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Discovery of a New Capsular Serotype (6C) within Serogroup 6 of *Streptococcus pneumoniae*▽

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Using two monoclonal antibodies, we found subtypes among pneumococcal isolates that are typed as serotype 6A by the quellung reaction. The prevalent subtype bound to both monoclonal antibodies and was labeled here 6Aα, whereas the minor subtype bound to only one monoclonal antibody and was labeled 6Aβ. To determine the biochemical nature of the two serologically defined subtypes, we purified capsular polysaccharides (PSs) from the two subtypes and examined their chemical structures with gas-liquid chromatography and mass spectrometry. The study results for 6Aα PS are consistent with the previously published structure of 6A PS, which is →2) galactose (1→3) glucose (1→3) rhamnose (1→3) ribitol (5→phosphate. In contrast, the 6Aβ PS study results show that its repeating unit is →2) glucose 1 (1→3) glucose 2 (1→3) rhamnose (1→3) ribitol (5→phosphate. We propose to continue referring to 6Aα as serotype 6A but to refer to 6Aβ as serotype 6C. Serotype 6C would thus represent the 91st pneumococcal serotype, with 90 pneumococcal serotypes having previously been recognized. This study also demonstrates that a new serotype may exist within an established and well-characterized serogroup or serotype.

Streptococcus pneumoniae is a major human pathogen that is responsible for a large percentage of cases of pneumonia, meningitis, otitis media, and sepsis (6). All pathogenic pneumococci are known to display one of many structurally diverse carbohydrate capsules, which shield pneumococci from host phagocytes and increase their pathogenicity (2). Antisera to a capsule type can be used to treat patients infected with the pneumococci expressing that capsule type (4). Consequently, for the past century, the serological types of pneumococcal capsules have been extensively investigated with quellung reactions. These studies have culminated in identifying 90 different pneumococcal capsules with distinct serological patterns (9) and chemical structures (10).

Not all 90 serotypes are equally pathogenic. For instance, serotypes 6A and 6B account for 4.7% and 7%, respectively, of cases of invasive pneumococcal disease in the U.S. population (19, 20). Because of their medical importance, the molecular natures of serotype 6A and its related serotype, serotype 6B, have been studied extensively. Biochemical studies found that the capsular polysaccharides (PSs) of serotypes 6A and 6B are linear polymers with a repeating unit containing four monosaccharides/alditols: rhamnose, ribitol-phosphate (P), galactose, and glucose (10). The two PSs are identical except for a difference in the linkage between rhamnose and ribitol (see Fig. 6).

Currently available pneumococcal vaccines are designed to elicit antibodies to the capsular PSs of the most common pneumococcal serotypes. Since vaccine-induced immunoprotection is serotype specific, serotyping pneumococcal isolates from patients is an important tool for monitoring the effectiveness of pneumococcal vaccines (3). Because the classical quellung reaction with rabbit antisera is tedious to perform (13), we have developed a new serotyping system based on mouse monoclonal antibodies (mAbs) and a multiplexed immunoassay (27). While validating the new system, we found that a minor fraction of the isolates determined to be serotype 6A by quellung reaction bound to one 6A-specific mAb (Hyp6AG1) but not to the other (Hyp6AM3), whereas the majority of the serotype 6A isolates bound to both mAbs (12). To distinguish between the isolates, we have labeled the isolates reacting with both mAbs as 6Aα and those reacting with only Hyp6AG1 as 6Aβ in this report. To investigate the significance of this serological difference, we studied the chemical structures of the 6Aα and 6Aβ PSs.

MATERIALS AND METHODS

Bacterial isolates. Two serotype 6Aβ Brazilian isolates (BZ17 and BZ650) were previously described (12). Four serotype 6Aα strains (SP85, ST558, KK58, and CHPA378) and two serotype 6B strains (ST400 and ST518) were from our laboratory collections. All pneumococcal isolates studied had colony morphology typical of pneumococci, were optochin sensitive, and were bile soluble.

6A subtyping assay. The subtyping assay used in this study is an inhibition-type enzyme-linked immunosorbent assay (ELISA). Briefly, the wells of ELISA plates (Corning Costar Corp., Acton, MA) were coated at 37°C with 5 μg/ml of 6Aα capsular PS (a gift of G. Schiffman, Brooklyn, NY) overnight in phosphate-buffered saline. After washing the plates with phosphate-buffered saline containing 0.05% of Tween 20, a previously diluted bacterial culture supernatant (or lysates) was added to the wells along with an anti-6A antibody. Pneumococcal lysates were prepared by growing pneumococci in 10 ml of Todd-Hewitt broth supplemented with 0.5% yeast extract without shaking until the tubes became

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turbid and then incubating the tubes for 15 min at 37°C with a lysis buffer (0.1% sodium deoxycholate, 0.01% sodium dodecyl sulfate, and 0.15 M sodium citrate in deionized water). mAb Hyp6AG1 was used at a 1:250 dilution, and mAb Hyp6AM3 was used at a 1:100 dilution. Pool Q and factor "6b" rabbit antisera from Staten Serum Institute (Copenhagen, Denmark) were used at a 1:500 dilution. After 30 min of incubation in a humid incubator at 37°C, the plates were washed and incubated for 2 h with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Sigma, St. Louis, MO) or alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Biosource, Camarillo, CA). The amount of the enzyme immobilized to the wells was determined with paranitrophenyl phosphate substrate (Sigma) in diethanolamine buffer. The optical density at 405 nm was read with a microplate reader (BioTek Instruments Inc, Winooski, VT).

