Discovery of *Streptococcus pneumoniae* Serotype 6 Variants with Glycosyltransferases Synthesizing Two Differing Repeating Units*

Received for publication, April 23, 2013, and in revised form, July 26, 2013. Published, JBC Papers in Press, July 29, 2013, DOI 10.1074/jbc.M113.480152

Melissa B. Oliver†, Mark P. G. van der Linden§, Sharon A. Küntzel‡, Jamil S. Saad¶, and Moon H. Nahm†‡¶

From the Departments of †Microbiology and ‡Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294 and the †Department of Medical Microbiology, German National Reference Center for Streptococci, University Hospital Rheinisch-Westfälische Technische Hochschule, D-52074 Aachen, Germany

---

**Background:** Two atypical serogroup 6 strains have been discovered.

**Results:** They express capsular polysaccharide with two different repeating units and share A150T mutation in WciNα.

**Conclusion:** This mutation shifts WciNα from a galactosyltransferase to a bi-specific glycosyltransferase, creating two new hybrid serotypes: 6F and 6G.

**Significance:** Pneumococci can change capsule serotypes by point mutations and may alter their interactions with the immunity of the host.

*Streptococcus pneumoniae* is a persistent, opportunistic commensal of the human nasopharynx and is the leading cause of community-acquired pneumonia. It expresses an anti-phagocytic capsular polysaccharide (PS). Genetic variation of the capsular PS synthesis (*cps*) locus is the molecular basis for structural and antigenic heterogeneity of capsule types (serotypes). Serogroup 6 has four known members (6A–6D) with distinct serological properties, homologous *cps* loci, and structurally similar PSs. *cps* of serotypes 6A/6B have *wciNα*, encoding α-1,3-galactosyltransferase, whereas serotypes 6C/6D have *wciNβ* encoding α-1,3-glucosyltransferase. Two atypical serogroup 6 isolates (named 6X11 and 6X12) have been discovered recently in Germany. Flow cytometric studies using monoclonal antibodies show that 6X11 has serologic properties of 6B/6D, whereas 6X12 has 6A/6C. NMR studies of their capsular PSs revealed that 6X11 and 6X12 have two different repeating units with a distribution of ~40:60 6B:6D and 75:25 6A:6C PS, respectively. Sequencing of the *wciNα* gene in 6X12 and 6X11 revealed single and double nucleotide substitutions, respectively, resulting in the amino acid changes A150T and D38N. Substitution of alanine with threonine at position 150 in a 6A strain was associated with hybrid serologic and chemical profiles like 6X12. The hybrid serotypes represented by 6X12 and 6X11 strains are now named serotypes 6F and 6G. Single amino acid changes in *cps* genes encoding glycosyltransferases can alter substrate specificities, permit biosynthesis of heterogeneous capsule repeating units, and result in new hybrid capsule types that may differ in their interaction with the immune system of the host.

---

*Streptococcus pneumoniae* (pneumococcus) is an opportunistic commensal of the human nasopharynx and is the leading cause of community-acquired pneumonia, otitis media, bacterial sepsis, and meningitis (1). Pneumococcal pathogenesis is primarily associated with the polysaccharide (PS)² capsule, which shields pneumococci from phagocytosis and greatly enhances virulence (2, 3). More than 90 different capsule types (serotypes) have been determined, each of which has a unique capsular PS synthesis (*cps*) locus, chemical structure, and serologic property (4–6).

Some serotypes are serologically cross-reactive and can be grouped into one serogroup. For instance, serogroup 6 traditionally has had two cross-reactive serotypes, 6A and 6B, that have identical PSs except for the linkage between rhamnose and ribitol caused by genetic differences in the rhamnosyltransferase gene *wciP* (Fig. 1) (7). Recently, two new members were added to serogroup 6 when serotypes 6C and 6D were discovered among the isolates that were respectively typed as 6A and 6B by Quellung reaction (8–10). 6C and 6D PSs differ from 6A and 6B PSs, respectively, by having glucose (Glc)’ in place of galactose (Gal) (Fig. 1). Reflecting this structural difference, the capsule gene (*cps*) loci of serotypes 6A and 6B have *wciNα* encoding a galactosyltransferase, whereas serotypes 6C and 6D have *wciNβ*, which is distinct from *wciNα* and encodes a glucosyltransferase (11).

Although there are many pneumococcal serotypes, only a few serotypes are primarily associated with invasive disease, and current pneumococcal vaccines target those serotypes (12). For instance, serogroup 6 includes commonly pathogenic serotypes and is targeted in all pneumococcal vaccines (13). Widespread pneumococcal vaccination exerts a selection pressure against the serotypes in the vaccine and promotes the emergence of novel, nonvaccine serotypes (14, 15). Thus, serotype

---

* This work was supported, in whole or in part, by National Institutes of Health Grants R01 AI-31473 (to M. H. N.) and P30CA013148 (to the University of Alabama at Birmingham Comprehensive Cancer Center). This work was also supported by the Robert Koch-Institut and Bundesministerium für Gesundheit Project 1369-235.

1 To whom correspondence should be addressed: Dept. of Pathology, University of Alabama at Birmingham, BBRB 614, 1720 2nd Ave. S., Birmingham, AL 35294-2170. Tel.: 205-934-0163; Fax: 205-975-2149; E-mail: nahm@uab.edu.

