Lipooligosaccharide biosynthesis genes of nontypable *Haemophilus influenzae* 2019

Na-Gyong Lee[1], Melvin G. Sunshine[2], Jeffery J. Engstrom[3], Bradford W. Gibson[3] and Michael A. Apicella[2].

Laboratory of Microbiology, College of Pharmacy, Seoul National University, Seoul 151-742, Korea[1], Department of Microbiology, University of Iowa, Iowa City, IA 52242, U.S.A.[2] and Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143, U.S.A.[3]

Haemophilus influenzae is a causative agent of many childhood disease, including meningitis and respiratory tract infections. In developed countries, *H. influenzae* type b strain (Hib), a capsular serotype, is the leading cause of bacterial meningitis in young children. The capsular polysaccharide of this organism plays a critical role in mediating the virulence of Hib. Nontypable 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. Since NTHi lacks a capsular polysaccharide, its pathogenicity is mediated more directly by surface components of the bacterial outer membrane, including proteins and lipooligosaccharides (LOS). The LOS of *H. influenzae* is analogous to the lipopolysaccharide (LPS) of enteric gram-negative bacteria; it contains lipid A linked by 3-deoxy-D-manno-octulosonic acid (KDO) to a heterogeneous sugar polymer, but the LOS lacks the repeating O-antigen units (Fig. 1) (Gibson *et al.*, 1993). The physicochemical analysis of the structure of oligosaccharide of NTHi LOS has shown a unique deep core structure with variable branched chains which makes it more complex than LOS from *Neisseria* and *Bordetella* species. The lipid A portion of LOS is responsible for the toxicity associated with this organism, but the role of the oligosaccharide portion of LOS in pathogenesis of NTHi is less clear. An understanding of structural and genetic features of NTHi LOS is essential not only to identifying surface-exposed epitopes available for host/pathogen interactions but also to developing vaccines for this organism.

Isolation and characterization of the *rfaD* (ADP-heptose epimerase) and *rfaF* (heptosyltransferase) gene cluster

The ability to study the genes required for LOS biosynthesis has been hampered by the lack of genetic tools for *Haemophilus* species. Transposon mutagenesis has been used to clone the LOS biosynthesis genes by generating corresponding phenotype mutants, but the low efficiency of mutagenesis and the

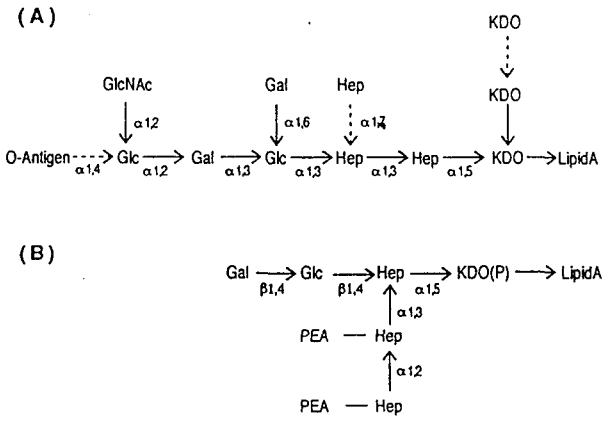


Fig. 1. Schematic illustration of the structures of *S. typhimurium* LPS (A) and NTHi 2019 LOS (B). Abbreviations: Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, L-glycero-D-mannoheptose; KDO, 3-deoxy-D-manno-octulosonic acid; P, phosphate; PEA, phosphoethanolamine. Possible partial substitutions are indicated with dashed arrows. The higher-molecular-weight forms of NTHi 2019 LOS have not been fully characterized.

regional preference of insertion sites have limited its success in identifying LOS biosynthesis genes. Furthermore, it is often difficult to assign the functions of identified genes (McLaughlin *et al.*, 1994). The genetics of *Salmonella* LPS biosynthesis has been studied extensively during last two decades, and a great number of LPS mutant strains have been isolated and characterized. The *rfa* genes, which encode LPS core biosynthesis enzymes, are present as a cluster on *S. typhimurium* chromosomes, and several *rfa* genes have been cloned (Schnaitman and Klena, 1993). The LPS of *Salmonella* and the LOS of *Haemophilus* share common structure in the deep core region, which made it possible to isolate genes responsible for biosynthesis of this region from *Haemophilus* species by complementing *Salmonella* LPS deep core mutants.

