Characterization of Selected Strains of Pneumococcal Surface Protein A*

Received for publication, April 13, 2001, and in revised form, June 5, 2001
Published, JBC Papers in Press, June 18, 2001, DOI 10.1074/jbc.M103304200

Mark J. Jedrzejas‡‡, Ejvis Lamani‡, and Robert S. Becker¶

From the ‡Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294 and ¶Aventis Pasteur, Swiftwater, Pennsylvania 18370

Several proteins, in addition to the polysaccharide capsule, have recently been implicated in the full virulence of the Streptococcus pneumoniae bacterial pathogen. One of these novel virulence factors of S. pneumoniae is pneumococcal surface protein A (PspA). The N-terminal, cell surface exposed, and functional part of PspA is essential for full pneumococcal virulence, as evidenced by the fact that antibodies raised against this part of the protein are protective against pneumococcal infections. PspA has recently been implicated in anti-complementary function as it reduces complement-mediated clearance and phagocytosis of pneumococci. Several recombinant N-terminal fragments of PspA from different strains of pneumococci, Rx1, BG9739, BG6380, EF3296, and EF5668, were analyzed using circular dichroism, analytical ultracentrifugation sedimentation velocity and equilibrium methods, and sequence homology. Uniformly, all strains of PspA molecules studied have a high α-helical secondary structure content and they adopt predominantly a coiled-coil structure with an elongated, likely rod-like shape. No β-sheet structures were detected for any of the PspA molecules analyzed. All PspAs were found to be monomeric in solution with the exception of the BG9739 strain which had the propensity to partially aggregate but only into a tetrameric form. These structural properties were correlated with the functional, anti-complementary properties of PspA molecules based on the polar distribution of highly charged termini of its coiled-coil domain. The recombinant Rx1 PspA is currently under consideration for pneumococcal vaccine development.

Streptococcus pneumoniae bacterial pathogen causes life-threatening diseases in humans like pneumonia, bacteremia, and meningitis (1, 2). In addition, this pathogen causes less serious but prevalent diseases such as sinusitis and otitis media (1, 3). The recent increase in penicillin-resistant strains of pneumococci (4, 5), together with only moderate effectiveness, at best, of the current pneumococcal vaccine reinforces the need for an improved cure and for the investigations of various aspects of the pathogenesis of S. pneumoniae, especially bacteria-host interactions. The precise knowledge of how S. pneumoniae and other bacteria interact with host tissues is still largely speculative. It is known, however, that pneumococci produce several antigens responsible for various processes during colonization of the host. Such antigens include but are not limited to the polysaccharide capsule and proteins such as pneumococcal surface protein A (PspA) (6, 7) and C (PspC) (7, 8), hyaluronate lyase (3, 9), and pneumolysin (10). These antigens directly contribute to the invasive capability of the bacteria by allowing, for example, greater microbial access or migration between host tissues or by compromising the host defense mechanisms (7, 10–12).

PspA is a surface protein of S. pneumoniae (13) found in every characterized pneumococcal strain (14). Its size is strain-dependent and varies from ~67 to 99 kDa (15). It is attached to pneumococci through noncovalent interactions of the C-terminal repeat region with the terminal choline residues of the teichoic or lipoteichoic acids present on the pneumococcal cell wall (16) and classified as a choline-binding protein. The PspA molecule is built from four distinct domains which include the antigenic N-terminal part followed by a highly flexible, tether-like proline-rich region, a repeat region which is responsible for the attachment to the choline residues, and a C-terminal hydrophobic tail (Fig. 1) (17). The N-terminal moiety likely protrudes outside of the capsule, interacts with all antibodies reactive to PspA, and has been described as the functional part of this protein (the PspA function was defined as its ability to elicit in host protective antibodies) (18, 19). This part of PspA is essential for full pneumococcal virulence; antibodies raised against the N-terminal part of PspA are protective against pneumococcal infections (18). This domain for the Rx1 strain has been shown earlier to have an α-helical coiled-coil structure with a seven-residue (heptad) repeat of its sequence which is characteristic of coiled-coils (19–22). Previous structural studies of this domain of Rx1 PspA indicated its highly charged and polar character which has been shown to, on the one hand, stabilize PspAs interactions with the electronegative capsule through interactions with the electro-positive part of this domain and, on the other hand, points the electronegative end of PspA away from the bacterial cell wall (19). This electronegative part has already been implicated in PspAs anti-complementary properties which prevent the host complement system from attaching to S. pneumoniae (7, 19). The proline-rich region of PspA likely serves as a flexible tether anchoring PspA to the cell wall through the choline-binding region (repeat region).

