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Structural Studies of Lipooligosaccharides from *Haemophilus ducreyi* ITM 5535, ITM 3147, and a Fresh Clinical Isolate, ACY1: Evidence for Intrastrain Heterogeneity with the Production of Mutually Exclusive Sialylated or Elongated Glycoforms

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The structures of the lipooligosaccharides (LOSs) from *Haemophilus ducreyi* ITM 5535 and ITM 3147 and a fresh clinical isolate, ACY1, have been investigated. Oligosaccharides were obtained from phenol-water-extracted LOS by mild acid hydrolysis and were studied by methylation analysis, fast atom bombardment and electrospray ionization mass spectrometry, and nuclear magnetic resonance spectroscopy. The major oligosaccharide obtained from all strains was a nonasaccharide with the structure β-D-Gal*p*-(1→4)-β-D-GlcNA*c*p-(1→3)-β-D-Gal*p*-(1→4)-D-α-D-Hep*p*-(1→6)-β-D-Glc*p*-(1→[L-α-D-Hep*p*-(1→2)-L-α-D-Hep*p*-(1→3)]4)-L-α-D-Hep*p*-Kdo (Kdo stands for 3-deoxy-D-*manno*-octulosonic acid) and is thus identical to that identified as the major oligosaccharide in *H. ducreyi* ITM 2665 (E. K. H. Schweda, A. C. Sundström, L. M. Eriksson, J. A. Jonasson, and A. A. Lindberg, J. Biol. Chem. 269:12040–12048, 1994). Electrospray ionization mass spectrometry on O-deacylated LOS from *H. ducreyi* ITM 5535 obtained after treatment with anhydrous hydrazine gave evidence for the presence of a sialylated major compound, Neu5Acα(2→3)-β-D-Gal*p*-(1→4)-β-D-GlcNA*c*p-(1→3)-β-D-Gal*p*-(1→4)-D-α-D-Hep*p*-(1→6)-β-D-Glc*p*-(1→[L-α-D-Hep*p*-(1→2)-L-α-D-Hep*p*-(1→3)]4)-L-α-D-Hep*p*-Kdo(P)-O-deacylated lipid A (Neu5Ac stands for *N*-acetylneuraminic acid). However, an even larger oligosaccharide could be isolated from all strains as a minor component, viz., the undecasaccharide β-D-Gal*p*-(1→4)-β-D-GlcNA*c*p-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNA*c*p-(1→3)-β-D-Gal*p*-(1→4)-D-α-D-Hep*p*-(1→6)-β-D-Glc*p*-(1→[L-α-D-Hep*p*-(1→2)-L-α-D-Hep*p*-(1→3)]4)-L-α-D-Hep*p*-Kdo, which represents an *N*-acetyl lactosamine disaccharide unit elongation of the LOS outer core. No sialylation of this latter minor component undecasaccharide was detected.

Although *Haemophilus ducreyi* outer membrane lipooligosaccharide (LOS) has been shown to cause extensive tissue necrosis if injected into animals (1), its role in the pathogenesis of chancroid is not clearly understood. When extracted from the bacterium, it may, like the analogous structures from other gram-negative microorganisms, be split by mild acid hydrolysis into a lipid A part and an oligosaccharide part. The lipid A part has been shown to be identical to that of *Haemophilus influenzae* and is composed of a glucosamine disaccharide bearing four 3-hydroxymyristic acids, two myristic acids, and two phosphate groups (16). Lipid A entails the endotoxic activity involved in the pathogenesis of fever, inflammation, and related events. The relative importance of lipid A in subacute or chronic disease remains to be settled. The oligosaccharide part is presumed to be atoxic, variable, and nonessential for bacterial survival in vitro (except for 3-deoxy-D-*manno*-octulosonic acid [Kdo]), yet it is closely related to the virulence of the organism. The few structures that have been determined all show the typical constellation of three heptoses linked to Kdo (found as the inner core for all different LOS structures identified so far for *H. influenzae* and *H. ducreyi*) to which a variable oligosaccharide outer core, mostly consisting of hexoses (11, 21), is attached. In this context, we have described two structures for *H. ducreyi*, viz., ITM 4747 LOS and ITM 2665 LOS (21).

The major LOS component from strain ITM 4747 is shown below:

β-D-Gal*p*-1→4-β-D-Glc*p*-1→4-L-α-DHep*p*-Kdo(P)-lipid A
3
↑
L-α-D-Hep*p*-1→2-L-α-D-Hep*p*-1

The lactosyl disaccharide outer core of ITM 4747 LOS has also been observed for *H. influenzae* 2019 (15). The only recognized difference between the two LOSs is phosphoethanolamine groups linked to two heptoses of the inner core region of *H. influenzae* 2019 LOS (21).

