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The Two Murein Lipoproteins of *Salmonella enterica* Serovar Typhimurium Contribute to the Virulence of the Organism

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Septic shock due to *Salmonella* and other gram-negative enteric pathogens is a leading cause of death worldwide. The role of lipopolysaccharide in sepsis is well studied; however, the contribution of other bacterial outer membrane components, such as Braun (murein) lipoprotein (Lpp), is not well defined. The genome of *Salmonella enterica* serovar Typhimurium harbors two copies of the lipoprotein (*lpp*) gene. We constructed a serovar Typhimurium strain with deletions in both copies of the *lpp* gene (*lpp1* and *lpp2*) by marker exchange mutagenesis. The integrity of the cell membrane and the secretion of the effector proteins through the type III secretion system were not affected in the *lpp* double-knockout mutant. Subsequently, the virulence potential of this mutant was examined in a cell culture system using T84 intestinal epithelial and RAW264.7 macrophage cell lines and a mouse model of salmonellosis. The *lpp* double-knockout mutant was defective in invading and inducing cytotoxic effects in T84 and RAW264.7 cells, although binding of the mutant to the host cell was not affected when compared to the wild-type (WT) serovar Typhimurium. The motility of the mutant was impaired, despite the finding that the number of flagella was similar in the *lpp* double knockout mutant and the WT serovar Typhimurium. Deletion in the *lpp* genes did not affect the intracellular survival and replication of *Salmonella* in macrophages and T84 cells. Induction of the proinflammatory cytokines tumor necrosis factor alpha and interleukin-8 (IL-8) was significantly reduced in macrophages and T84 cells infected with the *lpp* double-knockout mutant. The levels of IL-8 remained unaffected in T84 cells when infected with either live or heat-killed WT and *lpp* mutant, indicating that invasion was not required for IL-8 production and that Toll-like receptor 2 signaling might be affected in the Lpp double-knockout mutant. These effects of the Lpp protein could be restored by complementation of the isogenic mutant. The *lpp* double-knockout mutant was avirulent in mice, and animals infected with this mutant were protected from a lethal challenge dose of WT serovar Typhimurium. The severe combined immunodeficient mice, on the other hand, were susceptible to infection by the *lpp* double-knockout mutant. The serovar Typhimurium mutants from which only one of the *lpp* (*lpp1* or *lpp2*) genes was deleted were also avirulent in mice. Taken together, our data indicated that Lpp specifically contributed to the virulence of the organism.

Salmonella enterica serovar Typhimurium is frequently implicated in causing outbreaks of enteric disease due to the consumption of contaminated food (58). In mice, serovar Typhimurium elicits systemic infection, providing an experimental model of typhoid fever (23). In the United States, the number of prevalent cases of salmonellosis has been estimated to range from 80,000 to 3,700,000 annually (12, 69). An important feature of pathogenesis of *Salmonella* infections is the ability of the organism to attach to and invade host epithelial cells (74). Serovar Typhimurium colonizes intestinal mucosa and transcytosis M cells and then is phagocytized by resident macrophages, where they multiply and further invade internal organs, such as the liver and spleen (32, 35). Invasion of host cells by serovar Typhimurium induces cytoskeletal rearrangement accompanied by membrane ruffling and cytotoxicity (32, 50).

One of the most serious complications of serovar Typhimurium infection is septic shock, which is mediated in part by lipopolysaccharide (LPS). It has been estimated that more than 600,000 cases of sepsis and septic shock occur annually in the United States, resulting in more than 100,000 deaths (66). LPS exerts its biological effect via lipid A-dependent induction of cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and IL-6 from macrophages and neutrophils; activation of the complement cascade; and by the induction of other inflammatory mediators (36, 60, 79). Recently, we reported that the Braun (murein) lipoprotein (Lpp) (8, 9), a major bacterial outer membrane component of gram-negative bacteria in the family *Enterobacteriaceae*, also contributed significantly to the development of septic shock (85). In that study, Lpp purified from *Escherichia coli* was shown to induce the in vivo production of TNF-α and IL-6 in LPS-nonresponsive mice (85). Furthermore, Lpp synergized with *E. coli* LPS to produce proinflammatory cytokines leading to septic shock, indicating that Lpp and LPS activate cells through different mechanisms (85). LPS activates cellular responses via both Toll-like receptor 4 (TLR4) and CD14 and LPS-binding protein (77). In contrast to LPS, Lpp purified from *E. coli* activated cells via toll-like receptor 2 (TLR2) (1). The lipid moiety, attached at the N-terminal end of the protein back-

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bone of Lpp, is essential for its immune and inflammatory activities, as shown for LPS (66).

Various functionally distinct species of lipoproteins exist in gram-negative bacteria (3). These lipoproteins are either structural proteins, e.g., Lpp, peptidoglycan-associated lipoprotein (designated Pal), and outer membrane lipoprotein LolB precursor, enzymes, receptors, invasion-associated type III secretion system (TTSS) (e.g., InvH), or transporters performing crucial functions in the bacterial cell envelope. Organisms such as *Borrelia burgdorferi* and *Treponema pallidum*, which lack LPS, induce inflammatory mediators through lipoprotein, which indicates that it plays an important role in bacterial pathogenesis (85).

As with LPS, murein Lpp is synthesized in the cytoplasm as a precursor and undergoes sequential posttranslational modifications (lipolization) catalyzed by glyceryl transferase (Lgt), *O*-acyl transferase, prolipoprotein signal peptidase II, and *N*-acyl transferase (Lnt) to form mature Lpp (38). Mature Lpp is then transported across the cytoplasmic membrane by protein translocation machinery (55). Recent studies indicated that mutation in genes coding for Lgt and Lnt reduced the growth and motility of serovar Typhimurium (19, 31). Likewise, Neilsen et al. (63) reported that a naturally occurring Lpp$^-$ mutant of *E. coli* JE5505 (44) was less inflammatory than its parental strain. However, it is not known whether this *E. coli* mutant had alterations in other genes as well, which could affect its virulence. Therefore, targeted mutants for Lpp needed to be developed to precisely define its role in pathogenesis, and they formed the basis of this study.

We showed previously that Lpp and LPS from *E. coli* and *Yersinia enterocolitica* were equally potent with regard to host cellular responses (84, 85). Further, the release of LPS and Lpp from bacteria and their synergistic effect (84, 85) warrant further investigation of the critical role Lpp plays in bacterial virulence and induction of the proinflammatory cytokines that lead to septic shock.

In this study, we report, for the first time, the construction of a serovar Typhimurium strain in which two highly homologous murein-lipoprotein genes (*lpp1* and *lpp2*) located in tandem in the *Salmonella* genome were deleted. Subsequently, the *lpp* double-knockout mutant was used to study the role of Lpp in *Salmonella* virulence using both in vitro and in vivo models. Compared to the wild-type (WT) and complemented strains of serovar Typhimurium, the *lpp* double-knockout mutant was defective in invasion and less cytotoxic and produced reduced levels of proinflammatory cytokines, possibly by altering TLR2 signaling. Importantly, the Lpp$^-$ mutant was avirulent in mice, and mice infected with this mutant were resistant to a lethal challenge of WT serovar Typhimurium. The severe combined immunodeficient (SCID) mice lacking immune cells, on the other hand, died after infection with the Lpp mutant of serovar Typhimurium. To define the role of each of the *lpp* genes in serovar Typhimurium virulence, we constructed mutants of *Salmonella* in which the *lpp1* and *lpp2* genes were individually inactivated or deleted by marker exchange mutagenesis. The *lpp1* and *lpp2* isogenic mutants were similarly avirulent in mice, as noted with the *lpp* double-knockout serovar Typhimurium mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. Serovar Typhimurium strain 14028 was described elsewhere (50), and a spontaneous nalidixic acid resistance strain of serovar Typhimurium was generated in our laboratory. The *E. coli* strain JE5505 was a naturally occurring Lpp$^-$ mutant. This *E. coli* strain grew and divided normally (44). The suicide vector pDMS197 had a conditional R6K origin of replication (*ori*), a levansucrase gene (*sacB*) from *Bacillus subtilis*, and a tetracycline resistance (Tc^r) gene (21). The suicide vector pJQ200SK (67) contained P15A *ori*, a *sacB* gene, and a gentamicin resistance (Gm^r) gene. The *E. coli* and *Salmonella* strains were grown either in Luria-Bertani (LB) medium (2) or on *Salmonella-Shigella* (SS) agar plates (Difco, Detroit, Mich.). A list of bacterial strains and plasmids used in this study is provided in Table 1.

Cell culture. RAW264.7 murine macrophage and T84 intestinal epithelial cell lines were obtained from American Type Culture Collection (Manassas, Va.). Macrophages were maintained in Dulbecco's modified eagle medium with 10% fetal bovine serum supplemented with penicillin-streptomycin (Invitrogen, Carlsbad, Calif.). T84 cells were grown in Dulbecco's modified eagle medium–F-12 medium with 5% fetal bovine serum supplemented with penicillin-streptomycin. Both types of cells were incubated at 37°C with 5% CO_2.

Construction of serovar Typhimurium *lpp* isogenic mutants by using suicide vectors. Based on our studies and subsequently on the basis of the genomic sequence of serovar Typhimurium, two copies of the *lpp* gene (designated *lpp1* and *lpp2*), separated by 82 bp of DNA, are present in the chromosome (Fig. 1) (56). A double-knockout mutant, in which both copies of the *lpp* gene from strain 14028 were deleted, was first generated by homologous recombination using the suicide vector pJQ200SK. Briefly, the 5′- and 3′-flanking DNA sequences to the *lpp* genes were amplified from the chromosomal DNA of serovar Typhimurium by the PCR, utilizing two pairs of specific primers (F2/R5 and F6/R2) with appropriate restriction enzyme sites (Fig. 1A and Table 2). A common XhoI restriction enzyme site was introduced into flanking DNA fragments of the *lpp* genes during PCR. The resulting flanking DNA fragments were ligated through the XhoI site and cloned into a pBluescript vector at the BamHI/ApaI sites to produce a recombinant plasmid, pBlue*lpp*. Subsequently, a kanamycin resistance (Kn^r) gene cassette (obtained from the plasmid pUC4K; Amersham Pharmacia, Piscataway, N.J.) was inserted at the XhoI restriction enzyme site to generate a recombinant plasmid, pBlue*lpp*K. The plasmid, pBlue*lpp*K, was digested with BamHI/ApaI restriction enzymes and ligated into a suicide vector, pJQ200SK, to produce a recombinant plasmid, pJQ200*lpp*K, which was then transformed into *E. coli* S17-1. This *E. coli* strain contains a chromosomal λ*pir* gene required for the replication of the suicide vector pJQ200SK. Since serovar Typhimurium does not have the λ*pir* gene, the recombinant suicide plasmid could not replicate in serovar Typhimurium. The suicide vector that contained a *sacB* gene caused lethality in serovar Typhimurium and *E. coli* when induced with 5% sucrose.

We used both a suicide plasmid and a lambda (λ) Red system for constructing *lpp1* and *lpp2* single-knockout mutants. To specifically truncate the *lpp1* gene of serovar Typhimurium, the coding region of the *lpp1* gene was PCR amplified by using primers LF and LR (Fig. 1A and Table 2). It was subsequently cloned into a pBluescript vector at the compatible sites. A kn^r gene cassette from the pUC4K plasmid was first digested with the PstI restriction enzyme, and its ends were made blunt by using a PCR polishing kit (Stratagene, La Jolla, Calif.). The kn^r gene cassette was then inserted into the *lpp1*-coding region at the HindII restriction enzyme site. This truncated version of the *lpp1* gene was subsequently cloned into a suicide vector, pDMS197, at the KpnI/XbaI restriction enzyme sites, generating a recombinant plasmid, pDMS197*lpp1*K.

