Mutation of the htrB Gene in a Virulent Salmonella typhimurium Strain by Intergeneric Transduction: Strain Construction and Phenotypic Characterization

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The htrB gene product of Haemophilus influenzae contributes to the toxicity of the lipooligosaccharide. The htrB gene encodes a 2-keto-3-deoxyoctulosonic acid-dependent acyltransferase which is responsible for myristic acid substitutions at the hydroxy moiety of lipid A β-hydroxymyristic acid. Mass spectroscopic analysis has demonstrated that lipid A from an H. influenzae htrB mutant is predominantly tetraacyl and similar in structure to lipid IVₐ, which has been shown to be nontoxic in animal models. We sought to construct a Salmonella typhimurium htrB mutant in order to investigate the contribution of htrB to virulence in a well-defined murine typhoid model of animal pathogenesis. To this end, an r₄ m₃ galE mutS recD strain of S. typhimurium was constructed (MGS-7) and used in inter- and intraspecies transduction experiments with both coliphage P1 and Salmonella phage P22. The Escherichia coli htrB gene containing a mini-Tn10 insertion was transduced from E. coli MLK217 into S. typhimurium MGS-7 via phage P1 and subsequently via phage P22 into the virulent Salmonella strain SL1344. All S. typhimurium transductants showed phenotypes similar to those described for the E. coli htrB mutant. Mass spectrometric analysis of the crude lipid A fraction from the lipooligosaccharide of the S. typhimurium htrB mutant strain showed that for the dominant hexaacyl form, a lauric acid moiety was lost at one position on the lipid A and a palmitic acid moiety was added at another position; for the less abundant heptacyl species, the lauric acid was replaced with palmitoleic acid.

Lipoooligosaccharide (LOS), a major component of the outer membrane of nontypeable Haemophilus influenzae, is a complex molecule that requires the functions of many genes for proper assembly. One gene, htrB, has recently been shown to play a role in the acylation of the lipid A portion of the LOS of H. influenzae (19). The lipid A of H. influenzae is typically hexaacyl, containing ester- and amide-linked 4-hydroxymyristic acids. The two β-hydroxymyristic acids on the second glucosamine are replaced at their hydroxy group with myristic acids. The two β-hydroxymyristic acids on the second glucosamine are replaced at their hydroxy group with myristic acids. In contrast, the lipid A of the H. influenzae htrB mutant is approximately 90% tetraacyl, with only four hydroxymyristic acid ester- and amide-linked fatty acids. This mutant lipid A is similar in structure to lipid IVₐ. The remaining 10% of the htrB lipid A is pentaacyl, with a single myristic acid substitution.

Recent studies of nontypeable H. influenzae have indicated that changes in the LOS structure affect bacterial virulence. The similarity of the LOS from the nontypeable H. influenzae htrB mutant to lipid IVₐ suggests that it binds to the CD14 receptor but does not initiate signalling which results in a macrophage cytokine response (18). Experimental evidence has shown that LOS from the nontypeable H. influenzae htrB mutant has reduced toxicity in an infant-rat model and elicits less tumor necrosis factor α from human macrophages than does wild-type LOS (unpublished data). In addition, Somerville et al. (31) have shown that an Escherichia coli msbB mutant which produces lipooligosaccharide (LPS) that lacks the myristoyl acid moiety of lipid A has a reduced ability to stimulate expression of E-selectin and tumor necrosis factor α in human endothelial cells.

htrB mutants of E. coli and H. influenzae exhibit a range of phenotypes. Karow and coworkers (13) have shown that mutations of the E. coli htrB gene render cells temperature sensitive and affect their ability to grow at temperatures above 32.5°C. Suppressors of this phenotype can be isolated at 42°C (15–17). E. coli htrB mutants are more resistant to deoxycholate than is wild-type E. coli (10 versus 2.5%). After a shift to 42°C, the morphology of the htrB mutant is altered, with the formation of filaments and bulges. Lee et al. (19) have shown that the H. influenzae htrB mutant is also initially temperature sensitive, but passage at 30°C results in the induction of undefined factors which allow growth at 37°C. The morphology of the H. influenzae mutant does not differ from that of the wild-type strain. Unlike the E. coli htrB mutant, the H. influenzae htrB mutant is more sensitive to deoxycholate than is the wild-type H. influenzae strain. Clementz et al. (6) have demonstrated that htrB mutants of E. coli contain no lauroyl transferase activity, resulting in underacylated lipid IVₐ precursors. The phenotypes associated with insertional inactivation of htrB in both E. coli and nontypeable H. influenzae could be related to changes in the lipid A structure and associated membrane alterations.

