The Putative Proteinase Maturation Protein A of *Streptococcus pneumoniae* Is a Conserved Surface Protein with Potential To Elicit Protective Immune Responses

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Surface-exposed proteins often play an important role in the interaction between pathogenic bacteria and their host. We isolated a pool of hydrophobic, surface-associated proteins of *Streptococcus pneumoniae*. The opsonophagocytic activity of hyperimmune serum raised against this protein fraction was high and species specific. Moreover, the opsonophagocytic activity was independent of the capsular type and chromosomal genotype of the pneumococcus. Since the opsonophagocytic activity is presumed to correlate with in vivo protection, these data indicate that the protein fraction has the potential to elicit species-specific immune protection with cross-protection against various pneumococcal strains. Individual proteins in the extract were purified by two-dimensional gel electrophoresis. Antibodies raised against three distinct proteins contributed to the opsonophagocytic activity of the serum. The proteins were identified by mass spectrometry and N-terminal amino acid sequencing. Two proteins were the previously characterized pneumococcal surface protein A and oligopeptide-binding lipoprotein AmiA. The third protein was the recently identified putative proteinase maturation protein A (PpmA), which showed homology to members of the family of peptidyl-prolyl *cis/trans* isomerases. Immunoelectron microscopy demonstrated that PpmA was associated with the pneumococcal surface. In addition, PpmA was shown to elicit species-specific opsonophagocytic antibodies that were cross-reactive with various pneumococcal strains. This antibody cross-reactivity was in line with the limited sequence variation of *ppmA*. The importance of PpmA in pneumococcal pathogenesis was demonstrated in a mouse pneumonia model. Pneumococcal *ppmA*-deficient mutants showed reduced virulence. The properties of PpmA reported here indicate its potential for inclusion in multicomponent protein vaccines.

Streptococcus pneumoniae is an important human pathogen which causes meningitis, otitis media, sepsis, and pneumonia. The precise molecular mechanisms by which the pneumococcus invades and damages host tissues are not fully understood. For many years, the polysaccharide capsule has been recognized as the major virulence factor and consequently was considered an important vaccine candidate (for reviews see references 5 and 34). The use of a 23-valent vaccine containing capsular polysaccharides from pneumococci commonly causing disease has had limited effect in reducing the morbidity and mortality associated with this organism (1, 16, 19, 41). The current pneumococcal vaccine strategy focuses on the use of conjugates, in which a limited number of capsular polysaccharides are linked to a carrier protein. The proteins in the conjugate vaccines cause a switch in the immune response to polysaccharides from T-cell independent to T-cell dependent. This results in an increase in the antibody response and the generation of memory T lymphocytes. Conjugate vaccines are more immunogenic in young children than polysaccharide vaccines (15, 18). Although the results of early trials look promising, the long-term efficacy is uncertain since large-scale vaccination may over time lead to a shift in serotype distribution towards capsular types that are poorly immunogenic or not included in the vaccine. Such a shift may be enhanced by the horizontal exchange of capsular genes, as described previously (8, 22, 23).

Over the past few years, much attention has been focused on the role of pneumococcal proteins in pathogenesis and protection. Proteins that are involved in the pathogenesis of infections by *S. pneumoniae* are considered interesting components for future conjugate or multicomponent protein vaccines. The immunological response against such proteins should provide protection against colonization and infection with *S. pneumoniae* strains of all capsular polysaccharide types. Immunization with pneumolysin (36), pneumococcal surface protein A (PspA) (33, 45, 53), pneumococcal surface adhesin A (PsaA) (44), and neuraminidase (28) clearly confers protection in animal models.

The purpose of this study was to identify additional pneumococcal proteins with abilities to elicit protective immune responses. We isolated a pool of hydrophobic, potentially surface-associated proteins of *S. pneumoniae* that were able to elicit cross-reactive, species-specific antibodies with opsonophagocytic activity. At least three distinct proteins contributed to the in vitro opsonophagocytic activity. Two proteins were the previously characterized surface proteins PspA and oligopeptide-binding protein AmiA. The third protein was identified as the putative proteinase maturation protein A (PpmA) (35a). The potential of PpmA to elicit protective immune responses and its role in the pathogenesis of pneumococcal infection are discussed.

