

## Genetic Alteration of Capsule Type but Not PspA Type Affects Accessibility of Surface-Bound Complement and Surface Antigens of *Streptococcus pneumoniae*

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**The *Streptococcus pneumoniae* capsular polysaccharides and pneumococcal surface protein A (PspA) are major determinants of virulence that are antigenically variable and capable of eliciting protective immune responses. By genetically switching the *pspA* genes of the capsule type 2 strain D39 and the capsule type 3 strain WU2, we showed that the different abilities of antibody to PspA to protect against these strains was not related to the PspA type expressed. Similarly, the level of specific antibody binding to PspA, other surface antigens, and surface-localized C3b did not depend on the PspA type but instead was correlated with the capsule type. The type 3 strain WU2 and an isogenic derivative of D39 that expresses the type 3 capsule bound nearly identical amounts of antibody to PspA and other surface antigens, and these amounts were less than one-half the amount observed with the type 2 parent strain D39. Expression of the type 3 capsule in D39 also reduced the amount of C3b deposited and its accessibility to antibody, resulting in a level intermediate between the levels observed with WU2 and D39. Despite these effects, the capsule type was not the determining factor in anti-PspA-mediated protection, as both D39 and its derivative expressing the type 3 capsule were more resistant to protection than WU2. The specific combination of PspA and capsule type also did not determine the level of protection. The capsule structure is thus a major determinant in accessibility of surface antigens to antibody, but certain strains appear to express other factors that can influence antibody-mediated protection.**

*Streptococcus pneumoniae* is a major cause of pneumonia, otitis media, and meningitis, especially in the elderly, young children, and immunocompromised individuals. The capsular polysaccharide and pneumococcal surface protein A (PspA) are important determinants of virulence for *S. pneumoniae*. Both molecules are surface components that exhibit a high degree of variability among strains and are capable of eliciting protective immunity. For the capsule, 90 different serotypes have been identified (20), and immunity to one capsule type is not cross-protective against other types (28). Strains expressing certain capsular serotypes appear to be better able to cause disease than others, and only a small number of serotypes account for the majority of pneumococcal infections in humans (8, 12, 15). Levels of virulence in mice have been shown to vary with the capsule type expressed, although the genetic background also plays an important role (2, 25, 26, 41, 44). The capsule inhibits phagocytosis by blocking access to cell wall-localized C3b (7, 42). Differences in virulence may be related to the ability of the specific capsular polysaccharide structure to block the accessibility of antibodies and complement to surface components or to mask cell-wall bound C3b from recognition by phagocytic receptors (6, 7, 11, 42). Hostetter demonstrated that strains with different capsular types differ in the

amount and site of bound C3b, as well as the C3b degradation products, potentially affecting opsonophagocytosis (23).

PspA occurs on all *S. pneumoniae* strains, and although variability exists, all PspAs appear to possess a signal peptide, an  $\alpha$ -helical N-terminal region that contains most of the immunogenic epitopes and antigenic variability, a proline-rich domain, a choline binding domain important in anchoring the protein to the bacterial surface, and a short C-terminal tail (21, 30, 31, 35, 43). PspA reduces complement activation (40) and binds lactoferrin (16). Based on reactivity with a panel of monoclonal antibodies, PspAs were originally divided into 31 serotypes (9). Subsequent analyses of the sequences encoding the N-terminal halves of the proteins resulted in assignment of PspAs to three families and further subdivision into six clades (21). In general, PspAs from one serotype can elicit protection against strains representing different capsule and PspA types, although the levels of protection may differ between strains (4, 30, 32, 34). However, PspA-elicited protection against the capsule type 2 strain D39 is poor, even when immunization is with PspA obtained from D39 derivatives (4, 30, 32, 34). These same PspAs elicit protection against a wide variety of strains, including the capsule type 3 strain WU2 (4, 32, 34). The poor protection against D39 is consistent with the observation that PspA<sup>-</sup> mutants of D39 remain virulent, whereas the absence of PspA in WU2 results in avirulence (5, 36, 47; Abeyta and Yother, unpublished data). This finding suggests that the background of the invading strain may be an important determinant of its resistance to anti-PspA-mediated protection.

Because the studies described above were based on work with strains having different genetic backgrounds, it is not clear