Polysaccharide isolation and purification. A pneumococcal strain (SP85 or BZ17) was grown in 2 liters of a chemically defined medium (26) from JRH Biosciences (Lenexa, KS), which was supplemented with choline chloride (1 mg/liter), sodium bicarbonate (2.5 mg/liter), and cysteine-HCl (0.73 mg/liter) and lysed with 0.05% deoxycholate. After removing cell debris by centrifugation, PS was precipitated in 70% ethanol and was recovered by dissolving it in 120 ml of 0.2 M NaCl. After dialyzing the PS in 10 mM Tris-HCl (pH 7.4), the PS was loaded onto a DEAE-Sepharose (Amersham Biosciences, Uppsala, Sweden) column (50 ml) and eluted with a linear gradient of NaCl from 0 to 2 M. The resulting fractions were tested for 6Aα or 6Aβ PS with the inhibition assay described above. The PS-containing fractions were pooled, concentrated by ethanol precipitation (70%), dialyzed, and lyophilized. The lyophilized PS was dissolved in 3 ml of water and loaded onto a gel filtration column containing 120 ml of Sephacryl S-300 HR (Amersham Biosciences). The PS was eluted from the column with water, and all the fractions were tested for 6Aβ PS with the inhibition assay. The fractions containing the first 6Aα or 6Aβ PS peak were pooled and lyophilized.

Monosaccharide analysis. The lyophilized capsular PS was subjected to methanolysis in 1.5 M methanolic HCl at 80°C for 16 h. After evaporating the methanolic HCl, the residue was trimethylsilylated by reacting with Tri-Sil reagent (Pierce Biotech Inc., Rockford, IL) for 20 min at room temperature. The reaction products were analyzed on a gas-liquid chromatograph/mass spectrometer (Varian 4000; Varian Inc., Palo Alto, CA) fitted with a 30-m (0.25-mm-diameter) VF-5 capillary column. Column temperature was maintained at 100°C for 5 min and then increased to 275°C at 20°C/min and finally held at 275°C for 5 min. The effluent was analyzed by mass spectrometry (MS) using the electron impact ionization mode.

Oxidation, reduction, and hydrolysis. Capsular PS (1 mg/ml) was treated with 40 mM sodium periodate in 80 mM sodium acetate buffer (pH 4) for 4 days at 4°C in the dark. After neutralizing the excess periodate with ethylene glycol, the sample was dialyzed and lyophilized (23). The PS (1 mg/ml) was reduced with 200 mg/ml of sodium borohydride ($NaBH_4$) or its deuterium form ($NaBD_4$) for 3 h at room temperature, dialyzed, and lyophilized. The oxidized/reduced 6Aβ PS was hydrolyzed in 0.01 M NaOH at 85°C for 30 min, neutralized by adding 0.01 M HCl, and then directly used for MS without desalting.

MS/MS. The tandem mass spectral analyses of native and oxidized/reduced 6Aβ were performed in the Mass Spectrometry Shared Facility at the University of Alabama at Birmingham with a Micromass Q-TOF2 mass spectrometer (Micromass Ltd., Manchester, United Kingdom) equipped with an electrospray ion source. The samples dissolved in distilled water were injected into the mass spectrometer along with running buffer (50/50 acetonitrile-water containing 0.1% formic acid) at a rate of 1 μl/min using a Harvard syringe pump. The injected sample was negatively ionized with electrospray (needle voltage of 2.8 kV) and detected with a time-of-flight mass spectrometer. For tandem MS (MS/MS), the parent ion was fragmented into daughter ions by energizing it to 40 eV before collision with argon gas. The daughter ions were analyzed with a time-of-flight mass spectrometer. The MS/MS spectra were processed using the Max-Ent3 module of MassLynx 3.5.

Smith degradation and glycerol detection. Periodate-treated 6Aα and 6Aβ PSs were reduced with 10 mg/ml $NaBD_4$ in 1 M ammonium hydroxide for 16 h. Excess $NaBD_4$ was removed by the addition of glacial acetic acid (4 to 5 drops) and 0.5 ml of methanol-acetic acid (9:1). Samples were dried under a stream of nitrogen and washed twice with 0.25 ml of methanol. Dried samples were suspended in 0.5 ml of 1.5 M methanolic HCl and incubated at 80°C for 16 h. Samples were dried under a stream of nitrogen and washed twice with 0.25 ml of methanol. Dried samples were suspended in 0.1 ml of Tri-Sil (Pierce) and incubated at 80°C for 20 min. One microliter of samples was injected into a Varian 4000 gas chromatograph/mass spectrometer (Varian 4000; Varian Inc. Palo Alto, CA) equipped with a 60-m VF-1 column. Helium was used as the carrier gas at a constant flow rate of 1.2 ml/min. The oven conditions were an initial temperature of 50°C held for 2 min, a temperature increase at 30°C/min to 150°C, and then another increase at 3°C/min to 220°C, which was held for 2 min. The injector temperature was kept at 250°C, and the MS transfer line was kept at 280°C. MS data acquisition parameters included scanning from *m/z* 40 to 1,000 in the electron impact mode or in the chemical ionization mode using acetonitrile.

RESULTS

Conventional serotyping rabbit antisera do not distinguish the subtypes, but monoclonal antibodies do. Unlike quellung reactions, our new serotyping method is quantitative. Since the qualitative nature of the quellung reaction may have prevented the detection of 6A subtypes, we sought to determine if subtypes might be distinguishable with a quantitative assay using the rabbit sera used for quellung reactions. To make this determination, we adapted the rabbit sera to an inhibition assay in which pneumococcal lysates were allowed to inhibit the binding of rabbit antisera to 6A PS immobilized on ELISA plates (Fig. 1). As a control, we first tested pneumococcal lysates for inhibiting the two mAbs Hyp6AG1 and Hyp6AM3 (Fig. 1A and B). Lysates of three 6Aα isolates (CHPA378 from the United States, KK58 from Korea, and ST558 from Brazil) inhibited both mAbs, and lysates of two 6B isolates (strains ST400 and ST518 from Brazil) inhibited neither mAb (Fig. 1A and B). Two lysates of 6Aβ isolates (strains BZ17 and BZ650 from Brazil) clearly inhibited the binding of Hyp6AG1, even at a 1:1,000 dilution (Fig. 1A). However, they showed almost no inhibition of Hyp6AM3, even at a 1:10 dilution (Fig. 1B).

