horizontal lines represent the means, and the error bars represent the standard errors of the means. ***, P < 0.001 compared with UK-1; **, P < 0.01 compared with UK-1; *, P < 0.05 compared with UK-1. Samples that were negative by direct plating and positive by enrichment in selenite cysteine broth were recorded as 1 CFU/organ.

FIG. 4. Colonization of mouse organs by /H9273/3761 and its isogenic mutants after intranasal (i.n.) administration with 10^9 CFU. Log_{10} CFU/g tissue were determined on day 6 postinoculation for the mutants and on day 3 postinoculation for the wild-type strain in Peyer’s patches (A), spleen (B), and liver (C) of BALB/c mice. The horizontal lines represent the means, and the error bars represent standard errors of the means.
Results of the experiments are shown in Figures 5A and 5B. The anti-PspA IgG titers were measured by ELISA. Data represent reciprocal anti-IgG antibody titers in pooled sera from mice orally immunized with attenuated Salmonella carrying either pYA4088 (pSpA) or pYA3493 (control) at the indicated number of weeks postimmunization. The error bars represent variations between triplicate wells. Mice were boosted at week 5.

**Effects of the mutations on the immunogenicity of recombinant S. Typhimurium strains by i.n. or i.p. administration.**

Based on our observation that i.n. inoculation provided better access of the S. Typhimurium strains to lymphoid tissues (Fig. 4), we wanted to assess the immunogenicity of these strains. We therefore, different doses were used to immunize the mice via different routes of administration. Four groups of mice were immunized i.n. with 1 × 10^7 CFU or i.p. with 2 × 10^6 CFU of strain χ9241 or its derivatives when administered i.n. or i.p. Both are routes that bypass the alimentary tract. LD_{50} values of Salmonella vary based on the route of administration used; therefore, different doses were used to immunize the mice via different routes of administration. Four groups of mice were immunized i.n. with 1 × 10^7 CFU or i.p. with 2 × 10^6 CFU of strain χ9241 or its derivatives, χ9241 carrying the empty vector pYA3493 served as negative controls. All mice survived immunization, and we did not observe any signs of disease in the immunized mice during the experimental period. We measured serum IgG antibody responses to rPspA (Fig. 5A) and to Salmonella LPS (Fig. 5B) in immunized mice. Parent strain χ9241(pYA4088) and its isogenic Δwzy-48 counterpart induced the highest levels of IgG against PspA and LPS among all the vaccine strains. At 8 weeks post-primary inoculation (3 weeks after the boost), the ΔwbaP45 derivative of χ9241 was significantly (P < 0.05) different from the ΔwaaL46 derivative. No immune responses were detected to PspA in mice immunized with χ9241 containing the control plasmid (reciprocal titer, <1:50). Immune responses (IgG, IgG1, and IgG2a) against PspA were below the level of detection in mice immunized with waaG42 and rfaH49 at 4 weeks (reciprocal titer, <1:50). Immune responses against LPS were below the level of detection in mice immunized with the ΔwaaG2, ΔwaaH3, Δrfah49, ΔwaaJ44, and ΔwaaL46 strains at 4 weeks and with the ΔwaaG2 strain at 8 weeks (reciprocal titer, <1:50).

Anti-PspA IgG titers in sera from mice immunized with the other vaccine strains were significantly lower (P < 0.05 to 0.001). Serum IgG against Salmonella LPS induced by each of the χ9241 derivatives was also significantly lower (P < 0.01) than that of the parent strain χ9241(pYA4088).

The immune responses to PspA were further evaluated by measuring the levels of anti-PspA IgG isotype subclasses IgG1 and IgG2a in each group of mice. At week 4, all strains elicited a balanced Th1/Th2 response, based on the fact that the mice produced similar titers of IgG1 and IgG2a (Fig. 5C and D), with the exception of the ΔwbaP strain, which elicited higher titers of IgG2a than IgG1 compared to the other mutants (P < 0.05), indicating a Th1-biased response. Interestingly, the wbaP strain elicited significantly higher titers of IgG1 and IgG2a than the waaL strain (Fig. 5C and D), though both mutations resulted in the same LPS structure (Fig. 1). By week 8, the titers of IgG2a in mice immunized with χ9241(pYA4088) and the ΔwaaI, ΔwaaL, ΔwbaP, and Δwzy mutants were higher than the IgG1 titers, indicating a shift to a Th1-biased response. The other mutants were poorly immunogenic, and the IgG1/IgG2 ratio was the same at weeks 4 and 8.
all carrying plasmid pYA4088, and boosted with the same dose of the same strain 5 weeks later. Serum IgG responses to PspA and to Salmonella LPS were measured by ELISA (Fig. 6A). The Δwzy-48 mutant induced the highest titers of anti-PspA IgG in the i.n. immunized groups, significantly higher than those for the parent strain /H9273 at week 8. Also at week 8, the anti-PspA titers in mice immunized with the parent strain /H9273 and the isogenic ΔwaaL46 mutant were identical.