Here, we report studies of N-terminal, functional modules of PspA from several different pneumococcal strains: BG6380, BG9739, EF3296, EF5668, and the vaccine candidate PspA strain Rx1. Our previous biophysical studies have been performed and reported only for a non-vaccine construct of Rx1

* This work was supported by a contract from Aventis Pasteur (to M. J. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Children’s Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609-1673. Tel.: 510-450-7932; Fax: 510-450-7910; E-mail: mjdjedz@choir.org.

¶ The abbreviations used are: PspA, pneumococcal surface protein A; CD, circular dichroism; MPSS, multiple protein sequence analysis; PspC, pneumococcal surface protein C.
Pneumococcal Surface Protein A

TABLE I
Calculated and measured properties and the secondary structure composition of five PspA constructs

| PspA construct | No. amino acids | Mol. mass | Homology to Rx1314 | α-Helix calculated/measured | Random coil calculated/measured | β-Sheet calculated/measured | Breaks in coiled-coil structure |
|----------------|----------------|-----------|--------------------|----------------------------|-------------------------------|-----------------------------|-------------------------------|
| Rx1314         | 314            | 34,720    | 100                | 82/63                      | 18/37                        | 0/0                         | Before Lys11 Val120, Pro123 Val173, Pro175 Gly205, Pro219 Past Pro290 Before Lys11 Val113, Pro117 Ser189, Pro202 Past Pro291 Before Lys11 Val117, Pro121 Past Gly246 |
| BG9739         | 301            | 33,837    | 78                 | 78/71                      | 22/29                        | 0/0                         |                       |
| EF5668         | 371            | 41,395    | 62                 | 80/64                      | 20/36                        | 0/0                         |                       |
| BG6380         | 425            | 47,657    | 48                 | 66/48                      | 34/52                        | 0/0                         |                       |
| EF3296         | 480            | 53,171    | 45                 | 74/71                      | 26/29                        | 0/0                         |                       |

*The percentage of the secondary structure elements such as α-helix and random coil and the coiled-coil structure were calculated based on the sequence data using the PHD program (26, 27) and Matcher software (28), respectively. No β-sheet structures were detected in any of the constructs. The BG9739 PspA construct together with the EF3296 PspA construct have the highest percentage of α-helical content, whereas the BG6380 PspA construct has the lowest.

*The data are based on the CD spectra analysis as described under “Experimental Procedures.”

PspA containing amino acids 1 through 303 (11 amino acids shorter than the vaccine construct termed here Rx1314) (19, 20). Circular dichroism, velocity and equilibrium sedimentation analysis, and sequence similarity studies of PspAs were used to characterize the structural properties of the functional part of this molecule for different pneumococcal strains in more detail. The functionality of PspA for this study was defined as its ability to elicit antibodies, which are protective against pneumococcal infection. All these properties are compared with the vaccine candidate recombinant protein construct Rx1314 (19, 20).

EXPERIMENTAL PROCEDURES

Overexpression and Purification of PspA Constructs—Different N-terminal PspA recombinant constructs/pneumococcal strains Rx1314 (amino acids 1–314), Rx1314MI (amino acids 1–314, M96I), BG6380 (amino acids 1–423), BG9739 (amino acids 1–300), EF3296 (amino acids 1–478), and EF5668 (amino acids 1–369) were obtained as previously described (20). These recombinant proteins contain the α-helical N-terminal portion of PspA of the appropriate strain and a part of the proline-rich region. The recombinant Rx1314 PspA has been shown to be protective against pneumococcal infection in mice (21). Briefly, the appropriate genes were cloned into a pET-9a expression vector which be protective against pneumococcal infection in mice (21). Briefly, the appropriate genes were cloned into a pET-9a expression vector which was used to transform Escherichia coli BL21 (DE3) pLysS cells. Each E. coli cells were grown and the PspA production was then induced with isopropyl-1-thio-β-D-galactopyranoside following standard procedures (20). The PspA molecules were purified as previously described (20, 22). No detergent was used to recover the overexpressed recombinant proteins from E. coli.

Analytical Methods—Electrophoresis was performed under reducing conditions in a 10% polyacrylamide gel using a Mini Protein II gel system (Bio-Rad) and the buffer system described by Laemmli (23). Coomassie Blue was used to stain the gels.