To delete the *lpp2* gene from serovar Typhimurium, we used a modified strategy similar to one employed in generating an *lpp* double-knockout mutant (Fig. 1A). The first of two modifications to the strategy included the use of a new 5′-flanking DNA fragment, containing the entire coding region of the *lpp1* gene that was generated by using a primer pair, F2 and R4. The pair of primers (F6 and R2) used to amplify the 3′-flanking DNA sequence was the same that was used for generating the *lpp* double-knockout mutant (Fig. 1A and Table 2). Secondly, we used suicide vector pDMS197 in *E. coli* strain SM10 (20) instead of pJQ200SK. The newly generated recombinant plasmid was designated pDMS197*lpp2*K. All of the DNA manipulations were performed as described previously (2).

The recombinant *E. coli* carrying pJQ200*lpp*K, pDMS197*lpp1*K, or pDMS197*lpp2*K was conjugated with serovar Typhimurium (nalidixic acid) resistance [Nal^r] (72). The Nal^r, Kn^r, Gm^s, and sucrose resistance transconjugants were selected as candidates for the *lpp* double-knockout mutants of serovar Typhimurium, while Nal^r, Kn^r, Tc^s, and sucrose resistance transconjugants were designated the candidates for *lpp* (*lpp1* or *lpp2*) single-knockout mutants of serovar Typhimurium. These selected transconjugants should represent genuine,

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Serovar Typhimurium strain 14028	*Salmonella enterica* serovar Typhimurium	50
STM-N	Nalidixic acid resistance (Nal^r) strain of serovar Typhimurium 14028	This study
Mutant 66	Isogenic mutant of serovar Typhimurium in which two copies of the lipoprotein gene (*lpp*) were deleted; Nal^r Kn^r	This study
Mutant 67	Isogenic mutant of serovar Typhimurium in which two copies of the *lpp* gene were deleted; Nal^r Kn^r	This study
lpp1 mutant	Isogenic mutant of serovar Typhimurium in which only one copy of the *lpp* gene (*lpp1*) was truncated by using suicide vector pDMS197; Nal^r Kn^r	This study
lpp2 mutant	Isogenic mutant of serovar Typhimurium in which only one copy of the *lpp* gene (*lpp2*) was deleted by using suicide vector pDMS197; Nal^r Kn^r	This study
*lpp1*R mutant	Isogenic mutant of serovar Typhimurium in which only one copy of the *lpp* gene (*lpp1*) was deleted using λ Red system; Nal^r Kn^r	This study
*lpp2*R mutant	Isogenic mutant of serovar Typhimurium in which only one copy of the *lpp* gene (*lpp2*) was deleted using λ Red system; Nal^r Kn^r	This study
Mutant 67/pBR*lpp*	Mutant 67 complemented with two copies of the *lpp* gene via pBR322 vector; Nal^r Kn^r Tc^r	This study
E. coli		
DH5α	*recA gyrA*	Laboratory stock
S17-1	Streptomycin and trimethoprim resistance; λ*pir*	Laboratory stock
SM10	Kn^r λ*pir*	21
JE5505	Naturally occurring Lpp-minus mutant of *E. coli*	44
Plasmids		
pBR322	Ap^r Tc^r	Amersham
pBluescript-SK	Ap^r	Stratagene
pUC-4K	Contains a 1.2-kb kanamycin^r gene cassette	Amersham
pJQ200SK	A suicide vector; P15A *ori sacB*, Gm^r	67
pDMS197	A suicide vector; R6K *ori sacB*, Tc^r	21
pBR*lpp*	STM *lpp* genes with its putative promoter region, cloned in pBR322 at the ScaI site	This study
pBlue*lpp*	pBluescript vector containing up- and downstream flanking DNA sequences to the *lpp* genes of STM	This study
pBlue*lpp*K	Kn^r gene cassette was inserted at the XhoI site in plasmid pBlue*lpp* that contained 5′- and 3′-flanking sequences to the *lpp* genes of serovar Typhimurium	This study
pJQ200*lpp*K	Suicide vector pJQ200SK containing the Kn^r gene cassette which was flanked by the up- and downstream flanking DNA sequences to the *lpp* genes of serovar Typhimurium; it was used to generate *lpp* double-knockout mutant of serovar Typhimurium	This study
pBlue*lpp1*	pBluescript vector containing *lpp1* coding region of serovar Typhimurium	This study
pBlue*lpp1*K	Kn^r gene cassette truncating the serovar Typhimurium *lpp1* gene at the HindII site in plasmid pBlue*lpp1*	This study
pDMS197*lpp1*K	Suicide vector pDMS197 containing the Kn^r gene cassette that interrupted serovar Typhimurium *lpp1* gene for generating the *lpp1* single-knockout mutant	This study
pBlue*lpp2*	pBluescript vector containing up- and downstream flanking DNA sequences to the *lpp2* gene of serovar Typhimurium	This study
pBlue*lpp2*K	Kn^r gene cassette was inserted at the XhoI site in plasmid pBlue*lpp2* that contained 5′- and 3′-flanking sequences to the *lpp2* gene of serovar Typhimurium	This study
pDMS197*lpp2*K	Suicide vector pDMS197 containing the Kn^r gene cassette which was flanked by the the up- and downstream flanking DNA sequences to the *lpp2* gene of serovar Typhimurium; it was used to generate *lpp2* single-knockout mutant of serovar Typhimurium	This study
pKD46	Temperature-sensitive plasmid expressing λ Red recombinase under control of arabinose; Ap^r	20
pKD4	Template plasmid in the λ Red system for Kn^r gene cassette; Kn^r Ap^r	20

double-crossover mutants (Fig. 1A). In such mutants, the Kn^r gene cassette or a truncated version of the *lpp* gene contained on the suicide vectors must have replaced the native *lpp* gene(s) on the chromosome of serovar Typhimurium by homologous recombination, with concomitant loss of the remaining suicide vectors containing the *sacB* and antibiotic resistance genes (Gm for pJQ200SK and Tc for PDMS197) (Fig. 1A).

The double-crossover mutants were identified as serovar Typhimurium by growth on MacConkey's and SS agar media to differentiate them from *E. coli* used for conjugation. The cultures were further identified as *Salmonella* by an automated identification system used in the Clinical Microbiology Laboratory, University of Texas Medical Branch (UTMB) (Galveston, Tex.). To demonstrate genuine, double-crossover events, the selected *lpp* isogenic mutants and the WT serovar Typhimurium were subjected to PCR analysis with different primer sets and/or Southern blot hybridization (as specifically performed for the *lpp* double-knockout mutants), using the *lpp* gene, Kn^r gene cassette, and pJQ200SK plasmid as probes.

Complementation of the serovar Typhimurium *lpp* double-knockout isogenic mutants. A DNA fragment containing both the *lpp1* and *lpp2* genes from WT serovar Typhimurium was PCR amplified by using a primer set, F3 and R3 (Table 2 and Fig. 1A). Subsequently, the ends of the fragment were made blunt with a PCR polishing kit and ligated into the blunt-ended ScaI-digested pBR322 vector. The recombinant plasmid then was transformed into *lpp* double-knockout

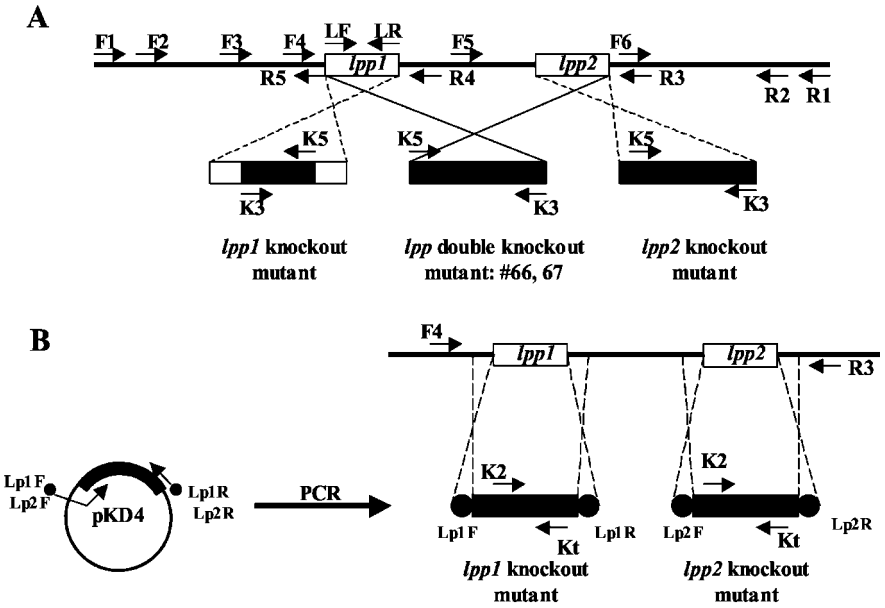

FIG. 1. Schematic diagram showing construction of *lpp* isogenic mutants of serovar Typhimurium via homologous recombination. In panel A, the Kn^r gene cassette-interrupted *lpp1* gene replaced the original *lpp1* gene on the genome of serovar Typhimurium and generated the *lpp1* mutant, while for the *lpp2* single-knockout mutant, the Kn^r gene cassette replaced the whole coding sequence of the *lpp2* gene. To generate the *lpp* double-knockout mutant, both copies of the *lpp* gene including the sequence between them were replaced by a Kn^r gene cassette. In panel B, the λ Red system was used, and the Kn^r gene cassette replaced the corresponding *lpp* copy on the genome of the single *lpp* knockout mutants of serovar Typhimurium. Arrows indicate the primers' position used for the generation and identification of the *lpp* knockout mutants. The exact DNA sequences of the primers were also shown in Table 2. The open bars indicate the copies of the *lpp* gene on the genome, and the solid bars indicate the Kn^r gene cassette used to replace or interrupt the *lpp* genes on the genome of serovar Typhimurium. The solid circles represent the flank DNA sequence to the *lpp* genes of serovar Typhimurium for homologous recombination in the λ Red system.

mutants by electroporation, following the manufacturer's instructions (Invitrogen).

Construction of *lpp1* and *lpp2* mutants using a λ Red system. A λ Red system, as described by Datsenko and Wanner (20), was also used to generate *lpp* single-knockout (*lpp1* or *lpp2*) mutants of serovar Typhimurium. Two specific primer sets (Lp1F, Lp1R and Lp2F, Lp2R) were synthesized and used with the λ Red system (Fig. 1B and Table 2). Each primer was 68 bp in length, and at the 5′ end of each, there was a flanking DNA sequence (46 to 48 bp in size; shown in nonitalic letters in Table 2) to the specific *lpp* copy that was targeted to be deleted from the genome of WT serovar Typhimurium. The DNA at the 3′ end of each of the primers had a sequence (shown in italic letters in Table 2) that bordered the Kn^r gene cassette on the plasmid pKD4 (20). By using each of the specific primer sets, a 1.3-kb DNA fragment could be amplified from the plasmid pKD4. These amplified DNA fragments contained a Kn^r gene cassette in the middle and a short flanking DNA sequence (46 to 48 bp on each side) to the specific *lpp* copy for homologous recombination. To increase the frequency of homologous recombination, an ampicillin resistance (Ap^r) plasmid, pKD46, which contained a phage λ Red recombinase gene, was first transformed into WT serovar Typhimurium. The λ Red recombinase gene could be hyperexpressed after induction of the culture with 1 mM arabinose. The presence of plasmid pKD46 in serovar Typhimurium also provided a temperature-sensitive phenotype, allowing bacteria to replicate normally at 30°C but not at 37°C due to curing of the plasmid with concomitant loss of the Ap^r phenotype.

Briefly, the serovar Typhimurium strain with the pKD46 plasmid was first grown at 30°C in the SOB medium (tryptone [20 g], yeast extract [5 g], and NaCl [0.5 g per liter] [pH 7.5]) in the presence of 1 mM arabinose until an optical density (OD) of 0.6 was reached. The bacteria were harvested, washed twice with ice-cold 10% glycerol, and electroporated with the 1.3-kb DNA fragment which was generated by PCR amplification with the primer sets Lp1F and Lp1R and Lp2F and Lp2R. The transformed cultures were screened on kanamycin (100 μg/ml) containing LB agar plates at 37°C. The Kn^r colonies were picked up for further PCR analysis with primer sets k2 and F4 and kt and R3 (Fig. 1B, Table 2). The colonies with the correct genotype were then examined for the Ap^r phenotype. The sensitivity of the culture for Ap would indicate successful curing of plasmid pKD46.

