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# Lack of Lipid A Pyrophosphorylation and Functional *lptA* Reduces Inflammation by *Neisseria* Commensals

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**The interaction of the immune system with *Neisseria* commensals remains poorly understood. We have previously shown that phosphoethanolamine on the lipid A portion of lipooligosaccharide (LOS) plays an important role in Toll-like receptor 4 (TLR4) signaling. For pathogenic *Neisseria*, phosphoethanolamine is added to lipid A by the phosphoethanolamine transferase specific for lipid A, which is encoded by *lptA*. Here, we report that Southern hybridizations and bioinformatics analyses of genomic sequences from all eight commensal *Neisseria* species confirmed that *lptA* was absent in 15 of 17 strains examined but was present in *N. lactamica*. Mass spectrometry of lipid A and intact LOS revealed the lack of both pyrophosphorylation and phosphoethanolaminylation in lipid A of commensal species lacking *lptA*. Inflammatory signaling in human THP-1 monocytic cells was much greater with pathogenic than with commensal *Neisseria* strains that lacked *lptA*, and greater sensitivity to polymyxin B was consistent with the absence of phosphoethanolamine. Unlike the other commensals, whole bacteria of two *N. lactamica* commensal strains had low inflammatory potential, whereas their lipid A had high-level pyrophosphorylation and phosphoethanolaminylation and induced high-level inflammatory signaling, supporting previous studies indicating that this species uses mechanisms other than altering lipid A to support commensalism. A meningococcal *lptA* deletion mutant had reduced inflammatory potential, further illustrating the importance of lipid A pyrophosphorylation and phosphoethanolaminylation in the bioactivity of LOS. Overall, our results indicate that lack of pyrophosphorylation and phosphoethanolaminylation of lipid A contributes to the immune privilege of most commensal *Neisseria* strains by reducing the inflammatory potential of LOS.**

The members of the genus *Neisseria* that colonize humans are classified into two pathogenic and eight commensal species. Infections due to the two pathogenic species, *N. meningitidis* and *N. gonorrhoeae*, represent a major public health problem around the world. *N. meningitidis* is the leading cause of epidemic meningitis, causing approximately 50,000 deaths worldwide each year (53). *N. gonorrhoeae* is a major cause of infections worldwide, estimated to be 60 million annually. In women, gonococcal infection can lead to pelvic inflammatory disease in 10 to 20% of those infected, causing chronic pain, infertility, and risk for ectopic pregnancy (22, 42, 50).

Although it can be a deadly human pathogen, *N. meningitidis* is carried in the nasopharynx by approximately 10% of the population and only results in disease characterized by septicemia and meningitis at low frequencies (65). Asymptomatic carriage of *N. meningitidis*, which is infrequent in infancy and peaks in early adulthood, is the mechanism by which the reservoir of meningococci is maintained within the population. Similarly, asymptomatic gonococcal infection of women plays an important role in maintaining transmission of the organism (16).

The eight commensal *Neisseria* species are *N. cinerea*, *N. elongata*, *N. flavescens*, *N. lactamica*, *N. mucosa*, *N. polysaccharica*, *N. sicca*, and *N. subflava*, with *N. perflava* and *N. flava* considered biovars of *N. subflava* (34). These species colonize the upper respiratory tract and are classified as part of the nonpathogenic normal flora, although scattered reports of infections by these organisms exist (17, 32). Historically, estimates of carriage rates ranged from approximately 3 to 40% depending on the species, and more recent data would suggest that the rates are even higher than previously estimated (35). In addition, high levels of colonization in

individuals have been reported. Using recent advances in sequencing technology, Zaura et al. found that five oral cavity sites sampled in three healthy individuals yielded sequences of *Neisseria* which together represented more than 10% of the 266 species-level phylotypes harbored at each site (66), with only streptococci being more predominant. This suggests that *Neisseria* species represent a major proportion of the core oral cavity microbiome in healthy individuals, and as such, they must have a considerable impact on the symbiotic colonization state of the respiratory tract mucosa maintained by the innate immune system.

Differences in the reactive and immunogenic potentials of the pathogenic and commensal *Neisseria* species have been examined in a few studies. For example, it was shown that *N. meningitidis* damaged ciliated cells in human nasopharyngeal organ culture, whereas *N. subflava* did not (54). In a study of cultured meningioma cells, Fowler et al. reported that although both *N. lactamica* and *N. meningitidis* were found to bind to the cells, only *N. meningitidis* induced meningioma cell death (18). Vaughan et al. dem-

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onstrated that at the peak period of colonization with *N. lactamica* in early childhood there was an absence of mucosal cell-mediated immunity to *N. lactamica* which generated only a mitogenic response associated with production of T cell-independent polyclonal IgM, unlike the response to *N. meningitidis* which primed for the development T or B cell memory (63). In a subsequent study, they reported that outer membrane vesicles of *N. lactamica* were B cell mitogens for cultured mucosal mononuclear cells; however, porin protein PorB and lipooligosaccharide (LOS) were determined to be unlikely mitogenic candidates (62). In contrast, PorB from *N. lactamica* has been shown to serve as a Toll-like receptor 2 (TLR2) agonist, similar to *N. meningitidis* PorB, although it was less bioactive for human airway epithelial cells than the meningococcal PorB variant (39).

Phenotypic relationships among *Neisseria* species have been analyzed using a taxonomic approach in the past (5). More recently, the availability of the complete genome sequence for *N. meningitidis* strains MC58 and Z2491 and *N. gonorrhoeae* strain FA1090 enabled a bioinformatic microarray analysis of *N. meningitidis* serogroup strains and commensal *Neisseria* to identify genetic variations conserved within the pathogens that were lacking in all the commensal strains (51). Using this definition, the analyses revealed 55 potential virulence genes in *N. meningitidis*, some of which were correlated with previously acquired data from other genetic typing methods. The genes implicated in pathogenicity included those involved in pilin biosynthesis, iron response, adherence to epithelial cells, and serogroup-specific capsule biosynthesis. Pilin proteins in the pathogenic *Neisseria* strains undergo antigenic variation, whereas the commensal *Neisseria* strains lack the required silent pilin gene copies (26, 43, 51). In addition, evidence has been obtained indicating that, whereas the commensal *Neisseria* strains have single chromosome copies, the pathogenic *Neisseria* are homozygous diploids, which could make the bacteria more resistant to DNA damage (60). The diversity of *Neisseria* species at mucosal surfaces provides a potential opportunity for genetic exchange among the bacteria, and comprehensive genome sequencing confirmed that this could occur (40).

We previously observed significant differences in the induction of proinflammatory cytokines mediated by TLR4 signaling in response to different strains of *N. gonorrhoeae* and *N. meningitidis* and to their LOS, a major inflammatory component of the bacterial outer membrane (30, 38, 45). In particular, we found that variations in acylation of the lipid A (LA) portion of the LOS and differences in the numbers of phosphate (P) and phosphoethanolamine (PEA) groups on the LA significantly influenced inflammatory signaling through TLR4. In this study, we postulated that the engagement of the innate immune system of the respiratory tract mucosa by the LOS of commensal *Neisseria* may elicit a weak response, thereby promoting persistent colonization. This concept is supported by the recent report that meningococcal LOS with hexaacylated LA was significantly more inflammatory in the lungs of an *in vivo* mouse model than LOS with pentaacylated LA (25). In addition, the study also suggested that, although bacterial products and cytokines can upregulate MD-2, low expression levels of MD-2 in the airway epithelia play a role in the respiratory response to neisserial LOS (29). Thus, we wanted to explore the relative inflammatory potentials of the commensal and pathogenic *Neisseria* strains and their LOS.

We report here that inflammatory signaling in the human THP-1 monocytic cell line, as well as polymyxin B resistance, is

TABLE 1 List of strains

Genomic group <sup>a</sup>	Species	Strain	Source <sup>b</sup> or reference
1	<i>N. meningitidis</i>	7889	WRAIR
	<i>N. meningitidis</i>	7946	WRAIR
	<i>N. meningitidis</i>	7946Δ <i>lptA</i>	This study
	<i>N. meningitidis</i>	MC58	28
	<i>N. meningitidis</i>	89I	WRAIR
2	<i>N. meningitidis</i>		NCBI
	<i>N. gonorrhoeae</i>	1291	13
	<i>N. gonorrhoeae</i>	F62	WRAIR
	<i>N. gonorrhoeae</i>	GC56	WRAIR
	<i>N. gonorrhoeae</i>	MS11	WRAIR
3	<i>N. gonorrhoeae</i>		NCBI
	<i>N. lactamica</i>	328	WRAIR
	<i>N. lactamica</i>	JJV8	WRAIR
	<i>N. lactamica</i>	4425	WRAIR
4	<i>N. lactamica</i>	NS19	WRAIR
	<i>N. cinerea</i>	14685	ATCC
	<i>N. cinerea</i>		NCBI
	<i>N. elongata</i>		NCBI
	<i>N. flavescens</i>	4322	WRAIR
	<i>N. flavescens</i>	NRL 30031	NCBI
	<i>N. flavescens</i>	SK114	NCBI
	<i>N. mucosa</i>	25996	NCBI
	<i>N. perflava</i>	10555	ATCC
	<i>N. polysaccharea</i>	ATCC 43768	NCBI
	<i>N. polysaccharea</i>	342	NCBI
	<i>N. sicca</i>	4319	WRAIR
	<i>N. sicca</i>	4320	WRAIR
	<i>N. sicca</i>	ATCC 29256	NCBI
	<i>N. sicca</i>	DS1	NCBI
	<i>Neisseria</i> species (oral)		NCBI
<i>N. subflava</i>	52	WRAIR	
<i>N. subflava</i>	NJ9703	NCBI	

<sup>a</sup> Genomic groupings are based on the microarray analyses of Stabler et al. (51).

