Structural characterization of the O-chain polysaccharide isolated from *Bordetella avium* ATCC 5086: variation on a theme[1]

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Abstract The O-chain polysaccharide (O-PS) of *Bordetella avium* was isolated from the lipopolysaccharide by mild acid hydrolysis to remove the lipid A, followed by hydrofluorolysis to remove the lipopolysaccharide core oligosaccharide leaving a residual O-PS for structural analysis. High resolution ^{1}H and ^{13}C NMR and MALDI studies showed the O-chain to be a polymer composed of 1,4-linked 2-acetamidino-3-[3-hydroxybutanamido]-2,3-dideoxy-β-D-glucopyranosyluronic acid residues.

Key words: Endotoxin; Lipopolysaccharide; O-chain; Nuclear magnetic resonance; Matrix-assisted laser desorption-ionization mass spectrometry; *Bordetella avium*

1. Introduction

Eight species have been described in the genus *Bordetella* [1] of which two, *Bordetella pertussis* and *B. parapertussis*, are agents of whooping cough. Although the former is an obligate pathogen for humans, there are strains of the latter that also cause pneumonia in sheep [2]. *B. bronchiseptica* infects many mammalian species including humans and causes respiratory diseases. The five other species included in the genus are: *B. hinzii* [3], *B. trematum* [4], *B. holmesii* [5] *B. petrii* [6], and *B. avium* [7]. *B. avium*, the subject of this study, is a selective pathogen for birds.

Involvement of lipopolysaccharide (LPS) in *Bordetella* infections was first shown with *B. pertussis*, the LPS damaging ciliated trachea cells by the induction of nitric oxide in synergy with tracheal cytotoxin [8]. *B. avium* binds to ciliated tracheal epithelial cells subsequently favoring lethal bacterial superinfection, often by *Escherichia coli* [9]. Interest in the mechanism of infection is driven by the economic stake in poultry production, especially turkey farming. More recently *B. avium* LPS has been described as an important virulence factor [10]. Mutants of *B. avium* with decreased binding to tracheal cells were found to have altered sodium dodecyl sulfate (SDS) gel electrophoresis profiles [11].

LPS O-chain structures found in *B. parapertussis* and *B. bronchiseptica* LPS consist of 1,4-linked 2,3-diacetamido-2,3-dideoxy-α-L-galactopyranosyluronic acid residues [12]. The *B. pertussis* LPS, which does not produce an O-chain, contains the *manno* isomer of the 2,3-diacetamido uronic acid in its distal trisaccharide [13]. The *B. hinzii* O-chain was found to be composed of trisaccharide repeating units of the *gluco* and *galacto* analogues of the same hexuronic acid [14,15].

We present here the analysis of the *B. avium* O-chain polysaccharide.

2. Materials and methods

2.1. Bacterial strains and cultures

B. avium smooth-type strain ATCC 5086 was grown as described [12]. The cells were killed in 2% phenol before harvesting.

2.2. LPS

The LPS was extracted by the modified phenol–water method [16], and purified as previously described [17].

2.3. Thin-layer chromatography (TLC)

Chromatography was performed on aluminum-backed silica TLC plates (Merck) and spots were visualized by charring (145°C after spraying with 10% sulfuric acid in ethanol). The solvent was a mixture of isobutyric acid:ammonium hydroxide (3:5, v/v for polysaccharides and 5:3, v/v for LPS) [17].

2.4. SDS–polyacrylamide gel of LPS

Gels were prepared and loaded with samples of 0.2–0.5 μg of the LPS preparations, electrophoresed as previously described [14], and then stained [18].

2.5. Detergent-promoted hydrolysis

The LPSs (200 mg) were cleaved by hydrolysis in 20 mM Na-acetate–acetic acid pH 4.5–1% SDS at 100°C for 1 h at a concentration of 5 mg/ml [19]. After centrifugation, the supernatant containing the polysaccharides (O-PS core+free core) was lyophilized (95 mg), taken up in 2 ml of column buffer (0.05 M pyridine acetate pH 5), and chromatographed on a Sephadex G-50 column (45×1.6 cm). Fractions containing the separated O-PS core (~50 mg) and core oligosaccharide, detected by TLC for PS content, were collected and lyophilized.

