PfbA, a Novel Plasmin- and Fibronectin-binding Protein of *Streptococcus pneumoniae*, Contributes to Fibronectin-dependent Adhesion and Antiphagocytosis*

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Masaya Yamaguchi†, Yutaka Terao‡, Yuka Mori‡, Shigeyuki Hamada*, and Shigetada Kawabata‡

From the †Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka 565-0871, Japan and the ‡Department of Life Science, Nihon University Advanced Research Institution for Sciences and Humanities, Chiyodaku, Tokyo 102-8251, Japan

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Avoiding phagocytosis is also important for bacterial survival, and a variety of mechanisms related to streptococcal survival strategies for avoiding host immunity have been reported. In particular, *S. pyogenes* expresses a number of proteins that are postulated to play a crucial role in its colonization and antiphagocytic activities (5–8). However, the mechanisms used by *S. pneumoniae* to escape phagocytosis remain unclear.

A polysaccharide-based vaccine against *S. pneumoniae* is presently used; however, it is ineffective in children younger than 2 years of age and only 60% effective in older children and adults (9, 10). A newer, conjugate vaccine consisting of a protein linked to the saccharides of seven major disease-causing serotypes has been licensed for use in infants. This vaccine is effective in preventing invasive diseases caused by pneumococci expressing the capsular serotypes contained in the vaccine. Nevertheless, it cannot be expected to provide protection against other serotypes. In addition, antibiotic-resistant strains are on the rise (9, 11, 12). Therefore, there is an urgent need to improve the characterization of *S. pneumoniae* surface proteins that could serve as candidate targets for protein-based vaccines and the development of new antibiotics (13–15). One of the most promising avenues for creating effective vaccines or drugs is the targeting of adhesins and invasins that promote the adhesion of pathogens to human tissues.

In the present study, we found that inactivation of the *pfbA* gene in *S. pneumoniae* significantly reduced the bacterial ability to bind human epithelial cells. We also showed that *pfbA* expression was involved in protecting pneumococci against phagocytosis. Finally, we found that anti-PfbA antibodies

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"Streptococcus pneumoniae* is a major causative agent of mortality throughout the world. The initial event in invasive pneumococcal disease is the attachment of pneumococci to epithelial cells in the upper respiratory tract. Several bacterial proteins can bind to host extracellular matrix proteins and function as adhesins and invasins. To identify adhesins or invasins on the pneumococcal cell surface, we searched for several proteins with an LPXTG anchoring motif in the whole-genome sequence of *S. pneumoniae* and identified one, which we called PfbA (plasmin- and fibronectin-binding protein A), that bound to human serum proteins. Immunofluorescence microscopy and fluorescence-activated cell sorter analysis revealed that PfbA was expressed on the pneumococcal cell surface. A ∆*pfbA* mutant strain was only half as competent as the wild-type strain at adhering to and invading lung and laryngeal epithelial cells. In addition, epithelial cells infected with ∆*pfbA* showed morphological changes, including cell flattening and a loss of microvilli, that did not occur in cells infected with the wild-type strain. The mutant strain also exhibited a weaker antiphagocytotic activity than wild type in human peripheral blood. Moreover, the growth of wild-type bacteria in human whole blood containing anti-PfbA antibodies was reduced by ~50% after 3 h compared with its growth without the antibody. These results suggest that PfbA is an important factor in the development of pneumococcal infections.

*S. pneumoniae*, which is also simply called pneumococcus, is a Gram-positive Diplococcus and the major pathogen of community-acquired pneumonia. It also causes meningitis, otitis, and septicemia, with a high incidence of morbidity and mortality throughout the world (1, 2). The initial phase of infection involves colonization followed by intimate contact..."
reduced pneumococcal growth in human whole blood by ~50% compared with its growth without antibodies.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Cells, and Reagents—S. pneumoniae strain R6 was kindly provided by Dr. Shinichi Yokota (Sapporo Medical University). The organism was grown in tryptic-soy (TS) broth (Difco) with spectinomycin (500 μg/ml) added to the medium to select an isogenic mutant strain. Escherichia coli strains XL-10 Gold (Stratagene) and BL21 (DE3) pLysE (Novagen) were grown in Luria-Bertani broth (Sigma) or on Luria-Bertani agar plates supplemented with 100 μg/ml of ampicillin and spectinomycin. Human laryngeal and alveolar cell lines HEp-2 (ATCC CCL-23) and A549 (ATCC CCL-185) were purchased from RIKEN Cell Bank (Japan).