To isolate genes involved in LOS biosynthesis of NTHi 2019, we undertook a series of complementation studies of *S. typhimurium* LPS mutants with defined enzymatic defects. To accomplish this, the NTHi 2019 plasmid library DNA was transformed into *rfaD*$^-$ strain SL3600. The *rfaD* gene encodes an ADP-heptose epimerase, and LPS from the *rfaD*$^-$ strain is truncated due to the lack of substrate. Transformants carrying a plasmid containing the *rfaD* gene were selected on the basis of the properties of *Salmonella* strains with a wild type LPS that are less permeable and thus more resistant to hydrophobic antibiotics than mutants strains with a defective LPS core structure (Roantree *et al.*, 1977). The complementing ability of the plasmid DNA was also confirmed by testing for sensitivity to the LPS-specific

phages. Plasmid DNA extracted from an transformant contained an insert of 4.8kb, which was designated pD41 (Fig. 2). SDS-PAGE analysis showed that the LPS of SL3600 complemented with plasmid pD41 was converted to the wild-type phenotype (Fig. 3).

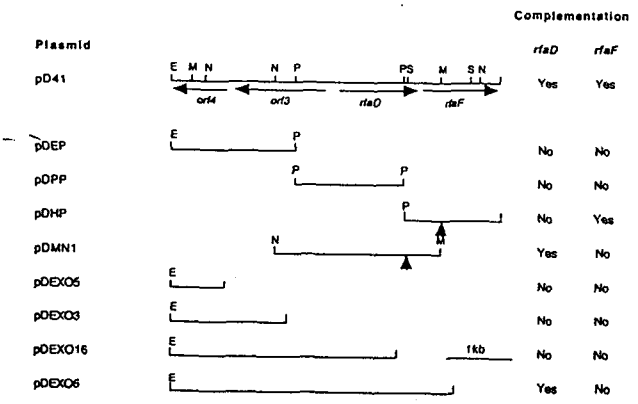
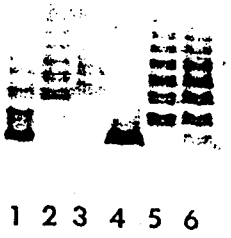


Fig. 2. Restriction map of the regions of the *rfaD* and the *rfaF* genes of NTHi 2019, and complementation of the *S. typhimurium* LPS mutants by plasmids carrying NTHi 2019 genomic DNA. The arrows indicate open reading frames. Two triangles marked on the map show kanamycin cassette insertion sites in the isogenic *rfaD* and *rfaF* mutants. E, *Eco*RI; M, *Mlu*I; N, *Nsi*I; P, *Pst*I; S, *Sty*I.

Fig. 3. Silver-stained LPS gel showing complementation of the *S. typhimurium* LPS mutants by plasmids carrying the NTHi 2019 *rfaD* and the *rfaF* genes. Lanes: 1, SL3600(rfaD$^-$); 2, SL3600 carrying pD41 ; 3, SL3600 carrying pDMN1; 4, SL3855(rfaF$^-$); 5, SL3855 carrying pD41; 6, SL3855 carrying pDHP.



