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Phosphorylation of the Lipid A Region of Meningococcal Lipopolysaccharide: Identification of a Family of Transferases That Add Phosphoethanolamine to Lipopolysaccharide

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A gene, NMB1638, with homology to the recently characterized gene encoding a phosphoethanolamine transferase, *lpt-3*, has been identified from the *Neisseria meningitidis* genome sequence and was found to be present in all meningococcal strains examined. Homology comparison with other database sequences would suggest that NMB1638 and *lpt-3* represent genes coding for members of a family of proteins of related function identified in a wide range of gram-negative species of bacteria. When grown and isolated under appropriate conditions, *N. meningitidis* elaborated lipopolysaccharide (LPS) containing a lipid A that was characteristically phosphorylated with multiple phosphate and phosphoethanolamine residues. In all meningococcal strains examined, each lipid A species contained the basal diphosphorylated species, wherein a phosphate group is attached to each glucosamine residue. Also elaborated within the population of LPS molecules are a variety of "phosphoforms" that contain either an additional phosphate residue, an additional phosphoethanolamine residue, additional phosphate and phosphoethanolamine residues, or an additional phosphate and two phosphoethanolamine residues in the lipid A. Mass spectroscopic analyses of LPS from three strains in which NMB1638 had been inactivated by a specific mutation indicated that there were no phosphoethanolamine residues included in the lipid A region of the LPS and that there was no further phosphorylation of lipid A beyond one additional phosphate species. We propose that NMB1638 encodes a phosphoethanolamine transferase specific for lipid A and propose naming the gene "*lptA*," for "LPS phosphoethenolamine transferase for lipid A."

Lipopolysaccharide (LPS) is found in all gram-negative bacteria and is usually composed of three regions: lipid A, core oligosaccharide (OS), and a polysaccharide molecule often referred to as the "O-antigen." LPS from *Neisseria meningitidis* lacks the polysaccharide and is sometimes referred to as "lipooligosaccharide" (LOS) to reflect this. The core OS unit of *N. meningitidis* LPS comprises an inner core diheptose-*N*-acetylglucosamine backbone, wherein the two L-*glycero*-D-*manno*-heptose (Hep) residues can provide a point of attachment for outer core OS residues (8). Meningococcal LPS has been classified into 12 distinct LPS immunotypes (L1 to L12), originally defined by monoclonal antibody (MAb) reactivities (21) but further defined by structural analyses (4, 5, 7, 9, 14, 16, 26). The structural basis of the immunotyping scheme is primarily governed by the location of a phosphoethanolamine (PEtn) moiety on the distal heptose residue (HepII) at either the 3- or 6-position or absent, but is also dictated by the length and nature of OS extension from the proximal heptose residue (HepI) and the presence or absence of a glucose at HepII (17 [Fig. 1]). The lipid A region of the LPS is responsible for much of the toxicity of the LPS molecule, and LPS is indeed sometimes referred to as "endotoxin." The lipid A region consists of a disaccharide of pyranosyl glucosamine residues, the reducing α-configured residue being substituted at the 6-position by the β-configured residue. Each glucosamine sugar is similarly acylated at the 2-position with N-linked β-hydroxymyristic acid (3-OH C14:0), with lauric acid (C12:0) residues in turn attached to the β-hydroxy group of the 3-OH C14:0 fatty acids (10). Additionally, the 3-position of each glucosamine residue is acylated with O-linked β-hydroxylauric acid (3-OH C12:0). The lipid A region is typically substituted by a phosphate group glycosidically linked at the 1-position of the α-configured glucosamine residue and another phosphate group linked at the 4-position (4′-) of the β-configured glucosamine residue (basal diphosphorylated species). Studies by Kulshin et al. (10) found that both phosphate groups of the lipid A region of the nonencapsulated variant of the meningococcal serogroup B strain M986 were mostly substituted by *O*-phosphorylethanolamine moieties. In a more recent study, Tzeng et al. (25) found that the 4′-phosphate group in the lipid A region of a 3-deoxy-D-manno-octulosonic acid (Kdo) transferase mutant of the strain NMB was substituted to some extent with *O*-phosphorylethanolamine, and in some cases the glycosidic phosphate group was absent. Previous studies from our laboratory have suggested the presence of more extensively phosphorylated lipid A molecules (17). The genetic basis of phosphorylation of lipid A to form the basal diphosphorylated species is well understood (28). LpxH cleaves the pyrophosphate bond of UDP-2,3-diacylglucosamine to generate UMP and 2,3-diacylglucosamine-