Sequence Analysis—The sequence data for all constructs were edited using the PHD program (26, 27) and Matcher software (28), respectively. No β-sheet structures were detected in any of the constructs. The BG9739 PspA construct together with the EF3296 PspA construct have the highest percentage of α-helical content, whereas the BG6380 PspA construct has the lowest.

Hydrodynamic Characterization—Band and boundary sedimentation velocity experiments were performed at 20 °C in an AN-60 Ti analytical rotor at 56,000 rpm using a Beckman XLA analytical ultracentrifuge. Radial scanning was performed at 280 nm. For the boundary experiments, the cell contained 0.4 ml of protein in a solution consisting of 145 mM NaCl and 50 mM sodium phosphate buffer (pH 7.2) and 145 mM NaCl.

Circular Dichroism Studies—The circular dichroism (CD) spectra for different strains of PspA were recorded using an AVIV 62DS spectropolarimeter interfaced to a personal computer. The measurements were performed on six PspA samples at protein concentrations 0.147 mg/ml for Rx1314MI, 1.124 mg/ml for Rx1314, 0.829 mg/ml for BG9739, 0.479 mg/ml for EF5668, 0.723 mg/ml for BG6380, and 0.350 mg/ml for the EF3296 strain. All protein samples were in 50 mM sodium phosphate buffer (pH 7.2) and 145 mM NaCl.

The CD spectra were measured every 0.5 nm from 260 to 190 nm with 2-nm bandwidth and 1-s averaging per point. For all runs, the baseline was corrected by subtracting a spectrum of the corresponding buffer from the one obtained for the protein sample in identical conditions. The temperature for each run was maintained at 25 °C by a Lauda RS2 circulating water bath, and the temperature of the quartz cell, with a path length of 0.1 mm, was measured using a thermosensor. The secondary structure analysis was performed using the program PROSEC (31) employing standard procedures.

Hydrodynamic Characterization—Band and boundary sedimentation velocity experiments were performed at 20 °C in an AN-60 Ti analytical rotor at 56,000 rpm using a Beckman XLA analytical ultracentrifuge. Radial scanning was performed at 280 nm. For the boundary experiments, the cell contained 0.4 ml of protein in a solution consisting of 145 mM NaCl and 50 mM sodium phosphate buffer (pH 7.2). Boundary sedimentation velocity data were analyzed using the time derivative software supplied by Beckman Instruments as part of the package for sedimentation velocity data analysis (32).

Band centrifugation was also employed (33). Uncorrected s values were corrected to siso using the standard formula (34). The correction values for 50% D2O (35) were 1.1116 and 1.0527 for the relative viscosity and buoyancy terms, respectively. For each run 20–30 µl of the protein samples was used. Partial specific volume, v, was calculated based on the sequence of the different PspA constructs. Program Sedweb2 was used to calculate diffusion coefficients and molecular weights for all PspAs. The calculations of the frictional and axial ratios was performed as described previously by Jedrzejas et al. (19).

a Published results.
RESULTS AND DISCUSSION

Sequence Analysis—The sequences of all known N-terminal functional PspA constructs (Rx1314, BG9739, EF5668, BG6380, and EF3296) were compared and analyzed with respect to their common properties. The functionality of PspA for this study was defined as its ability to elicit antibodies, which are protective against pneumococcal infections. The aligned sequence data were edited and analyzed using the program MPSA (Fig. 2) (24). All five sequences of PspA molecules ranging from 301 (BG9739) to 480 (EF3296) residues are very similar. Their homology to the Rx1314 strain ranges from 45 (EF3296) to 78% (BG9739) (Table I). The best results for the alignment of all sequences was accomplished using the Mul-  

Circular Dichroism—In order to confirm the sequence analyses, the experimental determination of the secondary structure of the recombinant N-terminal fragments of PspA was performed using the CD spectropolarimetric method. The samples characterized using CD as well as SDS-polyacrylamide gel electrophoresis analysis were as follows: Rx1314MI, Rx1314, BG9739, EF5668, BG6380, and EF3296 (Figs. 2 and 3). The information from the normalized CD spectra of the PspA construct, shown in Fig. 4, was used to evaluate the secondary structure of each PspA sample using the PROSEC program (31) as described under “Experimental Procedures.” The result of the quantitative analysis of the CD spectra of all six PspA constructs are highly consistent with the sequence analysis presented above (as shown in Table I) and also indicate a high α-helical content with no β-sheet present for the secondary structure (Table I). Constructs EF3296 and BG6380 have the highest and the lowest percentage of the α-helical content, respectively. The superposition of the normalized CD spectra of all constructs is shown in Fig. 4, A and B. All PspAs seem to have a very high helical content with varying content of random coils, presumably between the coiled-coils.

