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Chemical and serological characterization of the *Bordetella hinzii* lipopolysaccharides[1]

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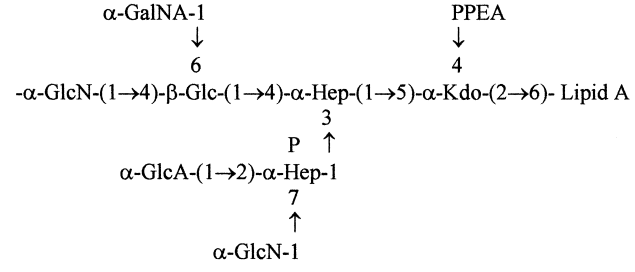
Abstract ***Bordetella hinzii*** **has recently been isolated from immunocompromised human hosts. The polysaccharides isolated from its endotoxin (lipopolysaccharide, LPS) were investigated using chemical analyses, NMR, gas-liquid chromatography/mass spectrometry and mass spectrometry by plasma desorption, matrix-assisted laser desorption/ionization and electrospray. The following structure for the O-chain-free LPS was deduced from the experimental results:**



Mass spectrometry and serology revealed that the O-chains were different from the homopolymer common to ***Bordetella bronchiseptica*** **and** ***Bordetella parapertussis*** **strains and were composed of a trisaccharide repeating unit. Masses up to 8 kDa were obtained for native LPS molecular species.**

Key words: Endotoxin; Structure; Mass spectrometry; Serology; *Bordetella hinzii*

1. Introduction

Bordetella pertussis, the Gram-negative pathogen responsible for whooping cough, is the most thoroughly studied member of the *Bordetella* genus. Its endotoxin, a major antigenic constituent on the bacterial surface composed of lipopolysaccharides (LPSs), has been serologically characterized, and the structures of the constituent lipids A and cores have been documented [1,2].

Bordetella bronchiseptica and *Bordetella parapertussis*, unlike *B. pertussis*, produce smooth-type LPSs [3]. All three have different and characteristic lipid A structures [4]. In *B. parapertussis*, the free cores, i.e. cores liberated from O-chain-free LPS by mild acid hydrolysis, were shorter than that of *B. pertussis*. Furthermore, the cores substituted by an O-chain always comprised additional glycoside residues compared to O-chain-deficient cores from the same preparation [5]. LPSs have been implicated as virulence factors in *Bordetella* infections [6], and more recently, in a process leading to damage of ciliated trachea cells by production and release of NO [7]. It is therefore important to establish a structure/activity relationship amongst LPSs of different *Bordetella* pathogens with those of their *pertussis* relative.

Monoclonal antibodies directed against well-characterized parts of the *Bordetella* LPS structures have been made [8]. These antibodies serve as markers for the typing of isolates [9]. They also defined substructures to be incorporated into a potential synthetic vaccine. In this study they have been used to characterize the reactivity of the *Bordetella hinzii* LPSs.

B. hinzii is a *Bordetella avium*-like Gram-negative bacterium which has been isolated from poultry and from immunosuppressed and cystic fibrosis patients [10,11]. Its LPS, unlike the O-chain-less LPS of *B. pertussis* is a smooth type. Its lipid A structure is unusual in that its C-2′ amide-linked 3-C_{14}OH is esterified by a 2-C_{14}OH [12]. This report gives spectra of unmodified LPS of high molecular weight. It describes the overall structure of this heterogeneous endotoxin confirmed by plasma desorption mass spectrometry (PDMS) and matrix-assisted laser desorption ionization (MALDI) spectra of the separated native O-chain-linked and O-chain-less LPSs and gives their serological reactivity with monoclonal antibodies.

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[1] Part of this work was presented at the 5th Conference of the International Endotoxin Society in Santa Fe, USA in September 1998.

Abbreviations: CE, capillary electrophoresis; ELISA, enzyme-linked immunosorbent assay; ES/MS, electrospray mass spectrometry; FAB, fast atom bombardment; Fuc2NAc4NMe, 2-acetamido-4-*N*-methyl-2,4,6-deoxy-galactose; GalNA, galactosaminuronic acid; GlcA, glucuronic acid; GlcNAc, *N*-acetyl glucosamine; GC, gas chromatography; Hep, heptose; LPS, lipopolysaccharide; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption/ionization; Man2NAc3NAcA, 2-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid; NMR, nuclear magnetic resonance; PDMS, plasma desorption mass spectrometry; PEA, phosphoryl ethanolamine; PPEA, pyrophosphoryl ethanolamine

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2. Materials and methods

2.1. Bacterial strains and cultures

B. hinzii smooth-type strain ATCC 51730 (NRCC 4794) was grown as described [3]. The cells were killed in 2% phenol before harvesting.

