The membrane phospholipids of *Neisseria meningitidis* and *Neisseria gonorrhoeae* as characterized by fast atom bombardment mass spectrometry

M. Mahbubur Rahman,¹ V. S. Kumar Kolli,¹ Charlene M. Kahler,² Giles Shih,² David S. Stephens² and Russell W. Carlson¹

Author for correspondence: Russell W. Carlson. Tel: +1 706 542 4439. Fax: +1 706 542 4412.
e-mail: rcarlson@ccrc.uga.edu

¹ Complex Carbohydrate Research Center, The University of Georgia, 220 Riverbend Road, Athens, GA 30602, USA

² Departments of Medicine and Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30303, and Department of Veterans Affairs Medical Center, Atlanta, GA, USA

The phospholipids of *Neisseria meningitidis* and *Neisseria gonorrhoeae* were characterized by fast atom bombardment (FAB)-MS and GLC-MS. The major phospholipids were phosphatidylethanolamine (PE), followed by phosphatidylglycerol (PG), with minor amounts of phosphatidic acid (PA) and trace levels of cardiolipin (DPG). All of the phospholipid preparations were variable in their fatty acyl substituents, which included C16:1, C16:0, C18:1, C14:0, C14:1 and C12:0. By MS/MS analysis, all pathogenic *Neisseria* spp. phospholipids contained a saturated fatty acyl substituent and either a saturated or unsaturated fatty acyl substituent in the *sn*-1 and *sn*-2 positions, respectively. Compared with enteric bacterial species, the phospholipids of *N. meningitidis* and *N. gonorrhoeae* have increased levels of phospholipids with short-chain fatty acyl residues (i.e. increases in C12:0, C14:1 and C14:0) and variable amounts of C18:1. The percentage of total PE and PG molecules with the shorter-chain fatty acids ranges from 35 to 47% and 42 to 66%, respectively, for *N. meningitidis* while these respective values are <10% and <5% for *Escherichia coli*. The variability and variety of meningococcal and gonococcal phospholipids suggest novel genetic mechanisms of neisserial phospholipid assembly and regulation, which may be important for the biology and pathogenesis of *N. meningitidis* and *N. gonorrhoeae*.

Keywords: *Neisseria meningitidis*, *Neisseria gonorrhoeae*, membranes, phospholipids

INTRODUCTION

Neisseria meningitidis and *Neisseria gonorrhoeae*, are obligate human pathogens that cause distinctly different disease syndromes. *N. meningitidis*, which normally colonizes the nasopharynx, is a major cause of sepsis and epidemic meningitis (Brandtzaeg *et al.*, 1989; Virji, 1996). In contrast, *N. gonorrhoeae* is a major sexually transmitted pathogen causing urethritis, epididymitis, salpingitis and infertility (Handsfield & Sparling, 1995). However, both pathogens are closely related genetically, colonize human mucosal surfaces and express a similar

array of outer-membrane proteins and lipooligosaccharide (LOS) structures that have defined roles in pathogenesis (Virji, 1996; Nassif & So, 1995; Kahler & Stephens, 1998). The contributions of membrane phospholipid structure to the virulence of meningococci and gonococci have not been investigated. Membrane phospholipids are increasingly recognized as important components of bacterial virulence mechanisms, including roles as signalling molecules and toxins (Moolenaar, 1995). The importance of phospholipid structure with respect to bacterial resistance to antimicrobial agents, humoral immune mechanisms and environmental stresses is also recognized (Hancock, 1997; Mold, 1989).

In Gram-negative bacteria, such as *Escherichia coli* and *Neisseria* spp., the outer membrane has an asymmetrical organization in which the outer leaflet is primarily composed of LPS and proteins and the inner leaflet

Abbreviations: DPG, cardiolipin; FAB, fast atom bombardment; LPA, lysophosphatidic acid; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

contains phospholipids (Nikaido, 1996). The role of phospholipids in maintaining the integrity of the outer membrane has been demonstrated. Perturbation of outer-membrane biogenesis in *E. coli* by mutations which decrease lipid A synthesis (Karow *et al.*, 1992) and suppress outer-membrane protein assembly (Kloser *et al.*, 1998) results in elevated levels of phospholipid synthesis and incorporation into the outer membrane. Since both LPS and phospholipid pathways share common fatty acid precursors, this effect may be mediated by the shift in the flow of these precursors from one pathway to the other. In *Proteus mirabilis*, it has been noted that LPS mutants contain an increased level of unsaturated fatty acid content which may compensate for fluidity changes in the outer membrane (Rottem *et al.*, 1978). Additionally, the export and trimerization of the major porins of the outer membrane rely upon a tight association with intact LPS, and these proteins do not localize to the outer membrane of *E. coli* mutants expressing LPS cores deficient in heptose (Sen & Nikaido, 1991). Interestingly, the relative abundance of membrane phospholipids also significantly affects the functional activities of outer-membrane proteins (Senff *et al.*, 1976; Wolf-Watz *et al.*, 1975).

