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A 43-Kilodalton Pneumococcal Surface Protein, PspA: Isolation, Protective Abilities, and Structural Analysis of the Amino-Terminal Sequence

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PspA is an antigenically variable surface protein of *Streptococcus pneumoniae* that appears to be essential for full pneumococcal virulence. In addition, monoclonal antibodies to PspA protect mice against infection with specific strains of pneumococci virulent for mice. In this study, we have isolated the 43-kDa N-terminal half of the native 84-kDa PspA and determined the sequence of the first 45 amino acids. This sequence, the first obtained for a pneumococcal surface protein, is consistent with that of an amphipathic coiled-coil alpha helix with a 7-residue periodicity common to fibrous proteins such as tropomyosin and streptococcal M protein. The 7-residue periodicity begins with residue 8 and extends throughout the remaining sequence for nearly 11 turns of the helix. Mice immunized with this purified PspA segment were protected from fatal pneumococcal challenge, thus demonstrating that those PspA epitopes eliciting protection were present in the N-terminal half of the molecule.

Anticapsular antibodies to *Streptococcus pneumoniae* have long been recognized as protective in normal individuals. However, children under 2 years of age and the immunocompromised elderly do not respond well to T-cell-independent antigens such as polysaccharides and therefore are not afforded optimal protection by the current pneumococcal vaccine (2, 5, 11). One approach to this problem would be to combine polysaccharides and proteins that elicit immune responses in a protein-polysaccharide conjugate vaccine that would elicit a T-cell-dependent cellular response.

In previous studies, we have reported on an antigenically variable surface protein of *S. pneumoniae* (24, 25), pneumococcal surface protein A (PspA), that appears to be essential for full pneumococcal virulence (26). Despite the lack of availability of purified PspA, several studies have provided indirect evidence that PspA can elicit protective antibodies. Monoclonal antibodies (MAbs) Xi126 (immunoglobulin G2b) and Xi64 (immunoglobulin M), which bind PspA, are able to protect mice against infection with specific strains of pneumococci virulent for mice (6, 24). Also, immunization with $PspA^+$ pneumococci, but not with their $PspA^-$ isogenic mutants, is able to protect X-linked immunodeficient (*xid*) mice from fatal challenge (26). When *xid* mice were immunized with recombinant PspA and challenged with four virulent pneumococcal strains, PspA elicited protection against three of the four strains (25a). PspA is found on the surface of all pneumococci studied to date (12) and appears to be required for full virulence of *S. pneumoniae* (26).

PspA is firmly attached to the pneumococcal cell (34a) and has proven difficult to purify. In the studies presented here, we have taken advantage of the fact that a particular mutant has been identified that produces only the N-terminal half of PspA. This partial 43-kDa PspA product is not attached to the cell but is released into the medium, and it retains the ability to react with protective MAbs Xi126 and Xi64. This report describes the purification of the 43-kDa PspA, the analysis of its N-terminal 45 amino acids, and its ability to elicit a protective immune response in *xid* mice.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* JY2008 carries an insertion-duplication mutation in *pspA* that results in the production of only the N-terminal half of native PspA. This N-terminal half of PspA is approximately 43 kDa when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and because of the loss of the C-terminal half, this partial product is not attached to the cell and consequently accumulates in the growth medium. This insertion-duplication mutation in JY2008 was constructed by introduction of the plasmid pKSD300 (26) into the *pspA* gene of pneumococcal strain Rx-1 (29). Plasmid pKSD300 is a derivative of plasmid pVA891 (21) and carries a 550-bp fragment of *pspA*. The construction of pKSD300 has been described in detail previously by McDaniel et al. (26).

Pneumococcal strain Rx-1 (29) is a highly transformable variant of strain R36A (14), and strain R36A is a nonencapsulated derivative of the virulent type 2 strain D39 (3). All three of these strains have native PspA's of approximately 84 kDa.

