Investigation of the Structural Heterogeneity of Lipooligosaccharides from Pathogenic *Haemophilus* and *Neisseria* Species and of R-Type Lipopolysaccharides from *Salmonella typhimurium* by Electrospray Mass Spectrometry†

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Received 6 November 1992/Accepted 1 March 1993

Heterogeneity in the lipooligosaccharides (LOS) of pathogenic *Haemophilus* and *Neisseria* species is evident from the multiplicity of components observed with electrophoretic analyses. Knowledge of the precise structures that make up these diverse LOS molecules is clearly the key to reaching an understanding of pathogenic processes such as phase variation and molecular mimicry. Except for a few cases, little is known about the specific structural features of LOS that underlie phase variation and molecular mimicry, partly because of the inherent difficulties in the structural elucidation of these complex glycolipids. In the lipopolysaccharides (LPS) from *Salmonella typhimurium* and *Escherichia coli*, rough, or R-type, mutants have been isolated that have provided insight into the biosynthetic pathways and associated genetics that control LPS expression. Nonetheless, recent work has shown that these R-type LPS are more complex than originally thought, and significant heterogeneity is still observed, primarily in their phosphorylation states. In order to investigate the structures of LPS and LOS in a more rapid fashion, we have determined the precise molecular weights of LOS (and LPS) preparations from various *Haemophilus*, *Neisseria*, and *Salmonella* species by electrospray ionization-mass spectrometry. The LOS (or LPS) were first O-deacylated under mild hydrazine conditions to remove O-linked esters primarily from the lipid A portion. Under negative-ion conditions, the O-deacylated LOS yield abundant multiply deprotonated molecular ions, $(M - nH)^{n-}$, where n refers to the number of protons removed and therefore determines the absolute charge state, $n = z$. Mass spectra from different LOS and LPS preparations have provided detailed information concerning the structural basis for LOS (and LPS) heterogeneity and corresponding saccharide compositions. The identification of sialic acid in the LOS of *Haemophilus* and *Neisseria* species and the variable phosphorylation of the core of *S. typhimurium* LPS have afforded insights into the biosynthetic pathways used by these organisms. Information of this type is important for understanding the underlying genetic and environmental factors controlling LOS and LPS expression.

Lipopolysaccharides (LPS) are one of the major outer-membrane constituents of all gram-negative bacteria. These glycolipids play key roles in the biology of these organisms (40) and have been found to be important virulence factors in pathogenic species (6, 8, 11). LPS are made up of a largely conserved lipid A moiety and a variable polysaccharide built up of repeating saccharide units off a much smaller core oligosaccharide (8, 50). These two regions are linked together through one or more acidic sugars identified as 3-deoxy-D-manno-2-octulosonic acid (KDO). The sensitivity of the KDO glycosidic linkage to mild acid has been routinely exploited to prepare and isolate separate pools of lipid A and polysaccharide for structural analysis (49). Rarely, however, are structural studies of the intact LPS carried out, with the noted exception of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, which remains the primary analytical tool for LPS molecular weight determination. SDS-PAGE analyses of LPS from numerous bacterial species have shown them to be heterogeneous populations with ladderlike molecular weight distri-

butions approaching $\geq 100,000$, depending on the degree of polysaccharide polymerization (32, 50).

Despite this generalized characterization of LPS, a subpopulation of LPS molecules exists in any population of LPS from an enteric organism that lacks the O antigen (12). These unsubstituted, or core, glycolipids are more hydrophobic than complete LPS molecules, and mutant bacteria that lack the ability to attach the O polysaccharide to the core LPS aggregate in aqueous solutions to form rough suspensions (23). The importance of these core LPS populations in wild-type bacterial populations remains unclear. It may be that they are merely incomplete structural intermediates, but recent evidence suggests that they are products of alternative biosynthetic pathways (22). In support of this latter possibility is the recent appreciation that a number of nonenteric gram-negative bacteria make only R-type LPS, lacking the polysaccharide structures that have been used to commonly define LPS serotypes in enteric species. Our own work as well as that of others on the structures of LPS from pathogenic *Neisseria* (9, 10, 16, 51) and *Haemophilus* (13, 28, 37) species has clearly shown that these LPS lack the repeating polysaccharide O antigen and instead contain a more variable and branched core oligosaccharide region linked to a relatively invariant lipid A. Indeed, the term

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† Report no. 60 from the Centre for Immunochemistry, University of California, San Francisco.

lipooligosaccharides (LOS) has now been adopted by many researchers to emphasize these important structural differences. SDS-PAGE analyses of species that produce only LOS, such as *Neisseria gonorrhoeae* (14, 24) and *Haemophilus influenzae* (2, 15), also show marked heterogeneity, but not a simple ladderlike pattern as seen in the LPS of enteric bacteria. Instead, the LOS patterns are quite complex, yielding multiple bands ranging from M_r s of 2,000 to over 7,000 that do not correspond to any obviously consistent set difference in mass. It is likely that this heterogeneity enables pathogenic *Haemophilus* and *Neisseria* species to adapt to changing and hostile environments, perhaps through rapid phase variation of these LOS structures (1, 21, 47).