<sup>b</sup> Abbreviations: WRAIR, Walter Reed Army Institute of Research; NCBI, National Center for Biotechnology Information; ATCC, American Type Culture Collection.

significantly greater when treated with the pathogenic *Neisseria* species than with the commensal *Neisseria* species. We found that the difference in induction of inflammatory signaling was characterized in most commensal *Neisseria* species by the absence of a functional *lptA* and the lack of phosphoethanolamylation and pyrophosphorylation of the LA component of the LOS.

## MATERIALS AND METHODS

**Bacterial strains and LOS extraction.** The sources of all of the bacterial strains, including those that were analyzed using a Blast search of the National Center for Biotechnology Information (NCBI) database, are listed in Table 1. Strains are presented as four genomic groupings based on the microarray analyses of *Neisseria* species by Stabler et al. (51). Briefly, *N. cinerea* strain 14685 and *N. perflava* strain 10555 were obtained in lyophilized form from the American Type Culture Collection (ATCC, Manassas, VA). *N. flavescens* strain 4322, *N. lactamica* strains 328, JJV8, 4425, and NS19, *N. sicca* strains 4319 and 4320, and *N. subflava* strain 52 were available in the Center for Immunochemistry from the collection of the late Herman Schneider, formerly of the Walter Reed Army Institute of Research. *N. meningitidis* serogroup A strain 7889, serogroup B strains MC58 and 7946, and serogroup C strain 89I and *N. gonorrhoeae* strains

1291, F62, GC56, and MS11 are clinical isolates that have been described previously (15, 30, 45). LOS was extracted and purified by a modification of the hot phenol-water method (3, 64).

**Bioinformatics analysis of *lptA* in *Neisseria* species.** The amino acid sequences encoded by *N. meningitidis* genes NMB1637 (57) (encoding a hypothetical protein), NMB1638 (*lptA*, encoding LPS phosphoethanolamine transferase for LA) (12), and NMB1640 (*serC*, encoding a putative phosphoserine aminotransferase) were used to perform a genomic Blast search ([http://www.ncbi.nlm.nih.gov/sutils/genom\\_table.cgi](http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi)) of all available *Neisseria* genomic DNA sequences present in the database (accession date, 31 March 2011). Genomic organizations of DNA sequences identified in individual searches were assembled by importing the DNA sequences into the computer program Geneious (Biomatters Ltd., Auckland, New Zealand).

**DNA extraction and Southern blot analysis.** Chromosomal DNA was isolated using Promega's Wizard genomic DNA purification kit (Promega Corp., Madison, WI). Chromosomal DNA was digested with SspI (New England BioLabs, Ipswich, MA) and electrophoresed on a 1.2% Tris-borate-EDTA (TBE) agarose gel (47), transferred onto a charged nylon membrane (DuPont, Wilmington, DE) using the alkaline transfer method of Reed and Mann (46), and fixed onto the membrane by UV exposure (1.5 J/cm<sup>2</sup>). Probe DNA was prepared by PCR amplification of the *lptA* gene using primers *lptAF* (ATGATAAACCGAACCTGAGGCCG) and *lptAR* (TCAGCGCGGACGGCGGCAGGCTGC). PCR was performed using the Expand long template PCR kit (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's specifications. Primers were purchased from Integrated DNA Technologies (Coralville, IA). Visualization of hybridization was accomplished with the use of Lumi-Phos and the Genius kit (Boehringer Mannheim, Indianapolis, IN). All of the steps followed the manufacturer's protocol except as follows: membranes were washed with 1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS, and 0.1 × SSC, 0.1% SDS at 65°C; 1% nonfat dry milk was used as the blocking agent and in the hybridization solution; and the antidigoxigenin antibody was diluted 1:10,000 instead of 1:5,000.

**Isolation of LA.** To hydrolyze the glycosidic bond between 3-deoxy-D-manno-octulosonic acid (Kdo) and LA with minimal dephosphorylation, the LOS (~1 mg/ml) was dissolved in 12.5 mM ammonium acetate (pH 4.5) and then stirred at 100°C for 60 min (8). After cooling the sample to room temperature, 5 parts (vol/vol) of chloroform-methanol solution (2:1, vol/vol) were added to two parts of the ammonium acetate solution. The mixture was stirred for 1 min and then centrifuged at ~5,000 × g for 20 min. The lower organic phase and interface were transferred into a glass vial and dried under a stream of nitrogen gas. The dry LA was stored at -20°C.

**Electrospray ionization (ESI)-MS and tandem MS (MS/MS).** The LA was dissolved in chloroform-methanol solution (1:2, vol/vol) at concentrations of ~0.2 to 1.0 mg/ml and analyzed by negative-ion nanoelectrospray using an LCQ Deca quadrupole ion trap mass spectrometer (MS) (Thermo Scientific, San Jose, CA). The needle voltage was typically set to 1.8 to 2.8 kV, and the temperature of the heated capillary was 150 to 180°C. For selection of parent ions for collision-induced dissociation, the isolation width parameter was set to 2 to 3.

**Negative-ion MALDI-TOF MS.** Negative-ion matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS was performed on a Synapt G2 high-definition (HD) MS system (Waters, Manchester, United Kingdom) with an orthogonal TOF mass analyzer in sensitivity mode. The Nd:Yag laser was operated with a wavelength of 355 nm at 100 to 200 Hz. MassLynx software was used to digitally smooth and correct the baseline of the spectra. The instrument was calibrated by using the exact mass for the monoisotopic (M-H)<sup>-</sup> ions for bovine insulin at *m/z* 5,728.5931, insulin β-chain at *m/z* 3,492.6357, renin substrate at *m/z* 1,756.9175, and angiotensin II at *m/z* 1,044.5267.

**Preparation of intact LOS for MALDI-TOF MS.** Intact LOS samples were prepared for MS analysis using a method described previously (55). Briefly, purified LOS (4 to 10 mg/ml) was suspended in a methanol-water

(1:3) solution with 5 mM EDTA, and an aliquot was desalted with a few cation exchange beads (Dowex 50WX8-200) that had been converted to the ammonium form. A spot containing a layer of matrix was formed by deposition of 1 or 2 drops (~1.0 μl each) of a solution composed of 2,4,6-trihydroxyacetophenone (200 mg/ml; Sigma-Aldrich, St. Louis, MO) in methanol, with nitrocellulose transblot membrane (15 mg/ml; Bio-Rad, Hercules, CA) in acetone-isopropanol (1:1, vol/vol) mixed in a 4:1 (vol/vol) ratio, within inscribed circles on the stainless steel sample plate. After the desalted sample solution was mixed with 100 mM dibasic ammonium citrate (9:1, vol/vol), 1.0 to 2.0 μl was deposited on top of the circular spot of dried matrix.

**TNF-α response of human monocytes.** The human monocytic leukemia cell line THP-1 was obtained from ATCC. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO<sub>2</sub> atmosphere and differentiated with 10 ng/ml phorbol myristate acetate (Sigma-Aldrich) for 18 h. Differentiated cells were seeded in 96-well plates at a concentration of 10<sup>4</sup> cells per well and treated for 18 h with LOS (100 ng/ml) or with live bacteria (multiplicity of infection of 1) grown overnight on plates with Difco GC medium base containing 1% IsoVitalX (Becton, Dickinson, Franklin Lakes, NJ) in the presence of 5% CO<sub>2</sub>. The doses of LOS and bacteria were based on our previous studies of their relative bioactivities (30, 31, 38). Cell culture supernatants were assayed for tumor necrosis factor alpha (TNF-α) levels by enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer (human TNF-α ELISA Ready-SET-Go; eBioscience, San Diego, CA).

