Production of a unique pneumococcal capsule serotype belonging to serogroup 6

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Serogroup 6 of *Streptococcus pneumoniae* contains three serotypes, named 6A, 6B and 6C, with highly homologous capsule gene loci. The 6A and 6B capsule gene loci consistently differ from each other by only one nucleotide in the *wciP* gene. The 6A capsule gene locus has a galactosyltransferase, which has been replaced with a glucosyltransferase in the 6C capsule gene locus. We considered that a new serotype named '6X1' would be possible if the galactosyltransferase of the 6B capsule gene locus is replaced with the glucosyltransferase of 6C. We demonstrate that this gene transfer yields a viable pneumococcal strain and that the capsular polysaccharide (PS) from this strain has the predicted chemical structure and serological similarity to the capsular PS of the 6B serotype. The new strain (i.e. serotype 6X1) is typed as 6B by the quellung reaction, but it can be distinguished from 6B strains with mAbs to 6B PS. Reexamination of 264 pneumococcal isolates that had been previously typed as 6B with classical typing methods revealed no isolates expressing serotype 6X1. Nevertheless, this study shows that this capsular PS is biochemically possible and could exist/emerge in nature.

Received 24 September 2008

Revised 4 November 2008

Accepted 12 November 2008

INTRODUCTION

Streptococcus pneumoniae is a major human pathogen commonly responsible for pneumonia, bacteraemia, meningitis and otitis media, especially among young children and older adults (Fedson, 1988). The most prominent virulence factor of the pneumococcus is the capsular polysaccharide (PS), which coats the surface of the bacterium to block antibodies and complement from binding to surface moieties and being recognized by phagocytic cells (Avery & Dubos, 1931). To avoid host immunity, *S. pneumoniae*, as a species, can express at least 91 distinct capsules which are chemically and serologically distinct (Henrichsen, 1995; Park *et al.*, 2007b). Antibodies against this PS have been shown to provide serotype-specific protection from infection, and vaccines against pneumococcus incorporate capsular PSs of the most prevalent strains (Cole, 1913). For example, serogroup 6 strains are very common in invasive pneumococcal disease and the current vaccines are formulated to protect against serogroup 6 infections (Hausdorff *et al.*, 2000).

Serogroup 6 has classically contained two serotypes, 6A and 6B (Kamerling, 2000). These two serotypes produce capsules with very similar structures. Both of these PSs have repeating units composed of galactose–glucose–

rhamnose–ribitol–phosphate, but the linkage between rhamnose and ribitol is 1→3 for 6A and 1→4 for 6B (Kamerling, 2000). The two serotypes also have very similar capsule gene loci, which are about 17 kb in size and contain all the genes for capsule biosynthesis (Jiang *et al.*, 2001; Park *et al.*, 2007a). The only genetic difference between 6A and 6B serotypes has been attributed to one nucleotide of *wciP*, which encodes a rhamnosyltransferase responsible for the rhamnose–ribitol linkage (Aanensen *et al.*, 2007; Mavroidi *et al.*, 2004). *WciP* with a codon for serine at residue 195 is associated with serotype 6A, but a codon for asparagine is associated with 6B (Mavroidi *et al.*, 2004).

Recently, a new serotype, 6C, was discovered using two mAbs (Lin *et al.*, 2006; Park *et al.*, 2007b). Although 6C was previously typed as 6A with classical serotyping tools, chemical analysis revealed that the galactose of the 6A PS is replaced by a glucose residue in 6C. Genetic studies of the 6A and 6C capsule gene loci have shown that *wciN* is responsible for the difference, as substitution of *wciN*_{6C} (*wciN* of 6C) for *wciN*_{6A} (*wciN* of 6A) through homologous recombination results in a serotype switch from 6A to 6C (Park *et al.*, 2007a).

The discovery of 6C led to the logical suggestion that recombination might also produce a new member of the serogroup 6 family, herein labelled '6X1', which would have *wciP* of 6B and *wciN*_{6C}. The designation 6X1 is used to distinguish this artificial serotype from the next naturally

Abbreviation: PS, polysaccharide.

