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# Study of the Role of the *htrB* Gene in *Salmonella typhimurium* Virulence

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**We have undertaken a study to investigate the contribution of the *htrB* gene to the virulence of pathogenic *Salmonella typhimurium*. An *htrB*::mini-Tn*10* mutation from *Escherichia coli* was transferred by transduction to the mouse-virulent strain *S. typhimurium* SL1344 to create an *htrB* mutant. The *S. typhimurium htrB* mutant was inoculated into mice and found to be severely limited in its ability to colonize organs of the lymphatic system and to cause systemic disease in mice. A variety of experiments were performed to determine the possible reasons for this loss of virulence. Serum killing assays revealed that the *S. typhimurium htrB* mutant was as resistant to killing by complement as the wild-type strain. However, macrophage survival assays revealed that the *S. typhimurium htrB* mutant was more sensitive to the intracellular environment of murine macrophages than the wild-type strain. In addition, the bioactivity of the lipopolysaccharide (LPS) of the *htrB* mutant was reduced compared to that of the LPS from the parent strain as measured by both a *Limulus* amoebocyte lysate endotoxin quantitation assay and a tumor necrosis factor alpha bioassay. These results indicate that the *htrB* gene plays a role in the virulence of *S. typhimurium*.**

Many of the factors required for the virulence of *Salmonella typhimurium* have been well studied. Pathogenic *Salmonella* strains carry a set of genes that encode an invasion-specific type III secretion system and the secreted invasion proteins that enable them to efficiently penetrate the intestinal epithelium of the host (2, 13, 17, 20, 28, 30, 31). Other determinants, which are still being identified, permit these pathogens to grow within the hostile environment of the host lymphatic system (9, 10, 19, 27, 57). In vitro experiments indicate that one mechanism that contributes to survival is the ability to modify the intracellular environment of the phagolysosome of a macrophage (1, 5, 15). Salmonellae also possess a virulence plasmid that carries a set of virulence-related genes that are required for growth of the bacteria in the lymphatic system of an infected host (22–24, 69). In addition, it has been clearly established that the lipopolysaccharide (LPS) of *Salmonella* strains is critical for the establishment of disease (45, 49). LPS seems to play multiple roles in *Salmonella* infection of a host. One of the contributions of the molecule to virulence is the protection of the bacterium from serum-mediated killing as well as from the hostile intracellular environment of the phagolysosome (7, 18, 21, 56, 58, 60, 61). In addition, it has been established that LPS induces the production of cytokines (12).

Recent work has demonstrated that the *htrB* gene plays an important role in the biosynthesis of the lipooligosaccharide (LOS) polymer in *Haemophilus influenzae* (35). The changes in LOS structure that occur as a result of the *htrB* mutation decrease the toxicity of the molecule and reduce the virulence of the organism (44). The ability of purified LOS from an *H. influenzae htrB* mutant to elicit inflammatory cytokines from human macrophages was significantly reduced compared to that of purified LOS from the parent strain. In addition, infection studies in an infant rat model revealed that the ability of the *htrB* mutant to colonize a host was reduced compared to that of the wild-type organism.

We have recently constructed and partially characterized an *S. typhimurium htrB* mutant (59). The strain was found to have two changes associated with the *htrB* mutation. The mutant grew normally at 30°C in broth or on agar and at 37°C in broth. However, the growth rate on agar was reduced at 37°C. We also found that the acylation pattern of lipid A of the LPS from the *htrB* mutant was altered compared to that of the LPS of the parent strain. One effect of the observed changes in the LPS of the *htrB* mutant was that its toxicity was reduced by 1 order of magnitude, compared to that of the wild-type *S. typhimurium* LPS, as determined both in a galactosamine mouse model and by a *Limulus* amoebocyte lysate (LAL) toxicity test. The changes in the LPS structure had no effect on the serum resistance of the *htrB* mutant, but they did appear to render the strain more sensitive to the intracellular environment of macrophages. In addition, we report the results of infection experiments in which the ability of the *S. typhimurium htrB* mutant to cause systemic disease when injected intraperitoneally (i.p.) into mice was found to be significantly reduced.