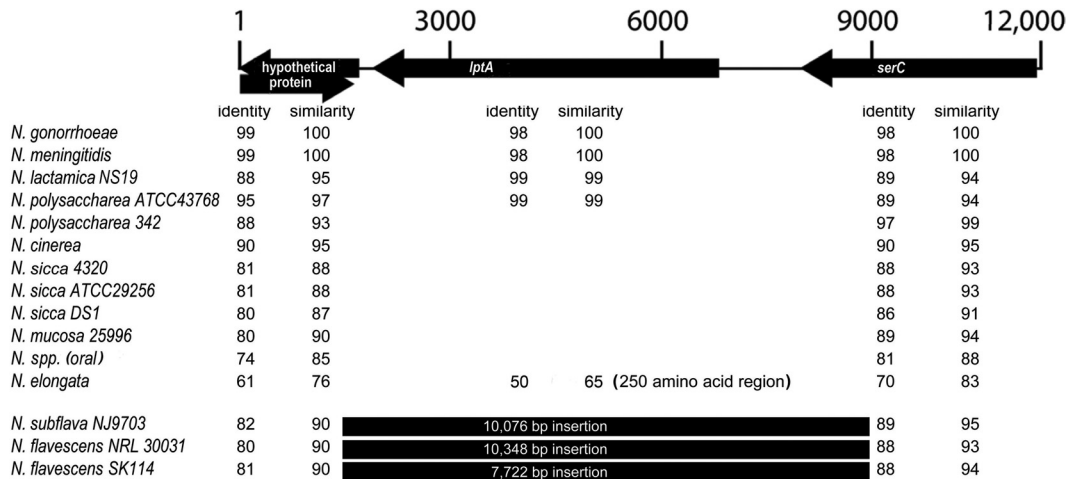
**Polymyxin B MIC assay.** The MICs of polymyxin B (Sigma-Aldrich) for *Neisseria* strains were determined by adding 2-fold dilutions of the antibiotic in a concentration range of 0.8 to 200 μg/ml to plates with Difco GC medium base containing Kellogg's supplements as we described previously (52). A single colony was struck onto each plate, and the plates were incubated for 24 h at 37°C and scored for growth. The MIC was defined as the lowest concentration of the antibiotic that prevented colony formation on the plate.

**Construction of *N. meningitidis* 7946 *lptA* mutant.** The *lptA* gene was amplified by PCR from strain MC58 using primers described by Cox et al. (12) and cloned into the EcoRI restriction sites of pUC19. The kanamycin resistance gene was amplified by PCR and inserted into the MluI restriction site. The plasmid carrying the inactivated *lptA* was then transformed into meningococcal strain 7946. Transformants were selected on GC agar plates that contained 50 μg/ml kanamycin, and mutants were confirmed by PCR and by MALDI-TOF MS analysis of purified intact LOS.

**Statistical analysis.** Statistical analyses were performed using Sigma-Stat for Windows version 3.10 (Systat Software, San Jose, CA). Groups of data were analyzed by the Student-Newman-Keuls analysis of variance test for multiple pairwise comparisons. *P* values of <0.05 were considered significant for all comparisons.

## RESULTS

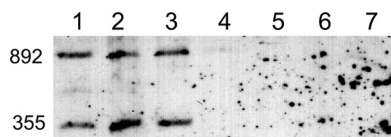
**Bioinformatics analysis of genomic sequences of commensal *Neisseria* strains for the presence of *lptA*.** We have previously shown that PEA moieties on the LA from *N. meningitidis* and *N. gonorrhoeae* LOS play an important role in activation of the TLR4 signaling pathway (31, 38). For pathogenic *Neisseria* strains, PEA residues are added to the LA by *LptA*, a PEA transferase specific for LA (12, 37). To investigate whether the genomes of commensal *Neisseria* strains contain *lptA*, the DNA and translated protein sequences of *lptA* were used to search the genomic sequences of numerous commensal *Neisseria* strains available at NCBI for the presence of *lptA*. Sequences corresponding to *lptA* were found in all gonococcal and meningococcal strains (Fig. 1). In addition, this gene was found in *N. lactamica*, but it was absent from all other commensal strains except one of two strains of *N. polysaccharea*. We used the flanking sequences of *lptA* to reconstruct the chro-



**FIG 1** Genomic organization of the *lptA* region of *Neisseria* species. The amino acid sequences encoded by *N. meningitidis* genes NMB1637 (hypothetical protein), NMB1638 (*LptA*), and NMB1640 (*SerC*) were used to perform a genomic Blast search, and the overall organization of these genes as found in *N. meningitidis* is shown at the top of the figure. The numbers across the top of the figure indicate the size of the region in nucleotides. If a number is seen in a row, it indicates that the gene was present in that strain (for gonococcal strains, the information was derived from 14 different strains; for meningococcal strains, the information was derived from 3 different strains). The column labeled “Identity” indicates the percent identity seen between the gene found in that strain and the probe sequence. Similarity indicates the degree of conserved substitution identity, also as a percentage.

mosomal organization of *lptA* and found that it was conserved in all strains. We also found that all commensal *Neisseria* strains possessed the two flanking open reading frames and that most of these were present as linked genes. In a small subset of strains, these genes were separated by an insertion of between ~8,000 and 10,000 bp. Taken together, the bioinformatics data indicate that *lptA* is absent in the majority of commensal strains.

**Southern hybridization to confirm the absence of *lptA* in various commensal *Neisseria* strains.** We performed Southern hybridization using the *lptA* sequence as a probe to confirm the absence of *lptA* in four commensal strains, *N. cinerea* strain 14685, *N. flavescens* strain 4322, *N. perflava* strain 10555, and *N. subflava* strain 52, as shown in **Figure 2**. NMB1638, the meningococcal *lptA*, is encoded by a 1,635-bp fragment of DNA (57). Analysis of the DNA sequence of MC58 indicated that *lptA* contains two internal SspI sites. The expected sizes of the bands generated after digestion are 355 and 892 bp. The data in **Figure 2** demonstrate that *N. gonorrhoeae* strain MS11 and *N. meningitidis* strains 89I and 7946 possessed the expected hybridization bands that correspond to the presence of an intact *lptA*. However, none of the four commensal strains analyzed possessed DNA sequences that hybridized with the probe. These data, in combination with the bioinformatics analyses described above, strongly support the



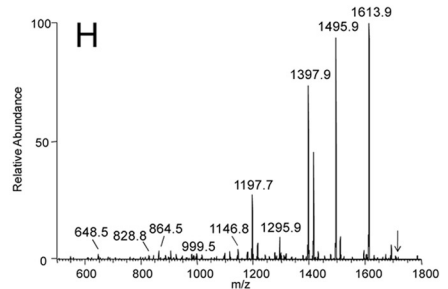
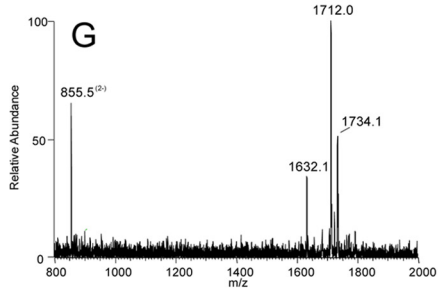
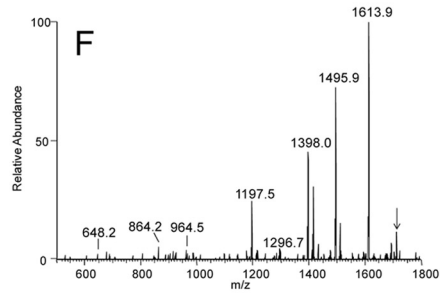
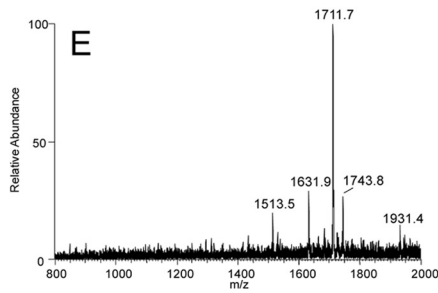
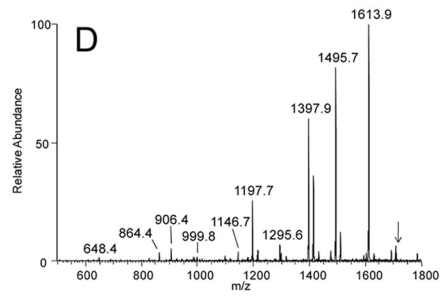
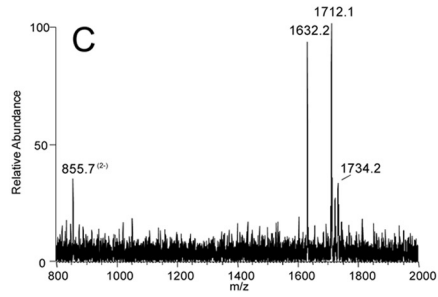
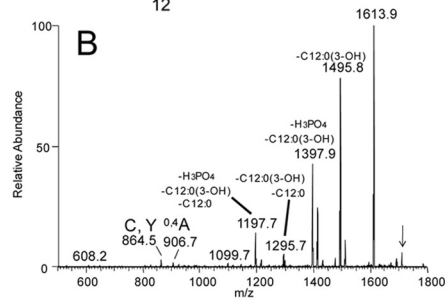
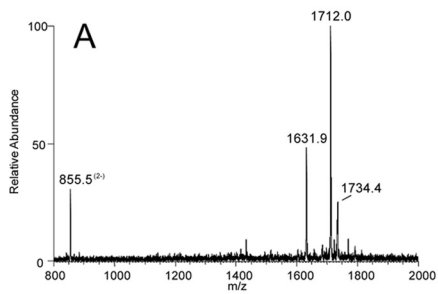
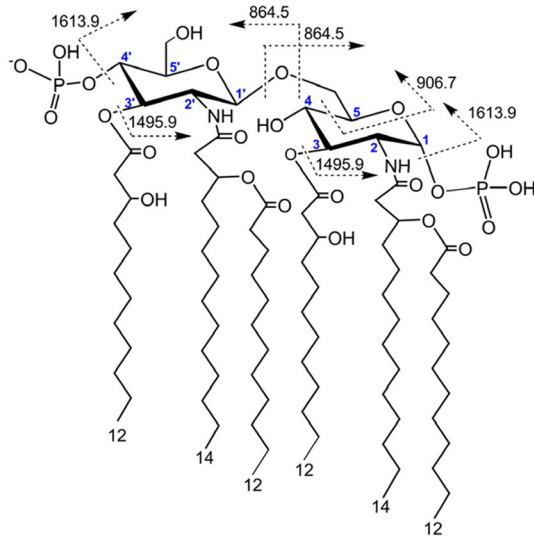
**FIG 2** Southern hybridization to detect *lptA*. After digestion of chromosomal DNA with SspI, the DNA was transferred to a charged nylon membrane and probed with a digoxigenin-labeled probe specific for *lptA*. The lanes represent DNA isolated from the following strains: lane 1, *N. gonorrhoeae* strain MS11; lane 2, *N. meningitidis* strain 7946; lane 3, *N. meningitidis* strain 89I; lane 4, *N. cinerea* strain 14685; lane 5, *N. flavescens* strain 4322; lane 6, *N. perflava* strain 10555; and lane 7, *N. subflava* strain 52. The numbers on the left side of the figure indicate the fragment sizes in base pairs.

conclusion that commensal *Neisseria* strains are characterized by the lack of *lptA*.