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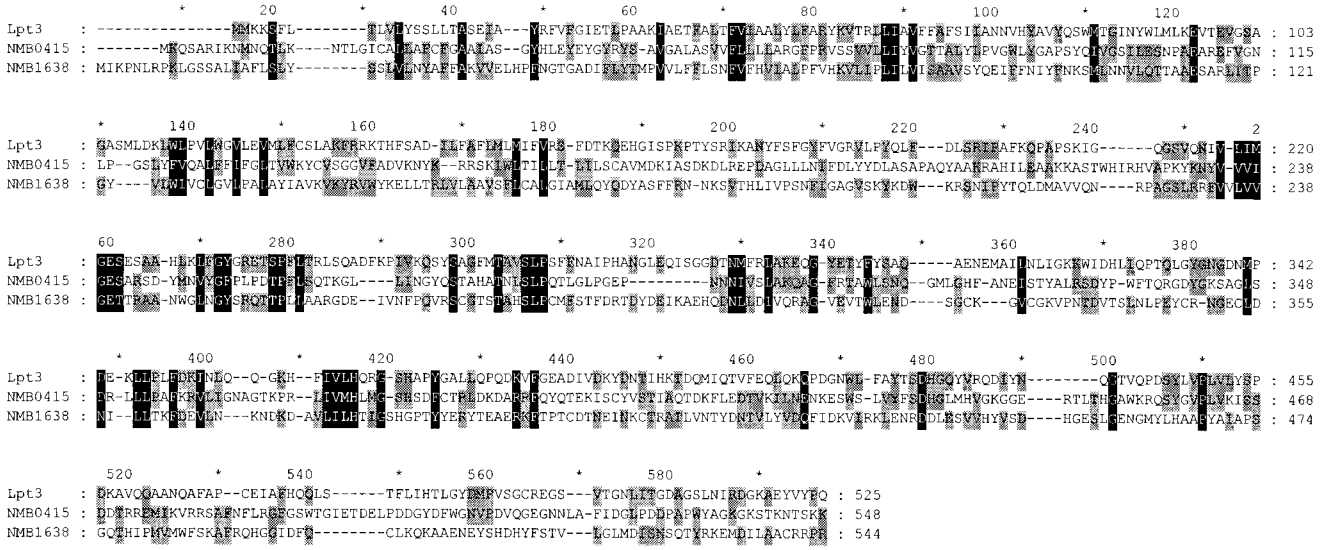

FIG. 1. Alignment of translated amino acid sequences of *lpt-3*, NMB0415, and NMB1638 by Clustal X. The shading indicates the residues that are conserved between the relevant sequences.

1-phosphate in the fourth step of lipid A biosynthesis, and LpxK transfers a phosphate group from ATP to the 4-position of the β-glucosamine residue to generate the lipid IV_A molecule in the fifth step of lipid A biosynthesis. However, nothing is known of the genetic basis of further phosphorylation of the lipid A molecule. Previous studies in our laboratories identified a gene, *lpt-3*, that encodes a protein that is responsible for the specific transfer of a PEtn residue to the 3-position of the HepII residue in the core OS (11). The knowledge that the lipid A region can contain PEtn residues led us to search for homologs to *lpt-3* in the meningococcal genome and to investigate any function these homologs may have in the phosphorylation of the lipid A region.

In the present study, complex phosphorylation patterns were identified in the lipid A region that are observed in all meningococcal strains examined following growth and LPS isolation under appropriate conditions. A gene that is involved in the transfer of PEtn to the lipid A molecule was identified, and this gene's product was found to be part of a family of proteins that may have conserved function across a wide range of gram-negative bacteria.

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MATERIALS AND METHODS

Strains and culture conditions. The *N. meningitidis* strains H44/76 (B:15:P1.7,16:L3) and MC58 (B:15:P1.7,16b:L3) have both been described previously (6, 13). Strains 35E and 89I are both serogroup C strains and are the reference strains for the L2 and L4 immunotypes, respectively. A collection of 53 diverse *N. meningitidis* strains including representatives of each capsular serogroup was used to investigate the distribution of target genes (12). The *Neisseria lactamica* strains used were recent isolates from human carriage.

All strains were grown overnight at 37°C on brain-heart infusion (BHI) medium (Oxoid), solidified with agar (1% [wt/vol]; Bioconnections), in an atmosphere of 5% CO_2. Kanamycin (100 μg ml^{-1}) or erythromycin (6 μg ml^{-1}) was added to the culture medium for selection of transformed strains as appropriate. *Escherichia coli* strain DH5α was used to propagate recombinant DNA constructs and was grown at 37°C on Luria-Bertani (LB) medium.

Recombinant DNA techniques. Recombinant DNA techniques were performed as described by Sambrook et al. (20). Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim or New England Biolabs and used according to the manufacturer's instructions. Oligonucleotide primers were synthesized by Sigma Genosys. PCR amplifications were performed with *Taq* polymerase (Invitrogen) in 50-μl reaction volumes in a Master-Cycler gradient thermal cycler. Thirty cycles of PCR were performed, each consisting of 1 min of denaturation at 94°C, 1 min of annealing at 54°C, and 1 min of extension at 72°C. Chromosomal DNA was prepared from *N. meningitidis* strains by a method described elsewhere (1).

To mutate NMB1638, the gene was first amplified by PCR from strain MC58 chromosomal DNA with oligonucleotide primers 1638-a (5′ CTGATTGCCTT CCTTTCC) and 1638-b (5′ TCAGCCCCAATACCGTGC) and cloned into plasmid pT7-blue (Invitrogen). A kanamycin resistance cassette was excised from pUC4kan by digestion with *Eco*RI and inserted into the *Mfe*I site within the cloned NMB1638 gene. The resulting construct was used to transform strains H44/76, 35E, and 89I, and transformants were selected on kanamycin. To make the H44/76::1638::lpt-3 double mutant, a previously described construct with an erythromycin resistance cassette inserted in the *lpt-3* gene (11) was used to transform the parental strain, H44/76. Chromosomal DNA was prepared from strain H44/76::lpt-3 and used to transform strain H44/76::1638, and transformants were selected on erythromycin. The insertional inactivation of the two genes was confirmed by PCR amplification with oligonucleotide primers 1638-f (5′ GCCTTCCTTTCCCTGTATTCC) and 1638-ri (5′ CTGTAACCGTTCAA ACCCC) for NMB1638 and primers H3r and H3f (11) for the *lpt-3* gene. The PCR conditions were as described above, but an annealing temperature of 50°C was used for primers 1638-f and 1638-ri.