Southern blot and PCR analyses on *lpp* knockout mutants of serovar Typhimurium. Chromosomal DNA from the WT and serovar Typhimurium *lpp* isogenic mutants was isolated using a QIAamp DNA mini kit (Qiagen, Inc., Valencia, Calif.). Southern blot analysis was performed with the *lpp* double-knockout mutants. Briefly, an aliquot (10 to 15 μg) of the chromosomal DNA from WT and *lpp* double-knockout mutants was digested with appropriate restriction enzymes (e.g., BglII and MluI) and subjected to 0.8% agarose gel electrophoresis (71). The digested total DNA was transferred to a nylon membrane and baked at 80°C for 2 h. The blots were prehybridized and hybridized using Quickhyb at 68°C, as described by the manufacturer (Stratagene). The Kn^r gene cassette, the *lpp* gene, and the suicide vector pJQ200SK labeled with [α^{32}P]dCTP (ICN, Irvine, Calif.) were used as probes. After hybridization, the blots were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) for 20 min, followed by washing with 1× SSC plus 0.1% SDS at 68°C for 20 min, and exposed to X-ray film at room temperature.

Next, PCR analysis was performed to identify the *lpp* (*lpp1* or *lpp2*) single-knockout mutants generated by a suicide vector, pDMS197. Different primer sets were used to verify the presence of an antibiotic resistance gene cassette (Kn^r) and junctional sequences in the mutants. These primers were designed either to the Kn^r gene cassette or to the genome sequence of WT serovar Typhimurium outside the flanking sequences that were used for homologous recombination. This strategy allowed us to correctly predict the genotypes of the mutants. For the *lpp1* single-knockout mutant, primer sets K3 and K5, F4 and R3, F4 and K5, and R3 and K3 were used. The primer set F5 and R3 was specifically designed for examining the integrity of the *lpp2* gene in the *lpp1* single-knockout mutant (Fig. 1A and Table 2). For the *lpp2* single-knockout mutant, primer sets K5 and K3, F1 and R1, F1 and K3, and R1 and K5 were employed, and primer set F4 and R4 was used to verify the presence of the *lpp1* sequence in the *lpp2* single-knockout mutant of serovar Typhimurium (Table 2 and Fig. 1A). We previously described the PCR program used in this study (15). The denaturation temperature varied between 94 and 96°C, and the annealing temperature varied between 55 and 68°C with different sets of primers used in the PCRs.

Preparation of outer membranes of various serovar Typhimurium strains. Our subsequent detailed studies were performed only with the *lpp* double-knockout mutants of serovar Typhimurium. The outer membrane proteins from various serovar Typhimurium strains were extracted as previously described (26).

TABLE 2. Sequences of the primers used in this study for preparing serovar Typhimurium isogenic mutants

Primer position	Primer name and sequence[ab]	Reference	Purpose
5′	F1: 5′ GATGCGTCTGAACTTCTCTC-3′	This study	PCR for identifing *lpp* knockout mutants of serovar Typhimurium
3′	R1: 5′ GCAATGCCGCTCATGGCCTG 3′	This study	
5′	F4: 5′ GCTACATGGAGATTAACTCA 3′	This study	
5′	F5: 5′ TGGCGCACATCGTGCGCCAT 3′	This study	
5′	F2: 5′ GTGGATCCTATGCAGAACACGGTCAGCG 3′ BamHI	This study	PCR amplification of 5′ flanking sequence of *lpp* gene
3′	R5: 5′ AGCTCGAGCTAGATTGAGTTAATCTCCA 3′ XhoI	This study	
3′	R4: 5′ CACTCGAGACGCAGGTACTATTACTTAC 3′ XhoI	This study	
5′	F6: 5′ ATCTCGAGTACTGCGAAGGCTACTGCGTCG 3′ XhoI	This study	PCR amplification of 3′ flanking sequence of *lpp* gene
3′	R2: 5′ AAGGGCCCTTCGCTGGCGATGTATAAC 3′ ApaI	This study	
5′	F3: 5′ ACAAGCTTGCCGCTGATCGTGGTAGCGAC 3′ HindIII	This study	PCR amplification of *lpp* genes for complementation
3′	R3: 5′ AGAAGCTTCGACGCAGTAGCCTTCGCAGTA 3′ HindIII	This study	
5′	LF: 5′ CACTCGAGATGAAAGCTACTAAACTGGTAC 3′ XhoI	This study	PCR amplification of *lpp1* coding region to generate *lpp1* single-knockout mutant of serovar Typhimurium
3′	LR: 5′ CCGAATTCTTACTTGCGGTATTTAGTAGCC 3′ EcoRI	This study	
5′	K5: 5′ CGCTGAGGTCTGCCTCGTGAAGAAGGTGTT 3′	72	Primers that specifically bind to the kn^r gene cassette, used to identify *lpp* knockout mutants of serovar Typhimurium
3′	K3: 5′ AAAGCCACGTTGTGTCTAAAATCTCTGATGT 3′	72	
5′	K2: 5′ CGGTGCCCTGAATGAACTGC 3′	20	
3′	Kt: 5′ CGGCCACAGTCGATGAATCC 3′	20	
5′	Lp1F: 5′ CTTGTAACGCTACATGGAGATTAACTCAATCTAGA GGGTATTAATA*ATGTGTAGGCTGGAGCTGCTT CG* 3′	This study	Primer set used to make *lpp1* single knockout mutant in the λ Red system
3′	Lp1R: 5′ ATGGCGCACGATGTGCGCCATTTTTATTACGCAGGTACTATTACTTAC*CATATGAATATCCTCCTTAG* 3′	This study	
3′	Lp2F: 5′ CCATTTTTTTTACCTATATAACCACACAAAATATAAGGTTATTGTT*TGTGTAGGCTGGAGCTGCTTCG* 3′	This study	Primer set used to make *lpp2* single-knockout mutant in the λ Red system
5′	Lp2R: 5′ TGGCGCACGATGTGCGCCATTTTATATCATGCGTCAAATCATTTACAG*CATATGAATATCCTCCTTAG* 3′	This study	

[a] Underlining indicates restriction enzyme sites in the primer.
[b] Italic sequence indicates DNA sequence that binds to plasmid pKD4 in the λ Red system.

Briefly, a 50-ml bacterial culture was centrifuged, and the pellet was resuspended in 9 ml of phosphate-buffered saline (PBS) containing DNase (30 μg/ml) and two proteinase inhibitor tablets (Caltag Laboratories, Burlingame, Calif.). After sonication of the cells, the debris was removed by centrifugation in a Sorvall RC-SB Plus (Kendro Laboratory Products, Ashville, N.C.) at 10,000 rpm for 20 min, and the supernatant was ultracentrifuged in an Optima L-90K (Beckman Coulter, Palo Alto, Calif.) at 35,000 rpm for 1 h. The pellet was resuspended in 5 ml of distilled water with one proteinase inhibitor tablet. Subsequently, sodium sarcosyl was added to a final concentration of 0.5%, and the preparation was incubated at room temperature for 20 min before ultracentrifugation at 35,000 rpm for 45 min. The supernatant contained the inner membrane proteins. The pellet containing the outer membrane proteins was resuspended in 200 μl of distilled water with proteinase inhibitor. The protein concentration was measured at 280 nm.

Western blot analysis. Western blot analysis was performed to evaluate the presence of Lpp in the WT serovar Typhimurium, its isogenic mutants (double knockout), and the complemented strain. An aliquot (20 to 25 μg) of total proteins from various strains of serovar Typhimurium, as well as their outer membrane protein preparations, was separated by SDS–15% polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose membranes. Membranes were blocked with 3% gelatin and washed in 1× Tris-buffered saline with 0.05% Tween twice for 10 min each. A primary monoclonal antibody to *Y. enterocolitica* Lpp (84) in 1% gelatin solution (prepared in 1× Tris-buffered saline with 0.05% Tween) was applied to the membranes, and then they were allowed to incubate for 2 h at room temperature. After washing, appropriate secondary horseradish peroxidase (HRP)-conjugated, antimouse antibodies diluted 1:25,000 in 1% gelatin were applied to the membranes. Subsequently, the membranes were washed, and an enhanced chemiluminescence substrate kit (Pierce, Rockford, Ill.) was used, according to the manufacturer's instructions, for detecting a signal on the X-ray film.

Examination of the cell membrane integrity of the serovar Typhimurium *lpp* double-knockout isogenic mutant. The integrity of the cell envelope of various bacterial cultures was examined by methods described below.

(i) Membrane blebbing. Both the WT and *lpp* double-knockout mutant were grown to exponential phase (OD at 600 nm of 0.4 to 0.5). The cells were washed and subjected to transmission electron microscopy. Briefly, bacterial culture suspensions were pelleted and fixed in a mixture of 1.25% formaldehyde and 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.3) to which 0.03% $CaCl_2$ and 0.03% trinitrophenol were added. They were postfixed in 1% OsO_4 in 0.1 M cacodylate buffer, en bloc stained with 2% aqueous uranyl acetate, dehydrated in ethanol, and embedded in Poly/Bed 812 (Polysciences, Warrington, Pa.). Ultrathin sections were cut on a Reichert-Leica Ultracut S ultramicrotome, stained with lead citrate, and examined in a Philips 201 electron microscope at 60 kV. The membrane blebbing was examined in a minimum of 100 bacterial cells (73).

(ii) Sensitivity to detergents. To demonstrate sensitivity of the WT serovar Typhimurium and *lpp* double-knockout mutant to the effect of detergent Triton X-100 (TX-100) and SDS, the bacterial cells were grown to an OD of 0.4 to 0.5 and diluted 50-fold, and then various concentrations of TX-100 (0.5 to 5%) and SDS (0.5 to 2%) were added. The cultures were incubated at 37°C for 3 h with shaking (180 rpm), and the OD was measured. A 50% reduction in the OD in three independent experiments indicated the sensitivity of the culture to the treatment, as previously reported (11). The samples were also plated on LB agar plates to determine numbers of CFU, allowing confirmation of a reduction in the absorbance values.

(iii) Sensitivity to antibiotics. To determine the effect of antibiotics, the WT and *lpp* double-knockout mutant of serovar Typhimurium were spread on LB agar plates, and filter paper disks containing 10 to 100 μg of rifampin were placed on the agar plates. The plates were incubated overnight at 37°C, and the zone of inhibition of bacterial growth was measured (78). A 50% increase in sensitivity of the culture to rifampin was considered positive. For the antibiotic vancomycin, various concentrations (100 to 200 μg/ml) were added to the culture, which was originally grown to an OD of 0.4 to 0.5 and then diluted 50-fold. After incubation at 37°C for 3 h, a decrease in the absorbance (by 50%) was recorded as described above.

(iv) Release of β-lactamase. We also determined outer membrane permeability of the WT and Lpp double-knockout serovar Typhimurium mutant by measuring resident (chromosomally encoded) β-lactamase activity in the total soluble, cell extract fractions and in the supernatant fractions of cells grown to an OD of 0.6 (4). Briefly, after growth, the bacterial cultures were centrifuged, resuspended in the original culture volume of fresh LB medium, and sonicated for 3 min with intermittent cooling on ice. After spinning, the clear cell extracts and the original culture medium were used to measure the enzymatic activity. To a 100-μl sample, we added 900 μl of chromogenic cephalosporin substrate (CENTA; Calbiochem, San Diego, Calif.) at a final concentration of 25 μg/ml and measured the absorbance at 410 nm after incubation at 37°C for 1 to 6 h (5, 11). The percentage of β-lactamase release in the culture supernatant was calculated based on total enzymatic activity in the WT and compared with that of the Lpp mutant of serovar Typhimurium. We used *E. coli* DH5α and *E. coli* JE5505 (Lpp^- mutant) as controls in these experiments (4).

