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A polysaccharide of *Alloiococcus otitidis*, a new pathogen of otitis media: chemical structure and synthesis of a neoglycoconjugate thereof

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Abstract—*Alloiococcus otitidis* is a recently discovered Gram-positive bacterium that has been linked with otitis media (middle ear infections). In this study, we describe the structure of a novel capsular polysaccharide (PS) expressed by the type-strain of *A. otitidis*, ATCC 51267, and the synthesis of a glycoconjugate composed of the capsule PS and bovine serum albumin (BSA). The capsule PS of *A. otitidis* type-strain was determined to be a repeating trisaccharide composed of 3-substituted *N*-acetyl-D-glucosamine (Glc*p*NAc), 6-substituted *N*-acetyl-D-galactosamine (Gal*p*NAc), and 4-substituted D-glucuronic acid (Glc*p*A), of which the majority was amidically decorated with L-glutamic acid (Glu): $\{\rightarrow 6)$-β-Gal*p*NAc-(1→4)-[Glu*p*→6]-β-Glc*p*A-(1→3)-β-Glc*p*NAc-$(1\}_n$. Monomeric analysis performed on other *A. otitidis* strains revealed that similar components were variably expressed, but Glu appeared to be a regular constituent in all the strains examined. Due to the suitable presence of Glc*p*A and Glu, our approach for glycoconjugate synthesis employed a carbodiimide-based strategy with activation of available carboxyl groups by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), which afforded direct coupling between the capsule PS and BSA. Analysis by mass spectrometry indicated that this *A. otitidis* capsule PS–BSA conjugate was composed of BSA units that carried up to seven capsule PSs. This work represents the first report in the literature describing an *A. otitidis* cell-surface carbohydrate and the synthesis of a glycoconjugate preparation thereof. Presently, we are formulating plans to immunologically evaluate this *A. otitidis* glycoconjugate vaccine in animals.

Keywords: *Alloiococcus otitidis*; Otitis media; Capsule polysaccharide; Neoglycoconjugate; Glutamic acid; Structural characterization

1. Introduction

Middle ear infections (otitis media) represent one of the most serious ailments that afflict children.[1] The pathogens, *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae*, and *Moraxella catarrhalis*, have become well recognized as those being associated with otitis media.[2] However, in the late 1980s, Faden and Dryja reported the identification of a new Gram-positive bacterium that showed a strong association with childhood otitis media.[3] Subsequently, this unknown cocci was given the name *Alloiococcus otitidis*.[4,5] A limited number of investigations have now furnished data demonstrating a direct connection between *A. otitidis* infections and otitis media, and in fact *A. otitidis* is now being more frequently detected in otitis media patients.[6–8] Moreover, it has been noted that the incidence of *A. otitidis* is significantly high in the nasopharynx of children prone to ear infections,[9] and that, even upon treatment with antibiotics, in most cases the pervasiveness of

Abbreviations: BSA, bovine serum albumin; EDC, 1-ethyl-3-(3 dimethylaminopropyl)carbodiimide hydrochloride; EI, electron impact; ESI, electrospray ionization; GLC, gas–liquid-chromatography; MALDI-TOF, matrix assisted laser desorption ionization; MS, mass spectrometry; NMR, nuclear magnetic resonance; PS, polysaccharide; TOF, time of flight

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A. otitidis remained remarkably high.[10] A recent study by Harimaya and co-workers[11] showed evidence of local antibody response against *A. otitidis* in the middle ear effusions of patients. The observed presence of IgG, secretory IgA, IgG2, and IgM against *A. otitidis* suggested that specific local immune response against *A. otitidis* is induced during middle ear infection with this organism.[11] The complete genome sequence of *A. otitidis* strain 1104-92 is available as a patent application,[12] with defined polynucleotide sequences encoding putative antigenic polypeptides encoded by *A. otitidis* strain 1104-92 open reading frames being described.

Similar to many other Gram-positive bacteria, *A. otitidis* should express polysaccharides (PSs) on its cell-surface, such as capsule, teichoic-, and lipoteichoic-acid PSs. Hence, we focused on the extraction, purification, and chemical structural determination of the covalent structure of *A. otitidis* carbohydrate polymers with the intention of using them in vaccine preparations. In this study, we describe the structural characterization of the capsule PS expressed by the type-strain of *A. otitidis*, ATCC 51267, and the synthesis of a glycoconjugate composed of the capsule PS and bovine serum albumin (BSA), whose efficacy as a vaccine is to be evaluated in animal models.

2. Experimental procedures

2.1. Bacterial growth conditions

A. otitidis type strain ATCC 51267 was isolated from a child with middle ear infection,[3] strains SS1337, 1514-89, 1515-89, 1518-89, 1996-94, and 2621-96 were also of a similar clinical origin. All strains were obtained from the Centre for Disease Control and Prevention (Atlanta, USA). *A. otitidis* were grown at 37 °C in a Bacto Todd Hewitt Broth and Tween 80 mixture with a stirring rate of 60 rpm for 3 days. Cells were harvested by centrifugation at 5000*g* at 4 °C for 30 min. On average, each 4 L batch yielded 2 g of cell paste. The harvested cells were killed by treatment with 1% phenol and kept in storage at −20 °C.

2.2. Extraction and purification of capsule PS

The lyophilized cells of *A. otitidis* ATCC 51267 cells were subjected to 2% acetic acid at 100 °C for 2 h to cleave the putative glycosyl phosphate bridge that links the cell-surface PSs of Gram-positive bacteria to the peptidoglycan layer. The supernatant, obtained after low-speed centrifugation, was dialyzed (1000 amu cut-off limit), lyophilized, and passed through a size exclusion column (1 m × 1 cm) of Bio-Gel P-6 with water as eluent. Carbohydrate containing fractions were detected by the phenol-sulfuric acid assay method,[13] and were further scrutinized by one-dimensional (1D) nuclear magnetic resonance spectroscopy (NMR) prior to any chemical manipulations. Typically, 1.5 mg of carbohydrate material was obtained from 2 g of cell paste.

2.3. Sugar composition analysis and linkage analysis

Monosaccharide composition analysis was performed by the alditol acetate method.[14] The glycosyl hydrolyses were carried out with 4 M trifluoroacetic acid at 105 °C for 5 h followed by reduction in H_2O with $NaBD_4$ overnight at room temperature, or at 80 °C to reduce the glycuronic acid unit, and subsequent acetylation by acetic anhydride with residual sodium acetate as the catalyst at 100 °C for 2 h. The alditol acetate derivatives were analyzed by gas liquid chromatography (GLC) using a Varian 3400 gas chromatograph equipped with a 30-m DB-17 capillary column [210 °C (30 min)→ 240 °C at 2 °C/min], and by GLC–mass spectrometry (GLC–MS) in the electron-impact (EI) and chemical-ionization (CI) modes in a ThermoFinigan PolarisQ instrument. Sugar linkage analysis was performed by the methylation procedure[15] ($NaOH–Me_2SO–CH_3I$) and with characterization of the permethylated alditol acetate derivatives by GLC–MS in the electron impact mode (DB-17 column, isothermally at 190 °C for 60 min). Relative retention times were assigned with respect to glucose (rrt^{Glc}). Enantiomeric configurations of the individual components were determined by the formation and characterization of the respective 2-(*S*)- and 2-(*R*)-butyl chiral glycosides.[16]