Strain ITM 2665 displayed a pentasaccharide branch as the outer core of its major LOS component: β-D-Gal*p*-1→4-β-D-GlcNA*c*p-1→3-β-D-Gal*p*-1→4-D-α-D-Hep*p*-1→6-β-D-Glc*p*-1→. The terminal trisaccharide is present both in *Neisseria gonorrhoeae* LOS and on the surface of human cells (in the lactoneo series of gangliosides) and would therefore, like the lactosyl disaccharide of ITM 4747 LOS (a structure that is often present in mammalian cell surface glycoproteins), qualify as molecular mimicry (10).

Melaugh et al. (11) have presented preliminary data suggest-

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ing a nonasaccharide LOS (excluding lipid A) for *H. ducreyi* 35000, similar to that of ITM 2665, although the reported phosphorylation patterns were different. Recently, a minor LOS component for the same strain was described with a further tentative β-D-Gal*p*-1→4-β-D-GlcNAc*p*1→ (*N*-acetyl lactosamine) disaccharide unit elongation of the outer core (12). Truncated forms were also found. The significance of this is unknown.

Unlike the case for *H. influenzae* and *N. gonorrhoeae*, high-frequency genetic structural (phase) variation of LOS has not been established for *H. ducreyi*. In fact, evidence to date suggests that *H. ducreyi* LOS does not undergo the phase variation seen for *H. influenzae* and *N. gonorrhoeae* LOSs. However, the question arises whether strains like ITM 4747 (representing a hexasaccharide LOS) can also express a larger LOS structure, e.g., like the one of ITM 2665, and vice versa.

In further analogy with *Neisseria* species, sialic acid (*N*-acetylneuraminic acid [Neu5Ac]) substitution has been demonstrated in *H. ducreyi* LOS (7). This is thought to modulate the immunogenic potential. Sialylation of *H. ducreyi* and *N. gonorrhoeae* LOSs results in an increase in the resistance to the bactericidal activity of normal human serum (13, 14). In both *Haemophilus* and *Neisseria* species, the sialic acid unit is linked to a terminal galactose residue in the outer core (7). Not only endogenous but also environmentally imposed sialylation of LOS has been demonstrated in *N. gonorrhoeae*. If Neu5Ac CMP (CMP-NANA), the substrate of gonococcal sialyltransferase, is provided in the growth medium, the organism may or may not sialylate the LOS, depending on whether the LOS synthesized is a competent acceptor structure (24). van Putten (24) showed that sialylation of gonococcal LOS has a marked inhibitory effect on epithelial cell invasion. He was therefore led to speculate that structural phase variation of the LOS may induce "a reversible switching between an invasive, barely sialylated phenotype that is susceptible to complement-mediated killing and a non-invasive highly sialylated phenotype that is not killed by antibodies and complement" (24). The mechanism seems feasible also for *H. ducreyi* LOS. However, the need for structural phase variation might well be influenced by endogenous production of CMP-NANA.

Because of the molecular heterogeneity caused by phase variation and variable substitutions like phosphorylation, acetylation, and sialylation, the determination of the native structure of LOS has proved to be a difficult problem in the *Haemophilus* group of bacteria. In this study, we have characterized, for three strains, the most commonly found *H. ducreyi* oligosaccharide structures as seen by nuclear magnetic resonance (NMR) combined with fast atom bombardment mass spectrometry (FAB-MS), electrospray ionization mass spectrometry (ESI-MS), and methylation analysis.

MATERIALS AND METHODS

Bacteria and cultivation. *H. ducreyi* ITM 5535 and ITM 3147, kindly supplied by P. Piot and E. Roggen, Antwerp, Belgium, and the fresh clinical isolate ACY1 (from A. C. Yemane, Stockholm, Sweden) were cultured in large petri dishes on a medium containing Columbia agar base (BBL Microbiology Systems), 5% fetal calf serum, 15% sheep blood, 3 mg of vancomycin per liter, and 1.5% IsoVitaleX. The plates were incubated in anaerobic jars containing GasPak (BBL Microbiology Systems) at 34°C. After 48 h of incubation, the bacterial colonies were harvested, suspended in Luria broth, and frozen at −70°C. LOS was extracted from lyophilized bacteria by the phenol-water method (25).

