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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 IV_A, 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 $r^- m^+$ *galE mutS recD* strain of *S. typhimurium* was constructed (MGS-7) and used in inter- and intrastrain transduction experiments with both coliphage P1 and *Salmonella* phage P22. The *Escherichia coli htrB* gene containing a mini-Tn*10* 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 lipopolysaccharide 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 heptaacyl species, the lauric acid was replaced with palmitoleic acid.

Lipooligosaccharide (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 acid. 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 IV_A. 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 IV_A 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 alpha 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 lipopolysaccharide (LPS) that lacks the myristoyl acid moiety of lipid A has a reduced ability to stimulate expression of E-selectin and tumor necrosis factor alpha 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 IV_A 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

Strain, plasmid, or primer	Genotype(s), phenotype(s), or sequence	Source or reference
Strains		
E. coli		
MLK2	W3110 *galE* [= *rph-1* IN(*rrnD-rrnE*) *galE*]	Strain B1789 (13)
MLK217	MLK2 *htrB1*::mini-Tn*10*	13
DH5α	ϕ80d*lacZ* ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 phoA hsdR17*($r^- m^+$) *supE44 thi-1 gyrA1 relA1*	Gibco BRL
S. typhimurium		
MST3063	*leuA414 hsdL mutS121*::Tn*10*	Stan Maloy
MST3488	Δ*mutS 280 recD542*::Tn*10d*, Cm^r	Stan Maloy
SL1344	*his*, Str^r, mouse virulent	36
LB5000	*trpC2 metA22 metE551 1iv-452 leu3121 xyl404 rpsL120* H1-b H2-e,n,x *nml* (Fels 2)$^-$ *fla66 hsdL hsdSA29 hsdSB121*	B. A. D. Stocker
SL5283	LB5000 *galE503*	B. A. D. Stocker
MGS-1	SL5283 *recD542*::Tn*10d*, Cm^r	This work
MGS-3	MGS-1 *mutS*::Tn*10*, Cm^r Tet^r	This work
MGS-7	MGS-3 cured of Tn*10*, $r^- m^+$ *galE recD* Δ*mutS*, Cm^r	This work
MGS-23	MGS-7 *htrB*::mini-Tn*10*, Tet^r	This work
MGS-31	SL1344 *htrB*::mini-Tn*10*, Tet^r	This work
MGS-39	Precise excision of Tn*10* from MGS-31, Tet^s	This work
MGS-43	MGS-31/pMGS1	This work
Plasmids		
pBDJ129	Single-copy plasmid, Cm^r	Brad Jones
pMGS1	pBDJ129 carrying an approximately 1.6-kb *Hin*dIII-*Bam*HI PCR fragment containing the intact *htrB* gene	This work
pCR2.1′	Amp^r Kan^r cloning vector	Invitrogen
Primers		
HtrBL1	5′-GCAAAAGCTTGCTTGCAGATACTCACCAAC-3′[a]	
HtrBR1	5′-CGAGGATCCGCAATCCAGAGAGCTTTTATCG-3′[a]	
217R1	5′-CGAATCTACCCAAGTTCTCCACC-3′	
217L2	5′-TCGACCACTTTGTTCATCCACGC-3′	

[a] The underlined nucleotides for primer HtrBL1 are the *Hin*dIII restriction site, and the underlined nucleotides for HtrBR1 are the *Bam*HI restriction site.

recombination of up to 6 orders of magnitude (26). To circumvent this, we constructed an *S. typhimurium* strain capable of serving as a recipient in intergeneric transductions from *E. coli* and subsequent transductions with phages P1 and P22 of the *htrB*::mini-Tn*10* gene from *E. coli* to a virulent *S. typhimurium* strain. In addition, we studied the *S. typhimurium htrB* mutant phenotype and chemical modification of the *S. typhimurium htrB* mutant lipid A. An analysis of the virulence of this *S. typhimurium htrB* mutant has been presented elsewhere (12).

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) when 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 Tn*10* curing were previously described (2).

DNA manipulations. *E. coli* phage 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 used were 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).

Construction 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 mutS recD* 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 (*recD542*::Tn*10d* [Cm^r]) and MST3063 (*mutS*::Tn*10* [Tet^r]) by selecting first a Cm^r clone (MGS-1) and then a Tet^r Cm^r 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 Tn*10* 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 recD mutS*.

LPS gel analysis. LPS was prepared by proteinase K digestion as previously described (9), separated 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 an 0.8% agarose gel, transferred to a Hybond-N membrane (Amersham Corp.) by vacuum blotting (Trans-Vac TE80; Hoefer Scientific), and cross-linked to the membrane with a Stratolinker (Stratagene). The blot was probed with a digoxigenin-labelled 700-bp *Cla*I-*Sac*II 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 *Hin*dIII restriction site, and primer HtrBR1 is tailed with the *Bam*HI restriction site. These sites were added to the PCR product to

allow cloning into *Bam*HI-*Hin*dIII-digested pBDJ129. 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 pBDJ129 were restricted with *Hin*dIII and *Bam*HI overnight at 37°C and ligated, resulting in plasmid pMGS1. pMGS1 was transformed into DH5α, and a miniplasmid preparation was prepared (QIAprep Spin miniprep kit) and electroporated into LB5000, an $r^- m^+$ *S. typhimurium* strain. The plasmid was electroporated into MGS-31, producing MGS-43.