2.4. Mass spectrometry

A fraction of the methylated sample was used for analysis by direct electron impact-MS (EI-MS) in the positive mode at 70 eV on an Agilent 6890 GLC–MS instrument coupled to a Micromass/Waters GCT time of flight mass spectrometer. The methylated sample was dissolved in CH_2Cl_2. The interpretations of the positive ions obtained from EI-MS of the methylated material were similar to those described previously by Dell.[17] Electrospray-MS (ES-MS) was obtained with a qTOF ULTIMA GLOBAL instrument (Waters) in the positive ion mode with a capillary (needle) voltage of 1.8 kV, a source temperature of 80 °C, a desolvation gas temperature of 200 °C, and a CID voltage for MS/MS of 30 V. The intact material was dissolved in 0.5% acetic acid in acetonitrile–water buffer, whereas the permethylated material was dissolved in a methanol–water–sodium acetate mixture. The matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS experiments were carried out in a MALDI Micro MX instrument operated in the linear mode with N_2 laser source (337 nm) and positive ion detection.

Samples for analysis were mixed with sinapinic acid matrix, and 1–2 μL was deposited on a plate to dry (dry droplet method) and then placed in the spectrometer.

2.5. Nuclear magnetic resonance spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 400 spectrometer at 293 K using standard Bruker software. Prior to performing the NMR experiments, the samples were lyophilized three times with D_2O (99.9%). The HOD peak was used as the internal reference at δ_H 4.821 for ^{1}H NMR spectroscopy, and orthophosphoric acid (δ_P 0.0) as external reference for ^{31}P NMR experiments. Just before the NMR experiments were carried out, a D_2O sample containing TMS (δ_H 0.00) was run to reference the HOD signal.

2.6. Conjugation of capsule PS to BSA

The capsule PS of *A. otitidis* ATCC 51267 was dissolved in 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer (pH 5.5), followed by the addition of 1-ethyl-3(3-dimethylamino-propyl) carbodiimide (EDC) in a 1:1 weight ratio to the capsule PS (adopted from Ref. 18).[18] Bovine serum albumin (BSA) was immediately added in the same weight ratio and the pH was maintained at 5.5 using MES-acid buffer (0.1 M). The reaction was carried out for 4 h at room temperature with stirring. The reaction was stopped by raising the pH value to 7 by sodium phosphate buffer (pH 8). The mixture was dialyzed against water for two days. The lyophilized material was analyzed by MALDI-TOF-MS. In this instance, due to the small quantities, no attempt was made to purify the glycoconjugate mixture by size exclusion chromatography.

3. Results

3.1. GLC–MS analysis of the water-soluble extract from *A. otitidis* ATCC 51267

As a starting point in our investigation, we decided to analyze the crude water-soluble preparation that was obtained from the mild-acid treatment of bacterial cells. Monosaccharide composition analysis of the water extract, performed by the characterization of alditol acetate derivatives by GLC–MS (EI and positive CI mode) and with comparison with well defined chemical standards, revealed the presence of *N*-acetyl-glucosamine (GlcNAc) and *N*-acetyl-galactosamine (GalNAc) as major components, with $rrt^{Glc} = 1.33$ and $rrt^{Glc} = 1.41$, respectively. Glycerol (Gro) at $rrt^{Glc} = 0.19$ and Glc ($rrt^{Glc} = 1.00$) were also observed. Upon analysis in CI mode it was observed that a portion of the Glc derivative contained two extra atomic mass units (amu), $[M+H-60]^+ = 378$ and $[M-H]^- = 436$, which inferred the presence of glucuronic acid (GlcA) (from the inclusion of two ^{2}H atoms during reduction). In the vicinity of the Gro derivative, two significant peaks at $rrt^{Glc} = 0.23$ and $rrt^{Glc} = 0.35$ were determined to be derivatives of glutamic acid (Glu). The monosaccharides mentioned above were determined to possess the D absolute configuration and the Glu residue was confirmed to be present as the L enantiomer.

Sugar linkage-type analysis carried out by the characterization of the permethylated alditol acetate derivatives (GLC–MS in EI mode) showed 3-substituted Glc*p*NAc [→3)-Glc*p*NAc-(1→] and 6-substituted Gal*p*NAc [→6)-Gal*p*NAc-(1→] as the dominant units, and, in lower concentrations, tri-substituted Gro, terminal Glc*p* [Glc*p*-(1→], 2-substituted Glc*p* [→2)-Glc*p*-(1→], 4-substituted Glc*p* [→4)-Glc*p*-(1→], and terminal Gal*p*NAc [Gal*p*NAc-(1→], and Glc*p*NAc [Gal*p*NAc-(1→]. Notably, the 4-substituted Glc*p* mentioned above appeared to emanate from a Glc*p*A residue in that the typical *m*/*z* 233, of a 4-substituted permethylated glucitol acetate, was substituted by *m*/*z* 235. This preliminary analysis showed that the water-soluble preparation of *A. otitidis* ATCC 51267 contained a variety of monosaccharide components, Gro, Glc, GlcA, GlcNAc and GalNAc, and one amino acid, Glu, and that GlcNAc and GalNAc appeared to be the main monosaccharide components.

3.2. GLC–MS analysis of *A. otitidis* ATCC 51267 cell-surface polysaccharides

After mild acetic acid treatment of cells, two main carbohydrate fractions, PS-1 (major) and PS-2 (minor), were obtained by purification of the hydrolysate by size exclusion chromatography. Monosaccharide composition and linkage-type analyses carried out on the fastest eluting fraction (PS-1) revealed that it contained Glc*p*NAc as [→3)-Glc*p*NAc-(1→] and Gal*p*NAc as [→6)-Gal*p*NAc-(1→]. Small amounts of terminal Gal*p*NAc and Glc*p*NAc were also observed to be present in PS-1. Some 4-substituted Glc*p* originating from Glc*p*A, as seen in the previous section, was also detected by GLC–MS here. No other recognizable peaks corresponding to a GlcA permethylated alditol acetate derivative were seen by GLC–MS; however, when the permethylated alditol acetate mixture was analyzed by ESI-MS, an *m*/*z* ion at 393.4 was readily observed, which revealed the presence of a di-substituted GlcA unit. Of much less intensity, the mono-substituted Glc*p*A derivative (that which yielded 4-substituted Glc*p*) was also observed at *m*/*z* 365.2. Analysis of the monosaccharide derivatives showed that PS-1 was composed of mono-substituted [→3)-Glc*p*NAc-(1→] and [→6)-Gal*p*NAc-(1→], and of a Glc*p*A residue, mainly present as a di-substituted residue.

The second fraction (PS-2) was composed of phosphate (detected by ^{31}P NMR), terminal and 2-substituted Glc*p*, and trisubstituted Gro. Because PS-2 contained emblematic units of teichoic-acid PSs, namely, Gro and phosphate, PS-2 was designated as being a teichoic-acid PS. Subsequently, we designated PS-1 as the capsule PS.