When administered i.p., the Δwzy mutant also induced higher anti-PspA serum IgG titers than the other mutants and titers were similar to those for the parent strain, /H9241 at week 8. Also at week 8, the anti-PspA titers in mice immunized with the parent strain /H9241(pYA4088) and the isogenic ΔwaaL46 mutant were identical.

Evaluation of immunogenicity against OMPs from different enteric bacteria. Previous work has shown that a complete lack of or regulated expression of dominant surface antigens, such as O-antigen, increases the levels of serum antibodies that are cross-reactive to conserved surface epitopes of other enteric bacteria (13, 35, 44). Therefore, we determined whether sera raised against our mutants that have truncated core and O-antigen had a similar effect. We evaluated the reactivity of pooled immune sera (n = 7) from orally immunized mice described in the previous experiment (Fig. 5) against purified OMPs from homologous and heterologous wild-type strains by ELISA (Fig. 7). The results roughly correlated with the results we obtained against PspA and LPS (Fig. 5). Antibodies raised against the parent vaccine /H9273 reacted more strongly to OMPs from three enteric strains tested than those induced by any of its mutant derivatives. In all eight vaccine strains, ΔwaaG and ΔwaaL derivatives consistently induced the lowest reactivity against OMPs, while the Δwzy derivative induced the highest cross-reaction against the OMPs (Fig. 7). Similar results were also achieved from the immunized mice by i.n. or i.p. route administration (Fig. 8), although the Δwzy mutant delivered i.p. elicited the highest level of antibodies cross-reactive to Salmonella enterica serotype Enteritidis (Fig. 8B).

DISCUSSION

Attenuated salmonellae have the ability to stimulate both cellular and humoral immunity against homologous or heterologous antigens after oral administration (9, 10, 12, 14). O-antigen and core sugar comprise key virulence determinants (47, 57, 60). An incomplete core or O-antigen of LPS may result either from deficiency in glycosyltransferase activity, from the inability to synthesize sugars that are part of the core or O-antigen chain, or from deletion of LPS processing genes such as...
In this study, we systematically investigated the relationship between LPS truncation and the second can be obtained by deleting either waa or wzy genes, such as waaI43, rfaH, and heterologous OMPs 8 weeks after the primary immunization by oral administration. Sera from mice immunized with S. Typhimurium strain (H9273) were each individually tested against purified outer membrane proteins from enteric bacteria S. Typhimurium (A), an avian pathogenic E. coli O78 strain (B), or S. Enteritidis 3550 (C). The error bars represent variations from triplicate wells. ***, P < 0.001; **, P < 0.01; *, P < 0.05 compared to titers from mice immunized with 9241(pYA088).

Deletion of the waa or wba genes targeted in this study resulted in truncated core structures (Fig. 1A and B) (31). rfaH mutants are reported to produce a mixed core structure containing the major Rb5, minor Rb, Rb1, Rb2, and some Ra being capped by shorter or longer polymers of O-antigen units (39, 56). Our results with the ΔrfaH49 mutant are consistent with the expected phenotype, as we observed primarily an Rb3 truncated core (similar to results with the ΔwaaI43 mutant) (Fig. 1A and B) in addition to O-antigen detectable only by our P22 phage transduction assay (Table 3). This is likely to be due to the fact that RfaH is a transcriptional anti-terminator that reduces the polarity of long operons by binding to the opsequence located in the 5′ regions of the waa, wba, and other operons (2). Therefore, the rfaH deletion does not fully abolish synthesis of full-length transcripts (1, 2). As expected, all mutants with truncated outer core and O-antigen were more sensitive to DOC, polymyxin B, and pooled human serum than wild-type UK-1, while all mutants were resistant to oxos bile (Table 3 and Fig. 2C). It is surprising that the ΔwaaG42 mutant that contains only the inner core was as resistant to PHS as the ΔwaaL46 mutant that produces a complete core and significantly (P < 0.01) more resistant to serum than the ΔwaaH44, ΔrfaH49, and ΔwaaI43 mutants (Fig. 2), each of which contains more sugar moieties attached to the terminal inner core (Fig. 1A). In addition, the waaG46 mutant was 2-fold more resistant to polymyxin B than the ΔwaaH44, ΔrfaH49, and ΔwaaI43 mutants (Table 3). One explanation for this result is that the loss of O-antigen in our mutants may

wzy, rfaH, or wxx (16, 20, 34, 35). The first can be achieved by deleting either waa or wba genes (31), leading to nonreversible truncation of LPS, and the second can be obtained by deleting genes such as galE or pmr, responsible for dNDF-sugar synthesis, leading to reversible LPS truncation (11, 13, 29, 37, 58).