## MATERIALS AND METHODS

**Bacterial strains.** *S. typhimurium* SL1344 is an invasive, mouse-virulent strain that has been described previously (70). *S. typhimurium* MGS-31, an *htrB* mutant of SL1344, was constructed and described in detail previously (59). Briefly, bacteriophage P1 was grown on *Escherichia coli* MLK217, which carries a Tn*10* transposon insertion in the *htrB* gene (33). The P1 lysate was plated on a *galE mutS recD S. typhimurium* strain that was restriction negative and modification positive ($r^- m^+$), and transductants were selected on L agar containing 20 μg of tetracycline per ml. Mutations in the *mutS* and *recD* genes decrease the level of homology required for recombination during transduction. Subsequently, *S. typhimurium* SL1344 was transduced to tetracycline resistance with a P22 lysate prepared on an *S. typhimurium* $r^- m^+$ *galE mutS recD* tetracycline-resistant (*htrB*::Tn*10*) transductant. This strain was designated MGS-31. SL1344 tetracycline-resistant transductants were confirmed to carry the *htrB*::Tn*10* insertion by a combination of PCR, Southern blot analysis, and phenotypic tests. An *S. typhimurium* SL1344 *htrB*$^+$ revertant (*S. typhimurium* MGS-39) was selected by plating the *S. typhimurium htrB*::Tn*10* mutant on Bochner selection medium (3).

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TABLE 1. Determination of the $LD_{50}$s of *S. typhimurium* SL1344 and derivative strains for BALB/c mice following intraperitoneal inoculation

| Strain | Relevant genotype | $LD_{50}$, CFU (i.p.) |
|---|---|---|
| SL1344 | Wild type | <50 |
| MGS-31 | *htrB*::mini-Tn*10* | $9.7 \times 10^6$ |
| MGS-39[a] | *htrB*$^+$ | <50 |
| MGS-31 pMGS1 | *htrB*::mini-Tn*10* (*htrB*$^+$) | <50 |

[a] Strain MGS-39 was obtained by selecting for a precise excision of the mini-Tn*10* from the *htrB* gene so that function was restored.

A functional copy of the *htrB* gene was amplified by PCR and cloned into the single-copy plasmid pBDJ129 to create pMGS1. This plasmid was introduced into *S. typhimurium* MGS-31.

**LPS purification.** LPS preparations were made according to the method of Galanos et al. (14).

**Animal virulence experiments.** Eight-week-old female BALB/c mice were purchased from Harlan-Sprague (Indianapolis, Ind.) and were used to determine the i.p. 50% lethal doses ($LD_{50}$s) of *S. typhimurium* SL1344 and derivative strains. Bacterial suspensions of each strain, with concentrations ranging from $5 \times 10^1$ to $5 \times 10^7$ CFU, were delivered into the peritoneal cavities of the mice by injection. Infected mice were observed for signs of systemic disease and death for 14 days. The method of Reed and Muench (52) was used to determine the $LD_{50}$ for each strain of *S. typhimurium*.

**LAL endotoxin quantitation assay.** The standardized endotoxic activity of LPS was determined by using an LAL gel clot kit from Associates of Cape Cod. The assay was performed according to the manufacturer's recommendations. The threshold sensitivity of reaction was 0.125 endotoxic unit (EU) per ml. Serial dilutions of purified LPS were made in endotoxin-free water and tested. Tests were performed three times.

**Galactosamine animal model.** Galactosamine-treated mice were used to evaluate the toxicity of LPS purified from wild-type *S. typhimurium* SL1344 and the *htrB* strain. Mice were injected simultaneously, i.p., with 8 mg of D-galactosamine and 10-fold dilutions of purified LPS in quantities ranging from 0.001 to 10 μg in a total volume of 500 μl of pyogen-free saline solution. Injected mice were observed for 24 h following injection.