2 The abbreviations used are: PS, polysaccharide; *cps*, capsular polysaccharide synthesis locus; RU, repeating units; HMQC, heteronuclear multiple quantum coherence; FCSA, flow cytometric serotyping analysis; HMB, heteronuclear multiple bond correlation; TOCSY, total correlation spectroscopy.
A Single Mutation Can Alter Capsular Polysaccharide

6A →2)-α-D-galp-(1→3)-α-D-glcp-(1→3)-α-L-rhap-(1→3)-D-rib-ol- (5→
6B →2)-α-D-galp-(1→3)-α-D-glcp-(1→3)-α-L-rhap-(1→4)-D-rib-ol- (5→
6C →2)-α-D-glc’p-(1→3)-α-D-glcp-(1→3)-α-L-rhap-(1→3)-D-rib-ol- (5→
6D →2)-α-D-glc’p-(1→3)-α-D-glcp-(1→3)-α-L-rhap-(1→4)-D-rib-ol- (5→

FIGURE 1. Structural models for the repeating units of serotypes 6A, 6B, 6C, and 6D. Glc’ indicates the second glucose residue in the repeating units of 6C and 6D PSs.

surveys of pneumococcal isolates are performed in many countries. In Germany, ~20,000 invasive pneumococcal disease isolates were collected from 1992 to 2012, and ~7% belong to serogroup 6 (16). However, two isolates (6X11 and 6X12) could not be assigned to one of the four known serotypes. Our studies are aimed at characterizing the serologic, genetic, and chemical basis of 6X11 and 6X12 capsules.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Other Reagents—Reference strains expressing serotypes 6A, 6B, 6C, or 6D or no capsule were TIGR6A, TIGR6B, TIGR6C, TIGR6D, and TIGRJS, respectively, which were produced in the genomic background of TIGR4 by previously described genetic manipulations (8, 17, 18). MNZ21 is a clinical isolate expressing serotype 6D (10). The 6X11 and 6X12 strains are clinical adult isolates from Germany named PS6657 and PS16864, respectively. The strains were rederived from a single colony on blood agar plates to avoid a potential mixture of two different serotypes. All pneumococci were grown at 37 °C in 5% CO2 in Todd Hewitt broth (BD Biosciences, San Jose, CA) containing 0.5% yeast extract (Becton Dickinson, Mountain View, CA) and 2% horse blood. Bacteria were harvested by centrifugation, washed, and resuspended in 20 mM Tris-HCl and 150 mM NaCl.

Flow Cytometry—Flow cytometric experiments were performed as described (10, 21). Frozen bacteria aliquots were thawed, washed, and resuspended in PBS containing 4% fetal bovine serum (Thermo Scientific, Waltham, MA). Cells were stained with phycoerythrin-conjugated goat anti-mouse IgM antibody diluted 1:1,000 or phycoerythrin-Cy7-conjugated goat anti-mouse IgG antibody diluted 1:3,000. After washing, bacteria were resuspended in FACS buffer and incubated with a culture supernatant of a hybridoma at 1:40 final dilution. After incubation for 30 min at 4 °C without shaking, the plates were washed with FACS buffer and incubated again for 30 min at 4 °C with phycocyanin-conjugated goat anti-mouse IgM antibody diluted 1:1,000 or phycocyanin-Cy7-conjugated goat anti-mouse IgG antibody diluted 1:3,000. After washing, bacteria were resuspended in FACS buffer and examined with a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA).

Capsular Polysaccharide Purification—Capsular PSs were purified from pneumococcal strains MNZ21, 6X11, and 6X12. In addition, four mutants (MBO172, MBO182, MBO184, and MBO177) were produced as described below. The strains were grown in 2 liters of chemically defined medium (JRH Biosciences, Lenexa, KS) supplemented with choline chloride (1 g/liter), sodium bicarbonate (2.5 g/liter), and cysteine-HCl (0.73 g/liter) and lysed with 0.1% deoxycholic acid. After removing cell debris by centrifugation, PS was precipitated stepwise in 30, 50, and 70% ethanol and was recovered by dissolution in 200 mM NaCl. After desalting by dialysis against MilliQ water, the PS was loaded onto a 60-mL column of DEAE-Sepharose (GE Healthcare) and eluted with a linear gradient of NaCl from 0 to 1 M. The resulting fractions were tested for PS using a multiplexed inhibition assay (22). The PS-containing fractions were pooled, desalted by dialysis, lyophilized, dissolved in 1–2 mL of water, and loaded onto a gel filtration column (120 mL of Sephacryl S-300 HR; GE Healthcare). Using a buffer containing 10 mM Tris-HCl and 100 mM NaCl, the PS was eluted from the column, and all fractions were tested for PS with the multiplexed inhibition type immunoassay (20). The fractions containing the first PS peak were pooled, lyophilized, and used for NMR studies.

NMR Spectroscopy—Briefly, 1–2 mg of lyophilized PS was dissolved in 0.6 mL of D2O for one- or two-dimensional NMR studies. The one-dimensional 1H NMR data of purified PSs were collected on a Bruker DRX (600 MHz) spectrometer equipped with a cryoprobe at 25 °C located in University of Alabama at Birmingham Comprehensive Cancer Center. The data were analyzed with the ACD/NMR Processor Academic Edition (Advanced Chemistry Development, Inc., Toronto, Canada). Assignments of 1H and 13C signals for 6X11, 6X12, 6A, 6B, and 6D PSs were achieved by two-dimensional nuclear Overhauser spectroscopy (1H-1H NOESY), correlation spectroscopy (1H-1H COSY), total correlation spectroscopy (1H-1H TOCSY), 1H-13C heteronuclear multiple quantum coherence (HMOC), and 1H-13C heteronuclear multiple bond correlation (HMBC) data collected at 45 °C on a Bruker Avance II (700 MHz) spectrometer equipped with a cryoprobe. The data were processed with NMRPipe (23) and analyzed with NMR-VIEW (24).

Site-directed Mutagenesis—Mutant pneumococcal strains were made by transforming TIGR6A with appropriate genetic constructs as described (11). An unencapsulated variant, named MBO163, was made by allelic exchange of the wchA-wcin-weiO gene region with a Janus cassette (Cassette 1) (see Fig. 6) as described (17, 27, 28). Additional genetic constructs with desired mutations at wciNa were generated by overlap extension PCR. Cassette 2 codes for WciNa with A150T mutation, cassette 3 codes for D38N and A150T mutations, cassette 4 D38N, and cassette 5 codes for A150S mutation. Replacement of the Janus cassette in MBO163 with cassettes 2, 3, 4, and 5 resulted in four recombinant strains, which were named MBO172, MBO182, MBO184, and MBO177, respectively (see Fig. 6). All mutations have been confirmed by DNA sequencing (Heflin Sequencing Core, University of Alabama at Birmingham). DNA sequences of TIGR6A, MBO163, MBO172,
MBO182, MBO184, and MBO177 were deposited in GenBank™ (accession numbers KC832412–KC832417).