The GenBank/EMBL/DBJ accession number for the TIGR6X1 sequence of *Streptococcus pneumoniae* is EU714777.

found serogroup 6 member, which, if discovered, might be designated 6D. Chemically, 6X1 PS may have glucose instead of galactose and have a 1→4 rhamnose–ribitol linkage. However, it was not clear whether the theoretical 6X1 serotype was indeed biologically feasible, nor was it clear whether it might already exist in nature. Therefore, we have produced a serotype 6X1 strain and examined our laboratory collection of pneumococcal isolates for a possible 6X1 strain.

METHODS

Bacterial strains and culture. To determine whether 6X1 exists in nature, 264 pneumococcal isolates that were previously serotyped as '6B' by classical means were reserotyped for serotypes 6B or 6X1 using mAbs. The isolates were a part of our laboratory collection of 6B isolates, which have originated from Africa, Asia, Australia, South America, North America and Europe. In addition to these, TIGR6A, TIGR6AX and TIGR6C, which are isogenic strains of TIGR4 expressing the 6A-type capsule, no capsule, and 6C-type capsule (Park *et al.*, 2007a), were used as assay controls or as a source of DNA. The source of the TIGR4 strain was the authors' laboratory (Tettelin *et al.*, 2001). Additional TIGR4 variants, TIGR6B, TIGR6BX and TIGR6X1, were prepared as described below. All bacteria were grown in Todd–Hewitt broth (BD Biosciences) supplemented with 0.5% yeast extract (THY) and kept frozen at –80 °C until used. The TIGR6X1 strain will be available upon request from the University of Alabama at Birmingham (UAB) to all qualified investigators for research purposes.

PCR and DNA sequencing. PCR mixtures contained 38.8 µl sterile water, 2 µl of each primer (5 pmol µl^{–1}), 2 µl 10 mM dNTP, 5 µl 10 × LA *Taq* buffer solution (Takara Biochemical), and 0.2 µl LA *Taq* polymerase (2.5 U µl^{–1}; Takara Biomedical). As template, either chromosomal DNA isolated with a Wizard genomic DNA purification kit (Promega) or colonies grown on blood agar plates were used. Thermal cycling conditions varied depending on the primer set used. PCR products were analysed by electrophoresis in 1% agarose gels. The primers used are listed in Table 1. PCR products were purified using the Wizard PCR Clean-up System (Promega), and the DNA sequencing was performed by the Genomics Core Facility at UAB. DNA sequences were analysed with Lasergene v5.1 software (DNASTAR) and were compared with the previously reported sequences of the 6B

and 6C *cps* loci in GenBank (accession nos CR931639 and EF538714, respectively) (Mavroidi *et al.*, 2004).

Construction of TIGR6X1 by replacing *wciN*_{6A} of TIGR6B with *wciN*_{6C}. Our strategy for creating TIGR6X1 is described in Fig. 1. First, TIGR6B expressing serotype 6B was prepared by inserting the 6B capsule gene locus region into the TIGR4 genetic background using the Janus-cassette system, as previously described in detail (Park *et al.*, 2007a). Second, the *wciN* gene was removed from TIGR6B (Park *et al.*, 2007a) by transforming it with cassette 1 and selecting for kanamycin-resistant isolates. Cassette 1 has the Janus cassette, which contains a kanamycin-resistance gene (*kan*^R) and a streptomycin-sensitivity gene (*rpsL*⁺), and two flanking regions designed for homologous recombination to the 6B capsule gene locus (Park *et al.*, 2007a). A kanamycin-resistant strain was obtained and back-crossed into TIGR6B three times using genomic DNA from this resistant strain. Back-crossing was performed in order to minimize the possibility of unwanted mutations in the TIGR6B background. The resulting strain, which was labelled TIGR6BX, lost *wciN* and did not produce capsular PS. To insert *wciN*_{6C}, TIGR6BX was transformed with Cassette 2. Cassette 1 and cassette 2 were prepared from the genomic DNAs of TIGR6AX and CHPA388, respectively, using primer set 5113 and 3102 (Park *et al.*, 2007a). While Cassette 2 contained a part of *wciP* in addition to *wciN*_{6C}, it did not contain the *wciP* codon responsible for distinguishing 6A and 6B serotypes (Fig. 1). After selection for streptomycin resistance and back-crossing against TIGR6BX three times, a streptomycin-resistant strain was produced, designated TIGR6X1. When the capsule gene locus of TIGR6X1 was sequenced from *wchA* to *wciP*, the sequence showed that *wciN*_{6B} had been replaced with the *wciN*_{6C} gene, as intended. For this sequencing, primer sets 5114–3141, 5138–3104 and 5106–3105 were used to produce amplicons, and primers 5103, 5108 and 5129 were used in sequencing (see Table 1 for primer sequences). The TIGR6X1 sequence has been deposited in GenBank (accession no. EU714777). TIGR6X1 was morphologically indistinguishable from TIGR6B when grown on blood agar plates. Also, TIGR6X1 grew as well as other pneumococcal strains in THY broth (data not shown).