2.2. LPS

The LPS was extracted by the modified enzyme–phenol–water method [13], and purified as described [14].

2.3. Gas chromatography

Alditol acetates were analyzed by gas chromatography on an HP 5 column (Hewlett Packard, 30 m×0.32 mm) using the program 180°C (2 min) to 240°C at 2°C/min.

2.4. Mass spectrometry

PDM spectra were obtained as previously described [15]. Resolution was ±1 U.

2.5. MALDI/MS

MALDI/MS was carried out on a Perseptive Voyager STR model (PE Biosystem, France) time-of-flight mass spectrometer. Gentisic acid (2,5-dihydroxybenzoic acid), 10 mM in water, was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used as matrix. Dowex 50 (H^+) decationized samples (0.5 μg/0.5 μl) were deposited on the target, covered with 0.5 μl of the matrix in aqueous solution and dried. Analyte ions were desorbed from the matrix with pulses from a 337 nm nitrogen laser. Spectra were obtained in the negative ion mode at 20 kV with an average of 128 pulses. Lipid A peaks were used as internal standards. The masses are average masses.

2.6. CE-ESMS

A Crystal model 310 CE instrument (ATI Unicam, Boston, MA, USA) was coupled to the mass spectrometer via a co-axial sheath-flow interface [16,17]. All mass spectral analyses were conducted using a Q-TOF (Micromass, Manchester, UK) hybrid quadrupole/time-of-flight instrument for high resolution and on-line tandem mass spectrometric experiments. A sheath solution (70:30, isopropanol:methanol) was delivered at a flow rate of 1.5 μl/min to a low dead volume tee. Separations were obtained on 90 cm length bare fused silica at an applied voltage of 30 kV with an electrolyte solution composed of 30 mM aqueous ammonium acetate pH 8.5, containing 5% methanol for positive ion detection. In combined CE-MS-MS analyses, collisional activation was performed using argon collision gas at an energy (laboratory frame of reference) of 60 eV.

2.7. Phosphoryl ethanolamine (PEA)

Phosphoryl ethanolamine (PEA) analyses were performed on a Biotronik LC 2000 analyser equipped with a Dionex DCGA resin column (Dionex Corporation) and a Spectra-Glo fluorometer (Gilson). The post-column detection was carried out by measuring the fluorescence intensity of isoindole derivatives obtained by the action of *o*-phtalaldehyde in the presence of 2-mercaptoethanol [18,19]. The results were compared with a D-7500 integrator (Merck Hitachi).

2.8. Absolute configurations

Absolute configurations of sugars were determined on the HF-treated LPS according to the method of Gerwig et al. [20] after hydrolysis of the polysaccharide (2 N HCl, 2 h) and *N*-re-acetylation in the case of GlcN.

2.9. Thin-layer chromatography (TLC)

Chromatography was performed on aluminum-backed silica TLC plates (Merck) and visualized by charring (145°C after spraying with 10% sulfuric acid in ethanol). The solvent was a mixture of isobutyric acid:M ammonia (3:5) for polysaccharides and (5:3) for lipopolysaccharides [14].

2.10. SDS–polyacrylamide gel of LPS

Gels were prepared and loaded with samples of 0.2–0.5 μg of the starting LPS preparation and its silica gel fractions, electrophoresed as previously described [21], and then stained [22].

2.11. Chemical analyses

Hexosamines were assayed as in [23], phosphate as in [24] and Kdo as in [25].

2.12. Dephosphorylation of the B. hinzii endotoxin

The endotoxin (10 mg) was dispersed in aqueous HF (48% HF, 0.5 ml) and stirred at 4°C for 48 h. The solution was evaporated under a stream of nitrogen until neutrality was obtained.

2.13. Detergent-promoted hydrolysis

The endotoxin (100 mg) was cleaved by hydrolysis in 20 mM Na acetate–acetic acid pH 4.5–1% Na dodecylsulfate at 100°C for 1 h at a concentration of 5 mg/ml [26]. The supernatant containing the polysaccharides (PSs) was lyophilized (38 mg), taken up in 1.9 ml, and chromatographed on a Sephadex G-50 column (45 cm×1.6 cm) in 0.05 M pyridine–acetate pH 5. Aliquots of fractions were tested by TLC for PS content and collected.

2.14. Monoclonal antibodies/ELISA

mAbs PP6, P1P3, D7, and 60.5 were prepared from hybridoma produced by fusion of SP2/0-Ag.14 myeloma cells with spleen cells of BALB/C mice immunized with *B. parapertussis* LPS [8], with *B. pertussis* LPS or with killed *B. pertussis* cells [9]. The binding of mAbs was analyzed by ELISA [9].