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid <sup>a</sup>	Derivation and properties <sup>b,c</sup>	Reference(s)
<i>S. pneumoniae</i> strains		
D39	Type 2 encapsulated; PspA <sup>+</sup> (84 kDa)	1, 33
GH1003	pGG108 × WU2; Em <sup>r</sup> ; insertion downstream of <i>pspA</i> ; PspA <sup>+</sup> (92 kDa)	This study
JD611	Nonencapsulated derivative of WU2; Cps3D <sup>-</sup> ; PspA <sup>+</sup> (92 kDa)	10
JD770	Type 3 encapsulated derivative of WU2 containing insertion-duplication of <i>cps3DSU</i> ; Em <sup>r</sup> ; PspA <sup>+</sup> (92 kDa)	10
JD803	JD770 × D39; Em <sup>r</sup> ; type 3 encapsulated; PspA <sup>+</sup> (84 kDa)	25
JD804	Same as JD803; independent derivative	25
JY1123	pKSD300 × WU2; Em <sup>r</sup> ; PspA <sup>tr</sup> (46 kDa)	45
MA101	pJY4173 × D39; Em <sup>r</sup> ; insertion downstream of <i>pspA</i> ; PspA <sup>+</sup> (84 kDa)	This study
MA139	GH1003 × D39; Em <sup>r</sup> ; PspA <sup>+</sup> (92 kDa)	This study
MA158	Same as MA139; independent derivative	This study
MA200	WG44.1 × R36A; Em <sup>r</sup> ; PspA <sup>-</sup>	This study
MA1024	MA101 × WU2; Em <sup>r</sup> ; PspA <sup>+</sup> (84 kDa)	This study
MA1025	Same as MA1024; independent derivative	This study
R36A	Nonencapsulated derivative of D39; PspA <sup>+</sup> (84 kDa)	1
Rx1	Avirulent derivative of D39; PspA <sup>+</sup> (84 kDa)	33, 39
WG44.1	PspA <sup>-</sup> derivative of Rx1 ( $\Delta$ <i>pspA</i> ); Em <sup>r</sup>	36, 45
WU2	Type 3 encapsulated, virulent; PspA <sup>+</sup> (92 kDa)	3, 33
Plasmids		
pGG103	pJY4163::1.8-kb <i>Sau3AI-KpnI</i> fragment containing 3' region of Rx1/WU2 <i>pspA</i>	This study
pGG108	pJY4163::1.2-kb <i>SacI-KpnI</i> fragment containing WU2 <i>pspA</i> 3' region	This study
pGH160	pUC18::1.2-kb <i>SphI-SalI</i> containing WU2 <i>pspA</i> 5' region; encodes PspA <sup>46</sup>	This study
pJY4163	Vector for insertion-duplication mutagenesis; replicates in <i>E. coli</i> , not in <i>S. pneumoniae</i> ; Em <sup>r</sup>	45
pJY4173	pJY4163 containing 3' region of Rx1 <i>pspA</i>	45
pJY4306	pUC18::1.0-kb <i>HindIII-DraI</i> fragment (Rx1 <i>pspA'</i> bp 1-997); encodes PspA <sup>42</sup>	45
pJY4310	pUC18:: <i>HindIII-SacI</i> (Rx1 <i>pspA'</i> bp 1-790); encodes PspA <sup>30</sup>	45
pKSD300	pVA891::0.55-kb internal <i>Sau3AI</i> fragment of Rx1 <i>pspA</i>	36, 43
pRS102	<i>HindIII</i> self-ligated fragment from JY1123 (in pVA891); C-terminal coding region of WU2 <i>pspA</i> ; Em <sup>r</sup>	This study
pVA891	Vector for insertion-duplication mutagenesis; replicates in <i>E. coli</i> , not in <i>S. pneumoniae</i> ; Cm <sup>r</sup> ( <i>E. coli</i> only); Em <sup>r</sup>	29

<sup>a</sup> Strains MA139, MA158, MA1024, MA1025, JD803, and JD804 are each the result of at least three backcrosses with the parental recipients.

<sup>b</sup> Crosses were done by transformation.

<sup>c</sup> The apparent molecular masses of PspA (in parentheses) were determined by SDS-PAGE and Western immunoblotting. PspA<sup>+</sup>, full-length PspA; PspA<sup>tr</sup>, truncated PspA.

how, or if, the capsule and PspA serotypes influence the results. Here, we examined isogenic strains to determine the effects of alterations in PspA and capsule type on the levels of anti-PspA-mediated protection and accessibility of antibodies to surface components and bound C3b. We used the capsule type 2 strain D39 and the capsule type 3 strain WU2 for comparison because of the differences in PspA-elicited protection noted above. The type 2 capsule is a branched structure with subunits comprised of a linear backbone of  $\rightarrow$ 4)- $\beta$ -D-Glcp-(1 $\rightarrow$ 3)- $\alpha$ -L-Rhap-(1 $\rightarrow$ 3)- $\alpha$ -L-Rhap-(1 $\rightarrow$ 3)- $\beta$ -L-Rhap-(1 $\rightarrow$  and a side chain of  $\alpha$ -D-GlcpUA-(1 $\rightarrow$ 6)- $\alpha$ -D-Glcp linked  $\alpha$ -(1 $\rightarrow$ 2) from the first rhamnose in the backbone to the side chain glucose (24). The type 3 capsule is a linear repeating unit of  $\rightarrow$ 3)- $\beta$ -D-GlcpUA-(1 $\rightarrow$ 4)- $\beta$ -D-Glcp-(1 $\rightarrow$ 3) (37). Based on a single difference in reactivity with a panel of monoclonal antibodies, the D39 and WU2 PspAs are classified in different serotypes (serotypes 25 and 1, respectively) (9). The *pspA* sequences of the D39 derivatives Rx1 and R6 have been reported previously and exhibit complete identity (22, 43). A comparison of the predicted N-terminal halves of the WU2 and Rx1 PspA sequences showed that despite a number of differences, the PspAs were closely related and were members of the same PspA clade (21). By independently altering the capsule and PspA types in D39 and WU2, we obtained additional insights

into the contributions of these surface structures to the pathogenesis of *S. pneumoniae*.

#### MATERIALS AND METHODS

**Bacterial strains and genetic manipulations.** The bacterial strains used are listed in Table 1. *S. pneumoniae* strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% yeast extract (THY) or on blood agar base no. 2 (Difco) supplemented with 3% sheep blood in a candle jar at 37°C. *Escherichia coli* strains were grown in L-broth or on L-agar at 37°C. Erythromycin was used at a concentration of 0.3  $\mu$ g/ml for *S. pneumoniae* and at a concentration 300  $\mu$ g/ml for *E. coli*. Ampicillin was used at a concentration 50  $\mu$ g/ml for *E. coli*. *S. pneumoniae* was transformed as previously described (19, 46).

