Mutation of the htrB Locus of Haemophilus influenzae Nontypeable Strain 2019 Is Associated with Modifications of Lipid A and Phosphorylation of the Lipo-oligosaccharide

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The HtrB protein was first identified in Escherichia coli as a protein required for cell viability at high temperature, but its expression was not regulated by temperature. We isolated an htrB homologue from nontypeable Haemophilus influenzae strain (NTHi) 2019, which was able to functionally complement the E. coli htrB mutation. The promoter for the NTHi htrB gene overlaps the promoter for the rfaE gene, and the two genes are divergently transcribed. The deduced amino acid sequence of NTHi 2019 HtrB had 56% homology to E. coli HtrB. In vitro transcription-translation analysis confirmed production of a protein with an apparent molecular mass of 32–33 kDa. Primer extension analysis revealed that htrB was transcribed from a promoter dependent consensus promoter and its expression was not affected by temperature. The expression of htrB and rfaE was 2.5–4 times higher in the NTHi htrB mutant B29 than in the parental strain. In order to study the function of the HtrB protein in Haemophilus, we generated two isogenic HtrB mutants by shuttle mutagenesis using a mini-Tn3. The htrB mutants initially showed temperature sensitivity, but they lost the sensitivity after a few passages at 30 °C and were able to grow at 37 °C. They also showed hypersensitivity to deoxycholate and kanamycin, which persisted on passage. SDS-polyacrylamide gel electrophoresis analysis revealed that the lipo-oligosaccharide (LOS) isolated from these mutants migrated faster than the wild type LOS and its color changed from black to brown as has been described for E. coli htrB mutants. Immunoblotting analysis also showed that the LOS from the htrB mutants lost reactivity to a monoclonal antibody, 6E4, which binds to the wild type NTHi 2019 LOS. Electrospray ionization-mass spectrometry analysis of the O-deacylated LOS oligosaccharide indicated a modification of the core structure characterized in part by a net loss in phosphoethanolamine. Mass spectral analysis of the lipid A of the htrB mutant indicated a loss of one or both myristic acid substitutions. These data suggest that HtrB is a multifunctional protein and may play a controlling role in regulating cell responses to various environmental changes.

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria. It consists of lipid A linked by 2-keto-3-deoxyoctulosonic acid (KDO) to a heterogeneous sugar polymer and repeating O-antigen units. LPS plays an important role in pathogenicity and virulence. It also serves as a building block for the outer membrane and permeation barrier to hydrophobic compounds (1). Salmonella typhimurium LPS deep core mutants show increased sensitivity to various hydrophobic reagents and to elevated temperatures.

The htrB gene was first identified in Escherichia coli as encoding a protein essential for cell viability at a temperature above 33 °C (2). Unlike other heat shock proteins, however, its expression is not regulated by temperature (3). Bacteria with a mutation in the htrB gene, when exposed to nonpermissive temperatures in rich media, cease to divide and lose viability within 2 h (2). They also show morphological changes similar to those of cells with double mutations in two of the cell wall synthesis genes, pbpA and pbpB, suggesting a role of the HtrB protein in cell wall synthesis (3, 4). The E. coli htrB mutation does not affect the mobility of LPS on SDS gels, but the silver-stained LPS has a dramatically reduced intensity and a conversion from a black to a brown coloration (1). One study of htrB suppressor genes has shown that htrB mutant bacteria accumulate a high level of phospholipid at 42 °C and that spontaneous mutations in the accBC operon, which is involved in fatty acid biosynthesis, cause a decrease in phospholipid biosynthesis, restoring the balance between the two (5). This study suggested that htrB may be part of a link between growth rate and the regulation of phospholipid biosynthesis.

Haemophilus influenzae is a causative agent of many childhood diseases, including meningitis and respiratory tract infections. Nontypeable strains of H. influenzae (NTHi), which are commonly present in the nasopharynx of 50–80% of healthy carriers, are recently recognized to be important human pathogens, and evidence has shown that their lipo-oligosaccharide (LOS) is important in pathogenesis. Haemophilus LOS differs from enterobacterial LPS in that it does not contain repeating O-antigen units and is more similar to those from Neisseria and Neisseria.
Haemophilus influenzae 2019 htrB Gene

Bordetella (6). We have been studying genes involved in LOS biosynthesis in Haemophilus. In the process of sequencing the upstream region of the NTHi rfaE gene, which is responsible for ADP-heptose synthesis, we identified the htrB homologue (7). These two genes have overlapping promoter regions and are transcribed into diverse orientations. In an effort to understand the function of HtrB in Haemophilus, we constructed and characterized NTHi 2019 htrB mutants. Structural analysis of the LOS from an htrB mutant revealed a modification of the ratio of hexose to phosphoethanolamine in the LOS core structure and the loss of one or both myristic acid substitutions of the lipid A.