Earlier studies conducted in the 1960s and 1970s using TLC indicated that the major phospholipid component of membranes isolated from *N. gonorrhoeae* consisted largely of phosphatidylethanolamine (PE) with varying amounts of phosphatidylglycerol (PG), cardiolipin (DPG) and lysophosphatidylethanolamine (LPE) (Guymon *et al.*, 1978; Sud & Feingold, 1976; Senff *et al.*, 1976; Wolf-Watz *et al.*, 1975; Lewis *et al.*, 1968; Moss *et al.*, 1970). Subsequently, it was shown that the LPE found in gonococcal membranes was due to the degradation of PE by phospholipase A (Cacciapuoti *et al.*, 1978, 1979). Lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1) and *cis*-vaccenic (C18:1) acids were the major fatty acids attached to gonococcal phospholipid head groups (Guymon *et al.*, 1978; Sud & Feingold, 1976; Wolf-Watz *et al.*, 1975; Wilkinson, 1988; Moss *et al.*, 1970; Lewis *et al.*, 1968). The total cellular fatty acids and extractable cellular lipids of *N. meningitidis* isolates were subsequently found to be similar to those of gonococci (Lewis *et al.*, 1968; Moss *et al.*, 1970; Wilkinson, 1988). However, the precise structures of *N. meningitidis* phospholipids, including their fatty acylation patterns, have not been examined in detail.

This study is the first to detail by MS the complete structure of the phospholipids of both *N. meningitidis* and *N. gonorrhoeae*. In addition, a series of meningococcal capsule and LOS mutants were studied to determine whether changes in the expression of these surface structures affected the phospholipid profile. Considerable heterogeneity was observed in the *N. meningitidis* phospholipid fatty acylation patterns when compared to the phospholipids from various enteric bacterial species. This heterogeneity is likely to be a result of novel genetic mechanisms of neisserial phospholipid assembly and regulation.

METHODS

Bacterial strains and conditions of growth. Four strains of *N. meningitidis* (NMB, F8229, F8239, 6940) and *N. gonorrhoeae* FA 1090 were used in these studies, in addition to six genetically defined mutants of meningococcal strain NMB (Table 1). The wild-type meningococcal and gonococcal strains and mutant derivatives of meningococcal strain NMB were grown for 16–18 h in GC broth at 37 °C with 3.5% CO₂ (Morse & Bartenstein, 1974). The increased CO₂ atmosphere was achieved by supplementation with 10 ml 4.3% sodium bicarbonate per litre of broth. The following antibiotic concentrations were used for growth of mutants when appropriate: 5 µg tetracycline ml⁻¹ or 60 µg spectinomycin ml⁻¹.

Preparation of total membranes. To prepare total membranes, strains were grown in 1 l GC broth for 16–18 h in an environmental shaker at 37 °C and 200 r.p.m. Cells were collected by centrifugation at 11000 g by using a JA-14 rotor (Beckman) and the total membranes were extracted by using the method of Clark *et al.* (1987). All glassware was baked at 180 °C and plasticware was washed in chloroform, then air-dried. In brief, the cell pellet was resuspended in 30 ml 50 mM Tris buffer (pH 8.0), transferred to 50 ml Oak Ridge tubes and collected in a pellet by centrifugation in a JA-17 rotor (Beckman) at 13000 g for 15 min at 4 °C. The supernatant was discarded and the cells washed again in 10 ml of the same buffer. The cells were lysed by suspension in 2 ml lysis buffer (50 mM Tris buffer, pH 8.0), 1 mM EDTA, 20% sucrose and 1 mg lysozyme ml⁻¹) and two freeze-thaw cycles using a dry-ice ethanol bath and a room temperature water bath. After the last thaw, 20 ml 50 mM Tris buffer (pH 8.0) was added and the mixture was thoroughly vortexed. The lysis mixture was sonicated in an ice bath three times (30 s burst, 30 s rest) using a Virtis Virsonic 300 with a micro-tip at setting 5. The sonicated cells were incubated on ice for 10 min. The cellular debris was pelleted from the lysis mixture by 15 min centrifugation at 10000 g in a JA-17 rotor (Beckman) at 4 °C. The supernatant was transferred to a clean tube and centrifuged again using the same conditions. This step was repeated twice more. The supernatant containing the membranes was loaded into preweighed polyallomer tubes, balanced in an SW28 rotor (Beckman) and ultracentrifuged at 100000 g for 1.5 h at 4 °C. The supernatant was carefully drained and the translucent pellet, which contained total membranes, was weighed, then stored at -70 °C.

Extraction of phospholipids. The phospholipids were extracted from total membranes isolated from each strain using a modified method of Radin (1969). Glassware was rinsed with chloroform, then air-dried and Teflon-lined caps were used to prevent contamination with plastics. The membranes were suspended in the polyallomer tubes by the addition of solvent (2 vols chloroform to 1 vol. methanol) in a 17:1 (v/w) ratio of solvent to pellet and stirred constantly using micro-stir bars for 30 min at room temperature. The suspension was transferred to Corex glass tubes and the debris was collected by 15 min centrifugation at 10000 g in a JA-17 rotor (Beckman) at 4 °C. The supernatant was collected and the pellet was again extracted with solvent as before. The two supernatants were combined and 2 vols Folch upper phase reagent (chloroform/methanol/water containing 0.74% potassium chloride; 3:48:47, by vol.) was added. The mixture was briefly vortexed to form a single phase. The phases were separated by low speed 1 min centrifugation in a clinical centrifuge. The upper phase and the precipitate at the interface were discarded. The lower phase was extracted with the Folch