The C-terminal coding region of WU2 *pspA* was targeted and cloned essentially as described previously for Rx1 *pspA* (45). The WU2 derivative JY1123 was constructed by transformation and insertion of pKSD300, which harbors an internal 550-bp fragment from the 5' half of Rx1 *pspA* in pVA891(Em) (36, 43, 45). Chromosomal DNA from JY1123 was digested with *HindIII*, self-ligated, and transformed into *E. coli*, with selection for erythromycin-resistant transformants. The resulting plasmid, pRS102, contained the C-terminus-encoding two-thirds of WU2 *pspA* and flanking DNA. A 1.8-kb *BamHI/KpnI* fragment containing the 3' half of WU2 *pspA* was subcloned into pJY4163 to generate pGG103, and subclones of this plasmid were used to obtain the WU2 *pspA* sequence. Sequencing was performed by the UAB Automated Sequencing Core Facility (University of Alabama at Birmingham, Birmingham) or by the Sanger dideoxy method (38) by using a Sequenase 2.0 kit (Amersham Life Sciences, Arlington Heights, Ill.). Plasmid and chromosomal DNAs were isolated by using Qiagen columns (Qiagen, Chatsworth, Calif.).

For construction of D39 derivatives that expressed the WU2 PspA, D39 was transformed with chromosomal DNA from GH1003, a WU2 derivative carrying an erythromycin resistance marker downstream of *pspA*. Erythromycin-resistant transformants were selected and backcrossed three times with the parental recipient strain. Two independently obtained derivatives, MA139 and MA158, were used in the subsequent studies. A similar procedure was used to introduce D39 *pspA* into WU2. The donor for this construction was MA101, and the independent derivatives obtained were MA1024 and MA1025. Switching of the *pspA* sequences was confirmed by Southern and Western analyses, as described below.

**Transcription analyses.** RNA was isolated from *S. pneumoniae* by using the FastPrep system (Bio 101). For Northern analyses, RNA was denatured, electrophoresed through a 1% agarose–2.2 M formaldehyde gel, transferred to a Duralon-UV membrane (Stratagene), hybridized, and developed by using the Genius system (Boehringer Mannheim Corp., Indianapolis, Ind.). DNA probes were made as described below for Southern analyses. The *pspA* probe was generated by incorporation of a labeled nucleotide during PCR amplification of the pJY4310 insert.

Primer extension was performed by a modification of a previously described method (27). Briefly, 20 µg of RNA was mixed with 5 ng of a 5'-biotinylated *pspA* primer (5'-TAGCGACGCTGGCTAGACTTG-3') in hybridization buffer (200 mM Tris hydrochloride [pH 8.0], 1 M NaCl, 1 mM disodium EDTA) in a 10-µl (final volume) reaction mixture and heated at 100°C for 2 min and then at 40°C for 2 h. After the mixture was cooled to room temperature, 5 µl of 2× reverse transcriptase buffer (0.1 M Tris hydrochloride [pH 8.2], 12 mM MgCl<sub>2</sub>, 20 mM dithiothreitol, 0.4 mM dATP, 0.4 mM dTTP, 0.4 mM dGTP, 0.4 mM dCTP), 0.2 µl of actinomycin D (5-mg/ml stock), 10 U of RNasin, and 5 U of avian myeloblastosis virus reverse transcriptase (Promega) were added. After incubation at 42°C for 1 h, 10 µl of sequencing dye was added to stop the reaction, and the tubes were stored at –20°C. Four microliters of each reaction mixture was electrophoresed on a 5% polyacrylamide sequencing gel along with a sequencing ladder from *pspA* generated with the same primer by using pJY4306 and pGH160 as templates. Primer extension products were visualized by exposure to X-ray film after chemiluminescent detection with the Plex system (NEB).

**Southern blot analysis.** Chromosomal DNA was digested with *Hind*III and *Kpn*I, electrophoresed through 0.8% agarose, and transferred to Duralon-UV membrane by using a Possiblot pressure blotter (Stratagene, La Jolla, Calif.). The *pspA*-specific probe was generated by PCR by using primers specific for the *pspA* leader sequence and 3' transcription termination site (5'-GTCTCAGCCTACTGTTGT-3' and 5'-GGGGTACCTAAAGAATACG-3', respectively) (43). *Taq* polymerase (Fisher Scientific, Pittsburgh, Pa.) was used for PCR amplification. The probe was labeled with digoxigenin-dUTP, and blots were developed by using the DIG/Genius system (Boehringer Mannheim).

**Western immunoblot analyses of PspA.** *S. pneumoniae* samples were prepared as previously described (45). Equivalent amounts of all samples (based on cell number) were electrophoresed in sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Western immunoblotting and processing were performed as previously described (45). The PspA-specific monoclonal antibody Xi126 was provided by David Briles (University of Alabama at Birmingham).

**Generation of PspA-specific polyclonal antiserum and protection studies.** BALB/cByJ mice, obtained from Jackson Laboratory, Bar Harbor, Maine, were immunized by subcutaneous injection with 1 µg of PspA purified from strain R36A as previously described (48) and emulsified in complete Freund's adjuvant. Control serum was obtained by immunization with an equivalent fraction from the PspA<sup>–</sup> R36A derivative MA200. After 14 days, the mice were boosted by intraperitoneal injection by using the same amount of antigen without adjuvant. Seven days later, blood was collected, and the serum was pooled and stored at –85°C. The serum was examined for reactivity with PspA in a Western immunoblot, as well as by an indirect enzyme-linked immunosorbent assay (ELISA), as previously described (34).

In protection studies, 8- to 10-week-old BALB/cByJ mice were passively immunized by intraperitoneal injection with 20 µl of PspA-specific polyclonal serum or control serum diluted in 200 µl of sterile lactated Ringer's solution. After 1 h, the mice were challenged by intravenous injection with 10<sup>7</sup> CFU of pneumococci. Survival was monitored for 21 days, and the results were compared by using Fisher's exact test.