**Materials and Methods**

**Strains, Plasmids, and Culture Conditions**—The bacterial strains and plasmids used in this study are described in Table I and Fig. 1. E. coli strains were grown in LB medium containing appropriate antibiotics at 30 or 37 °C. NTHi strains were cultured on brain-heart infusion medium agar plates supplemented with 2% Flisles reagent (Difco Laboratories) (sbhi) or in sbhi broth at 30 or 37 °C with agitation. E. coli strains, antibiotics were added when necessary at a final concentration of 50 μg/ml for ampicillin, 30 μg/ml for kanamycin, and 40 μg/ml for chloramphenicol. For NTHi strains, 1.5 μg/ml of chloramphenicol or 15 μg/ml of kanamycin was added when needed.

**DNA Manipulations**—Restriction and DNA-modifying enzymes were purchased from New England Biolabs and Promega. Standard DNA recombinant procedures were performed as described (8). For cloning of DNA fragments made by PCR into a vector DNA, the TA cloning system (Stratagene) was used. After prehybridization in 6× SSC, 1% SDS, 0.5% dry milk, the membrane was hybridized with a digoxigenin-dUTP-labeled DNA probe at 65 °C and washed. The hybridized probe was detected using the DIG Luminescent Detection Kit (Boehringer Mannheim), and the membrane was exposed to a Kodak X-OMAT AR film at room temperature.

**Primer Extension Analysis**—NTHi strains grown overnight in sbhi broth at 30 °C were diluted 10-fold with fresh medium, grown to OD600nm 0.2, and then shifted to the desired temperature and allowed to grow for 1 h. Cells were then harvested, and RNA was extracted as described by Verwoerd et al. (13), followed by DNase I treatment and ethanol precipitation. The purified RNA was quantitated spectrophotometrically, and the quality of RNA was confirmed on a formaldehyde-ethanol precipitation. The purified RNA was quantitated spectrophotometrically, and the quality of RNA was confirmed on a formaldehyde-ethanol precipitation. The purified RNA was quantitated spectrophotometrically, and the quality of RNA was confirmed on a formaldehyde-ethanol precipitation. The purified RNA was quantitated spectrophotometrically, and the quality of RNA was confirmed on a formaldehyde-ethanol precipitation.

**Immunoblot Analysis**—Immunoblot analysis of colonies lifted after overnight growth was performed with monoclonal antibody 6E4. mAb 6E4 recognizes a non-phase varying KDO-like epitope on the LOS of all Haemophilus influenzae strains we have studied (15). Colonies grown on sbhi plates were transferred onto a nitrocellulose filter (Micron Separations Inc.) and air-dried completely. The filter was blocked by soaking in 3% gelatin solution and probed with mAb 6E4 (15) overnight at room temperature. The filter was then incubated with protein A conjugated with horseradish peroxidase (Zymed Laboratories). A chromogenic substratc, 4-chloro-1-naphthol (Bio-Rad), was used as the developing reagent.

**LPS Gel Analysis**—LPS was prepared by proteinase K digestion as

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**Table I**

| Bacterial strains and plasmids used in this study | Strains or plasmids | Genotype or characteristic | Reference or source |
|-------------------------------------------------|---------------------|---------------------------|---------------------|
| H. influenzae 2019                             | wild type          | M. A. Apicella            |                     |
| B28                                             | 2019 htrB:Eco mini-T3 | This study                |                     |
| B29                                             | 2019 htrB:Eco mini-T3 | This study                |                     |
| E. coli K-12                                    | DH5α (pCRII)       | 9                         | Life Technologies, Inc. |
| MLK2                                            | B178               | Ref. 2                    |                     |
| MLK21                                           | B178 htrB::mini-T10 | Ref. 2                    |                     |
| MLK48                                           | B178 htrB::mini-T10 | Ref. 2                    |                     |
| Plasmids                                        | pCRII              | PCR cloning vector, Amp’, Kan’ | Invitrogen Inc. |
| pGEM-T                                          | PCR cloning vector, Amp’ | Promega                   |                     |
| pGB19                                           | Cloning vector, Cn’ | G. J. Barcak              |                     |
| pHS56                                          | Cloning vector, Kan’ | Ref. 12                   |                     |
| pSHlox-1                                        | Cloning vector, Amp’ | Novagen Inc.              |                     |
| pHIHO                                           | 4-kb DNA from NTHi 2019 in pSHlox | Ref. 7                   |                     |
| pHI2E                                           | 2.8-kb Bgl II fragment from pHI2E in pUC19 | Ref. 7                   |                     |
| pHI7E                                           | PCR fragment carrying rfaE in pGEM-T | Ref. 7                   |                     |
| pHI1H                                           | PCR fragment carrying htrB in pCRII | This study                |                     |
| pSK51                                          | E. coli htrB in pBluescript SK | Ref. 2                   |                     |
| pGHH                                           | htrB on BamHI–PstI fragment from pHIH in pGB19 | This study                |                     |
| pKHH                                           | kanamycin cassette inserted into PstI of pGHH | This study                |                     |
| pHBSgL11                                        | 2.4-kb Bgl II fragment carrying htrB and rfaE in pHS56 | This study                |                     |
| pB29                                           | mini-Tn3 inserted in htrB ORF in pHBSgL11 | This study                |                     |
| pB29                                           | mini-Tn3 inserted in htrB ORF in pHBSgL11 | This study                |                     |
described (16), separated on a 14% polyacrylamide gel containing SDS (14), and visualized by silver-staining as described previously (17). Large scale preparations of LOS were obtained using the procedure of Darveau and Hancock (18).