3. Results and discussion

The *B. hinzii* endotoxin is a mixture of O-chain-linked and O-chain-free lipopolysaccharides as shown by SDS–gel electrophoresis [12]. The O-chain-free LPSs appear to be shorter than the LPSs of *pertussis*. They were relatively homogeneous and contained only one major and some minor molecular species each differing by one O-chain subunit. In both *B. parapertussis* and *B. bronchiseptica*, the repeating unit is a polymer of 2,3-diacetamido-2,3-dideoxy-α-L-galactopyranosyluronic acid [3]. An identical O-chain structure for two distinct species of a genus is unusual.

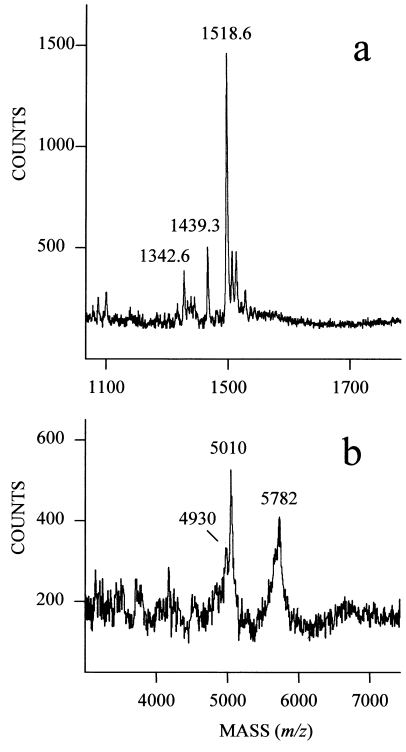

Fig. 1. Negative ion PDM spectra of: (a) the core oligosaccharides from *B. hinzii*, (b) the extended core linked to O-chains from *B. hinzii*.

