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## ***Salmonella* synthesizing 1-monophosphorylated LPS exhibits low endotoxic activity while retaining its immunogenicity**

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### **Abstract**

The development of safe live, attenuated *Salmonella* vaccines may be facilitated by detoxification of its lipopolysaccharide. Recent characterization of the lipid A 1-phosphatase, LpxE, from *Francisella tularensis* allowed us to construct recombinant, plasmid-free strains of *Salmonella* that produce predominantly 1-dephosphorylated lipid A, similar to the adjuvant approved for human use. Complete lipid A 1-dephosphorylation was also confirmed under low pH, low Mg<sup>2+</sup> culture conditions, which induce lipid A modifications. *lpxE* expression in *Salmonella* reduced its virulence in mice by five orders of magnitude. Moreover, mice inoculated with these detoxified strains were protected against wild-type challenge. Candidate *Salmonella* vaccine strains synthesizing pneumococcal surface protein A (PspA) were also confirmed to possess nearly complete lipid A 1-dephosphorylation. After inoculation by the LpxE/PspA strains, mice produced robust levels of anti-PspA antibodies and showed significantly improved survival against challenge with wild-type *Streptococcus pneumoniae* WU2 as compared to vector-only immunized mice, validating *Salmonella* synthesizing 1-dephosphorylated lipid A as an antigen delivery system.

### **Keywords**

Attenuated *Salmonella*; Dephosphorylation of Lipid A; LpxE; TLR4; PspA

### **Introduction**

Recombinant attenuated *Salmonella* vaccines (RASVs) can deliver antigens from a variety of different pathogens, generating a range of immune responses including serum antibodies, mucosal IgA and a panoply of cell-mediated immune responses at local and distal sites (1–4). However, one problematic issue in the field has been that while candidate RASVs are adequately attenuated in animal models, when administered to humans, these vaccines can produce unwanted side effects, including fever and intestinal distress (5, 6). One possible cause of this fever is the lipid A component of lipopolysaccharide (LPS), also known as endotoxin (7). This could be of particular concern when using live strains exhibiting regulated delayed lysis in vivo to deliver a bolus of recombinant antigen and/or to confer complete biological containment (8).

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LPS, the major surface membrane component present in almost all gram-negative bacteria, consists of lipid A, a core oligosaccharide, and a highly variable and immunogenic O-antigen polysaccharide. Lipid A (Fig. 1A) is responsible for the toxicity of LPS (9, 10). Lipid A is detected by the toll-like receptor 4 (TLR4)/myeloid differentiation factor 2 (MD-2) receptor complex of the mammalian innate immune system (11–17). The structure-activity relationship of lipid A has been extensively studied, and factors governing its immunological activity have been identified. The total number and length of the acyl chains and two phosphate groups at the 1 and 4' positions are critical factors for full lipid A activation of human TLR4/MD-2 (18–20). Hexa-acylated *E. coli* lipid A with both 1 and 4' phosphate moieties and acyl chains from 12 to 14 carbons in length has optimal pro-inflammatory activity, whereas altering the number or length of the attached fatty acids or altering the charge of lipid A can reduce the magnitude of the signal (18, 19, 21). The recent TLR4-MD-2-LPS crystal structure shows that the 1- and 4'-phosphate groups interact with a cluster of positively charged residues from dimeric TLR4 and MD-2 (20). Removal of the 1- or 4'-phosphate not only weakens the ligand affinity but may also induce structural rearrangement of the TLR4 MD-2 multimer (20).

Monophosphoryl lipid A (MPL) is used clinically as a vaccine adjuvant in Europe and Australia (22) and was recently approved for use in the United States. As an adjuvant, MPL improves vaccine efficacy, induces dendritic cell maturation, induces primarily a Th1 response, indirectly reduces the threshold for activation of Th1 cells, and upregulates MHC class II molecules, CD80, and CD86 (23–26). Lipid A activates both the TLR4–TRIF and TLR4–MyD88 pathways, while MPL selectively activates the TLR4–TRIF signaling pathway, leading to significantly lower secretion of pro-inflammatory cytokines, such as IL-6, IL-1 $\beta$  and IFN- $\gamma$  than wild-type lipid A, but robust induction of G-CSF, MCP-1, and IP-10 (15). The MPL vaccine adjuvant therefore maintains or enhances immuno-stimulatory benefits, but possesses reduced toxicity (21).

LpxE, an inner membrane phosphatase from *Francisella tularensis* subspecies novicida, strain Utah 112, can selectively remove the 1-phosphate group of lipid A in living cells of *E. coli* and *Salmonella* (Fig. 1B) (27), generating a close analog of MPL that remains covalently linked to LPS. In previous studies, *lpxE* was expressed from a multicopy plasmid, a method not ideal for use in vaccine strains due to potential stability issues and because target antigen genes are typically expressed from multicopy plasmids. In this study, we identified a unique chromosomal location for *lpxE* insertion that supports levels of *lpxE* transcription to provide levels of LpxE adequate for nearly complete 1-dephosphorylation of the lipid A in *Salmonella*. In addition, we demonstrate that *lpxE* expression from this chromosomal location in live *Salmonella* is attenuating. Our ultimate goal is to modify live attenuated *Salmonella* vaccine strains to produce dephosphorylated lipid A as an additional safety feature. Therefore, we introduced our optimal *lpxE* construct into an attenuated *Salmonella* strain. We found that chromosomal *lpxE* expression in a vaccine strain carrying additional attenuating deletions does not result in loss of efficacy when used to deliver a heterologous antigen.

## Materials and Methods

### Bacterial strains, plasmids, media, and growth conditions

*S. Typhimurium* cultures were routinely grown at 37°C in LB broth (28) or LB agar with or without 0.1% arabinose, nutrient broth (Difco) or in N minimal medium (29), pH 5.8, supplemented with 0.1% casamino acids, 38 mM glycerol and 10  $\mu$ M MgCl<sub>2</sub>. DAP was added to a concentration of 50  $\mu$ g/ml for the growth of  $\Delta$ *asd* strains (30). LB agar containing 5% sucrose was used for *sacB* gene-based counter selection in allelic exchange experiments. *S. pneumoniae* WU2 was cultured on brain heart infusion agar containing 5% sheep blood or

in Todd-Hewitt broth plus 0.5% yeast extract. MOPS minimal medium (31) with/without 10 µg/ml *p*-aminobenzoic acid was used to confirm the phenotype of  $\Delta pabA$   $\Delta pabB$  mutants.

### Plasmids and mutant strain construction

DNA manipulations were carried out as described (32). Transformation of *E. coli* and *S. enterica* was performed by electroporation. Transformants were selected on LB agar plates containing appropriate antibiotics. Selection for Asd<sup>+</sup> plasmids were done on LB agar plates. The primers used in this paper are listed in Supplemental Table 1. For construction of the  $\Delta pagL7$  mutation, which deletes the entire *pagL* open reading frame, the *S. Typhimurium*  $\chi 3761$  (33) genome was used as template for cloning. A 350-bp DNA fragment containing the region upstream of the *pagL* gene (from ATG start codon, but not including ATG) was PCR amplified using primers PagL-Del1 and PagL-SbfI2 (Supplemental Table 1); another 350-bp DNA fragment containing the region downstream of *pagL* gene (from TAA stop codon, but not including TAA) was PCR amplified using primers PagL-SbfI3 and pagL-del4. The two PCR fragments were purified in agarose gels, combined at a 1:1 molar ratio and joined by PCR using primers pagL-Del1 and pagL-Del4. The resulting PCR product was digested with KpnI and XmaI and ligated to KpnI-XmaI-digested pRE112 (34), resulting in plasmid pYA4284. The same strategy is used to construct pYA4288 and pYA4287, which were used to delete the entire *pagP* and *lpxR* open reading frames, respectively. There is an SbfI site at the point of each deletion in all three plasmids. The P<sub>lpp</sub> promoter from *E. coli* was amplified by P<sub>lpp</sub>-FSbfI and P<sub>lpp</sub>-R. The *lpxE* gene from pXYW-1 (pACYC184 carrying a 2.5-kb *F. tularensis* genomic DNA fragment containing *lpxE*) (27) was amplified using primers LpxE-F1 and LpxE-Rsbfi. The two PCR fragments were purified from agarose gels, combined at a 1:1 molar ratio and joined by PCR using primers P<sub>lpp</sub>-FSbfI and LpxE-RSbfI. The resulting PCR product was digested with SbfI and ligated with SbfI-digested, shrimp alkaline phosphatase-treated pYA4284 ( $\Delta pagL7$ ), pYA4288 ( $\Delta pagP8$ ), or pYA4287 ( $\Delta lpxR9$ ). Candidate plasmids were screened by PCR to verify the orientation of the insert. The resulting plasmids were pYA4291 ( $\Delta pagL72::P_{lpp}$  *lpxE*), pYA4473 ( $\Delta pagP82::P_{lpp}$  *lpxE*), and pYA4474 ( $\Delta lpxR92::P_{lpp}$  *lpxE*). The same strategy was used to construct suicide plasmids pYA4294, pYA4295 and pYA4296 for insertion of P<sub>lpp</sub> *lpxE* (codon-optimized) (Table 1).