**Negative-ion ESI-MS and MS/MS analyses of LA of *Neisseria* commensals reveal DPLA.** We performed negative-ion ESI-MS and MS/MS analyses of LA from *N. cinerea* strain 14685 (**Fig. 3A and B**), *N. flavescens* strain 4322 (**Fig. 3C and D**), *N. perflava* strain 10555 (**Fig. 3E and F**), and *N. subflava* strain 52 (**Fig. 3G and H**). An illustration of the conserved structure of the bisphosphorylated LA from *Neisseria*, with phosphates on the 1 and 4' positions of the diglucosamine disaccharide, is shown (**Fig. 3, top**). The base peak at  $m/z$  1,712 in all of the ESI-MS spectra (**Fig. 3A, C, E, and G**) is consistent with LA containing two phosphate moieties (diphosphoryl LA [DPLA]); a peak for LA containing one phosphate moiety (monophosphoryl LA [MPLA]) is also observed at  $m/z$  1,632. The peaks at  $m/z$  855 are consistent with the doubly charged DPLA (calculated exact mass at  $m/z$  855.6).

In addition, there are less-abundant peaks for sodium adducts of the DPLA ( $(M-2H+Na)^-$ ) at  $m/z$  1,734 in the spectra of *N. cinerea* (**Fig. 3A**), *N. flavescens*, (**Fig. 3C**), and *N. subflava* (**Fig. 3G**). The peak at  $m/z$  1,513.5 seen in the spectrum of the *N. perflava* LA (**Fig. 3E**) is in accord with prompt fragment ions from the molecular ions for the DPLA at  $m/z$  1,712 due to loss of hydroxylaurate ( $3-OH C_{12:0}$ ,  $-198$  Da). The peak at  $m/z$  1,931.4 in the *N. perflava* spectrum is consistent with the presence of a single Kdo on the DPLA that could occur from incomplete hydrolysis.

The MS/MS spectra of  $(M-H)^-$  ions at  $m/z$  1,712 (**Fig. 3B, D, F, and H**) are similar and help to confirm the identity of the structures as hexaacylated DPLA. Assignments for some ions are provided in the spectrum shown in **Figure 3B**. The base peak at  $m/z$  1,613.9 in each spectrum is due to loss of  $H_3PO_4$  (98 Da) from the parent ions. Prominent peaks are observed at  $m/z$  1,496 and  $m/z$  1,398 for elimination of hydroxylauric acid as a free acid ( $-216$  Da) and loss of hydroxylaurate with  $H_3PO_4$ , respectively. Less abundant are peaks at  $m/z$  1,296 due to loss of hydroxylaurate and laurate ( $C_{12:0}$ ,  $-200$  Da) and at  $m/z$  1,198 for loss of hydroxylaurate, laurate, and  $H_3PO_4$ . Peaks for fragments formed by glyco-



sidic bond cleavage that could be reducing terminal Y-type fragment ions or nonreducing terminal C-type fragment ions, as illustrated in the structure in Figure 3, can be observed in each spectrum at  $m/z$  864. Losses of hydroxylaurate from the fragment ions at  $m/z$  864 are in accord with the ions observed at  $m/z$  648 in the spectra in Figure 3D, F, and H. Cross-ring  $^{0,4}$ A-type fragment ions are observed at  $m/z$  906 in the spectra shown in Figure 3B and D. These glycosidic and cross-ring fragments are consistent with the bisphosphorylated LA structure. Taken together, the ESI-MS and MS/MS analyses of the purified LA show that all four commensal *Neisseria* strains primarily express 2P LA and do not pyrophosphorylate or phosphoethanolaminylate their LOS.

**MALDI-TOF MS of intact LOS from commensal and meningococcal *Neisseria* strains.** MALDI-TOF MS of intact LOS was performed to confirm *lptA* functionality or lack thereof and to evaluate phosphorylation of the unmodified LOS structures, as pyrophosphoryl bonds could be cleaved during acid hydrolysis, although the conditions used were chosen to minimize this possibility (8) and, in previous work, we readily detected PEA substituents on LA (30, 31). Spectra were acquired of the LOS from *N. cinerea* strain 14685, *N. flavescens* strain 4322, *N. perflava* strain 10555, *N. subflava* strain 52, and *N. meningitidis* strain 7946 using a methodology we described previously (30, 31). In addition, we analyzed the LOS from *N. lactamica* strain 328, since our bioinformatics analyses indicated that, although it is also a commensal *Neisseria* strain, it may express *lptA*.

High-resolution negative-ion mass spectra of intact LOS were acquired on a Synapt G2 HDMS with a MALDI source with an orthogonal TOF mass analyzer. Figure 4 illustrates spectra of intact LOS from *N. flavescens* strain 4322 (top), *N. lactamica* strain 328 (middle), and wild-type *N. meningitidis* strain 7946 (bottom). The observed masses of peaks for Y-type fragment ions containing LA are labeled in red. Table 2 presents calculated exact monoisotopic masses for peaks of LA fragment ions in the spectra that are consistent with the ions observed and our previously published data (10). The high mass resolution of the data obtained on the Synapt G2 HDMS is apparent from the inset with data for the intact *N. flavescens* LOS in the top spectrum in Figure 4, showing the molecular ion region for peaks with a monoisotopic mass at  $m/z$  4,167.88.

In the bottom panel of Figure 4, the peak for the molecular ions at  $m/z$  4,144.81 for the intact *N. meningitidis* 7946 LOS that we have analyzed previously is in accord with the peak observed at  $m/z$  1,915.10 for triphosphoryl LA (TPLA) with a single PEA and a lacto-*N*-neotetraose  $\alpha$ -chain with a single PEA, sialic acid, *O*-acetyl group, and glycine (Gly; calculated  $m/z$  4,144.79). These data are consistent with those we reported previously regarding the structure of the meningococcal 7946 LOS (10).

An advantage of this methodology of MALDI-TOF analysis of

intact LOS is that it produces molecular ions for intact LOS and in-source prompt fragment ions that correspond to both the oligosaccharide and LA domains. Thus, in addition to the peaks for Y-type fragment ions for LA in each of the spectra, there are peaks for apparent molecular ions for the intact LOS (Fig. 4, labeled in black). Peaks for some of the B-type oligosaccharide fragment ions predicted from the difference between the  $m/z$  values of the peaks for the molecular ions and the LA fragment ions were detected in each spectrum but have not been labeled.