FIG. 8. Cross-reactivity of serum IgG from intranasal (i.n.) and intraperitoneal (i.p.) immunized mice against OMPs (outer membrane proteins) from three enteric bacteria. IgG cross-reactivity of sera (1: 1,000 dilution) obtained from mice (n = 5) immunized with 9241(pYA088) or its mutant derivatives to homologous and heterologous OMPs 8 weeks after the primary immunization by i.n. route administration (A) and by i.p. route administration (B). Sera from mice immunized with 11314 (ΔwaaI43), 11317 (ΔwaaL46), 9885 (Δwzy-48), and 9241 (parent vaccine strain) were each individually tested against purified outer membrane proteins from S. Typhimurium strain 3761 (Typhimurium), avian pathogenic E. coli O78 strain 7122 (E. coli), and S. Enteritidis strain 3550 (Enteritidis). The error bars represent variations from triplicate wells. ***, P < 0.001; **, P < 0.01; *, P < 0.05 compared to titers from mice immunized with 9241(pYA088).
expose and/or activate outer membrane proteases that partially restore serum or polymyxin B resistance. It has been demonstrated that outer membrane proteins such as PgtE, PagC, and Rck contribute to serum and/or antimicrobial peptide resistance (21, 23, 26, 48). It is known that PgtE, a Salmonella β-barrel surface protease, can function to activate plasminogen in rough but not smooth strains (21, 36) and also to inactivate complement components (52). In addition, pgtE is regulated posttranscriptionally by PhoP (21, 36), a regulator known to function to activate plasminogen in rough but not smooth strains (21, 36) and also to inactivate complement components (52). In addition, pgtE is regulated posttranscriptionally by PhoP (21, 36), a regulator known to function to activate plasminogen in rough but not smooth strains (21, 36) and also to inactivate complement components (52).

postulated that the O-antigen and outer core are required for optimal invasion and are essential for the intestinal epithelial barrier and reach the internal lymphoid organs (Fig. 2) (6), could account for their observed inability to cross the intestinal epithelial barrier and reach the internal lymphoid organs. In all, the virulence and colonization data indicate that the rough mutants do not produce O-antigen (Fig. 1B) and the poor overall immunogenicity of the rough mutants due to their decreased colonizing ability. However, the ΔwabP mutant elicited detectable anti-LPS titers (Fig. 5B). Although we did not analyze the antibodies beyond what is shown in Fig. 5, it is likely that the responses detected were directed toward the lipid A and core regions of LPS, which were present in the ELISA coating antigen. This kind of response could be desirable if one is interested in producing a vaccine against other bacteria that share the same or similar core epitopes, such as in other Salmonella and Shigella spp. and pathogenic Escherichia coli (17, 51, 63).

Removal of the immunodominant O-antigen from the surface of Salmonella may result in enhanced immunogenicity of outer membrane proteins and other surface antigens (13, 44). In a previous study, we described a strain χ9241 derivative, χ9852, with arabinose-regulated expression of rfaH (35). This strain displayed a reversibly rough phenotype, dependent on arabinose availability. Mice orally immunized with strain χ9852(pYA4088) developed serum IgG antibodies with enhanced cross-reactivity against OMPs from a variety of enteric bacteria compared with mice immunized with the smooth parent strain, χ9241(pYA4088). This result has potential implications for developing a multivalent vaccine to protect against multiple pathogens in a single vaccine platform. In the current study, we found that mutations resulting in permanently rough χ9241 derivatives did not enhance production of cross-reactive antibodies to OMPs from any of the enteric bacteria tested (Fig. 7A to C), with the exception of the Δwzy strain at week 8, although, while statistically significant, the enhancement was much less than we observed previously with the regulated rfaH strain χ9852 (35), or even with an arabinose-regulated wzy strain (34). This most likely is due to the poor colonization and rapid elimination of the rough vaccine strains by the host, leading to their overall poor immunogenicity when administered orally.

In summary, we systematically examined the relationship between S. Typhimurium LPS length and virulence, colonization potential, and immunogenicity. We conclude that mutants with truncated core and O-antigen colonize mice poorly and thus elicit a suboptimal humoral response against a vectored antigen. However, the wzy-48 mutant was roughly comparable to the smooth parent strain χ9241 in most studies and therefore may have potential to be used, along with other attenuating mutations, in developing RASVs capable of delivering heterologous or homologous antigens to induce protective immunity. However, these results, combined with our previous studies, lead us to recommend the use of reversibly rough strains as a preferred mode of driving the production of cross-reactive core and/or OMP antibodies, due to the inability of permanently rough strains to colonize and elicit high-titer antibody responses.

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