Isolation of oligosaccharide preparations from ITM 5535, ITM 3147, and ACY1. Oligosaccharide material was obtained after mild hydrolysis (1% acetic acid, 100°C, 2 h) of LOS (132 mg from ITM 5535, 67 mg from ITM 3147, and 200 mg from ACY1). The insoluble lipid A (30, 14.4, and 78 mg, respectively) was separated from the hydrolysis mixture by centrifugation. The water-soluble part (85, 53, and 97 mg, respectively) was freeze-dried, reduced with sodium borodeuteride (10 mg/ml) overnight, and purified by gel permeation chromatography on a Bio-Gel P4 column (2.5 by 90 cm) with pyridinium acetate buffer (0.1 mM, pH 5) as the eluant. Fractions were monitored with a differential refractometer (R403; Waters-Millipore Corporation, Bedford, Mass.). From ITM 5535, one oligosaccharide fraction, OS 5535 (11 mg), was obtained. From ITM 3147, we isolated fractions OS 3-1, OS 3-2, OS 3-3, OS 3-4, and OS 3-5 (2, 10, 2, 2, and 2 mg, respectively). From ACY1, we isolated fractions OS A-1, OS A-2, OS A-3, and OS A-4 (5, 32, 10, and 9 mg, respectively).

O-deacylation of LOS from *H. ducreyi* ITM 5535. LOS (40 mg) was incubated with 2.5 ml of anhydrous hydrazine for 30 min at 37°C. The sample was cooled to −20°C, and acetone (2.5 ml, −20°C) was added to precipitate the O-deacylated LOS. After centrifugation, the supernatant was removed and the pellet was washed again with cold acetone and centrifuged again. The precipitate was then resuspended in water and lyophilized to give O-deacylated LOS (24 mg).

NMR spectroscopy. NMR spectra of solutions in deuterium oxide were recorded at 40°C. Chemical shifts are reported in parts per million, with internal sodium 3-trimethylsilylpropanoate-d_4 (δ 0.000, ^{1}H) and external 1,4-dioxane (δ 67.40, ^{13}C) as the references.

Analytical and spectroscopic methods. For gas-liquid chromatography (GLC), a Hewlett-Packard 5890 instrument fitted with a flame ionization detector was used. Separation of alditol acetates and partially methylated alditol acetates was performed on DB-5 and DB-1 fused-silica capillary columns, respectively, with a temperature gradient of 160 (2 min) to 220°C at 4°/min. GLC-MS was performed with a Hewlett-Packard 5890 gas chromatograph equipped with an Hewlett-Packard 5970 electron impact mass spectrometer, using the above conditions. FAB mass spectra were recorded on a NERMAG R10-10L quadrupole instrument. Ions were produced from a matrix of triethanolamine. O-deacylated LOS from *H. ducreyi* ITM 5535 was analyzed by ESI-MS on a VG/Fisons Quattro II quadrupole mass spectrometer in the negative mode. The sample (1.3 mg) was first dissolved in 1 ml of a mixture of water-acetonitrile-methanol (4:1:1) with 0.1% ammonia solution added. Ten microliters of this solution was injected via a syringe pump into a running solvent of H_2O-CH_3CN (1:1) at a flow rate of 50 μl/min. A general approach to the analysis of electrospray data is as follows: during the ionization process, a molecular species of mass M may produce a series of ion species, $(M - nH)^{n-}$, of mass M − nH, that vary in their degree of deprotonation. ESI-MS of the molecular species (negative-ion mode) will yield a series of peaks with mass/charge (*m*/*z*) ratios of $(M - nH)^{n-}/n$. After identification of an ion series pair of charge of n_1 and $n_2 = n_1 + 1$ with corresponding *m*/*z* ratios of $(M - n_1H)^{n1-}/n_1$ and $(M - n_2H)^{n2-}/n_2$, respectively, mass M and charge state n_1 can be determined. The evaluation of M was done according to this by Fisons software.

Other analytical procedures. Sugars were identified as their alditol acetates (18) by using authentic standards. Methylation analysis was performed essentially as described by Levery and Hakomori (8). The relative proportions of the various alditol acetates and permethylated alditol acetates obtained in sugar and methylation analyses discussed below correspond to the detector response of the GLC-MS. Phosphorus analysis was performed by the method of Chen et al. (4).