Structural analysis of the LPS derived from wild-type strains and genetic constructs has become essential to understanding the molecular basis of LPS and LOS biosynthesis. While sophisticated chemical and physical methods have been developed for the separate analyses of the lipid A (4, 19, 43) and oligosaccharide portions (9, 10, 39, 51), there has been a lack of more precise and sensitive tools for assaying structural heterogeneity in LOS preparations that have not first undergone acetic acid hydrolysis. Recently, we have shown that liquid secondary ion mass spectrometry (LSIMS) can be used to analyze LOS that have first been treated with hydrazine to remove O-linked fatty acids on the lipid A region followed by treatment with aqueous HF to remove phosphate and phosphate esters (25, 39). The removal of these groups makes the LOS amenable to mass spectrometric analysis, presumably by increasing their water solubility and decreasing their propensity to aggregate in solution (39).

In this report, we describe a more sensitive and general approach for the analysis of LOS (and LPS) in their O-deacylated forms by electrospray ionization-mass spectrometry (ESI-MS). This method requires only a few micrograms (and sometimes considerably less) of material for the final mass spectrometric analysis. These studies show that the LOS (and LPS) are often more heterogeneous than analyses of the lipid A and oligosaccharide portions have previously revealed. Furthermore, the molecular weight data obtained from these ESI-MS spectra have provided mass measurements with sufficient accuracy in most cases to establish preliminary monosaccharide compositions. These molecular weight data have allowed us to identify unique structural elements in these LOS preparations, such as acid-labile phosphate attached to KDO in the core regions of *H. influenzae* and *Haemophilus ducreyi*, and to define important biosynthetic pathways, such as the termination of lactosamine-containing oligosaccharide branches with sialic acid in both *Neisseria* and *Haemophilus* species.

MATERIALS AND METHODS

Materials. Anhydrous hydrazine was obtained from Sigma (St. Louis, Mo.), and aqueous HF (48%) was purchased from Mallinckrodt (Muskegon, Mich.). Water and acetone were obtained from Burdick and Jackson (Muskegon, Mich.). All other reagents and solvents used were of reagent grade. LPS from *Salmonella typhimurium* TV119 (Ra mutant) and SL1181 (Re mutant) were obtained from Sigma and analyzed for purity by composition analysis, methylation analysis, and separate mass spectrometric analyses of their corresponding lipid A and oligosaccharide moieties as previously described (39). LOS from *H. ducreyi* 35000 and *Neisseria meningitidis* B6275 were isolated by using a modified phenol water extraction procedure of Westphal and

Jahn (48). The LOS from *H. influenzae* 2019 and A2 were prepared by the procedure of Darveau and Hancock (5). LOS from *N. gonorrhoeae* FA5100 and F62 were a generous gift from H. Schneider (Walter Reed Army Institute of Reserve).

O-Deacylation of LOS. LOS were O-deacylated according to the procedure of Helander et al. (13). Briefly, 0.5 to 2 mg of LOS was incubated with 100 to 200 μ l of anhydrous hydrazine for 20 min at 37°C. The samples were cooled to -20°C, and 0.5 to 1 ml of chilled acetone was added dropwise to precipitate the O-deacylated LOS. The sample vials were then centrifuged at 12,000 $\times g$ for 20 min at 5°C. The supernatant was removed, and the pellet was washed again with cold acetone and centrifuged. The precipitated O-deacylated LOS was then resuspended in 500 μ l of water and lyophilized.

Purification of O-deacylated LOS. After acetone precipitation, O-deacylated LOS was in most cases pure enough to be analyzed directly by mass spectrometry. In some cases, however, gel filtration chromatography was carried out to provide a more purified final preparation. For gel filtration chromatography, typically 1 mg of O-deacylated LOS was dissolved in 100 μ l of column buffer consisting of 50 mM triethylamine acetate (pH $\approx$ 7). The sample was then loaded onto a Bio-Gel P-4 column (1.5 cm inside diameter by 25 cm; 400 mesh) and eluted isocratically at a flow rate of 0.4 ml/min with refractive index detection.

Mass spectrometric analysis. O-Deacylated LOS was analyzed by ESI-MS (7) with a VG Bio-Q mass spectrometer with an electrospray ion source. Scans were taken in the negative-ion mode over the m/z range of 300 to 2,000 at a resolution of $\sim$ 500. LOS samples were dissolved in distilled water at a final concentration of 1 to 3 μ g/ μ l, and 3- μ l aliquots were injected via a Rheodyne injector into a constant stream of H₂O-acetonitrile (1/1, vol/vol) containing 1% acetic acid or 0.1% triethylamine. A flow rate of 2 to 4 μ l/min was maintained throughout the analysis via an Isco syringe pump. Mass calibration was carried out with an external horse heart myoglobin reference by using the supplied VG Bio-Q software, and the instrument was tuned up in the negative-ion mode with sulfated cholecystokinin-8 (Peninsula Laboratories). Spectra were averaged over several scans and digitally smoothed with a 5- or 10-point smoothing routine. For relatively abundant peaks, the accuracy was $\pm$ 0.3 m/z , but accuracy was worse for the poorly defined peaks of lower abundance ($\pm$ 0.4 to 0.8 m/z). For calculating the predicted molecular weights of these LOS, the following interval average mass values were used: hexose, 162.1424; L- or D-glycero-D-manno-heptose, 192.1687; N-acetylhexosamine, 203.1950; KDO, 220.1791; sialic acid, 291.2579; phosphoethanolamine (PEA) 123.0483; phosphate, 79.9799. Average masses for the O-deacylated LOS were calculated by adding the appropriate interval mass values to the average molecular weight of the conserved diphosphoryl diacyl lipid A moiety containing two N-linked β -hydroxymyristic acids with an M_r of 953.0089.