3.3. Mass spectrometry analysis of *A. otitidis* ATCC 51267 capsule PS

A portion of the intact capsule PS was analyzed by ESI-MS in combination with a TOF component. The ESI/TOF-MS experiment yielded a variety of multiple charged *m*/*z* ions, of which the quadruply charged *m*/*z* 1427.08 ion was selected for further MS/MS experiments. Figure 1 shows the ESI/TOF-MS/MS experiment carried out on *m*/*z* 1427.08^{+4} ion, which provided *m*/*z* ions of defined composition at *m*/*z* 305.96 that represented a GlcA carrying the amino acid Glu; *m*/*z* 379.98 for a disaccharide composed of one HexNAc and one GlcA without Glu; *m*/*z* 508.98 for a disaccharide composed of one HexNAc and one GlcA-(Glu) unit; *m*/*z* 582.99 for a trisaccharide containing two HexNAc units and one GlcA residue; *m*/*z* 711.99 for a trisaccharide containing two HexNAc units and one GlcA(Glu) residue; *m*/*z* 786.52 for a tetrasaccharide composed of three HexNAc units and one GlcA; *m*/*z* 914.98 for a tetrasaccharide composed of three HexNAc units and one GlcA(Glu); *m*/*z* 1090.99 for a pentasaccharide of three HexNAc units, one GlcA, and one GlcA(Glu) residue; *m*/*z* 1220.00 for a pentasaccharide composed of three HexNAc units and two GlcA(Glu) residues; and *m*/*z* 1423.05 for a hexasaccharide containing four HexNAc residues and two GlcA(Glu) units. To confirm the presence of a GlcA(Glu) residue, an ESI-MS was performed on the fully methylated monomeric units, which were obtained by methylation after acid hydrolysis of the capsule PS, and it showed an *m*/*z* at 422.3 corresponding to the MH^+ of the fully methylated GlcA(Glu). The data obtained from this ESI/TOF-MS/MS experiment afforded structural data indicative of a capsule PS composed of a trisaccharide with two HexNAc units (GlcNAc and GalNAc as shown by GLC–MS), and one GlcA which was non-stoichiometrically substituted by the amino acid Glu. The relative intensities between ions *m*/*z* 379.98 [GalNAc–GlcA] and 508.98 [GalNAc–GlcA(Glu)] suggested that the majority of the GlcA units may be substituted by the Glu moiety.

To obtain monosaccharide sequence information, an EI-MS experiment (Fig. 2) was performed on the methylated capsule PS preparation that yielded *m*/*z* ions from the non-reducing end of the capsule PS. Primary type glycosyloxonium ions, and secondary ions (in brackets) arising from β-elimination (−32 amu) and double-cleavage (−14 amu) processes, were observed at *m*/*z* 260 (228; 246) for [HexNAc], 505 (473; 491) for [HexNAc→HexNAc], 635 (603; 621) for [HexNAc→GlcA(Glu)], and at 880 (848; 866) for [HexNAc→HexNAc→GlcA(Glu)] or [HexNAc→GlcA(Glu)→HexNAc], which were indicative of a trisaccharide repeating block composed of two HexNAc units (GalNAc and GlcNAc) and one GlcA-(Glu) residue. A secondary ion at *m*/*z* 691 (from 723-32) was also observed, which indicated the presence of a trisaccharide repeat at the non-reducing end in which the GlcA residue did not carry a Glu unit [HexNAc→HexNAc→GlcA→...]. No *m*/*z* ions belonging

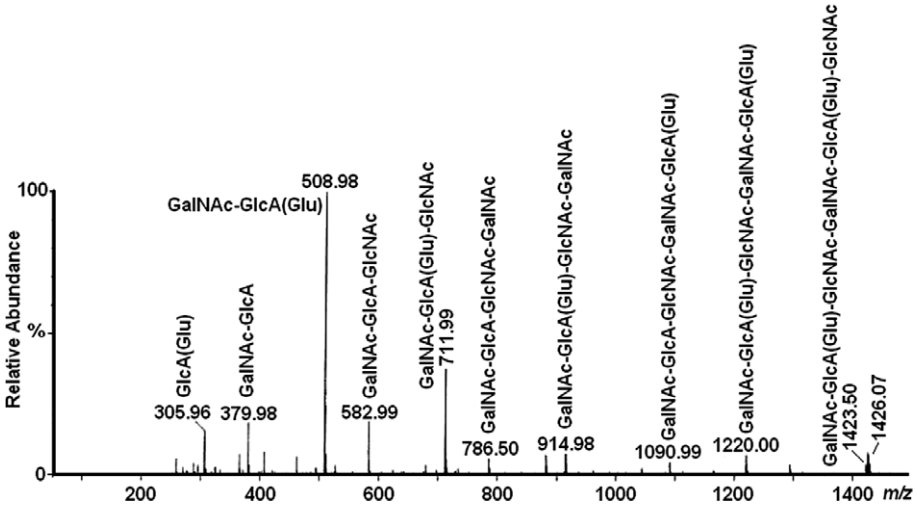


Figure 1. Spectrum of ESI/TOF-MS/MS experiment carried out on *m*/*z* 1427.08 showing daughter *m*/*z* ions of defined composition that revealed the presence of a chain block composed of *N*-acetyl-hexosamine (GalNAc and GlcNAc) and hexuronic acid (GlcA), which could be substituted by a glutamic acid (Glu) residue. The sequences shown contain the GalNAc, GlcNAc, GlcA, and Glu designations (the units previously characterized by GLC–MS).