PCR. To amplify the 3.9-kb fragment containing the mini-Tn*10* element within the *htrB* gene or the 834-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 68°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% OsO_4, dehydration through an ethanol series, and final drying in hexamethyl-disilazone (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 SL1344, two ($htrB^+$) control strains 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 precipitation, centrifugation at 0°C, and final fractionation in $CHCl_3$-methanol-H_2O (10/5/6 [vol/vol/vol]). The $CHCl_3$-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 were taken under negative-ion conditions by using a Cs^+ beam of 10 keV and 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 CsI 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 was dried and redissolved in 400 μl of 14% BF_3–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 response factors and retention times. For the identification of hexadecanoic acid found in the MGS-31 strain, a commercial mixture of bacterial fatty acid methyl esters containing *cis*-Δ^9-hexadecanoic 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), *galE* $r^- m^+$ *recD mutS*, fulfills these requirements. Rough variants of *S. typhimurium* lacking O side chains on their LPSs are sensitive to coliphage P1 (23) and can be used for generalized transduction by this phage. Phage P22, however, requires the presence of these O side chains (smooth strains) for adsorption. Thus, strains carrying the *galE* mutation are particularly suitable as hosts for both phage P1 and phage P22. When *galE* mutants are grown in the presence of high concentrations (~1%) of galactose plus glucose, the LPS produced is the smooth form (P22 sensitive), but when they are grown in the absence of galactose, the LPS produced is rough (P1 sensitive) (23, 29). *S. typhimurium* strains which lack the LT2 host restriction systems, i.e., $r^- m^+$, are able to accept DNA from a strain other than a *S. typhimurium* strain without first having to be modified by a *Salmonella* strain (4). However, interspecific recombination between *E. coli* and *S. typhimurium* is up to 6 orders of magnitude lower than intraspecific recombination between either two *E. coli* or two *S. typhimurium* strains (26). This is primarily due to a 20% divergence in DNA sequences between the two species. Rayssiguier et al. (27) have shown that when one of the host mismatch repair functions (encoded by *mutL* and *mutS*) is inactivated, then recombination between divergent species is only about 10-fold lower than that between strains from the same genus. They have also shown that intergeneric recombination is dependent upon the RecBCD pathway. However, the results of experiments by Biek and Cohen (1) suggested that intermediate structures in recombination produced as a result of unwinding by RecBCD and normally degraded by *Exo*V are longer lived in *recD* mutants; thus, intergeneric recombination is more likely to occur.

TABLE 2. Phenotypic characteristics of the *S. typhimurium htrB*::Tn*10* strain

Characteristic	MLK2 ($htrB^+$)[a]	MLK217 (*htrB*::Tn*10*)[b]	SL1344 ($htrB^+$)[c]	MGS-31 (*htrB*::Tn*10*)
Growth on LB agar at (°C)[d]:				
30	+	+	+	+
37	+	−	+	−
42	+	±	+	±
Morphology at (°C):				
30	Normal	Normal	Normal	Long/normal
42	Normal	Long	Normal	Long/normal
Deoxycholate resistance or sensitivity[e]	Sensitivity	Resistance	Sensitivity	Resistance
Growth on minimal medium at 37°C	+	+	+	+

[a] Wild-type *E. coli*.
[b] *E. coli htrB*::Tn*10*.
[c] Also for MGS-39 (SL1344 *htrB*::Tn*10* cured of the Tn*10* element) and MGS-43 (SL1344 *htrB*::Tn*10*/pMGS1).
[d] +, growth; −, no growth; ±, growth (at 42°C) due to *htrB* suppressors (15–17, 19) (not tested for SL1344 *htrB*::Tn*10*).
[e] sensitivity, growth on 2.5% but not 5.0% deoxycholate; resistance, growth on 10% deoxycholate.

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 $r^- m^+$ *galE recD mutS S. typhimurium* strain MGS-7 was accomplished by using phage P1*cmclr100* with selection for Tet^r at 30°C. Tet^r transductants were found at frequencies of 5×10^{-7} and 7×10^{-7} per PFU. No transductants were observed (frequency of less than 5×10^{-9}) when the recipient was either SL5283 (rec^+ mut^+) or MGS-1 (*recD* mut^+). Tet^r transductants (e.g., MGS-23) were tested for the *htrB* phenotypes associated with the