**TNF-α bioassay.** The murine macrophage-like cell line J774 was stimulated by the addition of dilutions of whole-cell bacteria or acellular LPS preparations. One hour after the addition of bacteria or LPS, gentamicin was added to a final concentration of 25 μg/ml. The cells were stimulated for 18 h at 37°C in a $CO_2$ tissue culture incubator. Cell supernatants were collected, and the remaining cells were removed by centrifugation at 5,000 × *g* for 10 min. Tumor necrosis factor alpha (TNF-α) levels in cell culture supernatants were quantitated by the use of the TNF-α-sensitive cell line WEHI 164 as described by Espevik and Nissen (8). Alamar Blue vital dye was used to determine WEHI 164 cell viability, which was compared to that of cells exposed to serial dilutions of purified recombinant TNF-α. Plates were read at 570- and 600-nm wavelengths.

**Serum resistance assays.** A modification of the serum sensitivity assay described by Ross et al. (54) was used. Log-phase *S. typhimurium* cells ($2 \times 10^5$ to $5 \times 10^5$ CFU) were added to 50% mouse or human serum and incubated at 37°C with gentle agitation for 60 min. Duplicate samples were removed at time zero for determination of the initial CFU for each assay. At 60 min, duplicate samples were removed and dilutions were plated on agar plates to determine the number of organisms that survived exposure to complement.

**In vitro macrophage survival assay.** Macrophages were obtained from BALB/c mice by peritoneal lavage with sterile phosphate-buffered saline (PBS) (47). Isolated macrophages were washed once with PBS and resuspended in RPMI 1640 plus 10% fetal calf serum at $5 \times 10^5$ macrophages per ml. One milliliter of cells ($5 \times 10^5$ macrophages) was seeded into each well of a 24-well plate, and the cells were allowed to adhere. Bacteria were added to each well at a ratio of 10 to 20 organisms per macrophage, and the plates were incubated at 37°C in 5% $CO_2$ for 30 min to allow internalization of the bacteria. External bacteria were then removed by washing with PBS three times. Fresh medium with 10 μg of gentamicin per ml was added to each well to inhibit extracellular growth of bacteria, and the plates were incubated at 37°C in 5% $CO_2$ for 15 h. At the end of the 15-h period, the medium was removed by aspiration, the monolayers were washed three times with PBS, and the macrophages were lysed with 0.2 ml of 1% Triton X-100 in PBS for 10 min. L broth (0.8 ml) was added to each well, and dilutions were plated to quantitate the percent survival of each bacterial strain. Triplicate samples were plated individually, and the means and standard deviations for the output numbers of bacteria were determined.

## RESULTS

**Analysis of the role of *htrB* in mouse virulence.** One measure of the virulence of pathogenic *Salmonella* strains is the ability of the bacteria to establish a lethal systemic infection in mice. To assess the contribution of the *htrB* gene to *S. typhimurium* virulence, we determined the i.p. $LD_{50}$s of mouse-virulent *S. typhimurium* SL1344 and derivative strains by using a mouse model of infection (Table 1). Consistent with previous findings, the *S. typhimurium* parent strain SL1344 was found to have an i.p. $LD_{50}$ of less than 50 organisms for BALB/c mice (30). In contrast, the i.p. $LD_{50}$ of *S. typhimurium* MGS-31 was found to be ~100,000-fold higher than that of SL1344, or $9.7 \times 10^6$ CFU. Mouse virulence was completely restored in strain MGS-39 (*htrB*$^+$), which has a precise excision of the Tn*10* transposon insertion in the *htrB* gene ($LD_{50}$, <50 organisms). Importantly, we found that the $LD_{50}$ of strain MGS-31 carrying a single-copy plasmid with the PCR-amplified coding region of the functional *htrB* gene was also less than 50 organisms. This last result confirmed that the loss of virulence observed in the strain with the Tn*10* insertion in the *htrB* gene was directly due to disruption of the *htrB* gene and not to downstream polar effects on transcription attributable to the transposon insertion.