The mutations were introduced into *S. Typhimurium* by allelic exchange by conjugation with *E. coli* strain  $\chi 7213$  (35) harboring suicide vectors, pYA4284, pYA4288, pYA4287 to generate the  $\Delta pagL7$ ,  $\Delta pagP8$  and  $\Delta lpxR9$  mutations, respectively. Plasmids pYA4291, pYA4473 and pYA4474 were used to generate strains  $\chi 9437$  ( $\Delta pagL72::P_{lpp}$  *lpxE*),  $\chi 9701$  ( $\Delta pagP82::P_{lpp}$  *lpxE*), and  $\chi 9703$  ( $\Delta lpxR92::P_{lpp}$  *lpxE*), respectively. Plasmids pYA4294, pYA4295 and pYA4296 were used to generate  $\chi 9440$  ( $\Delta pagL71::P_{lpp}$  *lpxE*),  $\chi 9732$  ( $\Delta pagP81::P_{lpp}$  *lpxE*) and  $\chi 11092$  ( $\Delta lpxR91::P_{lpp}$  *lpxE*), respectively.

Some mutations were also introduced into the attenuated *S. Typhimurium* vaccine strain  $\chi 9241$  using the same methods. The presence of the  $\Delta pabA1516$  and  $\Delta pabB232$  mutations in strain  $\chi 9241$  were verified by the inability of the strains to grow in MOPS minimal medium without *p*-aminobenzoate. The presence of the  $\Delta asdA16$  mutation was confirmed by inability to grow in media without DAP and by PCR. The  $\Delta araBAD23$  mutation was verified by a white colony phenotype on MacConkey agar supplemented with 1% arabinose and by PCR. LPS profiles of *Salmonella* strains were examined by previously described methods (36).

### Isolation of lipid A

Strains were grown in LB or N media modified to 10 µM Mg<sup>2+</sup> and pH 5.8 (37). Typically, 200 mL cultures were inoculated from overnight cultures of like media to A<sub>600</sub> 0.02 and

grown to  $A_{600}$  0.8–0.9 for LB and 0.4–0.6 for N medium. The cells were pelleted by centrifugation at  $3,500 \times g$  for 20 min at  $4^{\circ}\text{C}$ , washed with phosphate-buffered saline (PBS), and pelleted again. The cell pellets were resuspended in 20 mL of PBS and transferred to chloroform-safe Nalgene bottles. Chloroform and methanol were added to a final ratio of 1:2:0.8 (chloroform:methanol:PBS, v/v), forming a single-phase Bligh/Dyer mixture (38). The cell suspensions were agitated by stirring for 1 h at room temperature, followed by centrifugation at  $2,500 \times g$  for 20 min. The supernatants containing phospholipids were discarded, and the pellets were resuspended by vortexing in 40 mL of a single-phase Bligh/Dyer mixture and transferred to a 50-mL Pyrex tube with a Teflon-lined cap. The tubes were centrifuged again and the supernatants discarded. The air-dried pellets were resuspended in 25 mL of 50 mM sodium acetate pH 4.5 by vortexing and probe sonication (39). After sonication, the pH was adjusted to 4.5 with acetic acid if necessary. The tubes were then placed in boiling water for 30 min to release the lipid A from the LPS. After cooling and vortexing, the solutions were transferred to chloroform-safe Nalgene bottles. Chloroform and methanol were added to a final ratio of 2:2:1.8 (chloroform:methanol:aqueous sodium acetate, v/v), forming a two-phase Bligh/Dyer mixture (38). The mixture was vigorously shaken to extract the lipid A, and the bottles were centrifuged at  $2,500 \times g$  for 20 min at  $20^{\circ}\text{C}$  to separate the two phases. The lower (chloroform) phase from each bottle was carefully removed to a round bottom flask and dried down on a rotary evaporator. The remaining upper phase was extracted again with fresh pre-equilibrated lower phase from a two-phase Bligh/Dyer mixture (2:2:1.8, chloroform:methanol:PBS, v/v). After centrifugation, the lower phase was added to the same flask and dried down again. The dried lipid A species were redissolved in chloroform:methanol (2:1, v/v) by vortexing and sonication, transferred to a glass tube, dried down, sealed with a Teflon-lined cap, and stored at  $-80^{\circ}\text{C}$ .

### Mass spectrometry (MS) of lipid A

Isolation of lipid A is described in SI text. Spectra were acquired on an ABI QSTAR XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Toronto, Canada), equipped with an electrospray ionization (ESI) source. Spectra were acquired in the negative-ion mode and typically were the accumulation of 60 scans collected from  $m/z$  200–2000. The dried lipid A species were prepared for MS by dissolving them in 1.5 mL of chloroform:methanol (2:1, v/v). An aliquot of 10–20  $\mu\text{L}$  was diluted 10-fold in the same solvent and supplemented with 2  $\mu\text{L}$  piperidine (final concentration 1%, v/v). Immediately after preparation, each sample was directly infused into the ion source at 5–6  $\mu\text{L}/\text{min}$ . The negative ion ESI was carried out at  $-4200$  V. Data acquisition and analysis were performed using the Analyst QS software (ABI/MDS-Sciex).

### LPS purification and LPS concentration determination

For tissue culture experiments, LPS was prepared from 20 ml of bacterial culture with Tri-reagent (Sigma) as described previously (40). The samples were repurified using the DOC-phenol method to remove trace protein (41). LPS preparations were quantitated using the Kdo method according to published procedures (42). Briefly, 3-deoxy-D-manno-octulosonic acid (Kdo) is released from LPS after 0.5 M  $\text{H}_2\text{SO}_4$  hydrolysis. Each LPS molecule contains 3 Kdo moieties. After determining the Kdo concentration, we then calculated the LPS concentration and then diluted the samples to the concentration appropriate for each experiment.

### Determination of virulence in mice

Seven week old, female BALB/c mice were obtained from the Charles River Laboratories (Wilmington, MA). All animal procedures were approved by the Arizona State University Animal Care and Use Committees. Mice were acclimated for 7 days after arrival before

starting the experiments. For determination of the 50% lethality dose (LD<sub>50</sub>), bacteria were grown statically overnight at 37°C in LB, diluted 1:50 into fresh LB media, and grown with aeration (180 rpm) at 37°C. When the cultures reached an OD<sub>600</sub> = 0.8–0.9, they were harvested by room temperature centrifugation at 4,000 rpm, washed once, and normalized to the required inoculum density in BSG by adjusting the suspension to the appropriate OD<sub>600</sub> value. Groups of five mice each were infected orally with 20 µL containing various doses of *S. Typhimurium*  $\chi$ 3761 or its derivatives, ranging from  $1 \times 10^3$  CFU to  $1 \times 10^9$  CFU. Oral infections were performed using a 20 µL pipette. Animals were observed for 4 weeks post infection, and deaths were recorded daily. Surviving mice in some groups were challenged with  $1 \times 10^9$  CFU of wild type strain  $\chi$ 3761. Animals were observed for 4 weeks post-infection and deaths were recorded daily.

To evaluate colonization, mice were orally inoculated with 20 µL of BSG containing  $1 \times 10^9$  CFU of each strain. At days 3 and 6 after inoculation, three animals per group were euthanized and Peyer's patches (PP), spleen and liver samples were collected. Each sample was homogenized in BSG at a final volume of 1 ml. Dilutions of  $10^{-1}$  to  $10^{-6}$  (depending on the tissue) were plated onto MacConkey and LB agar to determine the number of viable bacteria. Twenty colonies from each animal were randomly selected to confirm genotypic markers by PCR. Each experiment was performed twice.

### Cell line culture and LPS stimulation

The murine macrophage cell line RAW264.7 (ATCC, Rockville, MD) was maintained in DMEM (Invitrogen, San Diego, CA) supplemented with 10% FBS and 100 µg/mL each of gentamicin and penicillin. The human monocytic leukemia cell line Mono Mac 6 (MM6) (Lonza, Germany) was cultured in RPMI1640 containing sodium bicarbonate (2 g/L), insulin (10 µg/mL) and oxalacetic acid (1 mM), 100 µg/mL each of gentamicin and penicillin, glutamine (2 mM), nonessential amino acids for minimum essential medium (MEM; 1% v/v), sodium pyruvate (1 mM), folic acid (40 µg/mL) and 15% FBS. Cells were seeded in 96 well microtiter plates ( $2.5 \times 10^5$ /well) in 150 µL of the above medium and cultured at 37°C with 5% CO<sub>2</sub>. After 6 h, various dilutions of LPS preparations were added in 16 µL ( $10 \times$  dilution). 24 h later, culture supernatants were collected, centrifuged to remove contaminating cells and stored at -80°C until determination of cytokine content. All experiments were performed at least twice.

### Cytokine assay

Cytokine concentrations (human IL6, mouse TNF- $\alpha$  or mouse multiple cytokines) were determined using the Bio-Plex Protein Array System (Bio-Rad) according to the manufacturer's recommendations. Serum samples were diluted with specific serum dilution buffer (Bio-Rad). Peritoneal washes were centrifuged at 4,000 rpm for 10 min and the supernatants were assayed for cytokines. Samples were incubated with antibody-coupled beads for 30 min with continuous shaking. The beads were washed 3 times with 100 µL wash buffer to remove unbound protein and then incubated with biotinylated detection cytokine-specific antibody for 30 min with continuous shaking. The beads were washed three times and incubated with streptavidin-phycoerythrin for 10 min after incubation. The beads were washed three times in washing buffer, resuspended in 125 µL assay buffer, and the constituents of each well of microtiter plate were drawn up into the flow-based Bio-Plex suspension Array System. Cytokine concentrations were automatically calculated by Bio-Plex Manager software by using a standard curve derived from a recombinant cytokine standard, two readings were made on each bead set.