Electrospray ionization-Mass Spectrometry of O-Deacylized LOS—O-Deacylized LOS was prepared from approximately 2–3 mg of LOS from NTHi 2019 and the isogenic htrB mutant B29 by mild hydratone treat-ment (37°C, 20 min) as described previously (19). To determine molecular masses, O-deacylated LOS from both strains were subjected to mass analysis by electrospray ionization-mass spectrometry (ESI-MS) (20). Briefly, O-deacylated LOS was first solubilized in water at a concentration of ~10 μg/μl; a 1-μl aliquot of this solution was then taken and diluted with 4 μl of the ESI-MS running solvent prior to injection. Mass analysis was performed using a VG/Fison Platform single quadrupole mass spectrometer operation in the negative ion mode with a running solvent of H2O/acetonitrile (1:1, v/v) containing 1% acetic acid at a flow rate of 10 μl/min. Mass calibration was accomplished with an external mass reference of CsNO3, which provided cluster masses from m/z 256.9, [Cs(NO3)2]+ to m/z 2011.1, [Cs25NO41(NO3)1]+. Preliminary compositions were assigned to the LOS by computer search of potential monosaccharide compositions using the program Greta Carbo developed by W. Hines.

Fractionation and Mass Spectrometric Characterization of Lipid A—Approximately 32 mg of LOS from NTHi strain 2019 and 6.6 mg of LOS from the isogenic mutant B29 were hydrolyzed in 1% acetic acid for 2 h at 25°C at a concentration of 2 mg/ml. The hydrolysates were centrifuged at 5,000 × g for 20 min at 4°C, and the supernatants were removed. The pellets were washed twice with 0.5 ml of H2O followed by centrifugation at 5,000 × g for 20 min at 4°C. The water-insoluble crude lipid A fractions were suspended in chlorform/methanol/H2O (2:1:1, v/v/v) and centrifuged at 5,000 × g for 15 min, and the lower organic layer plus the middle emulsion layer containing the lipid A were removed and evaporated to dryness.

Both lipid A samples were then directly analyzed by liquid secondary ion mass spectrometry (LSIMS) in the negative ion mode. Lipid samples were redissolved in CH3Cl/CH3OH (3:1, v/v), and 1 μl of nitrobenzyl-lactol/thiethandamine (1:1, v/v) was applied as the liquid matrix. Samples were then analyzed on a Kratos MS 50S mass spectrometer (Ultramark® 1206 was used for manual calibration to an accuracy better than ± 0.2 Da.

RESULTS

Cloning and Identification of the NTHi 2019 htrB Gene—We isolated the rfaE gene from NTHi 2019 by complementing an rfaE mutant of S. typhimurium LT2 (7). Sequence analysis of the upstream region of the rfaE gene revealed an open reading frame (ORF) highly homologous to the E. coli htrB gene. The deduced amino acid sequences of these two genes shared homology (56% identity and 73% similarity). The NTHi 2019 HtrB homologue also showed homology (27% identity and 54% similarity) with the E. coli MsbB protein, which is a multicytoplasmic suppressor of the htrB mutation (21). This is similar to the homology between the E. coli HtrB and MsbB proteins.