RESULTS

R-type LPS from *S. typhimurium*. As part of our initial strategy, O-deacylated R-type LPS from *S. typhimurium* Ra and Re mutants were prepared from commercial sources. Because of their size and lack of a polysaccharide O antigen, these R-type LPS more closely resemble the LOS of *Neisseria* and *Haemophilus* species and were therefore considered appropriate standards in the initial phases of this work.

FIG. 1. Electrospray mass spectrum of *O*-deacylated LPS from *S. typhimurium* Re LPS. Structures for the different LOS species (A through C) are listed in Table 1.

In Fig. 1 and 2, the electrospray mass spectra of the *O*-deacylated *S. typhimurium* Re and Ra LPS are shown. In these two cases, the major deprotonated molecular ions are present in the doubly, $(M - 2H)^{2-}$; triply, $(M - 3H)^{3-}$; or quadruply, $(M - 4H)^{4-}$, charged states. These deprotonation states appear to be dependent on the number of strongly acidic unsubstituted phosphate esters on these molecules. In order to generate average M_r s for these species, the m/z values are first multiplied by the number of charges (z) and then the masses of the same number of protons are added. When more than one charge state is present, an average of the values is taken.

For example, *S. typhimurium* Re LPS has been reported to consist of a hexaacyl diphosphorylated lipid A attached to two KDOs (Table 1). After *O*-deacylation, the lipid A moiety

FIG. 2. Electrospray mass spectrum of *O*-deacylated LPS from *S. typhimurium* Ra LPS. Structures for the different LOS species (A and B) are listed in Table 1.

should contain only two N-linked β -hydroxymyristic acid chains as shown in Fig. 3, with an expected M_r of 1,393.4. As seen in Fig. 1, the electrospray spectrum of this Re LPS yielded a major doubly charged ion, $(M - 2H)^{2-}$, at m/z 791.6, and a much smaller singly charged peak, $(M - H)^{-}$ at m/z 1583.6, yielding an averaged M_r of 1584.9 ± 0.3 . This mass is ~ 192 Da larger than the expected mass value for Re LPS (calculated $M_r = 1,393.4$), indicating the additional presence of heptose ($+192.2$ Da). Composition analysis also indicated the presence of heptose (molar ratio of glucosamine to heptose, ≈ 1.7 to 1). Mass spectrometric analysis of the isolated oligosaccharide fraction after acetic acid hydrolysis (1% acetic acid, 2 h, 100°C) identified the major glycan as heptose-KDO ($[M + Na]^+ = 452.9$). This commercial R-type LPS should, therefore, be more correctly classified as an Rd₂ type mutant (42).

In addition to these two major peaks, smaller sets of peaks were also seen in the m/z 800 to 860 and 1,050 to 1,100 regions. The two largest peaks in the m/z 800 to 860 region, m/z 831.0 and 852.9, would nominally correspond to the addition of phosphate and PEA to the major doubly charged LOS peak at m/z 791.6 (M_r of 1584.9) under the assumption that they too were doubly charged peaks containing the expected diphosphoryl lipid A moiety (see Table 1). This interpretation is reasonable in light of the presence of heptose, which is known to be an acceptor for phosphate and PEA. Closer inspection of the peaks in the 1,050 to 1,100 region revealed them to be triply charged dimers of the major species, i.e., $(M_2 - 3H)^{3-}$. Such multiply charged dimers have only been observed when a dominant peak is encountered and relatively high concentrations of LOS (≥ 2 $\mu\text{g}/\mu\text{l}$) are used in the ESI-MS experiment.

The larger *S. typhimurium* Ra LPS gave an ESI-MS spectrum (Fig. 2) with two dominant triply charged peaks,