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TABLE 1. Bacterial strains used in this study

Species and strain	Relevant characteristic(s)	Source[a] or reference
S. pneumoniae		
FT231	Serotype 19, clinical isolate	F. Tenover, CDC, Atlanta, Ga.
D39	Serotype 2, clinical isolate	7
CDC205	Serotype 3, clinical isolate	J. Butler, CDC, Atlanta, Ga.
EF3296	Serotype 4, clinical isolate	C. Svanborg Eden, University of Göteborg, Sweden
ATCC6306	Serotype 6A, clinical isolate	American Type Culture Collection
911320	Serotype 6A, clinical isolate	Our laboratory
950357	Serotype 9V, clinical isolate	Our laboratory
S1003	Serotype 11, clinical isolate	Our laboratory
ATCC6314	Serotype 14, clinical isolate	American Type Culture Collection
950312	Serotype 14, clinical isolate	Our laboratory
S1001	Serotype 15, clinical isolate	Our laboratory
800129	Serotype 18C, clinical isolate	Our laboratory
19F G	Serotype 19F, clinical isolate	Our laboratory
ATCC6323	Serotype 23F, clinical isolate	American Type Culture Collection
950110	Serotype 23F, clinical isolate	Our laboratory
S3003	Serotype 38, clinical isolate	Our laboratory
19F K	Unencapsulated variant of 19F G	Our laboratory
Rx1	Unencapsulated variant of D39	42
P376	Serotype 6A, opaque variant of clinical isolate p303	27
P765	Serotype 6B, transparent variant of clinical isolate p314	26
P62	Serotype 9V, opaque variant of clinical isolate p10	51
R189	*ermAM*-containing strain	2
MS9	D39, *ppmA::ermAM*	This study
S. bovis 961008	Clinical isolate	Our laboratory
E. faecalis ATCC29212	Clinical isolate	American Type Culture Collection

[a] CDC, Centers for Disease Control.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and growth medium. The pneumococcal strains used in this study are described in Table 1. Pneumococcal strain FT231 was used for protein purification. Bacteria were grown to logarithmic growth phase (optical density at 550 nm 0.3) in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% yeast extract (Difco Laboratories) (THY broth) at 37°C.

Extraction of surface-associated hydrophobic proteins of *S. pneumoniae*. Bacterial cells were harvested by centrifugation (1,500 × *g*, 15 min, room temperature) and washed with an equal volume of phosphate-buffered saline (PBS, pH 7.5). After the final wash, the bacteria were resuspended in 1/25 the volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA). The cells were disrupted by ultrasonic treatment (Branson sonifier 250; Branson Ultrasonics, Danburry, Conn.). The method for extraction with sulfobetaine 14 (SB14) was adapted from that of Schouls and colleagues (40). In brief, cell walls, membranes, and other particulate material were collected by centrifugation at 48,400 × *g* for 20 min. The water-soluble cytoplasmic proteins were removed by washing the bacterial lysates five times with PBS. Pellets were resuspended in 150 mM NaCl and centrifuged for 20 min at 48,400 × *g*. The pellets were incubated for 2 h at room temperature with 0.25% *N*-tetradecyl-*N*,*N*-dimethylammonio-1-propanesulfonate (SB14; Serva, Heidelberg, Germany) in the presence of 150 mM NaCl–10 mM MgCl$_2$–10 mM Tris-HCl (pH 8.0) with constant stirring. The hydrophobic, membrane-associated proteins were recovered as described by Wessel and Flügge (52). Protein concentrations were determined by the method of Bradford (13).

Protein gel electrophoresis and staining. One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out in the Bio-Rad minigel system with 13% polyacrylamide gels. The samples were dissolved in sample buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, 10 mM dithiothreitol [DTT], 10% glycerol, 0.01% bromophenol blue indicator [Merck, Darmstadt, Germany]), boiled for 5 min, and subjected to electrophoresis (39).