(v) Secretion of TTSS effector proteins. To examine TTSS effector proteins, the WT serovar Typhimurium and its *lpp* double-knockout mutant were grown to an exponential phase (OD 0.4 to 0.5), and the culture supernatants (1 liter each) were precipitated with 10% trichloroacetic acid for 1 h at 4°C (81). The experiment was designed to monitor activity of only *Salmonella* pathogenicity island 1 (SPI-1)-encoded TTSS. After centrifugation, the trichloroacetic acid precipitates were dissolved in Laemmli's sample buffer (2) and subjected to SDS–12% PAGE. The gels were stained with either Coomassie blue or SYPRO RUBY (Bio-Rad, Hercules, Calif.). Alternatively, the protein bands were transferred to the polyvinylidene difluoride membrane, and the latter was stained and destained (2). The selected bands from the membrane were subjected to NH_2-terminal sequence analysis at the Protein Chemistry Core Laboratory, UTMB (Galveston, Tex.).

In vitro binding assay. The binding assay for various serovar Typhimurium strains was performed as previously described (25). Approximately 5×10^5 T84 intestinal epithelial cells/well were seeded into 24-well tissue culture plates and incubated overnight at 37°C with 5% CO_2. Cells were infected with serovar Typhimurium strains (grown to an OD of 0.4 to 0.5) at a multiplicity of infection (MOI) of 10:1 and incubated at 4°C (to inhibit invasion) for 1 h. Binding of the bacteria to the host cell monolayer was facilitated by centrifugation in an IEC Centra-7 (International Equipment Company, Needham Heights, Mass.) at 1,500 rpm for 10 min (22). Unbound bacteria were aspirated, cells were washed four times with PBS and lysed with 0.1% TX-100, and various dilutions of the cell lysates were plated onto SS agar plates for determining numbers of CFU.

Invasion assay. Invasion of T84 cells by the *lpp* double-knockout mutant, WT serovar Typhimurium, and the complemented strain was measured as described previously (14). Briefly, cells were seeded and infected as described for the binding assay. After 1 h of incubation at 37°C following infection, cells were washed three times with PBS and incubated at 37°C for an additional hour with gentamicin-containing medium (100 μg/ml) to kill extracellular bacteria. Following incubation, gentamicin-containing medium was removed, and the cells were washed six times with PBS and lysed with 0.1% TX-100. Then, various dilutions of the cell lysates were plated onto SS agar plates for determining numbers of CFU. For both binding and invasion assays, single colonies of various bacterial strains were grown in LB medium overnight at 37°C with shaking (180 rpm) in the presence of appropriate antibiotics. The cultures were diluted (50 to 100-fold) in the fresh medium and grown to an OD of 0.4 to 0.5. We used a well-characterized, nonflagellated and invasive mutant of *Salmonella enterica* serovar Dublin SL5928 and its respective WT as controls in our invasion assays (64). The mutated strain was nonmotile because of the inactivation of the flagellin gene *fliC* by transposon insertion (64).

Cytotoxicity and cell death. The release of lactate dehydrogenase (LDH) enzyme from the host cells was used to measure cytotoxicity of various serovar Typhimurium strains by employing the CytoTox 96 LDH assay kit (Promega, Madison, Wis.). The cell death was examined by following the described method (13). RAW264.7 and T84 cells were seeded into six-well tissue culture plates (4×10^6 cells/well) and incubated overnight at 37°C. Cells were infected with various serovar Typhimurium strains at an MOI of 10:1 and incubated at 37°C for 1 h. After incubation, medium containing unbound bacteria was aspirated, and cells were washed three times with PBS and incubated in gentamicin-containing medium for 1 h at 37°C. Following incubation, cells were washed and incubated in a fresh antibiotic-free medium for 24 h, and their morphology was examined under a Zeiss LSM 510 meta laser scanning confocal microscope (Infectious Disease and Toxicology Optical Imaging Core Facility, UTMB, Galveston, Tex.). The average percentage of dead cells was estimated in 20 microscopic fields. The supernatants from RAW264.7 and T84 cells were examined for the release of LDH at various time points (0, 6, 12, and 24 h) after infection.

Intracellular survival. The assay was performed using RAW264.7 and T84 cells, following the described method (49). Murine macrophages and human T84 cells were seeded in 24-well tissue culture plates (5×10^5 cells/well) and incubated overnight at 37°C with 5% CO_2. Cells were infected with the *lpp* double-knockout mutant and its complemented strain, as well as the WT parental strain, at an MOI of 10:1 and incubated for 1 h. After incubation, the extracellular bacteria were removed by aspiration, and cells were washed three times with PBS and incubated in gentamicin (100 μg/ml)-containing medium for 1 h. The gentamicin-containing medium was aspirated, and cells were washed three times with PBS and then incubated in a fresh medium containing a minimum concen-

tration of gentamicin (5 μg/ml) for 1, 6, 12, and 24 h. After three washes with PBS, cells were lysed by using 0.1% TX-100, cell lysates were plated on SS agar plates, and the survival and growth of the *Salmonella* strains inside T84 and RAW264.7 cells were assessed.

Motility assay. LB medium with 0.35% agar was used to characterize the motility phenotype of the *lpp* double-knockout mutant, along with the WT and the complemented strain of serovar Typhimurium (19). The overnight culture of each *Salmonella* strain was adjusted to the same optical density, and equal numbers of CFU (10^6) were stabbed into 0.35% LB agar. Plates were incubated at 37°C overnight, and the motility was assayed by examining the migration of bacteria through the agar from the center towards the periphery of the plate.

In vitro cytokine production. RAW264.7 and T84 cells were seeded into six-well plates at a concentration of 5×10^6 cells/well. RAW264.7 cells were infected with heat-killed (incubated at 65°C for 30 min) *lpp* double-knockout mutants or the complemented or WT strain at an MOI of 0.1:1 and incubated for 8 h at 37°C with 5% CO_2. After incubation, culture supernatants were collected, centrifuged at 10,000 rpm for 10 min, and saved at −20°C. Heat-killed bacteria were used to prevent invasion-induced cytokine production in RAW264.7 cells. The T84 cells were similarly infected with either live (at an MOI of 10:1) or heat-killed (MOI of 0.1:1) bacteria. Cells infected with live bacteria were incubated for 1 h, washed, and incubated for another hour with medium containing gentamicin (100 μg/ml). After incubation, cells were washed again and incubated with fresh medium without the antibiotic for 12 h. T84 cells treated with killed bacteria were incubated for 8 h at 37°C, as described for the macrophages. Culture supernatants were collected and stored at −20°C. These supernatants were used for measuring TNF-α and IL-8 levels.

ELISA. Levels of TNF-α and IL-8 were determined in the tissue culture supernatant of RAW264.7 and T84 cells infected with various serovar Typhimurium strains using Enzyme-linked immunosorbent assay (ELISA) (15). Briefly, the purified anticytokine capture antibodies (Pharmingen, San Diego, Calif.) were diluted to 1 to 4 μg/ml in binding buffer (0.1 M sodium bicarbonate buffer [pH 9.0]). The diluted antibodies were added to the wells of ELISA high-binding microtiter plates (Corning Costar, Corning, N.Y.) and incubated overnight at 4°C. Next, the capture antibodies were removed, and any nonspecific binding was blocked by adding 200 μl of blocking buffer (10% fetal bovine serum in PBS) to each well. The plates were incubated at room temperature for 2 h. After incubation, wells were washed three times with PBS-Tween (0.05%) (PBST) buffer.

A 100-μl aliquot of samples or standards was added, and the plates were incubated overnight at 4°C. After incubation, plates were washed six times in PBST. Detection antibodies (biotinylated anticytokine; Pharmingen) were diluted to 0.5 to 2 μg/ml in blocking buffer and added to the wells. Plates were incubated for 45 min at room temperature. After the plates were washed six times in PBST, an enzyme conjugate (streptavidin-conjugated HRP) was diluted to an optimal concentration in blocking buffer and added to the wells. The plates were incubated at room temperature for 30 min and washed six times with PBST. Next, 2,2′-azin-bis-(3-ethylbenzthiazoline-6-sulfonic acid) substrate solution [150 mg of 2,2′-azin-bis-(3-ethylbenzthiazoline-6-sulfonic acid) in 0.1 M anhydrous citric acid, adjusted to pH 4.35 with sodium hydroxide] was mixed, and 100 μl of 3% H_2O_2 was added to each 11 ml of substrate solution. A 100-μl aliquot was dispensed in each well, and the plates were incubated (for 5 to 80 min) for color development. The color reaction was stopped by adding 50 μl of stopping solution (20% SDS–50% diethyl formamide). Finally, the optical density was read with a microtiter ELISA plate reader (Molecular Devices Corp., Sunnyvale, Calif.) at 405 nm.

Mouse inoculation. C57/BL6 female mice (6 to 8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, Maine). They were challenged orally or intraperitoneally (i.p.) with various doses either of WT serovar Typhimurium or with the *lpp* (single- or double-knockout) isogenic mutants. Mice were observed daily for signs of distress and mortality for up to 2 months. BALB/c SCID mice were similarly purchased from Jackson Laboratory and challenged with various doses of the *lpp* double-knockout mutant by the i.p. route. As a control BALB/c mice were infected with similar doses of either WT or the *lpp* double-knockout mutant. The animals were observed for mortality for 1 month.

Statistical analysis. Where appropriate, the data were analyzed using Student's *t* test, and *P* values of ≤ 0.05 were considered significant.

Nucleotide sequence accession number. The DNA sequence of the *lpp* genes and the flanking DNA was submitted to GenBank under accession number AY333760.

RESULTS

Cloning and sequence analysis of the serovar Typhimurium *lpp* gene. Murein Lpp was shown by us and other investigators to induce in vitro and in vivo cytokine production and toxic shock (63, 84, 85). However, detailed studies in which targeted null mutants were developed using marker exchange mutagenesis have not been performed with any enteric pathogen to demonstrate the role of Lpp in bacterial pathogenesis. It is known that a single copy of the Lpp-encoding gene (*lpp*) located at 36 min on the chromosome exists in *E. coli* (62). By using specific primers to the *lpp* gene of *E. coli* (7), we amplified the *lpp* gene from the chromosomal DNA of serovar Typhimurium strain 14028. This PCR fragment was cloned into a TA cloning vector (Stratagene) and subjected to automated DNA sequence analysis in the Protein Chemistry Core Laboratory, UTMB. Our subsequent studies demonstrated that two copies of the *lpp* gene, separated by 82 bp and designated *lpp1* and *lpp2*, existed in the chromosome of serovar Typhimurium (Fig. 1). The presence of two *lpp* gene copies was confirmed by the recent genome sequencing of *S. enterica* serovar Typhi CT18 and of serovar Typhimurium LT2 (56, 65). The availability of the entire genome sequence of *Salmonella* strains allowed us to amplify the flanking DNA sequences to the *lpp* genes for preparing isogenic mutants by double-crossover homologous recombination.

The DNA and amino acid sequence homologies between *E. coli* Lpp and serovar Typhimurium Lpp1 and Lpp2 are shown in Fig. 2. The homology between the *E. coli lpp* gene and the serovar Typhimurium *lpp1* gene was 96%, whereas the homology between *E. coli lpp* and serovar Typhimurium *lpp*2 was 79% at the DNA level. The DNA homology between two copies of the *lpp* gene (*lpp1* and *lpp2*) of serovar Typhimurium was 79%. At the amino-acid level, *E. coli* Lpp and serovar Typhimurium Lpp1 exhibited 97% homology. The homology was 84% between *E. coli* Lpp and serovar Typhimurium Lpp2. The two copies of Lpp (Lpp1 and Lpp2) shared 86% homology (Fig. 2). The Lpp2 copy of serovar Typhimurium contained an additional amino acid residue, asparagine (N), at position 5, which was missing in *E. coli* Lpp and serovar Typhimurium Lpp1.