The insertional inactivation of NMB0415 was performed in a similar manner. Oligonucleotide primers 0415-A (5′ ATTTGCGCGCTTTTAGCC) and 0415-B (5′ CTTTTCCCGCATACCACG) were used to amplify the gene from strain MC58 chromosomal DNA, with an annealing temperature of 50°C, and the product was cloned into pT7-blue. A kanamycin resistance cassette was excised from pUC4kan by digestion with *Hinc*II, inserted into a site cut with *Bst*EII, and then end filled with Klenow enzyme (20) within the cloned NMB0415 gene. The resulting construct was used to transform strains 35E, 89I, and H44/76, and transformants were selected on kanamycin. Insertional inactivation of the gene was confirmed by PCR amplification with oligonucleotide primers 0415-A and 0415-B.

Tricine-SDS-PAGE. Whole-cell lysates were prepared from *N. meningitidis* strains grown overnight by harvesting and resuspending cells in phosphate-buffered saline (PBS) and then adding an equivalent amount of dissociation buffer and heating at 100°C for 5 min. Samples were then separated on 16.5% Tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels run at 30 mA at 4°C for 18 h.

Preparation of LPS. LPS samples were obtained from *N. meningitidis* strains grown overnight as described previously (17). Typically bacteria were scraped from 40 BHI plates and resuspended in 30 ml of 1.8% phenol in PBS. LPS was extracted by the hot phenol-water method of Westphal and Jann (27). Contaminating RNA was removed from the crude LPS following the first phenol extraction by incubation with 1,000 U of RNase (Promega) for 1 h at 37°C. A second phenol extraction was performed before precipitation of the LPS overnight with 0.5 M NaCl in 3 volumes of ethanol. The LPS was recovered by centrifugation at 4,000 × *g* at 4°C for 25 min, washed with 70% ethanol and then freeze dried for 6 h. O-deacylated LPS was prepared as described previously (17).

MS. Capillary electrophoresis (CE)-mass spectrometry (MS) and tandem MS (CE-MS/MS) analyses of O-deacylated LPS samples were carried out as described previously (3).

RESULTS

Identification of Lpt3 homologues. We have recently reported the identification of a gene, *lpt-3*, that is required for the addition of PEtn to the 3-position on the distal heptose (HepII) residue of the inner core of *N. meningitidis* LPS (11). Structural analysis has shown that PEtn is present at other positions within the LPS of *N. meningitidis*—e.g., at the 6-position of HepII (5, 9) or on the lipid A moiety (10)—but currently the genes required for transfer of PEtn to these positions have not been identified. The sequencing of the genome of the *N. meningitidis* serogroup B strain MC58 (24) has allowed us to search for homologs of the *lpt-3* gene within the same strain. Searches were performed with the TBLASTX algorithm, which compares an amino acid sequence against a nucleotide sequence, translated into all six reading frames. Two open reading frames with homology to Lpt3 were identified: NMB1638 (e value, 5.2×10^{-13}) and NMB0415 (e value, 4.8×10^{-9}). NMB1638 has been annotated as belonging to the same protein family as *lpt-3*, while NMB0415 is a conserved hypothetical protein, disrupted by an authentic frame shift, caused by a poly(C) tract (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gnm). Alignment of the amino acid sequences encoded by *lpt-3*, NMB1638, and NMB0415 showed each to be of a similar length and that a number of conserved residues were present across the entire length (Fig. 1). Further homology searches against completed genome sequences in the public database revealed that the three sequences formed part of a likely family of proteins that were conserved across a wide range of gram-negative bacteria.

Distribution of NMB1638 and NMB0415 among *Neisseria* strains. To investigate the distribution of NMB1638 and NMB0415, oligonucleotide primers were designed to amplify the genes by PCR from 53 *N. meningitidis* strains, including representatives from serogroups A, B, C, X, Y, and W-135 and the immunotyping strains (Table 1). Each strain tested gave a PCR product of the expected size for both NMB1638- and NMB0415-specific primers, except BZ147 and M981, which gave no product when the NMB0415-specific primers were used. Thus, both genes are widely distributed; NMB1638 is present in all *N. meningitidis* strains, while NMB0415 is present, as tested by PCR amplification, in 96% of *N. meningitidis* strains. The distribution of these two genes implies a general role regarding function that is not restricted by the immunotype of the LPS. A collection of 28 *N. lactamica* strains was similarly tested, and just one strain gave a PCR product with the NMB1638-specific primers, while no products were obtained with the NMB0415-specific primers, suggesting that these two genes were not conserved in this commensal species.