Two-dimensional SDS-polyacrylamide gel electrophoresis includes separation of proteins by isoelectric point and by molecular weight, respectively. Isoelectric focusing (pI, 4 to 7) was performed with a Multiphor II electrophoresis unit and Immobiline DryStrips (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's recommendations, but as modified by Rabilloud and colleagues (37). The proteins were separated in the second dimension by gradient (12 to 20%) polyacrylamide gel electrophoresis. Polyacrylamide gels were stained with Coomassie brilliant blue (CBB) (39).

Hyperimmune rabbit antiserum. Hyperimmune antiserum was raised against the hydrophobic, surface-associated protein pool and distinct CBB-stained two-dimensional protein gel spots. Shortly after introduction into a one-dimensional SDS-polyacrylamide gel, the SB14-extracted protein pool of *S. pneumoniae* FT231 was stained with CBB and excised from the gel. The total protein fraction,

as well as the individual protein spots cut from the two-dimensional polyacrylamide gel, were washed three times with 0.1 M sodium acetate–96% ethanol, ground into a fine suspension in 0.5 ml of PBS, and subsequently mixed with 0.5 ml of Freund's incomplete adjuvant (Pierce, Rockford, Ill.). New Zealand White rabbits were injected subcutaneously in four or five places. The primary injection was followed by three booster injections at 4-week intervals.

Indirect immunocytometric assay. Pneumococci were grown to logarithmic phase in THY broth at 37°C and washed three times in ice-cold PBS. The bacterial pellet was dissolved in 5% rabbit serum in PBS (10^7 bacteria in a 20-μl final volume) and incubated for 15 min at 4°C with shaking. After being washed twice with ice-cold PBS, the bacteria were incubated for 15 min at 4°C with 20 μl (1:5 dilution) of fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories, West Grove, Pa.) with shaking. Finally, the bacteria were washed twice with ice-cold PBS and resuspended in 100 μl of ice-cold fresh paraformaldehyde (0.5%) in PBS. The samples were analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

Phagocytosis assay. Analysis of the opsonophagocytic activity of the sera was performed as described by Alonso DeVelasco et al. (4) and adapted by Jansen et al. (24). In our assay, *S. pneumoniae* was grown to logarithmic phase in THY broth without heat-inactivated human pooled serum, and the bacteria were not inactivated. Phagocytosis was defined as the uptake and binding of fluorescein isothiocyanate-labeled bacteria by human polymorphonuclear cells (PMNs) because of opsonization with antiserum. The opsonophagocytic activity is defined as the reciprocal of the serum concentration at which 25% (unless otherwise stated) of the human PMNs were fluorescent.

Western blot analysis. The proteins separated by one-dimensional (0.5 μg) and two-dimensional (2.5 μg) SDS-polyacrylamide gel electrophoresis were transferred to Immobilon-P membranes (Millipore Corporation, Bedford, Mass.) as described by Sambrook et al. (39). The immunological detection of immobilized proteins was performed as described elsewhere (35a).

Amino acid sequence analysis. For N-terminal amino acid sequence analysis, the proteins were separated by two-dimensional SDS-polyacrylamide gel electrophoresis as described above with a few modifications. Recrystallized SDS (Serva) was used for preparing the electrophoresis buffers, and sodium thioglycolate (100 mM) was added to the cathodal buffer compartment. The proteins in the gel were blotted to a Problot membrane (Applied Biosystems, San Jose, Calif.) with the Multiphor II system (Pharmacia Biotech) according to the manufacturer's instructions except that 0.02% β-mercaptoethanol was added to the blotting buffer. The blots were stained with amido black (Merck). Amino acid sequence analysis was performed with a model 473A protein sequenator (Applied Biosystems) as recommended by the manufacturer.