JY2008 was grown in 6 liters of a chemically defined medium reported by van de Rijn and Kessler (32) and prepared for us by Hazleton Research Products, Inc., Denver, Pa. The medium was supplemented with 0.1% choline chloride, 0.075% L-cysteine hydrochloride, (Sigma Chemical Co., St. Louis, Mo.), and 0.25% $NaHCO_3$ (Fisher Scientific, Fair Lawn, N.J.). For isolation of the 43-kDa PspA, JY2008

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was grown to mid-log phase, and the supernatant fluid was harvested with a 0.22-μm-pore-size membrane (Millipore Pellicon system) and concentrated 60-fold with Pellicon and Minitan 10K membranes (Millipore Corp., Bedford, Mass.). WU2 is an encapsulated type 3 strain originally isolated from a patient at Washington University (7). Strain WU2 was grown to mid-log phase in Todd-Hewitt medium supplemented with 0.5% yeast extract (both from Difco Laboratories, Detroit, Mich.), harvested by centrifugation at 4,000 × *g* for 15 min, and resuspended in Ringer's lactate for injection into mice.

Purification of 43-kDa PspA. Concentrated supernatant fluid from strain JY2008 was dialyzed in 0.1 M phosphate-buffered saline (pH 7.2) (PBS) and ultracentrifuged at 196,000 × *g* for 1 h. This supernatant fluid was diluted 1:5 in 20 mM L-histidine–NaCl buffer (Sigma), adjusted to pH 6.0, and injected into a DEAE-fibered Isonet-D2 ion-exchange column (Kinetek Systems, Inc., St. Louis, Mo.). A stepwise NaCl gradient from 80 mM to 2 M was applied to the column, and fractions containing PspA were collected and identified by dot blot analysis (33) with MAb Xi126. Appropriate fractions were pooled and analyzed by SDS-PAGE. The proteins were stained with Coomassie brilliant blue R-250, and the PspA band was excised and electroeluted with an Elutrap with a BT1 exclusion membrane and a BT2 prefilter (Schleicher & Schuell, Inc., Keene, N.H.). The eluted protein was precipitated in a solution containing equal volumes of methanol and acetone and resuspended in PBS. Protein purity was confirmed by protein staining and Western immunoblotting with MAb Xi126.

Amino acid sequencing. One hundred picomoles of the purified 43-kDa PspA was electrophoresed through 9% resolving gels containing recrystallized SDS by using the Laemmli buffer system (18). Following electrophoresis, the method of Matsudaira (23) was followed with minor modifications. The gels were soaked twice in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11.0) (CAPS) buffer (Sigma) containing 10% methanol for 10 min each. A Millipore polyvinylidene difluoride membrane (19) was wetted completely for several seconds in 100% methanol and then washed in CAPS buffer for 10 min. PspA was electrotransferred to the polyvinylidene difluoride membrane in CAPS buffer at 0.5 A for 1 h by using established procedures (13, 23). After PspA was transferred, the membrane was washed two times in deionized water for 5 min and stained with 0.1% Coomassie blue R-250 in 50% methanol for 20 min. The section of the membrane containing PspA was excised and destained in 40% methanol–10% acetic acid for 5 min. The membrane was cut into small segments and stored in sterile Eppendorf tubes until sequencing. The high-performance liquid chromatography hardware, data acquisition, sequencing procedure, and determination of amino acid yields observed at a sequencing cycle have been described in detail (13).

Computer analyses of sequence. Computer software (DNA STAR, Inc., Madison, Wis.) was used to apply the Garnier-Robson (16) and Chou-Fasman (9) algorithms to secondary-structure analysis of the sequence. The Kyte-Doolittle model was used to analyze relative hydrophobicity (17).

Immunization and challenge studies. Sixteen 7-week-old CBA/N mice carrying the *xid* mutation (1, 34) were obtained from Jackson Laboratories, Bar Harbor, Maine. These mice were chosen because they cannot respond to T-cell-independent antigens such as pneumococcal polysaccharide. They were bled via the infraorbital sinus to establish preexposure levels of antibody to PspA. Purified 43-kDa PspA was emulsified in complete Freund's adjuvant (GIBCO, Grand Island, N.Y.) and injected subcutaneously into the inguinal and axillary regions, giving an approximate dose of 5 μg of protein per mouse. Fourteen days later, the mice were injected intraperitoneally with 5 μg of 43-kDa PspA. Control mice were immunized identically via the same routes with sterile buffer instead of the 43-kDa PspA. Seven days following the last immunization, all mice were bled and challenged intravenously with 300 CFU of the type 3 pneumococcal strain WU2.