**Antibody binding and complement assays.** Indirect ELISAs to detect binding of antibodies to surface antigens were performed essentially as described previously (18). *S. pneumoniae* cultures were grown to mid-exponential phase (~3 × 10<sup>8</sup> CFU/ml) in THY, heat killed by incubation at 65°C for 20 min, and centrifuged (14,000 × g, 20 min). The pellets were washed once and resuspended in the original culture volume of phosphate-buffered saline (PBS) (140 mM NaCl, 3 mM KCl, 5 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>; pH 7.4). The volumes were then

adjusted so that the optical densities (OD<sub>600</sub>) of all samples were equivalent. Microtiter plates were coated overnight at 4°C with 50-µl portions of samples for use in ELISAs. The PspA-specific polyclonal antiserum described above (diluted 1:1,000) was used to detect PspA. For detection of other surface antigens, a polyclonal antibody specific for serotype 19 (typing serum obtained from Statens Seruminstitut, Copenhagen, Denmark; diluted 1:5,000) was used. Because whole cells are used as immunogens for this antiserum and because of the poor immunogenicity of the type 19 capsule, the majority of the antibodies are reactive with noncapsular antigens (18). The antiserum can thus be used to detect general exposure of surface antigens to antibody. The results of the antibody binding assays were normalized to the binding observed with JD611, a nonencapsulated derivative of the type 3 strain WU2. The anti-type 19 polyclonal antiserum reacts with JD611 at antiserum dilutions of >2 × 10<sup>5</sup> (18).

The amount of type 3 capsule on the cell surface does not affect binding of the bacteria to a microtiter plate (18). Likewise, strains expressing type 2 and type 3 capsules bound equivalently to microtiter plates. Two methods were used to determine the latter result. First, heat-killed bacteria (~3 × 10<sup>8</sup> CFU/ml) were fluorescein labeled (13) by incubation in a 0.05 M sodium carbonate–0.1 M NaCl buffer containing 1 mg of fluorescein (Sigma Chemical Co., St. Louis, Mo.) per ml for 30 min at room temperature. The suspensions were centrifuged, washed, and resuspended in PBS; serial dilutions were then coated on microtiter plates. Following incubation overnight at 4°C, the plates were washed, and the percentage of bacteria bound to each plate was determined by dividing the amount of fluorescence bound by the total fluorescence in the coating sample. Fluorescence was determined by using a Victor2 multilabel counter (Perkin-Elmer) at excitation and emission wavelengths of 485 and 535 nm, respectively. Comparable levels of binding were observed for WU2, D39, and JD803. As a second test, the binding assays were carried out in suspension. Live and heat-killed bacteria were incubated with antisera (as described above) and live bacteria were incubated with complement (as described below). Samples were then centrifuged, washed, and resuspended in buffer containing the secondary antibody. Following this incubation, samples were centrifuged, washed, and resuspended in buffer containing the detection substrate. The results were normalized to the OD<sub>595</sub> of the final suspension. The results were comparable to those obtained by using bacteria bound to microtiter plates.

Complement assays were performed by a modification of the method of Gordon et al. (14). For opsonization, serum from BALB/cByJ or CBA/N mice was pooled and stored at –85°C. Bacteria were grown in THY to a concentration of ~3 × 10<sup>8</sup> CFU/ml. Cells in 0.5-ml samples were harvested by centrifugation, washed once in gelatin Veronal buffer (Sigma), and resuspended in 100 µl of gelatin Veronal buffer. Serum was added to the bacterial suspension at a level that was 10% of the final volume, and opsonization was carried out for 30 min at 37°C. Bacteria were then washed three times in PBS and resuspended in 100 µl of PBS, and 50 µl of a bacterial suspension containing ~2 × 10<sup>7</sup> CFU of bacteria was added in duplicate to 96-well microtiter plates (Falcon; Becton Dickinson, Oxnard, Calif.). The wells were coated overnight at 4°C, rinsed three times with PBS containing 0.05% Tween 20, and blocked for 1 h at room temperature with 1% bovine serum albumin in PBS. Samples were incubated at room temperature for 1 h with 50 µl of peroxidase-conjugated goat antiserum to C3 (anti-C3-HRP; Cappel Research Products, ICN Biomedicals, Costa Mesa, Calif.) diluted 1:500 in 1% bovine serum albumin in PBS. After three washes with PBS containing 0.05% Tween, color development with 2,2'-azino-di-(3-ethylbenzthiazolinesulfonic acid) (ABTS) peroxidase substrate (Fisher) and H<sub>2</sub>O<sub>2</sub> was measured at OD<sub>415</sub>. Nonspecific binding was determined by incubation of bacteria in heat-inactivated (30 min, 56°C) serum. The amounts of bound C3 were calculated relative to the amount of a normal mouse standard consisting of serum amyloid P component (Calbiochem, La Jolla, Calif.) coated on ELISA wells and processed as described above. For determination of the total C3 bound, 20-µl suspensions containing bacteria opsonized as described above were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (4 to 15% polyacrylamide gradient gels) and transferred to nitrocellulose. C3 was detected by using anti-C3-HRP diluted 1:1,000 and development with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill.). The ratios of total C3 binding were determined by densitometric analysis of the immunoblot. For both the accessibility and total C3 binding assays, comparable results were obtained with BALB/cByJ and CBA/N mouse sera. The latter serum contains an X-linked immunodeficiency that results in poor antibody responses to polysaccharide antigens and low levels of intrinsic antibody to the phosphocholine component of the pneumococcal cell wall (3).

In studies examining the effect of antibody on opsonization, bacteria (~2 × 10<sup>7</sup> CFU) were incubated with heat-inactivated (30 min, 56°C) PspA-specific polyclonal serum or control serum diluted 1/10 in 100 µl (final volume) of PBS.