To determine whether the loss in virulence was due to an inability of the *S. typhimurium htrB* strain to grow in the host, the number of viable bacteria in the spleens and livers of groups of mice infected with either the SL1344 parent or the *htrB* mutant were determined. As shown in Table 2, mice that were injected i.p. with $4 \times 10^2$ CFU of SL1344 had approximately $4 \times 10^4$ bacteria in the liver and $2 \times 10^4$ bacteria in the spleen at 1 day. By day 3 postinfection, the spleen and liver had ~$5 \times 10^7$ and ~$10^7$ organisms, respectively. By day 5, two mice in the group had died and the surviving mouse had more than $10^8$ CFU in both the spleen and liver. In contrast, mice infected with either $8 \times 10^2$ or $8 \times 10^4$ of the *S. typhimurium htrB* mutant appeared much more capable of controlling infection. Animals inoculated with $8 \times 10^2$ CFU had ~$10^4$ organisms in the liver at day 1 but less than $10^2$ in the spleen. At day 3 and day 5, the number of bacteria in the liver had not increased

TABLE 2. Growth of *S. typhimurium* SL1344 and *S. typhimurium* MGS-31 (*htrB*) in the organs of mice[a]

| Strain | Genotype of strain | Inoculum (CFU) | No. of bacteria in organ on day: | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 | | 3 | | 5 | |
| | | | Liver | Spleen | Liver | Spleen | Liver | Spleen |
| SL1344 | Wild type | $4 \times 10^2$ | $3.8 \times 10^4$ | $1.7 \times 10^4$ | $5.5 \times 10^7$ | $1.4 \times 10^7$ | >$1 \times 10^8$[b] | >$1 \times 10^8$[b] |
| MGS-31 | *htrB*::mini-Tn*10* | $8 \times 10^2$ | $1.0 \times 10^4$ | <$1.0 \times 10^2$ | $3.3 \times 10^3$ | $9.0 \times 10^2$ | $1.7 \times 10^4$ | $1.5 \times 10^4$ |
| MGS-31 | *htrB*::mini-Tn*10* | $8 \times 10^4$ | $6.7 \times 10^4$ | $2.0 \times 10^3$ | $1.3 \times 10^4$ | $4.5 \times 10^3$ | $4.8 \times 10^4$ | $1.8 \times 10^4$ |

[a] The inoculum of each strain was administered i.p. to groups of three mice. On the indicated day, the mice in a group were sacrificed and the livers and spleens were removed and homogenized. Dilutions of the homogenates were plated to obtain the numbers of bacteria per organ.

[b] Two mice inoculated with SL1344 ($4 \times 10^2$ CFU) died on day 5.

TABLE 3. Determination of the $LD_{50}$s of purified LPS species from different *S. typhimurium* strains injected i.p. into galactosamine-treated mice

| LPS source | Relevant genotype of strain | $LD_{50}$, μg (i.p.) |
|---|---|---|
| SL1344 | Wild type | 0.01[b] |
| MGS-31 | *htrB*::mini-Tn*10* | 0.1[b] |
| MGS-39[a] | *htrB*$^+$ | 0.01 |

[a] Strain MGS-39 was obtained by selecting for a precise excision of the mini-Tn*10* from the *htrB* gene so that function was restored.
[b] Pairwise analysis of the $LD_{50}$s of SL1344 and MGS-31 gave a *P* value of <0.01.

substantially, while the number of bacteria isolated from the spleen had increased to $9 \times 10^2$ at day 3 and $1.5 \times 10^4$ at day 5. Animals that received $8 \times 10^4$ organisms had little net growth in either the spleen or liver at day 1, 3, or 5. In a separate experiment, two mice that received $6 \times 10^6$ CFU of the *S. typhimurium htrB* mutant were each found to have $\sim 6 \times 10^6$ CFU in their livers and $\sim 5 \times 10^5$ cfu in their spleens 14 days postinoculation. These data demonstrate that the *S. typhimurium htrB* mutant is able to persist in mice but has a clearly reduced capacity to multiply compared to the virulent SL1344 strain.