To construct a plasmid containing the htrB ORF, we carried out PCR using two oligonucleotides, one upstream of the promoter region (5′-aagcatacatgatgctataaca-3′) and the other the universal forward primer, with pHE12 as a template (7). The resulting 1.3-kb PCR fragment was cloned into a vector, pCR1, yielding pH1H. When the E. coli htrB mutant strains, MLK48 and MLK217, were transformed with pH1H, they were able to grow at 37°C as well as the wild type parent MLK2, indicating that the function of NTHi 2019 HtrB is analogous to that of E. coli HtrB. Disruption of the htrB ORF in pB28 and pB29 by mini-Tn3 abolished the complementing ability of these plasmids (data not shown).

In pHE10 (Fig. 1), we also found a partial ORF located downstream of the htrB gene. A homology search of data banks revealed that the deduced amino acid sequence of this partial ORF was highly homologous to E. coli topoisomerase IV subunit B (73% identity and 86% similarity over 100 amino acids) by BESTFIT software analysis. A rho-independent transcription termination sequence was found between these two ORFs, suggesting that htrB is transcribed as a monocistronic message.

Identification of the htrB Gene Product—To confirm that the htrB ORF synthesizes a protein with the molecular mass estimated from the nucleotide sequence, we carried out in vitro transcription-translation analysis (Fig. 2). pCRII, the vector used to construct pH1H, produced two bands corresponding to the 31.5-kDa β-lactamase precursor from its ampicillin resistance gene and the 29.6-kDa aminoglycoside phosphotransferase from its kanamycin resistance gene (Fig. 2, lane 2). pH1H produced one more band, presumably from the htrb gene, above the β-lactamase precursor (Fig. 2, lane 3). The estimated molecular mass of HtrB was 36 kDa, but the peptide synthesized from pH1H migrated with an apparent molecular mass of 32–33 kDa (Fig. 2, lane 3). Based on complementation data (see below), we believe that this is the authentic HtrB. pH1E0 produced three proteins in addition to β-lactamase from the vector pS9-lox-1 (Fig. 2, lane 4), one corresponding to HtrB and another corresponding to RfaE as seen with pHE17 (Fig. 2, lane 5).

Construction of NTHi 2019 htrB Mutants—In order to investigate the function of HtrB in Haemophilus, we constructed isogenic htrB mutants by shuttle mutagenesis. The 2.4-kb BglII fragment from pH1E0 was cloned into pH5S6, resulting in pH5S5H1, which was used for mini-Tn3 mutagenesis (12). Two plasmids, pB28 and pB29, each with a mini-Tn3 transposon inserted into the htrB ORF at a different location, were obtained (Fig. 1). Sequence analysis and restriction mapping of these plasmids indicated that in both plasmids, mini-Tn3 was inserted with the chloramphenicol acetyltransferase gene in the same orientation as htrB. Both plasmids were used to transform NTHi 2019, and cells were selected on chloramphenicol (1.5 μg/ml) plates. Because the E. coli htrB mutant is temperature-sensitive above 33°C, we incubated cells at 30°C. Mutant strains from each plasmid were designated NTHi B28 and B29, respectively. The locations of the mini-Tn3 insertions in the NTHi 2019 chromosome were confirmed by genomic Southern hybridization using the 2.4-kb BglII fragment as a probe. BglII digestion yielded a 2.4-kb fragment with NTHi 2019 DNA but produced 4.0-kb fragments with NTHi B28 and B29 DNAs, indicating that they contained the 1.6-kb transposon (data not shown). The 2.4-kb band remained the same in NTHi 2019 DNA upon BglII and EcoRI double digestion, but the 4.0-kb BglII bands of NTHi B28 and B29 were cut into two fragments by EcoRI, which is present in mini-Tn3.

mRNA Expression of htrB and rfaE in htrB Mutant Strains—Primer extension analysis was carried out to determine the promoter region of the htrB gene (Fig. 3A), and a single transcription start site was found using a primer (5′-
expression increased in
scription start site contained a sequence (TAAACT) similar to
translation start site ATG. The region upstream of the tran-
csor residue located at 21 base pairs upstream of the putative
CA). Similar results were observed for
itated using an Ambis 4000 gelscanner (Ambis Inc., San Diego,
with NTHi 2019 at both temperatures by 4–6-fold whenquan-
did not affect
growth rates of B28 and B29 were
showed growth at 37°C. The growth rates of B28 and B29 were
grown at 33°C, but slower growth was
htrB 2019 grown at 30°C; lanes 4, NTHi
B29 at 30°C; lanes 5, NTHi B29 grown at 37°C.