RESULTS AND DISCUSSION

Structure determination. *H. ducreyi* ITM 5535, ITM 3147, and ACY1 LOS were extracted from lyophilized bacteria grown on solid-phase agar medium. Mild acetic acid hydrolysis of LOSs to cleave the ketosidic linkage to lipid A followed by borohydride reduction and gel filtration gave the preparations OS 5535 (ITM 5535), OS 3-1, OS 3-2, OS 3-3, OS 3-4, OS 3-5 (ITM 3147), OS A-1, OS A-2, OS A-3, and OS A-4 (ACY1). The ^{1}H and ^{13}C NMR spectra of the major oligosaccharides, OS 5535, OS 3-2, and OS A-2, were virtually identical with those from the major oligosaccharide obtained from *H. ducreyi* ITM 2665 (21), which strongly indicated identical structures for the oligosaccharides. This was further supported by sugar and methylation analyses of the samples which gave results similar to those obtained from ITM 2665. Thus, hydrolysates of OS 5535, OS 3-2, and OS A-2 contained D-glucose, D-galactose, 2-amino-2-deoxy-D-glucose, L-*glycero*-D-*manno*-heptose (LD-Hep), and D-*glycero*-D-*manno*-heptose (DD-Hep) (analyzed as alditol acetates), in the typical relative proportions 1:2.0:0.5:3.0:1.0. The absolute configurations of the sugars are anticipated to be the same as earlier. The presence of reduced, anhydro-Kdo (Kdo-anh-ol) was indicated by FAB-MS (discussed below), but no phosphate was detected in the preparations. It was evident from both ^{1}H (δ 2.04, 3H) and ^{13}C NMR data (δ 22.78 and 175.4) that the 2-amino-2-deoxy-D-glucose is N-acetylated.

Methylation analysis of OS 5535, OS 3-2, and OS A-2

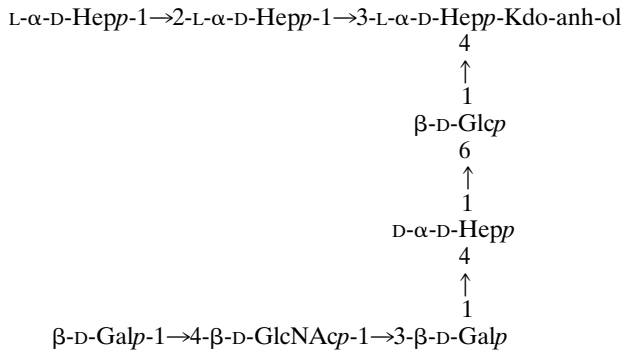
TABLE 1. Ions observed in the FAB mass spectra of different *H. ducreyi* oligosaccharide fractions[a]

Fraction	Mass (kDa) of ion
OS 5535	2,045, 1,882, **1,679**, 1,517, 1,487, 1,314, 1,152
OS 3-1	2,045, **1,882**, 1,679, 1,517, 1,487, 1,314, 1,152
OS 3-2	**1,679**, 1,517, 1,487, 1,314, 1,152
OS 3-3	1,679, **1,517**, 1,314, 1,152
OS 3-4	**1,314**
OS 3-5	1,314, **1,152**, 960, 798
OS A-1	2,045, **1,882**
OS A-2	**1,679**, 1,517
OS A-3	1,679, **1,517**

[a] The most abundant ions are shown in boldface type. The masses shown have been rounded off. In addition to $(M-H)^-$ ions, +22 Da ions were also observed.

showed terminal D-galactose, terminal LD-heptose, 4-substituted 2-amino-2-deoxy-D-glucose, 3-substituted galactose, 4-substituted DD-heptose, 6-substituted D-glucose, 3,4-substituted LD-heptose, and 2-substituted LD-heptose in the typical relative proportions 1.1:1.4:0.6:2.4:1.9:1.0:1.4:0.8.

The ^{1}H NMR spectra of OS 5535, OS3-2, and OS A-2 showed signals in the anomeric region at δ 5.73, 5.70, 5.75 (1H together, $J < 2$, not resolved), 5.08 (1H, $J < 2$, not resolved), 5.03, 5.06, 5.12, 5.30 (1H, $J < 2$, not resolved), 4.92 (1H, $J < 2$, not resolved), 4.72 (1H, $J = 7.8$ Hz), 4.49 (2H, $J = 7.8$ Hz), and 4.47 (1H, $J = 7.8$ Hz). The signals at δ 4.72, 4.49, and 4.47 were assigned to the hexose and hexosamine residues because of their large J coupling constants. The remaining signals were accordingly assigned to the heptoses. The chemical shifts of most of the proton signals could be assigned from the combined results of the double quantum filtered correlation spectroscopy and total correlation spectroscopy experiments and were identical to those found for the major oligosaccharide from ITM 2665, structure 1 (4), as follows:



The ^{1}H NMR spectra of OS 3-1 and OS A-1 were virtually identical to each other and showed the same anomeric signals for four α-linked heptoses as those observed for OS 5535, OS 3-2, and OS A-2. In addition, the signals for the anomeric proton and the *N*-acetyl methyl group of the GlcNAc residue were observed at δ 4.72 and 2.04, respectively, as well as overlapping signals centered at δ 4.5 corresponding to β-linked hexoses. It was indicated by the integral of the signals from the *N*-acetyl methyl group that more than one β-linked GlcNAc residue was present.