Since there is no animal model for nontypeable H. influenzae, we were interested in examining the contribution of htrB to virulence by investigating the role of htrB in the pathogenicity of a virulent Salmonella typhimurium strain in a well-characterized murine typhoid model of animal pathogenesis. One way to accomplish this was to transfer a known htrB mutation from E. coli to a virulent S. typhimurium strain. Such interspecies transfers are extremely inefficient due to divergency of up to 20% in the respective DNA sequences and a reduction in

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TABLE 1. Bacterial strains, plasmids, and primers

<table>
<thead>
<tr>
<th>Strain, plasmid, or primer</th>
<th>Genotype(s), phenotype(s), or sequence</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli MLK2</td>
<td>W3110 galE [= rph-1 IN(rmdD-rmeE) galE]</td>
<td>Strain B1789 (13)</td>
</tr>
<tr>
<td>E. coli MLK217</td>
<td>MLK2 htrB::mini-Tn10</td>
<td>13</td>
</tr>
<tr>
<td>S. typhimurium DGSa</td>
<td>808hlocZ ΔM15 ΔlocZYA-argF]U169 deoR recA1 endA1 phoA hsdR17(r-m')</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>S. typhimurium MST3063</td>
<td>leuA141 hisD100 mutS121::Tn10</td>
<td>Stan Maloy</td>
</tr>
<tr>
<td>S. typhimurium MST3488</td>
<td>ΔmutS 280 recD52::Tn10d, Cm'</td>
<td>Stan Maloy</td>
</tr>
<tr>
<td>S. typhimurium SL1344</td>
<td>his, Str', mouse virulent</td>
<td>36</td>
</tr>
<tr>
<td>S. typhimurium LB5000</td>
<td>tpc2 metA22 metE51 lij-452 leuA121 syl404 rpsL120 H1-b H2-c,n,x nml (Fels 2)</td>
<td>B. A. D. Stocker</td>
</tr>
<tr>
<td>S. typhimurium SL5283</td>
<td>LB5000 galE503</td>
<td>B. A. D. Stocker</td>
</tr>
<tr>
<td>S. typhimurium MGS-1</td>
<td>SL5283 recD52::Tn10d, Cm'</td>
<td>This work</td>
</tr>
<tr>
<td>S. typhimurium MGS-3</td>
<td>MGS-1 mutS::Tn10, Cm' Tet'</td>
<td>This work</td>
</tr>
<tr>
<td>S. typhimurium MGS-7</td>
<td>MGS-3 cured of Tn10, r-m' galE recD ΔmutS, Cm'</td>
<td>This work</td>
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<tr>
<td>S. typhimurium MGS-23</td>
<td>MGS-7 htrB::mini-Tn10, Tet'</td>
<td>This work</td>
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<tr>
<td>S. typhimurium MGS-31</td>
<td>SL1344 htrB::mini-Tn10, Tet'</td>
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<tr>
<td>S. typhimurium MGS-39</td>
<td>Precise excision of Tn10 from MGS-31, Tet'</td>
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<tr>
<td>S. typhimurium MGS-43</td>
<td>MGS-31::pMGS1</td>
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<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBDJ129</td>
<td>Single-copy plasmid, Cm'</td>
<td>Brad Jones</td>
</tr>
<tr>
<td>pMG51</td>
<td>pBDJ129 carrying an approximately 1.6-kb HindIII-BamHI PCR fragment containing the intact htrB gene</td>
<td>This work</td>
</tr>
<tr>
<td>pCR2.1'</td>
<td>Amp', Kan' cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HtrBL1</td>
<td>5'-CGAAGAGCTTGCAGATACCTACCCAC-3'</td>
<td></td>
</tr>
<tr>
<td>HtrBR1</td>
<td>5'-CGGAGATCCGAATCCAGAGGCCTTTATCG-3'</td>
<td></td>
</tr>
<tr>
<td>217'R1</td>
<td>5'-CGAATCTACACAGGTCATCAACG-3'</td>
<td></td>
</tr>
<tr>
<td>217L2</td>
<td>5'-TCGACCACTTTGTCATCAACCG-3'</td>
<td></td>
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</tbody>
</table>

* The underlined nucleotides for primer HtrBL1 are the HindIII restriction site, and the underlined nucleotides for HtrBR1 are the BamHI restriction site.

DYES MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. E. coli and S. typhimurium strains were grown in Luria-Bertani (LB) broth, LB broth solidified with 1.5% agar, or Davis minimal medium (Difco). Media were supplemented with ampicillin (50 or 100 µg/ml), tetracycline (20 µg/ml), streptomycin (100 µg/ml), and chloramphenicol (20 µg/ml) appropriate. Cultures were grown at 30°C unless otherwise indicated. Sodium deoxycholate at 2.5, 5.0, 7.5, or 10.0% was added to LB agar prior to autoclaving. Plates for the selection of Tn10 curing were previously described (2).