When the pneumococcal lysates were examined for inhibiting pool Q (a rabbit antiserum often used for serotyping) (22), both lysates of 6Aα and 6Aβ could inhibit equally well, but the 6B lysates could not inhibit (Fig. 1C). When a "6b" factor-specific rabbit serum was tested, we found that all 6Aα, 6Aβ, and 6B isolates could inhibit the factor serum equally well (Fig. 1D). Since the 6b factor serum is designed to be 6A specific, this was somewhat unexpected. However, the factor serum is designed to be specific in quellung reactions but not in this inhibition assay. Nevertheless, this experiment shows that rabbit antisera commonly used for pneumococcal typing do not distinguish between the two 6A subtypes.

Monosaccharide/alditol compositions of 6Aα and 6Aβ capsular PS differ. To investigate whether the 6Aα and 6Aβ PSs differ chemically, we determined their monosaccharide compositions. To do this, we purified the two PSs from strains SP85 (6Aα strain) and BZ17 (6Aβ strain) by ethanol precipitation, ion-exchange chromatography, and gel filtration and subjected the PSs to methanolysis. The resulting methyl glycosides were trimethylsilylated and analyzed by gas-liquid chromatography–MS. The chromatograms of 6Aα PS showed peaks that eluted and had MS fragmentation patterns consistent with ribitol, rhamnose, glucose, and galactose (Fig. 2A). These data are consistent with those reported previously (11). When the 6Aβ PS chromatogram was examined, characteristic peaks of ribitol, rhamnose, and glucose were found, but the galactose peaks were absent. When the areas of each monosaccharide peak were normalized to the rhamnose peak area and 6Aα and 6Aβ were compared (Fig. 2B), the ribitol peaks of 6Aα and 6Aβ PS were found to have equivalent areas. However, the glucose peak area of 6Aβ was about twice that of 6Aα (Fig. 2B). This finding suggests that the repeating unit of 6Aβ has one ribitol molecule and one rhamnose molecule just as 6Aα

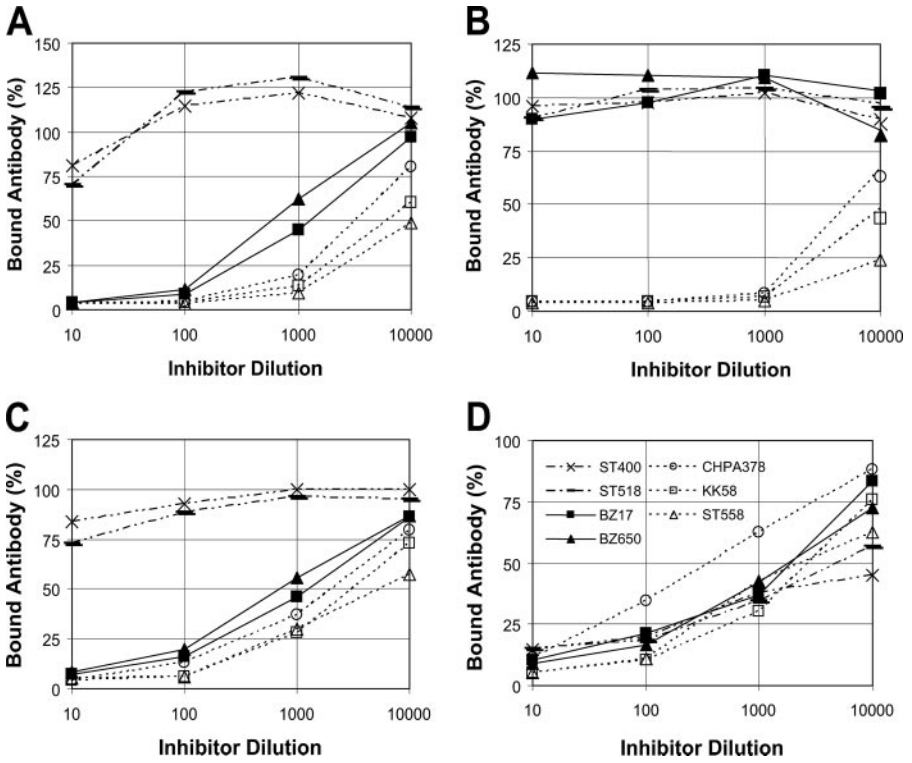


FIG. 1. Antibody bound to ELISA plates (*y* axis) against the dilution of pneumococcal lysates (*x* axis). Lysates include two 6Aβ isolates (solid symbols with continuous lines), three 6Aα isolates (open symbols with dotted lines), and two 6B isolates (dashed connecting lines). Antibodies used for the assay were Hyp6AG1 (A), Hyp6AM3 (B), rabbit serum pool Q (C), and rabbit "factor 6b" serum (D).

does but that the 6Aβ repeating unit has two glucose molecules instead of the glucose and galactose molecules seen for 6Aα.

To further investigate the two glucose molecules presumed to be present in 6Aβ PS, we treated 6Aα and 6Aβ PSs with periodate, which selectively destroys vicinal glycols. As expected from the previously published structure of 6A PS (10, 11, 18), we found that the galactose and ribitol peaks of 6Aα PS became undetectable, while the glucose and rhamnose peaks were undisturbed. When 6Aβ PS was treated with periodate, its ribitol became undetectable, and its glucose peak was reduced by about half, while its rhamnose peaks remained undisturbed (Fig. 2B). These findings strongly suggest that the 6Aα PS structure is identical to the previously published 6A PS structure. Also, it indicates that 6Aβ PS is chemically different from 6Aα PS and that 6Aβ PS has two glucose molecules, one of which is sensitive to periodate.