	Proline A			
Rx1	PEKPA	PAPET	PAPEAPAEQPKPAPAPQAPAPAPKPEKPAEQPKPEKTDDQQAEDYARRSEEEYNRLTQQQ	
WU2	···S·E·EPSQ·	·KPAEEA·	···Q·T·PTQ·GKPAEET·	·······A·
	Choline Binding Domain			
	Proline B		Repeat 1	
	PPKAEK	PAPAPK	TGWKQENGMWYFYNTDGSMA	2
	·····PAPAPQPEQ·	·····	·····	TGWLQNGSWYYLNSGAMA
				·····
	3	4	5	6
	TGWLQYNGSWYYLNANGAMA	TGWAKVNGSWYYLNANGAMA	TGWLQYNGSWYYLNANGAMA	TGWAKVNGSWYYLNANGAMA
	·····	·····S·	·····KDGD·	·····E·S·
				·····KA
				SQ·F·
				SDK·
				···V·S·P·
	7	8	9	10
	TGWLQYNGSWYYLNANGAMA	TGWAKVNGSWYYLNANGAMA	TGWVKDGD·TWYYLEASGAMKA	SQWFKVSDKYYVNGLGALA
	·····	·····S·	·····	·····S·S·
				·····
	C-terminus			
	VNTTVDGYKVNANGEWV			
	·····T·E·			

FIG. 1. Comparison of the predicted sequences of the C-terminal halves of the Rx1 and WU2 PspAs. The sequence begins at the proline A domain (amino acid 289 of the mature Rx1 PspA) and continues to the end of the protein. The individual sequences are contiguous throughout, with spacing added for clarity of alignment. Amino acids in the WU2 sequence that are identical to those in the Rx1 sequence are indicated by dots.

After incubation for 30 min on ice, the suspension was centrifuged, and cells were washed three times with PBS prior to opsonization as described above.

**Nucleotide sequence accession numbers.** The WU2 and Rx1 *pspA* nucleotide sequences have been deposited in the GenBank database under accession numbers AF516671 and M74122, respectively.

**RESULTS**

**Characterization of PspA.** Analysis of the C-terminal half of the predicted WU2 PspA sequence revealed a small number of differences with the sequence of PspA from the D39 derivative Rx1. In both, the proline-rich domain contains two segments, proline A and proline B, separated by a short spacer sequence. Both proline regions are larger in WU2 than in Rx1 (Fig. 1). The choline-binding domain of Rx1 PspA contains 10 nearly identical repeats of 20 amino acids each. The 9th and 10th repeats differ slightly from the other repeats, and an additional amino acid is present in the 9th repeat (Fig. 1) (43). This same pattern occurs in WU2 PspA. Additionally, repeats 7 through 10 in WU2 PspA are effectively a duplication of repeats 3 through 6 (Fig. 1). The C-terminal tail in both Rx1 PspA and WU2 PspA is 17 amino acids long, and only two residues differ (Fig. 1).

To aid in genetically switching the D39 and WU2 *pspA* sequences, an erythromycin resistance marker was integrated downstream of the putative *pspA* transcription terminator (43), as described below. To determine whether the insertions might cause polar effects on downstream genes, we performed Northern and primer extension analyses to determine the sizes of the transcripts containing *pspA*. Northern blotting revealed *pspA*-specific transcripts of approximately 1.9 and 2.0 kb for Rx1 and WU2, respectively (Fig. 2A). These sizes are consistent with those predicted for monocistronic transcripts and reflect the slightly larger size of WU2 PspA (see below). As previously reported for WU2 (17), the amount of the *pspA* transcript was constant from early exponential phase through early stationary phase (data not shown). Using primer extension analysis, we located the site of transcription initiation for the WU2 (Fig. 2B) and D39 (data not shown) *pspA* genes. For both genes,

initiation occurred 7 nucleotides downstream of the -10 site (at an A residue), thus confirming the location of the putative promoter sequence (Fig. 2C).

**Effect of alterations in PspA and capsular serotype on anti-PspA-mediated protection.** Derivatives of the capsule type 2 strain D39 and the capsule type 3 strain WU2 in which the

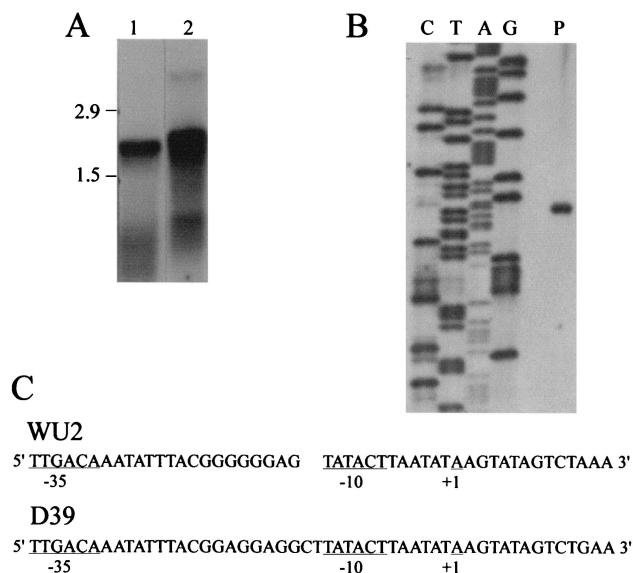


FIG. 2. Transcription of *pspA*. (A) Northern analysis. Lanes were loaded with 20 µg of RNA and probed with a *pspA*-specific DNA probe. Lane 1, Rx1; lane 2, WU2. (B) Primer extension analysis of WU2. Nucleotides are indicated above the lanes. The ladder reads 5' to 3' from bottom to top. The initiating nucleotide was confirmed by spiking the A sequencing reaction mixture with the primer extension reaction mixture. Lane P contained the primer extension product. (C) Nucleotide sequences of the WU2 and D39 *pspA* promoter regions. The transcription initiation site is indicated (+1). The D39 sequence contains two additional nucleotides in the spacer region between the -35 and -10 sequences. A gap was therefore introduced into the WU2 sequence for clarity of alignment.