All oligosaccharide preparations were analyzed by negative FAB-MS, and observed ions are summarized in Table 1. The FAB mass spectrum of OS 5535 showed a main peak at *m/z* 1,679. The molecular mass of a nonasaccharide containing three hexoses (Hex), four heptoses (Hep), one 2-acetamido-2-deoxy-hexose (HexNAc), and sodium borohydride-reduced Kdo is 1,698. It is suggested that the signal at *m/z* 1,679 corresponds to $(MW-18-1)^-$. A difference of 18 Da in molecular mass between the expected pseudomolecular ion and the observed one has been reported for Kdo-containing oligosaccharides isolated from *Haemophilus* LOS by us and others (11, 15, 16, 20, 21). The explanation for this would be that a number of anhydro-Kdo moieties are formed during the course of delipidation by β-elimination of a phosphate group from C-4 of Kdo (2). Observed ions at *m/z* ratios of 1,517, 1,487, 1,314, and 1,152 are consistent with either fragment ions (or the structures) $(M-Hex-1)^-$, $(M-Hep-1)^-$, $(M-HexHexNAc-1)^-$, and $(M-Hex_2HexNAc-1)^-$, respectively.

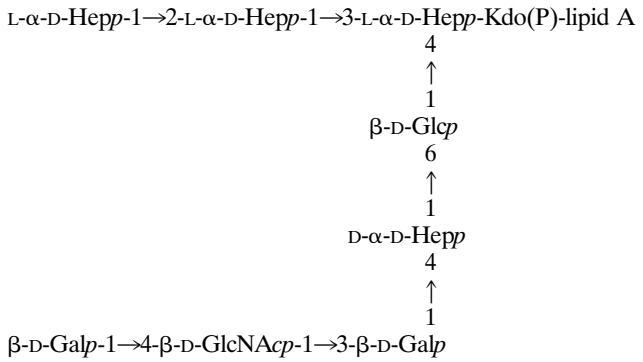
The FAB mass spectra of the different preparations from *H. ducreyi* ITM 3147 and ACY1 were very similar to that observed from *H. ducreyi* ITM 5535 (Table 1). For OS 3-1 and OS A-1, a major ion was observed at *m/z* 1,882 as well as a somewhat less abundant ion at *m/z* 2,045. The increment of 203 Da between *m/z* 1,679 and 1,882 and the increment of 163 Da between *m/z* 1,882 and 2,045 indicated that either a HexNAc and a Hex residue or a hexose residue carrying an ethanolaminepyrophosphate residue was linked to the major saccharide represented by *m/z* 1,679. The latter alternative was not likely, since treatment of the material with 48% HF did not affect the FAB mass spectrum. The mass 2,045 is one mass unit higher than expected for an addition of a hexosyl residue of 162. We attribute this to imperfections in the mass spectrometer calibration. Methylation analysis of OS 3-1 and OS A-1 gave terminal D-galactose, terminal L,D-heptose, 4-substituted 2-amino-2-deoxy-D-glucose, 3-substituted galactose, 4-substituted D,D-heptose, 6-substituted D-glucose, 3,4-substituted L,D-heptose, and 2-substituted L,D-heptose in the relative proportions 0.9:1.1:1.0:2.6:1.4:1.0:1.0:1.0. Thus, the amounts of 4-substituted 2-amino-2-deoxy-D-glucose and 3-substituted galactose had increased. Only trace amounts of terminal 2-amino-2-deoxy-D-glucose were detected. It was thus reasonable to assume that the major oligosaccharide isolated from the strains was elongated with another GalGlcNAc moiety, that the ion at *m/z* 2,045 is a pseudomolecular ion, and that the ion at *m/z* 1,882 is a fragment ion. The FAB mass spectrum of OS 3-5 revealed minor ions at *m/z* 960 and 798 for OS 3-5 which were consistent with $HexHep_3$-Kdo-anh-ol and Hep_3-Kdo-anh-ol.