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[1] This paper is dedicated to the memory of Dr. Alexandre Haeffner (Wissembourg, France) who inspired M.C. to join the world of research.

Abbreviations: COSY, correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; HSQCTOCSY, HSQC with TOCSY step; MALDI, matrix-assisted laser desorption-ionization; NOESY, nuclear Overhauser enhancement spectroscopy; PS, polysaccharide; TLC, thin-layer chromatography; TOCSY, total correlated spectroscopy

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2.6. Hydrofluorolysis of the O-PS core

O-PS cores (50 mg) were dissolved in cold, liquid anhydrous hydrogen fluoride (4 ml) and stirred at room temperature for 3 h [12]. HF was removed by a stream of nitrogen gas. The residue taken up in water was neutralized with dilute ammonia, deionized with Resin 101 (H^+) and AG 3-X4 (OH^-), reduced with $NaBH_4$, and chromatographed by Sephadex G-50 gel filtration to yield O-PS (30 mg).

2.7. Gas chromatography

Alditol acetates were analyzed by gas chromatography on HP-5 (Hewlett Packard, 30 m×0.32 mm) capillary column programmed for 180°C to 220°C (2°C/min) and 0.6 kPa.

Optical rotation was determined in 10 cm capillary tubes using a Perkin Elmer 243 polarimeter.

2.8. Mass spectrometry

Matrix-assisted laser desorption-ionization mass spectrometry (MALDI/MS) was carried out on a Perseptive Voyager STR model (PE Biosystem, France) time-of-flight mass spectrometer (IBBMC, Orsay). 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. The masses are average masses.

2.9. Nuclear magnetic resonance (NMR) spectroscopy

^1H and ^{13}C NMR spectra were acquired using a Varian Inova 500 or 600 MHz spectrometer with the standard Varian software. The ^{13}C NMR spectra were acquired on a Varian Mercury 200 MHz spectrometer. Spectra of a D_2O solution of the HF-treated O-chain (10 mg/ml) were recorded at 25°C and at pH 7 (uncorrected for D_2O). For the detection of NH protons, spectra were recorded at 25°C in 90% H_2O/10% D_2O with the pH adjusted to 4.5 with 1 N HCl. The experiments were performed with suppression of the HDO signal at 4.79 ppm. Acquisition and processing of respective two-dimensional (2D) experiments in D_2O (COSY, TOCSY, NOESY, HSQC, HMBC and HSQCTOCSY) and in 90% H_2O/10% D_2O (COSY, TOCSY, NOESY) were performed as described previously [20,21]. The observed ^1H and ^{13}C chemical shifts are reported relative to the methyl group of internal or external acetone (δ_H 2.225 ppm, δ_C 31.07 ppm). The proton and ^{13}C chemical shifts given in Table 1 were measured from the C–H cross-peaks in the HSQC spectrum. The ^{13}C chemical shifts of the non-protonated carbons were obtained from the HMBC spectrum. The NOESY 2D experiments were recorded with mixing times of 50 and 100 ms. The 1D TOCSY experiments with various spin-lock times from 30 to 150 ms were performed as reported previously [20].

3. Results and discussion

Preliminary composition and NMR analyses of the native and O-deacylated PS were unsatisfactory. We previously found [12] that treatment of *B. bronchiseptica* and *B. parapertussis* PS with anhydrous HF, followed by reduction and gel filtration, got rid of the core domain and resulted in clear NMR spectra of the resulting O-PS. In a similar fashion the current analysis was improved by the removal of core oligosaccharide by selective hydrofluorolysis.