Analysis of the *lpp* isogenic mutants of serovar Typhimurium. As shown in Fig. 1A, two copies of the *lpp* gene (*lpp1* and *lpp2*) were replaced by a Kn^r gene cassette in the *lpp* double-knockout mutant. Deletion of the *lpp* genes from serovar Typhimurium double-knockout mutants was confirmed by Southern blot analysis using the *lpp* gene, Kn^r gene cassette, and pJQ200SK plasmid vector as probes. By using the Kn^r gene cassette as a probe, the size of the radioactive band in the digested (BglII and MluI enzymes) chromosomal DNA of two selected serovar Typhimurium *lpp* double-knockout mutants (designated mutants 66 and 67) was 0.6 kb larger (3.0 kb) than that seen in WT serovar Typhimurium (2.4 kb). This increase in size was due to the insertion of the Kn^r gene cassette (1.2 kb) in the chromosome of the *lpp* double-knockout mutants. The increase in size was by only 0.6 kb and not 1.2 kb, because 559 bp of the DNA containing two copies of the *lpp* gene and 82 bp of the DNA fragment between the two copies of the *lpp* gene were deleted from the *lpp* double-knockout mutants. As expected, the digested chromosomal DNA from WT serovar Typhimurium did not react with the Kn^r gene probe (data not shown).