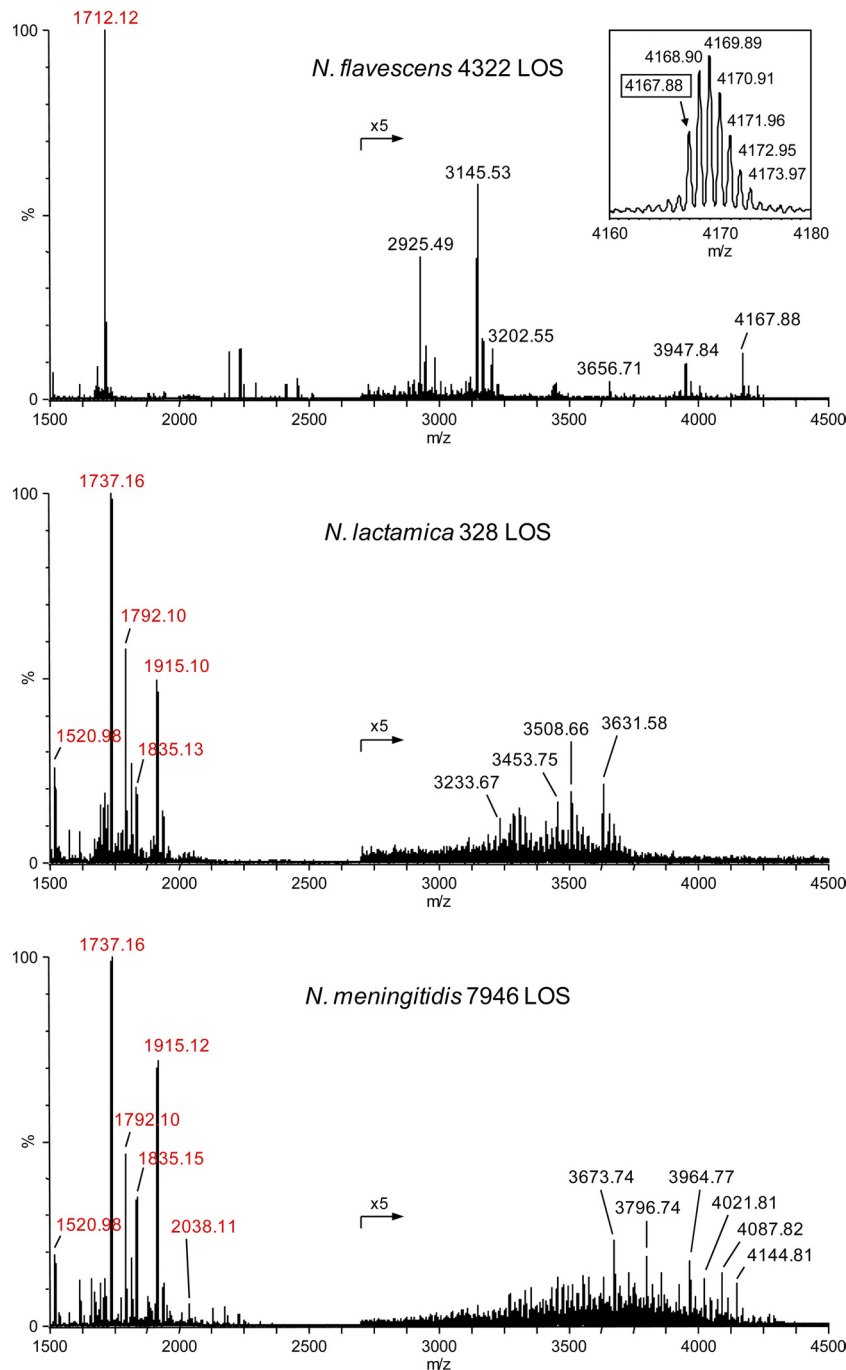
In the spectra of the LOS from *N. lactamica* strain 328 and *N. meningitidis* strain 7946, the base peaks at  $m/z$  1,737.16 (calculated  $m/z$  1,737.15) are consistent with LA fragment ions that contain a single P and a PEA but that have lost  $H_2O$ . This is probably due to facile loss of phosphate ( $H_3PO_4$ ,  $-98$  Da) from the DPLA with a single PEA or loss of diphosphate ( $H_4P_2O_7$ ,  $-178$  Da) from TPLA with a single PEA (33). Prominent peaks for Y-type LA fragment ions can be observed in the spectra from both *N. lactamica* and *N. meningitidis* at  $m/z$  1,792.1, 1,835.1, and 1,915.1 for TPLA, DPLA with a PEA, and TPLA with a PEA, respectively. Interestingly, in the spectrum of *N. meningitidis* strain 7946, there also is a peak at  $m/z$  2,038.11 that is consistent with a TPLA with two PEA moieties (calculated  $m/z$  2,038.10).

The base peak observed in the spectrum of the intact *N. flavescens* LOS at  $m/z$  1,712.12 is consistent with Y-type LA fragment ions of LOS with a DPLA moiety. This was also observed as the base peak in the spectra of the LOS from *N. cinerea* strain 14685, *N. perflava* strain 10555, and *N. subflava* strain 52 (data not shown). Absent from the spectra of all four of these commensal strains were peaks at  $m/z$  1,792.1 for fragment ions of TPLA, at  $m/z$  1,737.1, 1,756.1, or  $m/z$  1,835.1 for fragment ions of MPLA or DPLA with a PEA substituent, and at  $m/z$  1,915.1 for TPLA with a PEA. Thus, the MALDI-TOF analyses of intact LOS from *N. cinerea* strain 14685, *N. perflava* strain 10555, *N. flavescens* strain 4322, and *N. subflava* strain 52 further confirm that these four commensal *Neisseria* strains primarily express DPLA and do not pyrophosphorylate or phosphoethanolaminylate their LOS.

Overall, the LA peaks observed are consistent with our previous MS analyses of pathogenic *Neisseria* (30, 31). As shown in Figure 4, there were prominent peaks for LA with 3 or more phosphoryl moieties in the spectrum of the pathogenic *N. meningitidis* strain 7946. In contrast, as illustrated by the spectrum of the commensal *N. flavescens* strain 4322 LOS, there was a dearth of peaks for other than DPLA in the spectra of the LOS from the commensal strains. This difference is indicative of the higher degree of phosphorylation and phosphoethanolaminylation that appears to be characteristic of pathogenic *Neisseria* strains as compared to most commensal *Neisseria* strains.

The similarity between the abundance of the peaks for LA ions in the spectra of *N. lactamica* strain 328 and *N. meningitidis* strain

**FIG 3** Negative-ion ESI-MS and MS/MS analyses of LA of *Neisseria* commensals. ESI-MS and MS/MS spectra of LA from *N. cinerea* strain 14685 (A and B, respectively), *N. flavescens* strain 4322 (C and D), *N. perflava* strain 10555 (E and F), and *N. subflava* strain 52 (G and H) have peaks for DPLA and MPLA [calculated exact masses of the monoisotopic peaks for (M-H)<sup>-</sup> at  $m/z$  1,712.1 and 1,632.2, respectively], as well as peaks for the doubly charged DPLA (calculated exact mass at  $m/z$  855.6). The MS/MS spectra (B, D, F, and H) of (M-H)<sup>-</sup> ions at  $m/z$  1,712 (indicated by arrows) of the species are very similar and confirm that the LA moieties are DPLA. Fatty acid (hydroxylaurate,  $-216$  Da, and laurate,  $-200$  Da) and phosphate ( $H_3PO_4$ ,  $-98$  Da) losses and cross-ring fragment ions are noted on the MS/MS spectrum of *N. cinerea* (B). These data support diphosphoryl substitution and lack of PEA on the LA of commensal *Neisseria*. Top, illustration of the conserved structure of the diphosphorylated LA from *Neisseria* ( $m/z$  1,712.1), shown with phosphates on the 1 and 4' positions of diglucosamine. The dotted lines in the structure indicate the potential cleavage products of LA. The numbers shown in blue for the carbon atoms on the *N*-acetylglucosamine moieties are used to identify proposed sites of cross-ring cleavage, such as that producing the  $^{0,4}$ A-type fragment ions observed at  $m/z$  906 in the spectra shown in panels B and D.



**FIG 4** High-resolution negative-ion MALDI-TOF mass spectra of intact LOS from *N. flavescens* strain 4322 (top), *N. lactamica* strain 328 (middle), and *N. meningitidis* strain 7946 (bottom). The labeled masses represent the monoisotopic ions, as illustrated by the peak at 4,167.88 in the inset showing the envelope of  $(M-H)^-$  ions for the intact LOS from *N. flavescens* 4322. The numbers presented in red in each of the spectra label peaks for prompt fragment ions for LA. The phosphoryl substitution of LA from *N. lactamica* was very similar to that of LA from *N. meningitidis*. The base peak in the spectrum of *N. flavescens* at  $m/z$  1,712.12 is consistent with prompt fragment ions composed of DPLA. In the spectrum for *N. lactamica* 328 LOS, peaks for prompt fragment ions for LA are observed at  $m/z$  1,915.10 for TPLA with one PEA, 1,835.13 for DPLA with one PEA, and 1,792.10 for TPLA. The base peak at  $m/z$  1,737.16 is due to additional loss of  $H_4P_2O_7$  from 1,915.10 or of  $H_3PO_4$  from 1,835.13.

7946 is striking. The phosphoryl substitution on the LOS from *N. lactamica* strain JJV8 also appeared very similar based on negative-ion MALDI analysis of the intact LOS (data not shown). These data are consistent with the expression of *lptA* in *N. lactamica* as shown by the bioinformatics data and, furthermore, indicate that the gene is functional, as there are PEA moieties on the LA.