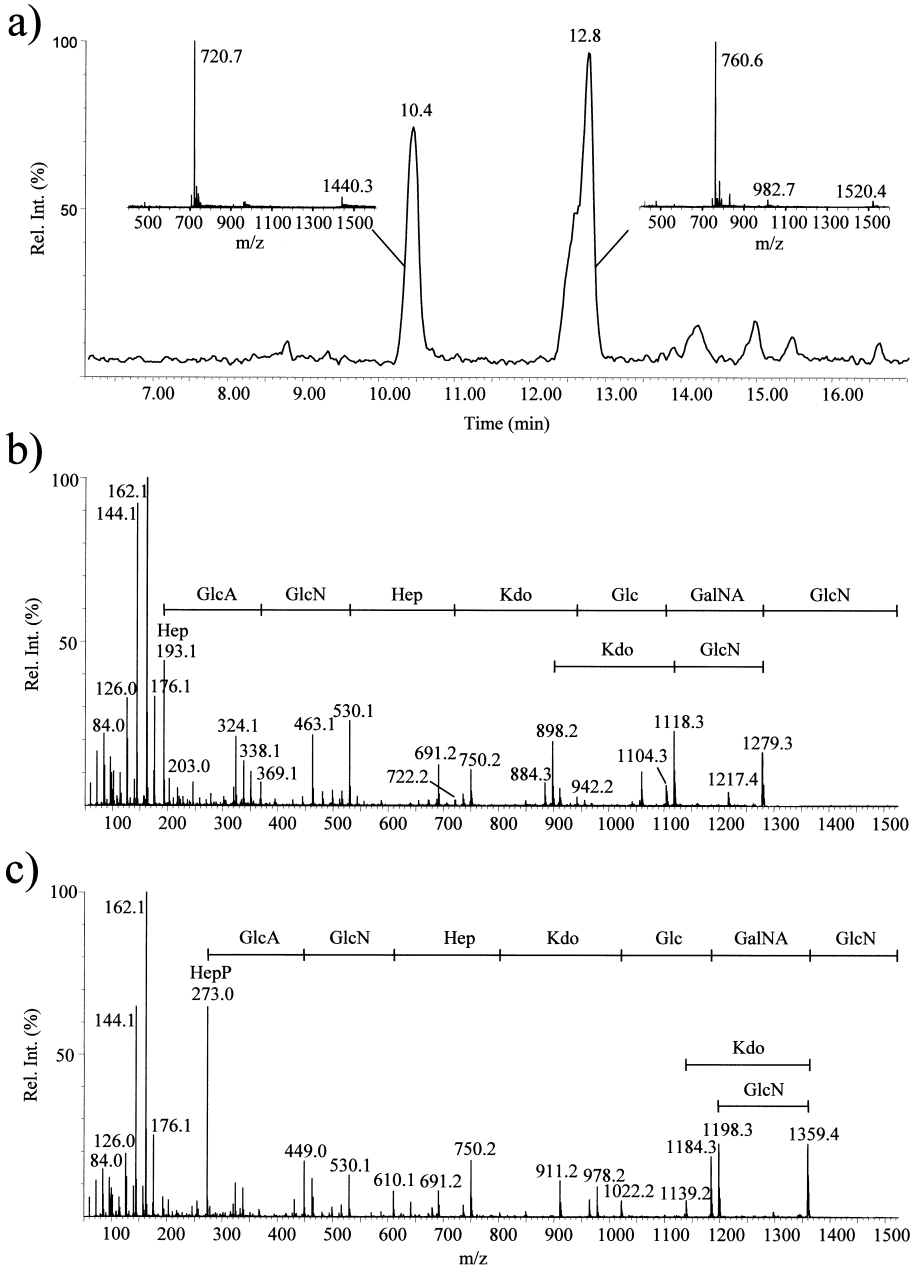

Fig. 2. CE-ESMS analysis of the core oligosaccharide from *B. hinzii*: (a) total ion electropherogram (*m/z* 400–1600), insets show the extracted mass spectrum for peaks migrating at 10.4 and 12.8 min, respectively, (b) product ion spectrum of *m/z* 720.7, (c) product ion spectrum of *m/z* 760.6.

3.1. Analysis of the glycosidic fractions separated after mild acid hydrolysis of the endotoxin

Chromatography on a Sephadex G-50 column of the supernatant obtained by mild acid hydrolysis gave two major and one minor fractions as visualized on TLC [14]. They corresponded to a major and a minor core oligosaccharide and to a core carrying the O-chain repeating units. The values obtained by PDMS (Fig. 1a) as well as by MALDI (not shown) were *m/z* 1518.6 and 1965 for the main and minor core fractions, and *m/z* 5782 respectively. The PD mass spectrum showed some heterogeneity due to minor molecular species missing either a phosphate group at *m/z* 1439.3 or a GalNA residue at *m/z* 1342.6. Proton NMR analysis of the *B. hinzii* O-chain gave three anomeric signals at δ4.48, δ4.55 and δ5.05, and signals for acetates and carboxylic groups. This concurred with the mass of the subunit, 772 Da, estimated by mass differences between two consecutive signals (PDMS and MALDI) found in one polysaccharide fraction from the Sephadex column (Fig. 1b). This mass corresponded approximately to three times that of a diacetamido acid hexose (258×3). This type of sugar was found in the LPS of different members of the genus. Treatment of the isolated O-chain with mild alkali in order to reduce heterogeneity by removal of potential O-acetyl groups for NMR analysis resulted in the fragmentation of this polymer indicating that at least one of the residues was susceptible to β-elimination and would therefore be different from the above-mentioned sugars in structure and mass.

The main free core oligosaccharide was shown by colorimetric assay to be phosphorylated and had a mass (1519 Da) consistent with that of an octasaccharide. ^{1}H NMR, PDMS, MALDI, ES/MS analyses of the native core as well as GC/MS and FAB confirmed that this octasaccharide was similar to the first eight sugars proximal to the lipid A of *B. pertussis* LPSs [2]. ^{1}H NMR analyses gave seven protons in the anomeric region for the free core signals that had values identical to those present in the *B. pertussis* parent [27]. They appeared at δ4.48, δ5.09, δ5.14, δ5.25, δ5.29, δ5.41, and δ5.52 and corresponded, respectively, to β-Glc, α-Hep 2, α-GlcA, α-GlcN 1, α-GalNA, α-Hep 1 and α-GlcN 2. Both heptose residues of *B. hinzii* LPS had the LD configuration and Glc, GlcN, and GlcA had the D configuration. Additional signals corresponding to Kdo were found in the hydrazine-treated O-chain-free LPS. In all these analyses, the mutant *B. pertussis* A100 core nonasaccharide was taken as a reference [1].

The minor underivatized core fraction gives a signal in MALDI at *m/z* 1965 (not shown) which was consistent with the mass of the main core plus that of two additional sugars: Fuc2NAc4N and di-NAc-diamino hexuronic acid. This minor fraction was also observed in the CE-MS analysis with a signal appearing at *m/z* 982.7, migrating at 12.6 min (Fig. 2a). The observed mass of this oligosaccharide is 1963.4 Da consistent with the previous interpretation.