For mass spectrometric analysis, the proteins of interest were purified from the gel, digested with trypsin, and analyzed by mass spectrometry as described elsewhere (35a). With Peptide Search (29), the deduced (partial) amino acid se-

quences were analyzed for matching sequences in all possible translation products of the December 1998 version of the unfinished pneumococcal genome released by the Institute for Genomic Research (TIGR; http://www.tigr.org/data/s_pneumoniae/) to identify the proteins. With the BLAST algorithm (6), putative pneumococcal proteins were analyzed for similarity to sequences deposited in the November 1999 version of the nonredundant protein database at the National Center for Biotechnology Information (Washington, D.C.).

Immunoelectron microscopy. Colonies of *S. pneumoniae* strain FT231 were fixed in a mixture of 4% formaldehyde, freshly prepared from paraformaldehyde, and 0.1% glutaraldehyde in sodium cacodylate buffer, pH 7.4. The bacteria were embedded in 1.5% low-gelling-temperature agarose (Sigma), and fixation was continued for a total of 24 h. Small pieces of the agarose-embedded bacteria were dehydrated in ethanol in a CS Auto apparatus (Reichart, Vienna, Austria) with a progressive lowering of temperature and embedded in Lowicryl HM20 resin (TAAB, Reading, United Kingdom) at −40°C. Ultrathin sectioning was performed with an Ultracut E microtome (Reichart). Sections were collected on gold grids and immunolabeled by incubation with anti-PpmA rabbit serum and anti-PspA rabbit serum, respectively, diluted 1:200 with buffer A (1% normal goat serum, 1% bovine serum albumin [Biocell], 0.1% Tween 20 [Sigma] in Tris-buffered saline [pH 8.2]) for 2 h at room temperature. Control specimens were incubated with normal rabbit serum and in buffer A without primary antibody. Sections were washed in five 50-μl droplets of buffer A for 5 min each and incubated for 3 h at room temperature in goat anti-rabbit IgG antibody labeled with 5-nm-diameter colloidal gold (Biocell, Cardiff, United Kingdom) diluted 1:100 with buffer A. Final washing was done twice with buffer A and five times in distilled water. Sections were stained with uranyl acetate and examined with a Philips CM12 electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV.

Nucleotide sequence analysis of *ppmA*. Nucleotide sequencing was performed on *ppmA* from the pneumococcal strains D39, CDC205, EF3296, P376, P765, 950357, P62, S1003, 950312, S1001, 800129, FT231, 19F G, 950110, S3003, and Rx1. Two PCR products of 640 and 643 nucleotides that cover the whole gene were generated with an overlap of 140 nucleotides. The primers 5′-TCTCATG CTTCGTAAAAATG-3′ and 5′-AGCAAAATCAGCACCTTCTG-3′ were used to amplify the 5′ part of *ppmA*. The primers 5′-CTGAATTGACAGATGAAG CC-3′ and 5′-CCTTGTACTATGCGTTTTATTG-3′ were used to amplify the 3′ part of *ppmA*. PCR amplification of *ppmA* was performed in a 100-μl PCR mixture as described for penicillin-binding protein genotyping (35). The PCR products were purified by sodium acetate precipitation to remove the unincorporated nucleotides and primers (39). Purified templates were sequenced with the Thermo Sequenase dye terminator cycle sequencing premix kit (Pharmacia Amersham, Roosendaal, The Netherlands) and 50 pmol of each PCR primer. Sequencing was performed on the Applied Biosystems Prism 377 (PE Applied Biosystems, Nieuwerkerk, The Netherlands).