Immunoblot analysis of mouse immune sera. Preimmunization and prechallenge sera were analyzed by Western immunoblots to establish baseline and postimmunization response to PspA. Purified 43-kDa PspA and a lysate (24) of strain WU2 were electrophoresed and transferred to nitrocellulose membranes by using established procedures (8, 31). The membranes were separated into strips and probed with the appropriate mouse antisera at a 1/50 dilution for 2 h, incubated with biotinylated goat anti-mouse immunoglobulin (Southern Biological Associates, Birmingham, Ala.) for 1 h, washed, and incubated with alkaline phosphatase-conjugated streptavidin (Sigma). The membranes were developed with 5-bromo-4-chloro-3-indolyl phosphate toluidine salt with 0.01% Nitro Blue Tetrazolium (Sigma).

Protein sequence accession number. The sequence data have been filed with GenBank and have been assigned accession no. A33134.

RESULTS

Purification of 43-kDa PspA. Because the intact native 84-kDa PspA has proven difficult to isolate from whole cells (unpublished observations), we purified the N-terminal half of PspA from culture supernatants of pneumococcal strain JY2008. This strain produces a truncated PspA that is not attached to the cell and that is about one-half the molecular mass of the PspA of parent strain Rx-1 (43 kDa as compared with 84 kDa). Figure 1 shows a Coomassie blue-stained polyacrylamide gel of the spent medium containing the protein prior to purification (lane A) and the isolated product following chromatography and electrophoresis (lane B). The Western immunoblot of this purified product obtained with MAb Xi126 shows that this isolated protein is the 43-kDa PspA (lane C). (Not shown is the strong reactivity of this PspA with MAb Xi64.)

Structural analysis of the amino-terminal sequence of PspA. The isolated 43-kDa PspA was sequenced directly from polyvinylidene difluoride membranes. The N-terminal 45-residue amino acid sequence is shown in Fig. 2. It is striking that 51% of this sequence is composed of alanine (27%) or lysine (24%). The sequence contains nearly a 1:1 ratio of charged to apolar amino acids.

Analyses of the secondary structure of this sequence were computed separately with Chou-Fasman (9) and Garnier-Robson (16) algorithms. The Chou-Fasman algorithm predicted an alpha-helical structure following a 4-residue beta turn including the proline in position 4, giving a prediction of an 89% alpha-helical structure. The Garnier-Robson algorithm predicted an entirely alpha-helical structure with no extended chain, turn, coil, or beta-sheet interruptions.

Figure 2 also shows the 7-residue periodicity of this PspA sequence. The 7-residue extreme N terminus consists primarily of uncharged and apolar amino acids. The portion of the sequence exhibiting the periodicity begins with residue 8 (Gln-8), and the repeating heptad of hydrophobic and hydrophilic amino acids continues throughout the remainder of the

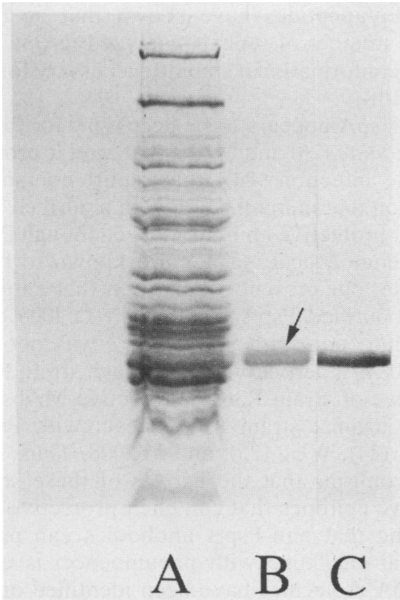

FIG. 1. Purification of PspA. Lane A, Coomassie blue stain of spent medium containing the 43-kDa PspA prior to purification; lane B, Coomassie blue stain of the isolated 43-kDa PspA (arrow); lane C, Western immunoblot of the isolated PspA obtained with MAb Xi126. Ten percent gels were run with 100 μg of protein per well.

TABLE 1. Immunization with 43-kDa PspA and challenge with pneumococcal type 3 strain WU2

Immunogen	No. of mice[a]:		
	With antibody to PspA[b]	Alive at 2 days postchallenge	Alive at 14 days postchallenge[c]
Isolated PspA	8	8	8
Sterile buffer	0	2[d]	2

[a] Mice ($n = 8$) were challenged with 300 CFU of type 3 pneumococcal strain WU2 (the 50% lethal dose of this strain is 10 CFU).