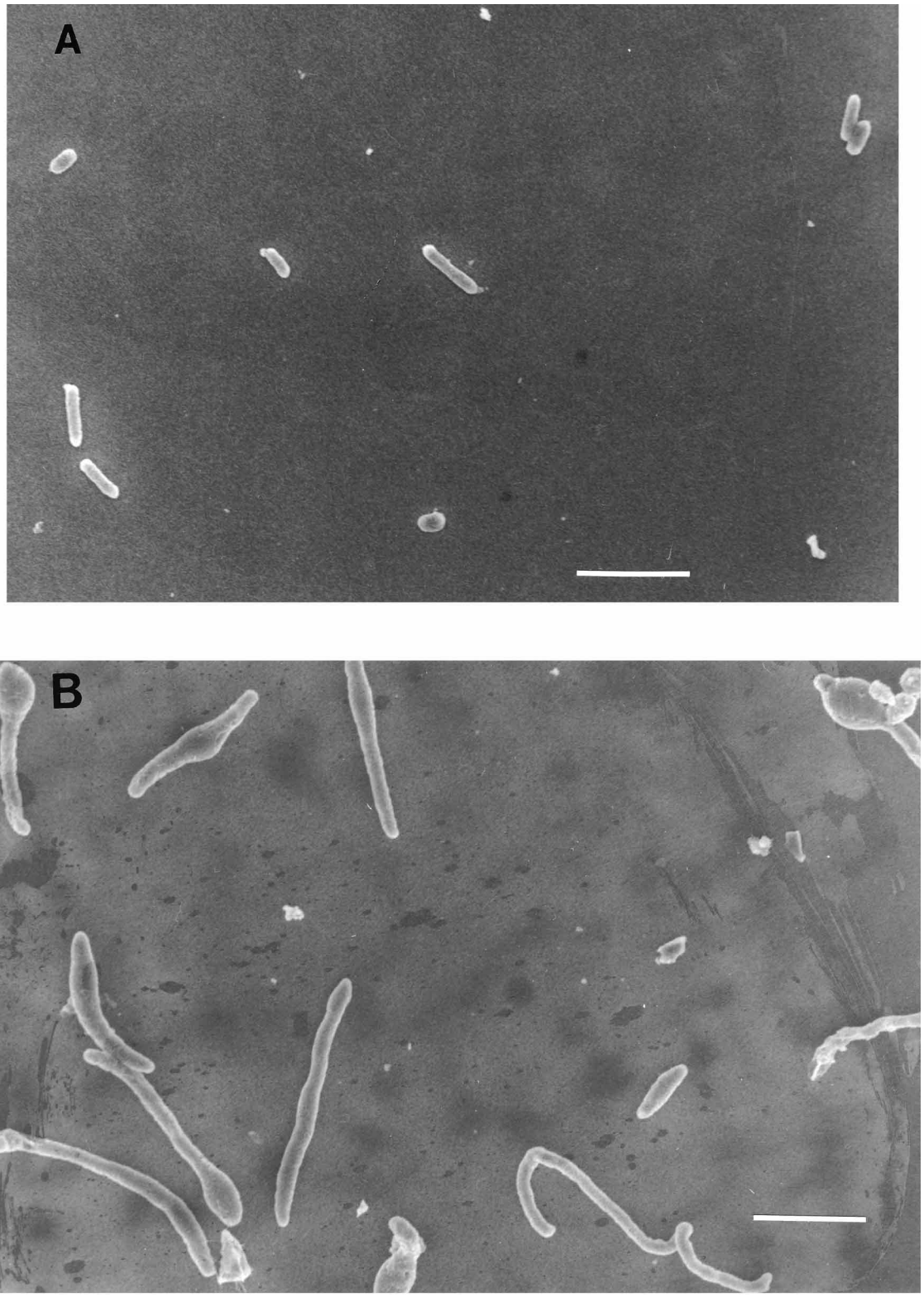


FIG. 1. Scanning electron (A and B) and transmission electron (C and D) micrographs of SL1344 (A and C) and *htrB*::mini-Tn*10* SL1344 (B and D) cells after growth at 30°C. Panel B shows snake forms and bulges. Panel D shows hyperflagellation. Bar, 5 (A and B) or 1 (C and D) μm.

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-

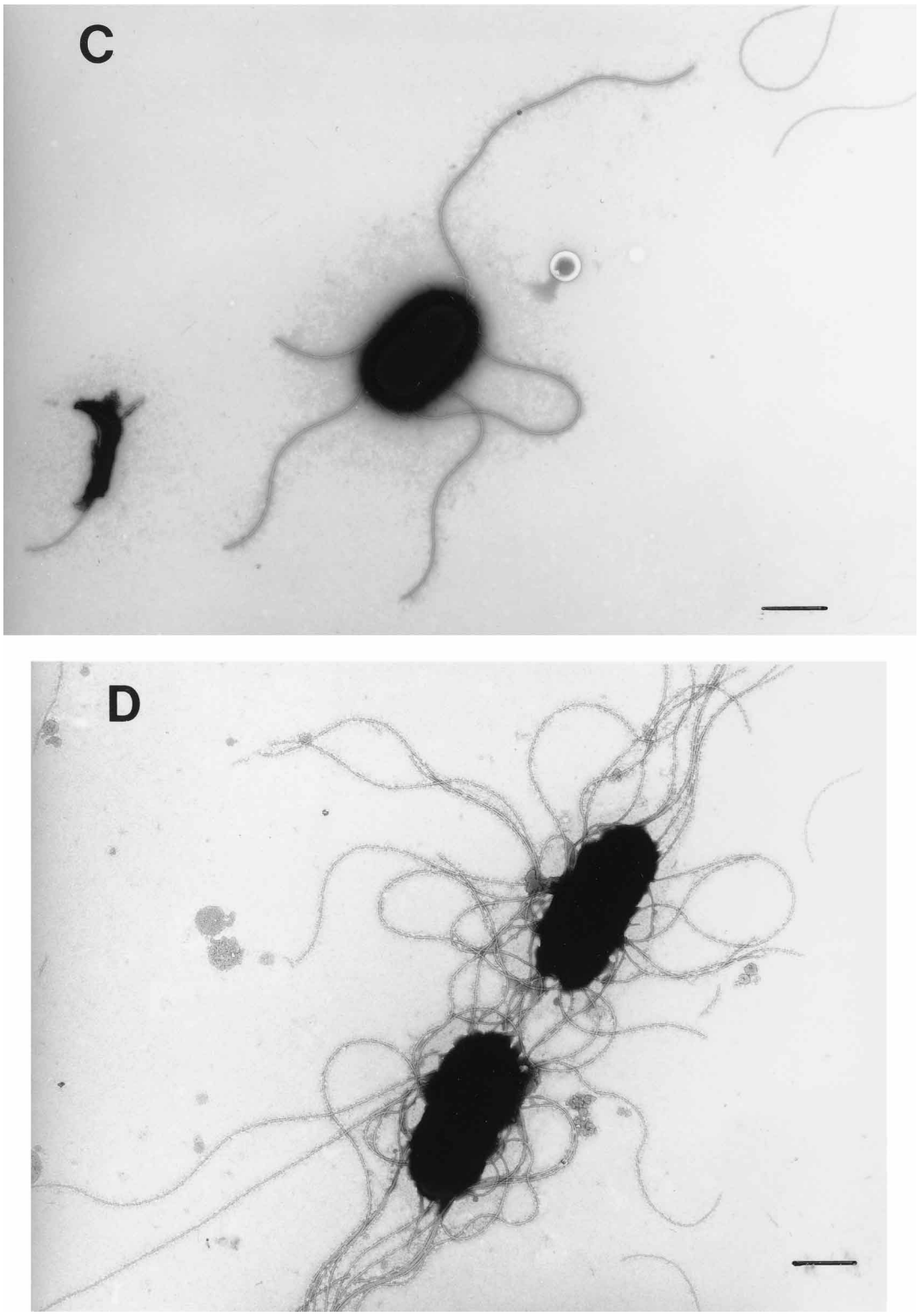


FIG. 1—*Continued.*

ferent 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*).