**Sandwich ELISA**—Flat-bottom 96-well ELISA plates (Corning Costar Corp., Acton, MA) were coated for 5 h with an IgG mAb Hyp6CG6 (2 μg/ml) in PBS. All incubations were done at 37 °C in a humid incubator, unless otherwise stated. After washing with PBS containing 0.05% Tween 20, the plates were blocked with 5% skim milk (BD Difco, Sparks, MD) in PBS for 2 h. After washing, previously diluted purified PSs were added to the wells at concentrations ranging from 3000 to 0.03 ng/ml and incubated for 1 h. The 6A and 6C PSs were from Statens Serum Institute, whereas the 6B PS was from ATCC. The 6X12 and 6D PSs were purified from strains 6X12 and TIGR6D, respectively. After washing, an IgM mAb Hyp6DM5 (specific for 6C and 6D) or Hyp6AM3 (specific for 6A) at 1:50 dilution was added to the wells. After incubation for 1 h, the plates were washed, and alkaline phosphatase-conjugated goat anti-mouse IgM antibodies (Sigma) were added to the wells at a 1:10,000 dilution and incubated for 1 h. The amount of enzyme immobilized to wells was determined by adding paranitrophenyl phosphate substrate (Sigma) (1 mg/ml) in diethanolamine buffer and incubated at room temperature for 2 h. The optical density at 405 nm was read with a microplate reader (BioTek Instruments Inc, Winooski, VT).

**RESULTS**

**Serologic Characterization of Two Serogroup 6 Variants**—To clearly define the serologic properties of the two German isolates with ambiguous serotypes, we rederived strains from single colonies and used flow cytometry (8, 10, 29) to determine their binding to five serogroup 6-specific mAbs Hyp6BM8, Hyp6AM3, Hyp6BM1, Hyp6DM5, and Hyp6AG1, which respectively react with serotypes 6A/6B/6C/6D, 6A, 6B, 6C/6D, and 6A/6C. As serogroup 6 reference strains, we used cps variants of TIGR4 (TIGR6A, TIGR6B, TIGR6C, and TIGR6D) (8, 10, 29). Although the reference strains showed expected binding patterns (Fig. 2), 6X11 and 6X12 showed unexpected patterns. 6X11 reacted with Hyp6BM8 and Hyp6DM5 as serotype 6D does, but it also weakly but reproducibly reacted with Hyp6BM1, a 6B-specific marker. Thus, 6X11 simultaneously expressed serologic properties of both 6B and 6D. Similarly, 6X12 displayed serologic properties of serotypes 6A and 6C by reacting with Hyp6BM8, Hyp6AM3, Hyp6AG1, and Hyp6DM5. These unique serologic findings of 6X11 and 6X12 were confirmed with inhibition ELISA using pneumococcal lysates and Hyp6AM3 and Hyp6BM1 (data not shown). Thus, 6X11 and 6X12 were serologically distinct from serotype 6A, 6B, 6C, and 6D strains.

**Structural Characterization of 6X11 and 6X12 PS Capsules**—To explain the unusual serologic behavior of the two German strains, we characterized the molecular structure of their capsular PS using NMR spectroscopy. The one- and two-dimensional NMR data revealed that the 6X12 PS contains two distinct forms of capsule repeating units (RUs) and that the 1H and 13C chemical shifts of these two forms are essentially identical to those of 6A and 6C PSs. Because the anomeric signals in the 1H NMR spectra correspond to residues in PS RUs, we first compared the anemonic signals of 6X12 PS with those of 6A and 6C PSs. Three signals have been observed in the anemonic region of 6A (5.60, 5.10, and 5.02 ppm), corresponding to the anemonic protons of αGal, αGlc, and Rha (26). Similarly, the...
one-dimensional $^1$H spectrum of 6C has three signals (5.57, 5.10, and 5.02 ppm), which respectively correspond to the anomeric protons of $\alpha$Glc', $\alpha$Glc, and Rha. In contrast, 6X12 PS had the three anomeric signals of 6A PS, as well as a fourth signal at 5.57 ppm, which corresponds to $\alpha$Glc' of 6C PS (Fig. 3A). Thus, 6X12 PS appears to contain RUs of both 6A and 6C PSs, even though it is purified from a single bacterial colony.

To unambiguously determine the structure of 6X12 PS, we collected a set of two-dimensional NMR data (NOESY, COSY, TOCSY, $^1$H-$^1$C HMQC, and $^1$H-$^1$C HMBC) for 6X12 and 6A PSs. Signal assignments for 6A and 6C PS are described elsewhere and were used as a guide to assign $^1$H and $^1$C signals of 6X12 (26). As shown in Fig. 4A, $^1$H-$^1$C HMQC NMR spectra of 6X12 and 6A are essentially identical except for six additional signals assigned to $\alpha$Glc' in 6C ($^1$H/$^1$C (ppm): 5.57/99.37, 4.01/77.32, 3.84/73.70, 3.52/70.91, 4.05/73.34, and 3.80/62.05). $^1$H and $^1$C chemical shifts are summarized in Table 1. Based on the signal assignment of the second monosaccharide in 6A and 6C RUs, $\alpha$Gal, and $\alpha$Glc', respectively, we determined that 6X12 PS is a mixture of two RUs: ~75% 6A and 25% 6C. We conclude that 6X12 strain produces a novel “hybrid” capsular PS (Fig. 4C).