[b] Antibody at time of challenge detected by Western immunoblot analysis with a 1/50 dilution of serum.

[c] There was a significant difference between immunized mice and controls ($P < 0.003$) by chi-square analysis with the continuity correction of Cochran (35).

[d] The two surviving mice were both sick as judged by ruffled fur, arched back, and decreased movement during days 2 and 3 postchallenge.

sequence; this periodicity is indicated by the letters *a* through *g* at the top of Fig. 2. Positions *a* and *d* of the repeat contain predominantly hydrophobic amino acids. Positions *b*, *c*, *e*, and *f* contain predominantly charged or polar amino acids. This 38-residue repeating sequence corresponds to nearly 11 turns of the helix.

A prediction of local hydrophobicity was made from the Garnier-Robson secondary-structure analysis. At its N terminus, PspA appears to be very hydrophilic in nature, primarily in the regions which are entirely and unambiguously alpha helical. Toward residue 45, the hydrophobicity potential increases. Because of the hydrophilicity of the N-terminal sequence, we would expect this portion of PspA to be highly exposed to the external environment.

Protection with purified 43-kDa PspA. Of eight CBA/N *xid* mice immunized with purified PspA, all were alive 14 days after challenge with 300 CFU of strain WU2 (the 50% lethal dose of WU2 in *xid* mice is approximately 10 CFU), and none showed any signs of illness following challenge. Of the eight control mice immunized with buffer, six were dead by 2 days postchallenge (Table 1). The two remaining control mice appeared very sick (ruffled fur, closed eyes, arched backs, and decreased movement) at 2 to 3 days following challenge but survived. Chi-square analysis indicated that there was a significant difference ($P < 0.003$) in survival between the immunized and control groups.

To determine whether the immunization with the purified product elicited anti-PspA antibody, preimmunization and prechallenge sera were analyzed by Western immunoblotting. Neither preimmune sera nor sera from control mice contained detectable antibodies reactive with the 43-kDa PspA or with the full-length WU2 PspA. Postimmunization sera from seven of eight PspA-immunized mice contained detectable antibodies to 43-kDa PspA, and all eight immunized mice had antibodies that were highly cross-reactive with the WU2 PspA epitopes (Table 1). These serologic results are consistent with the hypothesis that the protection elicited by immunization with the 43-kDa PspA is due to the production of anti-PspA antibodies.

DISCUSSION

Although PspA has been difficult to purify from cell wall extracts (data not shown), we were able to isolate the N-terminal half of PspA from a strain carrying an insertion-duplication mutation in *pspA*; this partial product fails to

							a	*b*	*c*	*d*	*e*	*f*	*g*	
GLU	**GLU**	**ser**	*pro*	*val*	*ala*	**ser**	**gln**	**ser**	**LYS**	*ala*	**GLU**	**LYS**	**ASP**	14
							tyr	**ASP**	*ala*	*ala*	**LYS**	**LYS**	**ASP**	21
							ala	**LYS**	**asn**	*ala*	**LYS**	**LYS**	*ala*	28
							val	**GLU**	**ASP**	*ala*	**gln**	**LYS**	*ala*	35
							leu	**ASP**	**ASP**	*ala*	**LYS**	*ala*	*ala*	42
							gln	**LYS**	**LYS**					45

FIG. 2. N-terminal amino acid sequence of PspA. Amino acids at positions *a* through *g* form the 7-residue periodicity of hydrophobic and hydrophilic residues extending from residue 8 (Gln-8) to the end of the sequence at residue 45. Bold capital letters denote charged, hydrophilic amino acids; bold lowercase letters denote uncharged, polar, hydrophilic amino acids; italicized letters designate apolar, hydrophobic residues.

attach to the cell. This PspA had a molecular mass of 43 kDa as determined by SDS-PAGE, approximately half the size of the native 84-kDa PspA of strain Rx-1. This purified PspA was partially sequenced, analyzed, and used as an immunogen in mouse protection studies.