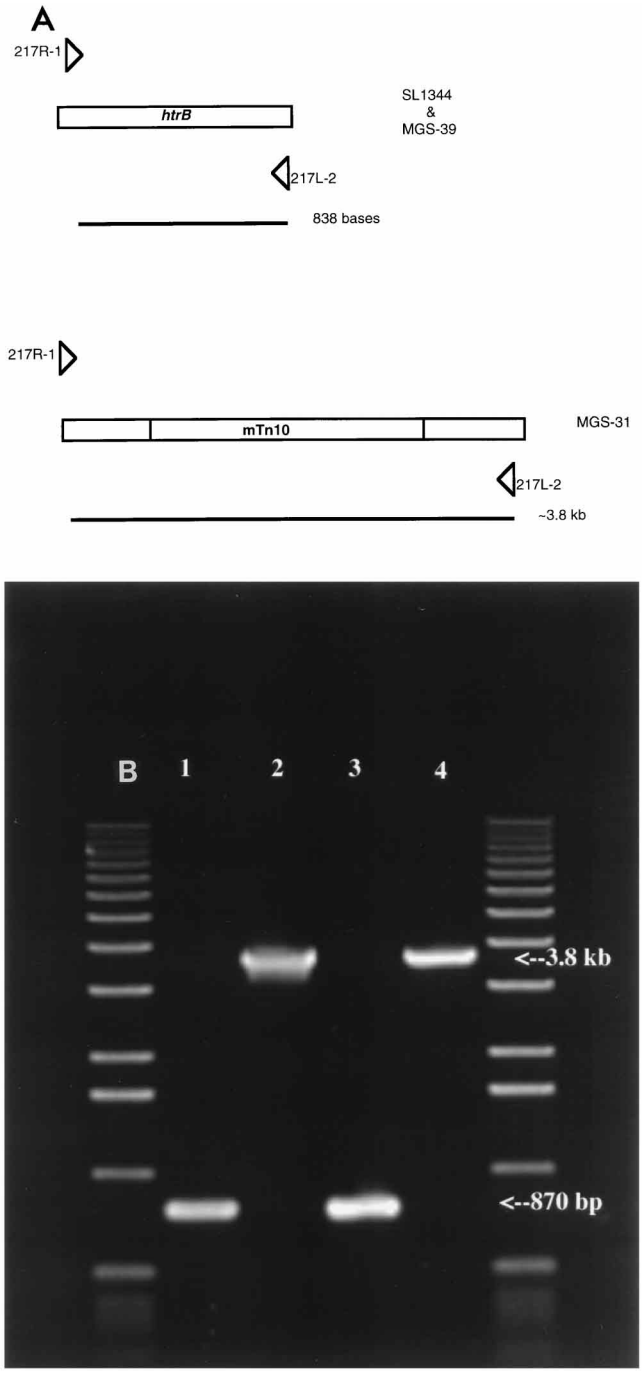


FIG. 2. Agarose gel electrophoretic analysis of amplified *htrB*$^+$ and *htrB*::Tn*10* DNA molecules. (A) Diagrams of amplified DNAs from the *htrB*$^+$ strain SL1344, the mini-Tn*10* (mTn10)-cured strain MGS-39 (*htrB*$^+$), and the *htrB*::mini-Tn*10* strains MLK217 and MGS-31. Arrowheads indicate the positions of primers. (B) Agarose gel. Lanes: 1, MLK2; 2, MLK217; 3, MGS-39; 4, MGS-31. SL1344 is not shown since its amplification was very weak. The SL1344 band always corresponded to that of MLK2 and MGS-39. Molecular size standards are given on the left and right.

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 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-Tn*10* 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-Tn*10* 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^r 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^r 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-Tn*10* 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-Tn*10* 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-Tn*10* 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. This generated an approximately 850-bp product which was cloned and sequenced. Sequencing of both strands showed that this strain was a precise eductant, i.e., no alterations extended into the chromosome from the mini-Tn*10* insertion site. MGS-39 was tested and shown to exhibit wild-type phenotypes, i.e., temperature resistance, normal cell morphology, and deoxycholate sensitivity. Thus, restoration to the wild-type phenotype in the mini-Tn*10*-cured strain indicated that the mutant *htrB* phenotypes observed were not caused by the presence of other *E. coli* genes in the *Salmonella* strains.

The possibility that the mutant phenotypes observed for MGS-23 and MGS-31 were due to polarity caused by the inserted mini-Tn*10* 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 (pBDJ129). 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-Tn*10*, 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-

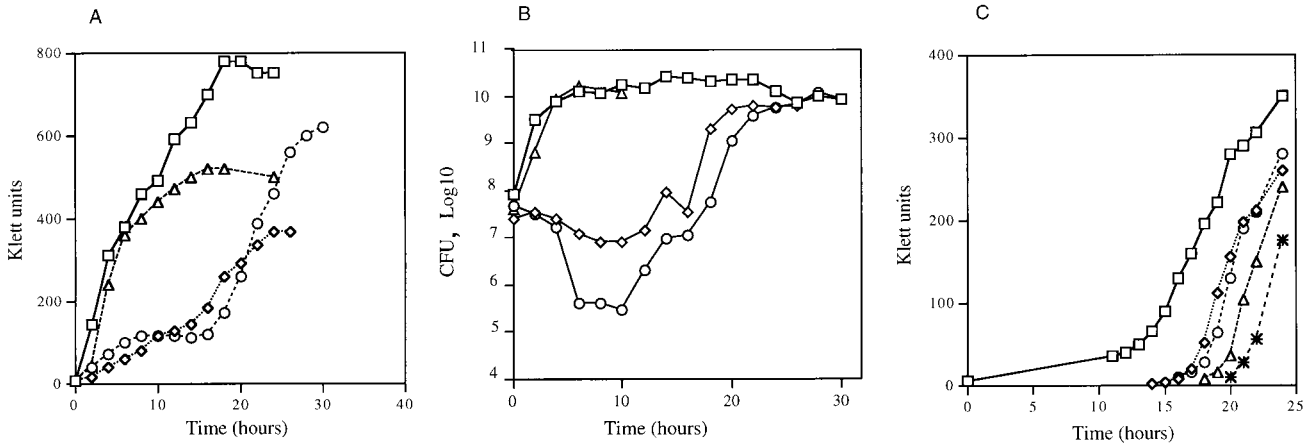


FIG. 3. Growth properties of SL1344 *htrB*::mini-Tn*10* bacteria in LB broth at 37 and 38.5°C. Overnight static cultures at 30°C were diluted to 5 Klett units, placed at the indicated temperatures, and shaken at 75 rpm. (A) Increases in culture mass, as determined by Klett measurements. (B) Viable cells per ml of culture. Dilutions of each culture were plated on LB agar containing 20 μg of tetracycline per ml at 30°C, and colonies were counted. △, *htrB*$^+$ at 37°C; ○, *htrB*::mini-Tn*10* at 38.5°C; □, *htrB*$^+$ at 38.5°C; ◇, *htrB*::mini-Tn*10* at 37°C. (C) Increases in culture mass at 38.5°C after 1:25 dilution into prewarmed LB broth. □, *htrB*::mini-Tn*10*; samples diluted at 14 (◇), 16 (○), 18 (△), and 20 (∗) h.