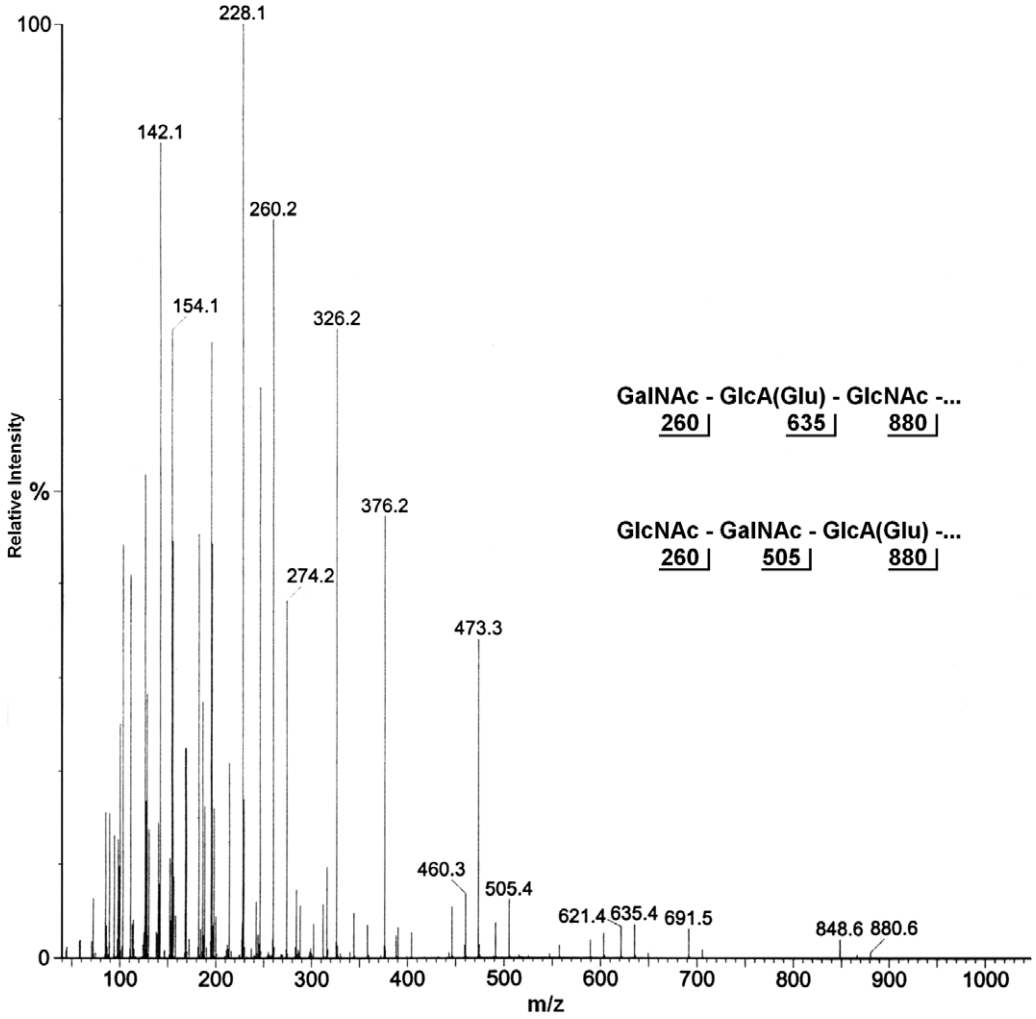


Figure 2. EI-MS spectrum of the methylated capsule PS showing primary glycosyl oxonium ions and respective secondary *m*/*z* ions emanating from the non-reducing-end, which specify the triglycosyl sequence of the capsule PS. The two trisaccharide structures shown represent the termini blocks present at the non-reducing-end of the capsule PS.

to terminal GlcA (*m*/*z* 233) or GlcA(Glu) (*m*/*z* 390) were observed, which pointed to the fact that these units may not be present as termini at the non-reducing end of the capsule PS.

With the intent of obtaining data pertaining to the molecular weight of the capsule PS, a portion of intact material was subjected to MALDI-TOF-MS analysis. Two capsule PS preparations, obtained from two separate growth batches, were analyzed. One capsule PS preparation, the same used in the ESI/TOF-MS study described above, afforded a high mass *m*/*z* ion at 5714, the *m*/*z* ion from which the quadruply charged *m*/*z* ion 1427.08^{+4} (ESI/TOF-MS) originated. A second capsule PS preparation (Fig. 3) afforded several *m*/*z* ions ranging from 5700s to 6900s with the most intense being observed at *m*/*z* 6554. The observed *m*/*z* ion at 13175 represents a non-covalent cation-bound dimer species, $[2M+H]^{+}$, which are characteristic artifacts of MALDI-TOF-MS experiments. The MALDI-TOF-MS data indicated that the capsule PS was composed of approximately 8 (5714 ÷ 712) or 9 (6554 ÷ 712) repeating trisaccharide units fully substituted with Glu: [HexNAc→GlcA(Glu)-(1→3)-HexNAc].[8,9]

3.4. Nuclear magnetic resonance spectroscopy analysis of *A. otitidis* ATCC 51267 capsule PS

The ^{1}H NMR spectrum (Fig. 4) of the capsule PS showed solely β anomeric proton resonances between δ_H 4.32 and δ_H 4.62. No α anomeric resonances were observed. Also readily observed were sharp singlets typical of *N*-acetyl moieties between δ_H 2.03 and δ_H 2.06, and unresolved multiplets characteristic of deoxy protons at δ_H 1.95 and at δ_H 2.27. An array of 2D NMR experiments were carried out to assign the capsule PS fine structural features and to re-affirm the structural conclusions previously obtained by MS analysis.

A 2D ^{1}H–^{1}H COSY spectrum (Fig. 5) revealed the presence of five anomeric-related $H_{1,2}$ cross-peak resonances, which were labeled A, B, C, D, and E. The 2D

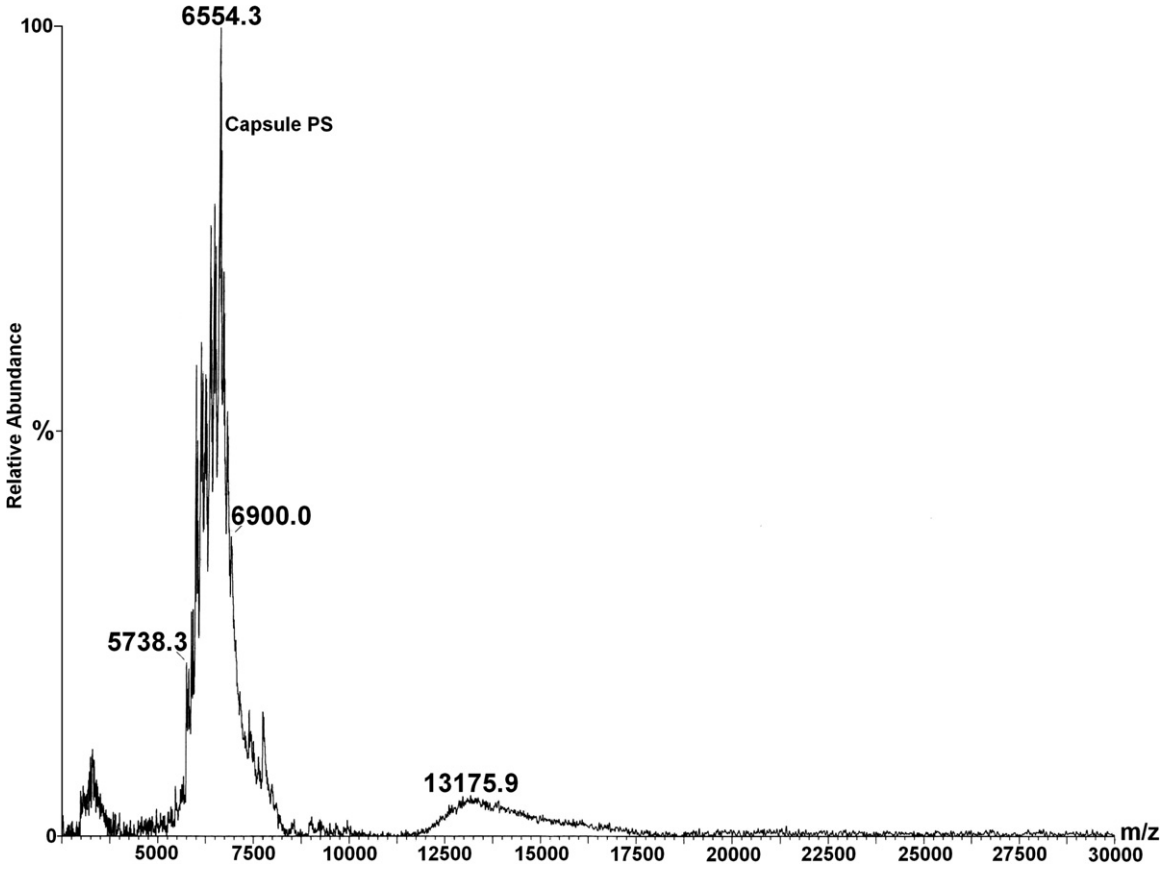


Figure 3. MALDI-TOF-MS spectrum of the capsule PS obtained from mild-acid treatment of cells.