### Rabbit ileal loop experiment

New Zealand White rabbits were fasted overnight and then anesthetized with isoflurane through an endotracheal tube, and the ileum was exposed and then ligated into several loops 3 to 5 cm long using 1-cm spacers. *S. Typhimurium* strains were injected into separate loops in a volume of 1 ml at a titer of  $1 \times 10^9$  CFU. LB broth was injected into one of the loops as a control. The abdominal musculature was closed using 3-0 chromic gut sutures, and the skin was closed with 3-0 ethilon sutures. Rabbits were maintained in a thermal blanket at 37°C. After 8 h, each rabbit was euthanized with an overdose of sodium pentobarbital. The abdomen was reopened, and the fluid within the ligated loops was collected, the volume measured, and the bacterial content enumerated. The loops were fixed in 10% formalin and subjected to H&E staining histopathological examination.

### Antigen preparation

rPspA protein was purified as described (43). *S. Typhimurium* LPS was obtained from Sigma. The rPspA clone was kind gift from Dr. Susan Hollingshead at the University of Alabama at Birmingham.

### SDS-PAGE and western blot analyses

Protein samples were boiled for 5 min in loading buffer and separated by SDS-PAGE. For western blotting, proteins separated by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 3% skim milk in 10 mM Tris-0.9% NaCl (pH 7.4) and incubated with rabbit polyclonal antibody specific for PspA (44) or anti-GroEL (Sigma, St. Louis, MO) antibodies. The secondary antibody was an AP-conjugated goat anti-rabbit immunoglobulinG (IgG) (Sigma). Immunoreactive bands were detected by the addition of BCIP/NBT solution (Sigma). The reaction was stopped after 2 min by washing with large volumes of deionized water several times.

### Immunogenicity of vaccine strains in mice

RASV strains were grown statically overnight in LB broth with 0.1% arabinose at 37°C. 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  $OD_{600}$  of 0.8 to 0.9. Cells were harvested by room temperature centrifugation at 4,000 rpm for 15 min and the pellet resuspended in 1 mL of BSG. Mice ( $n = 13$  per group) were orally inoculated with 20  $\mu$ l of BSG containing  $1 \times 10^9$  CFU of each strain on day 0 and boosted at 5 weeks with the same dose of the same strain. Blood was obtained by mandibular vein puncture at biweekly intervals. Following centrifugation, the serum was removed from the whole blood and stored at  $-20^\circ\text{C}$ .

### Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to assay serum antibodies against *S. Typhimurium* LPS, rPspA and whole cell bacterial suspensions ( $1 \times 10^9$  CFU/ml) as previously described (45). Color development (absorbance) was recorded at 405 nm using an automated ELISA plate (model SpectraMax M2e; Molecular Devices, CA). Absorbance readings 0.1 higher than PBS control values were considered positive reactions.

### Pneumococcal challenge

We assessed the protective efficacy of the attenuated *Salmonella* expressing *pspA* at week 8 by intraperitoneal challenge of immunized mice with  $4 \times 10^4$  CFU of *S. pneumoniae* WU2 (46) in 200  $\mu$ l of BSG (47). The  $LD_{50}$  of *S. pneumoniae* WU2 in BALB/c mice ( $n = 13$  per group) was  $2 \times 10^2$  CFU by intraperitoneal administration (data not shown). Challenged mice were monitored daily for 30 days.

## Statistical analysis

Numerical data were expressed as means  $\pm$  SEM. A two way ANOVA analysis followed by Bonferroni's multiple comparison test was used to evaluate differences in antibody titer data. One way ANOVA analysis followed by Dunnett's multiple comparison test was used to evaluate cytokine level and colonization for multiple comparisons among groups. The median lethal dose (LD<sub>50</sub>) was estimated using a probit analysis based on the XLSTAT. The Kaplan-Meier method was used for survival, and differences were analyzed by the log-rank sum test. All analyses were performed using GraphPad PRISM 5.0.  $P < 0.05$  was considered statistically significant.

## Results

### *Francisella* LpxE can efficiently remove the 1-phosphate group from *Salmonella* lipid A

To evaluate the effect of chromosomally-expressed *lpxE* in *Salmonella*, we inserted *F. tularensis lpxE* under constitutive transcriptional control of the strong *E. coli* promoter P<sub>Ipp</sub> into several different positions of the *Salmonella* chromosome. Lipid A was isolated from each strain after mild acid hydrolysis at pH 4.5 to cleave the Kdo-lipid A linkage (48) and subjected to electrospray ionization mass spectrometry (ESI-MS). Consistent with previous reports, the lipid A of the wild-type strain contains predominantly hexa-acylated lipid A (Fig. 1A and supplemental Fig. 1A, [M-2H]<sup>2-</sup> species near  $m/z$  897.6). Our initial results indicated that the amount of LpxE synthesized in each strain was not adequate to 1-dephosphorylate more than 50% of the lipid A in the cell (Fig. 1B and Supplemental Fig. 1A, [M-2H]<sup>2-</sup> species with  $m/z$  near 857.6). To reduce the levels of inflammatory lipid A further and increase the levels of 1-dephospho-lipid A, the *lpxE* sequence was codon-optimized for high-level expression in *Salmonella*. Three mutant strains expressing codon-optimized P<sub>Ipp</sub> *lpxE* were constructed and designated  $\chi$ 9440 ( $\Delta$ pagL71::P<sub>Ipp</sub> *lpxE*),  $\chi$ 9732 ( $\Delta$ pagP81::P<sub>Ipp</sub> *lpxE*) and  $\chi$ 11092 ( $\Delta$ lpxR91::P<sub>Ipp</sub> *lpxE*). ESI-MS of the lipid A for the mutant strains demonstrated that codon-optimization led to more efficient lipid A 1-dephosphorylation than in isogenic strains expressing the native *lpxE* sequence (Supplemental Fig. 1B). The level of P<sub>Ipp</sub> *lpxE* expression was dependent on the chromosomal location, because only strain  $\chi$ 9732 ( $\Delta$ pagP81::P<sub>Ipp</sub> *lpxE*) produced lipid A that was essentially completely 1-dephosphorylated (Fig. 1B and Supplemental Fig. 1B, [M-2H]<sup>2-</sup> species near  $m/z$  857.6). Based on these results, the *pagP81::P<sub>Ipp</sub> lpxE* cassette was chosen for further evaluation.

The structure of *Salmonella* lipid A is nearly identical to that of *E. coli* (9, 10). However, *Salmonella* lipid A can be covalently modified in response to environmental conditions. For example, the 4'-phosphate group can be covalently modified with a 4-amino-4-deoxy-L-arabinose (L-Ara4N) residue, and the 1'-phosphate can be modified with a phosphoethanolamine moiety in response to specific environmental signals (9, 10). In addition, the fatty acyl chains can be modified by hydroxylation of the 3' secondary myristate chain by LpxO, the addition of a secondary palmitate chain at the 2-position by PagP, or the removal of the 3 or 3' acyl chain(s) by PagL or LpxR, respectively (Fig. 1A) (10, 49). To evaluate the effect of deleting *pagL*, *pagP* and *lpxR* genes *in vitro* and in the presence or absence of *lpxE*, we constructed the following mutant strains:  $\chi$ 9434 ( $\Delta$ pagP8) and  $\chi$ 9732 ( $\Delta$ pagP81::P<sub>Ipp</sub> *lpxE*), and triple mutant strains:  $\chi$ 9485 ( $\Delta$ pagL7  $\Delta$ pagP8  $\Delta$ lpxR9) and  $\chi$ 9705 ( $\Delta$ pagL7  $\Delta$ pagP81::P<sub>Ipp</sub> *lpxE*  $\Delta$ lpxR9). As expected, the  $\Delta$ pagP strains  $\chi$ 9434 ( $\Delta$ pagP8) and  $\chi$ 9485 ( $\Delta$ pagL7  $\Delta$ pagP8  $\Delta$ lpxR9) lack the palmitate-containing peak seen in the wild-type strain  $\chi$ 3761 (Fig. 1C, [M-H]<sup>-</sup> species near  $m/z$  1016.66, and data not shown). Because LpxR and PagL are latent in normal laboratory growth conditions, no other differences were seen for  $\chi$ 9485 ( $\Delta$ pagL7  $\Delta$ pagP8  $\Delta$ lpxR9) compared to  $\chi$ 3761. In  $\chi$ 9732 ( $\Delta$ pagP81::P<sub>Ipp</sub> *lpxE*), the four major peaks detected are consistent with MPL ( $m/z$  857.6),

LpxO-modified MPL ( $m/z$  865.5) and their acetate adducts ( $m/z$  887.6 and 895.6, respectively) (Fig. 1C). The lipid A structures in strains  $\chi$ 9485 ( $\Delta pagL7 \Delta pagP8 \Delta lpxR9$ ) and  $\chi$ 9705 ( $\Delta pagL7 \Delta pagP81::P_{1pp} lpxE \Delta lpxR9$ ) are similar to those in strains  $\chi$ 9434 ( $\Delta pagP8$ ) and  $\chi$ 9732 ( $\Delta pagP81::P_{1pp} lpxE$ ), respectively. The small MPL peak ( $m/z$  857.6) seen in strains  $\chi$ 3761 (wild-type),  $\chi$ 9434 ( $\Delta pagP8$ ), and  $\chi$ 9485 ( $\Delta pagL7 \Delta pagP8 \Delta lpxR9$ ) is due to minor chemical 1-dephosphorylation as a result of the mild acid hydrolysis step employed to liberate lipid A from LPS by cleavage of the Kdo-lipid A linkage.