TABLE 1. Structures and molecular masses of the major *O*-deacylated LPS and LOS

LOS source (form)	Structure	Calculated M_r^a	Exptl M_r	Charge state ^b
(A) <i>S. typhimurium</i> Re (Rd ₂) LPS ^c	(KDO) ₂ → <i>O</i> -deacyl-lipid A	1,393.4	ND	
	Hep→(KDO) ₂ → <i>O</i> -deacyl-lipid A	1,585.5	1,584.9	2, 1 (A)
		1,665.5	1,664.0	2 (B)
		1,708.6	1,707.8	2 (C)
(B) <i>S. typhimurium</i> Ra LPS	$ \begin{array}{c} \text{Gal} \quad \text{P} \\ \downarrow \quad \downarrow \\ \text{GlcNAc} \rightarrow \text{Glc} \rightarrow \text{Gal} \rightarrow \text{Glc} \rightarrow \text{Hep} \rightarrow \text{Hep}(\text{KDO})_2 \rightarrow \text{O-deacyl-lipid A} \\ \uparrow \quad \uparrow \\ \text{Hep} \quad \text{P(PEA)}_{0-1} \end{array} $	2,981.6	2,979.9	3, 4 (A)
		3,104.6	3,101.8	3, 4 (B)
<i>N. gonorrhoeae</i> FA5100 ^d	$ \begin{array}{c} \text{KDO} \\ \downarrow \\ \text{Hep} \rightarrow \text{KDO} \rightarrow \text{O-deacyl-lipid A} \end{array} $	1,585.6	1,585.2	2 (A)
		1,665.5	1,664.6	2, 3 (B)
		1,708.6	1,708.0	2 (C)
		1,788.6	1,788.3	3 (D)
(D) <i>N. gonorrhoeae</i> F62	$ \begin{array}{c} \text{PEA} \quad \text{KDO} \\ \downarrow \quad \downarrow \\ (\text{GalNAc})_{0-1} \rightarrow \text{Gal} \rightarrow \text{GlcNAc} \rightarrow \text{Gal} \rightarrow \text{Glc} \rightarrow \text{Hep} \rightarrow \text{KDO} \rightarrow \text{O-deacyl-lipid A} \\ \uparrow \\ \text{GlcNAc} \rightarrow \text{Hep-PEA} \end{array} $	2,754.5	2,753.7	3 (A)
		2,916.6	2,916.3	3 (B)
		3,119.8	3,118.8	3 (C)
(E) <i>N. meningitidis</i> B 6275 ^d	$ \begin{array}{c} \text{KDO} \\ \downarrow \\ (\text{NeuNAc})_{0-1} \rightarrow \text{Gal} \rightarrow \text{GlcNAc} \rightarrow \text{Gal} \rightarrow \text{Glc} \rightarrow \text{Hep} \rightarrow \text{KDO} \rightarrow \text{O-deacyl-lipid A} \\ \uparrow \\ \text{GlcNAc} \rightarrow \text{Hep} \cdots (\text{PEA})_n \end{array} $	2,793.6		
		2,916.6	2,915.7	3 (A)
		3,084.8		
		3,207.9	3,208.0	3, 2 (B)
(F) <i>H. influenzae</i> 2019	$ \begin{array}{c} \text{Gal} \rightarrow \text{Glc} \rightarrow \text{Hep} \rightarrow \text{KDO(P)} \rightarrow \text{O-deacyl-lipid A} \\ \uparrow \\ \text{PEA-Hep} \\ \uparrow \\ \text{PEA-Hep} \end{array} $	2,237.9	2,238.9	3 (A)
		2,400.0	2,399.7	3, 2 (B)
		2,523.1	2,523.0	3 (C)
		2,601.3	2,601.6	3 (D)
		2,724.3	2,724.6	3 (E)
		2,886.5	2,888.7	3 (F)
(G) <i>H. influenzae</i> A2 ^e	$ \begin{array}{c} (\text{Hex})_x \rightarrow \text{Hep} \rightarrow \text{KDO(P)} \rightarrow \text{O-deacyl-lipid A} \\ \uparrow \\ (\text{NeuNAc})_{0-1}(\text{GlcNAc})_{0-1} \quad (\text{Hex})_y \rightarrow \text{Hep} \rightarrow \text{PEA} \\ \uparrow \\ \text{Hep} \end{array} $	2,277.0	2,277.9	3, 2 (A)
		2,439.1	2,438.4	3, 2 (B)
		2,601.3	2,600.8	3, 2 (C)
		2,724.3	2,723.1	3 (D)
		2,763.4	2,762.4	3 (E)
		2,925.6	2,925.9	3 (F)
		3,087.7	3,086.4	3 (G)
		3,249.9	~3,249	3 (H)
		3,257.9	3,256.2	3 (I)
(H) <i>H. ducreyi</i> 35000	$ \begin{array}{c} \text{Gal} \rightarrow \text{GlcNAc} \rightarrow \text{Gal} \rightarrow \text{Hep} \rightarrow \text{Glc} \rightarrow \text{Hep} \rightarrow \text{KDO(P)} \rightarrow \text{O-deacyl-lipid A} \\ \uparrow \\ (\text{PEA})_{0-1} \rightarrow \text{Hep} \\ \uparrow \\ \text{Hep} \end{array} $	2,549.3	2,548.8	3 (A)
		2,711.5	2,711.0	3, 2 (B)
		2,834.5	2,833.5	3 (C)
		2,914.7	2,913.9	3 (D)
		3,002.7	3,002.4	3, 2 (E)

^a The M_r s of the major predicted *O*-deacylated LOS forms are shown in boldface.^b Charge states refer to the $(M - nH)^+$ ions observed for each LOS form. Only those peaks $\geq 5\%$ of the base peak were used to calculate the molecular weights. The $n = 2$ charge state listed for *H. ducreyi* for component E was below this cutoff but still distinguishable from the background.^c Abbreviations: P, phosphate; Hep, heptose; Hex, galactose or glucose; NeuNAc, sialic acid. Note that structures are shown for both *S. typhimurium* Re LPS and the heptose-extended Rd₂ form.^d No information on the PEA content of the *N. gonorrhoeae* FA5100 and *N. meningitidis* B 6275 LOS was available.^e The precise structures of many of the *H. influenzae* A2 oligosaccharide moieties are not known ($x + y = 2$ to 8).

FIG. 3. Conserved structure of *O*-deacylated lipid A in *Salmonella* LPS and *Neisseria* and *Haemophilus* LOS. R represents the position of oligosaccharide substitution.

($M - 3H$)³⁻, at m/z 1,032.5 and 992.5, and somewhat less abundant quadruply charged peaks, ($M - 4H$)⁴⁻, at m/z 774.8 and 743.8. These peaks arise from two LPS components with M_r s of 3,101.8 and 2,979.9, respectively, differing within experimental error by the mass of PEA. This molecular weight is consistent with the expected mass of the Ra LOS as shown in Table 1, and no additional peaks were present in this spectrum that might arise if a third KDO or PEA-substituted KDO were present. A third KDO has been reported to exist in *Salmonella* Ra LPS, but in less than stoichiometric amounts (42). As expected from published structures of the Ra LPS, the PEA heterogeneity is presumably associated with the phosphate on the first inner-core heptose, existing either as a simple unsubstituted phosphate or as pyrophosphorylethanolamine. Although structural studies carried out on the oligosaccharide fraction of this LPS also showed the presence of a single PEA, we have previously observed two PEA moieties in other commercial preparations of *Salmonella* Ra LPS (18).