Determination of monosaccharide/ribitol sequence within the repeating units. Mild alkali hydrolysis of 6A PS breaks the phosphodiester bond in each repeating unit and produces a repeating unit with a negative charge, which can then be examined with tandem mass spectrometry. The hydrolysis product of 6Aα PS (from strain SP85) showed three well-defined peaks with a negative charge: *m/z* 683.21, 701.21, and 759.19 (Fig. 3A). The peak at *m/z* 683.21 represents an anhydrous form of the peak at *m/z* 701.21, and the peak at *m/z* 759.19 represents the molecule with *m/z* 701.21 complexed with NaCl. This indicates that the mass of the repeating unit is *m/z* 683.21, as previously described (10, 11). When the daughter ions (product ions) of the *m/z* 701.21 peak were examined, we found daughter ions with *m/z* 539.14, 377.08, and 231.01, which correspond to the masses of glucose-rhamnose-ribitol-P, rhamnose-ribitol-P, and ribitol-P fragments, respectively (Fig. 3C). We also found their anhydrous counterparts at *m/z* 521.13, 359.07, and 212.99. Additional peaks at *m/z* 96.94 and 78.94 represent $H_2PO_4^-$ and PO_3^- ions (Fig. 3C).

When 6Aβ PS was analyzed by following the same procedure as that used for 6Aα PS, we found three major peaks at *m/z* 683.24, 701.25, and 759.22, which correspond to the three major peaks found for 6Aα PS (Fig. 3B). Also, the 6Aβ cleavage products had a mass spectrum identical to those of 6Aα (Fig. 3D). This finding indicates that the mass of the repeating unit of 6Aβ PS is *m/z* 683.2 and that the carbohydrate sequence of the 6Aβ repeating unit is glucose 1-glucose 2-rhamnose-ribitol-P (to distinguish between the two glucoses, we labeled them glucose 1 and glucose 2; glucose 1 corresponds to the galactose of 6Aα). Thus, the monosaccharide sequence of 6Aβ is identical to that of 6Aα except for the replacement of galactose with glucose 1.

Determination of the linkages between carbohydrate and ribitol of the 6Aβ repeating unit. To identify the 6Aβ glucose that is periodate sensitive, we oxidized and reduced 6Aβ PS, obtained repeating units by mild alkali hydrolysis, and studied the repeating units with tandem mass spectrometry. Their mass spectra showed several major (and dominant) peaks between *m/z* 650 and 700 (Fig. 4A). The dominant peaks were at *m/z* 655.23, 659.73, 661.24, 664.25, 673.25, and 675.24. Due to natural isotopes, each dominant peak has satellite peaks with one or two additional mass units, and these satellite peaks can be

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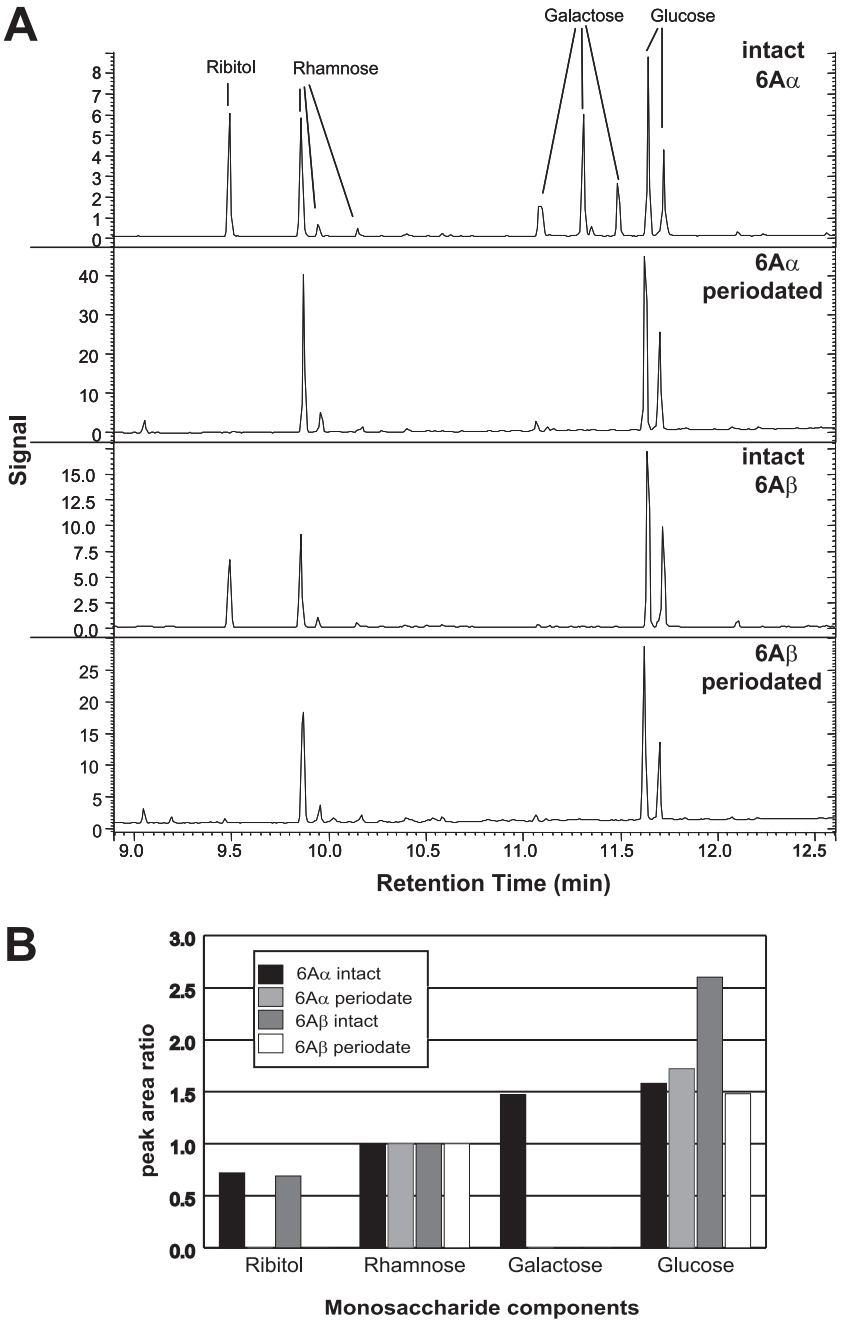