*pspA* sequences were exchanged were constructed by transforming the recipient strain with donor chromosomal DNA carrying an insertion downstream of *pspA*, located at a *KpnI* site approximately 50 bp downstream of the *pspA* transcription terminator. Following selection for the erythromycin resistance marker carried on the insertion and three additional backcrosses, replacement of the recipient *pspA* with *pspA* of the donor was confirmed by Southern analyses, in which differences in restriction fragment sizes between the two *pspA* sequences were apparent. The procedure used for construction of D39 derivatives carrying WU2 *pspA* and the results of the subsequent Southern blot analysis are shown in Fig. 3A and B. A similar procedure and confirmation analysis were used for construction of WU2 derivatives carrying D39 *pspA* (data not shown). The D39 and WU2 PspAs differ in size, and this difference in the derivatives was confirmed by Western immunoblot analyses (Fig. 3C). Because D39 and WU2 differ in the time required to kill BALB/cByJ mice following intravenous infection (25), we examined the virulence of the derivatives administered by this route. None of the derivatives differed from the corresponding parent strain, indicating that the insertions downstream of *pspA* did not affect virulence and the differences in virulence between D39 and WU2 are not attributable to PspA (data not shown).

To examine the effect of alterations in PspA type on the protective ability of antibody to PspA, mice passively immunized with PspA-specific or control serum were challenged intravenously with the derivatives or their parents. The PspA-specific antiserum was obtained by using PspA isolated from R36A, a nonencapsulated derivative of D39. The control serum was obtained by immunizing mice with a similarly isolated fraction from a PspA-negative derivative of R36A. As shown in Table 2, mice challenged with the type 3 strain WU2 or its derivative expressing D39 PspA (MA1025) were fully protected from fatal infection. When mice were challenged with the type 2 strain D39 or its derivatives expressing WU2 PspA (MA139 and MA158), however, there was no significant difference in survival between mice that received PspA-specific antibody and mice that did not. Thus, the specific PspA type is not responsible for the differences in anti-PspA-mediated protection.

To test the effect of alteration in capsule type on anti-PspA-mediated protection, we used the D39 derivatives JD803 and JD804, in which the type 2 capsule locus was replaced with the WU2 type 3 capsule locus. The amount of type 3 capsule produced by these strains is equivalent to the amount produced by WU2, but the virulence remains the same as that of D39 (25). These derivatives were as resistant to anti-PspA-mediated protection as D39 was (Table 2). Thus, capsule type alone does not determine the level of protection. In addition, the WU2 derivative expressing D39 PspA (MA1025) and the D39 type 3 derivatives (JD803 and JD804) had identical PspA and capsule serotypes yet had very different levels of resistance to anti-PspA-mediated protection. Thus, the specific combination of PspA and capsule type of the invading strain also does not determine resistance to protection. The effect of expressing the type 2 capsule in the type 3 background was not tested in these assays because such derivatives produce only small amounts of type 2 capsule and exhibit severely reduced virulence (unpublished data).