***N. gonorrhoeae* LOS.** The LOS from *N. gonorrhoeae* are likely to play important roles in the pathology of this sexually transmitted disease (11). Alterations in the LOS, or LOS from different gonococcal strains, have been shown to correlate with serum sensitivity and serum resistance (35, 44). The electrospray spectra of two gonococcal LOS, one from a genetic construct originally thought to contain only KDO and lipid A, strain FA5100 (36), and the serum-sensitive wild-type strain F62 (51), are shown in Fig. 4 and 5.

On the basis of partial LOS structure determined earlier for the *N. gonorrhoeae* FA5100 LOS (39), the *O*-deacylated form would be predicted to yield the same mass as that observed in the ESI-MS spectrum of *S. typhimurium* Rd₂ LPS (Table 1). Within experimental error, the ESI-MS spectrum shown in Fig. 4 does in fact show the largest peak to have the same doubly charged ion, ($M - 2H$)²⁻ at m/z 791.6, as seen in the *S. typhimurium* Re LOS spectrum, yielding a molecular weight of 1,585.2. Slightly less abundant peaks are also seen at m/z 782.5 and 853.0, corresponding to components with masses 18 Da smaller (M_r of 1,567.0) or 123 Da larger (M_r of 1,708.0) than the major LOS component. The lower mass peak is likely to originate from the loss of water from one of the two KDOs and has been previously observed in LSIMS spectra (17). The lack of such an anhydro form in the isobaric *S. typhimurium* Re LPS suggests that there may be a difference in the linkage between the KDOs that makes the gonococcal LOS more susceptible to undergo the loss of water. The presence of an LOS species 123 Da higher in mass than that expected for the gonococcal FA5100 LOS is almost certainly due to the presence of a PEA moiety. Previous LSIMS analysis of this same *O*-deacylated LOS did not detect the mass for this PEA-containing species, but given the low signal abundance for even the major LOS species in the LSIMS spectrum, it is

FIG. 4. Electrospray mass spectrum of *O*-deacylated LOS from *N. gonorrhoeae* FA5100. Insets for the m/z regions 525 through 625 and 1,025 through 1,175 are scaled up five times. Structures for the different LOS species (A through D) are listed in Table 1.

FIG. 5. Partial electrospray mass spectrum (m/z 900 through 1,100) of *O*-deacylated LOS from *N. gonorrhoeae* F62. Structures for the different LOS species (A through C) are listed in Table 1.

unlikely that this minor PEA-containing species would have been observed above the background (17). The peak at m/z 830.8 would correspond to phosphate addition to the major LOS species. In support of this interpretation, two small peaks at m/z 554.2 and 595.1 were also observed, the former of which corresponds to the triply charged state of the same component that gave the doubly charged peak at m/z 830.8, yielding an average M_r of 1,664.6. The peak at m/z 595.1, if it too is a triply charged peak, would yield an LOS with an M_r of 1,788.3, a value consistent with an LOS containing an additional PEA as well as phosphate (calculated M_r = 1,788.6). These higher charged states are only present for the two species containing additional phosphate groups, which would be expected to be capable of stabilizing higher charged (or deprotonation) states. Therefore, in addition to the major species heptose-KDO₂-*O*-deacyl-lipid A, three additional phosphorylated species could be assigned, containing an additional phosphate, PEA, or phosphate plus PEA (or pyrophosphorylethanolamine).

Like the ESI-MS spectrum of the *S. typhimurium* Re LPS, a set of peaks were also seen in the m/z 1,050 to 1,150 region, but these were considerably more complex. This increased complexity arises from the formation of both homo- and heterodimers of the three major LOS species as their triply charged ions, i.e., $(M_A M_A - 3H)^{3-}$, $(M_A M_B - 3H)^{3-}$, $(M_A M_C - 3H)^{3-}$, $(M_B M_B - 3H)^{3-}$, etc. These dimeric species were not observed in any other LOS preparations except the *S. typhimurium* Re LPS, and their presence in these two isolates is probably dependent on the greater signal abundance and dominance of one major LOS component.

The ESI-MS spectrum of the LOS from *N. gonorrhoeae* F62 shown in Fig. 5 represents the first of the wild-type strains and, not too surprisingly, gives a more complex

FIG. 6. Partial electrospray mass spectrum of *N. meningitidis* type B strain 6275. Structures for the two major LOS species (A and B) are listed in Table 1. Higher charge states for these two LOS were observed only for the more abundant sialylated species: $(M - 4H)^{4-}$ = 801.4 (not shown).

spectrum than the previous mutant FA5100 strain. In this strain, the *O*-deacylated LOS gave two major peaks at m/z 971.1 and 1,038.6, representing the triply charged deprotonated ions, $(M - 3H)^{3-}$, of LOS species with M_r s of 2,916.3 and 3,118.8, respectively. The mass difference between these two species corresponds to a single *N*-acetylhexosamine (Δm ~203 Da) and agrees with the dephosphorylated oligosaccharide structures previously reported for this strain by Yamasaki et al. (51), but the electrospray spectrum clearly shows the presence of two PEA moieties. Next to each of these two major peaks are two pairs of triply charged peaks at m/z 1,033.0 and 997.9 and m/z 965.5 and 930.3 that correspond to LOS components that are 18 Da ($-H_2O$) and 123 Da ($-PEA$) smaller in mass. The species with components that are 123 Da smaller in mass indicate that there are some LOS that contain only one of the two PEA groups. Last, two small peaks can be seen at m/z 916.9 and 911.0, which represent a third LOS species with an M_r of 2,753.7 and its anhydro counterpart with an M_r of 2,736.0. This third LOS is almost certainly a truncated analog of the LOS with an M_r of 2,916.3, containing one less hexose residue, and has been confirmed in studies of the oligosaccharide fraction from *N. gonorrhoeae* F62 LOS (17).