The *phoPQ* and *pmrAB* two-component systems play a key role in controlling the remodeling of lipid A (10). The *phoPQ* regulon (which includes *pmrAB*) is turned on during invasion of host tissues in response to conditions that can include low pH and/or a low concentration of  $Mg^{2+}$  (10) or sub-lethal concentrations of cationic peptides (50, 51). Among other modifications induced, the addition of phosphoethanolamine (pEtN) to the 1-phosphate by EptA is upregulated (10). It is possible that these PhoPQ-regulated modifications may interfere with the action of LpxE.

To evaluate whether LpxE can efficiently remove the 1-phosphate group from lipid A when the *phoPQ* regulon is induced, strains were grown in media at low pH and low  $Mg^{2+}$  concentration, conditions that upregulate *phoPQ* (52), and the lipid A analyzed. ESI-MS data for  $\chi$ 9732 ( $\Delta pagP81::P_{1pp} lpxE$ ) showed that LpxE can efficiently remove the 1-phosphate from lipid A when the *phoPQ* regulon is activated (Fig. 1D). In addition to the major peaks seen in cells grown on LB medium, the peaks observed in the low pH, low  $Mg^{2+}$  medium are consistent with 1-dephospho-lipid A, modified by 4-amino-4-deoxy-L-arabinose (L-Ara4N, Fig. 1D). Lipid A modification by ArnT (adding L-Ara4N to the 4' phosphate) and by EptA (adding phosphoethanolamine to the 1-phosphate) each take place on the periplasmic surface of the inner membrane, the same location as 1-dephosphorylation by LpxE (10). The prevalence of L-Ara4N-modified 1-dephospho-lipid A species suggests that either LpxE acts prior to lipid A modification by ArnT or efficiently utilizes modified lipid A as a substrate. More importantly, despite the upregulation of EptA, LpxE clearly acts prior to addition of pEtN to the 1-position, suggesting LpxE will function under the range of conditions experienced in the host.

### ***Salmonella* with 1-dephosphorylated lipid A is attenuated without loss of colonization efficiency**

We next determined the role of the individual mutations in mouse virulence (Table 2). The oral 50% lethal dose ( $LD_{50}$ ) of wild-type strain,  $\chi$ 3761,  $1.0 \times 10^4$  CFU, was similar to that previously observed (53). The  $\Delta pagP8$  mutant strain  $\chi$ 9434 ( $\Delta pagP8$ ) had the same oral  $LD_{50}$  as  $\chi$ 3761 (wild-type), extending the previous observation that a *pagP* mutant is unaltered for virulence in mice when introduced by the intraperitoneal route (54). The  $LD_{50}$  of  $\chi$ 9485 ( $\Delta pagL7 \Delta pagP8 \Delta lpxR9$ ) was increased 10-fold compared to the wild-type strain. However, the  $LD_{50}$  of  $\chi$ 9732 ( $\Delta pagP81::P_{1pp} lpxE$ ) was approximately  $10^5$ -fold greater than  $\chi$ 3761 (wild-type), although at the highest doses we observed mild to severe clinical manifestations of disease (scruffy coat, lethargy) from which some mice recovered (Table 2). Strain  $\chi$ 9705 ( $\Delta pagL7 \Delta pagP81::P_{1pp} lpxE \Delta lpxR9$ ) was completely avirulent, and no disease symptoms were observed even at the highest dose, indicating an  $LD_{50}$  value at least  $10^5$ -fold greater than the wild type.

While expression of *lpxE* in strains  $\chi$ 9732 ( $\Delta pagP81::P_{1pp} lpxE$ ) and  $\chi$ 9705 ( $\Delta pagL7 \Delta pagP81::P_{1pp} lpxE \Delta lpxR9$ ) was attenuating, this phenotype was not due to a general growth defect, as each mutant strain had growth characteristics nearly identical to wild-type strain  $\chi$ 3761 when grown in LB medium (data not shown). Strains  $\chi$ 9732 ( $\Delta pagP81::P_{1pp} lpxE$ ) and  $\chi$ 9705 ( $\Delta pagL7 \Delta pagP81::P_{1pp} lpxE \Delta lpxR9$ ) exhibited an LPS profile in silver-stained SDS-PAGE gels similar to  $\chi$ 3761 (Supplemental Fig. 2A). In addition,  $\chi$ 9732



( $\Delta pagP81::P_{Ipp} lpxE$ ) and  $\chi 9705$  ( $\Delta pagL7 \Delta pagP81::P_{Ipp} lpxE \Delta lpxR9$ ) were 2-fold more sensitive to the bile salt deoxycholate and SDS than other strains (data not shown), which may affect their survival in the intestinal tract, but the strains remained resistant to ox bile (data not shown).

Because the ability to colonize lymphoid tissues (e.g. Peyer's patches, spleen, liver) is an important attribute for live attenuated *Salmonella* vaccines (47, 55–57), we evaluated the effect of these mutations on tissue colonization. After oral dosing, each mutant strain was able to colonize mouse lymphoid tissues, although there were significant differences among some of the strains (Fig. 2). Notably, strain  $\chi 9434$  ( $\Delta pagP8$ ) achieved higher titers than the other mutants in the spleen and liver at 3 days post-infection. The titer of strains  $\chi 9732$  ( $\Delta pagP81::P_{Ipp} lpxE$ ) and  $\chi 9705$  ( $\Delta pagL7 \Delta pagP81::P_{Ipp} lpxE \Delta lpxR9$ ) in spleen and liver did not increase at 6 days post infection, resulting in significantly lower numbers than strains  $\chi 9434$  ( $\Delta pagP8$ ) and  $\chi 9485$  ( $\Delta pagL7 \Delta pagP8 \Delta lpxR9$ ).

Mice that survived inoculation with  $1 \times 10^6$  to  $1 \times 10^9$  CFU of the *lpxE*-expressing strains  $\chi 9732$  ( $\Delta pagP81::P_{Ipp} lpxE$ ) and  $\chi 9705$  ( $\Delta pagL7 \Delta pagP81::P_{Ipp} lpxE \Delta lpxR9$ ) were orally challenged with  $1 \times 10^9$  CFU ( $1 \times 10^5$  LD<sub>50</sub>s) of the wild-type strain  $\chi 3761$  thirty days after inoculation with the attenuated strains. All immunized mice survived challenge (Table 2). Thus the presence of LpxE attenuates the strains without compromising their ability to transiently colonize lymphoid tissues or their immunogenicity.

To evaluate the stability of the *P<sub>Ipp</sub> lpxE* gene *in vivo*, mice were orally inoculated with approximately  $1 \times 10^9$  CFU of either  $\chi 9705$  ( $\Delta pagL7 \Delta pagP81::P_{Ipp} lpxE \Delta lpxR9$ ) or  $\chi 9732$  ( $\Delta pagP81::P_{Ipp} lpxE$ ). After three days, spleens were harvested from the mice and colonizing *Salmonella* isolated. Spleen isolates were grown in LB and used to inoculate more mice. Our results indicated that the *P<sub>Ipp</sub> lpxE* gene was stable in these strains after three mouse passages as determined by PCR analysis of 38 colonies followed by DNA sequence analysis of three PCR-positive colonies isolated after the third passage (data not shown).

### ***Salmonella* synthesizing 1-monophosphorylated LPS exhibits low endotoxic activity *in vivo* and *in vitro***

We determined the effect of modified lipid A on cytokine stimulation in tissue culture (Fig. 3A, 3B) and in animals (Fig. 3C). In tissue culture, 0.1 pmol/ml of LPS purified from strains expressing *lpxE* ( $\chi 9732$  and  $\chi 9705$ ) induced significantly lower levels of the pro-inflammatory cytokines IL-6 in the human cell line MM6 (Fig. 3A) and TNF- $\alpha$  in the mouse macrophage cell line RAW264.7 (Fig. 3B) than other strains. However, there were no differences in induction of either cytokine when cells were stimulated with 10 pmol/ml (data not shown). When sera from immunized mice were analyzed for cytokine production, we found that immunization with  $\chi 9705$  ( $\Delta pagL7 \Delta pagP81::P_{Ipp} lpxE \Delta lpxR9$ ) induced lower levels of all cytokines tested except IL-17 (Fig. 3C).