DNA manipulations. E. coli plasmid P1 transductions were performed by the method of Miller (22), and Salmonella phage P22 transductions were performed by the method of Davis et al. (7). Restriction and DNA-modifying enzymes were purchased from New England Biolabs and Promega. The standard DNA procedures were used those of Sambrook et al. (28). For cloning of PCR-amplified DNA fragments into vector DNA, the TA cloning system from Invitrogen was used. Transformations of S. typhimurium with plasmid DNA were performed by electroporation (330 µF, 4 k Ω; 2.4 kV) with a Life Technologies cell porator. DNA sequencing was performed at the University of Iowa DNA Core Facility by using dye terminator cycle sequencing chemistry with AmpliTaq DNA polymerase-FS enzyme and was analyzed on a stretch fluorescent automated sequencer (model 373A; Applied Biosystems).

Constitution of an S. typhimurium strain able to function as a transductional recipient from E. coli. We sought to construct an S. typhimurium strain which would allow the transfer of chromosomal DNA from E. coli to S. typhimurium by transduction and efficient recombination into the genomic DNA of the recipient. An r-m' galE mutant 280 recD52::Tn10d strain of S. typhimurium was constructed to accomplish this. By using an r-m' galE derivative of S. typhimurium (SL5283) as the recipient, sequential P22 transductions were performed from donors MST3488 (recD52::Tn10d [Cm']) and MST3063 (mutS::Tn10 [Tet']) by selecting first a Cm' clone (MGS-1) and then a Tet' Cm' clone (MGS-3). Since a tetracycline-sensitive recipient was needed to transduce the htrB mutation from E. coli to S. typhimurium, MGS-3 was cured of its Tn10 element by growth on fusaric acid plates, which selects for spontaneous deletion of tetracycline resistance (2). A colony which grew on these plates and showed the same sensitivity to UV as that of the parental strain MGS-3 (data not shown) was selected. This strain (MGS-7) is r-m' galE recD3 Cm' with restriction enzymes, resolved on a 16% polyacrylamide gel containing sodium dodecyl sulfate (SDS), and visualized by silver staining as described previously (33).

Genomic Southern hybridization. S. typhimurium genomic DNA was digested with restriction enzymes, resolved on a 0.8% agarose gel, transferred to a Hybond-N membrane (Amersham Corp.) by vacuum blotting (Trans-Blot TE80; Hoefer Scientific), and cross-linked to the membrane with a Stratolinker (Stratagene). The blot was probed with a digoxigenin-labelled 700-bp ClaI-SauII fragment. This fragment was obtained by restriction endonuclease cleavage of plasmid pSKS1.

Cloning of the htrB gene into a single-copy plasmid. The complete htrB gene, including approximately 625 bp upstream of the start codon, was amplified by PCR with MLK2 genomic DNA and primers HtrBL1 and HtrBR1. Primer HtrBL1 is tailed with the HindIII restriction site, and the underlined nucleotides for HtrBR1 are the BamHI restriction site. This product was then amplified in E. coli BL21 (DE3) pLysS (Novagen). A Cm' clone (MGS-1) and then a Tet' Cm' clone (MGS-3). Since a tetracycline-sensitive recipient was needed to transduce the htrB mutation from E. coli to S. typhimurium, MGS-3 was cured of its Tn10 element by growth on fusaric acid plates, which selects for spontaneous deletion of tetracycline resistance (2). A colony which grew on these plates and showed the same sensitivity to UV as that of the parental strain MGS-3 (data not shown) was selected. This strain (MGS-7) is r-m' galE recD3 Cm' with restriction enzymes, resolved on a 16% polyacrylamide gel containing sodium dodecyl sulfate (SDS), and visualized by silver staining as described previously (33).
allow cloning into BamHI-HindIII-digested pBD129. PCR was performed with the following parameters: 10 cycles of 1 min each at 94, 60, and 72°C, followed by 22 cycles of 1 min each at 94, 65, and 72°C and final elongation at 72°C for 10 min. The amplified DNA and pBD129 were restricted with HindIII and BamHI overhangs, 37°C and ligated, resulting in plasmid pMG51. pMG51 was transformed into DH5α, and a miniplasmid preparation was prepared (QIAprep Spin miniprep kit) and electroporated into LB5000, an r e− s. S. typhimurium strain. The plasmid was electroporated into MGS-31, producing MGS-43. PCR. To amplify the 3-kb fragment containing the mini-Tn10 element within the htrB gene or the 843-bp wild-type fragment, PCR was performed with an Expand long-template kit (Boehringer Mannheim) and the following parameters: 2 min at 92°C, 10 cycles of 10 s at 92°C, 30 s at 65°C, and 3 min at 66°C, and 18 cycles where the elongation time was extended by 20 s for each cycle, with a final elongation of 8 min at 68°C. The primers used were 217R1 and 217L2.