**Endotoxic activity assays.** As described in a separate report (59), the LPS of the *S. typhimurium htrB* mutant has lipid A acylation changes that could reduce the toxicity of the LPS. These differences could contribute to the loss of virulence observed for the *S. typhimurium htrB* mutant. To address this possibility, LPS toxicity was quantitated for strains SL1344 and MGS-31. The toxicity of purified LPS from SL1344 was quantitated in an LAL endotoxin assay and found to have an activity of 10 EU/ng of LPS, while the LPS of the *S. typhimurium htrB* mutant had a reduced activity of 1.25 EU/ng of LPS. These in vitro experiments indicated that the LPS from the *htrB* mutant was less toxic than endotoxin from the parent strain.

**Analysis of LPS bioactivity in a galactosamine mouse model.** The virulence studies clearly indicated that the *S. typhimurium htrB* mutant was attenuated for virulence in a murine infection model. To determine whether changes in the LPS structure might play a role in the loss of virulence, we examined the effect of endotoxin from SL1344 and MGS-31 in mice treated with galactosamine. Injection of galactosamine into the peritoneal cavities of mice has been shown to make the animals exquisitively sensitive to low levels of LPS (11, 36). Using this model, the calculated $LD_{50}$ for the LPS from the *htrB* mutant was found to be 0.1 μg per galactosamine-treated mouse (Table 3). This value was 10-fold higher than that observed for the LPS of SL1344, which was 0.01 μg per galactosamine-treated mouse. Thus, LPS produced from an *S. typhimurium htrB* mutant is 10-fold less toxic in the galactosamine mouse model than is LPS from the parent strain.

**LPS-induced TNF-α production.** Another approach to assessing the activity of LPS is to measure the ability of endotoxin to stimulate the production of TNF. To perform these assays, LPS was isolate from *S. typhimurium* SL1344 and MGS-31 and used to induce production of the cytokine in the macrophage-like cell line J774. LPS isolated from the wild-type SL1344 induced a response of 9.0 pg of TNF-α per ng of LPS, while LPS obtained from the *htrB* mutant yielded only 1.2 pg of TNF-α per ng of LPS. Pairwise analysis of the data resulted in a statistically significant *P* value of less than 0.03. Thus, LPS from the *S. typhimurium htrB* mutant is less able to induce a cytokine response in cultured macrophages than is the LPS of the wild-type strain SL1344.

TABLE 4. Survival of *S. typhimurium* in murine peritoneal macrophages

| Strain | CFU | | % Macrophage survival |
|---|---|---|---|
| | Inoculated | Recovered | |
| SL1344 | $9.5 \times 10^6$ | 12,350 ± 2,670 | 0.13 ± 0.03 |
| MGS-31 | $7.0 \times 10^6$ | 2,800 ± 600 | 0.04 ± 0.009 |

**Macrophage survival of *S. typhimurium* SL1344 and MGS-31.** Another possible factor contributing to the reduced virulence of the *S. typhimurium htrB* mutant is a reduced capacity to survive interactions with professional killing cells within mice. To directly address this possibility, we examined the abilities of SL1344 and MGS-31 to survive the intracellular environment of murine macrophages in vitro. Cultured peritoneal macrophages isolated from BALB/c mice were infected with either *S. typhimurium* SL1344 or MGS-31 for 15 h, and the numbers of surviving bacteria were quantitated. As shown in Table 4, 0.13% of the pathogenic *S. typhimurium* SL1344 inoculum survived in the presence of murine peritoneal macrophages after 15 h. In comparison, the ability of the *S. typhimurium htrB* mutant MGS-31 to survive interactions with primary macrophages was reduced about three-fold (0.04% survival) after 15 h.