Table 1. Meningococcal and gonococcal strains used in this study

Strain	Genotype	Phenotype	Reference
Wild-type isolates			
<i>N. meningitidis</i> NMB	Wild-type	Serogroup B	Stephens <i>et al.</i> (1991)
<i>N. meningitidis</i> F8229	Wild-type	Serogroup A	Swartley <i>et al.</i> (1998)
<i>N. meningitidis</i> F8239	Wild-type	Serogroup A	Swartley <i>et al.</i> (1998)
<i>N. meningitidis</i> 6940	Wild-type	Serogroup B	Zollinger & Mandrell (1977)
<i>N. gonorrhoeae</i> FA 1090	Wild-type		Dempsey & Cannon (1994)
LOS mutants			
<i>N. meningitidis</i> M7	NMBsynX::Tn916	cap ⁻ , LOS ⁺	Stephens <i>et al.</i> (1994); Swartley & Stephens (1994)
<i>N. meningitidis</i> SS3	NMBgalE::Tn916	cap ⁺ , LOS ⁻	Stephens <i>et al.</i> (1994); Lee <i>et al.</i> (1995)
<i>N. meningitidis</i> CMK2	NMBlgtF::aphA-3	cap ⁺ , LOS ⁻	Kahler <i>et al.</i> (1996a)
<i>N. meningitidis</i> R6	NMBpgm::Tn916	cap ⁺ , LOS ⁻	Zhou <i>et al.</i> (1994)
<i>N. meningitidis</i> CMK1	NMBrfaK::Ω	cap ⁺ , LOS ⁻	Kahler <i>et al.</i> (1996b)
<i>N. meningitidis</i> 469	NMBnlaBgmh::Tn916	cap ⁺ , LOS ⁻	Shih <i>et al.</i> (1998)

Fig. 1. FAB mass spectrum for phospholipids of *N. meningitidis* NMB. See Tables 2 and 3 for peak identities.

upper phase reagent at least three more times or until there was no precipitate at the interface. After the final extraction, the lower organic phase was transferred to a glass vial and the solvent evaporated under a stream of nitrogen. The dried phospholipids were stored at -70°C under a nitrogen atmosphere.

Fatty acid analysis. Total fatty acids were released by methanolysis of phospholipid with methanolic 1 M HCl at 80°C for 4 h and were trimethylsilylated. The resulting fatty acid methyl esters were analysed by GLC-MS (York *et al.*, 1985). Trimethylsilylation allows one to observe any hydroxy

fatty acyl residues, if present. The double bond location of unsaturated fatty acids was determined by the preparation and GLC-MS analysis of dimethyl disulfide fatty acid methyl esters as described by Yruela *et al.* (1990).

MS. Fast atom bombardment (FAB)-MS was performed in negative-ion mode with a JEOL SX/SX 102A tandem mass spectrometer, which was operated at 10 kV accelerating potential. Ions were produced by FAB with xenon, using a JEOL FAB gun operated at 6 kV in a conventional FAB ion source. Spectra acquired for the first MS are averaged profile data of three scans as recorded by a JEOL XMS data system.

Fig. 2. FAB mass spectrum for phospholipids of *N. gonorrhoeae* FA 1090. See Tables 2, 3 and 4 for peak identities.

These spectra were acquired from 100–2000 m/z at a rate that would scan the mass range from 0 to 2500 in 1 min. A filtering rate of 100 Hz was used in acquiring these spectra. The samples were dissolved in chloroform/methanol (2:1, v/v) solution and 1 μ l aliquots were mixed with an equal volume of the FAB matrix, *m*-nitrobenzyl alcohol, on an MS probe tip. After permitting solvent evaporation, the probe was placed into the sample port of the mass spectrometer. The MS/MS spectrum was obtained by using a linked scan (B/E constant) at a rate that would scan the mass range from 5 to 2400 in 1 min with a 300 Hz filter.

Quantification of phospholipids by FAB-MS. Dipalmitoyl PE, PG and phosphatidic acid (PA) were purchased from Sigma. Two series of 1 ml solutions of these phospholipids were prepared. One series consisted of four different solutions varying in both PE and PG (10, 30, 60 and 90 μ g) but with each containing a constant 100 μ g amount of PA as an internal standard. The second series varied in both PA and PG (10, 30, 60 and 90 μ g) with a constant 100 μ g of PE as an internal standard. Each solution was analysed by FAB-MS as described above. The data were used to (a) determine if the intensity of the molecular ion for each phospholipid was proportional to the amount of that phospholipid, and (b) determine the FAB-MS response of PE relative to PG and of PA relative to PG. Analysis of the first series of solutions allowed the correlation of the molecular ion intensity with mass for both PG and PE, and also allowed the calculation of the FAB-MS response factor of PE relative to PG. The second series of solutions allowed such correlation for both PA and PG, and also the calculation of the FAB-MS response factor of PA relative to PG. Using these normalized response factors, the relative amounts of the various phospholipids for each membrane preparation were calculated, assuming that all of the various PE, PG and PA molecules have the same, or very similar, FAB-

MS responses to those of the respective dipalmitoyl PE, PG and PA standards. This approach overcomes the discrepancies in phospholipid quantification that were noted by Smith *et al.* (1995) where a comparison of MS techniques and conventional methods of analysis were shown to disagree.

RESULTS

The genotypes and phenotypes of the *N. meningitidis* and *N. gonorrhoeae* strains and mutants used for the analysis of total membrane phospholipids are shown in Table 1. The composition of membrane phospholipid molecules was determined by FAB-MS performed in negative-ion mode. The FAB-MS spectra for the phospholipids from *N. meningitidis* NMB and *N. gonorrhoeae* FA 1090 are shown in Figs 1 and 2, respectively. The spectra of the phospholipids from the other strains are not shown but the results are included in Table 4 and are discussed below.