PCR. To amplify the 19-kb fragment containing the mini-Tn10 element within the htrB gene or the 843-bp wild-type fragment, PCR was performed with an Expand long-template kit (Boehringer Mannheim) and the following parameters: 2 min at 92°C, 10 cycles of 10 s at 92°C, 30 s at 65°C, and 3 min at 66°C, and 18 cycles where the elongation time was extended by 20 s for each cycle, with a final elongation of 8 min at 68°C. The primers used were 217R1 and 217L2.

**Electron microscopy.** For transmission electron microscopy, bacterial cells were suspended in phosphate-buffered saline and deposited on Formvar-coated, glow-discharged nickel grids. After 5 min, the excess volume was drained away from grids; whole-cell mounts were stained with 2% phosphotungstic acid for 30 s. These negatively stained samples were viewed on a Hitachi H-7000 transmission electron microscope at 75-kV accelerating voltage. For scanning electron microscopy, bacterial cells were fixed with 4% paraformaldehyde-phosphate-buffered saline. Samples were prepared by standard techniques for scanning electron microscopy, including treatment with 1% OsO4, dehydration through an ethanol series, and final drying in hexamethyldisilazane (Polysciences, Inc.). Specimens were viewed on a Hitachi S-4000 scanning electron microscope at 5.0-kV accelerating voltage.

**Structural characterization of lipid A.** In order to determine the structural changes in the lipid A moiety of S. typhimurium htrB mutant MGS-31, 2 to 3 mg (each) of LPS from wild-type strain MGS-39 and MGS-43, and htrB mutant MGS-31 was subjected to mild acid hydrolysis (1% acetic acid [2 mg/ml] for 2 h at 100°C). After hydrolysis, the lipid A fraction was separated from the soluble oligosaccharide fraction by centrifugation, precipitation, centrifugation at 0°C, and final fractionation in CHCl3-methanol-H2O (10/5/6 [vol/vol/vol]). The lipid A fraction was separated from the soluble oligosaccharide fraction by precipitation, centrifugation at 0°C, and final fractionation in CHCl3-methanol-H2O (10/5/6 [vol/vol/vol]). The CHCl3-methanol layer containing crude lipid A was saved for mass spectrometry (MS) and fatty acid analysis as described below. For MS characterization of crude lipid A fractions, small aliquots of the four lipid A preparations (~2 µg [each]) were dissolved in 1 µl of a nitrobenzyl alcohol-triethanolamine liquid matrix (1/1 [vol/vol]) and analyzed by liquid secondary ionization-MS (LSIMS) as previously described (19, 21, Spectra). These were taken under negative-ion conditions by using a Cs+ beam of 10 keV and analyzed by the front end of a four-sector Kratos Concept magnetic-sector mass spectrometer. Approximately 10 scans each were taken at a scan rate of 1 s/decade over the mass range of m/z 80 to 3,000, and the resulting mass spectra were calibrated with an external Ca reference by using the manufacturer’s Mach 2 data system.

For the analysis of straight-chain, nonhydroxylated fatty acids, ~100 µg (each) of the four crude lipid A fractions were dried and redissolved in 400 µl of 14% BF3-methanol. These solutions were heated at 100°C for 6 h, cooled to room temperature, and concentrated to near dryness in a stream of nitrogen. The resulting fatty acid methyl esters were taken up in hexane, separated, and analyzed by gas chromatography (GC)-MS with a VG70S mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph. Samples were injected via an on-column injector onto a DB-1 capillary column (J&W Scientific) at 40°C and immediately ramped to 120°C at 20°C/min, followed by a program of 5°C/min that terminated at 240°C. A standard fatty acid mixture containing equimolar amounts of laurate, myristate, and palmitate was prepared to establish relative retention times for the identification of hexadecenoic acids. When used in the MGS-31 strain, a commercial mixture of bacterial fatty acid methyl esters containing cis-Δ4-hexadecenoic acid (palmitoleic acid) was obtained from Matreya, Inc. (Pleasant Gap, Pa.).

**RESULTS**

In order to study the role of the htrB gene in Salmonella pathogenicity and virulence, we constructed a strain of S. typhimurium (MGS-7) which would allow us to transfer a known htrB mutation via phage-mediated transduction from E. coli to a virulent S. typhimurium strain. MGS-7 needed to (i) be sensitive to coliphage P1 or Salmonella phage P22, (ii) accept foreign DNA without degrading it, (iii) allow recombinational intermediate structures to survive longer, and (iv) have a relaxed requirement for DNA sequence homology to allow intergeneric recombination to occur. Strain MGS-7 (and MGS-3, goE-) was found in the MGS-31 strain, a commercial mixture of bacterial fatty acid methyl esters containing cis-Δ4-hexadecenoic acid (palmitoleic acid) was obtained from Matreya, Inc. (Pleasant Gap, Pa.).