Preparation of Pneumococcal Recombinant Proteins—The expression vectors were constructed as follows with the primers listed in Table 1. The eno, spr1345, spr1652, and spr1806 genes were amplified by PCR, and the resultant PCR fragments were cloned into pQE-30 vector (Qiagen). Recombinant proteins which eliminated an N-terminal signal peptide sequence were cloned into pYT339 vector (Qiagen). Recombinant proteins were expressed in Escherichia coli strains XL-10 Gold (Stratagene) and BL21 (DE3) pLysE (Novagen) were grown in Luria-Bertani broth (Sigma) or on Luria-Bertani agar plates supplemented with 100 μg/ml of ampicillin and spectinomycin. Human laryngeal and alveolar cell lines HEp-2 (ATCC CCL-23) and A549 (ATCC CCL-185) were purchased from RIKEN Cell Bank (Japan).

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Mutant Construction—Inactivation of the spr1652 gene in S. pneumoniae was performed as described previously (16). pYT339 was constructed by inserting the aad9 gene into the BamHI and XbaI sites of pUC19. To construct the Δspr1652 mutant strain MY7, PCR products from the upstream and downstream regions of spr1652 were ligated into the pYT339 vector, and the resultant plasmids were linearized with HindIII and used to transform competent cells of the S. pneumoniae strain R6. To prepare competent cells, 0.5 ml of exponential-phase organisms in TS broth were added to prewarmed TS broth (9.5 ml) and incubated at 37 °C for 30 min. A portion (1 ml) of the culture was then removed and placed in a tube containing 100 ng of competence-stimulating peptide (17). After further incubation at 37 °C for 15 min, 0.2-ml portions were removed, placed in fresh tubes containing ~0.1-μg of linearized plasmid (10 μl), and incubated at 37 °C for 2 h. Thereafter, each culture was plated onto TS blood agar and incubated at 37 °C for 24 h. Inactivation of the spr1652 gene in the mutant strain MY7 was confirmed by reverse transcription-PCR amplification using the spr1652D, Spn9802, or Spn9828 primer pairs (Table 1).

RNA Isolation and Reverse Transcription-PCR—Pneumococcal RNA was extracted from exponentially growing cultures in TS broth. Total RNA was prepared from cells using a TRizol Max Bacterial RNA Isolation kit (Invitrogen), and then 0.8 μg of the total RNA was reverse-transcribed in the presence of random hexamers using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions.

Preparation of Rabbit Anti-PfbA Serum—All animal procedures were conducted in compliance with the Osaka University Graduate School of Dentistry guidelines and approved by the institutional Animal Care and Ethics Committee (accession number 05-018). On days 0, 21, 42, and 63, New Zealand White rabbits (Charles River Laboratories) were anesthetized with pentobarbital, as previously described (18), then given an injection of 500 μg of recombinant protein in Freund’s complete or incomplete adjuvant. Serum was collected on day 84 and stored at ~80 °C until use.

Ligand Blotting—Ligand blot analysis was performed as described previously (18). Briefly, human fibronectin (Fn, Sigma), human plasmin (Sigma), human plasminogen (Sigma), and human serum albumin (HSA, Sigma) were biotinylated using an ECL protein biotinylation kit (GE Healthcare), and the concentrations were adjusted to 10 μg/ml. Recombinant proteins were separated by electrophoresis on 10% SDS-PAGE gels, then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 10% membrane