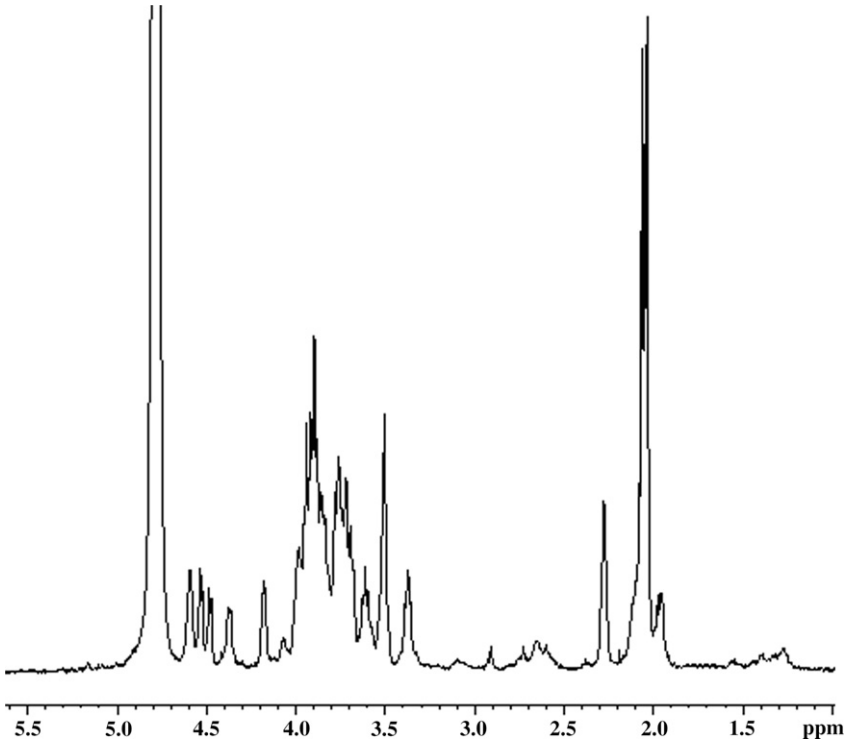


Figure 4. ^{1}H NMR spectrum of capsule PS showing the presence of β anomeric resonances (δ_H 4.35–4.65), of singlets belonging to methyl groups of *N*-acetyl-hexosamines (δ_H 2.06), and of methylene moieties of Glu amino acid (δ_H 1.96) and (δ_H 2.26).

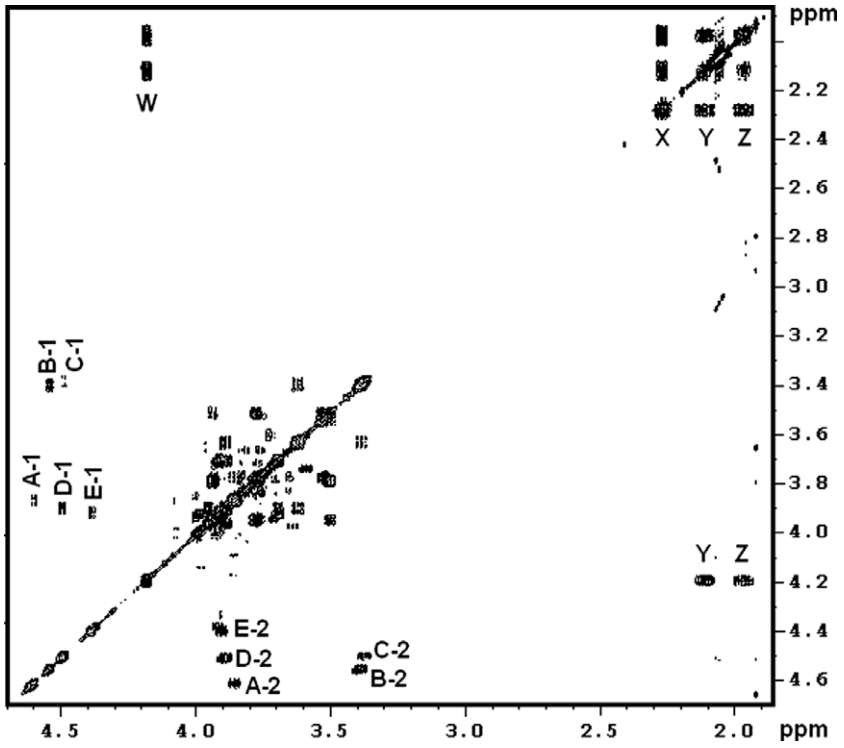


Figure 5. 2D ^{1}H–^{1}H COSY spectrum of capsule PS showing five distinct β anomeric resonances (labeled A, B, C, D, and E), and also the spin-system belonging to the Glu residue (W, X, Y, and Z).

^{1}H–^{1}H COSY spectrum also showed a correlation between δ_H 4.17 (W) of a CH unit, and those of methylenes (CH_2) at δ_H 2.27 (X), δ_H 2.08 (Y), and δ_H 1.95 (Z), which revealed that these resonances shared a structural relationship. A total correlation spectroscopy experiment (2D ^{1}H–^{1}H TOCSY) was performed, which provided data that readily established the relationship previously observed between the W, X, Y, and Z protons, and assigned the resonances of the monosaccharide ring proton resonances, which are individually shown in Table 1.

A 2D ^{1}H–^{13}C HSQC experiment (Fig. 6A) afforded distinct anomeric carbon resonances at δ_C 99.6 (E), δ_C 100.8 (A), δ_C 101.0 (D), δ_C 102.8 (C), and δ_C 103.0 (B). With the aid of the previously described 2D ^{1}H–^{1}H correlation experiments, most of the monosaccharide carbon resonances were assigned (Table 1). The ^{1}H–^{13}C HSQC spectrum also showed carbon signals belonging to the GlcNAc and GalNAc units, with resonances at δ_C 52.2 (unit D/E) and δ_C 54.4 (unit A), which are characteristic of C-2 of *N*-acetyl-hexosamine units, and a broad carbon resonance at δ_C 21.3 for the *N*-acetyl moieties. Of particular note, carbon resonances at δ_C 55.8 (correlating to proton resonance W), δ_C 34.0 (correlating to proton resonance X), and δ_C 28.1 (correlating to proton resonances Y and Z) confirmed that

Table 1. ^{1}H NMR and ^{13}C NMR chemical shifts (ppm) of monosaccharide units present in *A. otitidis* type-strain ATCC 51267 capsule PS

	H_1/C_1	H_2/C_2	H_3/C_3	H_4/C_4	H_5/C_5	H_6/C_6	$H_{6'}/C_6$
A →3)-β-D-GlcNAc-(1→	4.600/100.8	3.865/54.4	3.775/82.5	3.518/68.6	3.470/75.5	3.750/60.6	3.920/60.6
B →4)-β-D-GlcA(Glu)-(1→	4.535/103.0	3.389/72.0	3.615/73.6	3.880/73.3	3.940/74.3	–/169.0	
C →4)-β-D-GlcA-(1→	4.485/102.8	3.376/72.0	3.600/73.6	3.720/80.0	3.700/76.2		
D →6)-β-D-GalNAc-(1→	4.500/101.0	3.900/52.2	3.660/70.9	3.890/67.5	3.750/72.5	4.030/66.0	3.940/66.0
E →6)-β-D-GalNAc-(1→	4.380/99.6	3.915/52.2	3.700/70.9	3.890/67.4	3.710/72.5	3.870/66.8	4.020/66.8