A similar strategy has been utilized to characterize the molecular structure and sugar composition of 6X11 PS. Complete assignment of $^1$H and $^1$C signals for 6B and 6D PS has been achieved using homonuclear and heteronuclear two-dimensional NMR data as described above. We describe in detail the assignment strategy of the 6D PS. Three $^1$H signals of anomeric proton have been observed at 5.56, 5.14, and 5.10 ppm. The anomeric proton at 5.56 ppm, which is connected to a carbon signal at 99.35 ppm in the two-dimensional HMBC spectrum, is correlated to proton signals at 3.98, 3.84, and 4.05 ppm in the two-dimensional TOCSY spectrum. Strong NOE cross-peaks have been observed between anomeric signal at 5.56 ppm and two signals at 3.84 and 3.98 ppm. Medium or weak NOEs have also been observed between the anomeric signal at 5.56 ppm and signals at 3.53 and 3.81 ppm. $^1$H-$^1$C HMBC data show correlation between the anomeric signal at 5.56 ppm and carbon signals at 77.34, 73.27, 73.66, and 81.46 ppm. In the HMQC data, the carbon signals at 77.34, 73.27, and 73.66 ppm are connected to the proton signals at 3.98, 4.05, and 3.84 ppm, respectively. The carbon signal at 81.46 ppm is connected to a proton signal at 3.94 ppm. These correlations and others identified from the COSY data allowed for the unambiguous assignment of the proton signals at 5.56, 3.98, 3.84, 3.53, 4.05, and 3.81 to the H1, H2, H3, H4, H5, and H6 protons of the $\alpha$Glc' moiety, respectively.

The signal of anomeric proton at 5.11 ppm, correlated to a carbon signal at 97.07 ppm in the HMQC spectrum, has cross-peaks to proton signals at 3.67, 3.94, 3.70, and 3.97 ppm in the TOCSY spectrum. The signal at 3.94 ppm, which is connected to a carbon signal at 81.46 ppm in the HMQC, is assigned to the H3 proton of the $\alpha$Glc because it is linked to the anomeric proton of $\alpha$Glc' in the HMBC spectrum. The pattern of cross-peak correlations in the COSY, NOESY, and HMBC is very similar to that observed for the $\alpha$Glc' moiety, indicating that the proton signals at 3.67, 3.94, 3.7, 3.97, and 3.78 ppm belong to the H2, H3, H4, H5, and H6, respectively, of the $\alpha$Glc moiety.

The third anomeric proton at 5.14 ppm belonging to the Rha moiety has cross-peaks to proton signals at 4.26, 3.87, 3.58, 3.79, and 1.3 ppm in the TOCSY spectrum. NOE cross-peaks have also been observed between the signal at 5.14 ppm and signals at 4.26, 3.87, 3.58, 3.79, and 1.3 ppm. Numerous NOE, COSY, and TOCSY cross-peak correlations between these five proton signals have also been identified. In the HMBC, the anomeric proton at 5.14 ppm has long range cross-peaks to carbon signals at 68.63, 71.16, 76.85, and 78.66 ppm. These observations allowed for the assignment of the Rha moiety as shown in Table 1. The carbon signal at 78.7 is connected to a proton signal at 4.10 ppm in the HMBC spectrum, which in turn has cross-peaks to proton signals at 3.63, 3.79, 3.83, 4.09, and 4.23 ppm in the TOCSY spectrum. In the HMBC spectrum, proton signals at 3.63 and 3.79 ppm have cross-peaks to the same carbon signal at 64.46 ppm, and signals at 4.09 and 4.23 ppm have cross-peaks to the same carbon signal at 66.30 ppm. These observations and others from the NOESY, COSY, and HMBC data led to the assignment of signals of the ribitol moiety as shown in Table 1.

As expected, serotype 6B PS had three $^1$H resonances at 5.59, 5.16, and 5.14 ppm, assigned to $\alpha$Gal, Rha, and $\alpha$Glc, respectively (25). Serotype 6D PS also had three anomeric $^1$H signals at 5.56, 5.14, and 5.11 ppm, which respectively correspond to $\alpha$Glc', Rha, and $\alpha$Glc (Fig. 3B). Interestingly, 6X11 PS had four anomeric signals: three identical to those of 6D PS and a small fourth signal at 5.59 ppm, assigned to $\alpha$Gal of 6B PS (Fig. 3B). Thus, 6X11 PS purified from a single bacterial colony appears to contain RUs composed of 6D and 6B PS (Fig. 3B). As shown in Fig. 4B, the $^1$H-$^1$C HMQC NMR spectra of 6B and 6X11 PSs are identical except for the additional six signals in 6X11 ($^1$H/$^1$C (ppm): 5.56/99.35, 3.98/77.34, 3.85/73.69, 3.82/62.13, 3.53/70.96, and 4.06/73.26; Table 1). These signals are assigned to $\alpha$Glc' in 6D as described above. Collectively, the NMR data demonstrate that 6X11 PS contains two different RUs (~40%...
6B and 60% 6D), confirming that 6X11 is a new “hybrid” capsule type. The structural model of 6X11 is summarized in Fig. 4C.

cps Loci of 6X11 and 6X12 Are Nearly Identical to Those of 6A—To determine the genetic basis for the two anomalous German strains, we determined their capsule gene loci (cps) sequences from dexB to allA (Fig. 5; GenBank™ accession numbers KC832410 and KC832411). Compared with a serotype 6A cps sequence (GenBank™ accession number CR931638), 6X11 and 6X12 sequences were 99.9 and 98.9% identical, respectively. The sequence differences were limited to ~10–100 individual nucleotides that were randomly distributed (Fig. 5). wciP allelism can distinguish serotypes 6A/6C from 6B/6D; the former group has wciPa, whereas the latter has wciPB (7). When the wci alleles of the two German strains were examined, the 6X12 sequence was identical to a typical 6A wciPa (GenBank™ accession number CR931638), but the 6X11 sequence was identical to the wciPB of a typical serotype 6B isolate (GenBank™ accession number JF911503) (30). Thus, this finding explains the association of 6X12 with serotypes 6A/6B and 6X11 with 6B/6D.