TABLE 1. *N. meningitidis* strains used to screen for the presence of NMB1638 and NMB0415 by PCR amplification

Strain	Serogroup/Immunotype
106	A
243	A
255	A
393	A
690	A
1014	A
7891	A
92001	A
00153	A
11-004	A
120M	A
14/1455	A
322/85	A
A4/M1027	A
C751	A
D8	A
F4698	A
F6124	A
S5611	A
528	B
1000	B
3906	B
297-0	B
44/76	B
AK22	B
B6116/77	B
BZ147	B
BZ198	B
BZ232	B
EG328	B
M40/94	B
MC58	B
NGE28	B
NGE30	B
NGE31	B
NGH15	B
NGH36	B
94/155	C
F1576	C
L91/4286	C
MA5756	C
SB25	C
7880	L10
35E	L2
89I	L4
M981	L5
M992	L6
6155	L7
M978	L8
A22	W
E26	X
860800	Y
E32	Z

Construction of insertional mutants. To determine the role of NMB1638 and NMB0415, strains were mutated by insertional inactivation of the relevant open reading frame with a kanamycin resistance cassette. These mutations were made in strains with three different LPS immunotype backgrounds: 35E (L2), H44/76 (L3), and 89I (L4). Strains 35E and 89I have PEtn at the 6-position of HepII in the inner core of the LPS, while in H44/76, PEtn is present at the 3-position. Additionally, a double mutant, H44/76::lpt3::1638, was constructed. The

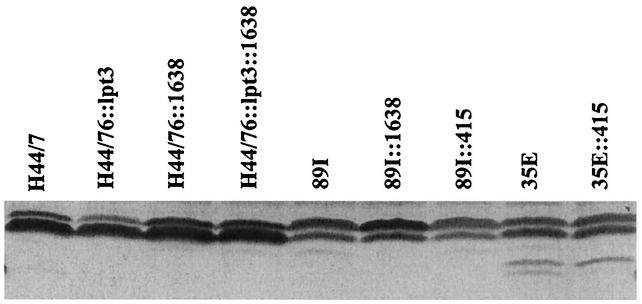

FIG. 2. Profiles of LPS from *N.* meningitidis strains mutated in *lpt-3*, NMB0415, and NMB1638 fractionated on Tricine-SDS-PAGE gels, and stained with silver.

LPS profiles of the mutants and wild-type parental strains were analyzed by gel electrophoresis and silver staining (Fig. 2). Migration patterns of LPS from both NMB1638 and NMB0415 mutants were unchanged compared to those of the parental strains. However, for NMB1638 mutants, the quality of color after staining was altered, a phenomenon that had previously been observed when comparing LPS from wild-type and *lpt-3* mutant strains (11).

Structural characterization of lipid A regions of O-deacylated LPS derived from wild-type and mutant strains. (i) NMB0415 mutants. LPS was prepared from strains 35E and 35E::0415. The poly(C) homopolymeric tract of NMB0415 in strain 35E contains eight C's, allowing a full-length product to be produced (data not shown). The electrospray (ES)-MS data obtained for the O-deacylated LPS were identical for both the mutant and parental strain (data not shown), consistent with the gel electrophoresis data, indicating that NMB0415 does not have an identifiable role in LPS biosynthesis in this strain.

(ii) NMB1638 mutants. LPS isolated from *N. meningitidis* strains H44/76 and H44/76::1638 was O deacylated and examined by ES-MS. A series of peaks were observed in the triply charged region of the spectrum of the parent strain at *m/z* 930, 957, 971, 998, and 1,039 (Fig. 3A) corresponding to mass differences of 80, 123, 203, and 326 average mass units (amu) from the base peak at *m/z* 930. A similar series of peaks at lower intensity were also observed in the triply charged region at *m/z* 1,027, 1,054, 1,068, 1,095, and 1,136 due to glycoforms bearing a sialic acid moiety in the outer core OS. When the mutant strain was examined (Fig. 3B), only two peaks at *m/z* 930 and 957 were observed in the triply charged region of the spectrum, corresponding to a mass difference of 80 amu with the corresponding sialylated triply charged pair also observed at *m/z* 1,027 and 1,054. In order to determine what modifications to the O-deacylated LPS structure were responsible for these mass differences, CE-MS/MS experiments were performed. In the negative-ion mode, the ketosidic bonds of the O-deacylated LPS molecule between the Kdo residues and the lipid A moiety fragment favorably, to give an intact O-deacylated lipid A species and the core oligosaccharide; the negative charge usually remains with the lipid A fragment, and therefore MS/MS experiments can readily be used to determine the size of the lipid A molecule for each peak observed in the original mass spectrum. In this way, each triply charged ion from the original mass spectrum of the parent strain corresponding to the glycoforms of interest was selected and fragmented: the resulting product ion spectra are shown in Fig. 4. It can be seen that the glycoform that gave rise to the triply charged ion at *m/z* 930 contained a lipid A species of size 952 amu by virtue of the doubly charged ion at *m/z* 475 and singly charged ion at *m/z* 952, which corresponds to the basal lipid A structure of a disaccharide of phosphorylated, N-acylated glucosamine molecules (Fig. 4A). Fragmentation of the triply charged ion at *m/z* 957 gave rise to a major doubly charged ion at *m/z* 515 that corresponds to a lipid A species of 1,032 amu that is 80 amu larger than the lipid A species of 952 amu, consistent with the presence of an additional phosphate residue (Fig. 4B). Fragmentation of the triply charged ion at *m/z* 971 gave rise to a major doubly charged ion at *m/z* 536 that corresponds to a lipid A species of 1,075 amu that is 123 amu larger than the lipid A species of 952 amu, consistent with the presence of an additional PEtn molecule (Fig. 4C). In a similar way, the size of the lipid A species for each glycoform was determined, and these data are summarized in Table 2. This extensive phosphorylation pattern of the lipid A moiety has been consistently observed for LPS from all strains of *N. meningitidis* we have examined, including immunotype strains L1, L2, L3, L4, and L8 and clinical strains BZ157, 1000, NGE30, and NGH15, as well as BZ157 and MC58 *lpt-3* mutants. The phosphorylation pattern of lipid A to give rise to a species of 1,278 amu was investigated by MS/MS techniques. In positive-ion mode, when the peak corresponding to the phosphoforms containing a lipid A species of 1,278 amu was fragmented, a dominant singly charged product ion of *m/z* 302 was observed (Fig. 5A). This peak was also seen in the product ion spectrum in positive-ion mode from fragmentation of the peak that corresponded to the phosphoforms that contained a lipid A species of 1,155 amu (data not shown). This ion of *m/z* 302 was thought to correspond to a novel hydrated molecule with a composition of P-P-PEtn. Similarly, in negative-ion mode, a