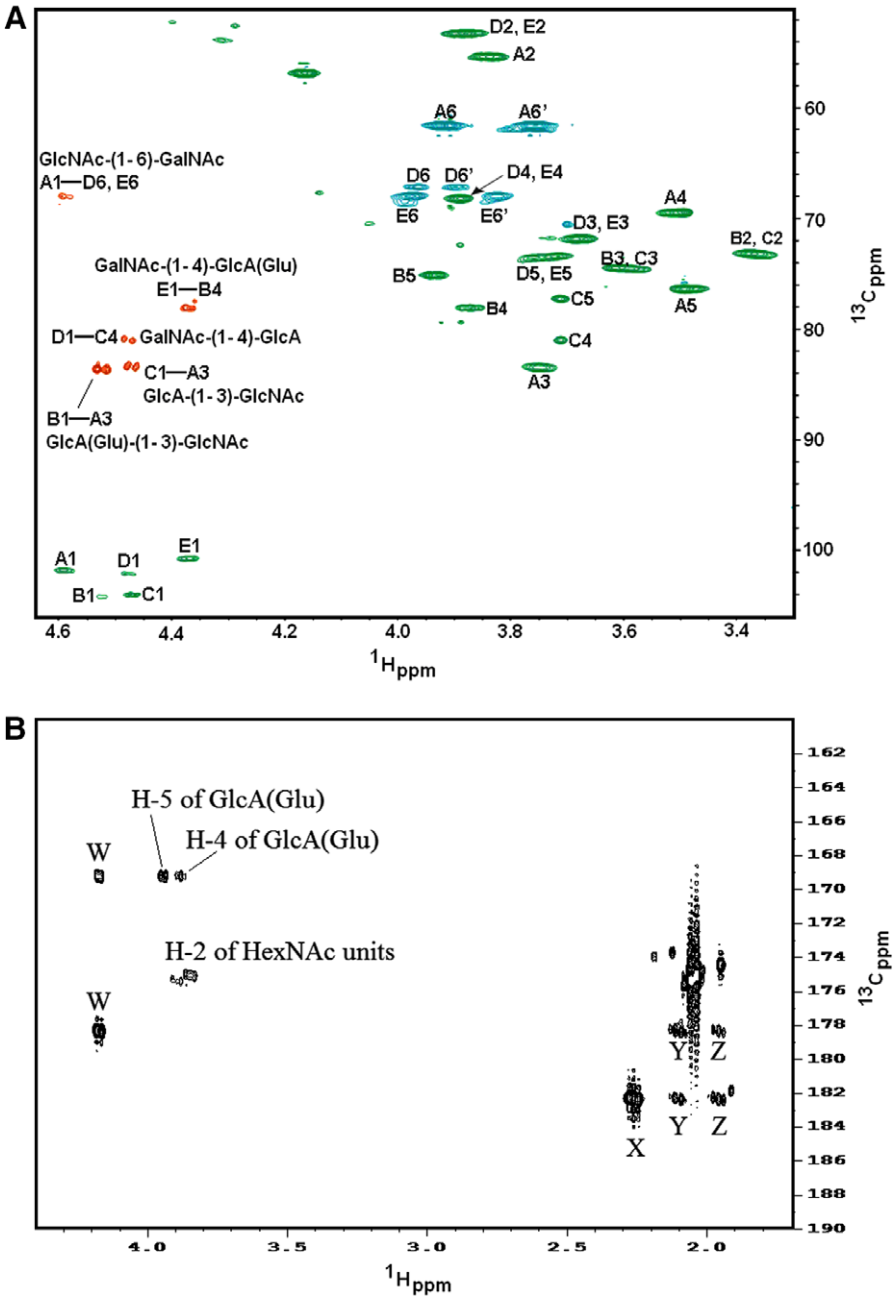


Figure 6. (A) The assignment of carbon resonances of capsule PS by ^{1}H–^{13}C HSQC (green and blue), and the detection of through-bond glycosyl linkages by ^{1}H–^{13}C HMBC (red). (B) Expansion of the ^{1}H–^{13}C HMBC spectrum displaying region containing the connectivities between the carbonyls of Glu and GlcA and neighboring protons.

these proton resonances emanated from CH (W) and CH_2 (X, Y, and Z) structural moieties. Specifically, the proton and carbon resonance of W suggested that the carbon atom in this CH moiety was attached to a nitrogen atom.

To investigate if there was a nitrogen-carrying moiety in the capsule PS, a ^{1}H NMR spectrum was performed in 90% H_2O and it showed a distinct NH resonance at 8.63, which was subsequently shown through a ^{1}H–^{1}H COSY correlation with proton resonance W (δ_H 4.17). These data revealed that indeed the capsule PS expressed a CH–NH moiety, and in turn, based on the previous correlations observed, the following moiety could be concluded to be present: HN–CH–CH_2–CH_2, the side-chain of the amino acid, Glu.

To gain more concrete information on the monosaccharide sequence, and to observe the carbonyl units of the Glu unit, a ^{1}H–^{13}C HMBC experiment (Fig. 6A and B) was carried out on the capsule PS. Connectivities (Fig. 6A) were observed between H-1 of B and C-3 of A

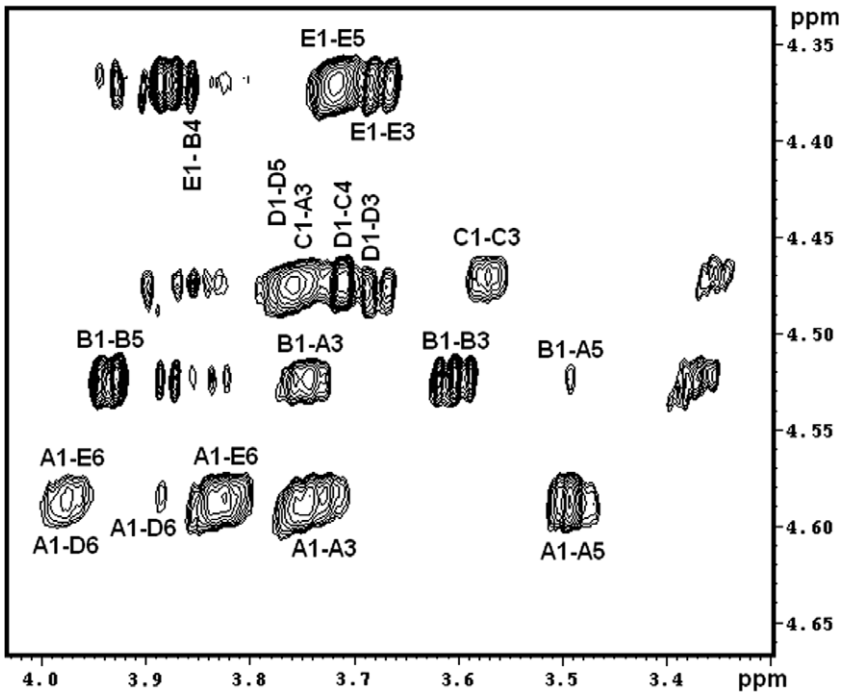


Figure 7. ^{1}H–^{1}H NOESY spectrum of capsule PS showing through-space NOE connectivities that substantiated the glycosyl linkages described previously.