Next we examined the sequences of wciN, which genetically distinguishes serotypes 6A/6B from serotypes 6C/6D. Serotypes 6A/6B have WciNa, which adds UDP-Gal, whereas serotypes 6C/6D have WciNb that is completely different from WciNa and adds UDP-Glc (9–11). When the two German strains were examined, they had wciNα but not wciNβ. Careful comparison of the 6X12 wciNa sequence to a canonical 6A wciNa sequence (CR931638) revealed a single nucleotide substitution (G to A) at position 488 of the coding strand, changing alanine 150 to threonine 150 (Fig. 5). 6X11 wciNa had two mutations comprised of a substitution (G to A) at position 113 changing aspartic acid 38 to asparagine 38, as well as the aforementioned G448A point mutation resulting in A150T of its corresponding gene product. The two mutations were highly unusual because they were absent among all wciNa sequences of strains expressing serotype 6A or 6B in the literature. One amino acid change can convert a mono-specific glycosyltransferase to a bi-specific transferase (31). Thus, the two mutations A150T and D38N may broaden the specificity of WciNa from UDP-Gal only to UDP-Gal and UDP-Glc and be responsible for the observed serologic and biochemical changes.

WciNa Residue 150 Mediates Substrate Specificity—To confirm the hypothesis described above, we used a site-directed mutagenesis strategy to generate four isogenic TIGR6A variants (MBO172, MBO177, MBO182, and MBO184) with mutations at residues 38 or 150 of WciNa (Fig. 6). MBO172 has WciNa (A150T), MBO182 has WciNa (D38N and A150T), MBO184 has WciNa (D38N), and MBO177 has WciNa (A150S). A150S variant was made because it is found in WciNα of serotype 33B and human glycogenin-1 (32). Flow cytometry
studies suggested that all strains expressed equivalent amounts of capsule as the parent strain (data not shown) and that the mutations do not alter the amount of capsule synthesized. The mutants were then studied for antigenic changes by flow cytometry using mAbs Hyp6AG4 and Hyp6DM5, which are specific for serotypes 6A and 6C, respectively. As expected, TIGR6A and TIGR6C strains reacted with only one of the two mAbs, whereas 6X12 reacted with both mAbs. Although MBO184 reacted only with the 6A-specific mAb like the 6A strain, MBO172, MBO182, and MBO177 reacted with both mAbs like 6X12 (Fig. 7A). These findings suggest that the D38N mutation alone does not significantly change Wci\(\alpha\) ligand specificity, but the A150T or A150S mutation can alter Wci\(\alpha\) specificity and capsular PS structure.

To determine the effect of mutations on the molecular structures of PS, we obtained \(^1\)H NMR spectra of all four isogenic mutants. The \(^1\)H NMR spectra of 6X12 and MBO172 were identical in all regions, providing evidence that A150T mutation alone was responsible for the altered capsule type seen for 6X12. In contrast, the spectrum of MBO184, which has a D38N mutation, was similar to that of 6A PS, whereas the spectrum of MBO182 with both D38N and A150T mutations showed a more prominent Glc\(^p\) peak compared with Gal peak. Thus, D38N mutation alone does not alter Wci\(\alpha\) specificity but

\[ \text{TABLE 1} \]

\(^{1}\)H and \(^{13}\)C chemical shifts (ppm) of 6A, 6B, 6D, 6X11, and 6X12 PSs obtained at 45 °C

| Sugar/ribitol position | 6A PS | 6B PS | 6C PS* | 6D PS | 6X11 PS | 6X12 PS |
|------------------------|-------|-------|--------|-------|---------|---------|
| C-1                    | 5.60/99.42 | 5.59/99.38 | None | None | 5.59/99.38 | 5.60/99.42 |
| C-2                    | 4.31/74.74 | 4.28/74.95 | None | None | 4.28/74.95 | 4.31/74.74 |
| C-3                    | 3.99/70.26 | 3.99/70.19 | None | None | 3.99/70.19 | 3.99/70.26 |
| C-4                    | 4.05/71.04 | 4.06/71.05 | None | None | 4.06/71.05 | 4.05/71.04 |
| C-5                    | 4.30/72.15 | 4.31/72.14 | None | None | 4.31/72.14 | 4.30/72.15 |
| C-6                    | 3.73/62.51 | 3.72/62.45 | None | None | 3.72/62.45 | 3.73/62.51 |

* 6C PS chemical shift data as reported by Ref. 26.
enhances its preference for UDP-Glc introduced by A150T mutation. When the 1H NMR spectra of MBO177 was examined, it had a bigger Glc/H1 peak than Gal peak, suggesting that it produces more 6C RUs units than 6A RUs (Fig. 7B). Thus, the A150S mutation is more effective than A150T mutation in altering WciN/substrate specificity. Taken together, our data indicate that A150 is the key residue responsible for WciN/substrate specificity observed in the two German strains.

**6X12 PS Is a Hybrid of 6A and 6C PS RUs**—To show that 6X12 PS is a hybrid composed of 6A and 6C RUs mixed together in a single polymer (and not a mixture of two different polymers: one composed of only 6A RUs and the other composed of only 6C RUs), we used two sandwich ELISAs. One ELISA is specific for 6C RU, and the other is specific for a polymer containing both 6A and 6C RUs (Fig. 8). As expected, the ELISA for 6C RU detected 6C PS, a mixture of 6A and 6C PS,
and 6X12 PS (Fig. 8A). A mixture of 6A and 6C PSs at a 3:1 ratio was used to mimic the binary capsule model based on the predicted percentage of each RU from the NMR data in Fig. 3. 6D PS gave a weak signal at a very high concentration (Fig. 8A) because of the cross-reactivity of the detection antibody (Hyp6DM5) with 6D PS. Interestingly, only 6X12 PS produced a positive signal in the ELISA designed to detect PS chains containing both 6A and 6C RUs (Fig. 8B). Thus, our findings demonstrate that 6X12 PS is a hybrid PS with two different RUs mixed together in a single polymer.