Sialylation of *N. meningitidis* LOS. Endogenous sialylation of *N. meningitidis* LOS has been noted recently (25) and is evident in the ESI-MS spectrum of strain B6275 as shown in Fig. 6. In this spectrum, a major peak is seen at m/z 1,067.8 (triply charged), with its smaller quadruply charged counterpart at m/z 801.4 (not shown). This yields an averaged M_r of 3,208.0 and is in agreement with the molecular weight of the sialylated LOS previously determined for the HF-treated component (25), but with the addition of two (lipid A) phosphates and two PEA moieties. The PEA moieties are presumably on the oligosaccharide portion, although definitive evidence has not yet been obtained from analysis of either the oligosaccharide or lipid A fractions. In addition to these major ions, a smaller peak is seen at m/z 970.9 which represents the triply charged ion of the nonsialylated counterpart, M_r of 2,915.7. On the basis of the relative peak abundances of the sialylated and asialo LOS species, the level of sialylation in this meningococcal strain is ≈ 85 mol%, considerably larger than that determined by weight (15.3 μg of sialic acid per mg of LOS, or ≈ 20 mol%) (25).

FIG. 7. Electrospray mass spectrum of *O*-deacylated LOS from *H. influenzae* nontypeable strain 2019 (A) and type b strain A2 (B). The peak at 1,084.8 is actually a poorly resolved doublet, containing a smaller peak at m/z ~1,082. The peak at m/z 1,142 labeled CCK is cholecystokinin-8, a sulfated peptide used as an internal standard (see Materials and Methods). Structures for the different LOS species are listed in Table 1.

***H. influenzae* A2 and 2019.** *H. influenzae* is primarily a respiratory pathogen, causing a host of infections in humans, including pneumonia, inner-ear infections, and meningitis (30, 46). Several *H. influenzae* strains were examined by electrospray mass spectrometry, and the ESI-MS spectra of two of them, nontypeable strain 2019 and type b strain A2, are shown in Fig. 7. In the ESI-MS spectrum of *H. influenzae* 2019 (Fig. 7A), two major peaks dominate at m/z 799.0 and 1,198.7 and belong to an LOS with an M_r of 2,399.7 as the triply and doubly charged molecular ions, respectively. This molecular weight is consistent with the structure of the lactose-containing hexasaccharide previously identified from the acid-released oligosaccharide fraction, i.e., $\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow (\text{Hep}\alpha 1 \rightarrow 2\text{Hep}\alpha 1 \rightarrow 3)4\text{Hep}\alpha 1 \rightarrow 5\text{KDO}$, containing

a phosphate on KDO and two PEA moieties (37). In fact, an earlier ESI-MS spectrum of this LOS preparation taken in the positive-ion mode provided the first clear evidence for a phosphate on KDO (37). This modified KDO was first identified as its anhydro form in the oligosaccharide fraction but was suspected of containing a 4-linked phosphate that underwent β -elimination during the mild acid hydrolysis step used to liberate the oligosaccharide from the lipid A (37, 39). In addition to this expected LOS species, there is evidence in this ESI-MS spectrum for at least five other triply charged LOS components with M_r s of 2,238.9 (m/z 745.3), 2,523.0 (m/z 840.0), 2,601.6 (m/z 866.2), 2,724.6 (m/z 907.2), and 2,888.7 (m/z 961.9). By mass difference, these latter five LOS species differ from the major LOS by a single hexose

FIG. 8. Electrospray mass spectrum of *O*-deacylated LOS from *H. ducreyi* 35000. Structures for the different LOS species (A through E) are listed in Table 1.

truncation (calculated $M_r = 2,237.9$), addition of PEA (calculated $M_r = 2,523.1$), addition of two or three hexoses (calculated $M_r = 2,724.3$ or $2,886.5$), or addition of two hexoses and loss of one PEA (calculated $M_r = 2,601.3$).

In the corresponding ESI-MS spectrum of *H. influenzae* A2 (Fig. 7B), a much more complex set of peaks is seen that represents a similar set of lower-mass components, as well as a unique series of larger ones. For example, the peak at m/z 906.7 (triply charged ion for M_r of 2,723.1) and the pair at m/z 866.1 and 1,299.2 (triply and doubly charged ions for M_r of 2,600.8), yield molecular weights of LOS that are within experimental error identical to two of the six components seen in the ESI-MS spectrum of *H. influenzae* 2019. Preliminary analyses of the oligosaccharide fraction revealed these two LOS components may represent different but isobaric structures (Table 1). Evidently, the LOS from the typeable A2 strain has a different branch structure than the nontypeable 2019 strain and is capable of making a series of hexose-extended analogs. These extended LOS species are evident from the sequential addition of 54 m/z (or 162 Da; Δm hexose) in this triply charged region to the base peak at m/z 866.1, yielding the ion series m/z 919.8, 974.3, 1,027.8, and 1,082, for a total of eight hexoses on the intact LOS. The peak at m/z 906.7 provides evidence for a second PEA group attached to the major LOS which had not been observed in previous LSIMS analyses of the oligosaccharide region (38). The PEA is also present in small amounts in the other hexose-extended (or truncated) LOS species as well but is less obvious because of the lower relative signal abundances. Perhaps most significant of all is the presence of a minor species at m/z 1,084.4 that would nominally correspond to an LOS containing, in addition to five hexoses, a single *N*-acetylhexosamine and sialic acid. The sialic acid

content of *H. influenzae* A2 LOS has been reported to be 0.4% by weight (27), but prior to these data there had been no convincing evidence that sialic acid was located on any specific LOS species.