**TABLE 2. Phenotypic characteristics of the S. typhimurium htrB::Tn10 strain**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MLK2 (htrB) a</th>
<th>MLK217 (htrB::Tn10) b</th>
<th>SL1344 (htrB) c</th>
<th>MGS-31 (htrB::Tn10) d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth on LB agar at (°C):</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>37</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>42</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Morphology at (°C):</td>
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<td>Normal</td>
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</tr>
<tr>
<td>30</td>
<td>Normal</td>
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</tr>
<tr>
<td>42</td>
<td>Normal</td>
<td>Long</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Decapoylcholate resistance or sensitivity</td>
<td>Sensitivity</td>
<td>Resistance</td>
<td>Sensitivity</td>
<td>Resistance</td>
</tr>
<tr>
<td>Growth on minimal medium at 37°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a. Wild-type E. coli.

b. E. coli htrB::Tn10.

c. Also for MGS-39 (SL344 htrB::Tn10 cured of the Tn10 element) and MGS-43 (SL344 htrB::Tn10pMGS1).

d. +, growth; ±, no growth; –, growth (at 42°C) due to htrB suppressors (15–17, 19) (not tested for SL344 htrB::Tn10).

S. TYPHIMURIUM htrB MUTANT 5523

S. TYPHIMURIUM htrB MUTANT 5523

T. C. Biek and S. L. Cohen (1) suggested that intermediate structures in recombination produced as a result of unwinding by RecBCD and normally degraded by ExoV are longer lived in recD mutants; thus, intergeneric recombination is more likely to occur.

Transduction of the htrB mutation from E. coli MLK217 to S. typhimurium SL1344. The transduction of htrB from E. coli to virulent S. typhimurium SL1344 was accomplished in the following two-step process: (i) transduction via coliphage P1 to MGS-7 and (ii) transduction via Salmonella phage P22 from MGS-23 to the virulent strain SL1344. The transduction of the htrB mutation from E. coli MLK217 to the r e− mutant recD mutS S. typhimurium strain MGS-7 was accomplished by using recD mutS, fulfilled these requirements for template identification of homologous sequences. When used in the MGS-31 strain, a commercial mixture of bacterial fatty acid methyl esters containing cis-Δ4-hexadecenoic acid (palmitoleic acid) was obtained from Matreya, Inc. (Pleasant Gap, Pa.).

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parental *E. coli* mutant (Table 2). In liquid culture, MLK217 cells grown at 30°C have normal cell morphology. Upon transfer to 42°C, they cease growing, form long filaments (some with bulges), and are dead after 3 h (15). MGS-23 cells grown in static culture at 30°C showed mostly normal cell morphology but also some longer cells. With aeration, many long filamentous cells, some with bulges, were observed (Fig. 1B). They were also seen at higher temperatures. Motility was also dif-

FIG. 1. Scanning electron (A and B) and transmission electron (C and D) micrographs of SL1344 (A and C) and *htrB*::mini-Tn10 SL1344 (B and D) cells after growth at 30°C. Panel B shows snake forms and bulges. Panel D shows hyperflagellation. Bar, 5 (A and B) or 1 (C and D) μm.
different for filamentous MGS-23 cells in that the normal alternating runs and tumbles of wild-type cells were not seen. This is possibly related to the differences in flagellation seen for htrB mutants in electron microscopy photos (Fig. 1D) and the increase in cell length of the htrB mutant. MLK217 cells are more resistant to deoxycholate (10%) than are wild-type cells (2%). The same response to deoxycholate was observed for MGS-23 cells compared with that of MGS-7 cells (wild type for htrB).
growth after being transferred to 30°C. A few colonies may have arisen in the heaviest part of the streak. However, plates incubated at 39 and 41°C showed good growth throughout the streak after being transferred to 30°C, indicating that MGS-23 cells were not killed at higher temperatures.

To confirm that MGS-23 carries the mini-Tn10 element within the htrB gene, a pair of primers (217R1 and 217L2 [Table 1]) was designed to allow direct amplification of an 880-bp PCR product from wild-type genomic DNA or an approximately 3.8-kb product from htrB::mini-Tn10 genomic DNA. Agarose gels showed that only a 3.8-kb product was amplified from MGS-23 DNA, whereas a product of approximately 850 bp was amplified from the parental-strain DNA (Fig. 2B).

The virulent strain SL1344 was transduced to tetracycline resistance with P22 grown on MGS-23. After isolation, Tet' clones showed the same phenotypic properties observed for MGS-23 and described above. They were temperature sensitive, formed filaments and bulges, and were deoxycholate resistant. PCR was performed with genomic DNA from MGS-31, one of the Tet' MGS-23 clones, and the primers described above (Fig. 2A), and only the 3.8-kb product was produced (Fig. 2B), indicating the presence of the mini-Tn10 element within the htrB gene in MGS-31. Southern hybridization studies (data not shown) demonstrated that a single DNA fragment from MGS-31 hybridized to a specific htrB DNA probe. These results clearly demonstrated that the htrB gene of S. typhimurium was replaced by the disrupted E. coli htrb gene and that a second htrB gene was not present in the genome. Thus, the htrB gene of S. typhimurium MGS-31 was replaced by the E. coli htrB::mini-Tn10 gene sequence.