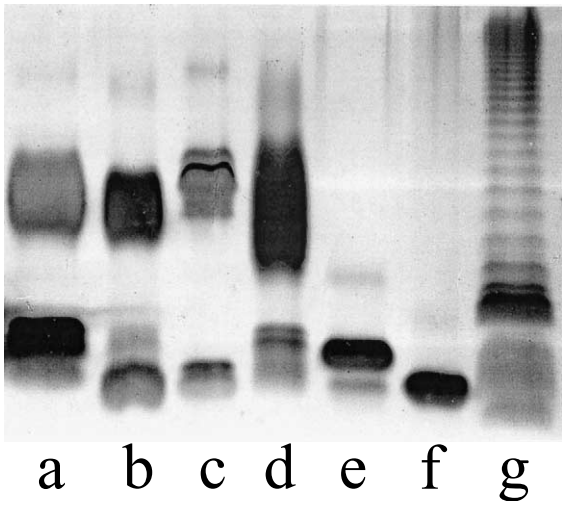

Fig. 1. SDS–PAGE of various *Bordetella* LPSs [1]: a, *B. bronchiseptica*; b, *B. parapertussis*; c, *B. hinzii*; d, *B. avium*; e, *B. pertussis* 1414; f, *B. pertussis* A100; g, *Salmonella minnesota*.

The SDS electrophoretic gel (Fig. 1) indicated that *B. avium* LPS had relatively short O-chains compared to those of *Salmonella*. Also, no ladder-like pattern of bands was obtained, suggesting that the repeating unit was a monosaccharide as found in *B. bronchiseptica* and *B. parapertussis* O-PS which have about 13 O-chain subunits. Compared to *B. hinzii* LPS which had a molecular weight of about 7000 Da including ~15 glycose O-chain residues [14], *B. avium* LPS with a similar core migrated slightly faster indicative of a shorter LPS.

The O-PS obtained by Sephadex G-50 column chromatography tested for optical rotation had $[\alpha]_D$ −77° (c 0.1, H_2O) and ^1H NMR showed the presence of one anomeric proton (4.65 ppm), and two methyl signals at 1.21 and 2.20 ppm. The sample was approximately 10% impure as judged by comparison of the integral of the resonances in the 1.9–2.1 ppm region with the integral of the methyl resonance at 1.21 ppm. The ^{13}C NMR spectrum (Fig. 2) indicated the presence of one anomeric carbon (O–C–O), two nitrogen-bearing car-

Table 1
^1H and ^{13}C NMR chemical shifts (ppm) for the HF-treated O-PS from *B. avium*

Residue		H1	H2 H2′	H3	H4	H5	H6	2-NH 3-NH	NH_a NH_b
		C1	C2	C3	C4	C5	C6		
a β-GlcA	δ_H	4.65	3.51	4.14	3.82	3.71		8.92 8.11	
	δ_C	102.7	56.6	51.8	78.8	78.3	172.9		
x 3-OH-Butanoic acid	δ_H		2.39 2.23	4.12	1.21				
	δ_C	175.2	45.3	65.2	22.4				
y Acetamidino	δ_H		2.20						8.72 8.29
	δ_C	167.0	19.3						

Spectra were obtained at 25°C in D_2O at pH 7 and in 90% H_2O/10% D_2O at pH 4.5. Error for δ_H is ±0.03 ppm and for δ_C ±0.08 ppm.

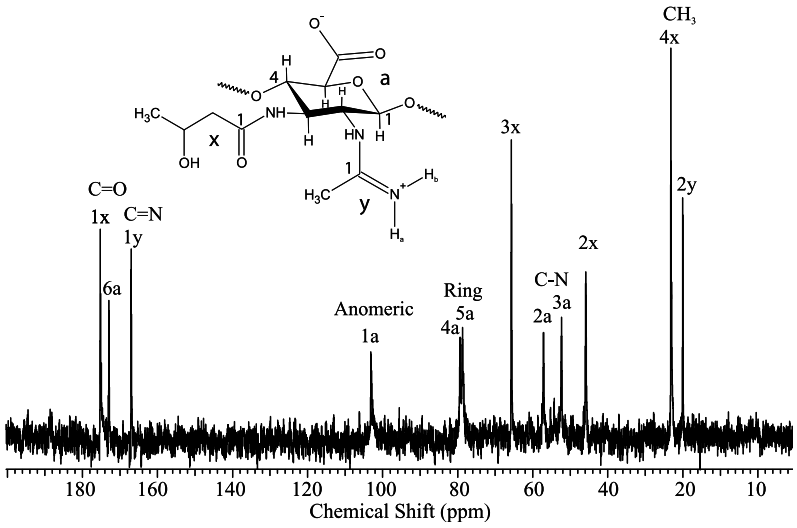

Fig. 2. 50 MHz ^{13}C NMR spectrum of the HF-treated O-chain of *B. avium* at 25°C in D_2O at pH 7. The glycose residue was designated residue **a**, 3-OH-butanoic acid **x** and the acetamidino group **y**.