***H. ducreyi* LOS.** *H. ducreyi* is a genital pathogen that can cause chancroid, a genital ulcer disease (29). The LOS from this organism can itself cause skin lesion formation when tested in the rabbit dermal model (3, 45), and differences in LOS composition have been linked to differences in the virulence of various strains (33). The *O*-deacylated LOS from one such strain, *H. ducreyi* 35000, is shown in Fig. 8. This ESI-MS spectrum contains almost all of the elements of heterogeneity that have been observed in the previous LOS and LPS preparations. Although there is a single dominant peak at m/z 902.9, there is also a series of extended and truncated analogs, one of which is sialylated. Indeed, it was the unexpected peak at m/z 999.8 that provided the first evidence that *H. ducreyi* LOS contained sialic acid. This spectrum, like the ESI-MS spectra of *H. influenzae* A2 and 2019, also provided evidence for the additional phosphate group on KDO, which is lost during the acid hydrolysis step used to isolate the oligosaccharide portion. Overall, this ESI-MS spectrum contains considerable information about the structure of these LOS. First, the base peaks at m/z 902.9 can be assigned as the triply charged ion for the previously identified major oligosaccharide component (see Table 1) (28). From there, it is relatively easy to assign the m/z 848.6 ion as containing one less hexose and the three higher-mass peaks at m/z 943.5, 970.3, and 999.8 as containing an additional PEA, *N*-acetylhexosamine, or sialic acid, respectively. It is worth noting that sialylated counterparts are not present for any of the extended or truncated species, clearly implying that the position of sialylation is on the terminal

galactose of this major lactosamine-containing LOS as shown in Table 1. In the double-charged region, peaks are seen that correspond to only the most abundant LOS species at m/z 1,354.1 and, to a much lesser extent, its sialylated counterpart at m/z 1,499.7.

DISCUSSION

Phosphorylation heterogeneity of *Salmonella typhimurium* R-type LPS. The ESI-MS spectra of the two commercial R-type LPS showed these preparations to be heterogeneous or incorrectly assigned. The Ra and Re LPS, for example, were both heterogeneous in their PEA content, containing either one or no PEA groups, an observation that we have previously made when employing oligosaccharides derived from Ra LPS as structural standards in oligosaccharide analyses (39). The so-called Re LPS was found to contain heptose and therefore is more correctly classified as an Rd₂-type LPS, although small amounts (<5%) may also contain PEA or phosphate. These negatively charged phosphate groups are likely to exert a significant effect on membrane permeability (31). Phosphate and PEA have already been implicated in conferring resistance to detergent and hydrophobic compounds, possibly by cross-linking LPS through divalent cations or polyamines or through salt bridges to positively charged groups on proteins or other LPS (34). In the case of the Re (or Rd₂) LPS preparation, the data clearly show that not all of the components are consistent with LPS that are thought to be associated with a deep-rough phenotype, i.e., free of phosphate.

Acid-labile phosphate in *Haemophilus* LOS. In our earlier investigations of the structures of the oligosaccharides from *H. influenzae* (37, 39) and *H. ducreyi* LOS (28), we encountered a reducing terminal anhydro KDO as a constant structural feature. However, HF treatment of the LOS prior to mild acid hydrolysis yielded oligosaccharides that contained unmodified KDO. Because Helander et al. (13) had previously reported the presence of phosphate at the 4 and 5 positions of KDO in a constructed mutant strain of *H. influenzae*, I-69 Rd⁻/b⁺, it seemed likely that a similarly linked phosphate on KDO was present in these wild-type strains prior to mild acid hydrolysis. The ESI-MS spectra of the *O*-deacylated LOS from both *H. influenzae* A2 and 2019, as well as that from *H. ducreyi* 35000, all gave molecular weights that showed them to contain a third phosphate in addition to the two phosphates already shown to exist on the lipid A portion (13, 28). So far, all *Haemophilus* strains examined by ESI-MS support the presence of a third phosphate, suggesting that it is likely to be an invariant feature of the LOS present in these bacteria. The biological importance of this phosphate is unknown, but given its location between the lipid A and the inner-core heptose region, it may exert an effect on LPS-LPS or LPS-protein interactions or possibly influence the conformation of the lipid moiety, which has at least one of its two phosphate groups in close proximity.