Quellung reaction. Bacterial colonies from blood agar plates were suspended in a small volume of PBS (0.137 M NaCl, 2.31 mM KH₂PO₄, 7.69 mM Na₂HPO₄ and 2.15 mM KCl), and 2 µl of this broth was combined with 2 µl serum and 2 µl methylene blue dye solution (3 mg ml^{–1} methylene blue, 1.5 mg ml^{–1} NaCl in sterile water) on a glass microscope slide. After adding a coverslip, mixtures were examined under bright-field microscopy using a ×100

Table 1. List of PCR primers

Primer	Sequence (5'–3')	Source or reference
Forward primers		
5103	AAACATGACATCAATTACA	Park <i>et al.</i> (2007a)
5106	TACCATGCAGGGTGAATGT	Park <i>et al.</i> (2007a)
5108	ATGGTGAGAGATATTTGTCAC	Park <i>et al.</i> (2007a)
5113	GGGAAAAATAAAAAATAGGTCGGG	Park <i>et al.</i> (2007a)
5114	TTAGTGACGGAGGCAGGTGAA	This study
5129	TCCTACTTACAGCAACTTCTCGTG	This study
5138	AAAGCTATGTCGCCTGCTAAAAAAGCAATGGCTA	This study
Reverse primers		
3102	CTGGCATGTCATCTTTAGAAAA	This study
3104	CCTGAAAAACAATACTACTT	This study
3105	TTCCCATCTCTAAACATTCTCCT	This study
3141	GGCGACATAGCTTTTCTTTCAATATCTT	This study

Fig. 1. Diagram of the *wciN* region exchange experiment; an asterisk at the *wciP* gene indicates the critical codon (serine versus asparagine) associated with the 6A/6B serotype difference (Mavroidi *et al.*, 2004). *kanA^R*, kanamycin-resistance gene; *rpsL⁺*, streptomycin-sensitivity gene.

oil-immersion lens. The rabbit antisera specific for serotypes 6A and 6B were prepared by the Centers for Disease Control and Prevention (CDC). Factor 6a serum made by Staten Serum Institut was purchased from Mira Vista Diagnostics.

Inhibition ELISA to distinguish serotypes 6B and 6X1. The two serotypes were distinguished using an inhibition-type ELISA. Briefly, the wells of ELISA plates (Corning Costar) were coated at 37 °C with 5 µg ml⁻¹ 6B capsular PS (ATCC) overnight in PBS. After washing the plates three times with PBS containing 0.05 % Tween 20, 50 µl of a previously diluted bacterial culture supernatant (or lysate) was added to the wells along with 50 µl anti-6B mAb. Pneumococcal lysates were prepared by growing pneumococci overnight in 1 ml THY broth without shaking and then incubating the tubes for 15 min at 37 °C with a lysis buffer (0.1 % sodium deoxycholate, 0.01 % SDS, 0.15 M sodium citrate in deionized water). Culture supernatants of 6B-specific hybridomas Hyp6BM7 and Hyp6BM8 were used at dilutions of 1:50 and 1:100, respectively. These hybridomas were produced from a fusion of myeloma cells with spleen cells isolated from mice immunized with 6B PS (Sun *et al.*, 2001). After 30 min incubation in a humid incubator at 37 °C, the plates were washed three times and incubated for 30 min with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Sigma). The plates were washed three times, and then 100 µl paranitrophenyl phosphate substrate (Sigma) in diethanolamine buffer at a concentration of 1 mg ml⁻¹ was added, and allowed to incubate at room temperature for 1–2 h. The A₄₀₅ was read with a microplate reader (BioTek Instruments).