Compared with that of 2019 at 30°C, but slower growth was
observed at 37°C. The temperature sensitivity of these htrB−
strains was greater at 38.5°C, so that their growth reached a
maximum at 6 h and then started to decrease, suggesting cell
pression is not under heat shock regulation (3). To determine if the
same is true in Haemophilus, we measured the htrB transcript
level in RNA from NTHi 2019 grown at 30 and 37°C by primer
extension analysis. The results indicated that the transcript
level of htrB was not affected by temperature (Fig. 3A, lanes 2
and 3). Because the E. coli htrB mutation phenotype is very
 diverse and complex (2, 3, 5, 21, 22) and our previous data
suggested that it may play a role in regulation of gene expres-
sion (7), we measured htrB and rfaE expression levels in RNA
from NTHi 2109 and B29 grown at 30 and 37°C. Temperature
did not affect htrB expression in either strain. The level of
expression of htrB, however, increased in B29 as compared with
NTHi 2019 at both temperatures by 4–6-fold when quan-
tiﬁed using an Ambis 4000 gel scanner (Ambis Inc., San Diego,
CA). Similar results were observed for rfaE in that rfaE expres-
ion increased in htrB− cells and was not dependent on temper-
Fig. 3. Primer extension analysis of the NTHi 2019 htrB (A)
and rfaE (B) genes. The position of the transcription start site is
indicated with an asterisk on the sequence, and the position for the
extended product is indicated with an arrow. The DNA sequencing
ladders shown (GATC) were obtained using the same primer as that
used in the primer extension reaction. Lanes 1, no RNA; lanes 2,
NTHi 2019 grown at 30°C; lanes 3, NTHi 2019 grown at 37°C; lanes 4,
NTHi B29 at grown 30°C; lanes 5, NTHi B29 grown at 37°C.
owns to deoxycholate was similar to kanamycin. At 30 and 37°C,
NTHi 2019 grew well in the presence of 1000 μg/ml of deoxycholate and
still showed some growth in the presence of 2500 μg/ml of deoxycholate
(data not shown). At 30°C, B28 and B29 cells grew as well as
the wild type cells in the presence of 250 μg/ml of deoxycholate
but began to show sensitivity at 500 μg/ml and failed to grow at
1000 μg/ml. At 37°C, B28 and B29 began to show growth
inhibition at 50 μg/ml of deoxycholate and almost complete
inhibition at 250 μg/ml of deoxycholate.

Characterization of the NTHi htrB Mutants—NTHi B28 and
B29 strains were initially selected at 30°C and were not able to
grow at 37°C. After they were passed a few times at 30°C,
however, they began to lose this temperature sensitivity and
showed growth at 37°C. The growth rates of B28 and B29 were
comparable with that of 2019 at 30°C, but slower growth was
observed at 37°C. The temperature sensitivity of these htrB−

ccaatggcacaataggataggaagac-3’ complementary to the 5’
region of the htrB ORF. The ﬁrst nucleotide transcribed was a
C residue located at 21 base pairs upstream of the putative
translation start site ATG. The region upstream of the tran-
scription start site contained a sequence (TAAACT) similar to
the consensus −10 region of the bacterial σ70-dependent pro-
moters at an appropriate distance (6 base pairs). An element
(TTACCA) resembling the consensus sequence of the
region (TTGACA) was found. There was a 16-base pair spacer
between this region and the
sion is not under heat shock regulation (3). To determine if the
expression is not under heat shock regulation (3). To determine if the
same is true in Haemophilus, we measured the htrB transcript
level in RNA from NTHi 2019 grown at 30 and 37°C by primer
extension analysis. The results indicated that the transcript
level of htrB was not affected by temperature (Fig. 3A, lanes 2
and 3). Because the E. coli htrB mutation phenotype is very
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tiﬁed using an Ambis 4000 gel scanner (Ambis Inc., San Diego,
CA). Similar results were observed for rfaE in that rfaE expres-
ion increased in htrB− cells and was not dependent on temper-

Fig. 2. In vitro transcription-translation analysis of the NTHi 2019 htrB gene. Lane 1, protein molecular mass standards; lane 2,
pCR11; lane 3, pHIH; lane 4, pHEG; lane 5, pHE7; lane 6, pGEM-
Zf(+) (amp). The open circle indicates the protein product corresponding to
rfaE; the solid circle indicates that corresponding to htrB; the open
square indicates that corresponding to the ampicillin resistance gene;
the solid square indicates that corresponding to the kanamycin resistance
gene. The sizes of the molecular mass standards are shown on the left.
from black to brown. Reconstitution of mutant strains B29 with acrylamide gels (Fig. 5), and the color of the band had changed faster than that from NTHi 2019 on silver-stained SDS-polyacrylamide gels was not affected (1). The LOS from B28 and B29 migrated weakly on SDS-polyacrylamide gels but its migration pattern described that LPS of the sensitivity to deoxycholate at both temperatures.