Cloning and insertional inactivation of *ppmA*. *ppmA* was amplified from *S. pneumoniae* strain D39 genomic DNA by PCR with primers pmpp-FW1 (5′-G TTTGGAATTCGCAAGCAAATCACTCTCC-3′), positioned at nucleotides 369 to 340 upstream of the ATG start codon, and pmpp-REV1 (5′-CAGTAG GATCCTTGTACTATGCGTTTTATTG-3′), positioned at nucleotides 1073 to 1104 downstream of the ATG start codon. The forward and reverse primers contain *Eco*RI and *Bam*HI recognition sequences. The amplified *Eco*RI-*Bam*HI-digested *ppmA* DNA fragment was cloned into pBluescript KS+ (pMS1) and transformed into *Escherichia coli* DH5α (39). A *ppmA* mutant was constructed by insertion of an erythromycin resistance cassette (*ermAM*) in the gene. *ermAM* was amplified from *S. pneumoniae* strain R189 genomic DNA with primers ermAM-FW1 (5′-AAAGTTCGAAGCTTAAGTTCAAAACTACTTG CCC-3′) and ermAM-REV1 (5′-AAAGCTGCAGTTCGAATGTCTTCTCAC CTTTAG-3′). The amplified *ermAM* DNA fragment was *Csp*45I-digested and cloned into the *Csp*45I site of *ppmA* (nucleotide position 64 downstream of the ATG start codon) of pMS1, forming pMS2. The *Eco*RI-*Bam*HI-digested pMS2 DNA fragment was used to transform *S. pneumoniae* D39 as described previously (54). To confirm that *ppmA* was inactivated by *ermAM*, chromosomal DNA was analyzed by PCR with primers pmpp-FW1 and pmpp-REV1. Expression of PpmA was assessed by Western blot analysis with PpmA antibodies. The *ppmA* mutant MS9 was used in the described experiments.

Mouse pneumonia model. Preparation of the challenge dose and intranasal challenge of mice were performed as described before (25). Significant differences in the survival time of mice challenged with the *ppmA* knockout mutants and parent strain D39 were determined by the nonparametric Mann-Whitney *U* test, with significance set at a value of *P* of ≤0.05.

RESULTS

Surface-associated protein fraction of *S. pneumoniae* is able to elicit opsonophagocytic activity. Approximately 30 polypeptides were isolated by the SB14 extraction procedure in relatively high concentrations, as shown by two-dimensional SDS-polyacrylamide gel electrophoresis (Fig. 1). Immunocytometric analysis demonstrated that serum raised against the extracted proteins recognized components at the surface of pneumococ-
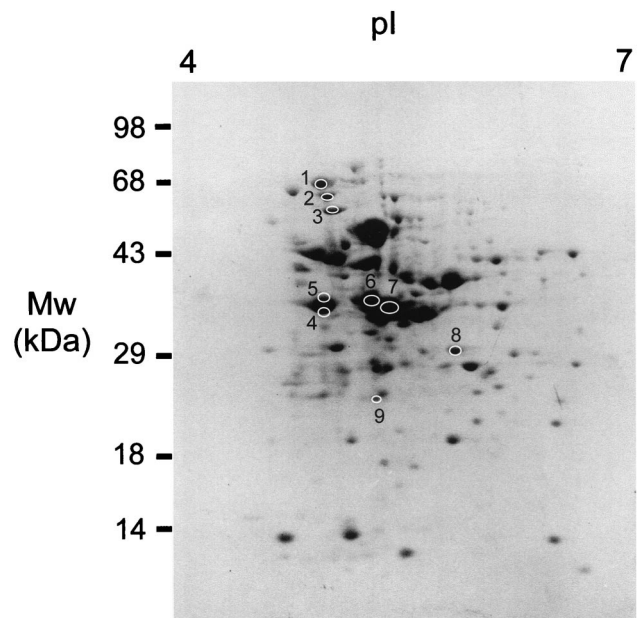

FIG. 1. Two-dimensional analysis of SB14-extracted surface-associated proteins from *S. pneumoniae* strain FT231. The proteins were separated by isoelectric focusing (pI, 4 to 7) and gradient SDS-polyacrylamide gel electrophoresis (10 to 100 kDa) and stained with CBB. Circles mark the proteins recognized by serum raised against the SB14-extracted proteins. The numbers 1 to 9 refer to the proteins discussed in the text.