As with *B. pertussis* A100 cores isolated after mild acid hydrolysis, the mass observed for the main *hinzii* core differed from the calculated mass by the mass of a molecule of water. This was a strong indication that a substituent was eliminated under these conditions from position 4 of the reducing Kdo in *B. hinzii*, leading to an olefinic derivative. This was also confirmed by the colorimetric test for Kdo on the native and HF-treated endotoxins, which was positive only after the dephosphorylation treatment [28].

3.2. CE-ESMS analyses of the main core oligosaccharide fraction

Analyses of the main core fraction separated on the Sephadex column gave two principal components migrating at 10.4 and 12.7 min, respectively. The first component gave doubly $[M+2H]^{2+}$ and singly protonated $[M+H]^+$ ions at *m/z* 720.7 and 1440.3, respectively, consistent with a core oligosaccharide having a molecular mass of 1439.4 Da. The second core component showed prominent $[M+H]^{2+}$ and $[M+H]^+$ signals at *m/z* 760.6 and 1520.4, respectively. The mass difference between these two oligosaccharides (80 Da) together with their migration order was consistent with a non-phosphorylated and a phosphorylated core glycan, respectively. As already mentioned the minor core oligosaccharide with two additional sugars also appeared in this analysis.

The MS/MS spectrum of *m/z* 720.7 (Fig. 2b) showed a number of structurally informative fragment ions corresponding to cleavage of each glycosidic bond. Because of the highly branched structure of this core glycan, fragmentation took place from different terminal residues thus resulting in a complex mass spectral pattern. However, sequence and branching information was obtained from the spacing between adjacent signals as indicated in the figure. For example, fragment ions at *m/z* 193, 369, and 530 were consistent with a Hep residue (oxonium ion at *m/z* 193) to which is linked both a GlcA and a GlcN residue. This trisaccharide is further attached to a central Hep residue as indicated by a fragment ion at *m/z* 722. To this central Hep is also linked an anhydro Kdo (*m/z* 942) and a Glc (*m/z* 1104) residue. Finally, fragment ions at *m/z* 1266 and 1279 supported the proposal of terminal GlcN and GalNA residues, respectively, linked to the Glc, consistent with the structure of *B. pertussis* [2].

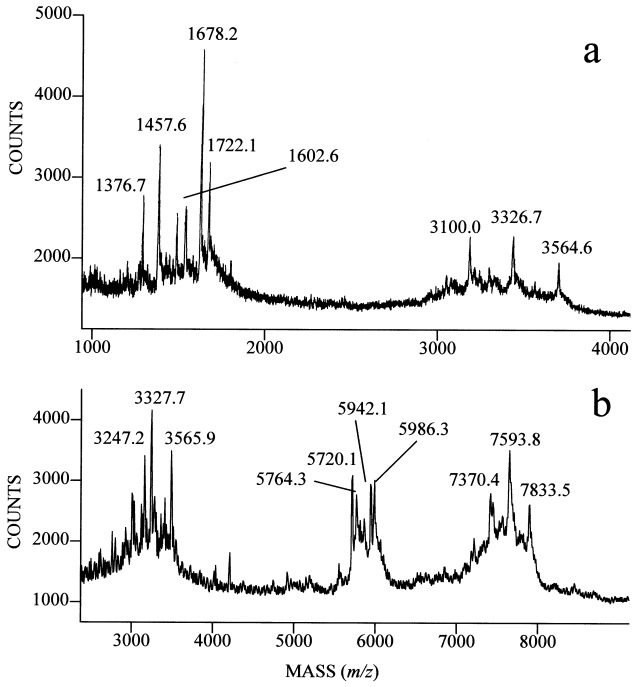

Fig. 3. Negative ion MALDI-TOF spectra of: (a) the separated O-chain-free LPSs from *B. hinzii*, (b) the native LPSs from *B. hinzii*.

The fragment ion spectrum of *m/z* 760.6 (Fig. 2c) enabled the location of the phosphorylated residue. In this case, the presence of a fragment ion at *m/z* 273 together with ions at *m/z* 449 and 611 supported the proposal of a phosphorylated side chain Hep residue.

3.3. MALDI spectra of the native and separated O-chain-free and O-chain-linked LPSs present in the B. hinzii endotoxin

The O-chain-free and O-chain-linked *B. hinzii* LPSs were separated on a silica gel column [12,21]. The O-chain-free LPS spectrum is presented (Fig. 3a) as well as the native LPS spectrum (Fig. 3b). The spectrum of O-chain-linked LPSs gave signals also seen in that of the native LPS (not shown).