Purification of capsular PS. Capsular PS expressing serotype 6X1 was purified in two different ways. One method was to purify the PS by ethanol precipitation, ion-exchange chromatography and molecular mass-sizing chromatography, as described previously (Park *et al.*, 2007b). The other method, which is faster than the first method, was to purify capsular PS after removing protoplasts, as described below. TIGR6X1 was grown in 1 l THY broth without shaking until the culture reached OD₆₀₀ ~0.4. The culture was then centrifuged at 15 000 g for 10 min. The cell pellet was washed twice with 1 l PBS, resuspended in 30 ml protoplast buffer [20 % sucrose, 5 mM Tris/HCl (pH 7.4), 2.5 mM Mg₂SO₄ in deionized water] with mutanolysin (Sigma) at a concentration of 20 U ml⁻¹, and allowed to incubate overnight at room temperature. The next day, the bacterial cells were examined under a phase-contrast microscope to ensure that

‘protoplasting’ had occurred, and then protoplasts were removed by centrifugation at 27 000 g for 15 min. The supernatant was sterilized through a 0.22 µm pore-size filter, diluted 1:1 in deionized water, and loaded onto a DEAE-Sepharose column (Amersham Biosciences) with a 2 ml bed volume. The column was washed with 4 ml 50 mM ammonium acetate, and the PS was eluted from the column with 4 ml 500 mM ammonium acetate. After lyophilization, the eluted PS was loaded on a Sephacryl S-300 HR column (Amersham Biosciences) with a bed volume of 130 ml and the PS was eluted with 10 mM Tris/HCl (pH 7.4). The fractions were tested for the presence of PS by an inhibition assay using Hyp6BM8. The first 10 ml of fractions, which contains most of the PS, were pooled and lyophilized.

Monosaccharide composition analysis of PS. A 1 mg sample of lyophilized capsular PS prepared by the protoplast method was dissolved in 500 µl of 1M HCl and incubated at 80 °C for 16 h. After drying the sample under a nitrogen stream, the remaining PS was washed twice with 250 µl methanol. The sample was then incubated with 200 µl Tri-Sil Reagent (Pierce Biotech) to trimethylsilylate all the monosaccharides. The reaction products were analysed on a gas-liquid chromatograph/mass spectrometer (Varian 4000) fitted with a 15 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⁻¹, and finally held at 275 °C for 5 min. The effluent was analysed by MS using the electron impact ionization mode. The area of each monosaccharide peak in GLC-MS was determined using Varian MS Workstation v6.5 software.

Analysis of PS by MS-MS. Intact capsular PSs prepared by the ethanol-precipitation method were hydrolysed to their repeating units before analysis by MS. A 2 mg sample of PS was hydrolysed in 1 ml 10 mM NaOH at 85 °C for 120 h, followed by another hydrolysis with 50 mM NaOH at 85 °C for 120 h. At the end of hydrolysis, all samples were neutralized with 0.1 M HCl.

MS-MS was performed at the Mass Spectrometry Shared Facility at the UAB with a Micromass Q-TOF2 mass spectrometer equipped with an electrospray ion source. The samples, dissolved in distilled water, were injected into the mass spectrometer with running buffer (50:50 acetonitrile:water containing 0.1 % formic acid) at a rate of 1 µl min⁻¹ using a Harvard syringe pump. The injected sample was negatively ionized with electrospray and detected with a time-of-flight

mass spectrometer. For MS-MS, the parent ion was fragmented into daughter ions by energizing it to either 35 or 40 eV before collision with argon gas. The daughter ions were analysed with a time-of-flight mass spectrometer. The MS-MS spectra were processed using the Max-Ent3 module of MassLynx 2.5.