Tn*10* 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-Tn*10*-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 ^{12}C-containing species.) In addition, two

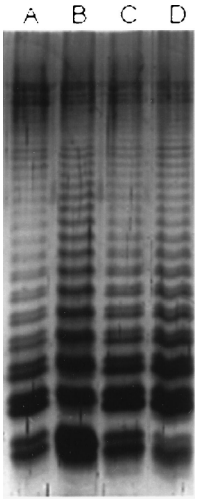


FIG. 4. SDS-polyacrylamide gel analysis of LPSs from SL1344 (*htrB*$^+$) and isogenic *htrB*::mini-Tn*10* strains. Lane 1, SL1344 (*htrB*$^+$); lane 2, MGS-31 (*htrB*::mini-Tn*10*); lane 3, MGS-39 (precise excision of mini-Tn*10* from MGS-31); lane 4, MGS-31 transformed with pMGS1.

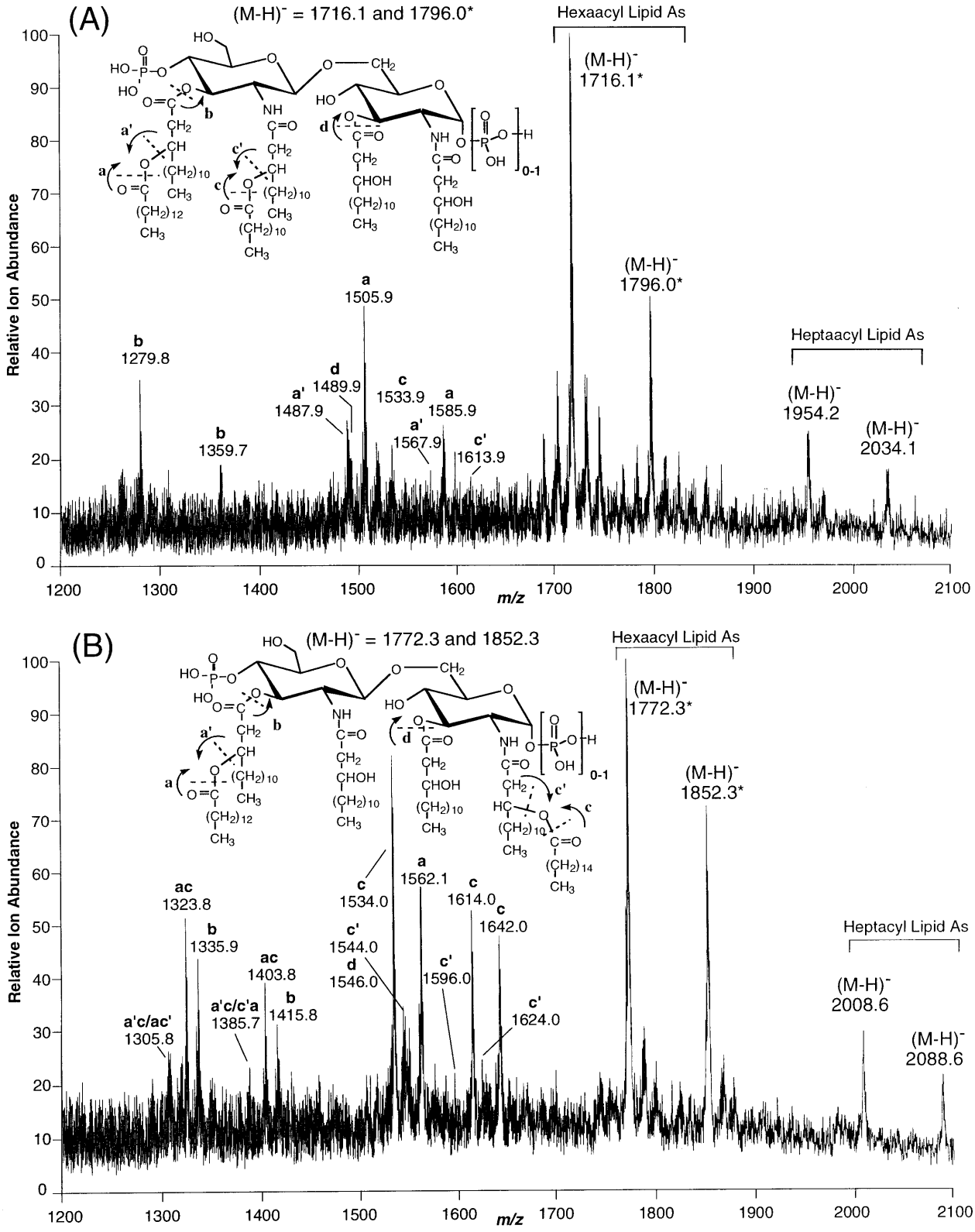


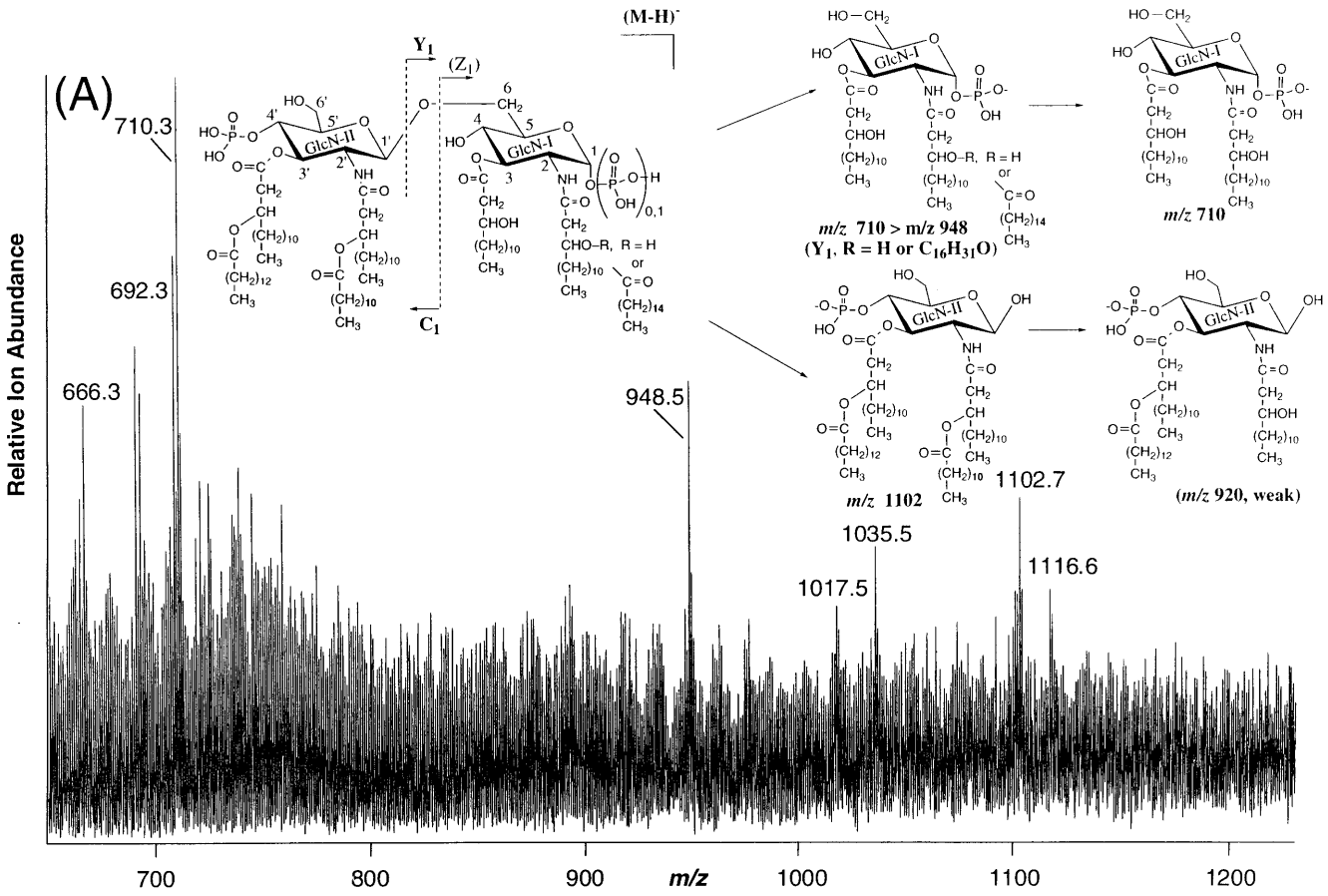
FIG. 5. Molecular ion region of the negative-ion LSIMS spectra of *S. typhimurium* lipid A preparations obtained from the cured mutant strain expressing wild-type lipid A structures (A) and the *htrB* mutant strain (B). Major deprotonated molecular ions were present at *m/z* 1,716.1 and 1,796.0 (wild 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′-, b′-, and c′-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.

less abundant $(M - H)^-$ peaks were present at *m/z* 1,954.2 and 2,034.1 and differed in mass by the addition of 238 Da relative to the two base molecular ion peaks (Fig. 5A). This quartet of peaks are consistent with the masses expected for mono- and diphosphoryl hexaacyl (M_r = 1,717.2 and 1,797.2) and heptaacyl (M_r = 1,955.4 and 2,035.4) lipid A species, as determined from previous published studies of *Salmonella* lipid A species (11, 25, 32). The 238-Da mass difference between the two sets of molecular ions is presumably due to further replacement of a β-hydroxymyristoyl group with palmitoyl, a fatty acyl substitution previously reported to occur at the amide-linked (second-position) β-hydroxymyristic acid on the reducing terminal glucosamine, GlcN-I (11, 32). In addition to these molecular ions, a number of