3.4. MALDI/MS of the O-chain-free LPS

An aqueous endotoxin suspension was analyzed after partial decationization without any modification or substitution of the native structure. The spectrum presented heterogeneity due to the tetra-, penta- and hexa-acylation of the lipid A portion, i.e. giving signals appearing at *m/z* 3100, 3326.7 and 3564.6, respectively, for the LPS molecular ions. Signals appeared at *m/z* 1376.7, and 1602.6 for the lipid A fragment ions, and *m/z* 1457.6, 1678.2 and 1722.1 for core fragment ions. The mass obtained for the intact hexa-acylated O-chain-free LPS (*m/z* 3565) differed from that of the sum of the masses of the lipids and cores isolated after mild acid treatment (1842 Da+1519 Da = 3361 Da). The difference was due to the presence of a substituent characterized as pyrophosphorylethanolamine (PPEA, 203 Da). In mass spectra

of the native LPS this substituent was preserved in the molecular ions as well as in the polysaccharide fragment ions. Such a substituent at the C-4 position of the Kdo molecule has already been found in half of the LPS molecular species of the *pertussis* endotoxin [25,29]. Thus, mass spectrometry of native LPSs helped to detect the presence of a substituent usually eliminated during the mild acid hydrolysis used to separate the lipid and glycosidic moieties. As in *B. pertussis*, PPEA was assumed to substitute the Kdo residue at the C-4 position, the only position that could undergo β-elimination at pH 4.5. The presence of PPEA was also demonstrated by chemical analysis. PEA was released from the endotoxin by a specific pyrophosphate cleavage in 1% pyridine in water at 50°C for 20 h [30]. PEA was first detected by ninhydrin reaction on TLC compared to a synthetic standard (R_f), and then confirmed by amino acid autoanalyser by fluorescence. This substituent was previously detected in the *B. pertussis* LPS strains 1414 and A100 in about 50% and 100% of the molecules, respectively.

3.5. MALDI/MS of the native endotoxin

The negative ion mass spectrum (Fig. 3b) also showed some heterogeneity as indicated previously in the SDS–electrophoresis gel [12]. The heterogeneity was compounded with prompt fragmentation between polysaccharides and lipid A molecules which resulted in four distinct regions in the spectrum. The lower mass region of the spectrum is not shown. It had peaks appearing between *m/z* 1300 to *m/z* 1900, corresponding to lipid A and free core ion fragments of O-chain-free LPS molecules as shown in Fig. 3a. The next group of peaks appeared at masses between *m/z* 3000–3600 corresponding to the sum of a lipid A plus free core, i.e. to intact O-chain-free LPS molecular species. They are the same as the masses observed in the separated O-chain-free LPS. The following group of peaks (*m/z* 5700 to *m/z* 6000) corresponded to O-chain-linked polysaccharides obtained by fragmentation of the LPSs. The last group at highest masses corresponded to the O-chain-linked LPS molecular ion species, their values according with the sum of the masses of the free lipid A plus polysaccharide moieties including PPEA. Interpretations of the signals are given in Table 1. As already mentioned, the mass of a repeating unit, i.e. 772 Da, was given by the difference between *m/z* values of consecutive peaks in the O-chain-linked polysaccharide fragment ion region (Fig. 1b) as well as in that of the O-chain-linked LPS molecular ions (not shown). From these masses, it was deduced that five repeating units of three sugars were present in the major molecular species. As already observed with *B. parapertussis* [5,31], the mass of the complete structure was accounted for by the sum of the masses of the lipid, the free core and the O-chains plus the mass of two additional sugars. The first of the extra residues was assumed to be the FucN4N earlier found in the O-chain-linked core of *B. parapertussis* and absent from the corresponding O-chain-free core [5]. However, unlike the FucNAc4NMe of *B. pertussis*, the residue present in the core linked to the *B. hinzii* O-chains was not acetylated and not methylated.

The difference in mass (258 Da) suggested that the second residue was a 2,3-diNAcA hexose. This kind of sugar was found in the *B. bronchiseptica* and *B. parapertussis* O-chains with a *galacto* configuration as well as in the *B. pertussis* core with a *manno* configuration. Thus, this residue could be either the last sugar of the core or the last sugar of the O-chain moiety.