These mutant strains were also evaluated in rabbit ileal loops. Cells were introduced into ligated loops and incubated for 8 hours. Histological samples from loops infected with the wild-type strain  $\chi 3761$  or mutant strains were stained with hematoxylin and eosin and examined microscopically (Fig. 4). The wild-type strain induced severe destruction of the mucosa including large areas of the necrotic epithelia, flattened villi, and heavy infiltration by polymorphonuclear leukocytes (PMN) (Fig. 4A and data not shown). In contrast, mutant strains  $\chi 9434$  ( $\Delta pagP8$ ) and  $\chi 9485$  ( $\Delta pagL7 \Delta pagP8 \Delta lpxR9$ ) induced some tissue destruction, although it was much milder than the wild-type strain (Fig. 4B and C). Loops injected with mutant strains  $\chi 9732$  ( $\Delta pagP81::P_{Ipp} lpxE$ ) and  $\chi 9705$  ( $\Delta pagL7 \Delta pagP81::P_{Ipp} lpxE \Delta lpxR9$ ) expressing *lpxE* were similar to the LB control loops, with no tissue

destruction or PMN infiltration apparent (Fig 4D, 4E). These data are consistent with the mouse cytokine data which indicated that the 1-monophosphorylated LPS in *Salmonella* leads to a reduction in inflammatory responses after infection.

### ***Salmonella* strains synthesizing monophosphoryl lipid A retain their ability to deliver a heterologous antigen**

*S. Typhimurium* strain  $\chi 9241$  ( $\Delta pabA1516 \Delta pabB232 \Delta asdA16 \Delta araBAD23 \Delta relA198::araC P_{BAD} lacI$  TT) is an attenuated vaccine strain that has been successfully employed to deliver the pneumococcal surface protein PspA and induce protective immunity against *S. pneumoniae* challenge (Table 1) (44, 53). Strain  $\chi 9241$  carries the  $\Delta relA198::araC P_{BAD} lacI$  TT deletion/insertion which provides arabinose-regulated delayed antigen synthesis (44). To evaluate the effect of lipid A modification on the efficacy of this strain, the single or triple mutations were introduced into  $\chi 9241$  to yield strains  $\chi 9844$  ( $\Delta pagP8$ ),  $\chi 9845$  ( $\Delta pagP81::P_{Ipp} lpxE$ ),  $\chi 9846$  ( $\Delta pagL7 \Delta pagP8 \Delta lpxR9$ ) and  $\chi 9881$  ( $\Delta pagL7 \Delta pagP81::P_{Ipp} lpxE \Delta lpxR9$ ) (Table 1), and the expected lipid A modifications were confirmed by ESI-MS (data not shown). Subsequently, the Asd<sup>+</sup> recombinant plasmid pYA3493 (vector control) or pYA4088, which encodes a recombinant *pspA* gene fused to DNA encoding the  $\beta$ -lactamase signal sequence (44), was introduced into each strain. In plasmid pYA4088, *pspA* expression is driven by the P<sub>trc</sub> promoter, and the *bla* signal sequence directs periplasmic secretion of PspA (Supplemental Fig. 2B).

Groups of mice were orally inoculated with  $1-2 \times 10^9$  CFU of each strain and boosted 5 weeks later with the same dose of the same strain. Consistent with the attenuation of  $\chi 9241$ , there were no significant adverse health effects on the inoculated mice. Blood was taken at various times, and serum IgG titers against PspA were measured (Fig. 5A). Serum IgG titers against PspA and *S. Typhimurium* LPS were below the limits of detection (<50) prior to immunization. Each strain synthesizing PspA triggered the production of significant levels of anti-PspA antibodies compared to the vector control strain  $\chi 9241$ (pYA3493) ( $P < 0.001$ ). Importantly, the triple mutant strain expressing *lpxE*,  $\chi 9881$ (pYA4088), induced anti-PspA titers comparable to the parent strain  $\chi 9241$ (pYA4088). All the strains triggered the production of serum IgG antibodies against *Salmonella* LPS (Fig. 5B). The anti-LPS responses were similar for all strains except  $\chi 9844$ (pYA4088) ( $\Delta pagP8$ ) and  $\chi 9881$ (pYA4088) ( $\Delta pagL7 \Delta pagP81::P_{Ipp} lpxE \Delta lpxR9$ ), which induced significantly lower titers than the others at week 4 (Fig. 5B). By week 8, the anti-LPS IgG responses in all immunized mice were similar. Analysis of the ratios of IgG2a/IgG1 titers against PspA (Fig 5C) indicated that all strains induced a Th1 response typical of *Salmonella* (58). All strains expressing *pspA* elicited anti-PspA mucosal IgA responses significantly greater than the negative control strain  $\chi 9241$ (pYA3493) (Fig. 5D).

### **RASV synthesizing MPL and PspA induce protective immunity against *Streptococcus pneumoniae* challenge**

To evaluate whether immunization elicited protective immunity, 9 weeks after the primary immunization, mice were challenged intraperitoneally with  $\sim 4 \times 10^4$  CFU of virulent *S. pneumoniae* strain WU2 ( $200 \times LD_{50}$ ). Some mice in all groups immunized with strains expressing *pspA* survived challenge (Fig. 5E) while no mice inoculated with the control strain  $\chi 9241$ (pYA3493) survived challenge ( $P < 0.01$ ). There was no statistical difference in the level of protection elicited by any of the strains expressing *pspA*.

## **Discussion**

The potential usefulness of *Salmonella* as an antigen delivery vector is predicated on its ability to invade and transiently persist in host tissues, thereby stimulating robust mucosal,

cellular and humoral immune responses. A virtual plethora of attenuating mutations and strategies have been described and shown to be effective at immunizing animals, particularly mice in the case of *S. Typhimurium* (1, 3). However, results in human trials using *S. Typhi* or *S. Typhimurium* as vectors have yet to demonstrate similar results, with vaccine strains being reactogenic and/or overattenuated, eliciting poor immune responses against vectored antigens. Rendering lipid A non-toxic may provide a means to safely increase the dose and/or reduce the level of attenuation to facilitate induction of a robust response to the vectored antigen. This may also provide an adequate level of safety to allow the use of live attenuated bacterial vaccines in infants and children, a target group that can benefit the most from this technology and for strains exhibiting regulated delayed lysis (8).

*Salmonella* lipid A is a mixture of closely related species that contain between 5–7 fatty acid moieties, and it may also be modified with other polar substituents (Fig. 1A). About 15% of *Salmonella* lipid A is hepta-acylated, while the most abundant species is hexa-acylated, as in *E. coli* (59). Dephosphorylation of the 1-phosphate group of lipid A is sufficient to reduce or eliminate its toxic effects (15, 60). The MPL used clinically consists primarily of 3-O-deacylated-1'-monophosphoryl lipid A (3D-MPL), which is obtained by sequential mild acid and mild base hydrolysis of *Salmonella enterica* serovar Minnesota R595 LPS (61). It is a mixture of penta- and hexa-acylated species. The hexa-acylated component of 3D-MPL lacks the hydroxymyristic acid moiety at position 3 and possesses a secondary palmitate chain on the 2 position *R*-3-hydroxymyristate chain (Fig. 1A). Initially, we attempted to generate 3D-MPL in *S. Typhimurium* by the combined over-expression of *pagL*, *pagP* and *lpxE*. However, we were unable to achieve PagP- and PagL-mediated lipid A modification above 50% of the total lipid A content under our standard laboratory growth conditions (data not shown). We then opted to eliminate *pagL* and *pagP* to engineer *Salmonella* synthesizing the 1'-monophosphoryl-hexa-acylated lipid A variant shown in Fig. 1B, which is also an excellent adjuvant.

In this report, we demonstrated that insertion of *lpxE* into the *Salmonella* chromosome resulted in dephosphorylation of the lipid A, although LpxE activity was dependent on the position of *lpxE* in the chromosome. These results are consistent with the observations of Beckwith et al. described over forty years ago in *E. coli* (62) and subsequently confirmed for *Salmonella* (63) demonstrating that the precise location in the bacterial chromosome can affect the level of gene transcription. Expression of codon-optimized *lpxE* inserted into chromosome at *pagP* resulted in complete removal of the lipid A 1'-phosphate group in living *S. Typhimurium* cells (Fig. 1) under a variety of growth conditions (Supplemental Fig 1B).

Expression of *lpxE* was attenuating for virulence (Table 2). This virulence reduction appears to be directly related to the production of detoxified lipid A, as a  $\Delta pagP$  mutant was not attenuated. Evidence supporting this correlation comes from the finding that the mutants expressing *lpxE* induced lower levels of cytokine secretion compared with the parent strain in *in vitro* and *in vivo* models (Fig. 3, 4). Inoculation of mice with strain  $\chi 9705$ , in which 1'-dephosphorylation was essentially complete, resulted in lower induction levels of a number of pro-inflammatory cytokines including TNF- $\alpha$  and IL-6 (Fig. 3), while retaining overall immunogenicity (Table 2). It is difficult to compare these results directly to what occurs when humans are vaccinated with a live *Salmonella* vaccine for two reasons. First, since *S. Typhi* does not infect mice, it is not possible to generate meaningful cytokine data with *S. Typhi* vaccines in mice. Second, cytokine measurements in circulating human blood are not typically reported in trials with attenuated *S. Typhi* vaccines. However, in one study, peripheral blood monocytes were isolated from persons vaccinated with one of several *S. Typhi* vaccine strains and the levels of cytokines produced in response to *S. Typhi* flagella and LPS were measured (64). In typhoid patients, infected with *S. Typhi*, TNF- $\alpha$  levels

ranging from 2609 to 6338 pg/ml and IL-6 levels ranging from 2416 to 7713 pg/ml of serum in the acute and convalescent phases, respectively, have been reported (65). These levels are substantially higher than those we observed in mice with any of our *S. Typhimurium* strains including the wild-type strain  $\chi$ 3761 (Fig. 3).