LOS of *H. ducreyi* ITM 5535 was O deacylated with anhydrous hydrazine, and the product was analyzed by negative-ion ESI-MS without further purification. A partial ESI mass spectrum is shown in Fig. 1. It was obvious that two major compounds were present in the sample which inter alia showed ions at *m/z* 902.7 and 999.7 and their sodium and potassium adducts (*m/z* 909.7, 915.3, 1,007.2, and 1,012.2). It was evident from the spectrum that the ions were triply charged and that the ion at *m/z* 902.7 corresponded to a molecular mass of 2,711.0 Da and was thus consistent with the structure Hex_3 $HexNAcHep_4Kdo(P)$-deacylated lipid A. The ion at *m/z* 999.7 corresponded to a molecular mass of 3,001.9 Da which strongly indicated the presence of a sialylated compound with the structure $Neu5AcHex_3HexNAcHep_4Kdo(P)$-lipid A. The ion at *m/z* 1024.9 corresponded to a molecular ion of 3,077.9 Da and was consistent with $Hex_4HexNAc_2Hep4Kdo(P)$-deacylated lipid A, thus demonstrating that part of ITM 5535 also is elongated with *N*-acetyl lactosamine. The doubly charged ion at *m/z* 1,273.2 corresponded to a molecular mass of 2,569.6 Da and was consistent with $Hex_2HexNAcHep_4Kdo(P)$-deacylated lipid A. The ions at *m/z* 1,354 and 1,499 are the doubly charged ions corresponding to the masses 2,711.0 and 3,001.9. It should be

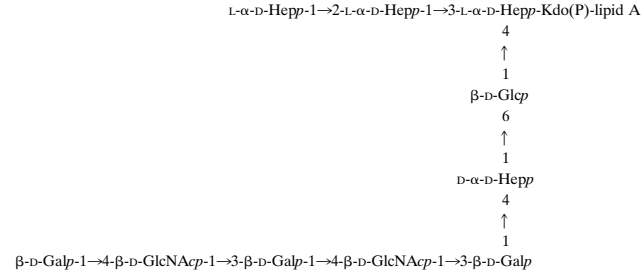
noted that ions indicating further substitution of phosphate or phosphoethanolamine on the molecules were not detected.

Methylation analysis of LOS from ITM 5535 revealed no other sugars than those observed for OS 5535. It is thus reasonable to assume that the structural element Neu5Acα2→3-β-D-Gal*p*-was present in the LOS.

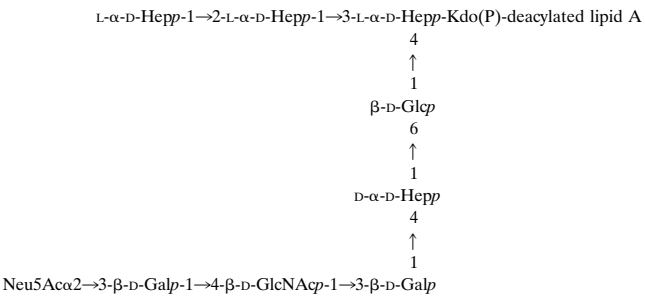
On the basis of the combined evidence presented above, structure 2 is suggested as the structure of one major LOS from *H. ducreyi* ITM 5535, ITM 3147, and ACY1. Structure 2 follows:



Another LOS, isolated as a minor compound from ITM 3147 and ACY1, and also observed in ITM 5535, is suggested to have structure 3 as follows:



The results from ESI-MS on *H. ducreyi* ITM 5535 revealed the sialylated component 4 as the second main component of the LOS mixture before acid hydrolysis. Structure 4 follows:



Structure 2 is identical to structure 1 of the LOS from *H. ducreyi* ITM 2665 studied by us (21). Presumably sialylated forms are present also in ITM 2665, ITM 3147, and ACY1. Recently, while this work was near completion, Melaugh et al. (12) published structures of oligosaccharides obtained from the LOS of *H. ducreyi* 35000. The structure of the main LOS from that strain is, as indicated by their preliminary study (11), identical to structure 1. Structures 2 and 3 are proposed as a minor and another major structure, respectively, for strain 35000. It is obvious that most of the strains studied so far have identical major structures in their LOSs. A different structure has been identified for *H. ducreyi* ITM 4747 (21). Whether this mutant strain is also capable of expressing by phase variation the outer core structure found in other strains remains to be elucidated.