In *S. typhimurium rfaD* is followed by *rfaF* and *rfaC*, constituting an operon (Schnaitman and Klena, 1993). The *rfaF* and *rfaC* genes code for heptosyltransferase II and I, respectively. We tested this possibility in NTHi 2019 by complementing the *rfaF* (SL3855) and *rfaC* mutant strains (SL1377) with pD41. pD41 was able to complement *rfaF* but not *rfaC* phenotype. Sequence analysis of an 4.8Kb insert revealed three complete and one partial open reading frames (ORF). One of them encodes a polypeptide of 308 amino acid residues highly homologous to that of *S.*

typhimurium (77% identity). Another ORF downstream encodes a polypeptide of 346 amino acid residues homologous to the counterpart of *S. typhimurium* (62% identity). The primer extension analysis performed to determine the promoter region showed that the transcription of the *rfaD* gene started at 26bp upstream of the putative translation start codon ATG and the promoter region contained the elements typical to bacterial σ^{70}-dependent promoters. *In vitro* transcription/translation analysis and SDS-PAGE of LOS isolated from *S. typhimurium* complemented with the NTHi *rfaD* and *rfaF* genes suggested that these two genes form an operon. To determine whether the *rfaC* gene is present downstream of these two genes as in *Salmonella*, we cloned the gene downstream of *rfaF*, which did not have complementing ability of *rfaC* mutation nor was homologous to the *Salmonella rfaC* gene.

To confirm the function of these genes in NTHi 2019, we constructed isogenic mutants of *rfaD* and *rfaF* by inserting a kanamycin cassette into each ORF. The *rfaD* mutant failed to grow at 37°C in the presence of kanamycin, but it was resistant to kanamycin at 30°C. The *rfaF* mutant did not show any temperature sensitivity regardless of the presence of kanamycin. The LOS isolated from these mutant strains migrated faster on SDS-polyacrylamide gel than that of the wild type (data not shown). Preliminary study of chemical structures of these mutants suggests that the *rfaD* mutant is capable of putting the D,D-heptose isomer on KDO. Chain extension stops because this appears to need the D,L-isomer. This explains why the SDS-PAGE pattern of the LOS of the *rfaD* and *rfaF* mutants migrated identically. These experiments demonstrated that the genetic organization of genes involved the LOS core biosynthesis of NTHi 2019 is similar but not identical to that of *S. typhimurium*.

Cloning and characterization of the ADP-heptose synthase gene (rfaE)

The *rfaE* gene of *S. typhimurium* is known to be located at 76 min on the genetic map outside of the *rfa* gene cluster. The *rfaE* mutant synthesizes heptose-deficient LPS, and the *rfaE* gene is believed to be involved in the formation of ADP-heptose, but cloning of the *rfaE* gene has not been reported yet (Sirisena *et al.*, 1992; Wilkinson *et al.*, 1972). A plasmid carrying the *rfaE* genes was isolated from NTHi 2019 by complementing SL1102, a *S. typhimurium rfaE* mutant strain with an NTHi 2019 plasmid library. Retransformation with the plasmid pHEI0 containing 4kb isolated from a reconstituted mutant into SL1102 gave wild-type LPS phenotypes. SDS-PAGE analysis confirmed the conversion of the *rfaE* mutant LPS to a wild-type LPS phenotype.

Sequence analysis of a 2.4 kb *Bgl*II fragment revealed two ORFs, and these two genes are transcribed from the same promoter region into opposite directions (Fig. 4) (Lee *et al.*, 1995a). One ORF encodes a polypeptide of 311 amino acid

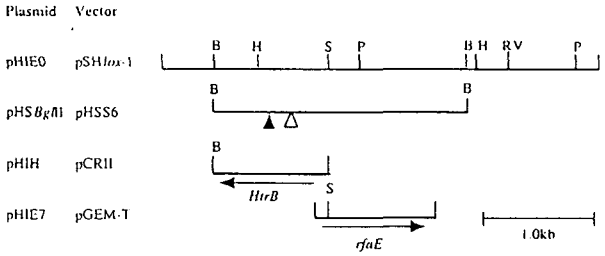


Fig. 4. Restriction map of the region of the NTHi 2019 *htrB* and the *rfaE* genes. The arrows indicate open reading frames. Two triangles marked on the map show mini-Tn3 insertion sites in isogenic *htrB* mutants. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; RV, *Eco*RV; S, *Sca*I.