Membrane fatty acid compositions

The fragment ions of m/z values from 199 to 300 represent fatty acid carboxylate anions. The fatty acids found in the various phospholipids from both wild-type and mutant strains are shown in Table 2. The most intense ions were: m/z 253, C16:1; 255, C16:0; and 227, C14:0. In addition, the following less-intense ions were also observed: 199, C12:0; 225, C14:1; 269, C17:0; 281, C18:1; 283, C18:0. These phospholipid-derived carboxylate fragment ions were as reported by Drucker *et al.* (1995).

Table 2. Carboxylate molecular species of the neisserial phospholipids observed as anions in FAB-MS spectra

Data are expressed as percentage total carboxylate anion peak. Bold type indicates the major components present in the respective samples.

Carboxylate ion		Relative ion intensity										
<i>m/z</i>	ID*	NMB	F8229	F8239	6940	FA 1090	M7	SS3	CMK2	R6	CMK1	469
199	12:0	8.4	8.0	6.2	6.6	3.1	9.7	8.4	8.5	10	10	8.7
225	14:1	3.1	2.7	2.5	3.4	1.6	2.6	2.7	2.5	2.6	2.6	2.6
227	14:0	17	18	20	12	6.7	20	18	20	20	13	16
251	16:2	3.2	2.4	1.9	5.2	4.0	2.0	2.9	2.2	2.1	3.1	2.9
253	16:1	30	29	25	35	42	24	29	27	25	31	30
255	16:0	33	35	40	29	29	37	34	35	35	34	33
281	18:1	4.8	5.5	4.3	8.2	14	4.5	5.2	5.5	4.4	5.3	7.1

† 12:0, Dodecanoate; 14:1, tetradecenoate; 14:0, tetradecanoate (myristate); 16:2, hexadecenoate; 16:1, hexadecenoate; 16:0, hexadecanoate; 18:1, octadecenoate.

Table 3. Fatty acid composition analysis of neisserial phospholipids

Data are expressed as percentage fatty acid methyl ester GLC total peak area. Bold type indicates the major components present in the respective samples. TR, trace.

ID*	NMB	F8229	F8239	6940	FA 1090	M7	SS3	CMK2	R6	CMK1	469
12:0	TR	TR	TR	TR	TR	TR	TR	TR	TR	TR	TR
14:1	TR	TR	TR	TR	TR	TR	TR	TR	TR	TR	TR
14:0	20	18	21	10	6	19	14	19	19	18	17
16:2	TR	TR	TR	TR	TR	TR	TR	TR	TR	TR	TR
16:1	28	26	30	31	37	23	27	27	24	25	22
16:0	47	49	43	44	41	52	52	47	51	50	54
18:1	5	7	6	15	16	6	7	7	6	8	7

* Fatty acids are designated as described for Table 2.

The total fatty acid composition of phospholipids from wild-type *N. meningitidis* and *N. gonorrhoeae* and meningococcal mutant strains were also analysed by methanolysis followed by trimethylsilylation and analysis by GLC-MS. The results of the GLC-MS analysis (Table 3) show that the major fatty acids observed by this method are consistent with the major fatty acids observed by FAB-MS analysis (Table 2.) The C12:0 fatty acid was observed in both the FAB-MS and GLC-MS analyses, but the high volatility of its methyl ester prevented accurate quantification by the GLC-MS procedure. However, the FAB-MS spectra clearly show that C12:0 was present in the phospholipids of all meningococcal strains examined (6.5–10 %) and a lesser amount (3.3 %) in *N. gonorrhoeae* FA 1090.

The phospholipids of *N. gonorrhoeae* FA 1090 contained significantly less shorter-chain fatty acids (e.g. C12:0, C14:0 and C14:1) compared to the *N. meningitidis* strains (Figs 1 and 2, Tables 2 and 3). The phospholipids from the three *N. meningitidis* isolates,

Fig. 3. Correlation of molecular ion intensity with the amount of phospholipid. (a) Variation in PG (■) and PA (◆) while holding PE at a constant level (100 µg). (b) Variation of PG (■) and PE (◆) while holding PA constant (100 µg).

Table 4. Neisserial phospholipid content as determined by FAB-MS analysis

Data are expressed as relative mass percentage of total phospholipid. The values were corrected for the different responses of PE, PG, and PA during FAB-MS analysis, and were normalized to PG that has the highest response. The NMB values and standard errors were calculated from the analyses of three different NMB phospholipid preparations.