(B/C-(1→3)-A), H-1 of D and C-4 of C (D-(1→4)-C), H-1 of E and C-4 of B (E-(1→4)-B), and H-1 of A and C-6 of D and E (A-(1→6)-D/E):

→6-E-(1→4)-B-(1→3)-A→

→6-D-(1→4)-C-(1→3)-A→

This experiment showed that the H-1 resonances of both B and C correlated to the C-3 of A. Unit A was thus designated as the 3-substituted GlcNAc to which both B and C could be connected, which implied that B and C were the same unit. The recognition of H-4 and H-5 of the GlcA(Glu) unit, characterized by 2D ^{1}H–^{13}C HMBC (Fig. 6B), belonging to residue B, allowed the assignment of B to GlcA(Glu). In this 2D ^{1}H–^{13}C HMBC, no resonances were readily observed for the H-4 and H-5 of GlcA. That a connectivity was seen between H-1 of A [→3)-Glc*p*NAc-(1→] and C-6 of both D and E implied that the 3-substituted Glc*p*NAc was glycosidically attached to the O-6 position of Gal*p*NAc (D/E). H-1 of D and E (6-substituted Gal*p*NAc) were in turn connected to the O-4 of B [Glc*p*A(Glu)] and C (which was assigned to Glc*p*A). The fact that the single 6-substituted Gal*p*NAc yielded both D and E anomeric resonances implied that the presence of the variably substituted Glc*p*A(Glu)$^{\pm}$ at its C-1 markedly influenced its chemical shifts. The same was not observed for the 3-substituted Glc*p*NAc (A) to which the Glc*p*A(Glu)$^{\pm}$ units (B and C) are attached.

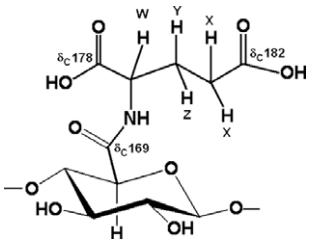
Additional spectroscopic information that pointed towards the existence of the linkage sites was obtained by a $^{1}H^{1}H$ 2D-NOESY (Fig. 7), in that *inter*-NOE connectivities were observed between H-1 of Glc*p*NAc (A) and H-6 protons of Gal*p*NAc (D and E), between H-1 resonances of Glc*p*A(Glu) (B) and Glc*p*A (C) and H-3 of Glc*p*NAc (A), H-1 of Gal*p*NAc (D) and H-4 of Glc*p*A (C), and H-1 of Gal*p*NAc (E) and H-4 of Glc*p*A(Glu) (B), identifying the capsule PS trisaccharide repeat

E B A

→6)-Gal*p*NAc-(1→4)-Glc*p*A(Glu)$^{\pm}$-(1→3)-Glc*p*NAc→

D C A

The ^{1}H–^{13}C HMBC spectrum (Fig. 6B) also revealed the presence of three distinct carbonyl moieties. The carbonyl at δ_C 169.0 correlated to H-4 and H-5 protons of Glc*p*A(Glu) and to the proton resonance W (CH) of the Glu residue. Another carbonyl at δ_C 178.0 correlated with protons W, Y, and Z, and the third carbonyl at δ_C 182.0 correlated with protons X, Y, and Z. The association between C-6 (δ_C 169) of Glc*p*A and the methine proton of Glu illustrated that Glu was amidically linked to the Glc*p*A residue (shown below):



3.5. Synthesis and characterization of *A. otitidis* ATCC 51267 capsule PS–BSA conjugate

As described above, the capsule PS of *A. otitidis* ATCC 51267 expressed carboxylic acids as structural motifs of the Glu and GlcA residues, and thus, we employed these available functional groups in the conjugation to a carrier protein in the design of an anti-*A. otitidis* glycoconjugate vaccine. The capsule-PS carboxylic acid moieties were transformed to activated carboxylates by EDC, and subsequently the activated capsule PS was directly conjugated to BSA protein to afford an *A. otitidis* capsule PS–BSA conjugate. The glycoconjugate preparation was analyzed by MALDI-TOF-MS that revealed the presence of a conjugate with an average molecular weight of 82 kDa, and a molecular weight range that spanned from 66 to 110 kDa (Fig. 8). When taking into account the average molecular weight of the capsule PS (6554 Da) (Fig. 3) and that of the BSA used here (66,349 Da) (determined by MALDI-TOF-MS), one could calculate that a BSA protein carried up to 7 capsule PSs. The MALDI-TOF-MS spectrum also showed peaks with average molecular weights of 41,040 and 164,244 kDa, which could be respectively assigned to a doubly charged species and a dimer species, whose origin could be artificial, as discussed previously. However, there is a possibility that the *m*/*z* 164244 ion could indeed represent a covalently linked species carrying 2 BSA units and the corresponding capsule PSs.

Parallel conjugation experiments were also carried out using solely GlcA, Glu, and also glutamine (Gln) units to compare their conjugation aptitude to BSA using the EDC procedure. The conjugates were analyzed by MALDI-TOF-MS, which showed that the GlcA, Glu, and Gln coupled to BSA, and in addition pointed to the fact that the Glu (68,830 Da) may couple to BSA to a higher degree than GlcA (68,359 Da) and Gln (67,936 Da).

3.6. Monomeric analysis of other *A. otitidis* strains

Monomer composition analyses were performed on the capsule PSs of strains SS1337 and 1518-89, and on the water-soluble preparations, obtained from mild-acid treatment of cells, of strains 1514-89, 1515-89, 1996-94, and 2621-96. The analyses showed that these strains expressed units similar to those found in the type-strain, namely, GlcNAc, GalNAc, GlcA, and Glu. Strain 1515-89 was also observed to contain mannose and galactose. Gro and Glc were also detected in the analyses of the water-soluble preparations. Linkage analysis on the capsule PSs of strains SS1337 and 1518-89 revealed that the GlcNAc and GalNAc units possessed linkage-types different from those found in the type-strain, which indicated that although the above strains contained similar constituents, they need not be arranged in the same manner. These preliminary composition data suggested that these monomeric components may be expressed in several *A. otitidis* strains, and, more interestingly, that the decoration of capsule PSs by Glu appears to be ubiquitous.