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**TABLE 1**

| Designation | Sequence (5’ to 3’) | References |
|-------------|---------------------|------------|
| For expression of recombinant proteins | | |
| SR20 | GATACCTTTGCAATTATCTACCATGACGTCGCG | This study |
| SB22 | AAAGTCTTTAAAAGTCGTGACTGTTT | This study |
| spr1345/BamF | CCGATCCCTGACAAAATAGCAGAACAGTTCGCG | This study |
| spr1345/PstR | AATCGACCTCTAGAACGACCTGTTTCCGACG | This study |
| spr1652/BamF | CCAAGCTTGTGTGCTATCACTACCTTTTGC | This study |
| spr1652/HindR | CCAAGCTTGTGTGCTATCACTACCTTTTGC | This study |
| spr1806/BamF | CCGATCCCTGAAAAAATAACAGATGTGGATAG | This study |
| spr1806/XmaR | TCCCTCGGAGATCTTGGCCGAGATGTG | This study |
| For deletional mutagenesis | | |
| aad9 BamF | CCGATCCCTGATTTTCTGCTAGATAC | This study |
| aad9 XbaR | GCTCTAGAGTAACTATTCTACATACCTTTTG | This study |
| 1652KOUecOf | CCGATCCCTGATTTTCTGCTAGATAC | This study |
| 1652KOU BamF | CCGATCCCTGATTTTCTGCTAGATAC | This study |
| 1652KODXbaF | GCTCTAGAGTAACTATTCTACATACCTTTTG | This study |
| 1652KODPrtR | AACTGAACTGAATGCACTACCATACACTTTCACG | This study |
| For reverse transcription-PCR | | |
| spr1652D/BamF | GATACCTTTGCAATTATCTACCATGACGTCG | This study |
| spr1652D/XmaR | CCGATCCCTGACAAAATAGCAGAACAGTTCGCG | This study |
| Sprn9802-143F | CGAATCTTTTTTTAGTTTACATCTACTATTTCTTTTG | This study |
| Sprn9802-304R | CGAATCTTTTTTTAGTTTACATCTACTATTTCTTTTG | This study |
| Sprn9828-19F | CGGATCCGAAGTTGTTACTAGTTCTTCACC | This study |
| Sprn9828-245R | CGGATCCGAAGTTGTTACTAGTTCTTCACC | This study |

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The abbreviations used are: TS, tryptic soy; Fn, fibronectin; HSA, human serum albumin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CFU, colony-forming units; FITC, fluorescein isothiocyanate.
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blocking agent (GE Healthcare) at 4 °C for 18 h and incubated with biotinylated proteins for 1 h and with horseradish peroxidase-labeled streptavidin at room temperature for another hour. Bands were detected by using ECL Western-blotting detection reagents (GE Healthcare) and exposing the membrane to x-ray film (Fuji photo film) at room temperature for 5 s.

Biacore Analysis—Biacore analysis was performed as described previously (19). Briefly, Fn, plasmin, plasminogen, or HSA was diluted to 100 µg/ml in 10 mM sodium acetate (pH 4.0) and immobilized on the surface of a CM5 sensor chip using an Amine-coupling kit (GE Healthcare). Lyophilized recombinant PfbA was suspended in HBS-EP buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Surfactant P20) and adjusted to 0.625, 1.25, 2.5, 5, and 10 µM. For binding analysis the recombinant PfbA was injected at a flow rate of 20 µl/min at 25 °C. A blank was used as the reference, the results of which were subtracted from all raw data. To fit the binding kinetics data to a model, BLA evaluation Version 3.0.2 software (GE Healthcare) was applied, and the 1:1 Langmuir binding model was chosen.

Immunofluorescence and Confocal Microscopic Analysis—Immunofluorescent staining was performed as described previously (20, 21). Briefly, streptococcal cells were washed with phosphate-buffered saline (PBS) and blocked with 10% goat serum (Tissue Culture Biologicals). To observe the localization of PfbA, the bacterial cells were stained with SYBR green I, and surface PfbA was visualized using rabbit anti-PfbA serum followed by Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen). The stained bacteria were analyzed using an LSM 510 confocal laser scanning microscope (Carl Zeiss).

Flow-cytometric Analysis of S. pneumoniae—S. pneumoniae cells were cultured to the mid-log phase and harvested by centrifugation then washed twice with PBS and blocked with PBS containing 10% goat serum and 5% bovine serum albumin (BSA) for 1 h at 4 °C. Next, antisera were diluted 1:100 and incubated with the bacterial cells on ice for 1 h, then FITC-conjugated goat anti-rabbit IgG (Invitrogen) was added, and the mixture was incubated on ice for another 1 h. After washing the samples were analyzed with a CyFlow flow cytometer (Partec) using FlowJo 8.3.2 software (Tree Star, Inc.).