FIG. 2. Carbohydrate composition (A) of capsular PS from 6Aα (top two graphs) and 6Aβ (bottom two graphs) before and after periodate treatment. The monosaccharides are identified in the top chromatogram. B shows normalized peak areas of each monosaccharide for 6Aα and 6Aβ. The peak areas of all monosaccharides from each PS are normalized to the peak area of the associated rhamnose. 6Aβ shows no galactose peaks but has about twice as much glucose as 6Aα does.

used to determine the charge states and the true masses of the dominant peaks (5). For instance, the dominant peak at *m/z* 661.24 has a satellite peak with *m/z* 661.57. Since these two peaks are separated by *m/z* 0.33, the *m/z* 661.24 peak represents a molecular ion with three negative charges and 1,983.72 atomic mass units (AMUs) {i.e., three repeating units with one water molecule [$(655.23 \times 2 + 673.76) = 1{,}983.72$]}. Similarly, the *m/z* 664.25 and 675.24 peaks represented two repeating units with two negative charges, but the *m/z* 675.24 peak has a sodium ion replacing a proton. The *m/z* 673.25 and 655.23 peaks represent one repeating unit with one negative charge with or without a water molecule. Since the mass of the anhydrous repeating unit prior to oxidation/reduction was 683.26, the repeating unit lost 28 mass units due to oxidation and reduction. To identify the periodate reaction products of ribitol and glucose, we named the ribitol fragment the Rx fragment and the two glucose fragments the Gx and Gy fragments (Fig. 5A).

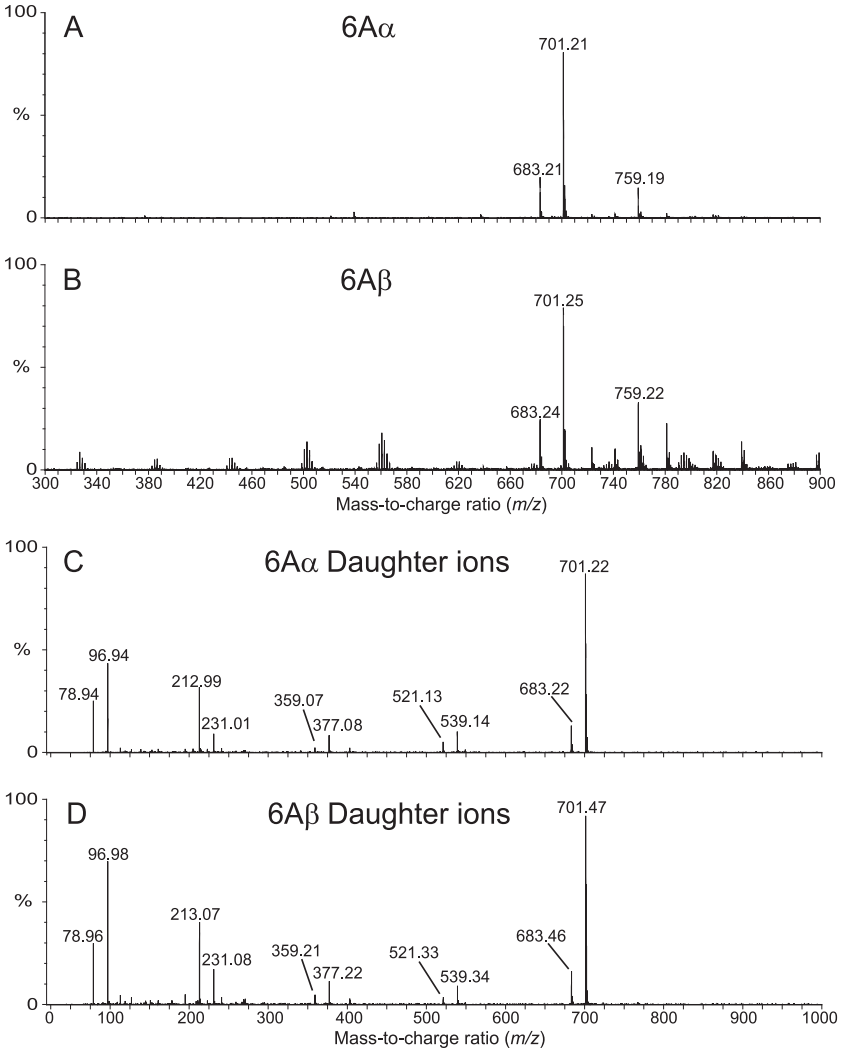


FIG. 3. Mass spectra of the repeating units of 6Aα (A) and 6Aβ (B) and their daughter ions (C and D, respectively). The mass-to-charge ratio (*m*/*z*) was rounded off to two decimal points.

Daughter ions were obtained by fragmenting the parent ion with *m*/*z* 673.25 (Fig. 4B). During the fragmentation, one fragment may exchange one AMU with the other fragment (7, 15). Also, molecular ions become variably hydrated within argon collision cells (25). Indeed, our daughter ions could be grouped into hydrated and anhydrous peaks based on differences of *m*/*z* 18 (Fig. 4B). The peaks found at *m*/*z* 673.25, 581.16, 509.13, 347.07, and 200.99 are hydrated peaks, each of which has a corresponding anhydrous peak that is *m*/*z* 18 less. Also, the peaks at *m*/*z* 200.99, 347.07, and 509.13 correspond to the fragments with 200, 346, and 508 AMUs with one hydrogen atom added to the fragmentation site (Fig. 5B) during the fragmentation. The peak at *m*/*z* 200.99 confirms that ribitol lost CH_2OH during the periodate treatment. The peaks at *m*/*z* 347.07 and 509.13 indicate that rhamnose and glucose 2 are periodate resistant. The presence of a peak at *m*/*z* 581.16 indicates that glucose 1 is cleaved by periodate.