Oxidation and reduction of PS. Capsular PSs were dissolved in 80 mM sodium acetate buffer (pH 4) at a concentration of 1 mg ml⁻¹. Sodium periodate was added to the PS solution to a final concentration of 40 mM and the reaction mixture was incubated in the dark at 4 °C for 72 h. Excess periodate was destroyed by adding ethylene glycol. To determine the intact monosaccharides of the oxidized capsular PS, PS was then lyophilized and analysed using GLC-MS as described above. To investigate the glycosidic bonds, the sample was reduced with sodium borohydride or sodium tetra-deuteroborate as previously described (Park *et al.*, 2007b), before being subjected to MS-MS as described above. 6X1 PS was prepared by the protoplast method, and 6B PS was obtained from the ATCC.

Hydrolytic stability assay. A 0.9 ml volume of PS (2 mg ml⁻¹) in water was mixed with 0.1 ml 0.1 M NaOH, and this solution was split into two Eppendorf tubes and incubated at 85 °C. At the indicated times, 0.1 ml was removed from these samples, neutralized with 0.1 M HCl, and then stored at 4 °C until used in the inhibition ELISA. Using the same buffers and incubation conditions described for the inhibition ELISA above, plates were coated with 100 µl 6A, 6B, 6C or 6X1 PS (5 µg ml⁻¹). The ELISA was performed with the hydrolysed samples on plates coated with their respective PSs. For 6A and 6C PSs, Hyp6AG1 was used as the primary antibody (as performed in Park *et al.*, 2007b), and for 6B and 6X1, Hyp6BM8 was used (as described above). Data shown are the average of samples run in duplicate.

RESULTS

6X1 capsular PS is structurally different from 6B capsular PS

Recently, we have demonstrated the structure of 6C capsular PS by identifying its monosaccharide composition by GLC-MS, and identifying the sequence of monosaccharides and their glycosidic linkages by MS-MS (Park *et al.*, 2007b). We used similar approaches to show that TIGR6X1 produces a capsular PS that is chemically different from that of TIGR6B. We first determined the monosaccharide/ribitol composition of TIGR6B and TIGR6X1 capsular PS by GLC-MS. The chromatogram for 6B showed peaks for ribitol, rhamnose, galactose and glucose, as expected for 6B capsular PS (Fig. 2). However, the 6X1 chromatogram did not show galactose peaks, although it showed peaks for ribitol, rhamnose and glucose (Fig. 2). Thus, TIGR6X1 produces a capsular PS different from that of TIGR6B. We then analysed the monosaccharide composition after treating TIGR6B and TIGR6X1 PSs with sodium periodate, which selectively destroys ribitol and monosaccharides with vicinal glycols. As expected for 6B PS, periodate treatment of TIGR6B PS eliminated the peaks for ribitol and galactose (Fig. 2), but the treatment did not alter the rhamnose and glucose peaks. In contrast, periodate treatment of TIGR6X1 PS extinguished the ribitol peak and reduced the glucose peaks, such that the glucose peak area became similar to the rhamnose peak

Fig. 2. GLC-MS chromatograms showing carbohydrate composition of capsular PS from pneumococcal strains expressing 6B and 6X1 serotypes and before and after periodate treatment. The monosaccharides are identified in the top chromatogram. Each panel is labelled with a description of the sample.

area. This suggested that the repeating unit of 6X1 PS has two glucose residues, whereas the repeating unit of 6B PS has one glucose residue and one galactose residue.