Since P1 transduction can transfer large regions of chromosomal DNA by recombination (20), it is conceivable that several E. coli genes flanking the htrB gene could have also been transferred. Thus, the phenotypes observed for MGS-23 and MGS-31 could have been due to the transfer of E. coli genes other than the mutated htrB gene. To address this question, we sought to isolate an htrB revertant from MGS-31 by selection for tetracycline sensitivity (loss of the mini-Tn10 element) on Bochner plates (2). One variant showed normal growth and morphology at 37°C and was deoxycholate sensitive. Genomic DNA from this clone (MGS-39) was subjected to PCR with the primers described above (Fig. 2A), and only the 3.8-kb product was produced (Fig. 2B). These results confirmed that the phenotypes associated with the mini-Tn10::mini-Tn10 element affecting some downstream gene(s) remained. To address this question, the intact htrB gene, including about 625 bases upstream of the start codon, was cloned from MLK2 genomic DNA (wild type for htrB) into a single-copy plasmid (pBD129). Karow and Georgopoulos (14) have shown that these upstream sequences give 100% promoter activity. This plasmid (pMGS1) was introduced into MGS-31 and was found to complement htrB::mini-Tn10, producing wild-type phenotypes, i.e., normal growth, normal cell morphology, and deoxycholate sensitivity at 37°C. These results confirmed that the phenotypes associated with the mini-

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MLK217 does not grow at temperatures above 32°C on rich medium. When plates that are incubated overnight at 39 or 41°C and show no visible growth are transferred to 30°C, no growth is seen. MGS-23 did not grow on plates at 37, 39, or 41°C. Plates incubated overnight at 37°C did not show visible growth.
Tn10 insertion in the htrB gene were due to disruption of the htrB gene, not to downstream polar effects caused by the transposon insertion.

Although the S. typhimurium htrB strains MGS-23 and MGS-31 showed temperature sensitivity when they were tested on solid media, it was observed that liquid cultures grew at 37°C and higher. The growth curves of MGS-31 at 37 and 38.5°C (average murine temperature range) were determined and showed that although MGS-31 grew more slowly than its wild-type parent did, it ultimately reached approximately 75% of wild-type turbidity at 24 h (Fig. 3A). A period of minimal change in turbidity was observed for MGS-31 at 38.5°C; it lasted between 8 and 14 h, at which point MGS-31 commenced growing at a rate comparable to that of its wild-type parent. Samples that were taken at various time points during this lag period and plated for CFU showed a 50% reduction in CFU for the culture grown at 37°C and a 2-log reduction in CFU for the culture grown at 38.5°C (Fig. 3B). When the Klett readings began to increase, at between 14 and 16 h, there was also a concomitant increase in CFU, with the htrB mutant reaching nearly the same level as that of the wild-type parent at 24 h. Samples that were taken from a culture grown at 38.5°C after the plateau period (14, 16, 18, and 20 h) and diluted into fresh prewarmed broth showed little or no lag in commencing growth (Fig. 3C). Suppression of the temperature-sensitive phenotype of E. coli and H. influenzae htrB mutant strains has previously been reported (15–17, 19). The growth curves in Fig. 2C can be explained by the induction of such suppressors. Restoration of the wild-type LOS did not occur upon suppression of the temperature-sensitive phenotype of the H. influenzae mutant (19).

LPS analysis of the SL1344 htrB mutant. It was previously reported that the LPS of the E. coli htrB mutant silver stained weakly on SDS-polyacrylamide gels but that its migration pattern was unaffected (30). Lee et al. (19) reported that the LOS of the H. influenzae htrB mutant silver stained more weakly and migrated faster on SDS-polyacrylamide gels than the corresponding wild-type LOS did. The LPS from the S. typhimurium htrB mutant was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining. LPS from SL1344, which is wild type for htrB, showed the wild-type phenotype (Fig. 4, lane A). LPSs from the htrB mutant MGS-31 (Fig. 4, lane B), the mini-Tn10-cured strain MGS-39 (lane C), and MGS-43, the htrB mutant carrying the single-copy plasmid containing the wild-type htrB gene (lane D) all showed a pattern similar to that of the wild-type strain. No decrease in staining intensity was observed for the S. typhimurium htrB mutant.