**Serum sensitivity of *Salmonella* strains.** *Salmonella* species are typically resistant to the killing activity of complement that is present in serum. In virulent *Salmonella* species, serum resistance is mediated by a plasmid-encoded protein, Rck (25, 29), and by LPS. Since we have demonstrated that an *htrB* mutation causes a modification of the *S. typhimurium* LPS, we investigated the effect of that structural change on serum sensitivity. *S. typhimurium* SL1344 and MGS-31 were exposed to normal or heat-inactivated mouse serum. An *S. typhimurium rfaE* strain, which has rough LPS, and *E. coli* HB101 were used as serum-sensitive controls. As shown in Table 5, none of the four strains was sensitive to the killing action of mouse serum. In contrast, when human serum was used in the killing assay, both the *S. typhimurium* rough mutant and *E. coli* HB101 were sensitive to killing (>100,000 reduction in CFU). However, neither parent strain *S. typhimurium* SL1344 nor *htrB* mutant MGS-31 was found to be sensitive to human complement.

## DISCUSSION

LPS is a complex, amphipathic molecule that is a large component of the outer leaflet of the outer membrane of gram-negative bacteria. The smooth version of LPS consists of a membrane-anchored lipid A moiety, a core oligosaccharide,

TABLE 5. Bacterial resistance to killing by human and mouse sera

| Bacterial strain (relevant genotype) | $Log_{10}$ kill for[a]: | |
|---|---|---|
| | Human serum | Mouse serum |
| *S. typhimurium* SL1344 (wild type) | 0.14 ± 0.01 | 0.03 ± 0.07 |
| *S. typhimurium* MGS-31 (*htrB*) | 0.03 ± 0.05 | 0.04 ± 0.06 |
| *S. typhimurium* SL3019 (*rfaE*) | 5.20 ± 0.20 | 0.01 ± 0.07 |
| *E. coli* HB101 | 6.01 ± 0.04 | 0.21 ± 0.04 |

[a] $Log_{10}$ kill was calculated as the $\log_{10}$ CFU per milliliter surviving in heat-inactivated human or mouse serum minus the $\log_{10}$ CFU per milliliter surviving in normal human or mouse serum.

and an O side chain (50, 51). It is well established that LPS and its individual components exert biological effects on eucaryotic cells that contribute to pathogenesis during infections with gram-negative organisms (50, 51). LPS has been shown to be responsible for early activation of the innate host immune response (50, 51).

The lipid A portion of LPS is an important contributor to the biological activity of the molecule. Lee et al. (35) have shown that the *htrB* gene of the nontypeable *H. influenzae* strain 2019 produces LOS with an altered lipid A structure. Other work indicates that the *htrB* gene in *H. influenzae* is a 3-deoxyD-manno-octulosonic acid-dependent acyltransferase (44). To determine whether the *htrB* gene is important in a relevant animal model of bacterial pathogenesis, an *S. typhimurium htrB* mutant has been constructed and partially characterized (59). Mass spectrometric analysis of purified LPS from the *S. typhimurium* mutant revealed that disruption of the *htrB* gene has an effect on the synthesis of the LPS. Lipid A from the *htrB* mutant was found to be predominantly hexacyl, like wild-type lipid A, but the 12-carbon lauric acid moiety that is usually attached to the hydroxy group of the amide-linked β-hydroxymyristic acid at the 2′ position of glucosamine II is absent. In addition, a 16-carbon palmitic acid moiety is attached at the analogous 2′ amide site in glucosamine I. In the minor heptaacyl lipid A species, the 12-carbon lauryl side chain which is normally present on glucosamine II of *S. typhimurium* lipid A is replaced with a 16-carbon unsaturated palmitoleic acid side chain. In addition to changes in LPS structure, the *S. typhimurium htrB* mutant displays some growth defects. The strain grows well at 30°C, more slowly at 37°C in broth, and poorly at 37°C on solid media.