**Differential induction of TNF- $\alpha$  by whole bacteria and LOS from commensal and pathogenic *Neisseria* strains.** The ability of various *Neisseria* strains to induce TNF- $\alpha$  in THP-1 cells was examined. As shown in **Figure 5**, *N. meningitidis* induced significantly higher levels of TNF- $\alpha$  than the two strains of *N. gonorrhoeae* ( $P < 0.01$  for 1291 and  $P < 0.001$  for GC56) or the six



TABLE 2 Masses of (M-H)<sup>-</sup> lipid A fragment ions of intact LOS

Strain	Experimental (M-H) <sup>-</sup>	Proposed composition	Calculated (M-H) <sup>-</sup>	Difference in ppm <sup>a</sup>
<i>N. flavescens</i> 4322	1,712.12	DPLA	1,712.12	0.0
<i>N. lactamica</i> 328	1,915.10	TPLA PEA	1,915.09	4.0
	1,835.13	DPLA PEA	1,835.13	2.2
	1,792.10	TPLA	1,792.08	9.0
	1,737.16	TPLA PEA-(2P+H <sub>2</sub> O) <sup>b</sup>	1,737.15	6.2
<i>N. meningitidis</i> 7946	2,038.11	TPLA 2PEA	2,038.10	4.5
	1,915.12	TPLA PEA	1,915.09	4.0
	1,835.15	DPLA PEA	1,835.13	13.1
	1,792.10	TPLA	1,792.08	9.0
	1,737.16	TPLA PEA-(2P+H <sub>2</sub> O) <sup>b</sup>	1,737.15	6.2

<sup>a</sup> ppm, parts per million.<sup>b</sup> Prompt fragmentation with loss of H<sub>4</sub>P<sub>2</sub>O<sub>7</sub>.

strains of commensals *N. lactamica*, *N. cinerea*, *N. flavescens*, *N. perflava*, and *N. subflava* ( $P < 0.001$  for all six comparisons). Both gonococcal strains also induced higher levels of expression of TNF- $\alpha$  than the commensals, except for *N. lactamica* ( $P < 0.05$  for all comparisons). Of particular note is that gonococcal strain GC56, which had the lowest induction potential for TNF- $\alpha$  of all the pathogenic *Neisseria* strains that we have studied to date (30, 38, 45), induced a level of TNF- $\alpha$  that was significantly higher than those induced by the commensal *N. cinerea*, *N. flavescens*, *N. perflava*, and *N. subflava* strains ( $P < 0.05$ ) but similar to those induced by both strains of *N. lactamica*. For the commensal bacteria *N. cinerea*, *N. flavescens*, *N. perflava*, and *N. subflava*, the TNF- $\alpha$  levels induced were, on average, approximately 12% of the level induced by meningococci. In contrast, the TNF- $\alpha$  levels for *N. lactamica* were significantly higher than those of the other commensals ( $P < 0.05$  for all comparisons), which is consistent with its relatively high levels of expression of LA PEA, TPLA, and TPLA with PEA as shown by the MALDI MS data. Interestingly, despite sharing LA structural similarities with meningococci and gonococci, the TNF- $\alpha$  levels for *N. lactamica* were, on average, approx-

imately 40% of the meningococcal level and 57% of the level of gonococcal strain 1291.

We tested the potential of purified LOS to induce TNF- $\alpha$  expression in THP-1 cells, as shown in Figure 5. LOS from *N. meningitidis* induced significantly higher levels of TNF- $\alpha$  than LOS from *N. gonorrhoeae* ( $P < 0.01$ ) and the commensal *N. cinerea*, *N. flavescens*, *N. perflava*, and *N. subflava* strains ( $P < 0.001$ ). The TNF- $\alpha$  levels induced by the LOS from these four commensals were, on average, approximately 35% of the levels induced by the LOS of the meningococci. Similarly, the gonococcal LOS induced higher levels of TNF- $\alpha$  than the LOS from these four commensal strains ( $P < 0.001$ ). These data are consistent with our previous data, which indicated that purified LOS induces similar relative amounts of TNF- $\alpha$  but lower absolute amounts of TNF- $\alpha$  than the whole bacteria (38). In contrast, the relative inflammatory potential of the *N. lactamica* LOS was similar to that of the LOS from *N. meningitidis* strain 891 and greater than that of the LOS from *N. gonorrhoeae* strain 1291 ( $P < 0.01$  for 1291). The similarity between the relative inflammatory potentials of the LOS from *N. lactamica* and from the pathogenic *N. meningitidis* is consistent with the similarity of the phosphoryl substitution on LA as shown in the MALDI-TOF analyses. Taken together, these data indicate that, compared to other *Neisseria* commensals that we tested, *N. lactamica* uniquely uses a mechanism other than expressing LA with an altered structure to reduce its inflammatory potential and maintain its commensalism.

**Sensitivity of commensal and pathogenic *Neisseria* strains to polymyxin B.** Previously, insertional inactivation of *lptA* in *N. gonorrhoeae* was shown to significantly increase the sensitivity of gonococci to polymyxin B, which indicated that PEA on the gonococcal LA influences the efficacy of polymyxin B (4, 37). Thus, we compared the polymyxin B sensitivities of the commensal species to those of *N. gonorrhoeae*, because our bioinformatics and MS data showed the presence of PEA on *N. lactamica* LA and its absence on the LA of all but one of the other commensal strains. As shown in Table 3, the MIC of polymyxin B for *N. gonorrhoeae*

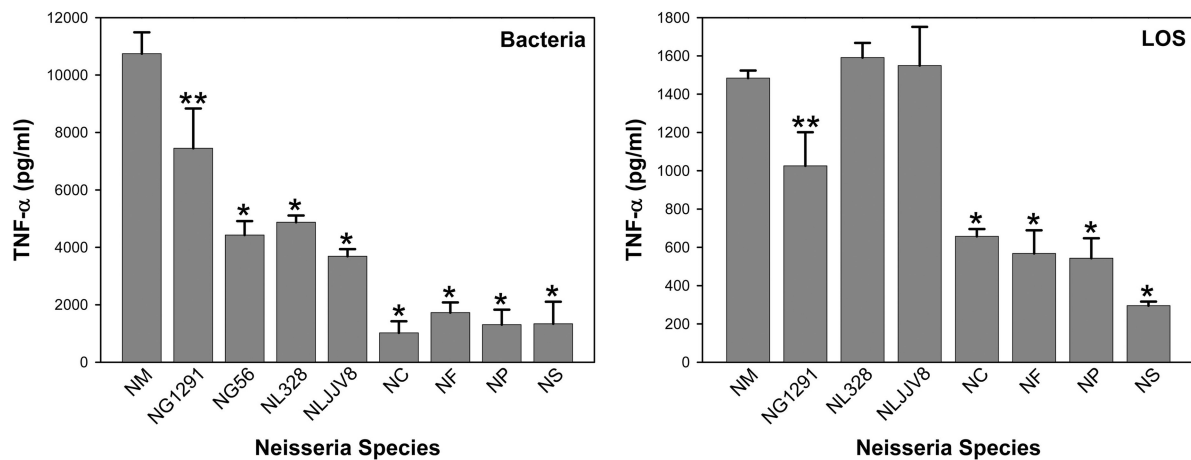


FIG 5 Induction of TNF- $\alpha$  in THP-1 cells by commensal and pathogenic *Neisseria* bacteria and LOS. Whole bacteria and LOS from *N. meningitidis* strain 891 (NM), *N. gonorrhoeae* disease isolate strains 1291 (NG1291) and GC56 (NG56), and commensals *N. lactamica* 328 (NL328), *N. lactamica* JJV8 (NLJJV8), *N. cinerea* 14685 (NC), *N. flavescens* 4322 (NF), *N. perflava* 10555 (NP), and *N. subflava* 52 (NS) were analyzed. The results presented are the means  $\pm$  standard deviations from at least three independent experiments (\*,  $P < 0.001$ , and \*\*,  $P < 0.01$ , compared with the results for 891). For bacteria, comparison of results between both gonococcal strains and the four commensal strains *N. cinerea*, *N. flavescens*, *N. perflava*, and *N. subflava* has a  $P$  value of  $< 0.05$ , and comparison of results between both strains of *N. lactamica* and the four commensal strains *N. cinerea*, *N. flavescens*, *N. perflava*, and *N. subflava* has a  $P$  value of  $< 0.05$ .

**TABLE 3** MICs of polymyxin B for various *Neisseria* species

<i>Neisseria</i> species and strain	Polymyxin B MIC ( $\mu\text{g/ml}$ )
<i>N. gonorrhoeae</i> MS11	50
<i>N. lactamica</i> NS19	25
<i>N. lactamica</i> 4425	25
<i>N. cinerea</i> 14685	1.6
<i>N. flavescens</i> 4322	1.6
<i>N. perflava</i> 10555	1.6
<i>N. sicca</i> 4319	<0.8
<i>N. sicca</i> 4320	<0.8
<i>N. subflava</i> 52	<0.8