Since both copies of the *lpp* gene (*lpp1* and *lpp2*) were deleted from serovar Typhimurium mutants 66 and 67, the *lpp*

```
                                15                  30                  45
E. coli Lpp                     MKAT-KLVLGAVILG STLLAGCSSNAKIDQ LSSDVQTLNAKVDQL
S. typhimurium Lpp1             MNRT-KLVLGAVILG STLLAGCSSNAKIDQ LSSDVQTLNAKVDQL
S. typhimurium Lpp2             MNRTNQLILGAVVLG STLLAGCSSNAKIDQ LSSDVQTLSAKVEQL
                                60                  75    79
E. coli Lpp                     SNDVNAMRSDVQAAK DDAARANQRLDNMAT KYRK
S. typhimurium Lpp1             SNDVNAMRSDVQAAK DDAARANQRLDNQAT KYRK
S. typhimurium Lpp2             SNDVNAMRSDVQAAK DDAARANQRLDNKVF RICK
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Organism	Nucleotide (*lpp*)	Amino Acid (Lpp)
***E. coli lpp* (Lpp)** ***S. typhimurium lpp1* (Lpp1)**	96%	97%
***E. coli lpp* (Lpp)** ***S. typhimurium lpp2* (Lpp2)**	79%	84%
***S. typhimurium lpp1* (Lpp1)** ***S. typhimurium lpp2* (Lpp2)**	79%	86%

FIG. 2. Nucleotide and amino acid sequence homologies between lipoproteins of *E. coli* and serovar Typhimurium 14028. *lpp*, lipoprotein gene; Lpp, lipoprotein. Both *E. coli lpp* and *S. enterica* serovar Typhimurium *lpp1* genes contained 237 nucleotides, whereas the *S. enterica* serovar Typhimurium *lpp2* gene contained 240 nucleotides.

gene probe did not react with the digested chromosomal DNA from these mutants. Neither digested chromosomal DNA from the double-knockout mutants nor that from WT serovar Typhimurium reacted with the plasmid pJQ200SK when it was used as a probe, indicating the complete loss of the suicide vector from the serovar Typhimurium *lpp* isogenic mutants 66 and 67 after homologous recombination. As a positive control, we used the pJQ200SK plasmid digested with BamHI, which exhibited a 5.4-kb band (data not shown).

The absence of Lpp protein in the mutant strains was confirmed by Western blot analysis using *Y. enterocolitica* anti-Lpp monoclonal antibody (85). As is evident from Fig. 3A, a 6.3-kDa band representing Lpp was visualized in the outer membrane preparation of WT serovar Typhimurium. The corresponding band in *lpp* double-knockout mutants 66 and 67 was missing. The *E. coli* JE5505 strain lacking Lpp similarly did not exhibit a 6.3-kDa band. These isogenic mutants synthesized similar amounts of LPS, as seen with WT serovar Typhimurium. The LPS levels were determined by the *Limulus* amebocyte lysate assay (84). We then examined whether the complemented strains of serovar Typhimurium could synthesize Lpp. As is evident from Fig. 3B, both WT serovar Typhimurium and the complemented strains (66-C and 67-C) synthesized Lpp, whereas the *lpp* double-knockout mutants (66 and 67) were negative for Lpp synthesis. *E. coli* JE5505 and *E. coli* DH5α were used as negative and positive controls, respectively. We noted that the *lpp* double-knockout mutants had the WT growth phenotype when grown in LB and M-9 synthetic medium, indicating no auxotrophy (data not shown).

To characterize the *lpp* single-knockout mutants of serovar Typhimurium prepared using a suicide vector, pDMS197, a PCR method was used. For the *lpp1* single-knockout mutant, the primer sets used were K3 and K5, F4 and R3, F4 and K5, R3 and K3, and F5 and R3 (Fig. 1A and Table 2). These primers amplified DNA fragments of the expected sizes of 1.2, 1.9, 1.3, 1.3, and 0.39 kb, respectively. Similarly, when primer sets K5 and K3, F1 and R1, F1 and K3, R1 and K5, and F4 and R4 were used for the *lpp2* single-knockout mutant, fragments of expected sizes of 1.2, 4.8, 3.0, 2.9, and 0.29 kb, respectively, were amplified (Fig. 1A and Table 2). These data indicated insertion of the kn^r gene cassette at the correct location, as well as the correct conjunctional sequences in the *lpp* single-knockout mutants of serovar Typhimurium.

Likewise, when primer sets, F4 and kt and R3 and K2, were used with the λ Red system, PCR products of 1.1 and 1.3 kb were amplified from the *lpp1* knockout mutant. With primer sets of F4 and kt and R3 and k2, 1.4- and 1.0-kb DNA fragments were amplified from the *lpp2*-knockout mutant, indicating the correct genotype of the mutant (Fig. 1B and Table 2).

Cell membrane integrity of the *lpp* double-knockout mutant. It has been reported that murein Lpp is involved in maintaining the structural integrity of the *E. coli* cell envelope (73, 83). Therefore, we examined whether the outer membrane integrity of the *lpp* double-knockout mutant (67) was altered, compared to that of WT serovar Typhimurium, by using different assays, such as analysis of outer membrane blebbing, sensitivity to detergents, permeability of bacterial cells to antibiotics, and the release of β-lactamase from the bacterial cells. We did not observe blebbing of the outer membrane in the mutant strain by transmission electron microscopy. There was no difference

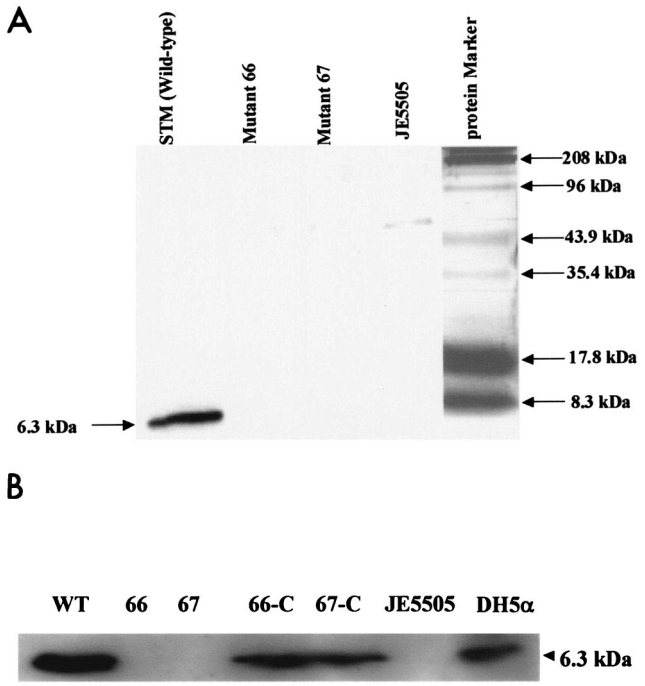

FIG. 3. (A) Western blot analysis on the *lpp* double-knockout mutants of serovar Typhimurium. The outer membranes of serovar Typhimurium WT, *lpp* double-knockout mutants (mutants 66 and 67), and *E. coli* JE5505 (Lpp$^-$) were isolated and separated by SDS–15% PAGE. The protein bands were then transferred to a nitrocellulose membrane and probed with *Y. enterocolitica* Lpp monoclonal antibody (1:1,000 dilution, determined empirically). The secondary antibodies (1:25,000 dilution) were goat antimouse conjugated with HRP. The blots were developed using an enhanced chemiluminescence kit. A band corresponding to the size of approximately 50 kDa in *E. coli* JE5505 appeared to be nonspecific and reacted with the antibodies. (B) Western blot analysis on the complemented *lpp* double-knockout mutants of serovar Typhimurium. The whole-cell lysates of serovar Typhimurium, *lpp* double-knockout mutants (66 and 67), the complemented *lpp* double-knockout mutants (66-C and 67-C), *E. coli* JE5505 (Lpp$^-$), and *E. coli* DH5α (Lpp positive) were isolated and separated by SDS–15% PAGE. The protein bands were then transferred to a nitrocellulose membrane and probed with *Y. enterocolitica* Lpp monoclonal antibody (1:1,000 dilution). The secondary antibodies (1:25,000 dilution) were goat antimouse conjugated with HRP. The blots were developed by using an enhanced chemiluminescence kit. The arrow indicates the correct size of Lpp.

in the sensitivity of the *lpp* double-knockout mutant to the effect of the detergent TX-100 (tested at concentrations of 0.5, 1, 2, and 5%) compared to results with WT *Salmonella*. The *E. coli* Lpp mutant JE5505, on the other hand, was sensitive to TX-100 at 5% compared to *E. coli* DH5α (Table 3). Similarly, both the WT and the *lpp* double-knockout serovar Typhimurium mutant were resistant to the effect of SDS at concentrations of 0.5, 1, and 2%. *E. coli* JE5505 was sensitive to SDS even at a concentration of 0.5% (Table 3).

The sensitivity of the *lpp* double-knockout mutant to the antibiotics rifampin (10, 50, and 100 μg) and vancomycin (100, 150, and 200 μg/ml) was similar to that of the WT serovar Typhimurium. The *E. coli* Lpp mutant was sensitive to both of these antibiotics (Table 3). Finally, the release of β-lactamase by the WT and the serovar Typhimurium Lpp mutant remained unaltered, while increased release of this enzyme was noted with the Lpp *E. coli* mutant (Table 3).

We also noted that the secretion of SPI-1-encoded TTSS effector proteins remained unaltered in the *lpp* double-knockout mutant versus WT serovar Typhimurium. We specifically examined secretion of SipA, SipB, SipC, and invJ in the culture supernatants. Bands of correct size for SipA (89 kDa), SipB (63 kDa), SipC (42 kDa), and invJ (40 kDa) were detected by SDS-PAGE. The identity of SipA and SipC was further confirmed by NH_2-terminal sequence analysis of 10 amino acid residues. These data suggested that the integrity of the cell envelope in the serovar Typhimurium *lpp* double-knockout mutant was not affected.

Invasive ability of various serovar Typhimurium strains. Infection of T84 intestinal epithelial cells with the *lpp* double-knockout mutant revealed that the deletion of *lpp* genes rendered *Salmonella* significantly reduced in invasive activity (Fig. 4). The invasive ability of the selected *lpp* double-knockout mutant (67) was reduced by 500- to 1,000-fold from that of WT serovar Typhimurium. However, the invasive capacity of the mutant was significantly restored after complementation (Fig. 4). The binding of the *lpp* isogenic mutant to host cells was minimally affected (data not shown). Studies have shown that *Salmonella* invades chicken ovarian cells, leading to contamination of egg follicles and vertical transmission of *Salmonella* (75). Further, *Salmonella* specifically targets and preferentially replicates within tumor cells (54). We, therefore, used A2780,

TABLE 3. Integrity of the membrane in the *lpp* double-knockout mutant of *Salmonella* is not affected

Strain	No. of OMV[a]	β-Lac[b] (% release)	Resistance to:			
			Triton X-100[c] (%)	SDS[d] (%)	Rif[e] (μg)	Van[f] (μg/ml)
WT serovar Typhimurium	0	48.4 ± 7.9	>5	>2	10	>200
Salmonella Lpp Mutant (67)	0	51.1 ± 7.3	>5	>2	10	>200
E. coli DH5α	0	15.0 ± 3.5	>5	>2	10	>200
Lpp mutant of *E. coli* (JE5505)	++	43.1 ± 6.8*	≤5	≤0.5	<10	≤100

[a] Number of outer membrane vesicles observed by electron microscopy in ultra-thin sections of bacteria. 0, no vesicle on cells; ++, many vesicles on all the cells (73).
[b] β-Lactamase activity present in the supernatant was indicated as percentage of the total activity (average values from triplicate experiments ± SD. The asterisk denotes statistically significantly values ($P \leq 0.05$) compared to the WT *E. coli* strain by Student's *t* test.
[c] Triton X-100 concentration (% vol/vol) leading to a 50% decrease in cell turbidity measured after 3 h of bacterial growth in the presence of TX-100. Three independent experiments were performed.
[d] SDS concentration (% weight/vol) leading to a 50% decrease in cell turbidity measured after 3 h of bacterial growth in the presence of SDS. Three independent experiments were performed.
[e] Rifampin concentration (μg/disk) leading to a more than 50% increase in the diameter of zone of bacterial inhibition with the Lpp mutants compared to their respective parental strains. Three independent experiments were performed.
[f] Vancomyin concentration (μg/ml) leading to a 50% decrease in cell turbidity measured after 3 h of bacterial growth in the presence of the antibiotic. Three independent experiments were performed.

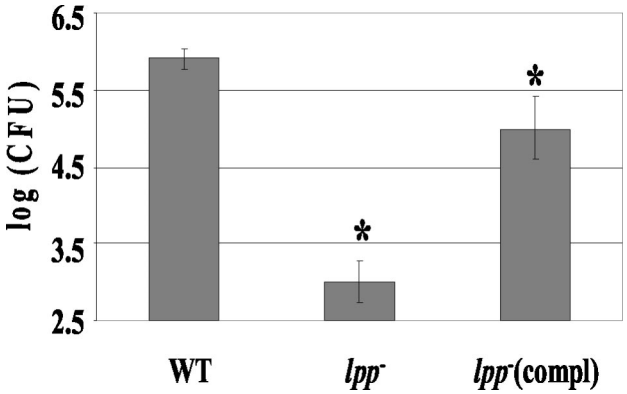

FIG. 4. Invasion of T84 cells by the *lpp* double-knockout mutant (*lpp*$^-$) (mutant 67), the WT, and the complemented (compl) strain of the *lpp* double-knockout mutant. The T84 cells were infected with *Salmonella* at an MOI of 10:1 for 1 h. The monolayers were washed and incubated with medium containing gentamicin for 1 h. After incubation, cells were washed, lysed with 0.1% TX-100, and plated on SS agar plates. The asterisk denotes statistical significance at *P* values of ≤0.05 by Student's *t* test, between the WT and mutant 67 (*lpp*$^-$) and between 67 (*lpp*$^-$) and 67-C [*lpp*$^-$compl]. The values for invasion between the WT and 67-C were not statistically significant. Arithmetic means ± standard deviations were plotted.

an ovarian tumor epithelial cell line, to confirm our invasion data obtained using T84 cells. Interestingly, the *lpp* double-knockout mutant did not invade A2780 cells, compared to the WT and the complemented strain of serovar Typhimurium. Likewise, minimal invasion was seen in HeLa cells infected with the *lpp* double-knockout mutant (data not shown). As with the T84 cells, we noted no significant effect on binding of the *lpp* double-knockout mutant to the tumor cell line and the HeLa cells.

We did not observe any difference in the binding of the *lpp* double-knockout mutant from that of WT serovar Typhimurium at 4°C, and no invasion was seen at 4°C with both the WT and *lpp* double-knockout mutant. The low invasion rate observed with the *lpp* double-knockout mutant was not because of lack of motility (see below, motility section), since in our invasion assays bacterial cells were centrifuged onto the monolayers. No statistically significant effect on the invasion rate was noted, whether or not the centrifugation step was included in the invasion assay. These data indicated that the defect in invasion was not the result of lack of motility or because of an inability of the mutant to bind to the host cells, but rather because Lpp had a putative role in cellular invasion. We used a well-characterized invasive and nonflagellated serovar Dublin mutant with its parental strain as controls in this assay. As expected, the WT serovar Dublin was as invasive for the T84 cells whether or not the bacterial cells were centrifuged onto the monolayer. However, with the serovar Dublin *fliC* mutant, a significant increase in invasion was noted when the bacterial culture was centrifuged onto the monolayer (data not shown).

Cytotoxicity and cell death associated with various serovar Typhimurium strains. To examine the role of Lpp in inducing cytotoxicity and cell death in host cells, we used RAW264.7

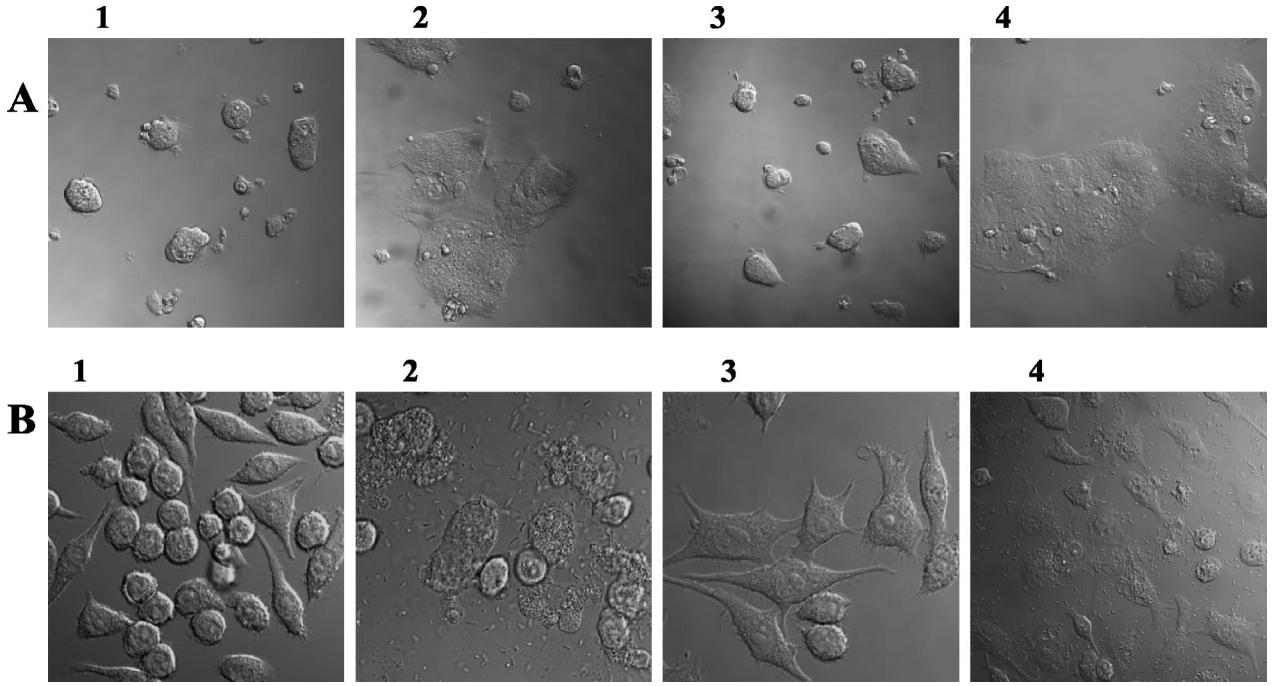