3.6. Reactivities with monoclonal antibodies

The core structure was found to be more conserved than the lipid A structure in the *Bordetella* species. However, a difference was always found in the length of the unsubstituted cores compared to the *pertussis* core. *B. pertussis* strain 1414 core has 12 sugars, *B. pertussis* A100, like *B. pertussis* 134, has

Table 1
Composition and assignments of signals observed in mass spectra of *B. hinzii* oligosaccharides (Figs. 2a and 3a), polysaccharides (Fig. 2b), and LPSs (Fig. 4A and B)

Calculated masses $(M-H)^-$	Fig. 2a	Fig. 2b	Fig. 3a	Fig. 4A	Fig. 4B	Interpretation
O-chain-linked LPSs						
7828.10					7833.5	LPS (six fatty acids)
7589.79					7593.8	LPS (five f.a.)
7363.33					7370.4	LPS (four f.a.)
PS						
5985.65					5986.3	$PS^5(-H_2O)$ (five units+extended core)
5942.58					5942.1	PS^5-EA($-H_2O$)
5766.45					5764.3	PS^5-EA-GlcA($-H_2O$)
5720.66					5720.1	PS^5-PPEA-P
5782.62		5782				PS^5-PPEA($-H_2O$)
5010.62		5010				PS^4-PPEA($-H_2O$)
4930.64		4930				PS^4-PPEA-P($-H_2O$)
O-chain-free LPSs						
3564.71				3564.6	3565.9	LPS (six f.a.)
3326.30				3326.7	3327.7	LPS (five f.a.)
3346.32					3247.2	LPS-P (five f.a.)
3099.94				3100.0		LPS (four f.a.)
Lipid A or core						
1722.26				1722.1		Core($-H_2O$)
1679.19				1678.2		Core-EA($-H_2O$)
1603.04				1602.6		Lipid A (five f.a.)
1457.27				1457.6		Core-PPEA-P
1376.68				1376.7		Lipid A (four f.a.)
1519.23	1518.6		1520.4			Core-PPEA($-H_2O$)
1439.25	1439.3		1440.3			Core-PPEA-P($-H_2O$)
1343.10	1342.6					Core-PPEA-GalNA($-H_2O$)

nine sugars [1], *B. parapertussis* core, seven sugars, and *B. hinzii*, eight sugars. The *B. bronchiseptica* cores, depending on the strain, were either similar to the *B. pertussis* dodecasaccharide core, bearing an additional phosphate group and/or had non-stoichiometric Fuc2NAc4NMe methylation and/or lacked the terminal heptose (M. Caroff, J.C. Richards, A. Martin, M.B. Perry and D. Karibian, unpublished data). The cores present in the O-chain-linked molecules all contained the FucNN residue.

To get additional comparative data on the LPSs isolated from *B. hinzii*, we used four mAbs (PP6, P1P3, D7, and 60.5) prepared and characterized previously in our laboratory [8,9]. All of these are directed against epitopes of *Bordetella* LPSs. Their reactivities with O-chain-free and O-chain-linked LPSs of *B. hinzii* were analyzed by ELISA and compared to that of the unfractionated LPSs. *B. bronchiseptica* LPS was also used for comparison since it has been shown to be the most antigenically polymorphic of the different *Bordetella* LPSs [32]. The results show indeed that the *B. bronchiseptica* LPS reacts with the four mAbs (Fig. 4A) whereas the unfractionated preparation of *B. hinzii* LPS reacts exclusively with mAb P1P3 (Fig. 4B). This antibody, which recognizes a structure present in the nine-sugar core of the *pertussis* A100 mutant and the seven-sugar core of *B. parapertussis*, also reacts with the O-chain-linked (Fig. 4C) and the O-chain-free (Fig. 4D) fractions of the *B. hinzii* LPS preparation, thus confirming the presence of a common conserved core substructure in all of the *Bordetella* genus, including *B. hinzii*. In contrast, mAb PP6, which reacts with the O-chains of *B. parapertussis* and *B. bronchiseptica*, did not react with the unfractionated preparation of *B. hinzii* LPS (Fig. 4B), or with the fraction consisting of the O-chain-linked LPSs (Fig. 4C), thus providing more evidence for the difference between the O-chain structures of *B. hinzii* and *B. parapertussis*/*B. bronchiseptica*. The two other mAbs, D7 and 60.5, did not react either with the unfractionated or with the O-chain-linked fraction of *B. hinzii* LPS. These antibodies have been shown to recognize distinct substructures of the distal trisaccharide (GlcNAc→Man2NAc3NAcA→Fuc2NAc4NMe) present in *B. pertussis* and *B. bronchiseptica*, but absent from *B. parapertussis* and from the *B. pertussis* A100 [9]. We found that mAb 60.5 reacts with the O-chain-free fraction of *B. hinzii* LPS (Fig. 4D). Because of the high sensitivity of ELISA, this observation can probably be ascribed not to the major core but to the above-mentioned minor core of higher mass and containing two additional sugars of the distal pertussis core as shown by MALDI and ES/MS. Fig. 5 summarizes the reactivity of the monoclonal antibodies with the different molecular species present in the preparation. The data also show that the use of our monoclonal antibodies in ELISA studies represents a valuable tool for rapid and sensitive detection of Bordetellae in patients, including *B. hinzii*, a relatively rare human pathogen.