Furthermore, the Rx1314 PspA construct was really a mixture of two PspA peptides overexpressed in E. coli (see “Experimental Procedures”) containing amino acids 1 to 314 of the Rx1 strain and a second peptide, coexpressed due to the presence of a secondary initiation site in the pspA gene at Met96, pspA domain architecture of PspA molecules. The recombinant constructs used in the analysis are also marked. B, elongated rod-like shape of the α-helical, antiparallel coiled-coil part of Rx1 PspA molecule. The drawing is based on the model of a PspA molecule containing amino acids 1 to 303 published by Jedrzejas et al. (19). The color coding of the surface corresponds to the magnitude of the electrostatic potential: blue, elec- 

Functional PspA fragments were also subjected to analytical ultracentrifugation studies in order to confirm their aggregation state and to investigate their shape. Based on our earlier work performed on the recombinant (not included in the vaccine under development) Rx1 PspA containing amino acids 1 through 303 (PspA303), all strains of PspA were expected to have an elongated rod-like shape (Fig. 1B and 5) (19). The results of band and boundary sedimentation velocity analyses are presented in Table II, and Figs. 6 and 7.

Globular proteins are known to be relatively compact and spherical with asymmetry factors (frictional ratios) below 1.2 (39). Fibrous proteins and nucleic acids have larger frictional ratios. The frictional ratios of all PspAs investigated are above 1.2 (1.53 to 1.91 for monomeric PspAs) with an axial ratio for monomer, defined as length/width of the protein molecule, ranging from 1:10 to 1:17, suggesting that all PspA constructs are elongated and non-globular in shape (Table II, Fig. 1B). The results are consistent with parallel aggregation of rod-like PspA molecules to create a bit bulkier but still elongated rod-like molecule. This construct was further investigated in order to describe the equilibrium properties of BG9739 using the sedimentation equilibrium methodology.

**Sedimentation Velocity Analysis**—Functional PspA fragments were also subjected to analytical ultracentrifugation studies in order to confirm their aggregation state and to investigate their shape. Based on our earlier work performed on the recombinant (not included in the vaccine under development) Rx1 PspA containing amino acids 1 through 303 (PspA303), all strains of PspA were expected to have an elongated rod-like shape (Fig. 1B and 5) (19). The results of band and boundary sedimentation velocity analyses are presented in Table II, and Figs. 6 and 7.

Globular proteins are known to be relatively compact and spherical with asymmetry factors (frictional ratios) below 1.2 (39). Fibrous proteins and nucleic acids have larger frictional ratios. The frictional ratios of all PspAs investigated are above 1.2 (1.53 to 1.91 for monomeric PspAs) with an axial ratio for monomer, defined as length/width of the protein molecule, ranging from 1:10 to 1:17, suggesting that all PspA constructs are elongated and non-globular in shape (Table II, Fig. 1B). The smallest frictional ratio observed was for the BG9739 PspA which self-associates to create tetramers with frictional ratios as small as 1.27 and an axial ratio of 1:5 (both ratios are for the tetramer only).

Table II as well as Figs. 6 and 7 show that the values of the sedimentation coefficient, s, from both types of sedimentation experiments, band and boundary, suggest that the aggregation for the BG9739 PspA N-terminal construct is consistent with the tetramer formation in solution (Table II). The low values of frictional ratios and the axial ratios calculated for the BG9739 tetramer suggest, as expected, that the aggregated protein is less elongated relative to the non-aggregated PspAs. The results are consistent with parallel aggregation of rod-like PspA molecules to create a bit bulkier but still elongated rod-like molecule. This construct was further investigated in order to describe the equilibrium properties of BG9739 using the sedimentation equilibrium methodology.

**Sedimentation Equilibrium Analysis of PspA BG9739**—The equilibrium sedimentation experiments for the BG9739 PspA construct were performed at room temperature for two sample concentrations, 1.09 and 0.36 mg/ml. The results for the 1.09 mg/ml sample are shown in Fig. 8 which represents the varying concentration of PspA BG9739 as a function of the radial distance of the exponential distribution of the protein concentration at 20°C and for the best model. The top part of the figure represents a distribution of the residuals (deviation of the concentration) from the fitted curve. The BG9739 sample at a lower concentration of 0.36 mg/ml afforded similar results (data not shown). The equilibrium data were fitted globally using the nonlinear least-squares program NONLIN (36). The data analysis showed that the monomer-tetramer model, no further, higher association was detected that would fit our data. It is clear that there is no evidence for higher, beyond the tetramer formation, association present for this PspA strain.
The sedimentation data for the BG9739 PspA strain from the equilibrium studies were used to calculate the association constant, $k_a$, between the monomer and the tetramer (40). Using this model, the global variable parameter, $H_{9262}$, was found to be 0.16689 and the association constant, $k_a$, was $4.7 \times 10^{10}$ M$^{-3}$.