Although the cytokine levels in the tissue culture assays were higher than expected, the IL-6 levels we obtained for  $\chi$ 9705 LPS stimulation of MM6 cells was similar to those reported previously for MM6 cells exposed to MPL (66). In another study using MM6 cells, there was about a 3-fold difference in TNF- $\alpha$  production by wild-type lipid A and MPL (67). The lipid A preparation from parent strain  $\chi$ 3761 elicited nearly two-fold greater levels of TNF- $\alpha$  than the monophosphoryl lipid A from  $\chi$ 9705 (Fig. 3). The difference in results may be due to effects caused by other structural differences between MPL and the lipid A from  $\chi$ 9705.

Interestingly, triple mutant strain  $\chi$ 9705 was slightly more attenuated than  $\chi$ 9732 ( $\Delta$ *pagP81::P<sub>1pp</sub> lpxE*), indicating that deletion of either *pagL* and/or *lpxR*, in the presence of  $\Delta$ *pagP81*, reduced virulence. We observed a similar correlation when comparing strains  $\chi$ 9434 ( $\Delta$ *pagP8*) and  $\chi$ 9485 ( $\Delta$ *pagL7*  $\Delta$ *pagP8*  $\Delta$ *lpxR9*) that did not express *lpxE* (Table 2). While it is not clear whether deletion of *pagL* or *lpxR*, or both is required for this small decrease in virulence, we speculate that the *pagL* deletion is responsible. The actions of PagL and PagP serve to decrease the interaction of lipid A with TLR4, which has been suggested to enhance the ability of *Salmonella* to adapt to the host environment (68). Importantly, 1-dephosphorylated lipid A did not alter the immunogenicity of attenuated *S. Typhimurium* strain  $\chi$ 9241 expressing the pneumococcal antigen gene *pspA* (Fig. 5) indicating that this modification may be used in conjunction with other attenuating mutations to reduce reactogenicity and enhance the safety profile of the vaccine.

Our finding that the production of monophosphoryl lipid A that induces a limited immune response in the host, reduced the virulence of *S. Typhimurium* is in contrast to what happens in *Yersinia pestis*, in which the production of strongly stimulatory LPS results in a significant reduction in virulence (69, 70). The different outcomes are due to the different pathogenesis mechanisms utilized by the two pathogens. *Y. pestis* employs a weakly stimulatory LPS synthesized at 37 °C to escape host detection and killing by local inflammatory responses when it infects the mammalian hosts via the bite of infected fleas. For *Salmonella*, induction of a local inflammatory response early in infection seems to be part of its survival strategy, providing one mechanism (among many) to facilitate its invasion of the gut mucosa (71). It is clear from research done with *Salmonella msbB* mutants, which produce penta-acylated lipid A, have a reduced affinity to TLR4 resulting in attenuation (72, 73).

Our results demonstrate that production of 1'-monophosphoryl lipid A by an attenuated *Salmonella* strain does not diminish its ability to elicit an immune response against its own LPS (Fig. 5B), or a heterologous antigen, PspA (Fig. 5A). Attenuated *Salmonella* strain  $\chi$ 9241(pYA4088) and its derivative,  $\chi$ 9881(pYA4088) that produces 1'-monophosphoryl lipid A, both induced a strong Th1 response, as determined by the high ratio of IgG2 to IgG1 (Fig. 5C). This is consistent with observations by our group and others, that *Salmonella* typically induces a Th1-biased response (43, 53, 74), although a more balanced Th1/Th2 response can be obtained by altering the *Salmonella* genotype or immunization strategy (43, 45).

Although strain  $\chi$ 9705 ( $\Delta$ *pagL7*  $\Delta$ *pagP81::P<sub>1pp</sub> lpxE*  $\Delta$ *lpxR9*) produces nearly all of its lipid A lacking the 1' phosphate group (Fig. 1) and is completely attenuated for virulence, production of monophosphoryl lipid A is not an acceptable attenuation strategy, as a single point mutation inactivating *lpxE* would render the strain as virulent as the wild-type parent

(compare strains  $\chi$ 9485 and  $\chi$ 9706 in Table 2). We have based our approach on the fact that commercial monophosphoryl lipid A (MPL) has been proven safe and immunogenic for use as an adjuvant in humans. Thus, *lpxE* expression may enhance the safety of a *Salmonella* vaccine strain attenuated by other means, for example, when using the regulated delayed lysis in vivo attenuation to deliver a bolus of protective antigen and confer complete biological containment (8, 75). We also want to prevent unwanted side effects that can be caused by lipid A, such as the mild diarrhea or fevers experienced by some vaccinees that have been observed in clinical studies (5, 76–79) using attenuated *S. Typhi* vaccines. This type of modification, though helpful and necessary, is likely to be incremental, particularly when evaluated in a mouse model, where these types of side reactions are typically not seen with attenuated *Salmonella* strains.

There have been a number of recent improvements in the design of *S. Typhi* strains for antigen delivery, including the use of promoters that allow regulated antigen expression (80, 81), refinement of attenuating mutations (3, 82) and regulated delayed attenuation (83). Now, in addition, lipid A detoxification by *lpxE* provides a new and useful tool for improving live *Salmonella* vaccines to prevent typhoid and paratyphoid fever (84) and for antigen delivery, thus improving the safety of the vaccine without compromising immunogenicity. This may permit the use strains that are more aggressive for colonizing mucosal tissues and thereby enhancing immune responses without compromising safety.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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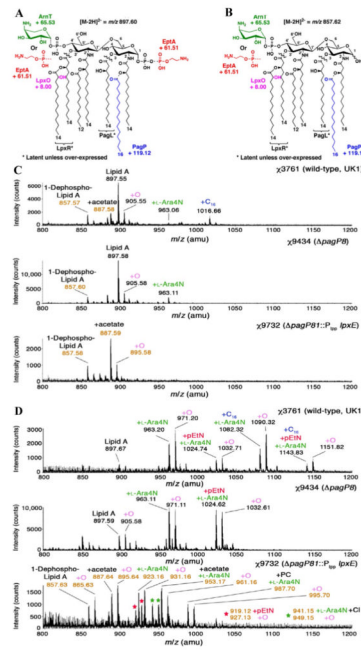
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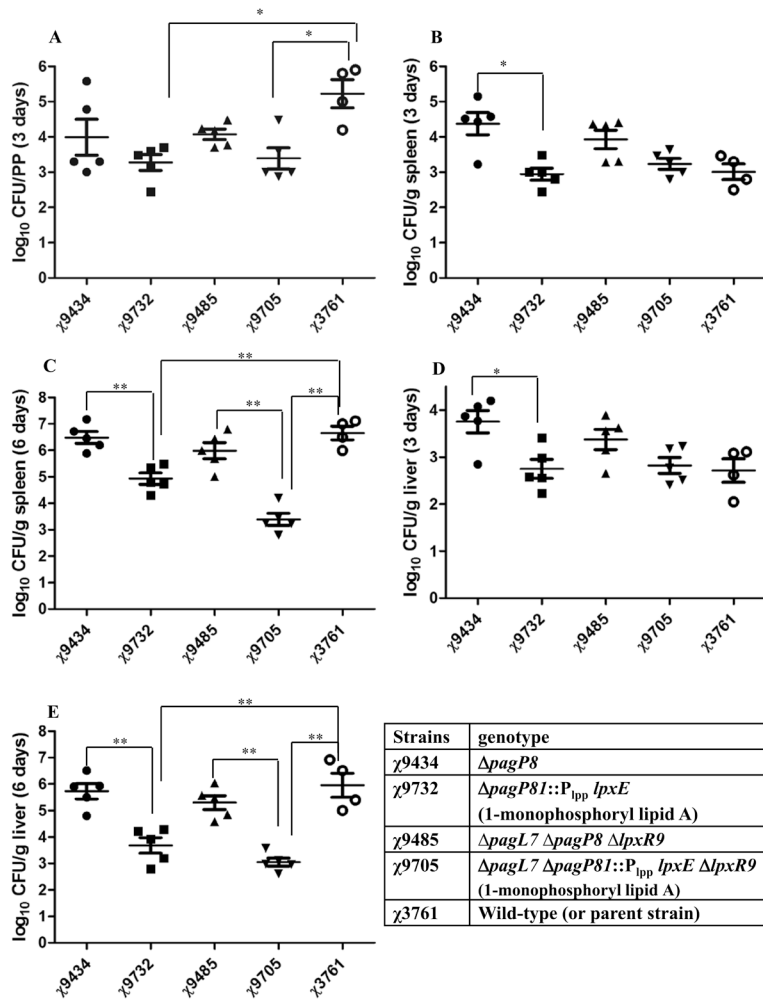
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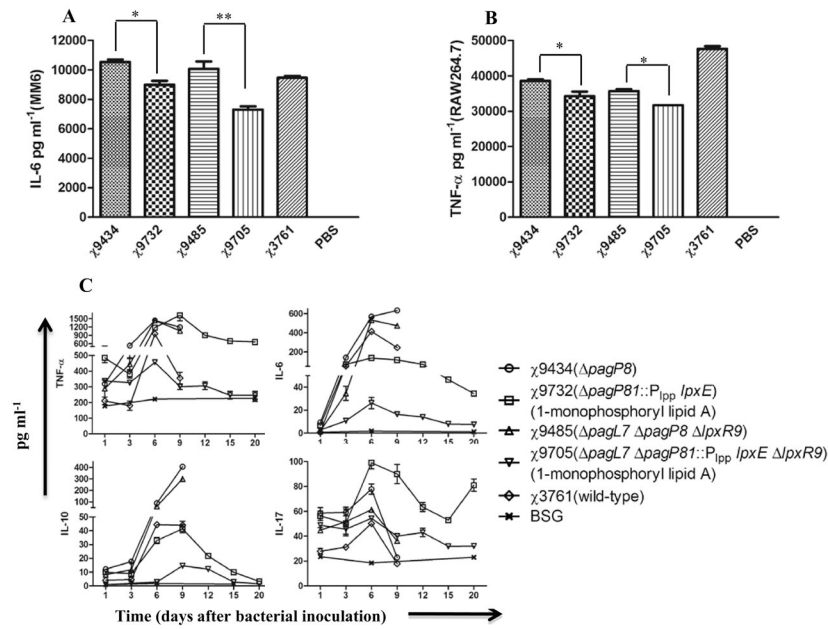
**Figure 1.**