We also performed immunoblotting analysis of LOS using the anti H. influenzae LOS mAb 6E4 (15) to determine if a change in LOS epitope structure occurred in the NTHi htrB mutants. Our studies demonstrated binding of this mAb to H. influenzae strain 2019 LOS at 30 and 37 °C. LOS from B29 and B28 did not bind to mAb 6E4 in the first few passages of the mutants. As the temperature sensitivity began to be repressed, we could show that mAb 6E4 reacted weakly with both B29 and B28 at 37 °C, whereas the antibody failed to react with the mutants at 30 °C. Reconstitution of B29 with pKHH reverted the mAb 6E4 phenotype of the corrected mutant to the wild type pattern.

Electrospray Ionization-Mass Spectrometry Analysis of NTHi htrB Mutant LOS—ESI-MS analysis of the O-deacylated LOS from the NTHi B29 strain provided a molecular mass profile of the LOS components that was similar but not identical to the wild type NTHi 2019 strain (see Fig. 6A). Despite their similarities, two differences can be readily discerned: one, a decrease in the level of LOS species containing two phosphoethanolamines and two, a shift to higher molecular mass LOS species containing more hexoses. This trend can be best seen by noting the most abundant species present in the B29 strain relative to the NTHi 2019 wild type. In the NTHi 2019 strain, this species is seen as the triply charged peak at m/z 799, (M-3H)−3, with a mass of 2,400 Da, corresponding to the previously reported LOS species containing a lactose disaccharide attached to a Hep3KDO(P)-Lipid A core with two PEA groups (24). In contrast, the ESI-MS spectrum of the B29 mutant O-deacylated LOS shows a triply charged base peak at m/z 853 (Mn = 2562), corresponding to a trihexose substituted core LOS with a much more prominent species at lower mass containing only one PEA group, i.e. (M-3H)−3 at m/z 812 with a M+ of 2439. Indeed, if one examines the relative proportion of LOS species containing one or two PEA groups between the wild type and mutant strains (Fig. 6, A and B, insets), it becomes clear that the PEA content has decreased significantly in the B29 strain. Further chemical analysis will be required to completely delineate the structural difference between these two strains, such as linkage positions of the singly and doubly phosphorylated core LOS and the nature of the LOS containing three or more hexoses. Nevertheless, the ESI-MS analysis clearly points to the major trends inherent in the htrB mutant LOS structures.

Mass Spectrometric Analysis of Lipid A—LSIMS analysis of the wild type 2019 strain produced a spectrum containing two abundant deprotonated molecular ions, (M-H)− at m/z 1823 and 1743, with several lower mass ions (see Fig. 7A). The two higher mass ions are consistent with a hexaacyl monophosphoryl lipid A, pKHH carrying the htrB gene restored the mobility and the color of LOS to those of the wild type (data not shown). To confirm that the htrB gene alone can complement the phenotypes of B28 and B29, we constructed a plasmid expressing htrB. The BamHI-PstI fragment carrying the htrB gene from pHIH was cloned into pGHH. Because strain B29 carried the chloramphenicol acetyltransferase gene in mini-Tn3, we cloned a kanamycin cassette into the PstI site of pGHH, resulting in pKHH. pKHH was transformed into B29 by electroporation and selected on kanamycin (15 μg/ml) at 30 °C to increase the probability to rescue transformants. pKHH carries the p15A origin, which is known to replicate in Haemophilus (23), but we were unable to detect any plasmid DNA in mini-plasmid DNA preparations from B29 cells transformed with pKHH. To detect any presence of plasmid DNA, we also transformed these mini DNA preparations into E. coli DH5α with selection for kanamycin resistance but did not get any transformants. A genomic Southern blot of the pKHH-transformed B29 cells, however, indicated that the kanamycin cassette had been integrated into the chromosome (data not shown). B29 transformed with pKHH behaved similarly to NTHi 2019 in sensitivity to deoxycholate at both temperatures.