bons (C–N), two methyl groups (CH_3), two signals in the carbonyl region (C=O) together with a signal at 167 ppm (C=N) indicative of an acetamidino group [22]. Assignments of the ^1H and ^{13}C NMR resonances of the HF-treated O-chain polysaccharide (Table 1) were made by 2D homonuclear and heteronuclear correlation and 1D selective NMR techniques [20]. For the ^1H NMR spectrum of the sample in H_2O, signals for four N-linked protons (NH) were observed in the region 8.1–8.9 ppm. Assignment of the NH resonances was done from COSY, TOCSY, and NOESY experiments as previously described [21–23]. The glycose residue was designated **a** and the two non-sugar components **x** and **y** and the atoms and resonances labelled as shown in Fig. 2.

3.1. NMR analysis of the monosaccharide residue (*a*)

Residue **a** was identified as a basic 2,3-dideoxy-β-glucopyranosyluronic acid residue. The correlations from H1 to H5 could be followed using COSY (Fig. 3) and TOCSY contour maps. The heterogeneity of the sample, already mentioned, did not allow measurement of coupling constants from the 1D TOCSY experiments. For 1D TOCSY experiments with a short 45 ms mixing time, the transfer of magnetization from H1 was relayed up to H3, confirming the assignment made by the COSY experiment. In the TOCSY 1D experiment with a mixing time of 75 ms, the transfer of the magnetization was relayed as far as H5, thus completing the assignment and indicating the *gluco* configuration of residue **a**. This is supported by the presence of the strong (H2, H4) and (H3, H5) NOEs (Fig. 3) due to the trans-diaxial orientation of these proton pairs in a glucose residue (Fig. 2). The strong intra-residue (H1, H3) and (H1, H5) NOEs indicated the β configuration. The (H1a, H4a) NOE was characteristic of a 1,4-glycosidic linkage in the homopolymer (Fig. 2). With the proton spectrum assigned, the ^{13}C chemical shifts of the protonated carbons were determined from the HSQC spectrum (Fig. 5). The resonance of the carboxyl group was located in the HMBC spectrum from the (H5–CO) correlation (Fig. 5). ^1H and ^{13}C chemical shifts were in accord with those of other 2,3-dideoxy-β-glucopyranosyluronic acid residues substituted at C-4 [21,23].

3.2. Identification and location of 3-hydroxybutanoic acid (*x*)

In the COSY spectrum, the methyl resonance (4x) at 1.21 ppm correlated with a methine signal (3x) at 4.12 ppm, which in turn correlated with signals for a methylene group at 2.39 and 2.23 ppm (2x and 2′x) (Table 1). The ^{13}C chemical shifts of the protonated carbons were assigned from the HSQC spectrum (Fig. 4). Assignment of the carbonyl resonance was from the isolated (2x, CO), (2′x, CO) and (4x, CO) correlations observed in the HMBC spectrum (Fig. 4). ^1H and ^{13}C chemical shifts were in agreement with those of (*S*)-3-hydroxybutanoic acid observed previously [22].