FIG. 5. Cytotoxicity and cell death induced by WT serovar Typhimurium and its various mutants in T84 (A) and RAW264.7 (B) cells. Cells were infected for 1 h with WT serovar Typhimurium, the *lpp* double-knockout mutant (67), or its complemented strain. Cells were washed and incubated with gentamicin-containing medium for 1 h. After incubation, cells were washed and further incubated with the antibiotic-free fresh medium for 24 h. Cells were gently washed twice in PBS and examined under a confocal microscope. Frame 2, cells infected with WT serovar Typhimurium (note dead cells); frame 3, cells infected with *lpp* double-knockout mutant (67) (note normal morphology of the cells); frame 4, cells infected with complemented strain (note dead cells); frame 1 represents a noninfected control.

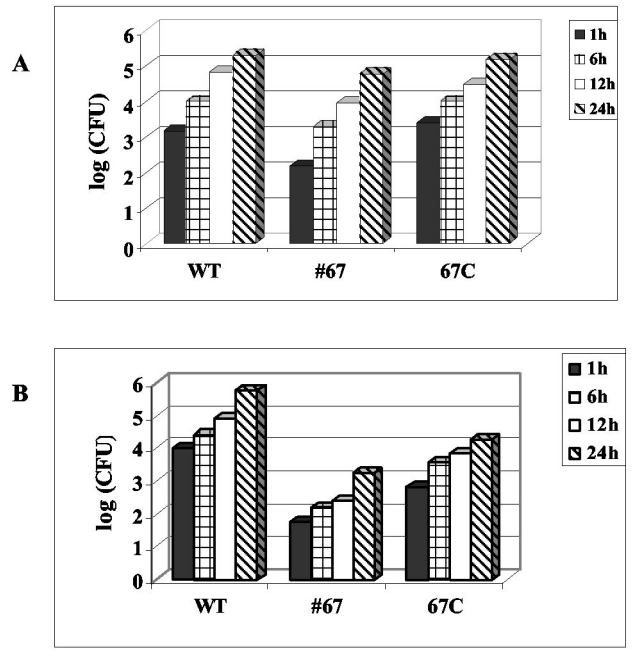

FIG. 6. Intracellular replication of the *lpp* isogenic mutant inside RAW264.7 cells (A) and T84 cells (B). Cells were infected with the *lpp* double-knockout mutant (67), the complemented *lpp* double-knockout mutant (67-C), and WT serovar Typhimurium 14028 for 1 h. Cells were washed and incubated for 1 h with gentamicin-containing (100 μg/ml) medium. After incubation, cells were washed and incubated in fresh medium containing a minimum concentration of gentamicin (5 μg/ml) for different time points (1, 6, 12, and 24 h). Finally, cells were lysed with 0.1% TX-100 and plated on SS agar plates to determine numbers of CFU. Three independent experiments were performed. Data from a representative experiment are shown here.

macrophage and T84 intestinal epithelial cell lines. Microscopic examination of T84 (Fig. 5A) and RAW264.7 (Fig. 5B) cells infected with the *lpp* double-knockout mutant revealed a reduction of about 80% in cell death (Fig. 5, frame 3), compared to cells infected with WT serovar Typhimurium (Fig. 5, frame 2). A large number of RAW264.7 and T84 cells infected with the WT strain exhibited features of apoptosis, such as condensation of chromatin, vacuolation, and membrane blebbing (data not shown). These data were confirmed by the LDH release assay performed on macrophages and T84 using a CytoTox 96 LDH assay kit (data not shown). The *lpp* double-knockout mutant of serovar Typhimurium caused a minimal release of LDH from the host cells. *Salmonella* strains complemented with *lpp* genes restored the WT phenotype (Fig. 5, frame 4), indicating that *Salmonella* Lpp played a role in the induction of death in the host cells. Frame 1 represented uninfected cells.

Intracellular survival of various serovar Typhimurium strains. Another virulence mechanism of *Salmonella* is the ability to survive the hostile environment inside professional phagocytes such as macrophages. Studies have shown that mutants of serovar Typhimurium that failed to replicate in cultured intestinal epithelial cells (e.g., Caco-2 and HeLa) and in macrophages were avirulent in a mouse model (16, 49, 50). To examine this, a RAW264.7 macrophage cell line was infected with the *lpp* double-knockout mutant, WT serovar Typhimurium, or complemented serovar Typhimurium strain for 24 h. As seen in Fig. 6A, the *lpp* double-knockout mutant was able to survive and replicate inside macrophages in a manner similar to that of WT serovar Typhimurium. We also examined the intracellular survival of serovar Typhimurium in T84 cells. Although the total number of *lpp* double-knockout mutants recovered from T84 cells was less than that of the WT, because of the decreased ability of the mutant to invade, the growth rate of the *lpp* double-knockout mutants was not significantly affected compared to those of the WT and complemented serovar Typhimurium strains in intestinal epithelial cells (Fig. 6B). These data indicated that Lpp was not required for the intracellular survival of *Salmonella* in macrophages and T84 cells.

Motility of various serovar Typhimurium strains. We performed a motility assay to test whether deletion of both of the *lpp* genes would have an effect on *Salmonella* motility. The *lpp* double-knockout mutant grew at the site of the initial inoculum but showed no motility, compared to the complemented and WT serovar Typhimurium, indicating that Lpp drastically affected *Salmonella* motility. To test whether this impairment in motility resulted from changes in flagellar production, we performed scanning electron microscopy. However, the electron micrograph (data not shown) indicated no change in the number of flagella per cell in the *lpp* isogenic mutant from that of the WT serovar Typhimurium.

In vitro cytokine production by various serovar Typhimurium strains. To test whether deletion of both of the *lpp* genes would affect the overall induction of TNF-α and IL-8, tissue culture supernatants from T84 and RAW264.7 cells stimulated with either heat-killed or live *Salmonella* were assayed for these cytokines. TNF-α production was significantly reduced in macrophages infected with the heat-killed *lpp*, double-knockout mutants, compared to results with the WT serovar Typhimurium (Fig. 7A). Neither the *lpp* double-knockout mutants nor the WT-infected T84 cells produced TNF-α; however, a similar reduction in IL-8 levels was observed in T84 cells infected with either live or heat-killed *lpp* double-knockout mutant, compared to the WT and complemented strains of serovar Typhimurium (Fig. 7B). These data indicated that *Salmonella* Lpp significantly contributed to the induction of inflammatory cytokines and that invasion of T84 cells was not required for IL-8 production, since IL-8 was also induced by heat-killed WT serovar Typhimurium. The reduced IL-8 production noted for the live or heat-killed *lpp* double-knockout mutant suggested a possible lack of signaling through the TLR2 rather than the lack of translocating TTSS-1 effectors.

Virulence in mice with various serovar Typhimurium strains. To investigate whether the Lpp deletion mutant (deletion of both Lpp1 and Lpp2) of serovar Typhimurium had altered virulence properties in vivo, we used a well-established mouse model. In initial experiments, we infected mice with various doses of either WT serovar Typhimurium or the Lpp double-knockout mutants to accurately calculate the lethal dose. Mice were challenged via the oral or i.p. routes and followed for 2 months for mortality. We determined that the lethal dose of WT serovar Typhimurium for oral challenge was 3,000 CFU, and that for i.p. challenge was 40 CFU. Results of a representative experiment are presented in Fig. 8A. Lpp-deficient serovar Typhimurium had no adverse health effects on mice following either oral challenge or i.p. challenge. In

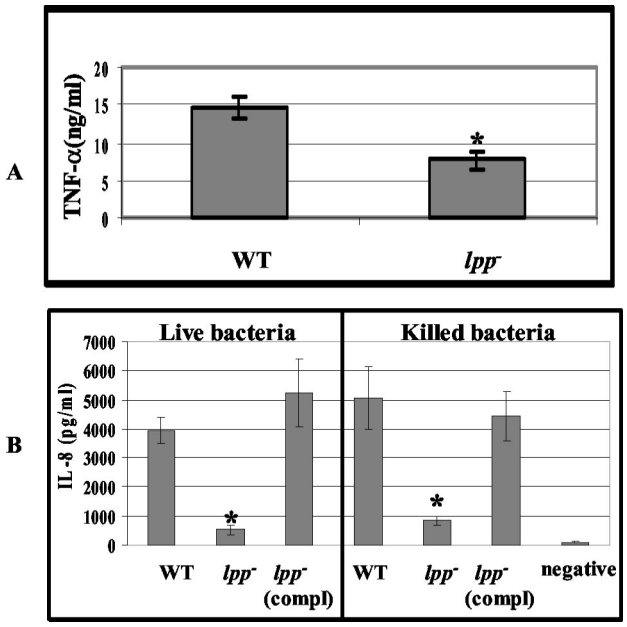

FIG. 7. TNF-α induced by *lpp* double-knockout mutant in RAW264.7 cells (A) and IL-8 production by T84 cells infected with live and heat-killed bacteria (B). Macrophages and T84 cells were stimulated with the heat-killed *lpp* double-knockout mutant or WT serovar Typhimurium at an MOI of 0.1:1 and incubated for 8 h. T84 cells were infected with live WT, the *lpp* isogenic mutant (67) (*lpp*$^-$), or the complemented *lpp* mutant [*lpp*$^-$ (compl)] at an MOI of 10:1 and incubated for 1 h. After incubation, cells were washed and incubated in gentamicin-containing medium for 1 h, after which cells were washed and incubated in antibiotic-free medium for 12 h. TNF-α and IL-8 levels were determined using ELISA as described in Materials and Methods. An asterisk denotes statistically significant data ($P \leq 0.05$) by Student's *t* test, between the WT and the *lpp* double-knockout mutant and between the *lpp* double-knockout mutant and its complemented strain. The values were not significant between WT and the *lpp* mutant complemented strain. Negative denotes no addition of the bacterial cells. The arithmetic means ± standard deviations were plotted. Compl denotes complemented strain.

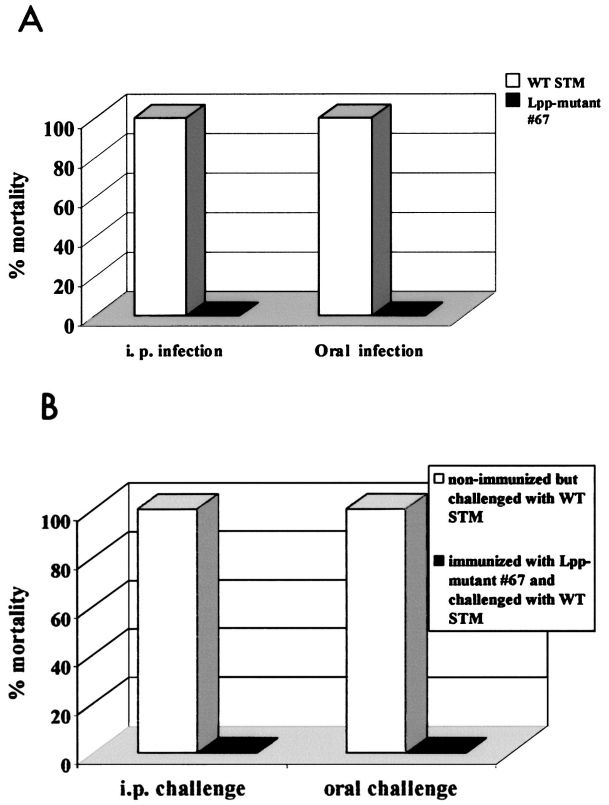

FIG. 8. (A) Mortality in mice following infection with the *lpp* double-knockout mutant (67) and WT serovar Typhimurium. Four groups of mice were used: two groups were infected orally with 3×10^3 CFU of the *lpp* mutant (67) or WT serovar Typhimurium. The other two groups were infected with 40 CFU of the *lpp* mutant (67) or WT serovar Typhimurium i.p. The group infected with WT serovar Typhimurium orally or i.p. showed 100% mortality compared to 100% survival in the groups that were infected with the mutant serovar Typhimurium. (B) Mortality in mice challenged with a virulent strain of serovar Typhimurium following immunization with the *lpp* double-knockout mutant of serovar Typhimurium (67). Out of four groups of mice used for the challenge study, two groups were immunized with mutant strain 67, and one group was orally inoculated (3×10^3 CFU) while the second group received i.p. (40 CFU) inoculation. The other two groups were left unimmunized as controls. After 2 months postimmunization, all groups of mice (immunized and nonimmunized) were challenged with a lethal dose of a virulent strain of serovar Typhimurium, either orally with 3×10^3 CFU or by the i.p. route with 3×10^3 CFU. Then, mice were observed for 4 weeks, and mortality was recorded.

contrast, mice challenged with WT serovar Typhimurium died within 10 to 12 days following oral or i.p. challenge. More importantly, mice infected with a two-logs-higher number of Lpp-deficient serovar Typhimurium were healthy and showed no signs of disease or discomfort (data not shown).

To investigate whether the Lpp-deficient serovar Typhimurium mutant might immunize mice to *Salmonella* infection, they were infected with the Lpp$^-$ mutant (double knockout) either orally (3,000 CFU) or i.p. (40 CFU) and then allowed to rest for 2 months. We noted that the *lpp* null mutant was cleared from mice within 2 to 3 weeks after infection (data not shown). Control mice were inoculated with saline. These mice were then challenged with WT serovar Typhimurium either orally (3,000 CFU) or i.p. (3000 CFU) and monitored for survival and disease. Mice immunized with Lpp-deficient serovar Typhimurium were completely protected from subsequent WT serovar Typhimurium challenge (Fig. 8B). These mice showed no signs of disease, and no deaths occurred over a 2-month period of observation. The unimmunized group of mice, which was given only saline, died within 10 to 12 days after challenge with WT serovar Typhimurium.

More importantly, 100% of the SCID mice lacking T, B, and NK cells inoculated i.p. with 500 CFU of the Lpp double-knockout mutant died. The death rate was 75% when mice were infected with 100 CFU, and 25% of the mice died at a dose of 10 CFU over a period of 1 month. In a parallel control experiment, in which BALB/c mice were infected with either 100 or 500 CFU of the Lpp mutant, 100% of the mice survived, while the death rate was 100% in mice infected with WT serovar Typhimurium. A total of four to five mice were used per group for these experiments. These data indicated a critical role of the immune system in providing protection against *Salmonella* infection.

We noted that infection of mice ($n = 5$ in each group) with the single *lpp* isogenic mutant (*lpp1* or *lpp2*) developed by using either the suicide vector or the λ Red system at a dose of 10^5

CFU by the i.p. route was not lethal as noted for the double-knockout mutant (mutant 67). Similarly, no lethality was noted with the mutants in mice when they were fed with 10^6 bacteria orally. All of the mice died within a week when challenged with WT serovar Typhimurium at these bacterial doses. We noted that the invasive ability of the *lpp1* and *lpp2* single-knockout mutants in T84 cells was approximately 2.4 logs higher than that of the Lpp double-knockout mutant, 67. The reduction in invasion by the double-knockout mutant from that by WT serovar Typhimurium was approximately 3.3 logs. Compared to the WT serovar Typhimurium, the single-knockout mutants exhibited 1.4 log less invasion in T84 cells. The invasive capacity of both the single-knockout mutants (*lpp1* or *lpp2*) was very similar. The use of this in vitro biological (invasion) assay with Lpp1 and Lpp2 mutants provided evidence that both of the *lpp* genes were functional. We opted for a biological assay for determining functionality of the *lpp1* and *lpp2* genes, since it is more accurate than Western blot analysis for evaluation of gene expression and function. As noted above, the invasive potential of Lpp1 and Lpp2 mutants was increased over that of the Lpp double-knockout mutant in T84 cells.

DISCUSSION

Murein Lpp from *E. coli* and *Y. enterocolitica* mimics many of the in vitro and in vivo properties associated with LPS (63, 84, 85). In this study, we provide for the first time conclusive evidence that Lpp plays an important role in *Salmonella* virulence and induction of systemic infections by developing *lpp* mutants through marker exchange mutagenesis. We used a suicide vector, pJQ200SK, for constructing the *lpp* double-knockout mutant, while both a suicide vector (e.g., pDMS197) and the λ Red system were employed for developing *lpp* single-knockout mutants of serovar Typhimurium. The rationale for selecting these two systems was that when the suicide vector pDM197 was employed, the *lpp1* gene was truncated with the Kn^r gene cassette, while the *lpp2* gene was deleted and replaced with the Kn^r gene cassette. Consequently, we were somewhat concerned about direct comparisons of our results obtained with the *lpp1*, *lpp2*, and *lpp1 lpp2* knockout serovar Typhimurium mutants, because of possible polar effects in the *lpp1* mutant.