The Fn labeling procedure was performed using a Fluoro-Reporter® FITC protein labeling kit (Invitrogen) according to the manufacturer’s instructions. S. pneumoniae cells were cultured to the mid-log phase and adjusted to 107 colony-forming units (CFU)/ml, then incubated with 0 or 10 µg/ml of FITC-labeled Fn for 30 min at 37 °C. The Fn binding activity on the surface of live bacterial cells was analyzed with a CyFlow flow cytometer.

Preparation of Streptococci for Adhesion and Invasion Assays—Pneumococci were cultured until the absorbance at 600 nm (A600) reached 0.6–0.7. The cells were then harvested by centrifugation and washed twice with PBS then resuspended in 1 ml of PBS. Biscarboxyethyl-carboxylfluorescein-pentaacetoxymethyl ester (Invitrogen), a nonvital intracellular dye, was added to the bacterial suspension to a final concentration of 1 mM. After a 30-min incubation at 37 °C, the fluorescent streptococci were washed 3 times with PBS and then used immediately in adhesion and invasion assays.

Streptococcal Adhesion and Invasion Assays—The bacterial adhesion to and invasion of human cells were quantified by standard procedures with minor modifications as described previously (22, 23). Briefly, HEp-2 and A549 cells were cultured in 24-well plates at a density of 1 x 104 cells per well and infected with 3 x 106 CFU of bacteria per well (multiplicity of infection, 1:30) for 1 h. To determine bacterial adhesion, the infected cells were washed 3 times with PBS and harvested with a trypsin solution. The fluorescence intensity in the cell lysates was measured in 96-well plates using a Wallac 1420 ARVO multilabel counter (PerkinElmer Life Sciences) to determine the number of S. pneumoniae. To measure bacterial invasion, the cells were washed 3 times and incubated for 1 h in Dubecco’s modified Eagle’s medium (Sigma) containing gentamicin (100 µg/ml) and penicillin (100 units/ml). The cells were washed and lysed, and then the fluorescence intensity was measured as described above to determine the number of invaded S. pneumoniae.

To determine the Fn-dependent adhesion activity, 24 h before the addition of bacteria the cells were rinsed with Dubecco’s modified Eagle’s medium and incubated with or without human Fn (10 µg/ml). Collagen-coated plates were used in the Fn-dependent adhesion experiment. The results are expressed as the mean ± S.D. of the percentage of S. pneumoniae recovered per well from six independent determinations. The assays were repeated three times, and representative results are shown. Statistical analysis was performed using a nonparametric Mann-Whitney U test. All tests were carried out using StatView J-5.0 software (SAS Institute Inc.).

Adhesion of PfbA-coated Fluorescent Beads to A549 Cells—Recombinant PfbA in BSA was covalently linked to 0.5-µm-diameter fluorescent beads (Invitrogen) according to the manufacturer’s instructions. A549 cells were grown to confluence in 24-well plates and then washed twice with PBS. Next, 1 ml of Dubecco’s modified Eagle’s medium containing 15 µl of PfbA-, BSA-, or non-coated fluorescent beads was added to the washed cells, and the plates were incubated at 37 °C, 5% CO2, for 1 h. The cells were washed 3 times with PBS and lysed with 200 µl of trypsin solution. To determine the number of fluorescent beads, the fluorescence intensity of the cell lysates was measured in 96-well plates using the Wallac 1420 ARVO multilabel counter with excitation at 485 nm and emission at 535 nm.

Scanning Electron Microscopic Analysis—A549 cells were grown to semi-confluence on coverslips and infected with the streptococcal strains as described above. The infected cells were incubated for 1 h, fixed with 2% glutaraldehyde for 1 h at room temperature, and washed with distilled water. The samples were dehydrated with 100% tert-butyl alcohol and freeze-dried. Finally, the samples were coated with platinum and examined with an emission-scanning electron microscope (JSM-6390LVZ, JEOL Ltd.).