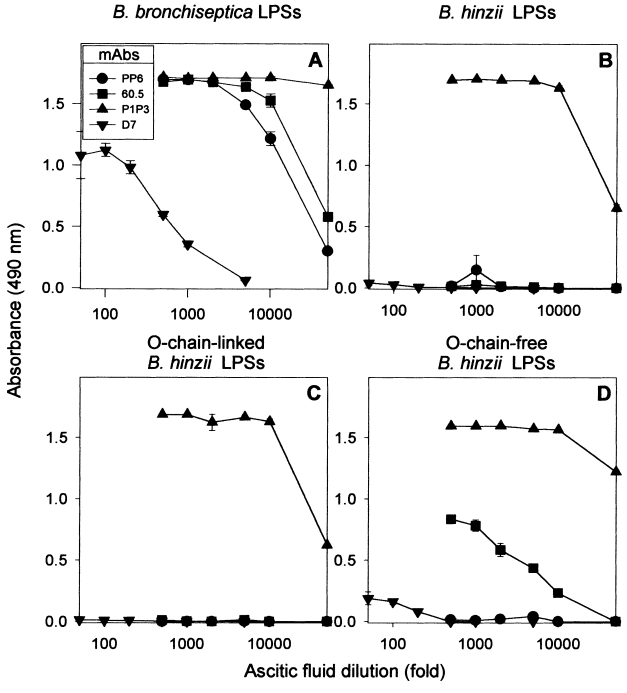

Fig. 4. Reactivities of anti-*Bordetella* mAbs with LPS preparations. LPSs from *B. bronchiseptica* (A), *B. hinzii* (B), and O-chain-linked (C) and O-chain-free (D) fractions of the *B. hinzii* LPSs, were incubated with different dilutions of ascitic fluids from clones PP6 (●), 60.5 (■), P1P3 (▲) and D7 (▼). The binding of the mAbs was determined by ELISA. Values represent the mean ± S.D. of triplicate determinations.

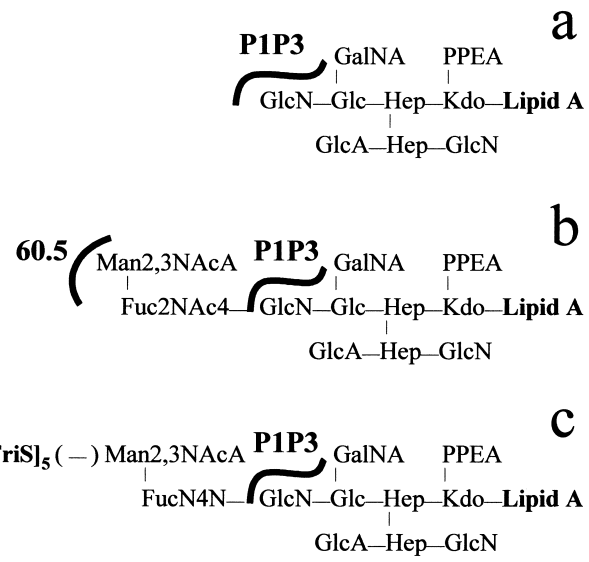

Fig. 5. Epitopes of the *B. hinzii* LPSs recognized by the mAbs P1P3 and 60.5. TisS = trisaccharide.

'Incompleteness' of free cores and 'near completeness' of O-chain-linked cores have already been observed in *B. parapertussis* LPSs [5]. Work on *hinzii* polysaccharides done simultaneously by Vinogradov [33] presents elegant NMR data on selected carbohydrate fragments obtained by strong alkaline degradation of the *hinzii* LPSs. The present work was done on the complete undegraded LPSs with intact substituents and therefore gives more information on the core structure itself. Alkali-labile substituents like PEA, acetate groups, and O-chains were preserved. Only mass spectrometry with native LPSs could positively indicate the association of the extended core with O-chains. The additional sugars, however, are always present in the related *B. pertussis* 1414 core structure. The latter was the only one having a terminal GlNAc residue and no O-chains. The presence of this terminal sugar could be interpreted as a signal preventing the addition of an O-chain. A similar hypothesis has already been presented in the case of

Pseudomonas aeruginosa in which a glucose residue present in the core of the deficient rough LPS was absent from the core of the S-type LPS [34]. The analysis of the endotoxin of this new member of the *Bordetella* genus gives additional comparative data on these interesting and complex structures.

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