Secondary-structure analyses of the 45-amino-acid N-terminal sequence predict that this PspA segment exists primarily as an alpha helix. The Garnier-Robson algorithm, which is thought to be a better indicator of secondary structure for fibrous proteins than the Chou-Fasman algorithm (15), predicts a 100% alpha-helical formation, whereas the Chou-Fasman algorithm predicts an initial beta turn including the proline in position 4 (Pro-4) before an uninterrupted alpha helix. The presence of serine and proline in positions 3 and 4 is consistent with reports that serine and proline frequently occupy the first and second positions of a beta turn (30), and serine, being slightly hydrophilic, can facilitate beta-turn formation and contribute to the stability of the local region (20). The nonhelical first seven residues are reminiscent of the nonhelical segment often found at the N terminus of alpha-helical proteins such as tropomyosin, laminin, intermediate filament protein, M protein, and paramyosin (10). Regardless of the conformation of the initial residues, the fact that there are no proline residues following Pro-4 is consistent with the predictions obtained with both algorithms that the overall conformation is an alpha helix.

The specific features of alpha-helical coiled-coil proteins have been studied in detail (10). They exhibit a unique heptapeptide repeat pattern, with primarily apolar residues occupying positions *a* and *d* (27). When arranged in the alpha-helical coiled-coil structure, the *a* and *d* residues of each helix are next to each other and allow the two helices to form stabilizing hydrophobic bonds. In this PspA sequence, apolar and uncharged polar amino acids occupy all *a* and *d* positions. The observation that alanine occupies all *d* positions is consistent with the fact that alanine is a favored residue for position *d* in a coiled-coil structure (10). The *f* position of coiled-coil helices is maximally exposed to the environment, and accordingly, charged residues are frequently found in this position; in this PspA sequence, charged lysines were present in four of the five *f* positions. The residues present in the *b* and *c* positions in coiled-coil helices also face outward into the environment and thus are hydrophilic and frequently charged. Of the 12 residues present in the *b* and *c* positions of PspA, 9 are charged, and 2 of the 3 remaining residues are uncharged polar residues that would interact well with water molecules.

The charged residues in the PspA sequence also exhibit the preferential distribution found in the sequence of other coiled-coil proteins such as M protein, in which predominantly positively charged residues are found in position *e* and predominantly negatively charged residues are found in position *g* (22). In this PspA sequence, lysine is the predominant charged amino acid in position *e*, and aspartic acid is found twice in position *g*, giving a 3:2 distribution of positive to negative charges in the *e* and *g* positions. An approximate 1:1 ratio of basic and acidic charges in the *e* versus *g* positions has been found in other coiled-coil proteins (22, 27, 28), and this distribution is thought to lend stability to the two helices by allowing the formation of salt bridges between them. On the basis of the features consistent with other coiled-coil proteins, it seems very likely that the N terminus of PspA has an alpha-helical conformation that could participate in coiled-coil interactions with other PspA molecules. In further support of this possibility, studies of synthesized repeating heptapeptides have shown that as few as five repeats (the number of repeats analyzed in this report) can provide the conformational stability necessary for coiled-coil structures (10).

Although PspA appears to be necessary for full virulence of *S. pneumoniae* (26) and MAbs that bind it protect against lethal mouse infection (24), this report contains the first demonstration that immunization with a purified PspA product can elicit protective immunity. Even though PspA's from different pneumococcal isolates are known to be serologically variable, our present observation that antibodies elicited against purified PspA from strain JY2008 can protect mice from infection with strain WU2 was not surprising. Strain JY2008 is a derivative of the rough strain Rx-1, which is a derivative of strain R36A. Protective MAbs Xi126 and Xi64, made against strain R36A, react with PspA's from strains Rx-1 (24), WU2 (25), and JY2008. Thus, our protection study confirms that the PspA's of these strains share cross-reactive epitopes that can elicit protective antibodies.

The finding that anti-PspA antibodies can protect mice against lethal challenge with pneumococci is encouraging because PspA molecules have been identified on almost all pneumococci studied to date (12). This suggests that the development of a PspA-based pneumococcal vaccine may allow broad protection against pneumococcal infections in the very young and immunocompromised elderly, populations that do not respond well to the current polysaccharide vaccine. The fact that at least a portion of PspA appears to be composed of an amphipathic helix indicates that it may also be a strong stimulator of T cells (4) and thus may be a candidate for the protein portion of a pneumococcal polysaccharide-protein conjugate vaccine.

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