Functional aspects of LOS outer core variation. The correlation of LOS structure with function has not yet been mapped to any greater extent. Molecular mimicry is usually implied as a mechanism for avoiding immunological host defenses. It is not known whether the lactosyl- and *N*-acetyl lactosamine disaccharide elements present in the outer core of *H. ducreyi* LOS have their main function as adherence-promoting compounds or whether their primary role is to modulate the immunogenic response through mimicry.

The adherence of *H. influenzae* to mammalian cells is partly mediated by high-molecular-weight proteins (22) closely related to *Bordetella pertussis* filamentous hemagglutinin, which is a 220-kDa protein with several binding sites, of which one binds galactose-containing glycoconjugates (e.g., lactose and lactosamine), thereby promoting adhesion to epithelial cells, and another recognizes the macrophage integrin complement receptor CR3, which is important for the intracellular uptake process (17). It is known that particles phagocytosed via CR3 do not engender an oxidative burst, suggesting that this route of entry might promote survival within the macrophage (17). Intracellular survival of *H. influenzae* in macrophage-like cells in human adenoid tissue has been demonstrated (6). Similarly, invasion of epithelial cells has recently been reported for *H. ducreyi* (23), and as judged from its hemagglutinating properties (1), there is reason to believe that filamentous hemagglutinin is also produced by this bacterium.

Carbohydrate-mediated recognition is crucially important not only for bacterial virulence but also for the adherence between eukaryotic cells. S-Lac lectins are a family of soluble lactose-binding lectins, some of which have been implicated in modulating cell-cell and cell-matrix interactions through specific carbohydrate-mediated recognition (9). Not only do they recognize lactose but they can also bind to other related structures like internal Galβ1→4GlcNAc elements of polysaccharides (9). Some have been localized to the adherence junctions (desmosomes) between animal cells (5). Interestingly, desmosome-like structures at points of interaction between *H. influenzae* and respiratory tract cells have been seen with an electron microscope (3). It is tempting to speculate that LOS might be involved in this interaction.

Like *Neisseria* species, *Haemophilus* species are also adapted to mucosal surfaces. Therefore, it is perhaps not surprising that similar structural LOS elements and variation can be observed in these phylogenetically separate groups of bacteria. The remarkable difference observed in this work between *H. ducreyi* and other bacteria is the relative paucity of LOS variation in *H. ducreyi*. Although intrastrain heterogeneity with sialylated and elongated glycoforms was demonstrated in all three *H. ducreyi* strains investigated, this bore little resemblance to the heterogeneity expressed by *H. influenzae* and *N. gonorrhoeae* with many different LOS variants. A speculative guess would be that this difference is somehow related to higher levels of endogenous production of CMP-NANA in *H. ducreyi* than in *H. influenzae*, which instead might depend on exogenous strategies to achieve sialylation of its LOS as described for *N. gonorrhoeae*. In this study, a major fraction (≈50%) of *H. ducreyi* ITM 5535 LOS was sialylated and showed very little variation of the kind observed in *H. influenzae* LOS, which on the other hand had very low levels (<5%) of sialylated LOS (data not shown). If there is no need for utilizing exogenous CMP-NANA, there might be no need for masquerading with high-frequency genetic phase variation of LOS as seen in *H. influ-*