fragment ions were present at lower masses in the *m/z* range from 1,200 to 1,600 that were consistent with the losses of the more labile O-linked fatty acids from the mono- and diphosphoryl hexaacyl lipid A species, including the loss of lauroyl, myristoyl, and myristoxymyristoyl groups. These molecular ions and fragment ions, which were present in wild-type and two control strains, are shown for the cured strain (MGS-39) in Fig. 5A.

LSIMS analysis of the lipid A fraction of the *htrB* mutant (MGS-31) also produced a spectrum that contained two abundant high-mass peaks at *m/z* 1,772.3 and 1,852.3, as well as two less abundant peaks of higher masses at *m/z* 2,008.6 and 2,088.6 (Fig. 5B). The two most abundant peaks, however, were shifted up by 56 Da relative to the two analogous peaks observed in the lipid A spectra from wild-type and control strains (i.e., mono- and diphosphoryl hexaacyl lipid A species at *m/z* 1,716 and 1,796 versus *m/z* 1,772 and 1,852). The masses of molecular ion peaks observed in the *htrB* mutant lipid A spectrum at *m/z* 2,008.6 and 2,088.6 were 236 Da higher (or were shifted up by 54 Da relative to the two heptaacyl peaks seen in the wild-type spectrum at *m/z* 1,954 and 2,034). This 236-Da increment is likely to correspond to the additional substitution of a hexadecanoic acid moiety. Moreover, fragment ions in the *m/z* range from 1,200 to 1,600 in strains producing wild-type or mutant lipid A structures showed that the 56-Da mass increase appeared to originate from one fatty acid difference between wild-type and mutant lipid A species, i.e., the replacement of lauric acid with palmitic acid, as fragment peaks in the *htrB* mutant spectrum that corresponded to the loss of myristoyl, palmitoyl, and myristoxymyristoyl groups, but not a lauroyl group, were identified (Fig. 5B).