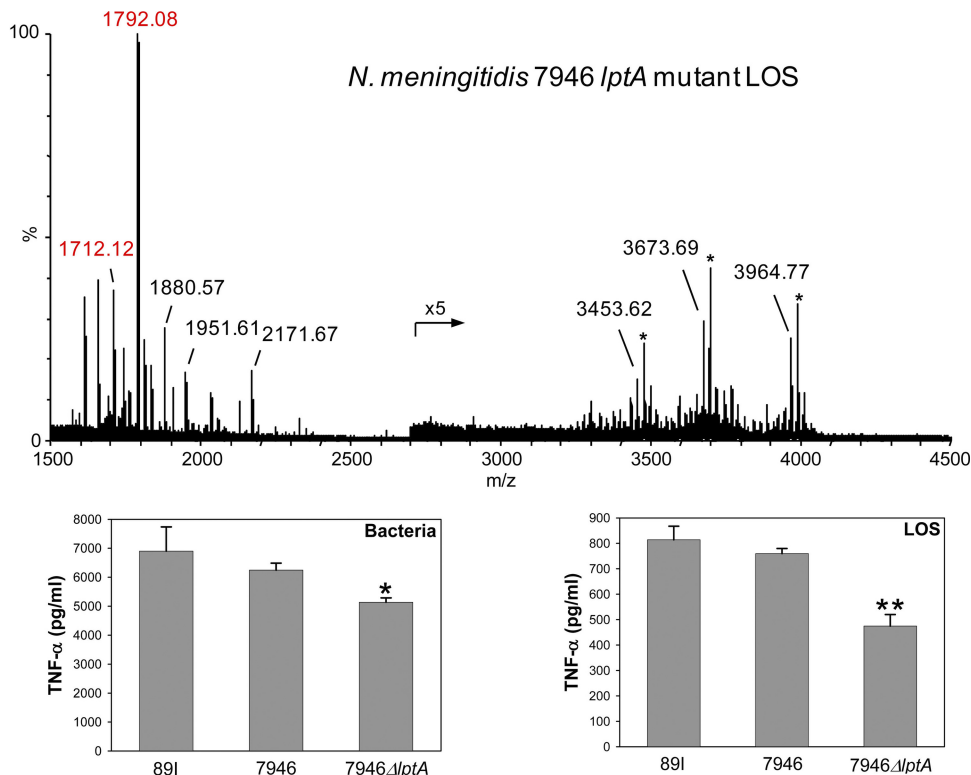
ranged from 32- to 64-fold greater than the MICs for the commensals *N. cinerea*, *N. flavescens*, *N. perflava*, *N. sicca*, and *N. subflava*. Similarly, the MICs for the two strains of *N. lactamica* were 16- to 32-fold greater than those for the other five commensal species, which is consistent with phosphoethanolaminylation of the *N. lactamica* LA. This suggests a role for PEA on the LA of *N. lactamica* in resistance to cationic antimicrobial peptides (CAMPs) such as polymyxin B, as shown previously for pathogenic *Neisseria* (4, 12, 37).

***lptA* null mutants of *N. meningitidis* 7946 bacteria and LOS have reduced inflammatory potential.** Our TNF- $\alpha$  and structural data showed that LOS and bacteria of the four commensal *N. cinerea*, *N. flavescens*, *N. perflava*, and *N. subflava* strains had re-

duced inflammatory potentials compared to those of LOS and bacteria of pathogenic *N. meningitidis* and *N. gonorrhoeae*, lacked PEA on LA, and did not express *lptA*. To confirm the biological significance of phosphoryl substitution and, specifically, phosphoethanolaminylation of LA in TNF- $\alpha$  induction, an *lptA* null mutant of *N. meningitidis* strain 7946 was generated and the effect of the mutation on the inflammatory potentials of the bacteria and LOS was investigated.

To confirm the absence of PEA in the LA of the 7946 *lptA* mutant, a high-resolution negative-ion MALDI-TOF mass spectrum of intact LOS from the mutant was obtained and is illustrated in Figure 6. As in the other MALDI spectra shown, observed masses of peaks for Y-type fragment ions containing LA are labeled in red. As expected due to the lack of expression of *lptA*, there are clear differences in the peaks observed for LA in the spectrum of the 7946 *lptA* mutant LOS and in the spectrum of the wild-type 7946 LOS shown at the bottom of Figure 4. In the spectrum of the mutant LOS, the base peak at  $m/z$  1,792.08 is consistent with Y-type LA fragment ions of LOS with a TPLA moiety. A less-abundant peak at  $m/z$  1,712.12 is in accord with a DPLA. Noticeably absent are peaks at  $m/z$  1,737, 1,835, or 1,915 due to DPLA or TPLA with a single PEA moiety. Also lacking are ions at  $m/z$  2,038 for a TPLA with two PEA groups.

The peak at  $m/z$  2,171.67 corresponds to oligosaccharide fragment ions and differs from those at 1,951.61 and 1,880.57 by the loss of Kdo ( $-220$  Da) and sialic acid ( $-291$  Da), respectively.



**FIG 6**  $\Delta$ *lptA* mutants of *N. meningitidis* strain 7946 bacteria and LOS induce less TNF- $\alpha$  in THP-1 cells than the wild-type 7946 strain or *N. meningitidis* strain 891 bacteria or LOS. In the high-resolution negative-ion MALDI-TOF mass spectrum of intact LOS from the mutant, the labeled masses represent the monoisotopic ions, and the numbers presented in red label peaks for prompt fragment ions for LA. Peaks at  $m/z$  1,792.08 and  $m/z$  1,712.12 are consistent with the expression of TPLA and DPLA. As anticipated, there was no evidence of PEA substitution on the LA. The graphs of TNF- $\alpha$  induction by 891, wild-type 7946, and 7946  $\Delta$ *lptA* mutant bacteria and LOS show that the absence of LA PEA significantly reduces the induction of TNF- $\alpha$  (\*,  $P < 0.01$ , and \*\*,  $P < 0.001$ , compared with the results for 7946 and 891). The results presented are the means  $\pm$  standard deviations from at least three independent experiments.

Similarly, observed at  $m/z$  3,964.77 are molecular ions that differ from the peaks observed at  $m/z$  3,673.69 by the loss of sialic acid and at  $m/z$  3,453.62 by the loss of both sialic acid and Kdo. Also, sodium adducts of the molecular ions (marked with asterisks) can be observed in this spectrum. The ions at  $m/z$  3,964.77 differ by 180.04 Da from the molecular ions observed for the wild-type 7946 LOS at  $m/z$  4,144.81, which corresponds to the expected loss of expression of PEA (123 Da) and the loss of 57 Da that is indicative of the loss of expression of the Gly moiety on the oligosaccharide. Thus, the spectrum of the intact 7946 *lptA* mutant LOS confirms the lack of expression of PEA on the LA. In addition, it indicates that the predominant form of the LA lacking PEA is triphosphorylated. The predominant substitution on the LA from wild-type 7946 also appeared to be triphosphoryl, which suggests that the expression of PEA and phosphorylation are independently regulated.

The 7946 *lptA* mutant bacteria induced significantly less expression of TNF- $\alpha$  than the wild-type 7946 or the 89I bacteria in THP-1 cells (Fig. 6;  $P < 0.01$  for both comparisons). In addition, the LOS isolated from the 7946 *lptA* mutant also induced less TNF- $\alpha$  than the LOS from wild-type 7946 and 89I ( $P < 0.001$  for both comparisons). These data provide strong support for our hypothesis that, in addition to its acylation state, the inflammatory potential of LA is positively correlated with the extent of phosphoryl substitution (30, 31).

## DISCUSSION

The relationship between the immune system and *Neisseria* commensals remains poorly understood. Previous studies from our laboratory have reported that specific structural features of the LOS of *N. gonorrhoeae* and *N. meningitidis* are associated with increased inflammatory signaling. In particular, we found that the degree of phosphorylation, phosphoethanolaminylation, and hexaacetylation of LA from the LOS of pathogenic *Neisseria* directly influences signal transduction through TLR4 and the expression of inflammatory cytokines (30, 31, 38) and may be of critical importance to the progression of the infection, or conversely, to the persistence of *Neisseria* commensalism. For commensal *Neisseria*, we postulated that the maintenance of symbiosis with the human respiratory tract mucosa is dependent on reduced agonistic activity of the LA portion of LOS for TLR4, thereby dampening innate immune sensing.

The results of this study show that whole bacteria of commensal *Neisseria* strains are substantially less inflammatory than those of pathogenic *N. meningitidis* and *N. gonorrhoeae* strains, as revealed by the induction of TNF- $\alpha$  in human THP-1 cells. MS analyses demonstrated that four commensal strains, one each of *N. cinerea*, *N. flavescens*, *N. perflava*, and *N. subflava*, expressed diphosphoryl LA without pyrophosphorylation or phosphoethanolaminylation, unlike the LA of pathogenic *Neisseria* species, which in addition to diphosphoryl substituents, has both pyrophosphorylation and phosphoethanolaminylation (30, 31, 36, 37). Similarly, the absence of LA phosphoethanolaminylation in a meningococcal *lptA* mutant resulted in significantly diminished induction of TNF- $\alpha$  both by bacteria and LOS. The mutant LOS was primarily triphosphorylated, in contrast to the diphosphorylated LA of the four commensal strains, and its induction of TNF- $\alpha$  was intermediate to the levels of induction of these commensal strains and the wild-type meningococcal LOS, supporting our hypothesis that increased phosphorylation of *Neisseria* LA,

either pyrophosphorylation or phosphoethanolaminylation, is correlated with greater inflammatory potential. In addition, we found that the gonococcal LOS was significantly less inflammatory than the meningococcal LOS, consistent with our previous results (30, 38). This difference may account for some of the substantially greater severity of morbidity and higher levels of mortality in cases of meningococemia than in cases of disseminated gonococcal infection (6, 53).