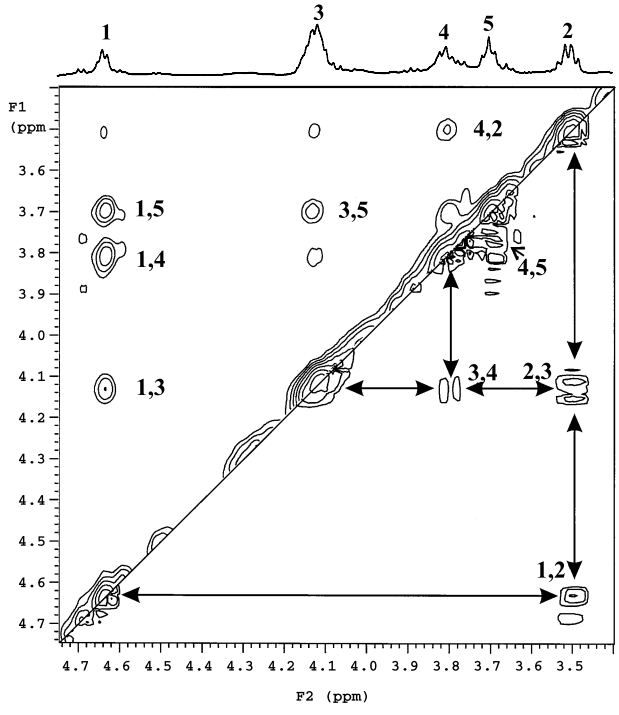

Fig. 3. 600 MHz 2D NMR spectra of the HF-treated O-chain of *B. avium* at 25°C in D_2O and at pH 7. The COSY (below the diagonal) and NOESY (above the diagonal) spectra are shown along with the corresponding proton spectrum.

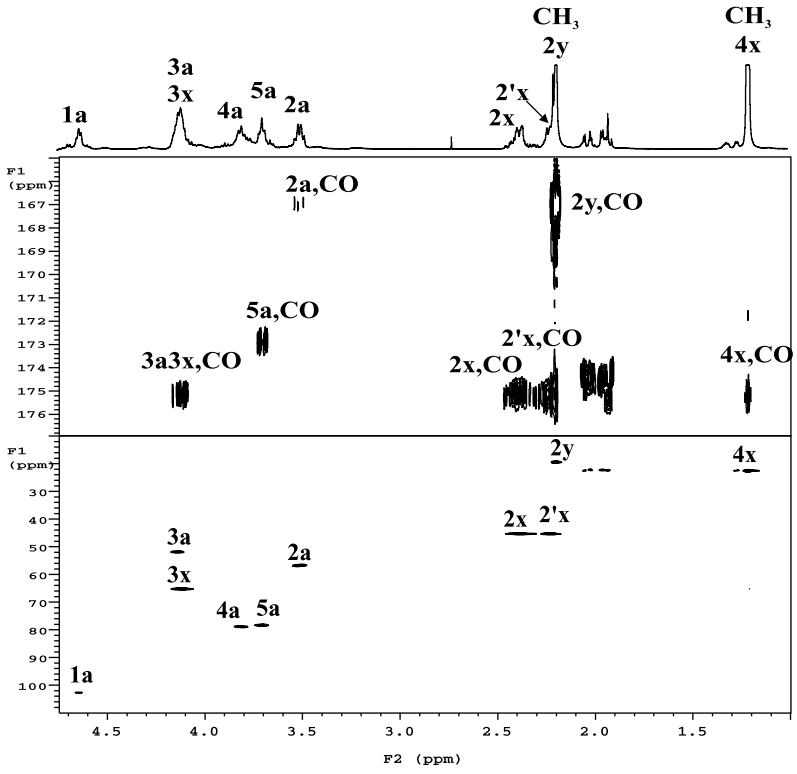

Fig. 4. 600 MHz 2D NMR heteronuclear spectra of the HF-treated O-chain of *B. avium*. The HSQC (bottom) and HMBC (top) spectra along with the corresponding proton spectrum are shown. F2 is for the ^{1}H chemical shift axis and F1 for the ^{13}C chemical shift axis.

The 3-hydroxybutanoic acid group was shown to be located on C3 of residue **a**. A correlation in the COSY and TOCSY spectra was observed between H3**a** and the NH at 8.1 ppm (3-NH) due to the large $J_{H,NH}$ coupling of 10 Hz brought about by the *trans* orientation for H–C–N–H. A NOE was then observed between the 3-NH and the 2′x and 2x resonances (Fig. 5). The *trans* orientation for H–C–N–H was also consistent with the strong (3-NH, H2a) NOE as seen in Fig.