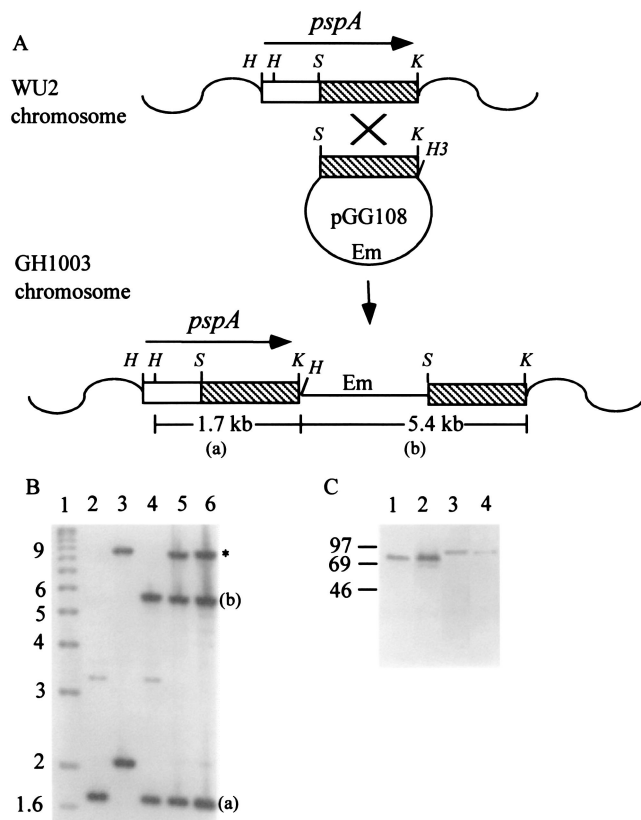


FIG. 3. Construction and characterization of derivatives expressing altered PspA serotypes. (A) Construction of WU2 derivatives carrying an erythromycin resistance insertion downstream of *pspA*. WU2 was transformed with pGG108, which contains the 3' 1.2 kb (▨) of WU2 *pspA*. Recombination resulted in insertion of the plasmid immediately downstream of *pspA*, with duplication of the 1.2-kb fragment (GH1003 chromosome). Chromosomal DNA from this strain was used to transform WU2 recombinant into D39, with selection for erythromycin resistance following recombination in homologous sequences flanking *pspA*. The positions of *HindIII* (H), *SacI* (S), and *KpnI* (K) restriction sites are indicated. (a) and (b) indicate restriction fragments shown in the Southern blot in panel B. (B) Southern blot analysis. *HindIII-KpnI*-digested chromosomal DNA was hybridized with a full-length *pspA*-specific probe, as described in Materials and Methods. The complete D39 *pspA* gene (lane 3) is present on a single 2.1-kb *HindIII-KpnI* fragment (43). The 8.9-kb band (indicated by an asterisk) in lanes 3, 5, and 6 results from hybridization of the probe with the closely related *pspC* gene, which is present in D39 but not WU2 (35). WU2 *pspA* (lane 2) contains an internal *HindIII* site, as shown in panel A. Not shown is the 350-bp *HindIII* fragment, which was detected in WU2 and all derivatives containing WU2 *pspA*. Lane 1, kilobase ladder; lane 2, WU2; lane 3, D39; lane 4, GH1003 (WU2 with insertion downstream of *pspA*); lane 5, MA139 (D39 with WU2 *pspA*); lane 6, MA158 (D39 with WU2 *pspA*). (C) Western immunoblot analysis of cell lysates from derivatives expressing an altered PspA serotype. Blots were reacted with the PspA-specific monoclonal antibody Xi126. The positions of molecular mass standards (in kilodaltons) are indicated on the left. Lane 1, D39; lane 2, MA1025 (WU2 derivative expressing D39 PspA); lane 3, WU2; lane 4, MA139 (D39 derivative expressing WU2 PspA).

#### Analysis of antibody binding to PspA and surface antigens.

A possible explanation for the results described above is that antibody binding to PspA varies with the background strain in a manner that is not dependent on capsule type. We therefore compared binding of the polyclonal PspA-specific antiserum to

TABLE 2. Protection of BALB/cByJ mice passively immunized with anti-PspA serum

Challenge strain	Capsule serotype	PspA serotype	No. of mice alive/ no. total <sup>b</sup>		<i>P</i> <sup>c</sup>
			Without antibody	With antibody	
WU2	3	1	0/12	12/12	<0.0001
MA1025	3	25	0/5	5/5	0.0079
D39	2	25	4/14	6/14	NS
MA139/MA158 <sup>a</sup>	2	1	3/14	6/14	NS
JD803/JD804 <sup>a</sup>	3	25	6/14	7/14	NS

<sup>a</sup> Data was combined for the two independently obtained derivatives, which did not differ from each other.

<sup>b</sup> Mice were infected intravenously with 10<sup>7</sup> CFU 1 h after intraperitoneal immunization with anti-PspA polyclonal antiserum (with antibody) or a control antiserum lacking PspA-specific antibody (without antibody).

<sup>c</sup> Calculated by using Fisher's exact test. NS, not significant.

strains D39 and WU2. In contrast to what might have been expected from the protection results, a higher level of binding was observed with D39 than with WU2 (Fig. 4A). Expression of D39 PspA in WU2 (MA1025) resulted in binding similar to that observed for the parent strain WU2, but the D39 derivative expressing WU2 PspA (MA139) bound more than twice as much antibody as the parent strain D39 (Fig. 4A). For the D39 derivative expressing the type 3 capsule (JD803), binding was similar to that observed with the WU2 (type 3) background strains.

The overall accessibility of the pneumococcal surface to antibody was assessed by using a polyclonal antiserum that reacted with surface antigens. The binding of this antiserum was generally similar to the binding observed with the PspA-specific antiserum; i.e., regardless of the genetic background, strains expressing the type 3 capsule bound less antibody than strains expressing the type 2 capsule (Fig. 4B). Switching the PspA types did not significantly affect the binding. Taken to-

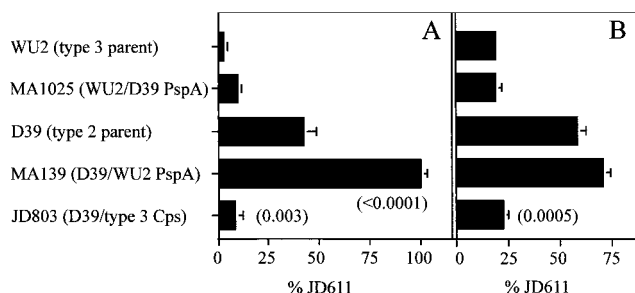


FIG. 4. Reactivity with polyclonal antisera to PspA and surface antigens. The data are the averages for at least three ELISA experiments in which plates were coated with whole cells and reacted with either PspA-specific antiserum (A) or antiserum reactive with noncapsular surface antigens (B). For MA1025, MA139, and JD803, the results for two independently obtained derivatives, which did not differ from each other, were combined. Binding was calculated relative to the binding of the capsule-negative strain JD611, which was defined as 100%. The results were compared by using Student's *t* test. *P* values for MA139 and JD803 are shown in parentheses for comparisons with D39. MA1025 and JD803 were not significantly different from WU2. The difference in antibody binding for D39 and WU2 was significant in both assays (*P* = 0.003 and *P* = 0.0009 for panels A and B, respectively).