Structural characterization of lipid A. MS analysis of lipid A fractions from the wild type (SL1344), two control strains (MGS-39 and MGS-43), and the htrB mutant (MGS-31) showed similar patterns of molecular ions and fragments but contained several important differences. In the negative-ion LSIMS spectra of crude lipid A fractions from wild-type and control strains, two abundant deprotonated molecular ion peaks were observed at (M - H)⁻ = m/z 1,716.1 and 1,796.0. (The molecular masses and molecular weights given are those for isotopically pure 12C-containing species.) In addition, two
FIG. 5. Molecular ion region of the negative-ion LSIMS spectra of *S. typhimurium* lipid A preparations obtained from the cured mutant strain expressing wild-type lipid A structures (A) and the *htrB* mutant strain (B). Major deprotonated molecular ions were present at *m/z* 1,716.1 and 1,796.0 (wild type) and 1,772.3 and 1,852.3 (mutant) for the hexaacyl mono- and diphosphoryl lipid A species from wild-type and mutant strains, respectively. Minor heptaacyl mono- and diphosphoryl lipid A species were also present in both wild-type-like and mutant strains at *m/z* 1,954.2 and 2,034.1 (wild type) and 2,008.6 and 2,088.6 (mutant), respectively. Abundant fragment ions corresponding to the loss of primarily O-acyl-linked fatty acids at their ketenes (a-, b-, c-, and/or d-type cleavages) or neutral fatty acids (a*-type cleavages) (inset structures) were present in both spectra. (These cleavages were accompanied by the transfer of hydrogen [curved arrows].) At lower masses (*m/z* 1,200; data not shown), peaks that corresponded to ions arising from glycosidic-bond cleavages (Fig. 6) (see text) were observed in the spectra of both types of lipid A.
acyl (Salmonella from previous published studies of less abundant (M

In the wild-type lipid A, the m/z 710 ion was present as the largest peak in the low-mass region, with a smaller ion at m/z 948, as would be expected for GlcN-I as primarily a diacyl species with only two unsubstituted β-hydroxymyristoyl moieties linked at the second position (amide linked) or third position (ester linked). The less abundant peak at m/z 948 in wild-type and control lipid A spectra can be explained as arising from partial substitution of the amide-linked β-hydroxymyristoyl group with palmitic acid at the β-hydroxy group. In contrast, the abundance of the m/z 948 peak was generally much larger in the mutant lipid A spectrum, suggesting that the GlcN-I moiety is primarily in a triacyl state, with a palmitoyl group in addition to the conserved amide- and ester-linked β-hydroxymyristic acids. There was also a peak at m/z 710 in the htrB mutant lipid A spectrum; this peak is likely to arise from further cleavage of the more abundant fragment at m/z 948 (or perhaps directly from a pentaacyl lipid A, which may be present to some degree at m/z 1,534). In addition to the m/z 710 and 948 peaks, unique fragments at m/z 1,102 (wild-type lipid A species) and m/z 1,156 (htrB mutant) were present. The latter fragment ions can be readily explained as arising from similar glycosidic-bond cleavages (C type, types), with charge retention on GlcN-II. Since the masses of these two sets of peaks differ by 54 Da, the structural difference between mutant and wild-type lipid A species in this distal portion of the structure (GlcN-II) consists of a moiety with an elemental composition of C54H93O29 (or CH2=CH=CH=CH=CH=). The presence of both neutral and ketene fatty acid losses from the hexaacyl lipid A species of a palmitoyl group for the mutant strain (--238 and --256 Da, respectively) and of a lauroyl group for the wild-type strain (--182 and --200 Da, respectively) in the high-mass region (c and c' cleavages [Fig. 5]) strongly suggests that this 56-Da difference is due to a replacement of lauric acid in the wild-type lipid A with hexadecanoic acid in the mutant lipid A.