Here, we have extended the study of this strain by comparing its virulence to that of the parent strain, *S. typhimurium* SL1344. Mouse infection studies demonstrated that the ability of the *S. typhimurium htrB* mutant to cause systemic infection was decreased more than 100,000-fold compared to that of the virulent parent. Thus, we conclude that a mutation in the *htrB* gene substantially reduces the ability of *S. typhimurium* to grow in the lymphatic system of the mouse.

To gain a better understanding of why this mutant has lost the ability to cause systemic disease, a number of different virulence properties have been examined. A series of experiments was performed to compare the toxicity of wild-type LPS to that of the *htrB* mutant. Two in vitro assays that quantitate LPS toxicity (the LAL quantitation assay and the TNF-α bioassay) revealed that LPS from the *S. typhimurium htrB* was approximately 10-fold less toxic than the parental LPS in vitro. Experiments with a galactosamine-treated mouse model, in which the subjects were injected with LPS from the wild type or the *htrB* mutant, revealed similar changes in the toxicity of the *htrB* LPS. These results established that the loss of *htrB* function in *S. typhimurium* results in changes in LPS structure that reduce its toxicity. This reduction in LPS toxicity may partially account for the loss of virulence of the *S. typhimurium htrB* mutant, since it is likely that the induction of inflammatory cytokines contributes to mortality in the murine model of typhoid fever.

The ability of *S. typhimurium* SL1344 and the isogenic *htrB* mutant to survive and grow within the lymphatic environment of the mouse was also examined. Those experiments revealed that there was persistence and net growth of *htrB S. typhimurium* organisms in the spleens and livers of mice. However, in comparison to the parent strain, growth was moderate, and it was clear that the mice, while not able to eliminate the organisms, were able to control the growth of the strain in the lymphatic organs. In vitro studies also indicated that the *htrB* strain did not survive as well as the wild-type parent within peritoneal macrophages of mice. While the rate of survival of MGS-31 was only three- to fourfold lower than that of SL1344 over a 15-h period, that disparity could explain the reduced growth rate of the mutant in the spleen and liver over the entire mouse infection period. Also of interest is the finding that the mutant persists in the mouse for long periods of time without causing an overwhelming systemic infection. These are properties that are considered to be important in the development of live vaccine strains. A future research direction might be to determine whether the *S. typhimurium htrB* strain could be developed as a live attenuated vaccine strain.

Finally, we have examined the effect of the *htrB* mutation on the sensitivity of *S. typhimurium* to complement-mediated killing. First, we examined the sensitivities of various bacterial strains to mouse serum. To our surprise, we were unable to demonstrate killing with mouse complement, even for a rough-LPS *S. typhimurium* mutant or *E. coli* HB101. This result suggests that mouse serum has very little activity against bacteria, even against strains that are considered to be serum sensitive. Others have also noted that mouse serum has little activity against microorganisms that are sensitive to human serum (34, 63). When we examined the effect of human serum on the bacterial strains, no difference was observed between SL1344 and MGS-31 while the rough-LPS *S. typhimurium* strain and *E. coli* HB101 were efficiently killed. These data indicate that complement killing may not be an active defensive mechanism in mice. However, if complement does play some role in murine immunity to bacterial infection, there is no difference in the sensitivities of the *htrB* mutant and SL1344 to serum killing.