**DISCUSSION**

Although the two German strains clearly belong to serogroup 6, our studies demonstrate that they have serologic properties, biochemical features, and genetic markers that are stable, unique, and distinct from the other four members in serogroup 6. It is well known that some established serotypes differ, unique, and distinct from the other four members in their **cps**. For instance, serotypes 9V and 9A differ by one single nucleotide (4), serotypes 15B/15C and 18B/18C differ by two nucleotides (4, 33), and serotypes 6A/6B differ by three nucleotides (7, 34). Thus, although the German strains genetically differ from serotypes 6A and 6B by one or two nucleotides, the genetic changes of the German strains alter the enzyme function, and therefore we propose the two German strains be recognized as representing two new serotypes, 6F and 6G. Serotype 6F is represented by 6X12 and has serologic properties of both serotypes 6A and 6C. Serotype 6G has properties of 6B and 6D and is represented by the strain 6X11.

The German strains provide important insights into the molecular basis of WciNa specificity. WciNa belongs to Pfam01501, which includes many glycosyltransferases used by viruses, bacteria, and eukaryotes and has the DXD motif well known for binding divalent cations (35, 36). A mutation of residue 38 of WciNa is present in only one of the two German strains, and its neighboring residues are not conserved among Pfam01501 members. Indeed, our data revealed that mutation of residue 38 does not alter ligand specificity, although it may augment the impact of mutation in residue 150. In contrast, the mutation at residue 150 is present in both German strains and is located within a highly conserved region. For instance, residues 148–152 of WciNa are conserved in human glycoegenin-1, except for residue 150 (FNAGV versus FNSGV) (32). Because residue 150 is variable, residues 149–151 are herein named as the NXG motif to simplify its description. Crystallographic studies found the NXG motif to form a part of the ligand-binding pocket: NXG of *Neisseria meningitidis* LgtC surrounds the “C1” of the donor ligand (37), and the NXG of human glycoegenin-1 interacts with the hydroxyl groups of “C2” and “C3” of Glc (32). Molecular modeling of WciNa using PHYRE2 (38) also predicted the NXG motif to form a ligand-binding pocket. Pfam01501 members with NAG are often galactosyltransferases like WciNa, whereas members with NSG, like glycoegenin-1, are often glucosyltransferases. Our studies clearly show that substitution of alanine in NAG to threonine or serine alters the ligand specificity of WciNa, making it capable of transferring both galactose as well as glucose. Taken together, we conclude that NXG is critical to WciNa ligand specificity and probably to the specificity of all Pfam01501 members.

More interesting is that both A150T and A150S mutations turn WciNa into bi-specific transferases, and the mutants produce novel PSs with two different RUs. Such a novel PS raises interesting points, such as the fact that one RU may favor termination of PS chains or require a higher substrate level than the other. Because of the interesting biochemical properties, characterization of bi-specific transferases is of high importance. To date, only two eukaryotic transferases have been reported, of which one is natural (39) and the other is artificially created (31). In contrast, studies of bacterial transferases suggest several examples of bi-specific transferases. Pneumococci (34) and meningococci (40) with mixed capsule types have been described. Although they may produce PSs with mixed RUs, their genetic and chemical bases have been incompletely characterized. Better described is LOS, which is produced by *Campylobacter jejuni* with a Cst-II variant (T51N) (41). The mutation was shown to be responsible for producing two RUs with different glycosidic linkages. Using WciNa variants, we have provided a clear example that can transfer two different ligands. mAbs that are specific for the different RUs are available, genetic manipulations are easily performed with pneumococci, and a simple *in vitro* substrate for WciNa was described recently (42). WciNa is therefore useful for studying the molecular basis of bi-specific transferases.
A Single Mutation Can Alter Capsular Polysaccharide

The significance of PS with multiple RUs in host-pathogen interactions is unclear at the moment. As the most exposed structure for bacteria, capsular PS is critical to host-pathogen interaction. Also a minor structural change can dramatically alter its interaction with the adaptive or innate immunity of the host. For instance, pneumococcal serotypes 19A and 19F, which differ by one linkage in their RUs, are starkly different in their cross-reactivity with vaccine-induced antibodies (43) and also binding of factor H (44). Also, C. jejuni strains with the Cst-II variant elicit a unique autoimmune disease (41). Thus, investigation of serotypes 6F and 6G will provide new insights in understanding survival advantages by comparing their complement binding and reaction with antibodies with other serogroup 6 members.

Because capsular PS is important in host-pathogen interaction, capsule evolution has been extensively studied. Most studies found that pneumococci regularly switch capsule types by acquiring new DNA from other bacteria through genetic recombination (45, 46). However, we show two single base mutations that are synergistic in capsule type alterations. Perhaps, there may be a third mutation that may complete serotype change from 6A to 6C. Thus, if point mutations give survival benefits to pneumococci, the presence of such mutational stepping stones would open an evolutionary pathway for pneumococci to alter their capsule structure without a source of foreign DNA. This serotype shift is probably useful in invading deeper tissues from the nasopharynx or in rapid responses to vaccination. Interestingly, evidence for such a capsule type shift by mutation has been recently described for Streptococcus iniae infecting vaccinated fish in fish farms (47).

Because protective immunity is associated with the surface PS, pathogens are commonly divided into discrete serotypes based on their surface PS structure. With increased knowledge of the genetic basis for PS synthesis, genetic markers are often used alone to determine serotypes (48–50). However, genetic differences between two serotypes may be only one or two nucleotides. Furthermore, the presence of PSs with mixed RUs blurs serologic boundaries and the definitions of serotypes. Thus, one should recognize that one typing method may be inadequate to identify new serotypes. Multiple analytical approaches will be required for accurate identification of serotypes useful for correct prediction of protective immunity.

Acknowledgments—We thank Dr. C. Abeygunawardana and Dr. William H. Benjamin, Jr., for critical readings and Dr. Ronald Shin for the one-dimensional NMR data.