At a protein concentration of 1.09 mg/ml at 20°C the majority of the protein in solution is present in a monomeric form (95%). The functional significance of the tetrameric aggregation of this PspA construct is not known at present. This aggregation might be, however, insignificant with respect to the function of PspA due to the very low occupancy of the tetrameric aggregation state in solution.

Structures of PspA Molecules—All strains of the N-terminal parts of PspA molecules investigated have been shown here to have a very high content of $\alpha$-helical structures arranged in monomeric coiled-coils. All PspA molecules with the exception of BG9739 are monomeric, and considering the sedimentation axial ratio data and standard dimensions of helical structure, such coiled-coil can only be created by the folding of these molecules back on themselves creating an anti-parallel coiled-coil. Only the BG9739 PspA aggregates to form tetrameric molecules, that based on the sedimentation data and the analysis of the size of helices, are also likely built from individual molecules folded on themselves to create anti-parallel coiled-coils that aggregate in tetramers. Formation of tetrameric coiled-coils (parallel or anti-parallel) arranged from fully extended molecules is unlikely as the resultant molecule would be extended to a significantly longer size than the sedimentation data supports (axial ratio for a tetramer higher than 1:10). Therefore, all PspA molecules analyzed in this study have a similar three-dimensional structural arrangement in the anti-parallel coiled-coil. The shape of such elongated molecules is similar to an elongated rod.

The sedimentation data for the BG9739 PspA strain from the equilibrium studies were used to calculate the association constant, $k_a$, between the monomer and the tetramer (40). Using this model, the global variable parameter, $\mu$, was found to be 0.16689 and the association constant, $k_a$, was $4.7 \times 10^{10}$ M$^{-3}$. At a protein concentration of 1.09 mg/ml at 20°C the majority of the protein in solution is present in a monomeric form (95%). The functional significance of the tetrameric aggregation of this PspA construct is not known at present. This aggregation might be, however, insignificant with respect to the function of PspA due to the very low occupancy of the tetrameric aggregation state in solution.

Structures of PspA Molecules—All strains of the N-terminal parts of PspA molecules investigated have been shown here to have a very high content of $\alpha$-helical structures arranged in monomeric coiled-coils. All PspA molecules with the exception of BG9739 are monomeric, and considering the sedimentation axial ratio data and standard dimensions of helical structure, such coiled-coil can only be created by the folding of these molecules back on themselves creating an anti-parallel coiled-coil. Only the BG9739 PspA aggregates to form tetrameric molecules, that based on the sedimentation data and the analysis of the size of helices, are also likely built from individual molecules folded on themselves to create anti-parallel coiled-coils that aggregate in tetramers. Formation of tetrameric coiled-coils (parallel or anti-parallel) arranged from fully extended molecules is unlikely as the resultant molecule would be extended to a significantly longer size than the sedimentation data supports (axial ratio for a tetramer higher than 1:10). Therefore, all PspA molecules analyzed in this study have a similar three-dimensional structural arrangement in the anti-parallel coiled-coil. The shape of such elongated molecules is similar to an elongated rod.

In addition, the evidence presented here supports the structural studies and the modeling of PspA deduced from our earlier analyses of the Rx1 strain PspA consisting of amino acids 1 through 303 (PspA303) (19). The similarity of all PspA...
molecules analyzed suggests their common functional properties, which at this time are not fully understood but it has been suggested that PspA have anti-complementary properties (19). However, all constructs analyzed here have the ability to induce protective antibodies in the host and these antibodies are broadly cross-reactive, including the tetrameric strain BG9739 (20). The similarity of the structural properties of all PspA strains discussed in this study are the likely rational for the cross-reactivity of antibodies raised against the single PspA strain.

### Analysis of the Genomic Sequence Data Bases

It would be surprising that PspA (or PspA-like molecules) would only be expressed by *S. pneumoniae* bacteria. In order to investigate this, the sequence data bases were analyzed using the BLAST program.