There is very little known about the regulation of pyrophosphorylation of LA in *Neisseria*, but a gene encoding the PEA transferase that is specific for LA has been identified. Using the *lptA* NMB1638 DNA sequence identified by Cox and coworkers as a probe for Southern hybridizations (12), we were able to confirm that *lptA* was absent in the four commensal strains that we had previously studied by MS. Furthermore, we sought to determine the genetic basis for phosphoethanolaminylation of LA in various *Neisseria* species by using bioinformatics to analyze *lptA*. We identified *lptA* in all gonococcal and meningococcal strains that have DNA sequences in the NCBI database (more than 20 sequences in total), and the nucleotide sequence of the gene was virtually identical in all strains, with only 4 nucleotide differences seen among all of the pathogens. The gene was similarly conserved in *N. lactamica*. Furthermore, the genomic organization among the gonococcus, the meningococcus, and *N. lactamica* was conserved. The conservation of *lptA* expression among the pathogenic *Neisseria* contrasted with the results from analysis of nearly all of the commensal strains other than *N. lactamica*, for which no genes with significant homology to *lptA* were found; one of two strains of *N. polysaccharea* was found to possess the gene, and one strain of *N. elongata* possessed a 750-bp region with 50% identity to a portion of *lptA* that would be unlikely to encode a functional transferase protein.

It is interesting to note that in a phenotypic taxonomic study of *Neisseria*, Barrett and Sneath found that *N. lactamica*, *N. polysaccharea*, and *N. elongata* grouped with *N. meningitidis* and *N. gonorrhoeae* and apart from the other commensal *Neisseria* species (5), suggesting a broader association in addition to *lptA*. A microarray genomic analysis of phylogenetic relationships between *Neisseria* species showed that *N. meningitidis* strains were clustered closely, with commensal *Neisseria* species at the greatest distance from them and *N. gonorrhoeae* between these two groups (51). The closer phylogenetic proximity of *N. gonorrhoeae* to the commensal strains than to *N. meningitidis* is significant in light of the results presented here, which show the inflammatory potential of gonococci to be intermediate to those of the meningococcal and commensal strains. Our report of the presence of *lptA* in *N. lactamica* is also consistent with the finding that *N. lactamica* is phylogenetically closer to *N. gonorrhoeae* than to any of the other commensal strains analyzed (51).

Our MS data showed that the LA from *N. lactamica* LOS was similar to LA from *N. meningitidis* in its phosphorylation and acylation. The potential of the LOS from *N. lactamica* to induce TNF- $\alpha$  was also similar to that of the LOS from *N. meningitidis*. However, live *N. lactamica* bacteria had significantly less inflammatory potential than *N. meningitidis* bacteria in THP-1 cells. The difference between the inflammatory potential of live *N. lactamica* bacteria and that of its LOS was striking. Our results are supported by recent data showing that live *N. lactamica* bacteria had significantly less inflammatory potential than killed bacteria in human nasopharyngeal epithelial cells, whereas live *N. meningitidis* bac-

teria were significantly more inflammatory than killed bacteria (58). The data suggest that live *N. lactamica* bacteria can specifically interact with host cells to dampen inflammatory responses in a manner that is distinct from the interactions of pathogenic *N. meningitidis* with host cells. This hypothesis is supported by the report that differential genetic regulation by *N. meningitidis* and *N. lactamica* was found after bacterial interaction with host epithelial cells (24) but that only 167 genes were common to the 347 and 285 genes differentially induced by *N. meningitidis* and *N. lactamica*, respectively.

Thus, overall, our results indicate that the lack of expression of PEA and pyrophosphoryl groups on LA reduces the inflammatory potential of most commensal *Neisseria* and, therefore, may facilitate their symbiotic relationship with human hosts, in which the bacteria are able to evade recognition by the innate immune system. In addition, our previous observation that two commensal strains (one *N. cinerea* and one *N. flavescens*) did not activate the inflammasome signaling pathway as robustly as *N. gonorrhoeae* can now be accounted for by LA structural data (14). Furthermore, our results suggest that the expression of LA with reduced inflammatory potential may similarly facilitate the ability of some carrier strains of meningococci or asymptomatic gonococcal strains to evade the immune system and survive within their human host for long periods of time. This implies that *Neisseria* may possess regulatory mechanisms that downregulate phosphorylation.

The presence of pentaacylated LA due to *lpxL1* mutations in a surprisingly large ~9% of meningococcal disease isolates from 464 patients was shown previously to be correlated with infections that were characterized by less rash and tissue factor-mediated coagulopathy (21). Studies of *lpxL1* mutant meningococcal strains in mice confirmed the significance of acylation in relation to inflammatory potential (20). Thus, our results showing that most strains of commensal *Neisseria* analyzed did not contain *lptA* are another example of naturally occurring variation in the LA of *Neisseria* species that may affect whether bacterial colonization of the nasopharynx progresses into a symptomatic clinical presentation.

Our analyses demonstrated that the five commensal species *N. cinerea*, *N. flavescens*, *N. perflava*, *N. sicca*, and *N. subflava*, none of which were found to contain *lptA*, were significantly more sensitive to polymyxin B than *N. gonorrhoeae* strain MS11. In contrast, *N. lactamica* was similar to *N. gonorrhoeae* in polymyxin B resistance, which is consistent with its expression of *lptA*. These findings expand on those in the study of Anand et al., which reported that *N. meningitidis*, *N. gonorrhoeae*, and *N. lactamica* were equally resistant to colistin (polymyxin E), whereas *N. subflava* was significantly more sensitive (2). Previously, *lptA* mutations in *N. meningitidis* were shown to increase sensitivity to polymyxin B (12, 37, 61). Sensitivity to CAMPs that are part of the innate immune system is thought to be reflected by sensitivity to polymyxin B (23, 37). In addition, an *lptA* null mutation in *N. gonorrhoeae* strain FA19 was shown to confer sensitivity to complement-mediated killing by normal human serum, and it was possible to restore serum resistance by complementation with wild-type *lptA* (37). Thus, our data regarding the sensitivity of commensal *Neisseria* strains to polymyxin B further confirm the absence of phosphoethanolaminylation of the LA of most strains and suggests that they are more likely to be more sensitive to CAMPs, as well as to complement-mediated lysis.

A previous report showed wide variations in sensitivity to colistin among clinical isolates of *N. polysaccharea* that could be divided into two groups, one of which displayed a 60-fold greater resistance to the antibiotic than the other (2). These data support our bioinformatic analysis of two strains of *N. polysaccharea*, which showed that only one of the two strains expressed *lptA*. Phosphoethanolaminylation of the LA in some strains of *N. polysaccharea* would be expected to greatly increase their resistance to polymyxin B compared to that of strains lacking PEA. Taken *in toto*, these data provide a genetic explanation for colistin resistance and suggest a mechanism by which *N. meningitidis*, *N. gonorrhoeae*, and *N. lactamica* can grow on Thayer-Martin medium (1, 59), which contains colistin, while the majority of commensal *Neisseria* strains cannot.

Using intestinal commensalism as a model, immune tolerance exists toward commensal bacteria, as they survive indefinitely within the mucosal environment of the gut without eliciting immune pathology. Several mechanisms have been proposed which suggest that this is an active process in which TLR signaling pathway molecules, such as TLR4 and TRAF6, are directly targeted by the gut flora, resulting in attenuated inflammatory responses (48). For example, the LA of the *Bacteroidetes* species that dominate the gut microbiota is pentaacylated, making it a weak agonist of TLR4/MD-2 on gut immune cells (27).

We have shown that due to the lack of a functional *lptA*, the LA of most strains of commensal *Neisseria* is not phosphoethanolaminylated and also lacks apparent pyrophosphorylation. Coupled with our previous results, these data indicate that the absence of these groups on the LA is critical to the reduced inflammatory potential of the commensal bacteria (30–32, 38). Our data support our hypothesis that the bisphosphorylated LA of the asymptomatic *Neisseria* commensal strains elicits a weak innate immune response that promotes persistent colonization. The potential significance of LA phosphorylation in physiological reactions is underscored by the finding that NF- $\kappa$ B activation by mono- but not diphosphorylated hexaacylated LA from *Salmonella enterica* serovar Typhimurium causes predominantly Toll-interleukin 1 receptor domain-containing adapter-inducing beta interferon (TRIF)-mediated rather than myeloid differentiation factor 88 (MyD88)-mediated signal transduction in mice (41). Monophosphoryl LA has been approved for use as a vaccine adjuvant in several products and is undergoing clinical trials as a component of cancer vaccines due to its stimulation of adaptive immunity and lack of proinflammatory activity (9, 11, 19). Recent data indicate that the balance between NF- $\kappa$ B signal transduction through the MyD88 or TRIF pathways may be affected by negative regulators of TLR signaling, including microRNA (44, 49).

Natural variations in pili, Opa proteins, porins, and capsules are thought to play important roles in the virulence and transmissibility of *N. meningitidis* (56). Our results indicate that lack of phosphoethanolaminylation and pyrophosphorylation of LA may be a structural component which contributes to the immune privilege of the *Neisseria* carriage state by reducing the inflammatory potential of the LOS and increasing the sensitivity of the bacteria to the activity of the host CAMPs. Given the importance of LOS in *Neisseria* infections (7), our data also pinpoint these structural differences in phosphoryl substitution as potentially one of the determinants of pathology in infections caused by *Neisseria*. A more complete understanding of the phenotypic and molecular basis of the diminished pathogenic potential of *Neisseria* commensals could inform efforts to define the relationships between

asymptomatic carriage and the virulence of disease caused by *N. meningitidis* and *N. gonorrhoeae*, to develop new prognostic indicators or targets for drug development, and to develop new adjuvants or vaccines.

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