REFERENCES

1. Mushler, D. M. (1992) Infections caused by Streptococcus pneumoniae. Clinical spectrum, pathogenesis, immunity, and treatment. Clin. Infect. Dis. 14, 801–807
2. Hyams, C., Camberlein, E., Cohen, J. M., Bax, K., and Brown, J. S. (2010) The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. Infect Immun 78, 704–715
3. Avery, O. T., and Dubos, R. (1931) The protective action of a specific enzyme against type III pneumococcus infection in mice. J. Exp. Med. 54, 73–89
4. Mavroidi, A., Aanensen, D. M., Godoy, D., Skovsted, I. C., Kaltsoy, M. S., Reeves, P. R., Bentley, S. D., and Spratt, B. G. (2007) Genetic relatedness of the Streptococcus pneumoniae capsular biosynthetic loci. J. Bacteriol. 189, 7841–7855
5. Calix, J. I., Porombo, R. J., Brady, A. M., Larson, T. R., Yother, J., Abeygunawardana, C., and Nahm, M. H. (2012) Biochemical, genetic and serological characterization of two capsule subtypes among Streptococcus pneumoniae serotype 20 strains. Discovery of a new pneumococcal serotype. J. Biol. Chem. 287, 27885–27894
6. Yother, J. (2004) Capsule, in The pneumococcus (Tuomanen, E. I., Mitchell, T. J., Morrison, D. A., and Spratt, B. G., eds), pp. 30–48, ASM Press, Washington DC
7. Mavroidi, A., Godoy, D., Aanensen, D. M., Robinson, D. A., Hollingshead, S. K., and Spratt, B. G. (2004) Evolutionary genetics of the capsular locus of serogroup 6 pneumococci. J. Bacteriol. 186, 8181–8192
8. Park, I. H., Pritchard, D. G., Cartee, R., Brandao, A., Brandileone, M. C., and Nahm, M. H. (2007) Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. J. Clin. Microbiol. 45, 1225–1233
9. Jin, P., Kong, F., Xiao, M., Offadeh, S., Zhou, F., Liu, C., Russell, F., and Gilbert, G. L. (2009) First report of putative Streptococcus pneumoniae serotype 6D among nasopharyngeal isolates from Ijkanchildren. J. Infect. Dis. 200, 1375–1380
10. Bratcher, P. E., Kim, K. H., Kang, J. H., Hong, I. Y., and Nahm, M. H. (2010) Identification of natural pneumococcal isolates expressing serotype 6D by genetic, biochemical, and serological characterization. Microbiology 156, 555–560
11. Park, I. H., Park, S., Hollingshead, S. K., and Nahm, M. H. (2007) Genetic basis for the new pneumococcal serotype, 6C. J. Infect. Immun. 75, 4482–4489
12. Robbins, J. B., Austrian, R., Lee, C. I., Rastogi, S. C., Schiffman, G., Henrichsen, J., Mäkelä, P. H., Broome, C. V., Facklam, R. R., and Tiesjema, R. H. (1983) Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. J. Infect. Dis. 148, 1136–1139
13. Hausdorff, W. P., Bryant, J., Paradiso, P. R., and Siber, G. R. (2000) Which pneumococcal serogroups cause the most invasive disease. Implications for conjugate vaccine formulation and use. Part I. Clin Infect Dis 30, 100–121
14. Steenhoff, A. P., Shah, S. S., Ratner, A. J., Patil, S. M., and McGowan, K. L. (2006) Emergence of vaccine-related pneumococcal serotypes as a cause of bacteremia. Clin. Infect. Dis. 42, 907–914
15. Singleton, R. J., Hennessy, T. W., Bulkow, L. R., Hammitt, L. L., Zulz, T., Hurlburt, D. A., Butler, J. C., Rudolph, K., and Parkinson, A. (2007) Invasive pneumococcal disease caused by nonvaccine serotypes among alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA 297, 1784–1792
16. van der Linden, M., Winkel, N., Küntzel, S., Farkas, A., Perniciaro, S. R., Reinert, R. R., and Imöhl, M. (2013) Epidemiology of Streptococcus pneumoniae serogroup 6 isolates from IPD in children and adults in Germany. PLoS One 8, e60848
17. Trzcinski, K., Thompson, C. M., and Lipsitch, M. (2003) Construction of otherwise isogenic serotype 6B, 7F, 14, and 19F capsule variants of Streptococcus pneumoniae strain TIGR4. Appl. Environ. Microbiol. 69, 7364–7370
18. Bratcher, P. E., Park, I. H., Hollingshead, S. K., and Nahm, M. H. (2009) Production of a unique pneumococcal capsule serotype belonging to serogroup 6. Microbiology 155, 576–583
19. Sun, Y., Hwang, Y., and Nahm, M. H. (2001) Avidity, potency, and cross-reactivity of monoclonal antibodies to pneumococcal capsular polysaccharide serotype 6B. Infect. Immun. 69, 336–344
20. Yu, J., Lin, J., Kim, K. H., Benjamin, W. H., Jr., and Nahm, M. H. (2011) Development of a multiplexed and automated serotyping assay for Streptococcus pneumoniae. Clin. Vaccine Immunol. 18, 1900–1907
21. Calix, J. I., Saad, J. S., Brady, A. M., and Nahm, M. H. (2012) Structural characterization of Streptococcus pneumoniae serotype 9A capsule polysaccharide reveals role of glycosyl 6-O-acetytransferase wjeE in serotype 9V capsule biosynthesis and immunogenicity. J. Biol. Chem. 287, 13996–14003
A Single Mutation Can Alter Capsular Polysaccharide

22. Yu, J., Carvalho Mda, G., Beall, B., and Nahm, M. H. (2008) A rapid pneumococcal serotyping system based on monoclonal antibodies and PCR. *J. Med. Microbiol.* 57, 171–178

23. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe. A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* 6, 277–293

24. Johnson, B. A., and Blevins, R. A. (1994) NMR View. A computer program for the visualization and analysis of NMR data. *J. Biomol. NMR* 4, 603–614

25. Talaga, P., Bellamy, L., and Moreau, M. (2001) Quantitative determination of C-polysaccharide in *Streptococcus pneumoniae* capsular polysaccharides by use of high-performance anion-exchange chromatography with pulsed amperometric detection. *Vaccine* 19, 2987–2994