Phospholipid		Phospholipid content										
<i>m/z</i>	ID*	NMB	F8229	F8239	6940	FA 1090	M7	SS3	CMK2	R6	CMK1	469
591	PA(28:0)	1.7 ± 0.2	1.8	2.1	1.6	0.7	1.7	1.7	1.7	2.0	1.9	1.4
617	PA(30:1)	1.4 ± 0.2	1.7	2.3	2.0	0.8	1.3	1.3	1.4	1.6	1.7	1.1
619	PA(30:0)	2.0 ± 0.2	2.1	3.1	1.8	0.8	2.4	2.0	2.2	2.4	2.5	1.6
645	PA(32:1)	3.7 ± 0.2	4.2	5.6	4.4	4.0	3.4	3.6	4.0	3.9	3.5	2.9
647	PA(32:0)	1.2 ± 0.1	1.2	2.1	1.4	0.8	1.3	1.2	1.2	1.1	1.8	0.9
	Total PA	10.0 ± 1.0	11.0	15.2	11.2	7.1	10.1	9.8	10.5	11.0	11.4	7.9
632	PE(28:1)	2.8 ± 0.2	2.8	2.7	4.2	1.3	2.5	2.9	2.1	2.7	3.9	3.2
634	PE(28:0)	11.2 ± 3.1	10.9	8.4	5.8	3.7	12.1	10.6	10.8	12.2	9.8	10.5
660	PE(30:1)	6.4 ± 1.1	7.4	7.3	7.1	4.9	5.8	5.8	5.9	6.3	6.6	6.6
662	PE(30:0)	12.7 ± 3.1	12.7	12.5	5.4	5.2	16.9	11.1	14.7	13.8	12.7	11.6
686	PE(32:2)	4.6 ± 0.4	4.9	5.4	9.1	7.9	3.6	4.4	4.1	4.2	4.3	5.3
688	PE(32:1)	20.7 ± 3.3	23.7	22.2	15.6	28.3	21.3	18.4	24.2	21.6	15.0	22.4
690	PE(32:0)	4.8 ± 0.6	5.7	7.1	4.0	4.2	6.6	4.4	5.3	4.5	7.1	5.3
714	PE(34:2)	1.5 ± 0.2	2.2	2.8	3.8	5.7	1.6	1.5	1.8	1.7	3.4	2.1
716	PE(34:1)	3.5 ± 0.4	4.7	4.0	4.7	8.2	4.3	3.5	5.1	4.2	5.3	4.8
718	PE(34:0)	2.6 ± 0.9	1.6	1.3	5.3	3.8	1.5	3.3	1.7	1.7	3.4	2.4
	Total PE	70.8 ± 10.7	76.6	73.7	65.0	73.2	76.2	65.9	75.7	72.9	71.5	74.2
661	PG(28:2)	0.9 ± 0.1	0.9	1.0	1.0	0.6	0.8	0.9	0.8	0.8	0.9	0.8
663	PG(28:1)	1.5 ± 0.2	1.3	1.3	1.1	0.5	1.7	1.5	1.5	1.5	1.5	1.3
665	PG(28:0)	2.0 ± 0.5	0.9	0.6	1.7	0.2	1.5	3.0	1.2	1.9	1.6	1.7
689	PG(30:2)	2.2 ± 0.3	2.5	2.4	1.9	2.9	2.2	2.1	2.5	2.2	1.7	2.4
691	PG(30:1)	1.3 ± 0.3	0.9	0.9	2.0	0.6	0.9	1.5	0.8	0.9	1.2	1.1
693	PG(30:0)	2.5 ± 0.7	1.2	0.9	2.2	0.5	1.8	3.6	1.5	2.1	2.1	1.9
717	PG(32:2)	1.1 ± 0.3	0.8	0.7	2.5	1.9	0.7	1.4	0.9	0.8	1.3	1.1
719	PG(32:1)	5.5 ± 2.1	2.5	1.5	7.0	7.8	2.5	7.2	3.1	3.8	3.9	4.9
721	PG(32:0)	1.1 ± 0.4	0.7	0.8	1.4	0.9	0.8	1.4	0.7	0.8	1.5	1.0
745	PG(34:2)	0.4 ± 0.2	0.2	0.2	1.1	1.4	0.2	0.4	0.2	0.3	0.5	0.4
747	PG(34:1)	1.1 ± 0.4	0.7	0.5	1.9	2.6	0.7	1.4	0.8	0.9	0.8	1.4
	Total PG	19.6 ± 4.5	12.6	10.8	23.8	19.9	13.8	24.4	14.0	16.0	17.0	18.0

* Example of abbreviations for phospholipids: PE(28:0) indicates a phosphatidylethanolamine in which the total number of carbons for both fatty acyl residues is 28.

NMB, F8229 and F8239, had similar fatty acid profiles. However, *N. meningitidis* 6940 had a fatty acid profile resembling that of *N. gonorrhoeae* FA 1090. Both 6940 and FA 1090 contained 1.5–3-fold more C18:1 and a corresponding 1.5–3-fold decrease of C14:0 when compared to the meningococcal isolates NMB, F8229 and F8239. No significant differences were observed in the fatty acid profiles of the capsule and LOS mutants compared to that of the parent strain, NMB.

Phospholipid compositions

The relative mass percentage of each phospholipid was determined from the molecular ion intensities of the various phospholipid molecules. To determine if ion intensity was proportional to the amount of a particular phospholipid, the molecular ion intensities were

measured for known amounts of dipalmitoyl PA, PG and PE by varying the level of two of the phospholipids while holding the amount of the third phospholipid constant. Under these conditions, the phospholipid that was held constant acted as an internal standard. In the first series, PG and PA were varied while PE was the constant internal standard. Fig. 3(a) shows that both the PG/PE and PA/PE molecular ion intensity ratios were proportional to the amount of PG and PA, respectively. Likewise, Fig. 3(b) shows that the PG/PA and PE/PA molecular ion intensity ratios were proportional to the amounts of PG and PE, respectively. By calculating the (PA/PE)/(PG/PE) and (PE/PA)/(PG/PA) values for each concentration from Fig. 3(a) and (b), respectively, the response of PA to PG and of PE to PG were determined to be 0.37 ± 0.06 and 0.24 ± 0.04 , respectively. Thus, the amount of each PA molecule can be

Fig. 4. Tandem MS/MS spectrum of the m/z 688 ion in the FAB-MS of *N. meningitidis* NMB phospholipid. The top shows the phospholipid structures and the expected fragmentation pattern for those structures.

normalized to PG by dividing its molecular ion intensity by 0.37 and the amount of each PE molecule can be normalized to PG by dividing its molecular ion intensity by 0.24.