LOS Analysis of NTHi 2019 htrB Mutants—It has been described that LPS of the E. coli htrB mutant silver-stained weakly on SDS-polyacrylamide gels but its migration pattern was not affected (1). The LOS from B28 and B29 migrated faster than that from NTHi 2019 on silver-stained SDS-polyacrylamide gels (Fig. 5), and the color of the band had changed from black to brown. Reconstitution of mutant strain B29 with
Mr = 1744) or two phosphates (hexaacyl diphosphoryl lipid A, Mr = 1824). Moreover, this spectrum is essentially identical to that previously reported for the major lipid A structure isolated from the LOS of *Haemophilus ducreyi* strain 35000 (25) and is also consistent with the hexaacyl lipid A structure determined by NMR from a deep rough mutant *H. influenzae* strain I-69 Rd²/B¹ (26). The lower mass fragment ions can be explained by the loss of myristic acid (−210 and/or 228 Da, m/z 1613/1595 and 1533/1515) and myristoylmyristic acid (−436 and/or 454 Da, m/z 1387/1369 and 1307/1289) as the ketene and/or protonated acids. These ions most likely arise through LSIMS-induced fragmentation of the higher mass mono- and diphosphorylated molecular ion species.

In contrast, the LSIMS spectrum shown in Fig. 7B for the lipid A preparation obtained from the mutant B29 strain lacks molecular ions corresponding to the wild type hexaacyl lipid A species. This spectrum contains two high mass ions at m/z 1613 and 1533 (Mr of 1614 and 1534) that correspond to the molecular ions for a di- and monophosphoryl pentaacyl lipid A species missing one myristic acid moiety. Because there are two myristic acid moieties (and four β-hydroxymyristic acids) on the parent wild type lipid A, it is not clear if these pentaacyl lipid A species originate from a single specific β-myristic acid deletion at one of two possible attachment sites or as a mixture formed by deletion at both sites. At lower masses, two additional molecular ions species are observed at m/z 1403 and 1323 that correspond to a mono- and diphosphoryl tetraacyl lipid A.
species lacking both myristic acid moieties. One should note that it is often difficult to distinguish between ions that are formed by LSIMS-generated fragmentation of higher mass parent ions losing fatty acyl groups as their corresponding ketenes from their isobaric molecular ion counterparts present in the original lipid A mixture lacking these same fatty acids. However, the presence of ions that are formed only through fragmentation processes can be used to help differentiate between these two possibilities. For example, the loss of myristic acid as the neutral free acid from the intact lipid A species (i.e. $-228$ Da as HOOCC(CH$_2$)$_{12}$CH$_3$) is characteristic of gas phase LSIMS fragmentation in the wild type lipid A spectrum. Ions of this type can be seen as ion pairs at m/z 1595/1515 and m/z 1385/1305 in Fig. 7A. These ions are largely absent in the LSIMS spectrum of the mutant B29 lipid A, supporting our interpretation that the tetraacyl lipid A ions at m/z 1403 and 1323 are formed primarily from molecular lipid A species (see Fig. 7B) and not as gas phase ketene losses of myristic acid (i.e. $-210$ Da as O=C=CH-(CH$_2$)$_{12}$CH$_3$) from the higher mass pentaacyl lipid A molecular ions.

**DISCUSSION**

We have identified a homologue of the E. coli htrB gene in NTHi 2019, which is transcribed in the opposite direction and immediately downstream of the rfaE gene. Studies of the htrB gene in E. coli have shown that this gene is associated with exquisite heat sensitivity but is not heat-inducible (3). Studies using E. coli htrB suppressor genes indicated that the phos-
pholipid content in the bacterial outer membrane is elevated and that the protein content, including porin proteins, is reduced at 42 °C in htrB mutant cells (5). This suggested that HtrB may be involved in phospholipid biosynthesis. Modifications in the lipid A structure of the htrB mutants might be a factor in the temperature sensitivity. Clementz and co-workers have shown that E. coli htrB and msbB encode KDO-dependent acyltransferases (27). Mass spectrometric analysis of the lipid A from the NTHi htrB mutant B29 indicated that modification of lipid A had occurred. The lipid A of the parent strain NTHi 2019 is hexaacyl. The analysis of the lipid A from B29 shows two species, a tetraacyl and a pentaacyl species, indicating loss of one or both of the myristic acid substitutions. It is interesting to speculate that in the low passage htrB mutants, the predominance of the tetraacyl lipid A species may account for the temperature susceptibility. As the suppressors, msbB (21) and msbA (22), of the htrB mutation are induced, they partially restore the myristic acid substitution and correct the temperature sensitivity.

The NTHi HtrB homologue has several other characteristics of the E. coli protein. It encodes a basic protein with an isoelectric point of 10.31 and molecular mass of 36,066 Da, which is slightly smaller than the E. coli HtrB protein. NTHi htrB mutants were temperature-sensitive upon initial isolation but, unlike E. coli mutants, upon passage at 30 °C developed a temperature profile similar to that of the wild type strain. This suggests that factor(s) similar to the E. coli msbA, msbB, and accBC genes may be operative that are suppressing the htrB mutation (5, 21, 22).