residues highly homologous to the *E. coli* HtrB protein (56% identity and 74% similarity). The other encodes a polypeptide of 342 amino acid residues with a molecular weight of 37.6 kDa, which was confirmed by *in vitro* transcription/translation analysis. Disruption of this ORF in pHIE7 by insertion of mini-Tn3 abolished the complementing ability of the plasmid, which indicated that this ORF was the authentic *rfaE* gene. A database search for sequence homology revealed that an ORF present downstream of the *E. coli* fructose phosphotransferase system genes exhibited homology with the RfaE (21% identity, 45% similarity). Currently, the function of this ORF is not known, but its location and the homology to the NTHi RfaE suggest it may have a function similar to that of the RfaE, i.e., phosphorylation of sugars. Since HtrB has been suggested to be involved in LPS biosynthesis (see below), it is interesting that in NTHi 2019 *rfaE* and *htrB* share the promoter region, suggesting that the mechanisms of regulation of their expressions are related. To determine the function of RfaE in *Haemophilus* LOS biosynthesis, a mutagenesis study with strains NTHi 2019 and Hib was performed, but *rfaE* mutants could not be recovered. This may be due to the lethal effect of accumulation of substrates in the *rfaE* mutant rather than modification of the cell wall since we could rescue an *rfaD* mutant which has the same LOS phenotype as would be expected with an ADP-heptose synthase mutant.

Roles of the HtrB protein in LOS biosynthesis

The HtrB protein was first identified in *E. coli* as a protein required for cell viability at a temperature above 33°C (Karow *et al.*, 1991) Unlike other heat shock proteins, however, its expression was not regulated by temperature. Bacteria with a mutation in the *htrB* gene, when exposed to nonpermissive temperature in rich media, cease to divide and lose viability with morphological changes. Many *htrB* suppressor genes have been isolated to elucidate the function of HtrB, but exact

function of HtrB is not known yet.

In the process of sequencing the upstream region of the NTHi 2019 *rfaE* gene, we identified the *htrB* homolog, which was able to functionally complement the *E. coli htrB* mutation. These two genes have overlapping promoter regions and are transcribed into diverse orientation (Fig. 4). The deduced amino acid sequence of NTHi 2019 HtrB shared 56% homology with *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 σ^{70}-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 investigate the function of HtrB in *Haemophilus*, we constructed two isogenic *htrB* mutants by shuttle mutagenesis using mini-Tn3 (Lee *et al.*, 1995b) 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. Previous reports (Karow and Georgopoulos, 1991) have suggested that HtrB is involved in LPS synthesis and\or cell wall formation, subsequently changing membrane permeability to various compounds. Therefore, we tested NTHi *htrB*$^-$ strains for sensitivity to kanamycin and deoxycholate. They showed hypersensitivity to both compounds compared with the wild type strain, which persisted on passage.

SDS-PAGE analysis revealed that the LOS isolated from these mutants migrated faster than the wild type LOS and its color changed from black to brown on silver staining (Fig. 5). 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, suggesting changes in the chemical structures of LOS in *htrB*$^-$ strains. Electrospray ionization-mass spectrometry analysis of the *O*-deacylated LOS oligosaccharide indicated a decrease in the level of LOS species containing two phosphoethanolamine (PEA) and a shift to higher molecular mass LOS species containing more hexose (Fig. 6), indicating a net loss in PEA on the core structure. 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

Fig. 5. SDS-polyacrylamide gel analysis of LOS from NTHi 2019 and isogenic *htrB* mutants. The arrow indicates the direction of sample migration. Lanes 1 and 4, NTHi 2019; lane 2, NTHi B29; lane 3, NTHi B28.