Lipid A structure of wild-type and *lpxE*-expressing *Salmonella*. **(A)** Covalent modifications of *Salmonella* lipid A. LpxR and PagL catalyze the removal of the 3'-acyloxyacyl and the 3-hydroxymyristoyl chains from lipid A, respectively, although these modifications are not seen in laboratory growth conditions. The lipid A species can be identified by ESI-MS in the negative ion mode, with unmodified lipid A as the  $[M-2H]^{2-}$  peak at  $m/z$  897.60. Various modifications shift the peak by the indicated  $m/z$  values. The addition of palmitate (+C<sub>16</sub>) to the 2 position *R*-3-hydroxymyristoyl chain catalyzed by PagP is indicated in blue. Other covalent lipid A modifications include the hydroxylation of the 3' secondary myristoyl chain by LpxO (+O, pink) the addition of L-Ara4N to the 4' phosphate by ArnT (+L-Ara4N, green), and the addition of pEtN to the 1 phosphate by EptA (+pEtN, red). **(B)** structure of 1-dephosphorylated lipid A. Heterologous expression of the *F. tularensis lpxE* leads to nearly quantitative 1-dephosphorylation of all *Salmonella* lipid A species, specifically shifting the lipid A  $[M-2H]^{2-}$  peak by  $m/z$  -39.98. **(C)** Lipid A profiles from ESI-MS analysis of χ3761(wild-type UK-1), χ9434(Δ*pagP8*), and χ9732(Δ*pagP81*::P<sub>1pp</sub> *lpxE*), grown in LB. Lipid A peaks are indicated with black  $m/z$  values, while 1-dephospho-lipid A peaks are indicated with orange  $m/z$  values. In each case, peaks labeled with +O are consistent with hydroxylated versions of the adjacent peak. For 1-dephospho-lipid A, peaks consistent with non-covalent acetate adducts are also detected. **(D)** ESI-MS analysis of lipid A purified from mutant strains χ3761(parent strain), χ9434 (Δ*pagP8*) and χ9732 (Δ*pagP81*::P<sub>1pp</sub> *lpxE*). Strains were grown at pH 5.8 in N-medium containing 10 μM Mg<sup>2+</sup> at 37°C.



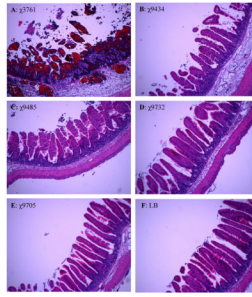
**Figure 2.**

Bacterial burden in (A) Peyer's patches (PP), (B, C) spleen and (D, E) liver of BALB/c mice ( $n = 4-5$  each group). Groups of mice were orally inoculated with  $\chi3761$  (wild-type UK-1),  $\chi9434$  ( $\Delta pagP8$ ),  $\chi9732$  ( $\Delta pagP8I::P_{lpp} lpxE$ ),  $\chi9485$  ( $\Delta pagL7 \Delta pagP8 \Delta lpxR9$ ) and  $\chi9705$  ( $\Delta pagL7 \Delta pagP8I::P_{lpp} lpxE \Delta lpxR9$ ); 3-5 mice per group were euthanized, and spleen, liver and PP were collected, homogenized, and plated onto MacConkey and LB plates at 3 days and 6 days post inoculation. *Salmonella* colonies were counted and expressed as CFU per gram of PP, spleen, or liver. Significant differences in mean between groups were determined using one-way ANOVA analysis followed by Dunnett's multiple comparison test. (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ).



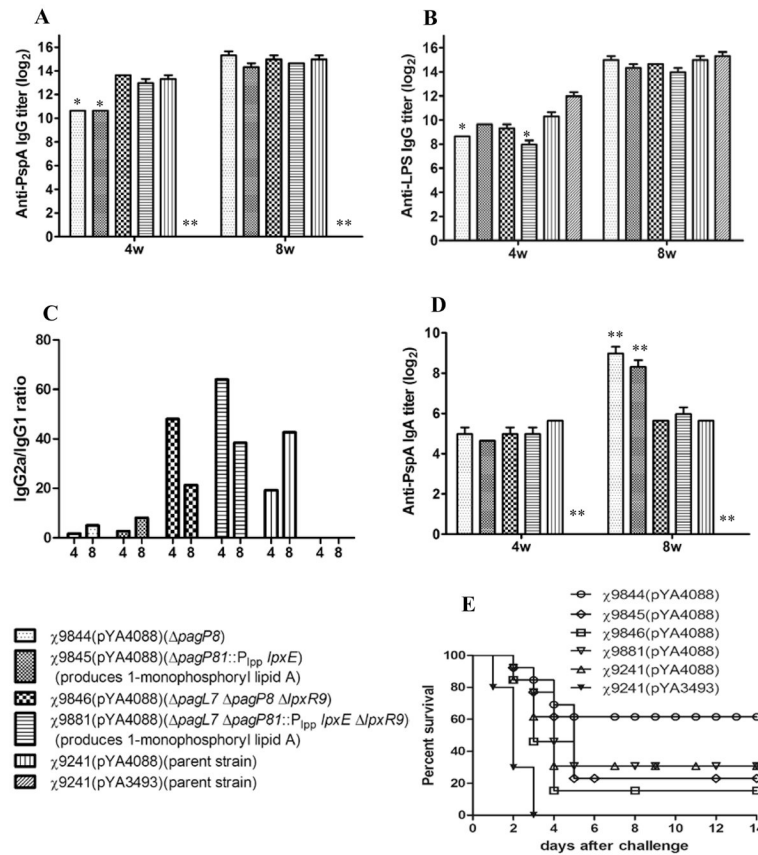
**Figure 3.**

Cytokine concentrations in tissue culture supernatants from cells stimulated by LPS and in sera from immunized mice. Supernatants from Mono Mac 6 human monocyte cell line (MM6) and RAW264.7 murine macrophages were collected 24 h after stimulation with 0.1 or 10 pmol/ml LPS and tested for the presence of human IL-6 in MM-6 (A) and murine TNF- $\alpha$  in RAW264.7 (B), respectively. Data represent the mean cytokine concentration from two independent assays. *P* values for all cytokine analyses were determined using one-way ANOVA followed by Dunnett's multiple comparison test. The multiple bioplex cytokine assay was used to detect and quantitate the amount of each cytokine in pooled sera collected from mice ( $n = 3$  mice/group) inoculated with  $\chi$ 3761 and its derivatives on different days (C). All mice in groups inoculated with strains  $\chi$ 9434,  $\chi$ 9485 and  $\chi$ 3761 succumbed to the infection by day 10. In the group inoculated with  $\chi$ 9732, one mouse died on day 12. All mice inoculated with  $\chi$ 9705 survived for the duration of the experiment. The error bars represent the Mean  $\pm$  SEM



**Figure 4.**

*S. Typhimurium* mutants with LpxE expression decrease inflammation but still induce fluid secretion in rabbit ileal loops. Three - to six-cm-long rabbit ileal loops were injected with  $10^9$  CFU of *S. Typhimurium* strains for 8 h, and the fluid secretion and histopathological changes in the ileum were examined. Histopathological staining of rabbit ileal loops injected with *S. Typhimurium* strains or the L broth control (H & E stain, 40 ×), wild-type induced flattened villi, marked infiltration of PMN, and thickening of endothelium of blood vessels that accompanies PMN infiltration, but mutants with 1-monophosphorylated LPS did not. Inflammatory response:  $\chi 3761$  (wild-type UK-1) >  $\chi 9485$  ( $\Delta pagL7 \Delta pagP8 \Delta lpxR9$ ) >  $\chi 9434$  ( $\Delta pagP8$ ) >  $\chi 9732$  ( $\Delta pagP81::P_{lpp} lpxE$ ) >  $\chi 9705$  ( $\Delta pagL7 \Delta pagP81::P_{lpp} lpxE \Delta lpxR9$ ).