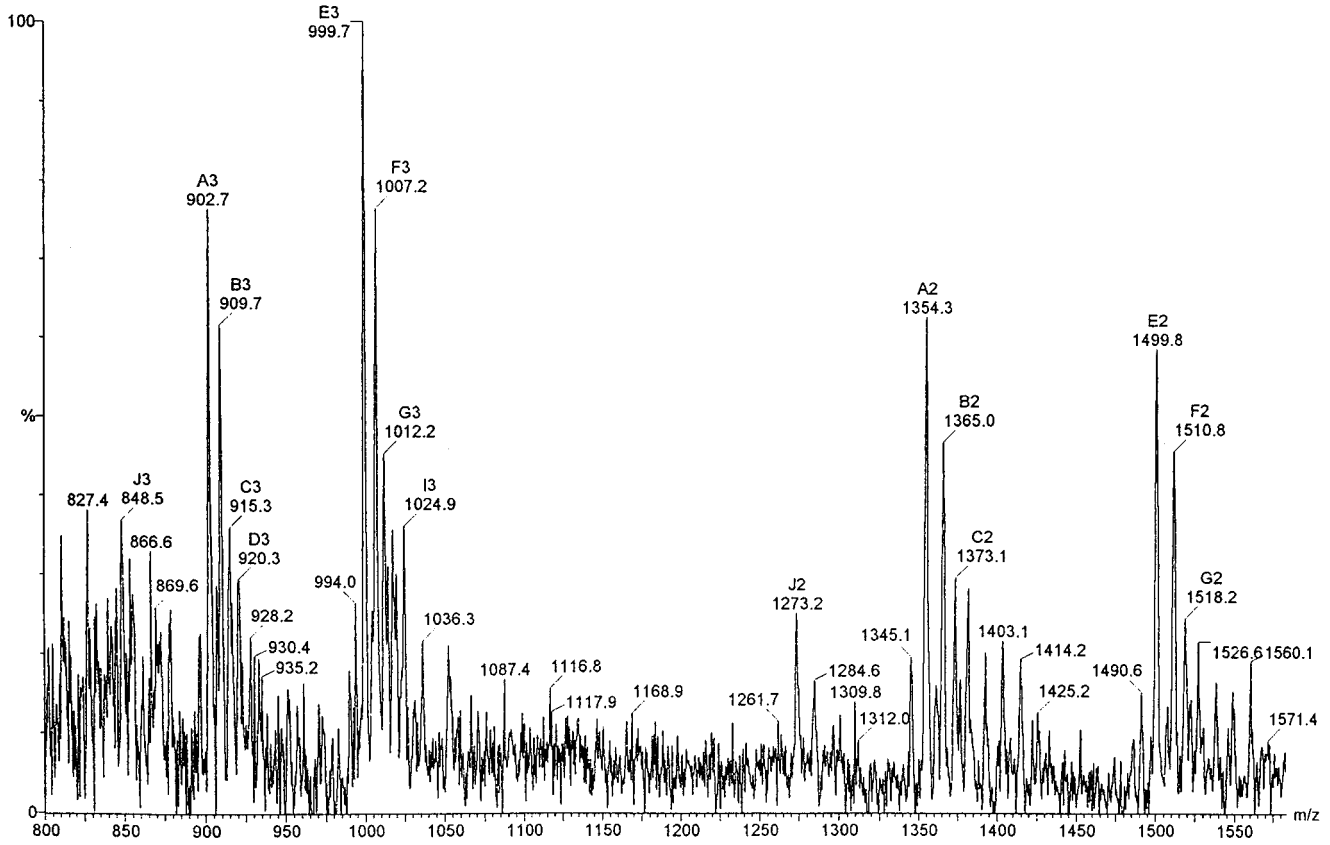


FIG. 1. Negative-ion ESI-MS of O-deacylated LOS from *H. ducreyi* ITM 5535. The LOS forms and charge states are indicated as follows: A2 is $(M_A - 2H)^{2-}$, B2 is $(M_B - 2H)^{2-}$, etc. LOS forms A, E, I, and J demonstrate the most significant structures and are described in the text. LOS forms B, C, F, and G are sodiated and potassiated adducts of forms A and E.

enzae and *N. gonorrhoeae*. Needless to say, other explanations are also possible.

The present data suggest that sialylation of structure 3 does not occur. The significance of the addition to structure 2 of a second *N*-acetyl lactosamine as an alternative to sialic acid is not clear. However, it might well correspond to phase variation in accordance with the theory of van Putten (24), suggesting that *N. gonorrhoeae* LOS variants which are not sialylated are sensitive to serum killing but enter mucosal epithelial cells more efficiently, whereas sialylation of LOS provides protection against phagocytosis and killing by antibodies and complement. Differences in *H. ducreyi* LOS composition have been linked to differences in the virulence of various strains when injected into the mouse (13). The total carbohydrate, hexose, and Neu5Ac contents of virulent strains were relatively higher than those of the avirulent strains. Virulence in the mouse model was associated with resistance both to the bactericidal action of human serum and to phagocytosis and killing by human polymorphonuclear leukocytes. A serum-resistant virulent strain was obtained by passage of an avirulent strain in the presence of increasing concentrations of normal human serum (13). Similar data from studies on gonococci show that in vivo phase shifts to higher-molecular-mass LOS are associated with the development of gonococcal leukorrhea (19). It is not known whether these observations correspond to the differences observed between the LOS structures expressed by *H. ducreyi* ITM 2665 and ITM 4747, respectively, although we suspect that they are more closely related to the intrastrain LOS heterogeneity observed in this study. It is tempting to speculate that switching between mutually exclusive elongated (structure 3) or sialylated (structure 4) LOS glycoforms derived from the same major compound (structure 1) might be a virulence factor that not only contributes to the bacterium's invasiveness but also induces resistance to the defense mechanisms of the host, particularly the classical complement pathway.

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