Periodate cleavage divides glucose 1 into two parts (which were named Gx and Gy in Fig. 5A). The combined mass of the two parts is 164 instead of 162 (mass of intact glucose) because glucose 1 lost no carbon but acquired two hydrogen atoms at the breakage site during the oxidation and reduction reactions. The mass spectrum shown in Fig. 4 is consistent with Gx and Gy having 91 and 74 AMUs, respectively. The peak at *m*/*z* 581.16 indicates that a repeating unit lost Gx and one extra proton (Fig. 5). The neutral loss of both Gx and Gy (74 AMUs) results in an additional loss of *m*/*z* 72 because Gy already lost one hydrogen to Gx and leaves one hydrogen with glucose 2. The same patterns were found for the anhydrous peaks, i.e., *m*/*z* 655.22, 563.16, and 491.12. Furthermore, when we reduced the 6Aβ PS with $NaBD_4$, we found that the two additional mass units were associated with glucose 1: the neutral loss of Gx fragment was *m*/*z* 93 instead of *m*/*z* 92, and that of Gy was *m*/*z* 73 instead of *m*/*z* 72 (Fig. 4C). These findings clearly indicate that glucose 1 is fragmented into Gx and Gy with the sizes shown in Fig. 5A.

The mass spectrum of daughter ions also provided information about the glycosidic linkages of 6Aβ PS. Glucose and rhamnose must be linked to the preceding carbohydrate at their first carbon (18). Also, they must be linked to the suc-

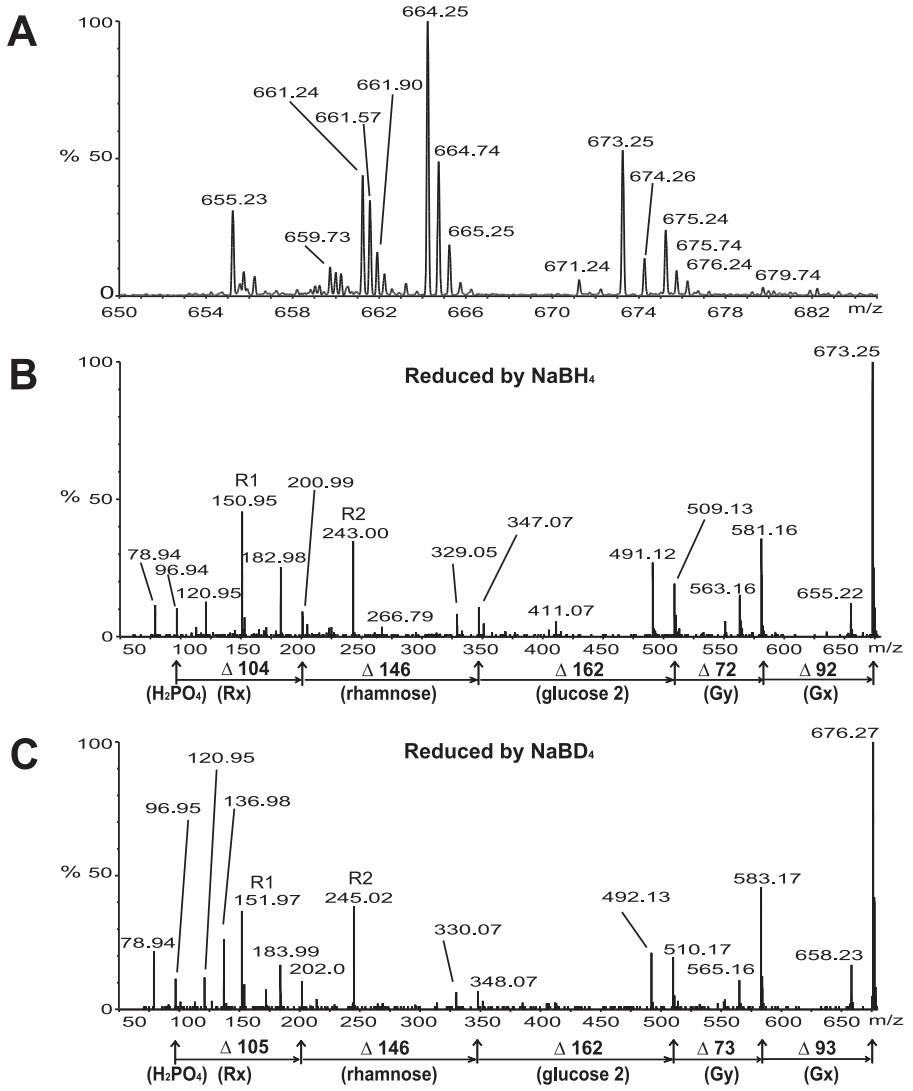


FIG. 4. Mass spectrum of the repeating unit of 6Aβ PS after oxidation and reduction (A) and daughter ions (B and C). The sample used for B was reduced with $NaBH_4$, and that used for C was reduced with $NaBD_4$. The mass-to-charge ratio (*m*/*z*) was rounded off to two decimal points. R1 and R2 (in B and C) indicate that the peaks corresponding to ions derived by reverse fragmentations. Numbers following the delta symbol indicate the *m*/*z* unit differences between the peaks and associated with the names of the fragments. All the peaks in C correspond to the peaks in B except for a peak at 136.98, which may be a contaminant.

ceeding carbohydrate at the third carbon in order to be resistant to periodate (18). Thus, 6Aβ PS must have glucose 1 (1→3) glucose 2 (1→3) rhamnose (1→). Further examination of the daughter ions shows that glucose 1 has the phosphodiester bond at its second carbon. To be periodate sensitive, glucose 1 must be phosphodiester linked at position 2, 4, or 6. The phosphodiester bond linkage cannot be at position 6 because the linkage at this position would result in a loss of a carbon atom in glucose 1 (Fig. 5F). If the phosphodiester linkage is at position 4, the periodate cleavage would occur between the second and the third carbons (Fig. 5E). Gx and Gy should then have masses of 120 and 42 AMUs, and a peak with *m*/*z* 552 should be detected instead of the peak at *m*/*z* 581.