Bactericidal Assay—Lancefield bactericidal assays were performed as described previously (23, 24). Strains R6 and MY7 were grown, washed, and resuspended in 1 ml of PBS as previously described. Diluted cultures (10 µl) were combined with fresh human blood with or without rabbit anti-PfbA IgG or preimmune IgG (final 100 µg/ml, 90 µl), and the mixtures were rotated at 37 °C for 1, 2, or 3 h. The IgG (2.0 mg/ml) from rabbit
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Ligand blotting among host proteins and pneumococcal proteins harboring LPXTG motif-containing proteins. Recombinant (r) SpR1345, rSpR1652, and rSpR1806 from *S. pneumoniae* were separated by SDS-PAGE then transferred to polyvinylidene difluoride membranes. Lanes 1–4 were stained with Coomassie Brilliant Blue (CBB). Lanes 5–7, 8–10, and 11–13 were incubated with biotinylated Fn, biotinylated plasmin, and biotinylated HSA, respectively, followed by horseradish peroxidase-labeled streptavidin. Lanes 14–16 were incubated with horseradish peroxidase (HRP)-labeled streptavidin alone. Bound biotinylated molecules were detected with ECL reagents.

![Image of ligand blotting](image)

**FIGURE 1.** Ligand blotting among host proteins and pneumococcal LPXTG motif-containing proteins. Recombinant (r) SpR1345, rSpR1652, and rSpR1806 from *S. pneumoniae* were separated by SDS-PAGE then transferred to polyvinylidene difluoride membranes. Lanes 1–4 were stained with Coomassie Brilliant Blue (CBB). Lanes 5–7, 8–10, and 11–13 were incubated with biotinylated Fn, biotinylated plasmin, and biotinylated HSA, respectively, followed by horseradish peroxidase-labeled streptavidin. Lanes 14–16 were incubated with horseradish peroxidase (HRP)-labeled streptavidin alone. Bound biotinylated molecules were detected with ECL reagents.

**RESULTS**

**PfbA Protein Binds to Fibronectin, Plasminogen, and Human Serum Albumin**—An LPXTG motif is the cell-anchoring sequence in Gram-positive bacteria, and most of the LPXTG-containing proteins work as virulence factors. Gram-positive bacterial adhesins/invasins commonly possess the motif. In the present study we selected three different LPXTG motif-containing proteins, SpR1345, SpR1652, and SpR1806, from the *S. pneumoniae* strain R6 genome data base because of their recombinant proteins were easily water-soluble. SpR1345 (Sp1492) is a mucin-binding protein (25), and SpR1806 (Sp1833), annotated as a right-handed β-helical protein, is a probable polysaccharide-modifying enzyme (14). SpR1806 (Sp1992) is a hypothetical protein. The 92–160-amino acid region of SpR1806 contained a domain of unknown function DUF1542 (E value 6.3 × 10⁻¹⁸), which is found in several cell-surface proteins. It has been suggested that some of these molecules function in antibiotic resistance and/or cellular adhesion.

Using ligand-blot analysis, we next investigated whether these recombinant proteins bound to host proteins. Our results showed that recombinant SpR1652 bound to Fn, plasmin, plasminogen, and HSA (Fig. 1, Table 2). We, therefore, focused our study on this protein, which we called PfbA (plasmin- and fibronectin-binding protein A). We next analyzed the binding constants using the surface plasmon resonance method with the Biacore system. The *K₆₅* values of PfbA binding to Fn, plasmin, plasminogen, and HSA indicated moderate affinities and specific interactions (Table 3) that were close to those of other Fn-binding proteins (26).

**Sequence Analysis of PfbA**—In the original genome annotation of *S. pneumoniae* strain R6 (27), PfbA (SpR1652) was characterized as a cell-wall surface anchor family protein. Indeed, PfbA possessed a typical LPXTG motif in its C terminus followed by a stretch of positively charged amino acid residues. In the N-terminal region, a deduced signal-peptidase cleavage site was located between amino acids 47 and 48. A BLAST search showed an Fn type III repeat in the 115–133-amino acid region of PfbA. In Fn itself, the first of several type III repeats binds to the type I repeat of another Fn molecule, and this interaction has been proposed as a point of regulation in Fn-Fn interactions (28).