Using the approach outlined above, the relative amounts of the various phospholipids for each membrane preparation were determined and are shown in Table 4. In all of the preparations, the amount of total PE was greatest, varying from 65 to 77%, followed by PG (14–20%) and then PA (8–11%). The standard error observed in total PE, PG and PA values for three different NMB preparations (see Table 4) suggests that there are significant differences in the levels of the various phospholipids among the *N. meningitidis* strains and the *N. gonorrhoeae* strain. That PE was the most abundant class of phospholipid in meningococci and gonococci is consistent with previous results (see review by Wilkinson, 1988). However, previous studies also indicated that *N. gonorrhoeae* membranes contain up to 20% DPG (see review by Wilkinson, 1988). The FAB-MS analysis shown in Figs 1 and 2 indicated only trace levels of DPG present in both the meningococcal and gonococcal isolates that we examined. In the case of *N. meningitidis* NMB the ions at m/z 1321 and 1293 probably represent DPG(C62:1) and DPG(C60:1), respectively. In the case of *N. gonorrhoeae*, ions of very weak intensities are observed at about m/z 1350 and 1378 (there may be more variability in these values due to their low intensities), which may represent DPG(C64:1) and DPG(66:1). It is possible that a portion of the ions

attributed to PA could be due to fragmentation of DPG. However, it is unlikely that a significant percentage of the PA ions is due to such fragmentation since (i) it has been shown that FAB-MS analysis of DPG results in a molecular ion that has an intensity about fivefold greater than any of the fragment ions that could be attributed to PA (Jensen *et al.*, 1987), and (ii) our data show DPG ions of only very weak intensities. In addition, the FAB-MS spectra (see Figs 1 and 2) all show lower intensity ions at m/z 391, 409, 434, 452 and 465. These ions could arise by molecular fragmentation of PE or PG molecules as described by Drucker *et al.* (1995), or may be due, in part, to the presence of lysophospholipid molecules.

A major difference between neisserial phospholipids and those from Gram-negative enteric bacterial species is the abundance of the short-chain fatty acyl substituents in the former (see review by Wilkinson, 1988), a result confirmed by this study. This increase in shorter-chain fatty acyl groups was reflected in the proportion of PE and PG molecules which contain C12:0, C14:0 or C14:1. The relative percentage of total PE and total PG which contain shorter-chain fatty acyl groups (i.e. fatty acyl chains in which the number of carbons for both fatty acids total 30 or less) varies from 21 to 47% for PE and from 24 to 66% for PG, while those same values for *E. coli* are 8.1 and 4.4% for PE and PG, respectively (calculated from the phospholipid data reported for *E. coli* in the review by Wilkinson, 1988). It is clear from these results that, in comparison to *E. coli*, all of the neisserial phospholipids are greatly enriched with

Table 5. The ratios of the various isobaric phospholipid molecules and the position of the fatty acyl substituents for each molecular species

Molecular ion*	Position of the fatty acyl substituents		Phospholipid	A:B ratio
	<i>sn-1</i>	<i>sn-2</i>		
719-A	C16:0	C16:1	PG	4:1
719-B	C14:0	C18:1	PG	
688-A	C16:0	C16:1	PE	6:1
688-B	C14:0	C18:1	PE	
662	C16:0	C14:0	PE	1:2:1
693-A	C13:0	C17:0	PG	
693-B	C16:0	C14:0	PG	1:3:1
634-A	C16:0	C12:0	PE	
634-B	C14:0	C14:0	PE	9:1
665-A	C16:0	C12:0	PG	
665-B	C14:0	C14:0	PG	

* A and B represent two molecular species of the same mass, e.g. 719-A and 719-B are two different PG molecules having the same [M-H] value of m/z 719.

molecules that contain shorter-chain fatty acyl substituents.

Isobaric phospholipid molecules and the positions of fatty acyl substituents

The structures of the various phospholipids were further characterized via tandem MS/MS analysis of single molecular ions which resulted in characteristic formation of fragment ions during collision-induced dissociation (CID). In addition, the relative intensities of the acyl carboxylate fragment ions from tandem MS/MS analysis indicate their positions in the phospholipid (Jensen *et al.*, 1987; Cole & Enke, 1991); i.e. at the *sn-1* or *sn-2* positions. The carboxylate fragment ion from the fatty acyl component at *sn-2* will form in preference to that at the *sn-1* position and will, therefore, have the greatest intensity (Jensen *et al.*, 1987; Cole & Enke, 1991). Fig. 4 shows the fragment ion spectrum obtained by tandem MS/MS analysis of a phospholipid from strain NMB. The structural basis for these assignments is also given in Fig. 4. These data are consistent with the m/z 688 ion being due to two isobaric PE molecules; one major PE component with C16:1 (m/z 253) and C16:0 (m/z 255) as the acyl substituents and a second minor component with C18:1 (m/z 281) and C14:0 (m/z 227) as the acyl substituents. Similarly, tandem MS/MS analysis of m/z 719 (spectrum not shown) suggests that this ion is due to two isobaric PG structures, the major component with C16:0 and C16:1 as the acyl substituents and a minor component substituted with C14:0 and C18:1. The MS/MS spectra of other major phospholipids having masses of 693, 665 and 635 were also obtained and the resulting data indicate that they have the structures

given in Table 5. According to these results, every phospholipid examined by tandem MS/MS contained a saturated fatty acyl substituent at the *sn-1* position and contained either a saturated or unsaturated fatty acyl substituent at the *sn-2* position.