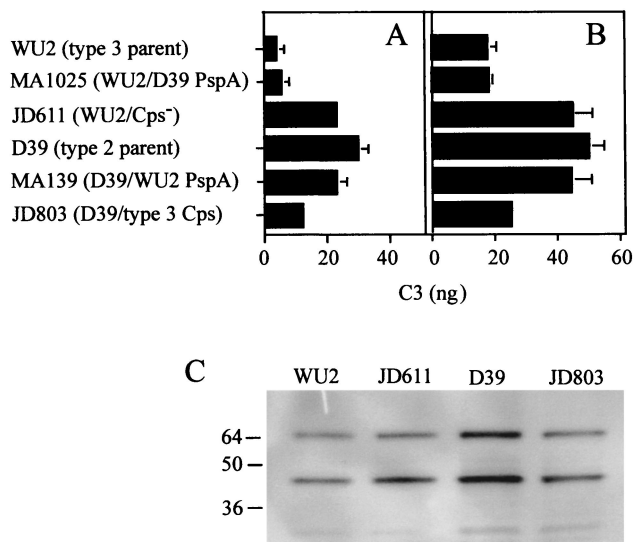


FIG. 5. Detection of complement bound to D39, WU2, and derivatives. (A and B) C3 bound to bacteria after opsonization in BALB/cByJ serum, detected by ELISA, as described in Materials and Methods. Comparable results were obtained following opsonization in CBA/N serum. The data are the averages for at least three experiments. For MA1025, MA139, and JD803, the data for two independently obtained derivatives were combined. The results were compared by using Student's *t* test. (A) Accessible surface-bound C3 after incubation in serum. MA1025 and MA139 were not significantly different from parent strains WU2 and D39, respectively. JD803 was significantly different from both WU2 and D39 (*P* = 0.02 and *P* = 0.002, respectively). WU2 and D39 were significantly different (*P* = 0.006). (B) Accessible surface-bound C3 after opsonization with the PspA-specific polyclonal antiserum followed by incubation in BALB/cByJ serum. JD803 was significantly different from D39 (*P* = 0.007) but not from WU2. D39 and WU2 were significantly different (*P* = 0.004). (C) Total bound C3. Equivalent numbers of bacteria opsonized in CBA/N serum were separated by SDS-PAGE and immunoblotted by using peroxidase-conjugated anti-C3. Comparable results were obtained following opsonization in BALB/cByJ serum. The bands represent the C3b  $\beta$ -chain (65 kDa) and iC3b  $\alpha$ -chain (46 kDa) fragments (40).

gether, the binding results suggest that accessibility of PspA and the entire pneumococcal cell surface is dependent upon the capsule type, but differences in accessibility between strains do not account for differences in anti-PspA protection levels.

**Effects of PspA serotype, capsular serotype, and PspA-specific antibody on complement binding.** Levels of accessible surface-bound C3b (and its degradation products) resulting from opsonization in mouse serum were measured by using C3-specific antiserum. More C3b was detectable on D39 than on WU2, and switching the WU2 and D39 PspAs did not alter these results (Fig. 5A). Alteration of the capsule type did have an influence, and the amount of detectable C3b on the D39 derivative expressing the type 3 capsule was intermediate between the amounts observed for two parents (Fig. 5A). The nonencapsulated D39 derivative R36A bound approximately 3.5-fold more anti-C3 than the WU2 nonencapsulated derivative JD611, indicating the importance of the genetic background in complement activation and/or deposition (data not shown). Incubation with PspA-specific antiserum enhanced complement fixation by all of the strains (Fig. 5B). Again,



however, less C3b was detectable on strains expressing the type 3 capsule than on strains expressing the type 2 capsule.

To determine whether the amount of C3b accessible to antibody was a reflection of the amount of C3b deposited, opsonized bacteria were examined by using SDS-PAGE and immunoblotting with the C3-specific antiserum. Consistent with the accessibility assay, more C3b products were present on D39 than on the other strains (Fig. 5C). However, the differences in accessibility were not completely accounted for by differences in total C3b binding (i.e., for WU2, JD611, D39, and JD803, the relative levels of total C3b binding were approximately 1:1.5:2.1:1.4, whereas those of accessible C3b were approximately 1:6.1:7.7:3.2). The capsule, its structure, and the genetic background thus appear to influence both the total amount of complement deposited and its accessibility to antibodies.

### DISCUSSION

The reasons for differences in virulence between *S. pneumoniae* strains are likely multifactorial. We previously observed that the effect of expression of the type 3 capsule on the virulence of recipients with different genetic backgrounds was unpredictable and could result in avirulence, enhanced virulence, or no change in virulence (25). Specific combinations of factors therefore appear to play an important role. Expression of the type 3 capsule in D39 did not alter the virulence of this strain (25), but as shown here, it decreased the ability of antibodies to bind surface components, including PspA and surface-localized C3b, and affected the amount of C3b deposited on the cell. Whether this effect is due solely to the alteration in capsule structure or is dependent on other factors present in the D39 background is not known. However, antibody binding to surface components of the D39 type 3 derivatives was observed at levels almost identical to the levels observed for WU2 and its derivatives that expressed the type 3 capsule, suggesting that the capsule structure itself is the major determinant of surface exposure.

Despite the binding of higher levels of antibody to PspA and surface-localized C3b by D39, this strain is much more resistant to anti-PspA-mediated protection than WU2. Neither the capsule type nor the PspA type appeared to be solely responsible for the resistance to protection, suggesting that strains such as D39 possess other factors that can impede antibody-mediated protection. Possibly, the C3b deposited on the D39 surface is localized at a site that is poorly accessible to phagocytic C3b receptors despite being available for antibody binding. Alternatively, the results could reflect differences between the in vitro assay and the in vivo situation. Whereas the virulence of PspA-negative mutants of WU2 is significantly reduced, mutants of D39 remain highly virulent (5, 36, 47; Abeyta and Yother, unpublished). PspA thus appears to be less important in D39 virulence, and reduced levels of PspA or reduced exposure on the surface during animal infections could explain both the minimal effect of mutations on virulence and the poor protection seen with antibody to PspA. Conversely, in vivo alterations in expression of capsule or other factors in the WU2 background may account for its sensitivity to protection.

Both capsule and PspA play important roles in resistance to complement. The capsule blocks access to cell wall-bound C3b

(7, 42; this study) and also appears to affect the amount of C3b deposited. PspA likewise reduces the amount of complement bound (40). Alteration of the PspA types examined in this study did not affect the function of PspA or the amount of C3b detected on the cell surface, suggesting that the functional domains may be conserved between PspA types. In contrast, the type 3 capsule was more effective than the type 2 capsule at blocking antibody access to surface antigens and surface-localized C3b. The structure of the capsular polysaccharide thus appears to play a major role in its ability to block accessibility to the pneumococcal surface.

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