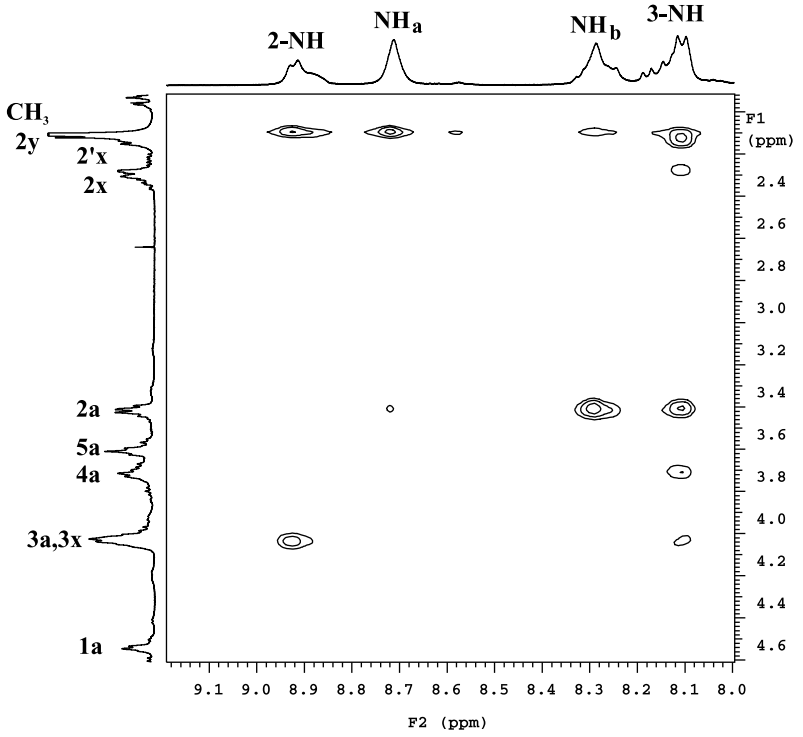

Fig. 5. 500 MHz 2D NMR NOESY spectrum of the HF-treated O-chain of *B. avium* at 25°C in 90% H_2O/10% D_2O at pH 4.5, along with the corresponding proton spectrum of the NH region.

5. The other weak NOEs to H4a and H3a are probably due to spin diffusion from H2a. A correlation between H3**a** and the carbonyl resonance at 175.2 ppm was also observed; however, this signal could also arise from the methine signal (3x) (Fig. 4).

3.3. Identification and location of the acetimidoyl group (**y**)

A long-range correlation between a carbon resonance at 167.0 ppm (C1**y**) and a methyl signal at 2.20 ppm (2y) (Fig. 4) indicated the presence of an acetimidoyl group [22]. At low pH, the amidino group ($C=N^+H_2$) is protonated and its proton resonances were observed in a spectrum acquired in 90% H_2O (Fig. 5). A strong NOE between the NH signal at 8.7 ppm (NH_a) and the one at 8.3 ppm (NH_b) was observed in the NOESY experiment due to the small interproton distance for NH_2. No COSY or TOCSY correlations were detected between these two resonances as expected from previous observations [22]. Another strong NOE was observed between NHa and the CH_3 2**y** resonance and a weaker one between NHb and 2y. This indicated a *cis* orientation for Ha–N–C–CH_3 and a *trans* for Hb–N–C–CH_3 as depicted in Fig. 2.

The 2-NH resonances were assigned from the COSY and TOCSY spectra where a crosspeak (2-NH, H2a) is observed due to the *trans* orientation for H–C–N–H and the resulting large $J^3_{H,NH}$ coupling of 9–10 Hz [22,23]. The (2-NH, CH_3 2y) NOE (Fig. 5) and the (H2a, CN) HMBC correlations (Fig. 4) were also indicative of an acetamidino group located at C2 of **a**.

The (2-NH, CH_3 2y) NOE observed in Fig. 5 is consistent with the H–N–C–CH3 having a *cis* orientation. Hence, from the other dihedral angles, H–C–N–H and Hb–N=C–CH_3, determined above, Hb will be in close proximity to H2a, consistent with the strong NOE observed between NHb and H2a (Fig. 5). Finally, the *trans* orientation for H3a–C3a–N–H explains the (2-NH, H3a) NOE (Fig. 5).

In the present investigation, it was shown by 1D and 2D NMR analyses of the HF-treated O-chain polysaccharide of *B. avium* that the PS is composed of a 1,4-linked 2-acetamidino-3-[3-hydroxybutanamido]-2,3-dideoxy-β-glucopyranosyluronic acid residue (Fig. 2).