DISCUSSION

In this study, FAB-MS, GLC-MS and tandem MS/MS techniques were used to analyse the structures of the phospholipids from four isolates of *N. meningitidis* and one isolate of *N. gonorrhoeae*, as well as from six LOS and capsule mutants of *N. meningitidis* NMB. The membrane preparations were extracted from stationary-phase cultures grown under conditions similar to those in all earlier studies of neisserial phospholipids (Wilkinson, 1988; Sud & Feingold, 1976; Wolf-Watz *et al.*, 1975; Senff *et al.*, 1976; Guymon *et al.*, 1978; Lewis *et al.*, 1968; Moss *et al.*, 1970). These earlier studies, most performed by using TLC, found that the fatty acyl groups of the phospholipids of *N. gonorrhoeae* and the fatty acids from cellular lipid extracts from *N. meningitidis* were more heterogeneous than those reported for other bacterial species (see review by Wilkinson, 1988), including *E. coli*, on which the standard model for the phospholipid and anaerobic fatty acid biosynthetic pathways is based (Rock *et al.*, 1996; Shibuya, 1992). The major phospholipids previously reported in *N. gonorrhoeae* were PE and PG, with up to 20% of total phospholipids reported as DPG. The most predominant fatty acids were C14:0, C16:0, C16:1 and C18:1 with lower, but significant levels of C12:0 (Guymon *et al.*, 1978; Sud & Feingold, 1976; Senff *et al.*, 1976; Wolf-Watz *et al.*, 1975; Wilkinson, 1988). Interestingly, a comparison of the results from these previous surveys indicated that the fatty acid profiles of most neisserial isolates were similar, even though cells were harvested from different growth conditions. The fatty acid profiles of bacteria from overnight broth cultures (Lewis *et al.*, 1968), overnight plate cultures (Moss *et al.*, 1970) or from different phases of growth (Sud & Feingold, 1976) were all remarkably similar. This behaviour is quite different from *E. coli* where the expression of DPG is dependent on the growth phase of growth (Rock *et al.*, 1996).

The results of the present study both support and are at variance with results of previous studies in gonococci. The major classes of phospholipids present in meningococci and gonococci were PE (65–77%), followed by PG (14–24%), confirming other work (Sud & Feingold, 1976; Wolf-Watz *et al.*, 1975; Senff *et al.*, 1976; Guymon *et al.*, 1978; Lewis *et al.*, 1968; Moss *et al.*, 1970). The present study also confirms that pathogenic *Neisseria* spp. contain significant levels of short-chain fatty acids, C12:0, C14:1 and in particular C14:0, which account for one-fifth of the total fatty acid composition of some meningococcal strains. However, in contrast to the earlier reports, we detected significant levels of PA (7–11%) and very low levels of DPG in all strains. The reason for the contrast between our results and those previously reported is not known; however, the contrast

in results could reflect real strain differences in phospholipid profiles or differences in the FAB-MS technique used in our work versus the TLC procedures used in the earlier work. Variability in amounts of PE, PG and PA was observed between the different *N. meningitidis* strains, including the LOS mutants of strain NMB and *N. gonorrhoeae*. The biological significance, if any, of these differences in overall phospholipid distribution requires further study, but illustrates the variability of meningococcal and gonococcal surface constituents.

Detailed structural analysis of the phospholipids of *N. meningitidis* and *N. gonorrhoeae* has not been previously reported (e.g. the fatty acylation patterns of the various phospholipids). The FAB-MS spectra of the phospholipid species produced by both meningococci and gonococci revealed a diverse array of acyl chains attached to each type of phospholipid head group. Tandem MS/MS was used to identify the acyl chain attachments at the *sn*-1 and *sn*-2 positions of the major phospholipids. This analysis confirmed that neisserial phospholipids contain a saturated fatty acyl substituent at *sn*-1 and either a saturated or unsaturated fatty acyl substituent at *sn*-2, verifying the diversity of these structures. The major phospholipids of *E. coli* have an asymmetric distribution with the *sn*-1 position of the glycerol phosphate backbone containing the saturated fatty acid C16:0, while *sn*-2 is occupied by unsaturated fatty acids C16:1 or C18:1 (Rock *et al.*, 1996; Wilkinson, 1988). Minor phospholipids from *E. coli* have C14:0, C16:1 or C18:1 in the *sn*-1 position (see review by Wilkinson, 1988). The predominant phospholipids of *N. meningitidis* NMB, PG(32:1) (28% of total PG) and PE(32:1) (29% of total PE), have a similar asymmetrical distribution of saturated (C16:0 or C14:0) and unsaturated (C16:1 or C18:1) acyl chains at the *sn*-1 and *sn*-2 positions, respectively, of the glycerol phosphate backbone. A major difference between the neisserial phospholipids and those from *E. coli*, is that the former have a much greater level of short-chain fatty acid substituents. This greater diversity of short-chain fatty acyl substituents is consistent with our previous report (Shih *et al.*, 1998) showing that strain NMB contains at least two lysophosphatidic acid (LPA) acyltransferases which catalyse the transfer from acyl-ACP to the *sn*-2 position of the glycerol phosphate backbone. In comparison, *E. coli* contains only one LPA acyltransferase (Coleman, 1992).