Several other research groups have also evaluated the effect of LPS defects on the virulence of *S. typhimurium*. Lyman et al. (38) examined *S. typhimurium* strains that had mutations in *rfaL*, *rfaJ*, *rfaG*, *rfaF*, or *rfaE* that resulted in a rough-LPS phenotype. The ability of each of the rough mutants to colonize the livers and spleens of mice and to induce systemic disease and death was significantly reduced. The virulence of an *S. typhimurium galE* mutant (rough-LPS phenotype) was also significantly compromised (46). Consistent with our results, the i.p. $LD_{50}$ of this strain was $10^6$ CFU per mouse, compared to $4 \times 10^2$ CFU per mouse for the parental strain. Another study (41) found that strains carrying a Tn*phoA* insertion that resulted in a discernible rough-LPS phenotype on plates had murine $LD_{50}$s that increased 100- to 10,000-fold. Others have noted that subtle changes in LPS structure can have a significant effect on mouse virulence (37, 64, 65). *Salmonella* strains carrying *S. typhimurium* O side chains were 10-fold more virulent than the same strains carrying *S. enteriditis* O side chains (39). The only difference between the strains tested was the dideoxyhexosyl group of the D-mannosyl residues; LPS from *S. typhimurium* has abequose, and LPS from *S. enteriditis* has tyvelose. Another strain of *S. typhimurium*, which had become resistant to phage FO as a result of a minor alteration in the LPS, had an $LD_{50}$ that was >100,000-fold higher than that of the parent strain (40). These studies highlight the fact that both large and small changes in the LPS can profoundly affect the ability of *Salmonella* strains to induce disease in mice.

While it is clear that LPS plays an essential role in *Salmonella* virulence, its precise function remains unclear. Many laboratories have demonstrated that *Salmonella* LPS is a potent stimulator of murine cytokines, including TNF-α, gamma interferon (IFN-γ), interleukin-1 (IL-1), IL-2, IL-6, IL-7, IL-10, and IL-12 (26, 32, 42, 48, 66). Many of these cytokines act to stimulate host immunity in either a general or a specific

manner. Early production of IFN-γ has been shown to result in the activation of macrophages and natural killer cells (55, 62), both of which are known to be important in the development of host immunity to *Salmonella* infection. In contrast, recent work has shown that IL-10 has an inhibitory effect on IFN-γ, TNF-α, and IL-12 (4, 6, 43, 62). Clearly, the net effect of LPS on the host immune response is the result of a multitude of antagonistic and protagonistic signals generated by immunocytokines. These complex cytokine activation pathways make it difficult to discern how a change in the structure of LPS might affect activation of host immunity.

Research aimed at understanding the effect of LPS on signal pathways in cells has begun to yield interesting results. LPS has recently been shown to have the ability to stimulate the activity of protein tyrosine kinases (67) and activate the mitogen-activated protein kinase cascade (53, 68). Activation of these signal transduction pathways by LPS may result in the production of cytokines. It is also quite possible that other, unidentified host responses are elicited. The results of a recent study may complicate our current view of LPS activity even further. Garcia-del Portillo et al. (16) have shown that *S. typhimurium* releases significant amounts of LPS after it is internalized by cultured epithelial cells. Neighboring cells that did not contain bacteria did not stain positive for LPS. These findings suggest that LPS may be primarily released from the surface of salmonellae after the bacteria are internalized. Future work is needed to determine whether the same cytokines are produced when LPS is delivered from an intracellular location rather than exogenously.

Our data clearly demonstrate that an *S. typhimurium htrB* strain is defective in establishing systemic disease of mice. The *htrB* mutation has an unknown effect on the ability of the strain to grow efficiently at 37°C on solid media, which may contribute to the loss of virulence. In addition, we have performed experiments that suggest that the inability of the bacteria to survive within macrophages also contributes to decreased virulence. Other experiments suggest that the LPS of the mutant may have an altered bioactivity, since it is less toxic as measured by in vitro assays and in an animal model of toxemia. We believe it is possible that an alteration in its signalling capacity also contributes to the increased $LD_{50}$ of the strain. Thus, a combination of changes caused by the *htrB* mutation contributes to the loss of virulence that we observe in the *S. typhimurium htrB* mutant.

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