26. Cai, P., Moran, J., Pavliak, V., Deng, C., Khoury, N., Marcq, O., and Ruppen, M. E. (2012) NMR structural analysis of the capsular polysaccharide from *Streptococcus pneumoniae* serotype 6C. *Carbohydr Res* 351, 98–107

27. Sung, C. K., Li, H., Claverys, J. P., and Morrison, D. A. (2001) An rpsL cassette, janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. *Appl. Environ. Microbiol.* 67, 5190–5196

28. Xayarath, B., and Yother, J. (2007) Mutations blocking side chain assembly, polymerization, or transport of a Wzy-dependent *Streptococcus pneumoniae* capsule are lethal in the absence of suppressor mutations and can affect polymer transfer to the cell wall. *J. Bacteriol.* 189, 3369–3381

29. Lin, J., Kaltoft, M. S., Brandao, A. P., Echaniz-Aviles, G., Brandileone, M. C., Hollingshead, S. K., Benjamin, W. H., and Nahm, M. H. (2006) Validation of a multiplex pneumococcal serotyping assay with clinical samples. *J. Clin. Microbiol.* 44, 383–388

30. Elberse, K., Witteveen, S., van der Heide, H., van de Pol, I., Schot, C., van der Ende, A., Berbers, G., and Schouls, L. (2011) Sequence diversity within the capsular genes of *Streptococcus pneumoniae* serogroup 6 and 19. *PLoS One* 6, e25018

31. Ramakrishnan, B., and Qasba, P. K. (2002) Structure-based design of LgtC from *Neisseria meningitidis* crystal structure of the retaining galactosyltransferase. *Biochemistry* 51, 7945–7950

32. Chaikuad, A., Froese, D. S., Berridge, G., von Delft, F., Oppermann, U., and Strynadka, N. C. (2001) Crystal structure of the retaining galactosyltransferase LgtC from *Neisseria meningitidis*. *J. Biol. Chem.* 276, 10000–10009

33. Persson, K., Hjorth, L. D., Dieckelmann, M., Wakarchuk, W. W., Withers, S. G., and Strynadka, N. C. (2001) Crystal structure of the retaining galactosyltransferase LgtC from *Neisseria meningitidis* in complex with donor and acceptor sugar analogs. *Nat. Struct. Biol.* 8, 166–175

34. Kelley, L. A., and Sternberg, M. J. (2009) Protein structure prediction on the Web. A case study using the Phyre server. *Nat. Protoc.* 4, 363–371

35. Ramakrishnan, B., and Qasba, P. K. (2007) Role of a single amino acid in the evolution of glycans of invertebrates and vertebrates. *J. Mol. Biol.* 365, 570–576

36. Claus, H., Stummeyer, K., Batzilla, J., Mühlenhoff, M., and Vogel, U. (2009) Amino acid 310 determines the donor substrate specificity of serogroup W-135 and Y capsule polymerases of *Neisseria meningitidis*. *Mol. Microbiol.* 71, 960–971

37. Yu, K. (2007) *Campylobacter* sialyltransferase gene polymorphism directs clinical features of Guillain-Barre syndrome. *J. Neurochem.* 103, 150–158

38. Han, W., Cai, L., Wu, B., Li, L., Xiao, Z., Cheng, J., and Wang, P. G. (2012) The wciN gene encodes an α-L-3-galactosyltransferase involved in the biosynthesis of the capsule repeating unit of *Streptococcus pneumoniae* serotype 6B. *Biochemistry* 51, 5804–5810

39. Lee, H., Nahm, M. H., Burton, R., and Kim, K. H. (2009) Immune response in infants to the heptavalent pneumococcal conjugate vaccine against vaccine-related serotypes 6A and 19A. *Clin. Vaccine Immunol.* 16, 376–381

40. Hyams, C., Trzciński, K., Camberlein, E., Weinberger, D. M., Chimalapati, S., Noursadeghi, M., Lipsitch, M., and Brown, J. S. (2013) *Streptococcus pneumoniae* capsular serotype invasiveness correlates with the degree of factor H binding and opsonisation with C3b/iC3b. * Infect. Immun.* 81, 354–363

41. Coffey, T. J., Enright, M. C., Daniels, M., Morona, J. K., Morona, R., Hryniecwich, W., Paton, J. C., and Spratt, B. G. (1998) Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype changes among natural isolates of *Streptococcus pneumoniae*. * Mol. Microbiol.* 27, 73–83

42. Wyres, K. L., Lamberts, L. M., Croucher, N. J., McGee, L., von Gottberg, A., Liliars, J., Jacobs, M. R., Kristinsson, K. G., Beall, B. W., Klugman, K. P., Parkhill, J., Hakenbeck, R., Bentley, S. D., and Brueggemann, A. B. (2013) Pneumococcal capsular switching. A historical perspective. *J. Infect. Dis.* 207, 439–449

43. Millard, C. M., Baiano, J. C., Chan, C., Yuen, B., Aviles, F., Landos, M., Chong, R. S., Benedict, S., and Barnes, A. C. (2012) Evolution of the capsular operon of *Streptococcus iniae* in response to vaccination. *Appl. Environ. Microbiol.* 78, 8219–8226

44. Pai, R., Gertz, R. E., and Beall, B. (2006) Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. *J. Clin. Microbiol.* 44, 124–131

45. Kong, F., Wang, W., Tao, J., Wang, L., Wang, Q., Sabananthan, A., and Gilbert, G. L. (2005) A molecular-capsular-type prediction system for 90 *Streptococcus pneumoniae* serotypes using partial cpsA-cpsB sequencing and wzy- or wzx-specific PCR. *J. Med. Microbiol.* 54, 351–356

46. Batt, S. L., Charalamous, B. M., McHugh, T. D., Martin, S., and Gillespie, S. H. (2005) Novel PCR-restriction fragment length polymorphism method for determining serotypes or serogroups of *Streptococcus pneumoniae* isolates. *J. Clin. Microbiol.* 43, 2656–2661