In two of the isolates, *N. meningitidis* 6940 and *N. gonorrhoeae* FA 1090, the amount of C14:0 was significantly decreased (1.5–3-fold) with a concomitant increase in C18:1 content compared to the other strains examined. Although Wolf-Watz *et al.* (1975) described multiple isolates of *N. gonorrhoeae* with high and low amounts of C18:1, this feature has not been previously reported for meningococcal isolates. In *E. coli*, the unsaturated fatty acids, palmitoleic acid (C16:1) and *cis*-vaccenic acid (C18:1), are synthesized by a branched pathway which diverges from that of the saturated fatty acids (Rock *et al.*, 1996). A 3-hydroxydecanoyl-ACP dehydrase encoded by *fabA* introduces a *trans* double

bond into the acyl chain of β -hydroxy-decanoyl-ACP, which is then isomerized to *cis*-3-decanoyl-ACP. This compound serves as the substrate for further elongation cycles catalysed by the β -ketoacyl-ACP synthetases (FabB and FabF) which extends the acyl chain to form palmitoleoyl-ACP (C16:1 Δ 9) and *cis*-vaccenoyl-ACP (C18:1 Δ 11). Therefore, in *E. coli*, C18:1 content increases at the expense of C16:1 primarily due to the activity of the temperature-regulated FabF (Rock *et al.*, 1996). However, we found the relative content of C16:1 did not vary significantly among the different meningococcal isolates with high or low amounts of C18:1, nor was the C18:1 content of meningococcal phospholipids temperature-regulated (data not shown). Instead, compared to the other neisserial phospholipids in this report, the phospholipids from *N. meningitidis* 6940 and *N. gonorrhoeae* FA 1090 appear to be increased in C18:1 at the expense of C14:0 and not at the expense of C16:1 as is the case for *E. coli*, suggesting that the enzymic activities of the meningococcal condensing enzymes will be different from those of *E. coli*.

The recent completion of the genome databases of *N. meningitidis* Z2491 (*N. meningitidis* sequencing group at the Sanger Center; <http://www.sanger.ac.uk>) and *N. gonorrhoeae* FA 1090 (Gonococcal Genome Sequencing Project; <http://www.genome.ou.edu>) has provided some insight into the genetic basis of the fatty acid biosynthesis pathway in these bacteria. The current model of fatty acid synthesis in *E. coli* requires two essential condensing enzymes (FabH and FabB), a β -ketoacyl reductase (FabG), two β -hydroxyacyl-ACP dehydrases (FabA and FabZ) and an enoyl-ACP reductase (FabI). Although homologues for these enzymes were found in the *N. meningitidis* Z2491 genome, only a single dehydrase homologue was present, presumably FabZ, since this enzyme is essential for the fatty acid elongation cycle. The absence of a FabA homologue in *Neisseria* spp. is not unique. A search for clusters of orthologous groups of proteins (COGs) in the complete genomes of 18 organisms (see Entrez genomes at <http://www.ncbi.nlm.nih.gov>) revealed that only three, *E. coli*, *Haemophilus influenzae* and *Bacillus subtilis*, contain two dehydrases. Six species, including *Rickettsia*, *Chlamydia*, *Synechocystis*, *Aquifex*, *Thermotoga* and *Helicobacter* species, contained a single *E. coli* dehydrase homologue, while the remainder did not contain any COGs related to the *E. coli* dehydrase. Therefore, it appears that the production of unsaturated fatty acids in these species, including *N. meningitidis* and *N. gonorrhoeae*, may proceed via different biochemical pathways to that of *E. coli*.

The variability of phospholipid structures suggests that outer-membrane biogenesis of meningococci and gonococci may differ from that of *E. coli*. As noted above, *E. coli* has one essential LPA acyl transferase, while at least two are found in *N. meningitidis*. The synthesis of lipid A is also essential for cell viability in *E. coli* (Nikaido, 1996; Raetz, 1996). In contrast, a meningococcal mutant has been reported to be without lipid A and remains viable (Steehgs *et al.*, 1998). Examination of the outer-

membrane profile of this meningococcal lipid-A-deficient mutant did not reveal any major alterations in outer-membrane protein expression, although the amount of PE containing shorter acyl chains was increased (Van der Ley *et al.*, 1998). Therefore, the ability of meningococci and gonococci to produce variable phospholipids containing short acyl chains may allow such mutants to functionally replace the absent lipid A and remain viable.

ACKNOWLEDGEMENTS

The work described in this report was supported by a grant from the National Institutes of Allergy and Infectious Diseases (PHS grant AI-33517 to D.S.S.) and by a grant from the Department of Energy (DE-FG09-93ER20097) to the Complex Carbohydrate Research Center.

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Received 6 March 2000; revised 11 May 2000; accepted 18 May 2000.