cal cells of the homologous strain FT231 (Fig. 2) and seven other pneumococcal strains (D39, EF3296, 911320, 950357, 800129, 19F, and 950110) that represent eight clinically important serotypes (types 2, 4, 6A, 9V, 14, 18C, 19F, and 23F, respectively) and display seven distinct genotypes (M. Sluijter, unpublished data). The in vitro serum opsonophagocytic activity was high (50/%) when determined with the homologous pneumococcal strain FT231 (Fig. 3A). In addition, the serum was invariably opsonophagocytically active against six genotypically distinct pneumococcal strains (EF3296, ATCC6306, ATCC6314, FT231, 19F G1.1, and ATCC6323) (M. Sluijter,
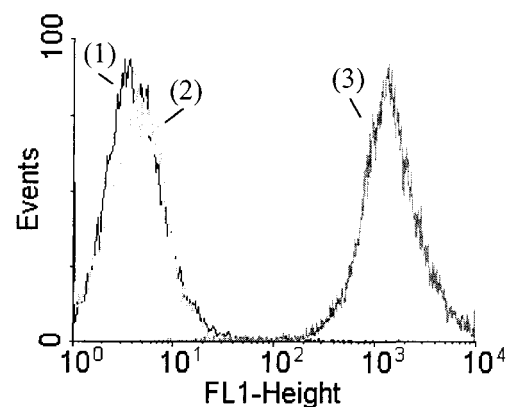

FIG. 2. Indirect immunocytometric analysis demonstrating the presence of surface-exposed epitopes in the SB14 protein fraction of *S. pneumoniae* strain FT231. *Y* axis, number of pneumococci analyzed; *x* axis, degree of immunofluorescence. Numbers: 1, bacterial autofluorescence; 2, nonspecific binding of fluorescein-conjugated goat anti-rabbit IgG; 3, specific binding of serum to components at the surface of pneumococci.
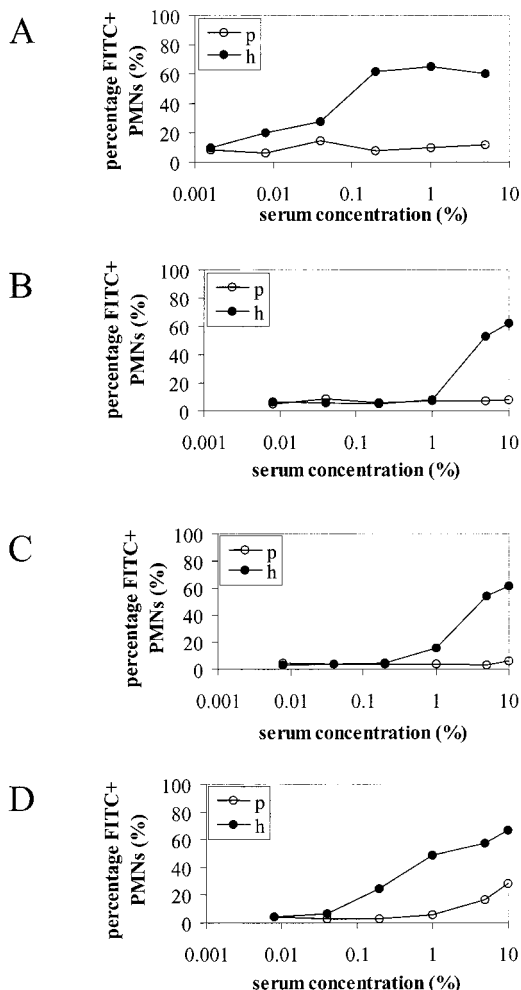
FIG. 3. Opsonophagocytic activity of hyperimmune rabbit sera raised against the surface-associated pneumococcal protein fraction (A) and sera raised against proteins 2 (B), 3 (C), and 4 (D) using the homologous strain FT231. The percentage of fluorescein isothiocyanate-positive (FITC+) human PMNs was determined at various serum concentrations. p, preimmune serum; h, hyperimmune serum.
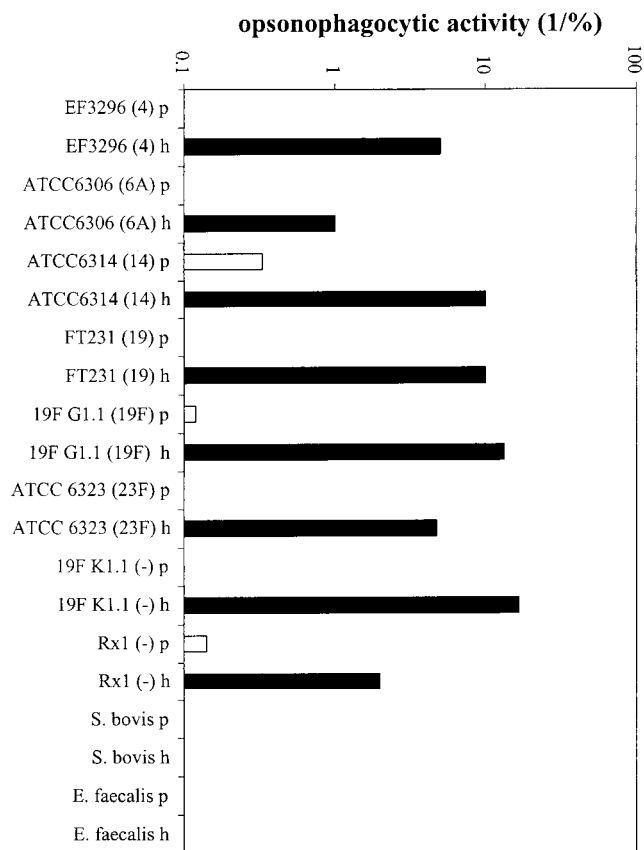