Sialylation of *Neisseria* and *Haemophilus* LOS. Previously, we reported on the sialylation of several meningococcal and gonococcal strains (25–27), an alteration that is likely to have important implications in the organism's ability to evade lysis by human serum (35, 44) or killing by human neutrophils (6, 20, 41). In one of these studies (25), an LSIMS spectrum of *N. meningitidis* B6275 was shown that was obtained after the LOS had first been dephosphorylated with aqueous HF and *O*-deacylated by mild hydrazine. This LSIMS spectrum showed the presence of an additional LOS component 291.1 Da larger in mass, (M – H)⁻, at m/z

2,799.2, than the expected molecular ion at m/z 2,508.1, consistent with the addition of sialic acid. Nevertheless, the signal abundances for the sialylated and asialo LOS species of *N. meningitidis* B 6275 were low and yielded molecular ions only after both *O*-deacylation and dephosphorylation (25). In contrast, the electrospray spectrum of the same *O*-deacylated LOS produced much more abundant and well-defined peaks for both the sialylated and nonsialylated LOS species. The ESI-MS spectrum also suggested a much higher level of sialylation (~85%) than that previously estimated from the LSIMS spectrum of the HF-treated LOS (~50%) or that based on the sialic acid content of LOS, i.e., 15.3 µg of sialic acid per mg of LOS, or ~20% (25). This is not entirely unexpected, because HF treatment used in the LSIMS analysis would cleave some portion of the acid-sensitive sialic acid glycosidic bonds and any measurement of sialic acid content based on the weight of LOS would also be expected to provide a potentially low estimate because of the presence of any impurities (e.g., salts) in the LOS preparation.

Sialylation has also been reported for *H. influenzae* and *H. ducreyi* LOS. We have found, for example, that *H. influenzae* A2 LOS contains 0.4% sialic acid by weight (or 3 to 5 mol% ratio) (27), but no structural data on an intact LOS species had been found to support this observation. The ESI-MS data from this LOS preparation, if they stand up to further experimental scrutiny, represent the first structural evidence based on an LOS species for the presence of sialic acid. Furthermore, the ESI-MS spectrum of *H. influenzae* A2 LOS provides a rough estimate of the sialylated LOS population (~4% mole ratio on the basis of relative peak areas) that is consistent with the previous weight estimate (27). The data on *H. influenzae* A2 also indicated a branching of the biosynthetic pathway, one path leading to the formation of lactosamine, which is terminated by sialylation, the other leading to a further extension of an all-hexose branch. Preliminary data have since been obtained that indicate the assignments of these crucial peaks in the *H. influenzae* A2 (as well as *H. ducreyi* 35000) LOS spectra are indeed correct, because treatment with neuraminidase removes the peaks corresponding to the sialylated LOS and shifts their observed molecular weights 291 Da lower (38).

Implications of the ESI-MS methodology. A mass spectrometric method for precise molecular weight determination of LOS mixtures from gram-negative bacteria has been presented. This method complements existing physical and chemical methods for the analysis of lipid A and oligosaccharides derived from LOS and R-type LPS. In terms of both mass accuracy and resolution, it is clearly a major improvement over the SDS-PAGE techniques employed for molecular weight analysis of LOS. ESI-MS analysis of the *O*-deacylated LOS provides an overall mass constraint on a relatively intact form, therefore allowing preliminary composition assignments to be made as well as identification of acid-labile moieties, such as sialic acid and phosphate linked to KDO.

In the cases studied here, *O*-deacylated LOS from a diverse set of bacterial sources have all yielded abundant deprotonated molecular ions under ESI-MS conditions. The most abundant charge states in virtually all cases were those that reflected the solution charge state under acidic conditions (pH ≈ 2 to 3). This generalization appears to be true whether or not the LOS is ionized under acidic or basic conditions and closely parallels the number of highly acidic unsubstituted phosphate groups. Because all of the LOS (and LPS) examined in this study appeared to contain

exclusively diphosphoryl lipid A, the major charge states observed in the negative-ion mode were at least the doubly deprotonated form $(M - 2H)^{2-}$. Additional phosphates on the oligosaccharide portion inevitably shifted the observed deprotonated molecular ions to the triply and quadruply charged states, as would be expected from the presence of additional sites for negative charge stabilization. Indeed, the shift to higher charge states in itself provided important information about the degree of phosphorylation of the LOS species. Although some LOS are capable of ionization in the positive-ion mode through the presence of primary amines such as a PEA group or groups on the oligosaccharide portions (37), the lack of more than one such site or absence of PEA groups altogether in many cases supports the use of negative-ion ESI-MS as the method of choice for routine LOS analyses. Although we have not attempted to use the ESI-MS spectra in any real quantitative sense, the ion abundances of the different LOS are in broad and often quite good agreement with the LOS populations expected from detailed analyses of the separated oligosaccharide moieties. Therefore, the ESI-MS data are likely to be a reasonably good estimate of differing LOS populations and can be used to help understand the molecular basis of differential and variable LOS expression.

This ESI-MS-based technique has also provided evidence showing many LOS preparations to be more heterogeneous in the oligosaccharide portion than might have been predicted on the basis of characterization of the component oligosaccharides alone. This was particularly true of the *Haemophilus* LOS species that had undergone previous and extensive oligosaccharide analyses. In many cases, a truncated or extended LOS analog was not seen in the oligosaccharide analysis, and this information could be of value in directing oligosaccharide characterization studies. The information contained in these spectra also has important ramifications in understanding LOS (and LPS) biosynthesis because it provides much more meaningful and interpretable data on probable sugar compositions and, therefore, a clearer picture of the biosynthetic pathways utilized by these organisms.

ACKNOWLEDGMENTS

We thank M. Tullius and D. Tang for technical assistance.

This work was supported by grants from the University of California Universitywide AIDS Program, R91SF243 (B. Gibson); and National Institutes of Health grants AI 21620 (M. Griffiss), AI 30006 (A. Campagnari), and AI 24616 and AI 18384 (M. Apicella). We also acknowledge the Mass Spectrometry Facility through grants from the National Center for Research Resources (RR 01614; A. Burlingame, Director) and NIH (ES04705).

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