Although alkaline hydrolysis cleaves the phosphodiester bond with glucose 1, it occasionally breaks the phosphodiester bond with ribitol instead. Examination of this cleavage product further confirms that the phosphodiester linkage must be at the second carbon of glucose 1. The peaks at *m*/*z* 150.95 and 243.00 are the reverse cleavage products of glucose 1 (labeled R1 and R2 in Fig. 4B) since products with these masses can be produced from glucose 1 with the phosphodiester bond at the second carbon and the 2-AMU loss to other fragments as described above (Fig. 5D), and these peaks have one (150.95→151.97) or two (243.00→245.02) more *m*/*z* units if reduction was performed with $NaBD_4$ instead of $NaBH_4$ (Fig. 4C). An ion at *m*/*z* 120.95 can also be obtained if the ion at *m*/*z* 150.95 loses the terminal methanol group. These peaks cannot be explained if the phosphodiester bond is at the fourth or the sixth carbon (Fig. 5E and F). Thus, the data with the reverse cleavage products also indicate that the phosphodiester bond is linked to the second carbon of glucose 1.

Additional examinations of the mass spectra showed that the

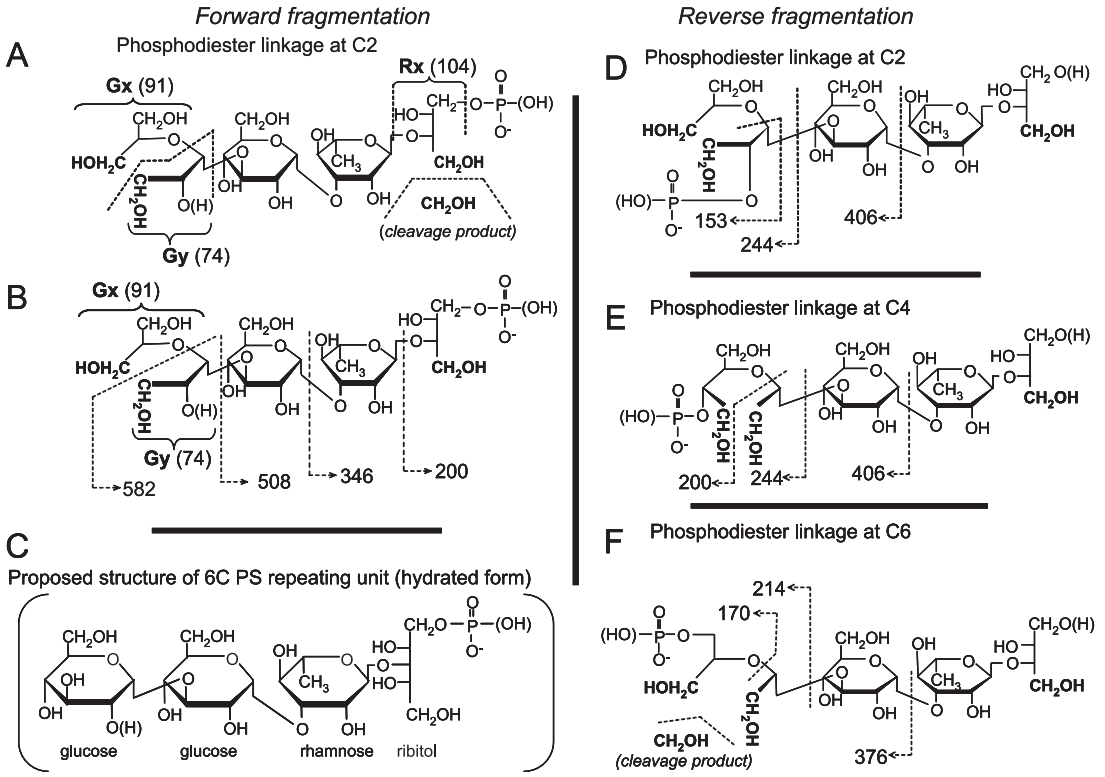


FIG. 5. Proposed chemical structures of 6C capsular polysaccharides and the structure of its cleavage products. The proposed structure of the 6C repeating unit is shown in C. A and B show possible molecular ions if the phosphate group is attached to ribitol and if the phosphodiester is linked to the second carbon of glucose 1. D, E, and F indicate potential cleavage patterns of the repeating unit if the phosphodiester is linked to the second (D), the fourth (E), or the sixth (F) carbon of glucose 1. Hydrated forms are shown, and the residues involved in hydration are shown in parentheses. Periodate-sensitive sites are shown in boldface type, and cleavage products are shown in A and F. Potential molecular ions are shown with dotted lines with arrows along with their AMUs. Gx and Gy are potential glucose 1 fragments, and Rx is the remaining ribitol fragment after oxidation and reduction reactions. Their AMUs are shown in parenthesis.

rhamnose-ribitol linkage must be (1→3). Since pneumococci use CDP-5-ribitol that is produced for teichoic acid synthesis for their capsule synthesis as well (17), the linkage between ribitol and glucose 1 must be ribitol (5→P→2) glucose 1. The peaks at *m/z* 78.94 and 96.94 correspond to PO_3^- and $H_2PO_4^-$, while the peaks at *m/z* 182.98 and 200.99 (Fig. 4B) correspond to the Rx fragment attached to PO_3^- and $H_2PO_4^-$ (Fig. 5A). Thus, ribitol must lose a hydroxyl methyl group during the oxidation and reduction reactions, and the linkage between rhamnose and ribitol must be (1→3). Considering all of the above-mentioned data, the 6Aβ repeating unit should be [P→2) glucose 1 (1→3) glucose 2 (1→3) rhamnose (1→3) ribitol (5→] (Fig. 5C).

When we analyzed 6Aα PS, we found peaks that were identical to the 6Aβ PS peaks (data not shown), which indicate that galactose and ribitol were destroyed by periodate but that glucose 2 and rhamnose remained intact. Thus, the structure of 6Aα PS must be [→2) galactose (1→3) glucose 2 (1→3) rhamnose (1→3) ribitol (5→P)], which is identical to the previously published 6A PS structure (Fig. 6) (10, 18). In summary, the only structural difference between 6A and 6C PS is the orientation of the hydroxyl group at the fourth carbon of glucose 1 (or galactose).