4. Discussion

Otitis media represents one of the major childhood health problems that, in addition to grave discomfort,

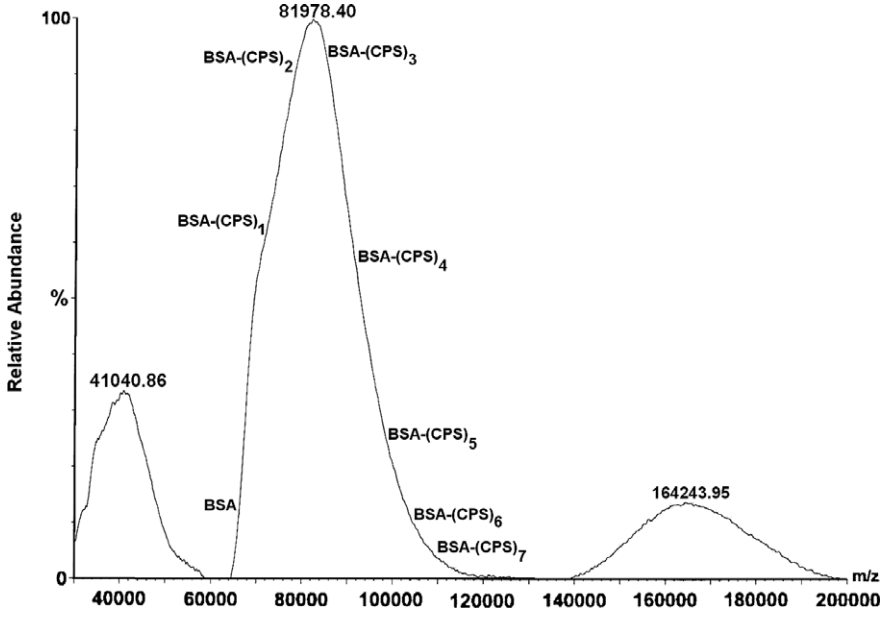
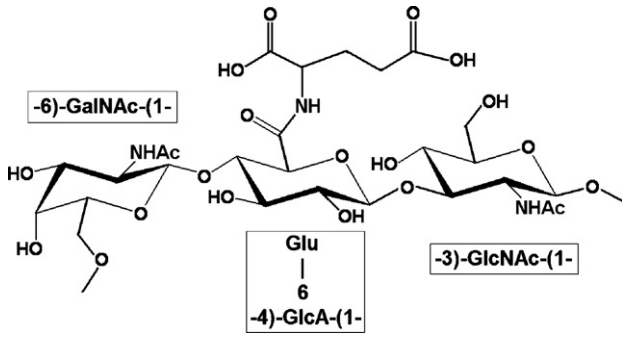


Figure 8. MALDI-TOF-MS spectrum of the capsule PS–BSA conjugate indicating that, on average, 3 capsule PSs were covalently appended to a BSA unit in this conjugate. This spectrum also showed the presence of conjugates containing BSA units carrying up to 7 capsule PSs.

can also affect the child's overall learning ability due to hearing loss and speech-delay issues. At the moment there is no vaccine directed solely at fighting otitis media, although Prevnar®, a multivalent glycoconjugate vaccine used primarily in the prevention of invasive pneumococcal disease in infants, is recognized as possessing some ability in reducing otitis media in children.[19] One of the main obstacles in curing otitis media is that the origin of this ailment is not always obvious because the causal agent in each situation is difficult to define.[20] Here, we investigated the cell-surface capsule PS of a newly discovered bacterial agent of otitis media, *A. otitidis*. In addition to identifying the structure of the capsule PS, our plans also included the use of the structurally defined capsule PS as a protective immunogen, in the shape of a glycoconjugate vaccine, to combat *A. otitidis* infections.

The capsule PS of *A. otitidis* type-strain ATCC 51267 was determined to be composed of trisaccharide repeating blocks composed of Glc*p*NAc, Gal*p*NAc, and Glc*p*A, in which the Glc*p*A units were non-stoichiometrically adorned by the amino acid, Glu (structure shown below):



The majority of Glc*p*A units appeared to carry a Glu residue, as observed by ESI-MS (Fig. 1) experiments and also NMR, in which the anomeric resonance of C (Glc*p*A) was observed to be weaker than that of B [Glc*p*A(Glu)] (Fig. 5). The most striking structural feature of this *A. otitidis* capsule PS is the decoration by the amino acid Glu. Inclusions of amino acid units in PSs are not found very frequently, but in fact Glu has been described previously as being a component of PSs produced by *Klebsiella*,[21] *Serratia marcescens*,[22] and *Streptomyces fulvissimus*.[23] Composition analysis performed on other *A. otitidis* strains also showed the presence of similar monomeric constituents, which implied that they may be common structural features of numerous *A. otitidis* strains.

The design of an anti-*A. otitidis* glycoconjugate vaccine, using the just-described capsule PS, utilized the carboxylic acid moieties of the Glu and GlcA units present in the capsule PS. The capsule PS–BSA conjugate was synthesized via a carbodiimide-based process and yielded glycoconjugates carrying 1–7 capsules on a BSA protein (Fig. 8). Conjugation experiments using the monomeric constituents suggested that the carboxylic acids of Glu may be more reactive than those of GlcA. Additionally, because the structural data obtained pointed to the fact that the majority of the Glc*p*A units are substituted by Glu, more carboxylic acids of Glu will be available for conjugation to BSA. Thus, conjugation between the capsule PS and BSA, in all probability, took place via the carboxylic acids of Glu. The fact that Gln was also observed to couple to BSA implied that both carboxylic acids of Glu could participate in conjugation to BSA. Most likely, several carboxylic acid moieties in each capsule PS were involved in the formation of a link to BSA, which, in all likelihood, yielded a multiple-point attachment conjugate. The average number of carboxylic acids in each capsule PS involved in attachment to BSA was not calculated here. The apparent regular expression of Glu in the capsule PSs could be particularly useful in the design of an anti-*A. otitidis* multivalent glycoconjugate vaccine, in that it may provide an accessible point of connection between *A. otitidis* capsule PSs and carrier protein(s), either by the strategy used here, or through a related coupling approach that exploits the presence of the Glu carboxylic acids.

The minor fraction (PS-2) contained phosphate, Gro, and Glc*p* (as terminal and 2-substituted) units. Gro and phosphate are usually found as the backbone of teichoic-acid PSs in Gram-positive bacterium,[24,25] and presumably the units observed in PS-2 are members of a teichoic-acid PS produced by *A. otitidis* ATCC 51267, in which a diglucosyl [Glc*p*-(1→2)-Glc*p*-(1→] moiety may be present as a side-branch emanating from the Gro unit of the Gro-phosphate backbone.

At the moment, due to the fact that *A. otitidis* is a fastidious grower, we are improving the growth conditions to obtain more cell-mass to scale-up glycoconjugate production for animal studies, and to carry out conjugation experiments with varied ratios of capsule PS and protein. Our plans also include the conjugation of the capsule PS to carrier proteins that are used in commercially available vaccines (i.e., tetanus toxoid and the genetically modified diphtheria toxin, CRM_{197}), and, more importantly, the design of a single-point attachment conjugate for immunological comparison with the multiple-point attachment glycoconjugate described in this work.

Here, we have revealed that the newly discovered bacterium, *A. otitidis*, expresses an acidic capsule PS that may afford a highly charged cell-surface environment, which in turn may play a role in making *A. otitidis* resistant to antibiotic attack. Also, it was shown that a glycoconjugate could be produced in which the capsule PS may be directly linked to a carrier protein. In the near future, the use of this glycoconjugate as an anti-*A. otitidis* vaccine will be tested in a mouse model to

evaluate its immunogenicity. The introduction of glycoconjugate vaccines against pneumococcal and influenza infections has been shown to be very effective in reducing disease; however, the niche left behind by the respective organisms may create an opening for bacteria such as *A. otitidis* to prosper, and thus the development of anti-*A. otitidis* vaccine should become a priority in the fight against otitis media.

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