Although we can rationalize the mass differences between wild-type and mutant hexaacyl lipid A species in terms of variations in fatty acid substitution, i.e., lauric acid versus palmitic acid, and between the corresponding heptaacyl lipid A species as palmitic acid versus hexadecanoic acid, the precise positions of these fatty acid differences were not known. As noted above, palmitic acid is known to be added in wild-type lipid A at the hydroxy group of the amide-linked β-hydroxymyristic acid at the second position of GlcN-I; such a hypothetical structure for the *htrB* mutant is shown above the corresponding mass spectrum in Fig. 5B. To support this assignment, a comparison of low-mass fragments in the wild-type-like and *htrB* mutant lipid A species (*m/z* < 1,200; Fig. 6) was carried out. This comparison showed that the 56-Da difference between wild-type and mutant hexaacyl lipid A species resides in a differential acylation pattern that subtracts a lauroyl group from nonreducing glucosamine from lipid A (GlcN-II) and places a palmitoyl group in GlcN-I. This change in acylation pattern is supported by the presence and absence of several low-mass peaks in each of the two spectral types. Specifically, peaks at *m/z* 710 and 948 were found in both mutant and wild-type lipid A spectra and are expected to originate from a single cleavage of the glycosidic oxygen (Y-type cleavage) (8), with charge retention on the reducing-terminal GlcN-I. In 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_1-2 and C_1 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 C_4H_6 (or $—CH_2—CH═CH—CH_2—$). 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 heptaacyl 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 diphosphoryl hexaacyl-substituted lipid A with a smaller amount of corresponding heptaacyl 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*-Δ^9-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 heptaacyl lipid A species. These proposed differences are shown in Fig. 7.


(A)
Relative Ion Abundance
710.3
692.3
666.3
948.5
1102.7
1035.5
1116.6
1017.5
(M-H)⁻
Y1
(Z1)
C1
GlcN-II
GlcN-I
m/z 710 > m/z 948
(Y1, R = H or C16H31O)
m/z 1102
m/z 710
(m/z 920, weak)
700
800
900
m/z
1000
1100
1200

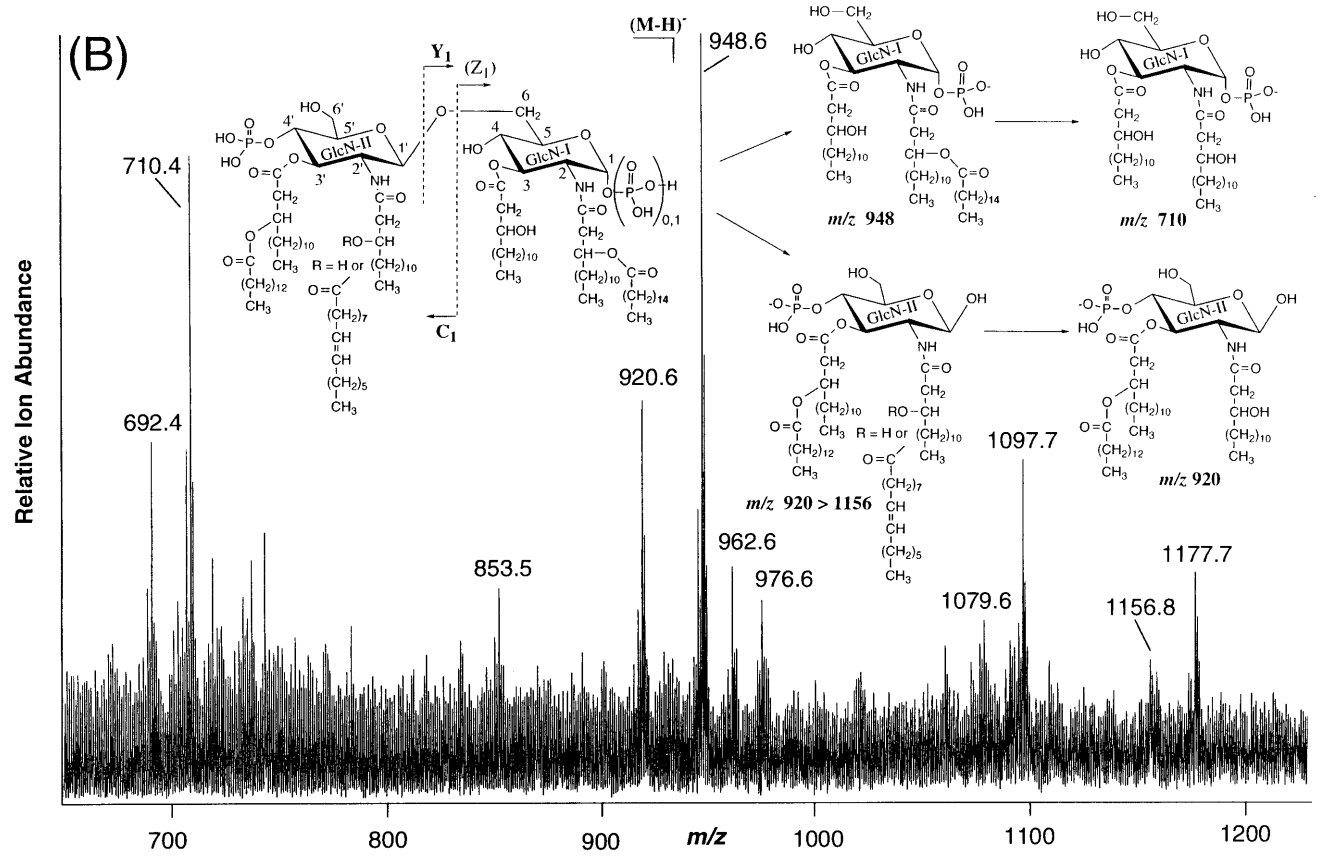

(B)
Relative Ion Abundance
710.4
692.4
948.6
920.6
853.5
962.6
976.6
1097.7
1079.6
1177.7
1156.8
(M-H)⁻
Y1
(Z1)
C1
GlcN-II
GlcN-I
m/z 948
m/z 710
m/z 920 > 1156
m/z 920
700
800
900
m/z
1000
1100
1200

FIG. 6. Low-mass region of preparations expressing wild-type lipid A structures (cured strain) (A) and *htrB* mutant lipid A structures (B). The origins of the low-mass fragments formed by cleavage of the glycosidic oxygen with charge retention on the reducing-terminal GlcN-I (Y type) or non-reducing-terminal GlcN-II (C type) are shown. Primarily four lipid A parent ions are observed in each spectrum (Fig. 5), based on the state of phosphorylation (mono- and diphosphoryl), substitution pattern (hexaacyl or heptaacyl), and/or the identity of the fatty acid substituted at the β-hydroxy group of the amide-linked β-hydroxymyristic acid (lauric or palmitoleic acid). Curved arrows indicate the directions of hydrogen transfer for the major C- and Y-type ions. More in-depth analyses of similar lipid A fragmentation pathways in *Salmonella minnesota* have been reported previously (5, 11).