In our study, NTHi htrB mutants showed hypersensitivity to kanamycin and deoxycholate compared with the wild type strain. In contrast to our results, E. coli htrB mutants exhibit higher resistance to deoxycholate than does the wild type (21). A difference in phenotype between Haemophilus and E. coli mutants and the corresponding wild type strains was also found in SDS-polyacrylamide gel patterns of LPS/LOS. LPS from both species were changed in color on silver-stained gels, indicating that their structures were modified. The LOS from the NTHi mutant, however, migrated faster than that from its parent strain, whereas LPS from the E. coli mutant strain did not show any change in migration on SDS gels (1).

ESI-MS analysis of LOS from the NTHi htrB mutant confirmed modification of the LOS and revealed a 50% reduction in the LOS species containing two phosphoethanolamines as well as a shift to higher molecular weight species not seen with LOS from the NTHi 2019 parent strain. These findings suggest that the degree of phosphorylation of heptose may be affecting chain progression from specific heptose moieties. In addition, elongation of these chains may be related to the degree of phosphorylation. A reduction in phosphate on heptose moieties has also been observed in LPS from rfaP mutant strains. rfaP mutants were initially isolated from S. typhimurium and characterized with respect to their sensitivity to hydrophobic antibiotics and detergents (26). The most striking feature of these mutants is the lack of a phosphate group linked to heptose I of the LPS core structure. RfaP is believed to have an LPS kinase-like function (28). There is no evidence that HtrB acts as an LPS kinase, but it may indirectly regulate phosphorylation of LOS. rfaP mutants are also moderately heat-sensitive, but the unique temperature profile of htrB mutant strains cannot be explained solely by dephosphorylation of LOS. At present, little is known about the enzymology or regulation of phosphate or phosphoethanolamine incorporation into LOS in H. influenzae. No one has identified an rfaP homologue in H. influenzae. We are currently pursuing studies in these areas.

Several studies indicate that the assembly and organization of the bacterial outer membrane require specific interactions between proteins and LPS and that the core structure is the region that is responsible for these interactions (29). It was also suggested that sensitivity to hydrophobic agents is not directly associated with truncations in the carbohydrate structure but with loss of phosphate groups on heptose moieties, indicating that the presence of phosphate residues may be even more important than that of the addition of saccharide groups. Nkaido and Vaara (29) also suggested that the bridging of negatively charged phosphate groups appears to be important in LPS-LPS interactions, which serve as a very effective barrier against hydrophobic molecules. The hypersensitivity of NTHi htrB mutants to kanamycin and deoxycholate could be explained in the context of dephosphorylation of LOS. This idea is also supported by the observation of Ray et al. (30) that LPS from Pseudomonas syringae, isolated in Antarctica, was more phosphorylated when grown at higher temperatures than when grown at lower temperatures and was more sensitive to cationic antibiotics such as kanamycin when grown at low temperatures. The greater sensitivity of NTHi htrB mutants to kanamycin at the higher temperature may be due to the synergistic effect of these two factors. The susceptibility of the htrB mutant to deoxycholate and kanamycin did not change with the restoration of the temperature stability. This indicates that the mechanisms controlling these phenotypes are different. The temperature sensitivity is related to modification in the lipid A and the changes in cell membrane permeability to modifications in phosphoethanolamine content of the LOS.

This hypothesis is also supported by our observation that the NTHi htrB mutants are more heat-sensitive than the NTHi rfaD mutant lacking heptose (and, thus, lacking a phosphate group)2 and that this heat sensitivity is lost upon passage that the temperature profile of htrB mutants may not be directly associated with dephosphorylation of LOS.

The increased expression of htrB and rfaE in the htrB strains cannot be explained at this time. Motif analysis of HtrB failed to indicate the presence of DNA binding domains. It is interesting, however, that the htrB gene shares the promoter region with the rfaE gene coding for ADP-heptose synthase involved in LOS biosynthesis and both of the gene expressions are elevated in htrB strains, suggesting that these two genes are transcriptionally related. It is also tempting to speculate that HtrB may down-regulate these genes (and others) and, thus, play an important role in the regulation of LOS biosynthesis.

As described, htrB mutant strains exhibit very diverse and complex phenotypes. It is not likely that the HtrB protein directly exerts all of these separate functions, but these may be the results of indirect effects regulated by HtrB. Our results strongly suggest that H. influenzae HtrB is a multifunctional protein with either acyltransferase activity or the ability to regulate this activity. In addition, it appears to play an important role in controlling cell responses to environmental changes including temperature.

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