**PfbA Is a Pneumococcal Cell-surface Protein**—Immunofluorescence microscopic analysis revealed that PfbA was localized to the pneumococcal cell surface (Fig. 2A; red in image). In addition, flow cytometric analysis showed that *S. pneumoniae* strain R6 was stained positively by the anti-PfbA serum, whereas ΔpfbA strain MY7 did not react with it (Fig. 2B). These findings indicated that PfbA is localized to the cell surface of live pneumococci. There was no significant difference in the surface expression of pneumococcal α-enolase, used as a negative control, between strains R6 and MY7 (Fig. 2B).

In addition, we examined the differences between strains R6 and MY7 using a fluorescence-activated cell sorter system and FITC-labeled Fn (Fig. 3). The fluorescence intensity was substantially lower for strain R6, incubated without FITC-labeled Fn, as compared with R6, incubated with FITC-labeled Fn, whereas the histograms for strain MY7 showed similar patterns regardless of the existence of FITC-labeled Fn. However, fluorescence intensity was substantially lower for strain MY7 than R6 when the bacteria were incubated with FITC-labeled Fn. These results suggest that PfbA is expressed and functions as an Fn-binding protein on the surface of *S. pneumoniae*.
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PfbA Facilitates Pneumococcal Adhesion to and Invasion of Epithelial Cells—To investigate the function of PfbA, the adhesion and the invasion efficiencies of strain R6 and its mutant, MY7, were compared using HEp-2 and A549 cells, which are human laryngeal and alveolar lung epithelial cell lines, respectively. Strain MY7 showed half the adhesion and invasion efficiencies of strain R6 and its mutant, but strain MY7 did not (Fig. 6). These results indicate that PfbA serves, at least in part, as an Fn-dependent adhesin and invasin.

Scanning Electron Microscopic Analysis of Morphologic Changes of A549 Cells Infected by Pneumococci—Scanning electron microscopic analysis showed that only a few microvilli had passive contact with strain MY7 cells at the point of their adhesion to the cell surface (Fig. 7, A and B). At the attachment point, elongated microvilli adhered to the bacterial surface and formed a tight network (Fig. 7B), which then fused to cover the streptococcal cells (Fig. 7C).

PfbA Possesses Antiphagocytotic Activity—We performed bactericidal assays to investigate the function of PfbA during the early phase of infection. A low number of bacteria (100 CFU) was incubated with 100 μl of human whole blood, and then antiphagocytic activities were determined based on the viability of the wild-type and ΔpfbA mutant strains in human blood. The bacterial growth activity of the ΔpfbA mutant strain MY7 in human whole blood was ~73, 57, and 57% after 1, 2, and 3 h, respectively, that of the wild-type strain R6 (Fig. 8).

Opsonic Activity of the Anti-PfbA Antibody—We also examined the efficacy of the anti-PfbA antibody for passive immunotherapy during the period in which the bacteria grew in the tissues. Briefly, a large number of S. pneumoniae (1000 CFU) was mixed with anti-PfbA or anti-PfbA IgG antibodies, after which the mixture was adjusted to 100 μl with human blood. Bacterial growth in human whole blood containing the anti-PfbA antibodies was ~107, 65, and 50% after 1, 2, and 3 h, respectively, of bacterial growth in the blood containing the preimmune IgG (Fig. 9).