To confirm that a lauric acid has been replaced with palmitic acid in the mutant hexaacyl lipid A structure, as suggested by LSIMS data, and to identify the hexadecanoic acid that forms the mutant heptacarboxyl species, GC-MS analysis was carried out to identify the precise fatty acid esters in crude lipid A preparations. GC-MS analysis of the three strains making wild-type lipid A identified the presence of laurate, myristate, and palmitate in an approximate molar ratio of 1/1/0.5. (The four conserved β-hydroxymyristic acids were not determined under these experimental conditions.) This result is in good agreement with the expected molar ratios, based on the presence of a dominant mono- and diposphoryl hexaacyl-substituted lipid A with a smaller amount of corresponding heptacarboxyl structures. In contrast, the lipid A fraction from the htrB mutant yielded no laurate but rather showed an increase in the molar ratio of palmitate to myristate (approximately 1/2/1), with a small amount of a hexadecanoate that coeluted with the methyl ester of cis-Δ9-hexadecanoic acid (or palmitoleic acid; 10% of the palmitate peak). The relative amounts of these straight-chain fatty acids are also in good agreement with the MS data that suggested that lauric acid is replaced quantitatively in the mutant hexaacyl lipid A structure with palmitic acid and that further (but nonquantitative, i.e., ~10%) substitution of this novel hexaacyl species with palmitoleic acid at the former lauric acid site gives rise to a new heptacarboxyl lipid A species. These proposed differences are shown in Fig. 7.
The results presented here demonstrate that coliphage P1-mediated transduction can be used to carry out intergeneric crosses between E. coli and S. typhimurium. The S. typhimurium strain described here, MGS-7 (galEr2m1mutSrecD), is capable of serving as the recipient for coliphage P1 and Salmonella phage P22 transductions. We used P1 transduction for DNA transfer only from E. coli to S. typhimurium. Using MGS-7 as the recipient, we transduced htrB::Tn10 from E. coli to S. typhimurium and characterized the resulting htrB mutant. Transduction frequencies of $5 \times 10^{-7}$ and $7 \times 10^{-7}$ per PFU were obtained. The construction of an E. coli strain similar to the S. typhimurium strain reported here, i.e., galEr2m1mutSrecD, should allow intergeneric transduction from S. typhimurium to E. coli by coliphage P1. In addition, transformation of such an E. coli strain with cosmid pPR1347, which carries the Salmonella enterica rfb genes and rfaC gene, would allow the E. coli strain to synthesize the S. enterica group B long-chain O antigen, thus conferring Salmonella phage P22 susceptibility and transducibility to the E. coli strain (24). Using such a strain, Horne et al. (10) have recently reported P22 transduction of a mutant fkpA gene from E. coli to S. typhimurium.

LPS is a major surface component of gram-negative bacteria. It is well established that LPS contributes substantially to microbial virulence. The lipid A moiety of LPS plays a role in inducing inflammation and shock. Previous studies (19) showed that a mutation of the htrB gene of H. influenzae 2019 reduced LOS with an altered lipid A structure. Wild-type lipid A is hexacyl, but the mutant lipid A was found to be tetraacyl.
having lost two myristoyl acid groups, making it similar in structure to the nontoxic, synthetic lipid IVₐ.

The htrB mutation in the nontypeable H. influenzae strain 2019 caused a defect in LOS biosynthesis (19). There was a net loss of phosphoethanolamine on the core heptoses and a loss of two myristic acid substitutions on the lipid A. SDS-PAGE analysis showed that LOS from the htrB mutant strain migrated faster than did LOS from the wild-type strain and stained a reddish brown instead of black. However, SDS-PAGE analysis of LPS from the wild-type strain and it contained the slower-migrating O side chain ladder. LPSs isolated from the Tn10-cured (MGS-39) and plasmid-containing htrB (MGS-41) strains gave the same results.

The MS structural data clearly show that the single ester-linked lauric acid moiety attached to the hydroxy group of the amide-linked β-hydroxyamyrinic acid at the second position (GlcN-II) is absent in the mutant organism and is modulated by the substitution of a palmitic acid moiety at the analogous amide site (second position) in GlcN-I. As noted previously, replacement of the N-linked β-hydroxyamyrinic acid with palmitic acid in GlcN-I is known to occur in wild-type Salmonella strains (32) but at low levels is usually found to form the less abundant heptaacyl lipid A species (14). The simplest explanation for this change in structure is that the htrB gene encodes a lauric acid-specific fatty acyltransferase. The quantitative addition of palmitic acid in GlcN-I in the hexaacyl species of this mutant can be rationalized as occurring from either increased efficiency or up-regulation of the palmitoyl transferase (3), which would normally not function to such a high extent in an htrB⁺ strain. Moreover, the formation of the less abundant heptaacyl lipid A species can be seen as arising through the addition of palmitoleic acid, which is added at the former (but unoccupied) lauric acid site. The replacement of a lauroyl group with the unsaturated extended palmitoleic acid moiety has been previously reported for temperature-sensitive Salmonella (35) and E. coli (34) strains and has been suggested as a mechanism for increasing membrane fluidity at low temperatures.

The htrB gene product functions as an acyltransferase in both nontypeable H. influenzae strain 2019 and E. coli, with lipid IVₐ as the acyl receptor. In H. influenzae, it functions as a myristoyl transferase (19), whereas in E. coli it functions as a lauroyl transferase (6). A mutation of the htrB gene in either organism causes temperature sensitivity, but other phenotypes, such as the response to deoxynicololate, differ between the two strains.

In summary, we have constructed a galE rhenus recD htrB strain that can serve as a P1 or P2 transduction recipient of E. coli DNA. This strain can serve as a tool for the transfer of E. coli genes and mutations into S. typhimurium for analysis. As described above, this technique was used to move an htrB::Tn10 mutation into S. typhimurium, where its effects on physiology, virulence, and LPS structure were analyzed in detail. We anticipate that these procedures can be applied generally to other genes of interest.

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