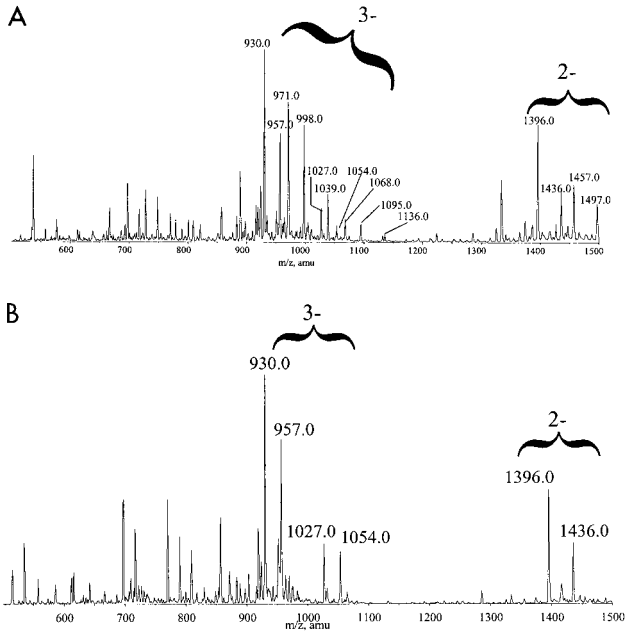

FIG. 3. Negative-ion ES mass spectrum of *O*-deacylated LPS from *N. meningitidis* strains H44/76 (A) and H44/76::1638 (B).