**Figure 5.**

ELISA measurements of serum IgG and mucosal IgA responses in immunized and control mice ( $n = 13$  per group) and protection against *S. pneumoniae* challenge. **(A)** Total serum IgG specific for PspA, No immune responses were detected to PspA in mice immunized with the *S. Typhimurium* vaccine strain with the vector control plasmid,  $\chi 9241(pYA3493)$  (reciprocal titer  $<1:100$ ); **(B)** anti-*S. Typhimurium* LPS serum IgG; **(C)** ratio of reciprocal anti-PspA IgG2a/IgG1 titers; **(D)** mucosal anti-PspA IgA. No anti-PspA IgG was detected in serum from mice immunized with the *S. Typhimurium* vaccine strain with the vector control plasmid,  $\chi 9241(pYA3493)$  (reciprocal titer  $<1:50$ ). The data represent reciprocal antibody titers (mean  $\pm$  SEM) in pooled sera or vaginal washes from mice orally immunized with attenuated *Salmonella* carrying either pYA4088 (PspA) or pYA3493 (control) and boosted 5 weeks later. \*  $P < 0.01$ ; \*\*  $P < 0.001$  compared to mice immunized with  $\chi 9241(pYA4088)$ . **(E)** Protection in orally immunized BALB/c mice challenged i.p. with  $4 \times 10^4$  CFU of *S. pneumoniae* WU2–9 weeks after the first immunization. All vaccine groups were significantly different from the  $\chi 9241(pYA3493)$  control ( $P < 0.01$ ),  $P$  values were determined using the log-rank test. There was no significant difference in protection among the other groups.



Table 1

Strains and plasmids used in this work.

Strain or plasmid	Description (phenotype information) <sup>a</sup>	Source or derivative
<b><u>S. Typhimurium</u></b>		
χ3761	Wild type, UK-1 (Virulent parent strain)	(33)
χ9437	Δ <i>pagL72</i> ::P <sub>lpp</sub> <i>lpxE</i> (native <i>lpxE</i> )	χ3761
χ9701	Δ <i>pagP82</i> ::P <sub>lpp</sub> <i>lpxE</i> (native <i>lpxE</i> )	χ3761
χ9703	Δ <i>lpxR92</i> ::P <sub>lpp</sub> <i>lpxE</i> (native <i>lpxE</i> )	χ3761
χ9440	Δ <i>pagL71</i> ::P <sub>lpp</sub> <i>lpxE</i> (codon-optimized <i>lpxE</i> )	χ3761
χ9732	Δ <i>pagP81</i> ::P <sub>lpp</sub> <i>lpxE</i> (codon-optimized <i>lpxE</i> ) (produces 1-monophosphoryl lipid A)	χ3761
χ11092	Δ <i>lpxR91</i> ::P <sub>lpp</sub> <i>lpxE</i> (codon-optimized <i>lpxE</i> )	χ3761
χ9704	Δ <i>pagL7</i> Δ <i>pagP82</i> ::P <sub>lpp</sub> <i>lpxE</i> Δ <i>lpxR9</i>	χ3761
χ9705	Δ <i>pagL7</i> Δ <i>pagP81</i> ::P <sub>lpp</sub> <i>lpxE</i> Δ <i>lpxR9</i> (produces 1-monophosphoryl lipid A)	χ3761
χ9434	Δ <i>pagP8</i>	χ3761
χ9485	Δ <i>pagL7</i> Δ <i>pagP8</i> Δ <i>lpxR9</i>	χ3761
<b><u>Vaccine strains</u></b>		
χ9241	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i> Δ <i>araBAD23</i> Δ <i>relA198</i> :: <i>araC</i> P <sub>BAD</sub> <i>lacI</i> TT (attenuated vaccine strain)	(44)
χ9844	Δ <i>pagP8</i>	χ9241
χ9845	Δ <i>pagP81</i> ::P <sub>lpp</sub> <i>lpxE</i> (produces 1-monophosphoryl lipid A)	χ9241
χ9846	Δ <i>pagL7</i> Δ <i>pagP8</i> Δ <i>lpxR9</i>	χ9241
χ9881	Δ <i>pagL7</i> Δ <i>pagP81</i> ::P <sub>lpp</sub> <i>lpxE</i> Δ <i>lpxR9</i> (produces 1-monophosphoryl lipid A)	χ9241
<b><u>E. coli</u></b>		
χ7232	<i>endA1</i> <i>hsdR17</i> ( <i>r<sub>K</sub></i> <sup>-</sup> , <i>m<sub>K</sub></i> <sup>+</sup> ) <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA</i> <i>relA1</i> Δ( <i>lacZYA-argF</i> ) <i>U169</i> λ <i>pir</i> <i>deoR</i> (φ80 <i>d</i> lac ( <i>lacZ</i> ) <i>M15</i> )	(35)
χ7213	<i>thi-1</i> <i>thr-1</i> <i>leuB6</i> <i>glnV44</i> <i>tonA21</i> <i>lacY1</i> <i>recA1</i> RP4-2-Tc::Mu λ <i>pir</i> Δ <i>asdA4</i> Δ <i>zhf-2</i> ::Tn10	(35)
<i>S. pneumoniae</i> WU2	Wild-type virulent, encapsulated type 3	(46)
<b><u>Plasmids</u></b>		
pRE112	<i>sacB</i> <i>mobRP4</i> R6K <i>ori</i> Cm <sup>R</sup>	(34)
pYA4284	<i>pagL7</i> deletion	pRE112
pYA4288	<i>pagP8</i> deletion	pRE112
pYA4287	<i>lpxR9</i> deletion	pRE112
pYA4291	<i>pagL</i> deletion, and P <sub>lpp</sub> <i>lpxE</i> (native) insertion	pRE112
pYA4473	<i>pagP</i> deletion, and P <sub>lpp</sub> <i>lpxE</i> (native) insertion	pRE112
pYA4474	<i>lpxR</i> deletion, and P <sub>lpp</sub> <i>lpxE</i> (native) insertion	pRE112
pYA4528	P <sub>lpp</sub> <i>lpxE</i> insertion into downstream of <i>bcfH</i> gene	pRE112
pYA4294	<i>pagL</i> deletion, and P <sub>lpp</sub> <i>lpxE</i> (codon-optimized) insertion	pRE112
pYA4295	<i>pagP</i> deletion, and P <sub>lpp</sub> <i>lpxE</i> (codon-optimized) insertion	pRE112
pYA4296	<i>lpxR</i> deletion, and P <sub>lpp</sub> <i>lpxE</i> (codon-optimized) insertion	pRE112
pXYW-1	<i>lpxE</i> in pWSK29a	(27)
pYA3493	Plasmid Asd <sup>+</sup> ; pBRori β-lactamase signal sequence for periplasmic secretion	(43)

Strain or plasmid	Description (phenotype information) <sup>a</sup>	Source or derivative
pYA4088	852 bp DNA encoding the $\alpha$ -helical region of PspA from amino acid 3 to amino acid 285 in pYA3493	(44)

<sup>a</sup>Strains carrying *lpxE* produce some level of 1-monophosphoryl lipid A. See text for details.

Table 2

Mouse survival after infection with the indicated *Salmonella* strains.

Dose	$\chi$ 3761 wild-type	$\chi$ 9434 $\Delta$ pagP8	$\chi$ 9732 <sup>c</sup> $\Delta$ pagP8I::P <sub>ppIpxE</sub> (m-lipid A) <sup>d</sup>	$\chi$ 9485 $\Delta$ pagL7 $\Delta$ pagP8 $\Delta$ pxR9	$\chi$ 9705 <sup>e</sup> $\Delta$ pagL7 $\Delta$ pagP8I::P <sub>ppIpxE</sub> $\Delta$ pxR9 (m-lipid A)
1 × 10 <sup>3</sup> CFU	4/5 <sup>b</sup>	6/7	-	2/2	-
1 × 10 <sup>4</sup> CFU	2/5	3/7	-	6/7	-
1 × 10 <sup>5</sup> CFU	0/5	0/7	-	3/7	-
1 × 10 <sup>6</sup> CFU	- <sup>c</sup>	0/2	2/2	0/7	2/2
1 × 10 <sup>7</sup> CFU	-	-	7/7	-	7/7
1 × 10 <sup>8</sup> CFU	-	-	6/7	-	7/7
1 × 10 <sup>9</sup> CFU	-	-	3/7	-	7/7
LD <sub>50</sub> (CFU) <sup>d</sup>	0.9 × 10 <sup>4</sup>	0.9 × 10 <sup>4</sup>	0.66 × 10 <sup>9</sup>	5.7 × 10 <sup>4</sup>	> 1 × 10 <sup>9</sup>

<sup>a</sup> 1-monophosphoryl lipid A

<sup>b</sup> Survivors/total number of mice

<sup>c</sup> Dose not tested

<sup>d</sup> LD<sub>50</sub> data were from probit analyses

<sup>e</sup> Surviving mice were challenged with 1 × 10<sup>9</sup> CFU of  $\chi$ 3761 thirty days after the initial inoculation. All challenged mice survived.