The structure of the O-chain subunit was confirmed from the mass data obtained in the negative ion MALDI spectra of the isolated HF-treated polysaccharide (Fig. 6). Molecular ion signals, multiples of 302 mass units, were observed up to 4000 Da. These masses (minus H_2O) were consistent with the calculated molecular weight of the sugar residue as deduced from NMR analysis. Two series of peaks were observed, adjacent peaks in each series differing by a mass of 302 u. The series starting at *m/z* 924 was made of polymers of the subunit plus a molecule of water. The second at *m/z* 748 contained in addition an unidentified fragment of the core. Major peaks of masses up to 12 repeating units were observed. The variation in chain length is due to natural heterogeneity.

The spectra of native O-chains also gave a series of peaks separated by the same mass increment (not shown). It was thus evident that no modification of the O-chains occurred during the HF treatment.

It is of note that the observed structure is the third one involving a monosaccharide repeating unit in the genus. It is also remarkable that the diversity of O-chain structures in *Bordetellae* seems to be restricted to 2,3-diaminohexuronic acid derivatives. *B. parapertussis* and *B. bronchiseptica* O-chains are composed of the same repeating unit of 2,3-diacetamido-2,3-dideoxy-galacturonic acid. *B. pertussis* does not have an O-chain, but its core is substituted by a trisaccharide containing a 2,3-diacetamido-2,3-dideoxy-mannuronic acid residue. The *B. hinzii* O-chain was found to be made of a repeating unit of *gluco* (one unit) and *galacto* (two units) isomers of the 2,3-diaminohexuronic glycose [14]. The structure determined in this report for the O-PS of *B. avium* repeating unit has the *gluco* configuration. Thus, the structural originality of the *Bordetella* LPS, already reported [1], seems to extend to O-chain structures which are highly similar, each being a homopolymer of one or two different isomers of un-

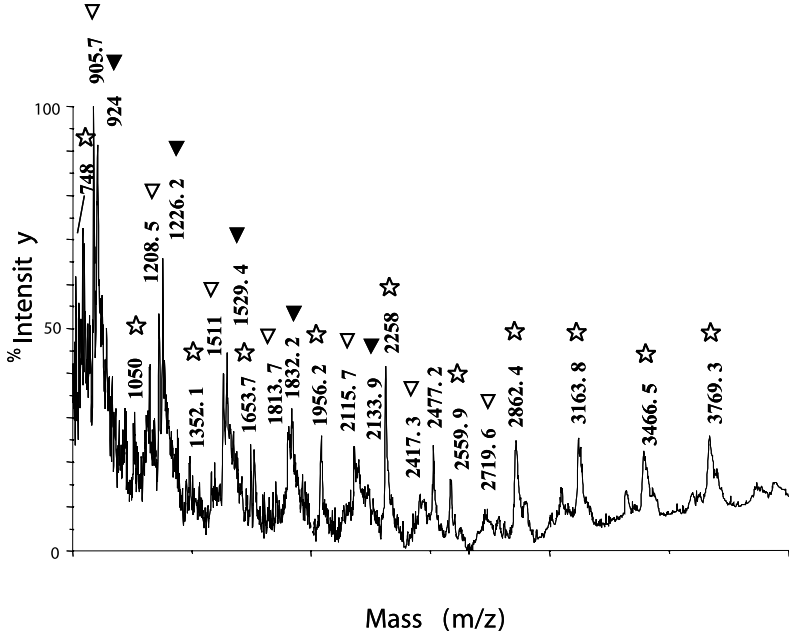

Fig. 6. MALDI-TOF negative ion spectrum of the HF-treated O-chain of *B. avium*. ▼, peaks corresponding to pure O-chain molecular species; ▽, peaks corresponding to the dehydrated form; ☆, peaks corresponding to O-chain units linked to a fragment of the core.

usual diaminohexuronic acids which carry different acyl substituents on the amino groups.

It will be interesting to see if the O-chains of other *Bordetella* species follow the same pattern, this will allow us to determine if this is a characteristic of the genus.

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