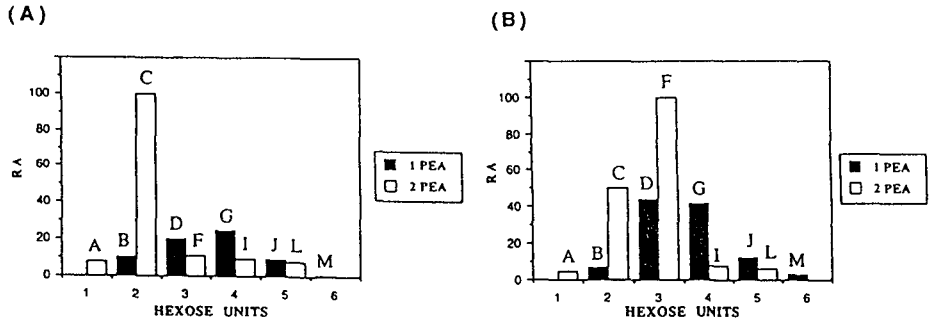


Fig. 6. Histrograms showing proposed composition differences among LOS glycoforms relative to the number of hexose (1-6) and phosphoethanlamine (PEA) (1-2) units. In both cases a core Hep3-KDO-lipid A is present. (A) NTHi 2019 LOS, (B) isogenic *htrB* mutant B29.

characterized with respect to their sensitivity to hydrophobic antibiotics and detergents (Helander *et al.*, 1992). 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. 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. The hypersensitivity of NTHi *htrB* mutants to kanamycin and deoxycholate could be explained in the context of dephosphorylation of LOS. This idea is supported by the observation of Ray *et al.* (1994) 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 at lower temperature. The susceptibility of the htrB mutant to deoxycholate and kanamycin did not change with the restoration of the temperature stability. This indicate that the mechanism controlling these phenotypes are different.

Mass spectrometric analysis of the lipid A portion of LOS from *htrB* mutant indicated a loss of one or both myristic acid substitutions (Lee *et al.*, 1995b). This observation is consistent with the finding that HtrB of NTHi 2019 has acyltransferase activity (unpublished data). It is, thus, interesting to speculate that in the low passage *htrB* mutants, the predominance of the tetraacyl or pentaacyl lipid A species may account for the temperature susceptibility and accumulations of suppressors, like *msbA* and *msbB* (Karow and Georgopoulos, 1992; 1993), may partially correct temperature sensitivity after several passages. 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

NTHi 2019 HtrB is a multifunctional protein with acyltransferase activity. In addition, it appears to play an important role in controlling cell responses to environmental changes including temperature.

Summary

LPS/LOS, the compound found only in gram-negative bacterial outer membrane, plays important roles in bacterial maintenance as well as its pathogenesis. We isolated and characterized several genes required for NTHi 2019 LOS biosynthesis, which encode enzymes required for sugar substrate synthesis or the transfer of substrates to receptor molecules. The *htrB* gene, however, appears to have more complex role. It has acyltransferase activity as well as various other activity, which may control regulation of LOS biosynthesis as well as its pathogenicity. Evidences supporting the latter come from the observations that the lipid A of the B29 induced significantly less TNF α from macrophages than that of the wild type LOS (unpublished data). *H. influenzae* A2-*htrB* mutant strain was also significantly less invasive than the wild type strain. The structural similarities of the enterobacterial LPS and the *Haemophilus* LOS enabled us to isolate the NTHi 2019 genes involved in LOS biosynthesis genes by using the *S. typhimurium* LPS deep core mutants. While a similar approach has been used for *E. coli*, this technique for selection of an LPS phenotype has not been applied to nonenterobacterial species. The difficulties inherent in the molecular manipulation of organism such as *Neisseria* and *Haemophilus* species make this approach particularly attractive in the identification and cloning LOS genes. Studies on genetic features of LPS/LOS biosynthesis would be useful for understanding bacterial pathogenesis as well as for developing vaccines for these gram-negative pathogenic bacteria.

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