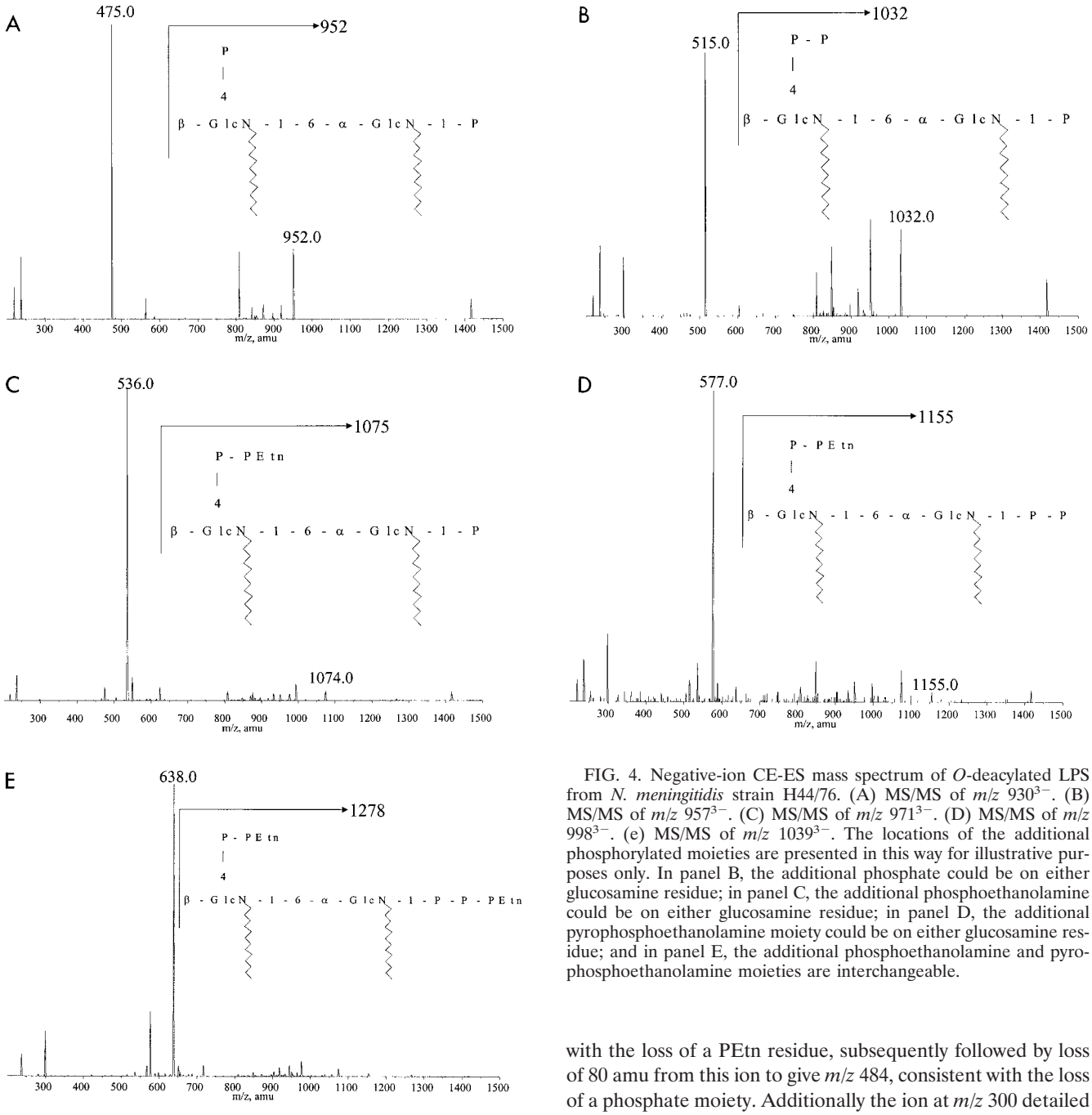

FIG. 4. Negative-ion CE-ES mass spectrum of *O*-deacylated LPS from *N. meningitidis* strain H44/76. (A) MS/MS of *m/z* 930^{3-}. (B) MS/MS of *m/z* 957^{3-}. (C) MS/MS of *m/z* 971^{3-}. (D) MS/MS of *m/z* 998^{3-}. (e) MS/MS of *m/z* 1039^{3-}. The locations of the additional phosphorylated moieties are presented in this way for illustrative purposes only. In panel B, the additional phosphate could be on either glucosamine residue; in panel C, the additional phosphoethanolamine could be on either glucosamine residue; in panel D, the additional pyrophosphoethanolamine moiety could be on either glucosamine residue; and in panel E, the additional phosphoethanolamine and pyrophosphoethanolamine moieties are interchangeable.

singly charged ion of *m/z* 300 was observed only from the fragmentation of peaks corresponding to the two largest lipid A species, 1,155 and 1,278 amu (Fig. 5B). When the lipid A species of 1,278 amu was fragmented, again a singly charged ion at *m/z* 300 was observed (Fig. 6A). Another ion at *m/z* 687 was produced in this spectrum and was assigned to one of the N-acylated glucosamine residues bearing the complex phosphorylated modification. When the ion at *m/z* 687 was fragmented, several product ions were produced that were consistent with this assignment (Fig. 6B). This was evidenced by loss of 123 amu from the ion at *m/z* 687 to give *m/z* 564, consistent with the loss of a PEtn residue, subsequently followed by loss of 80 amu from this ion to give *m/z* 484, consistent with the loss of a phosphate moiety. Additionally the ion at *m/z* 300 detailed above was observed, and this ion gave rise to ions at *m/z* 220 due to loss of a phosphate group and *m/z* 177 due to loss of a PEtn residue. Ions at *m/z* 79, 159, and 202, consistent with the presence of P, PP, and PPEtn groups, respectively, were also observed. These results are therefore consistent with the presence of a P-P-PEtn group on one of the N-acylated glucosamine residues of lipid A. When the O-deacylated LPS from the H44/76::1638 mutant strain was examined in MS/MS experiments, only two lipid A moieties were observed at 952 and 1,032 amu, because fragmentation of the triply charged ions at *m/z* 930 and 957 gave spectra identical to those in Fig. 4A and B. However, no larger lipid A species were observed in this mutant strain. This behavior was consistent for the O-deacy-

TABLE 2. CE-ES-MS and CE-ES-MS/MS data and proposed compositions of O-deacylated LPS from *N. meningitidis* strains H44/76 and H44/76::1638

Strain	MS ions observed (*m*/*z*)		MS/MS ions observed (*m*/*z*)		Size of lipid A species (amu)[a]	Lipid A composition
	3^-	2^-	2^-	1^-		
H44/76	930	1,396	475	952	952.26	2GlcN, 2 3-OH C14:0, 2P, H_2O
	957	1,436	515	1,032	1,032.24	2GlcN, 2 3-OH C14:0, 3P, H_2O
	971	1,457	536	1,074	1,075.31	2GlcN, 2 3-OH C14:0, 2P, PEtn, H_2O
	998	1,497	577	1,155	1,155.29	2GlcN, 2 3-OH C14:0, 3P, PEtn, H_2O
	1,039		638		1,278.34	2GlcN, 2 3-OH C14:0, 3P, 2PEtn, H_2O
H44/76::1638	930	1,396	475	952	952.26	2GlcN, 2 3-OH C14:0, 2P, H_2O
	957	1,436	515	1,032	1,032.24	2GlcN, 2 3-OH C14:0, 3P, H_2O

[a] Average mass units were used for calculation of molecular size based on proposed composition as follows: GlcN, 161.15 amu; 3-OH C14:0, 226.00 amu; P, 79.98 amu; PEtn, 123.05 amu; and H_2O, 18.00 amu.

lated LPS from several NMB1638 mutant strains that have been examined, including 89I and H44/76::lpt3 (data not shown). In each of the NMB1638 mutant strains examined, the PEtn residue elaborated in the core OS region was still present. Therefore, it could be concluded that NMB1638 was not involved in the general biosynthesis or incorporation of PEtn in the LPS. The structural data are consistent with the protein encoded by gene NMB1638 functioning as a PEtn transferase specific for the lipid A region. We therefore propose to name NMB1638 as "*lptA*," for "LPS PEtn transferase for lipid A."