DISCUSSION

Colonization and invasive infection by S. pneumoniae involve its expression of adhesins that target host components, and these proteins have been proposed to contribute to pathogen colonization or resistance to both the innate and adaptive immune systems (29). Several bacterial surface proteins, such as the choline binding proteins SpmA (also referred to as CbpA and PspC) (30–33) and PspA (34, 35), the ABC metal permeases Adc and PsaA (36–38), and the plasmin(ogen) binding proteins (39) Pava (16, 40) and SirA (41), are crucial for pneumococcal pathogenesis. These pneumococcal proteins adhere to a variety of extracellular matrix (42, 43) and serum proteins, such as factor H (44–46), immobilized Fn (16, 47), plasmin(ogen) (42, 48), and lactoferrin (34, 43), and these inter-
In the present study we demonstrated that PfbA possesses Fn-, plasmin(ogen)-, and human serum albumin-binding activities. Interestingly, our results also indicated that PfbA expression is important for pneumococcal colonization in cell culture and for antiphagocytic activity in bactericidal assays. Bacterial adhesion to and internalization into human epithelial cells were reduced in the absence of PfbA. The ability to bind Fn either in the fluid phase or when immobilized onto a surface is a common property of streptococci and has been postulated to accelerate the adhesion of \textit{S. pyogenes} to epithelial cells (49) and promote the binding of viridans streptococci in the thrombotic vegetations associated with infective endocarditis (50). Moreover, Fn binding activity is associated with the invasive properties of \textit{S. pyogenes}. The matrix form of Fn enhances the binding of \textit{S. pyogenes} to host cells (51), and PrtF1, a major Fn-binding protein, mediates this organism's invasion of human epithelial cells (22, 52, 53). Bacterial surface proteins bound to Fn form a bridge to integrins, which leads to the rearrangement of the cytoskeletal actin in the host cells and the uptake of the bacteria (54, 55). \textit{S. pneumoniae} strain TIGR4 is reported to possess two Fn-binding proteins, PavA and SP0082 (which is equal to SpR0075 in strain R6) (56). Although PavA seems not to function directly as an adhesin, it is probably involved in modulating other as yet unidentified virulence determinants of pneumococci (40). It remains to be determined whether SP0082 contributes to pneumococcal adhesion and invasion. The present results indicate that PfbA has a direct role as an adhesin and that it acts at least in part in the Fn-dependent adhesion to and invasion of epithelial cells. Scanning electron micrographs of A549 cells infected with pneumococci showed that infection with the \textit{pfbA} mutant strain resulted in morphological changes, including cell flattening and loss of microvilli, which were not observed in cells infected with the wild-type pneumococcal strain. These findings indicate that PfbA might mediate microvilli elongation or recruitment through binding to fibronectin.

Several Fn binding proteins are reported to play a role in phagocytosis resistance (57, 58). In the present experiments we found that the wild-type pneumococcal strain was more resistant to phagocytosis in human peripheral blood than the \textit{ΔpfbA} mutant strain. Several hypotheses could explain the antiphagocytosis effect of PfbA. A previous study demonstrated that plasmin,
formed by the conversion of plasminogen by staphylokinase, cleaves human IgG as well as human C3b from the bacterial surface, leading to impaired phagocytosis by human neutrophils (59). PfbA has plasmin(ogen)-binding activity and, therefore, its antiphagocytosis function might be related to the activity of plasmin. Additional possible mechanisms include the avoidance of phagocytosis by the molecular mimicry of host-protein-bound PfbA or by forming bacterial aggregates via an Fn-collagen complex, as previously reported for SfbI-expressing \textit{S. pyogenes} (57). Although the exact molecular mechanism is not yet understood, PfbA apparently represents a pneumococcal antiphagocytic effector molecule.

Studies have demonstrated that a protective immune response against \textit{S. pyogenes} can develop after immunization with the Fn-binding proteins SfbI (PrtF1) (60), Fbp54 (61), or FbaA (19). In addition, PfbA is predicted to belong to the $\beta$-helix group of proteins, which are rare and generally specific to the microbial world, and drugs fashioned to target them may benefit from their specificity toward infectious agents (62).

There has been growing concern about pneumococcal infection in the elderly, because the number of elderly has been increasing worldwide over the last century (63) and because aging is associated with the development of pneumococcal pneumonia (2). Therefore, the global morbidity and the mortality from pneumococcal infections may greatly increase in the near future. The identification of an antigenic protein that localizes to the pneumococcal cell surface would be useful for the development of vaccines and the prevention of pneumococcal infections. The present findings demonstrated that pneumococcal growth in human whole blood containing PfbA antibodies was reduced by 50% compared with its growth without PfbA antibodies. Thus, PfbA may be an attractive candidate for a component of a protein-based pneumococcal vaccine.