#### TABLE II

| PspA construct | Method       | Protein concentration (mg/ml) | Partial specific volume (v) | Sedimentation coefficient (s) | Frictional ratio (f/f<sub>c</sub>) | Axial ratio (a/b) | Diffusion coefficient (D<sub>a</sub>) | Molecular mass (Da) |
|----------------|--------------|-------------------------------|-----------------------------|------------------------------|-----------------------------------|------------------|-------------------------------------|-------------------|
| Rx1314M1       | Boundary band| 3.95                          | 0.736                       | 2.00                         | 1.58                              | 1:10             | 5.11                                | 38,594            |
| BG9739         | Boundary band| 1.09                          | 0.735                       | 1.85                         | 1.62                              | 1:12             | 6.32                                | 32,570            |
| Monomer        | Boundary band| 2.27                          |                            |                              |                                   |                  |                                     |                   |
| BG9739         | Boundary band| 6.30                          | 0.731                       | 1.98                         | 1.80                              | 1:15             | 4.99                                | 42,066            |
| Tetramer       | Boundary band| 6.79                          |                            |                              |                                   |                  |                                     |                   |
| EF6668         | Boundary band| 0.497                         | 0.731                       | 2.32                         | 1.89                             | 1:17             | 5.03                                | 43,803            |
| BG6380         | Boundary band| 0.878                         | 0.724                       | 2.61                         | 2.10                             | 1:20             | 6.79                                | 32,570            |
| EF6296         | Boundary band| 0.294                         | 0.736                       | 2.40                         | 1.53                             | 1:10             | 4.93                                | 53,757            |

* Partial specific volume, 30% hydration of protein was assumed for all PspA constructs.
* Sedimentation coefficient, s, has units in Svedbergs. For the boundary experiments, s was evaluated from several distribution data sets and then averaged.
* The frictional ratio is defined as the frictional coefficient of an ellipsoid of the given axial ratio divided by the frictional coefficient of a sphere of the same volume as the ellipsoid. The average of the band and boundary results is provided.
* Average axial ratio for a prolate ellipsoid for both boundary and band experiments (34).
* The molecular mass and diffusion coefficient were determined from the sedimentation data as described under "Experimental Procedures."

#### FIG. 6

**Boundary sedimentation distribution of the BG9739 PspA construct.** The distribution g(s) as a function of the sedimentation coefficient, s, shows the presence of a maximum peak height at an s value of 1.85 S, and a minor peak at 6.30 S suggesting that the BG9739 PspA construct may associate in tetrameric form.

#### FIG. 7

**Band sedimentation velocity analysis of the BG9739 PspA construct.** The dependence of the radial distance, r, as a function of time is shown. The measure of goodness of fit, R<sup>2</sup>, the angular velocity, ω (rad/s), slope of the best fit line, as well as the uncorrected a value are also shown. The band sedimentation experiment afforded an s<sub>20,w</sub> value of 2.27 S for the monomer and 6.79 S for the tetramer of BG9739 PspA, higher than the corresponding values obtained from the boundary experiment. A, movement of major band peak corresponding to the monomer. B, movement of minor band peak corresponding to the tetramer.
program to search for sequences homologous to the N-terminal functional part of the Rx1314 PspA (part  eliciting protective antibodies). The only highly homologous molecule found, in the known genes data bases and the finished microbial genomes, was PspC which was already known to be closely related in its properties to PspA (7). Low homology was also observed to other known highly α-helical molecules like myosin and tropomyosin (19, 41). However, BLAST searches of the yet unfinished microbial genomes at www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html resulted in multiple homology hits suggesting that PspA or PspA-like molecules might be present in other bacterial organisms (Fig. 9). Using Matcher program, the predominant coiled-coil pattern has been detected among all of these homologous proteins (data not shown) (19, 29). The coiled-coil pattern was seldom disrupted, as it was observed in PspA molecules examined in this study.

The already elucidated anti-complementary properties of PspA (19) might apply to similar proteins in other bacterial organisms in the *Streptococcus* genus such as *Streptococcus pyogenes* or other bacteria such as *Plasmodium falciparum*, *Enterococcus faecalis*, or *Staphylococcus aureus* (Fig. 9). More pathogenic bacterial organisms are likely to use the PspA mechanism to fight host defenses in order to accomplish the colonization of the host. The attachment of such molecules to bacteria might be different that that of PspA as choline residues of teichoic acids are not very common among bacterial organisms.