DISCUSSION

N. meningitidis elaborates LPS containing a lipid A region that is characteristically phosphorylated with multiple phosphate and PEtn residues. In all meningococcal strains examined, each lipid A species contains the basal diphosphorylated species, wherein a phosphate group is attached to each glucosamine residue. Also elaborated within the population of LPS molecules are a variety of "phosphoforms" that contain either an additional phosphate residue, an additional PEtn residue, additional phosphate and PEtn residues, or an additional phosphate and two PEtn residues in the lipid A. A gene, *lptA*, identified by its homology to a gene encoding an LPS PEtn transferase (*lpt-3*) has been shown to be responsible for the transfer of PEtn residues to the lipid A in several *N. meningitidis* strains. It is clear from the MS analyses that no PEtn residues are present in the lipid A region of the LPS derived from the *lptA* mutant. It is not clear, however, whether *lptA* is merely responsible for the addition of only one PEtn residue or whether this gene product is capable of adding both PEtn residues to the lipid A. It is possible that if one PEtn residue is not attached, then the required acceptor for the addition of the second PEtn residue is no longer available. *lptA* is universally present in the pathogenic *Neisseria* strains tested. A further gene present in the MC58 genome sequence with homology to *lpt-3*, NMB0415, had no identifiable role in *N. meningitidis* LPS synthesis. NMB0415 had previously been studied in an investigation of murein sacculus formation in neisseria and was inferred to play some role in natural competence in *Neisseria gonorrhoeae*, but the precise role was not clear (22).

The amino acid sequence of NMB1638 was used to search the National Center for Biotechnology Information database of microbial genome amino acid sequences and revealed 22 genes with high homologies (e values of $< 1 \times 10^{-74}$). The majority of these genes are of unknown function and occur in a wide range of gram-negative bacteria, including *Escherichia*

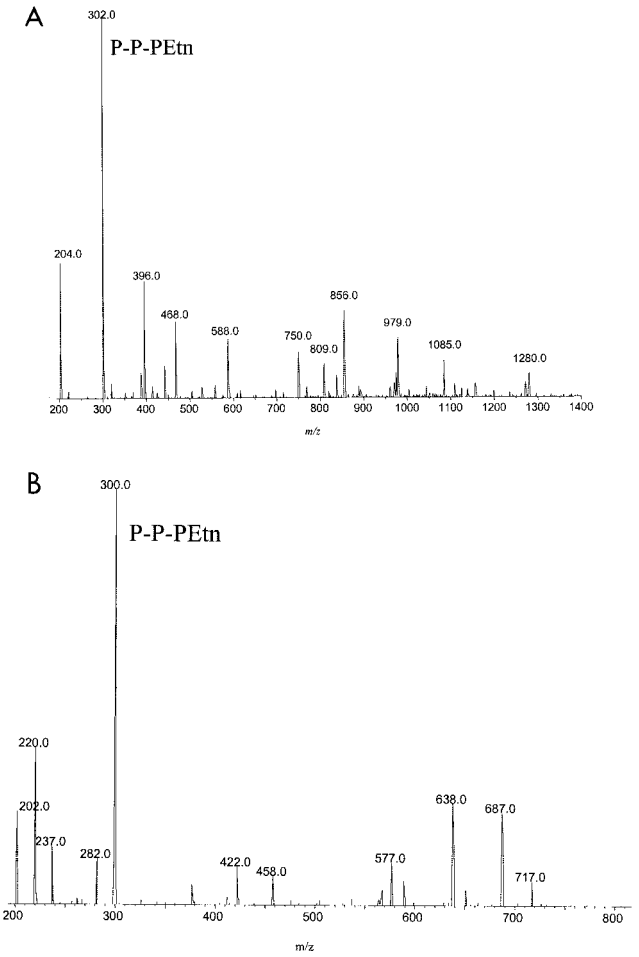

FIG. 5. CE-ES mass spectrum of *O*-deacylated LPS from *N. meningitidis* strain H44/76. (A) MS/MS of *m*/*z* $1{,}041^{3+}$. (B) MS/MS of *m*/*z* $1{,}039^{3-}$.

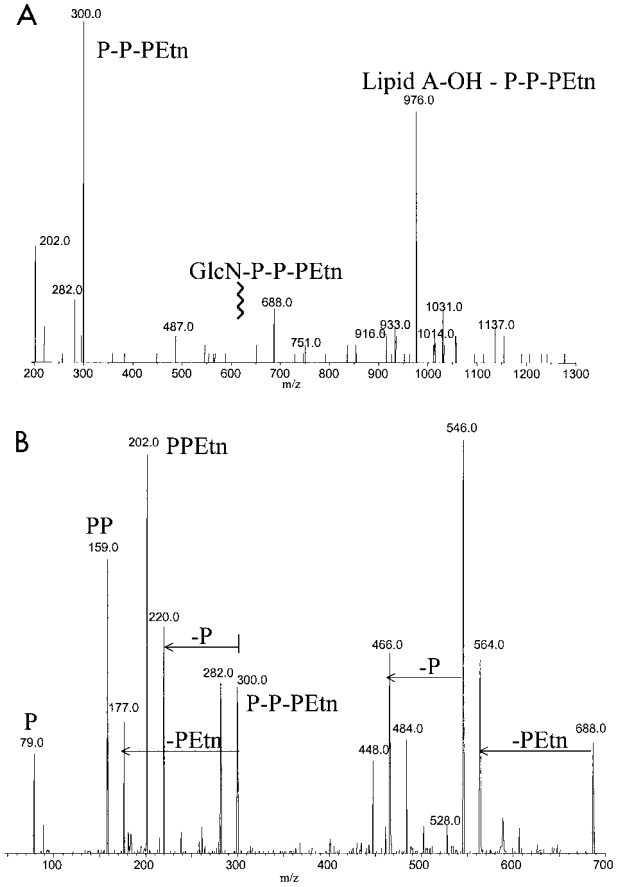

FIG. 6. Negative-ion CE-ES mass spectrum of *O*-deacylated LPS from *N. meningitidis* strain H44/76. (A) MS/MS of *m/z* 1278^{-}. (B) MS/MS of *m/z* 687^{-}.