We therefore also used the λ Red system for constructing the *lpp* single-knockout mutants. With this system, the *lpp1* and the *lpp2* genes were replaced with the Kn^r gene cassette as noted for the *lpp2* single-knockout and the *lpp* double-knockout mutants, resulting in direct comparison of the results. However, recently Murphy and Campellone (61) reported that extended expression of the recombination functions by using the λ Red system could induce a 10-fold increase in the rate of spontaneous mutations. We were therefore concerned about whether such mutations in vivo could lead to nonspecific effects rather than specific phenotypes associated with Lpp. Using different strategies to prepare the knockouts provided authenticity to our data, since the single-knockout mutants generated either by using the suicide vector or the λ Red system behaved very similarly in both in vitro and in vivo models of serovar Typhimurium infection.

Murein Lpp has been shown to play a role in stabilization of the bacterial cell envelope. An *E. coli* strain with a mutation in the *lpp* gene demonstrated outer membrane blebbing, sensitivity to TX-100, SDS, and antibiotics, and leakage of the enzymes RNase I and β-lactamase (11, 73, 83). Although mutation in the *E. coli lpp* gene caused the outer membrane to bleb outwards, the cytoplasmic membrane remained intact (83), thereby providing a barrier to some extent to the inner cellular components. Our studies with the *lpp* double-knockout mutant of serovar Typhimurium indicated no blebbing of the outer membrane, and the sensitivity of the *lpp* double-knockout mutant to detergent TX-100 and SDS was not altered. Further, the *lpp* double-knockout mutant was resistant to antibiotics, and the release of β-lactamase remained unaffected in the *lpp* double-knockout mutant compared to results for WT serovar Typhimurium (Table 3). The mechanism(s) of β-lactamase release in significant and comparable amounts from serovar Typhimurium and its *lpp* double-knockout mutant without indication of membrane vesicle formation is currently unclear (5).

Recently, several bacterial lipoproteins were identified that play an important role in bacterial pathogenesis (11, 18, 19, 27, 40, 82). Studies by Hazumi et al. (39) and Cascales et al. (11) demonstrated that Pal also was important in maintaining the outer membrane integrity of *E. coli* and that Pal contributed to bacterial virulence during sepsis. The Pal mutant of *E. coli* reduced mortality in mice and induced a low level of IL-6 compared to findings with mice infected with WT *E. coli* (40). Both Pal and murein Lpp are localized in the cell envelope, interact with the peptidoglycan layer, and have a common chemical structure at the amino terminus (47). Overproduction of Pal was shown to restore the outer membrane integrity of an *E. coli lpp* mutant; however, overproduction of murein Lpp did not complement the Pal mutant (11). Although we have not examined the expression level of gene-encoding Pal with our serovar Typhimurium *lpp* null mutant (double knockout), it is possible that Pal might compensate for the lack of Lpp, thereby providing stability to the outer membrane, as noted in this study.

Invasion of the host cells is an important virulence feature during systemic infection with *Salmonella*. This event is mediated by multiple bacterial virulence determinants, and the most important are the TTSS proteins encoded by the SPI-1 (17, 30, 46). The mechanism by which Lpp might affect *Salmonella* invasion (Fig. 4) is not known. Activation of a signal transduction pathway in the host cells involving Ca^{2+} and actin rearrangement, leading to membrane ruffling, is a key event in internalization of the bacteria (10, 32, 35, 68). Invasion-defective mutants of *Salmonella* were unable to induce membrane ruffling. Epidermal growth factor has been shown to rescue the ability of such mutants to invade host cells (28, 29). Therefore, it is possible that Lpp might be involved in triggering such signaling, most likely through TLR2, and this needs further investigation.

Recent studies also indicated that lipoproteins (other than Lpp and Pal) constitute a significant component of TTSS (e.g., PrgH, PrgK, and InvH), TTSS-associated invasion proteins (10, 17, 18), and the flagellar basal body (19). However, these lipoproteins are distinct from murein Lpp and Pal. Since in the serovar Typhimurium *lpp* null mutant (double knockout) the secretion of TTSS effector proteins encoded on the SPI-1 was not affected, these data suggested that Lpp might have a direct

or indirect (regulatory) role in modulating serovar Typhimurium virulence.

Cytotoxicity and the ability to induce cell death are important virulence mechanisms of *Salmonella* (13, 48, 52, 59). *Salmonella*-induced cytotoxicity has been shown to be triggered through LPS and components of the TTSS (30, 36, 52, 80). However, whether Lpp leads to cell toxicity and cell death is not known. Data from this study indicated that the ability of the *lpp* double-knockout mutant to induce cell death in RAW264.7 and T84 cells was significantly reduced (Fig. 5). Although *Salmonella*-induced cytotoxicity and cell death were shown to be triggered by transmembrane signaling, requiring no bacterial internalization, *Salmonella* strains incapable of efficiently invading host cells failed to induce cytotoxicity and cell death (59). We similarly observed that the *lpp* double-knockout mutant and WT serovar Typhimurium entered macrophages in almost similar numbers and both replicated intracellularly; however, significant cytotoxicity and cell death were observed only in macrophages infected with the WT and the *lpp*-complemented strain.

Studies of Chen et al. (13) and Santos et al. (69) noted that mutations in the invasion-associated type III secretion proteins InvJ, SpaO, SipB, SipC, and SipD encoded by SPI-1 abolished cytotoxicity in macrophages. Although the mechanism by which Lpp induces cytotoxicity is not fully defined, the possible involvement of a TLR-dependent stimulation of macrophages has been a matter of speculation (43). The reduced cytotoxicity induced by the *lpp* double-knockout mutant did not seem to be the result of a low bacterial load, as observed in T84 cells, since the reduction in cytotoxicity was also observed in RAW264.7 cells, which had an intracellular bacterial load similar to that seen with the WT-infected macrophages (Fig. 6A and B). These results suggested that Lpp directly contributed to the cytotoxicity and death of the host cells.

Survival of *Salmonella* inside macrophages is important during systemic infection and is dependent on the ability of the bacteria to modulate intracellular trafficking and to neutralize the activity of the antimicrobial effector mechanism (6, 16, 41). Therefore, the ability of the Lpp^- mutant (double knockout) to survive and replicate inside macrophages and T84 cells was examined. Effectors of SPI-2 TTSS have been implicated in the modulation of intracellular trafficking and have been shown to be required for intracellular growth and systemic infection (6, 37, 42, 76). Indeed, mutation in SPI-2 TTSS exhibited a defect in the intracellular growth of *Salmonella* in both macrophages and epithelial cells (16, 41). Our studies indicated that Lpp did not play a role in the intracellular survival and replication of *Salmonella*, since the Lpp mutant (double knockout) multiplied in a manner similar to that of WT serovar Typhimurium in both macrophages and epithelial cells (Fig. 6A and B).

The *lpp* double-knockout mutant displayed an impaired motility compared with the WT and the complemented strain, with no change in the number of flagella per cell in the *lpp* double-knockout mutant compared to that of the WT serovar Typhimurium, as determined by electron micrographs (data not shown). These data suggested that a mutation in Lpp might affect the composition rather than the flagellar number, which could alter flagellar motion. Indeed, mutations in Lnt and Lgt (enzymes required for biosynthesis of Lpp) were shown to affect flagellar assembly and motility (19).

The role of inflammatory cytokines has been established in the pathogenesis of systemic infection and septic shock (36, 60, 77). Studies from our laboratory showed that Lpp from *E. coli* induced TNF-α and IL-6 in macrophages from LPS-responsive and LPS-nonresponsive mice and acted synergistically with LPS to induce proinflammatory cytokine production (85). Neilsen et al. (63) reported that Lpp from *E. coli* DH5α caused the up-regulation of the same battery of cytokines induced by LPS. Reduction in the levels of TNF-α and IL-8 induced by the Lpp^- mutant (double knockout) indicated that *Salmonella* Lpp significantly contributed to the induction of inflammatory cytokines (Fig. 7).

Studies have shown that serovar Typhimurium induces the production of IL-8 in T84 cells through the NF-κB pathway (24, 34). Induction of IL-8 and NF-κB activation in intestinal epithelial cells infected with *Salmonella* was preceded by and required an increase in intracellular Ca^{2+} (34). Pretreatment of cells with p38 mitogen-activated protein kinase inhibitors prevented *Salmonella*-induced IL-8 production (45). Therefore, *Salmonella* induction of IL-8 may be mediated by Lpp activation of the mitogen-activated protein kinase pathway through TLR2 (1, 33, 45, 51, 70) and needs further investigation. This hypothesis appears attractive, since the heat-killed *Salmonella* WT serovar Typhimurium and complemented strain-infected T84 cells synthesized similar levels of IL-8, while the *lpp* (double-knockout) null mutants (live or heat-killed) were defective in IL-8 production, clearly establishing the role of Lpp in *Salmonella* virulence (Fig. 7). These data also suggested that for the *lpp* null mutant, TLR2 signaling associated with Lpp leading to the IL-8 production was affected.

Our data from in vitro experiments using RAW264.7 and T84 cells indicated that a deletion in the *lpp* genes rendered *Salmonella* defective in the invasion, cytotoxicity, and induction of cytokines. The in vivo studies performed with mice confirmed that the Lpp double-knockout mutant was indeed avirulent in mice (Fig. 8A). Further, mice immunized with the *lpp* null mutant were protected from subsequent challenge with WT serovar Typhimurium after the nonspecific immunity due to macrophage activation was returned to normal (Fig. 8B). Susceptibility of SCID mice to death with the *lpp* null mutant reflected the contribution of the immune response during salmonellosis.

Further, deletion of either one of the *lpp* genes was sufficient to render *Salmonella* avirulent in mice at the tested dose. Interestingly, the single-knockout mutants (*Lpp1* and *lpp2* mutants) were more invasive in vitro than the *lpp* double-knockout mutant; however, this difference in invasion did not alter lethality in mice. More detailed studies are needed, using higher bacterial doses and determining the mean time of death, to demonstrate whether Lpp1 or Lpp2 contributes more towards bacterial virulence.

The increased invasiveness of the single-knockout (*lpp1* and *lpp2*) mutants indicated that both copies of the *lpp* gene were functional. These data also suggested that attenuation of virulence observed in the double-knockout (*lpp1 lpp2*) mutant could be more related to alteration of TNF-α or IL-8 release

than to invasiveness and should be studied in detail in the future.

Interestingly, we determined that the *lpp* double-knockout mutant was resistant to bacteriophages P22 and ES18. While this work was in progress, Dailey and Macnab (19) reported that transposition within the *lpp* and *ltg* genes (encoding an enzyme required for the modification of Lpp) conferred resistance to a P22 phage. In addition, InvG, a component of the TTSS, has been shown to be required for f1 phage assembly (18). Our phage-binding assay (53) indicated a minimal reduction of P22 phage binding to the *lpp* double-knockout mutant, compared to that to WT serovar Typhimurium (data not shown), suggesting that Lpp might be involved in phage assembly.

In conclusion, the data presented in this study indicated that murein Lpp plays an important role in the virulence of serovar Typhimurium and that the effect of Lpp is not pleiotropic due to alteration in the cell envelope integrity. The ability of the *lpp* null mutant (double knockout) to survive and replicate inside macrophages and epithelial cells and to secrete SPI-1 TTSS effector proteins further indicated that the integrity of the cell envelope of the Lpp mutant remained intact. Although the precise mechanism of Lpp's action is not known, it is possible that Lpp mediates invasion and other cellular responses through activation of TLR2 or possibly indirectly by regulating serovar Typhimurium virulence factors (11). Our future studies will be targeted at defining the mechanism by which Lpp affects virulence of *Salmonella* and further exploring bacterium-host cell interaction with the *lpp* single- and double-knockout mutants. Further, it will be crucial to determine the importance of duplication of the *lpp* gene in *Salmonella*. Indeed, there are reports of duplication of the cholera toxin gene during infection through homologous recombination (57). Our future studies also will include delineating the mechanism of duplication of the *lpp* gene.

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