To determine whether the monosaccharide sequence of the 6X1 PS is as proposed in Fig. 3(a), we performed an alkali hydrolysis which breaks the phosphodiester bonds and produces repeating units. As previously observed for 6C PS (Park *et al.*, 2007b), the hydrolysis yields two types of repeating units of identical mass, one with the phosphate ion linked to ribitol (labelled forward fragmentation) and another linked to glucose (labelled reverse fragmentation). The phosphate ion endows the repeating unit with a negative charge. When the alkali hydrolysis product was analysed for negative ions by MS-MS, the results showed two prominent peaks of 683 and 701 amu (Fig. 3b), which were identical to the anhydrous and hydrated masses, respectively, of the predicted repeating unit of 6X1 PS (Fig. 3a). The peak at 260.902 amu was absent in other MS-MS attempts and may represent a contaminant.

We then subjected the ion of 683 amu (i.e. the intact repeating unit) to argon collision and identified its daughter ions by MS-MS analysis. We found daughter ions at 521, 359 and 213 amu, which respectively represent daughter ions that have lost the first glucose, the second glucose and the rhamnose (Fig. 3c). Also we observed peaks at 549, 403 and 241 amu, which also correspond to the daughter ions formed after reverse fragmentation by losing ribitol, ribitol-rhamnose and ribitol-rhamnose-glucose 2, respectively (Fig. 3c). Three peaks with 113, 127 and 145 amu were absent in other MS-MS analyses and may represent contaminants. Thus, the monosaccharide sequence of the 6X1 PS repeating unit is glucose 1-glucose

Fig. 3. (a) Proposed structure of the hydrated form of the repeating unit of 6X1 capsular PS. The calculated molecular mass is 701 amu. (b) Mass spectrum of the repeating units. The peaks at 683.3 m/z and 701.3 m/z correspond, respectively, to the anhydrous and hydrated forms of the repeating units. (c) Daughter ions of the ion of 683.3 amu shown in (b). Daughter ions are identified at the bottom. The peaks at 270.825, 574.758 and 632.756 amu and their satellite peaks (separated by 2 amu due to chloride isotopes) represent sodium chloride salt clusters (Hao *et al.*, 2001). The peaks at 270.825 represent $(\text{NaCl})_4\text{Cl}^-$. Peaks at 574.758 amu probably represent another salt cluster, $(\text{NaCl})_9\text{Cl}^-$, with a water molecule, like salt clusters with organic solvent molecules (Zhou & Hamburger, 1996). The peaks at 632.7 amu have one more NaCl (i.e. 58 amu) than the peaks at 574.758 amu.

2-rhamnose-ribitol, as proposed in Fig. 3(a). The two glucose residues were labelled 1 and 2 for clarity.

To determine the linkages between the residues of 6X1 PS, we examined the periodate-treated 6X1 repeating units with MS-MS as we have done for 6C PS (Park *et al.*, 2007b). This showed that ribitol and glucose 1 are cleaved

by periodate, while glucose 2 and rhamnose are not. The masses of daughter ions showed that the phosphodiester bond is made to the second position of glucose 1 and that all other glycosidic bonds are the same as in 6B PS (data not shown). The MS-MS studies supported the proposed structure shown in Fig. 3(a).

During the alkali hydrolysis experiments for MS, we observed that the 6X1 PS was very resistant to alkali hydrolysis. To measure resistance to hydrolysis, we examined the ability of 6A, 6B, 6C and 6X1 PSs to inhibit binding of Hyp6BM8 (for 6B and 6X1 PSs) or Hyp6AG1 (for 6A and 6C PSs) to target PS after alkali hydrolysis for various time periods. 6A and 6C PSs completely lost their ability to inhibit after only 1 h of hydrolysis. However, 6B PS lost 90 % of its inhibitory ability in 8 h and more than 100 h of hydrolysis was needed for 6X1 PS to lose 90 % of its inhibitory ability (Fig. 4). Thus, 6X1 PS is much more resistant than 6A and 6C PS to alkali hydrolysis, and may be more resistant than 6B PS.