DISCUSSION

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 (*galE* r^- m^+ *mutS recD*), 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*::Tn*10* from *E. coli* to *S. typhimurium* and characterized the resulting *htrB* mutant. Transduction frequencies of 5×10^{-7} and 7×10^{-7} per PFU were obtained. The construction of an *E. coli* strain similar to the *S. typhimurium* strain reported here, i.e., *galE* r^- m^+ *mutS recD*, 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 hexaacyl, but the mutant lipid A was found to be tetraacyl,

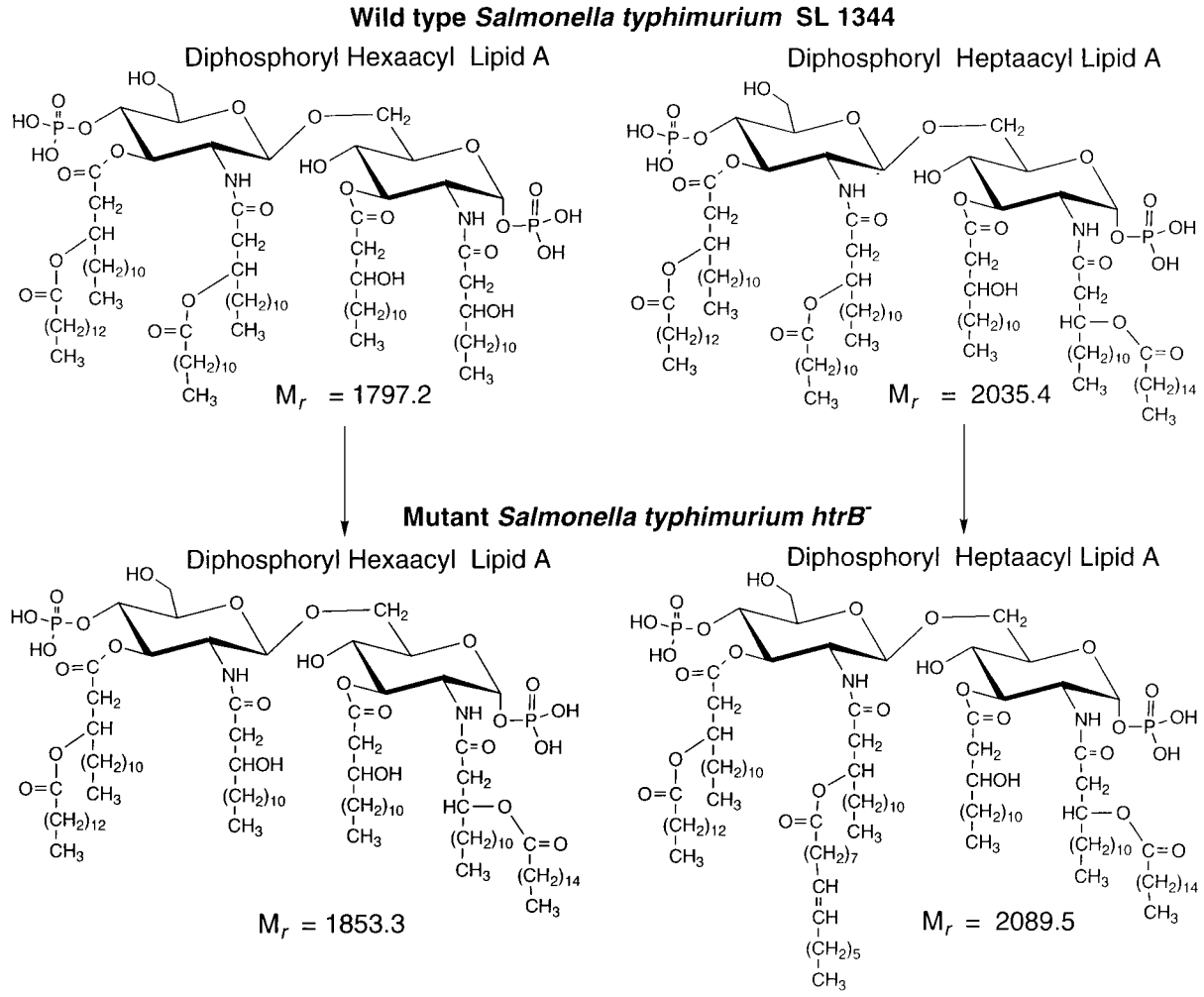


FIG. 7. Proposed structures and calculated isotopically pure masses of the hexa- and heptaacyl lipid A species from wild-type and *htrB* mutant strains. The structures are based on data obtained in this study and from lipid A structural data previously published for *S. typhimurium* and *S. minnesota* (11, 25, 32).

having lost two myristoyl acid groups, making it similar in structure to the nontoxic, synthetic lipid IV_A.

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 *S. typhimurium htrB* mutant strain (Fig. 4) showed that it did not migrate faster than did wild-type LPS, it stained the same color as did the wild type, and it contained the slower-migrating O side chain ladder. LPSs isolated from the Tn*10*-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-hydroxymyristic acid at the second position (GlcN-II) is absent in the mutant organism and is modified 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-hydroxymyristic 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 IV_A 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 deoxycholate, differ between the two strains.

In summary, we have constructed a *galE* r^- m^+ *mutS recD S. typhimurium* strain that can serve as a P1 or P22 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*::Tn*10* 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.

ACKNOWLEDGMENTS

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