Smith degradation of 6Aβ PS. Classically, the phosphodiester bond of 6A PS was determined to be at the second carbon of galactose by demonstrating that glycerol is released after a Smith degradation of the 6A PS that was oxidized and reduced (18). To confirm the position of the 6Aβ phosphodiester bond using this classical approach, we performed a Smith degradation of 6Aα and 6Aβ PSs after oxidation and reduction. When we analyzed the reaction products of 6Aα and 6Aβ PSs, we were able to detect glycerol from the two PSs (data not shown). Thus, glucose 1 has a phosphodiester bond at the second carbon.

DISCUSSION

We have found two subtypes (6Aα and 6Aβ) among pneumococcal isolates that have been previously typed as serotype "6A" by the classical serotyping method, the quellung reaction using polyclonal rabbit sera. Although these two "new" subtypes were initially defined by their binding to two mouse

6A →2) - Galactose - (1→3) - Glucose - (1→3) - Rhamnose - (1→3) - Ribitol - (5→P→
6B →2) - Galactose - (1→3) - Glucose - (1→3) - Rhamnose - (1→4) - Ribitol - (5→P→
6C →2) - Glucose - (1→3) - Glucose - (1→3) - Rhamnose - (1→3) - Ribitol - (5→P→

FIG. 6. Comparison of 6A, 6B, and 6C PS structures. 6B PS differs from 6A PS in its rhamnose-ribitol linkage. 6C PS differs from 6A PS by having a glucose residue in place of a galactose residue.

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mAbs, the two subtypes were found to produce capsular PSs with different chemical structures: 6Aα PS has the structure identified as 6A PS in the literature, but 6Aβ PS has glucose in place of galactose. Therefore, the two subtypes should be recognized as different serotypes. We propose to name the 6Aβ subtype serotype 6C while leaving the 6Aα subtype assigned to serotype 6A. Serotype 6C should be included as the third member of serogroup 6 in view of its serological and structural relation to serotype 6A. Serotype 6C would thus represent the 91st pneumococcal serotype, with 90 pneumococcal serotypes having previously been recognized (9).

Galactose and glucose molecules differ only in the orientation of the hydroxyl group attached to their fourth carbon, and the repeating units of 6A and 6C PS differ only in the orientation of one hydroxyl group. This small structural difference explains why 6C was not identified with polyclonal antisera in the past. With the elucidation of the chemical structure, 6C can be biochemically distinguished from 6A by carbohydrate composition analysis or by nuclear magnetic resonance (NMR). Pneumococcal capsular PS can be identified by simple proton NMR of anomeric protons (1). We found that although 6A and 6C NMR patterns do differ, the NMR pattern of the anomeric protons of 6C is very similar to that of 6A (data not shown). Although genetic tests would become available once the genetic basis is understood, serological methods would be the most useful way to identify 6C using either our monoclonal antibodies or polyclonal antisera made specific by absorption.

Serogroup 6 has been known to contain three epitopes: 6a, 6b, and 6c (9). Epitope 6a is known to be serogroup specific and to be present in both serotypes 6A and 6B, whereas epitopes 6b and 6c are found only in either serotype 6A or 6B, respectively. Discovery of the 6C serotype indicates the presence of additional epitopes within serogroup 6. mAb Hyp6AM3, which recognizes 6A but not 6B or 6C, should recognize epitope 6b. Since mAb Hyp6AG1 recognizes 6A and 6C, but not 6B, we may define it as recognizing a new epitope, "6d." We have another mAb binding to all three serotypes (6A, 6B, and 6C), and the shared epitope may be defined as the new serogroup-specific "6a." It is likely that some antibodies may recognize 6A and 6B but not 6C, and the epitope may then be labeled "6e." We also reported a conformation-dependent epitope for serotypes 6A and 6B (24). Our observation of so many epitopes for serogroup 6 is consistent with a previous observation that even a simple linear homopolymer of sialic acid can have at least three epitopes (21). We believe that pneumococcal PS has many more epitopes than previously defined (9) and that the presence of many epitopes increases the chances of altering epitopes during the manufacture of pneumococcal conjugate vaccines.

Our discovery of serotype 6C was quite unexpected because serogroup 6 has been studied extensively following its discovery in 1929 (8). We should therefore consider the possibility that additional subtypes (or serotypes) are present among even well-established and extensively characterized serogroups. For instance, one may need to consider the possible presence of subtypes among serotype 19A because two chemical structures for the 19A capsular PS have been reported (10). If 19A subtypes are found, their presence may help us explain the rapid increase in the prevalence of serotype "19A" seen after the introduction of the pneumococcal conjugate vaccine (16). In addition, we should consider the possibility that 6C may have arisen recently. Consistent with this possibility, our genetic studies suggest that the 6C serotype capsule gene locus is not as diverse (12; data not shown) as the 6A locus (14). It would be interesting to investigate the origin and spread of 6C strains by studying pneumococcal isolates obtained a long time ago (perhaps 50 to 100 years ago).

Currently available pneumococcal vaccines contain only 6B PS because it is presumed to induce cross-protection against 6A. As a part of pneumococcal vaccine efficacy surveys, all the pneumococcal isolates found in the United States are now tested for serotypes 6A and 6B. However, cross-protection against 6C may differ from that against 6A. Since 6C and 6B PSs have two structural differences, whereas 6A and 6B PSs have only one structural difference (Fig. 6), the cross-protection against 6C may be inadequate, and the currently available pneumococcal vaccines may reduce the prevalence of 6A but not 6C. In fact, current pneumococcal vaccines may help 6C become more prevalent than before, just as occurred for serotype 19A. Thus, all pneumococcal isolates should be tested for serotype 6C as well as for serotypes 6A and 6B.

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