**Conclusions Concerning the PspA Function and Protein-based Pneumococcal Vaccine Development**—As we have shown earlier for PspA303 of Rx1 strain based on the three-dimensional modeling, this molecule has high, polar charges accumulated on both terminal parts of the PspA N-terminal rod-like module presented on the surface of *S. pneumoniae* bacteria. Such charge polarization stabilizes the capsular structure on one end of the rod-like PspA module (positively charged C terminus) and prevents the host complement system from interacting with this pathogen on the other end of the module (highly negatively charged N terminus) (Fig. 5). By doing so, the negatively charged end of PspA extended outside of the capsule prevents interactions with complement molecules and thus

**Fig. 8.** Distribution of residuals (top) and absorbance (bottom) versus radial exponential distribution (radius) of the BG9739 PspA construct. The concentration of the protein as well as the residuals are expressed in $A_{280}$ units. These data were fitted to the monomer-tetramer model as explained in the text.

**Fig. 9.** Multiple alignment of the N-terminal part of Rx1314 PspA with sequences from selected microbial genomes. The sequences were aligned using Multalin (25) and displayed using MPSA (24). The sequence data represented correspond to unfinished genome sequences obtained from www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html: *S. pyogenes*, *S. pyogenes* Contig1; *E. faecalis*, *E. faecalis* gef_6217; *S. aureus*, *S. aureus* 4433; *P. falciparum*, *P. falciparum* Contig94. The color coding for amino acids is the same as described in the legend to Fig. 2.
prevents complement-mediated neutralization of pneumococci (19). The proline-rich region following the N-terminal coiled-coil structure of PspA acts as a tether which allows flexible attachment to the pneumococcal cell wall (Fig. 1). This attachment is accomplished through the choline-binding module interactions with teichoic and lipoteichoic acid structures on the pneumococcal cell wall. Such anti-complementary behavior of PspA was recently observed in an animal model (7).

The analysis of structural properties of all recombinant PspA from different strains analyzed in this work showed structural similarities of all these constructs. All PspA molecules have been found to be predominantly a-helical, having a coiled-coil conformation (likely anti-parallel), with a similar rod-like shape. The structural similarities of all these molecules shown are essential for the pneumococcal vaccine development as a simple uni-molecular vaccine composition, or composition with a limited number of proteins, is preferable. Such one molecule vaccine should, however, elicit protection against all strains or most of pneumococci present. The recently published studies by Nabors et al. (20) show initial data that the immunization of humans with recombinant Rx1314 PspA molecules caused an increase in circulating anti-PspA antibodies and these antibodies were cross-reactive to heterologous recombinant PspA molecules including those analyzed here (20). These studies suggest that PspA is a very likely candidate for a novel, solely protein antigen-based pneumococcal vaccine candidate.

Polysaccharide-based Vaccines and Other Approaches to Development of Pneumococcal Vaccines—Polyvalent vaccines composed from purified capsular polysaccharides of various numbers of serotypes are limited in their potency primarily due to their poor immunogenicity, predominantly in highly vulnerable populations of patients such as young children and the elderly over the age of 65 (42). The poor immunogenicity of the polysaccharide-based vaccines is due to their poor antibody response and because the T-cell independence of the response fails to induce memory. Finally, out of 90 pneumococcal serotypes known the available vaccines comprise only their limited number (up to 24). The development of protein-based or conjugated vaccines by coupling the polysaccharides with protein carriers should increase the potency of such vaccines. For the conjugated vaccines such an approach will also limit the serotypes included in the conjugate mixtures.

The combination of polysaccharides with a protein has been shown to significantly increase immunogenicity and memory to polysaccharide antigens. If the protein carrier(s) have the ability to induce additional protection (e.g. PspA), the resultant vaccine would be improved by the induction of anti-PspA antigen antibodies. Such additional protection might also be independent of the serotypes, as it seems to be the case for PspA at least in the investigated animal models (20). Therefore, the development of either a protein antigen(s)-based vaccine or a two-component vaccine comprising a polysaccharide and a non-polysaccharide part, such as a protein discussed above, PspA, might be the best approach (20, 43–46).

It is conceivable that vaccines against S. pneumoniae composed of mixtures of polysaccharides and protein antigens might provide better protection against this human pathogen than vaccines based only on one or limited mixtures of the possible single-type (polysaccharide or even possibly protein) protective components. Mixtures of selected possible and novel vaccine components have been shown to provide an additive attenuation of virulence (47). One of the potential protein antigen candidates for such vaccines, PspA, has been discussed above. More studies are, however, needed to assess the usefulness of PspA and other pneumococcal antigens, including polysaccharides, or their mixtures in various modes of pneumococcal challenge.