6X1 is serologically similar to, but distinct from, 6B

When the serological properties of TIGR6X1 were examined by the quellung reaction using polyclonal rabbit antisera, it was found to react with factor serum 6a and was typed as 6B. When we examined TIGR6X1 PS for binding various mAbs to 6A and 6B PS using an inhibition ELISA, we found it to be reactive with many mAbs to 6B PS. For instance, TIGR6X1 inhibited Hyp6BM8 binding to 6B PS. These observations clearly demonstrated that 6X1 PS is serologically very close to 6B PS. However, we also found a mAb specific to 6B PS (Hyp6BM7) but not reactive with 6X1 PS (Fig. 5). Thus, 6X1 PS is serologically distinct from 6B PS.

Fig. 4. Ability of various capsular PSs (2 mg ml^{-1}) to inhibit binding of mAb to ELISA plates (y axis) after the PSs were hydrolysed for various time periods (x axis); 'titre' indicates the dilution of a sample necessary to inhibit binding by 50%. For 6A and 6C PSs, ELISA plates were coated with 6A PS and mAb Hyp6AG1 was used. For 6B and 6X1 PSs, ELISA plates were coated with 6B PS and mAb Hyp6BM8 was used.

Fig. 5. Ability of lysates of various pneumococcal strains to inhibit the binding of mAbs Hyp6BM7 (a) and Hyp6BM8 (b) to 6B PS-coated ELISA plates. The amount of mAb bound (y axis) is shown in the presence of varying amounts of inhibitors (x axis). Lysates of pneumococcal strains were used as inhibitors. The pneumococcal strains are TIGR6A (◇), TIGR6B (■), TIGR6C (△), TIGR6X1 (●) and TIGR6BX (×). TIGR6BX does not produce capsular PS.

Pneumococcal isolates expressing serotype 6X1 PS were not found in nature

The serological studies described above showed that if pneumococcal isolates expressing 6X1 PS are present in nature, they would have been typed as serotype 6B. To look for the presence of serotype 6X1 isolates in nature, we examined 264 pneumococcal isolates that had been previously typed as serotype 6B using an inhibition ELISA capable of distinguishing between the 6B and 6X1 serotypes (Fig. 5). These 6B isolates came from six continents [North America (109), South America (94), Europe (24), Asia (17), Africa (12) and Australia (8)], and were isolated from patients with bacteraemia (38),

meningitis (19), pneumonia (40) and otitis media (17), as well as from healthy carriers (44) (106 isolates were from patients for whom the diagnosis is unknown). None of the 264 6B strains exhibited the antibody-binding profile of 6X1. Thus, the prevalence of serotype 6X1, if it exists, is much less than that of 6B.

DISCUSSION

The actual synthesis of the capsular PS requires cooperation among many different gene products. For instance, a new repeating unit made by a new glycosyltransferase must be compatible with the existing flippase as well as the polymerase before it can be expressed as a new capsule. Thus, to show that serotype 6X1 is possible, we produced a 'TIGR6X1' strain by inserting *wciN*_{6C} into a 6B capsule gene locus, and demonstrated that the new strain produces a capsular PS with the predicted structure, displays serological similarity to 6B, and can grow as well as other members of serogroup 6 in various growth conditions. Thus, serotype 6X1 could exist in nature.

Just as 6C was previously typed as '6A' by the classical typing method (Lin *et al.*, 2006; Park *et al.*, 2007b), the quellung reaction method typed the new 6X1 strain as serotype 6B. Thus, to identify natural isolates expressing serotype 6X1, we re-examined the isolates that had been classically defined as 6B using mAbs. Despite our testing more than 250 such isolates, we did not find 6X1 isolates in nature. Additionally, *wciN*_{6C} was not detected among the CDC isolates that were serotyped as '6B' with the classical typing method (Dr B. Beall, CDC, personal communication). Thus, pneumococcal isolates expressing serotype 6X1 may not exist in nature. If serotype 6X1 exists in nature, its prevalence is extremely low.