coli, *Salmonella enterica* serovar Typhi, *Yersinia pestis*, *Pseudomonas aeuroginosa*, *Agrobacterium tumefaciens*, *Xylella fastidiosa*, *Vibrio cholerae*, and *Campylobacter jejuni*. A second group of 29 genes with lower but significant levels of homology (e values ranging from 5×10^{-50} to 7×10^{-6}) were identified, including the gene *lpt-3* used in the original BLAST search. Indeed, the products of NMB1638 and *lpt-3* are annotated in the MC58 genome sequence as belonging to the same protein family. Alignments of the amino acid sequences from each of these genes showed conserved residues across the entire length, suggesting a similarity in protein structure and perhaps suggesting a conserved function. The structure of the LPS molecule has been characterized in detail for a limited number of bacterial species, and there is some correlation between the presence of PEtn on the LPS and possession of a homolog of NMB1638 in that species. In no other species has the gene(s) responsible for the addition of PEtn to the saccharide or lipid A moieties been identified, and we suggest that members of this family of genes are favorable candidates.

In a previous study on meningococcal lipid A, Kulshin et al. identified a structure that was not observed in our study, wherein two additional PEtn residues were present (10). This lipid A molecule would have a molecular size of 1,198 amu following O deacylation, and although several strains were examined, MS studies could not identify this structure. In a recent study by Tzeng et al., a meningococcal strain with the Kdo transferase gene mutated produced LPS that only contained the lipid A molecule (25). A relatively simple phosphorylation pattern was observed for this lipid A molecule. Only one of the phosphate groups was partially replaced by *O*-phosphorylethanolamine, and the glycosidic phosphate was sometimes absent. Clearly the strains used in the two studies mentioned above differ from those studied here. In our studies, growth conditions appear to be crucial to the lipid A phosphorylation pattern observed. Optimum conditions for the isolation of extensively phosphorylated lipid A were growth on solid media and LPS isolation without a prolonged dialysis step. Following phenol extraction of the LPS, ethanol precipitation of the combined aqueous phases resulted in isolation of LPS with extensively phosphorylated lipid A. However if the combined aqueous phases were dialyzed rather than ethanol precipitated, lipid A was found to contain only the basal diphosphorylated structure. Conversely, if meningococcal strains were grown in liquid media, the LPS isolated following ethanol precipitation contained only the basal diphosphorylated lipid A species (unpublished data). In the study by Kulshin et al. (10), the meningococcal strain was grown in broth, and the LPS was isolated by the Westphal method, involving a dialysis step. It is therefore surprising that *O*-phosphorylethanolamine groups were present in this LPS, considering the acidic conditions that were employed to isolate the lipid A molecule in this study. In the study by Tzeng et al. (25), again, the strains were grown in broth and LPS was isolated by a method that does not involve a dialysis step. However, because these studies were carried out with a mutant strain in which the LPS consisted of only the lipid A molecule, it is possible that the absence of Kdo and core OS could affect the phosphorylation of the lipid A species. In our laboratory, when cells were grown in broth and LPS was isolated without a dialysis step by ethanol precipitation, a small amount of lipid A species bearing PEtn groups was observed, suggesting that complex phosphorylation of lipid A can occur from broth-grown cells, although these growth conditions do not appear optimal. One interesting consideration regarding extensive phosphorylation of lipid A is that these modifications clearly do not preclude the formation of an intact LPS molecule. One must conclude that either the additional phosphorylation events occur late in LPS biosynthesis, or they do not affect any of the early crucial LPS biosynthetic steps. Perhaps the protein involved in the addition of the third phosphate group and the gene product of *lptA* follow the LpxK step and do not hinder the activity of KdtA. Further studies are required to determine when these additional phosphorylation reactions occur. Several studies have considered the biological effects of modifications to the basal lipid A structure. The endotoxic activity of *E. coli* lipid A was affected by variations in the phosphorylation pattern (18, 23), and the ability of meningococcal and gonococcal LPS to give a positive *Limulus* amebocyte lysate assay was possibly related to the amount of phosphorylated lipid A expressed (19). Furthermore, modifications to the lipid A region can affect the overall charge of the LPS molecule. *Burkholderia* (*Pseudomonas*) *cepacia* is notoriously resistant to cationic antibacterial agents and has been found to have a very low number of phosphate groups and contains a 4-amino-arabinose sugar in place of a phosphate group in the lipid A molecule (2). Polymyxin-resistant mutants of *E. coli*

K12 have been characterized and have been shown to contain increased amounts of PEtn in their lipid A molecules (15). The elaborate phosphorylation patterns seen in *N. meningitidis* lipid A are therefore of interest, and further studies are ongoing in an attempt to understand the sequence and specificities of lipid A phosphorylation in *N. meningitidis*. Preliminary data obtained by testing the resistance of wild-type and *lptA* mutants of *N. meningitidis* in serum showed no significant difference between isogenic pairs of strains (data not shown). Additionally, although not quantified, the temperature and antibiotic (kanamycin) sensitivities, total protein compositions, and yields of LPS were not significantly altered in the *lptA* mutants (data not shown). The significance of the presence or absence of particular phosphorylated residues in the lipid A region upon biological activity, particularly with respect to pathogenesis or resistance to antibacterial agents, therefore remains to be defined.

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