Acknowledgments—We thank Drs. Farhad Forohar for assistance, Patricia Jackson for help with collecting and interpreting the CD spectra, and Sambit R. Karr and Jacob Lebowitz for performing the sedimentation experiments and the necessary calculations.

REFERENCES

1. Mufson, M. A. (1990) in Principles and Practice of Infectious Diseases (Mandell, G. L., Douglas, R. G., Jr., and Bennett, J. E., eds) pp. 1539–1550, Churchill Livingstone, New York.

2. Lancet (1985) Lancet 2, 699–701.

3. Boulnois, G. J. (1992) J. Gen. Microbiol. 138, 249–259.

4. Sothern, R. B., Facklam, R. R., Pfikay, B. D., and Ostby, M. (1991) J. Infect. Dis. 163, 1273–1278.

5. Baquero, F., Martinez-Beltran, J., and Loza, E. (1991) J. Antimicrob. Chemother. 28, 31–38.

6. McDaniel, L. S., Sheffield, J. S., DeLaChic, P., and Bries, D. E. (1993) Infect. Immun. 61, 222–228.

7. Biles, D. E., Hollingshead, S. K., Swiatlo, E., Brooks-Walter, A., Szalai, A., Vihinen, A., McDaniel, L. S., Benson, W. P., Preller, K., Hermansson, A., Aerts, P. C., Van Dijk, H., and Crain, J. M. (1997) Microb. Drug Resist. 3, 401–408.

8. Brooks-Walter, A., Bries, D. E., and Hollingshead, S. K. (1999) Infect. Immun. 67, 6533–6542.

9. Linker, A., Meyer, K., and Hoffman, P. (1955) J. Biol. Chem. 219, 13–25.

10. Feldman, C., Munro, N. C., Jeffery, P. K., Mitchell, T. J., Andrew, P. W., Boulnois, G. J., Guerreiro, M., Deck, D. J., Todd, H. C., Cole, P. J., et al. (1992) Am. J. Respir. Cell Mol. Biol. 5, 416–423.

11. Lock, R. A., Paton, J. C., and Hansman, D. (1988) Microb. Pathog. 5, 461–467.

12. Sadowski, S., O’Connor, P., Stinson, A. R., Tharpe, J. A., and Rusell, H. (1994) Infect. Immun. 62, 319–324.

13. McDaniel, L. S., Scott, G., Kearney, J. F., and Bries, D. E. (1984) J. Exp. Med. 160, 586–597.

14. Crain, M. J., Waltham, W. D., IL Turner, J., Yather, J., Talkington, D. F., McDaniel, L. S., Gray, B. M., and Bries, D. E. (1990) Infect. Immun. 58, 3283–3299.

15. Yother, J., and White, J. M. (1994) J. Bacteriol. 176, 2976–2985.

16. Yother, J., and Bries, D. E. (1992) J. Bacteriol. 174, 601–609.

17. McDaniel, L. S., Ralph, B. A., McDaniel, D. O., and Bries, D. E. (1994) Microb. Pathog. 17, 323–337.

18. Jedrzejas, M. J., Hollingshead, S. K., Lebowitz, J., Chantatila, L., Bries, D. E., McDaniel, L. S., and Lamani, E. (2000) Arch. Biochem. Biophys. 374, 116–125.

19. Nabors, G. S., Braun, P. A., Herrmann, D. J., Heise, M. L., Pyle, D. J., Gravenstein, S., Schilling, M., Ferguson, L. M., Hollingshead, S. K., Bries, D. E., and Becker, R. S. (2000) Vaccine 18, 1743–1754.

20. Becker, R. S., Gray, M. L., Biscardi, K. S., Pyle, D. J., Huebner, R. C., and Nabors, G. S. (1997) in Vaccines 97, 599–604.

21. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997) J. Mol. Biol. 266, 498–500.

22. Johnson, M. L., Correia, J. J., Yphantis, D. A., and Halvorson, H. R. (1981) Anal. Biochem. 122, 423–428.
Characterization of Selected Strains of Pneumococcal Surface Protein A
Mark J. Jedrzejas, Ejvis Lamani and Robert S. Becker

J. Biol. Chem. 2001, 276:33121-33128.
doi: 10.1074/jbc.M103304200 originally published online June 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103304200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 11 of which can be accessed free at
http://www.jbc.org/content/276/35/33121.full.html#ref-list-1