Despite the fact that it is not detected in nature, the 6X1 serotype could emerge in nature by one of two mechanisms. One mechanism involves a mutation of the *wciP* gene of 6C, since the only difference between 6A and 6B serotypes appears to be one nucleotide in the *wciP* gene. The mutation rate for pneumococci is $\sim 1 \times 10^{-8}$ (del Campo *et al.*, 2005; Gould *et al.*, 2007; Morosini *et al.*, 2003) and a chronic obstructive pulmonary disease (COPD) patient with stable pneumonia may have 2.6×10^8 c.f.u. of pneumococci per millilitre of sputum (Sethi *et al.*, 2007). Thus, the correct mutation should arise in almost all cases of 6C pneumonia and often in other 6C infections with lower bacterial load. The alternative mechanism involves the lateral gene transfer of *wciN* from a 6C strain into a 6B strain as we have done here. This situation could actually occur in nature, since carriage of multiple pneumococcal serotypes can be relatively high among children (Gratten *et al.*, 1986; Hill *et al.*, 2008) and serotypes 6B and 6C are fairly common in some parts of the world (e.g. Brazil) (Lin *et al.*, 2006; Park *et al.*, 2007a). Furthermore, homologous recombination would occur easily, since the 17 kb capsule gene loci of 6B and 6C are

almost identical except for the *wciN* gene. These considerations strongly suggest that the circumstances for creating serotype 6X1 exist in nature.

Given that the circumstances for creating 6X1 do exist in nature, it is interesting to consider reasons for its absence. It is possible that 6C could have appeared so recently that there may not have been enough time for 6X1 to appear. Alternatively, there could be natural immune barriers against 6X1, but we found that pre-immune human sera do not kill or opsonize TIGR6X1 (data not shown). The most likely explanation is that there has not been enough biological pressure to select 6X1 over 6A, 6B or 6C. The need for selection pressure was recently demonstrated by an increase in the prevalence of serotypes 19A and 6C following the use of the conjugate vaccine (Nahm *et al.*, 2009; Park *et al.*, 2008). In the absence of a survival advantage, the 6X1 serotype may have appeared in nature (as it should in almost every case of a 6C infection, as mentioned above) but has not been propagated as a consequence of competition with more abundant 6A, 6B or 6C serotypes. In an analogous manner, antibiotic-resistant strains survive and propagate when antibiotics are used clinically, and disappear when antibiotics are discontinued (Katsunuma *et al.*, 2007).

6A PS was included in the original 14-valent PS vaccine, but it was replaced with 6B PS when the 23-valent vaccine became available in 1983, because 6B PS can elicit antibodies that cross-react with 6A PS and is much more resistant to hydrolytic breakdown than 6A PS (Zon *et al.*, 1982). In this study, we also discovered that 6X1 PS is as chemically stable as 6B PS and much more resistant to hydrolysis than 6C PS. Since 6X1 PS would likely elicit antibodies cross-reactive with 6C PS, 6X1 may be more useful as a vaccine component than 6C PS.

As our knowledge of pneumococcal capsule genetics, biochemistry and serology advances, we may wish to produce pneumococcal strains that express artificially created capsular PS for various reasons. We propose that these artificially created strains should be named in a systematic manner to avoid their names being confused with those of natural strains. We chose to use 'X' to denote such experimental strains. We chose '6X1' as the name for our strain because this experimental strain belongs to serogroup 6 and this is the first experimental strain within serogroup 6. This systematic approach should be applicable to any new strains created experimentally.

It is important to understand the evolution of the capsule gene locus, which encodes the most important virulence factor of pneumococci. Even when only two serotypes were known, the evolution of serogroup 6 was extensively studied (Mavroidi *et al.*, 2004; Robinson *et al.*, 2002). Serogroup 6 has become more interesting following the discovery of serotype 6C. Now, the serogroup would be even more interesting for evolution studies with a potential serotype, serotype 6X1.

ACKNOWLEDGEMENTS

The work was funded by a grant AI-031473 from the NIH and P. E. B. is supported by a training grant (T32 GM008111) from the NIH. UAB has applied for a patent covering the creation of 6X1. We thank Dr B. Beall and Mr R. Gertz at the CDC for assistance with the quellung reaction tests. We also thank Rob Cartee for performing GLC-MS and Marion Kirk for performing MS-MS analysis.

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Edited by: M. Kilian