FIG. 4. Opsonophagocytic cross-reactivity of hyperimmune rabbit serum raised against the pneumococcal protein fraction using the heterologous pneumococcal strains EF3296, ATCC 6306, ATCC 6314, FT231, 19F G1.1, ATCC 6323, 19F K1.1, and Rx1, *S. bovis* strain 961008, and *E. faecalis* strain ATCC 29212. The serotype of the pneumococcal strains is indicated in parentheses. *Y* axis, opsonophagocytic activity, defined as the reciprocal of the serum concentration at which 25% of the human PMNs were fluorescent by phagocytosis of fluorescein isothiocyanate-labeled bacteria. p, preimmune serum; h, hyperimmune serum raised against the surface-associated protein fraction.

unpublished data) representing serotypes 4, 6A, 14, 19, and 23F and two unencapsulated strains (19F K1.1. and Rx1). In contrast, a low serum opsonophagocytic activity was found against two strains of the genetically closely related species *Streptococcus bovis* and *Enterococcus faecalis* (Fig. 4).

PspA, AmiA, and PpmA contribute to serum opsonophagocytic activity. Two-dimensional Western blot analysis was performed to identify the proteins that were serologically recognized at the surface of pneumococci and responsible for the in vitro opsonophagocytic activity. The hyperimmune rabbit serum recognized nine proteins, designated 1 to 9 (Fig. 1). Monospecific rabbit sera were raised against the individual proteins. The monospecific sera raised against proteins 2, 3, and 4 were able to facilitate phagocytosis of pneumococci with an opsonophagocytic activity of 0.5/, 0.7/, and 5/%, respectively (Fig. 3B to D). Protein 2 was analyzed by N-terminal amino acid sequencing, and proteins 3 and 4 were analyzed by mass spectrometry. Eighteen of the first 21 amino acids of protein 2 were successfully identified (Table 2). The amino acid sequence was identical to that of PspA as published by McDaniel

and colleagues (GenBank accession number AAC62252) (31). The molecular size of PspA deduced from the two-dimensional protein gel was approximately 65 kDa and correlates with the size range of PspA (60 to 200 kDa) (17, 50). Mass spectrometric analysis of protein 3 resulted in three peptides that were identical to AmiA (GenBank accession number P18791) (Table 2) (30). Mass spectrometric analysis of protein 4 resulted in 12 peptides (Table 2) that were part of a hypothetical translation product present in the TIGR pneumococcal genome encoding a protein of 322 amino acids (7,659 to 8,597, contig 33). The calculated size of this hypothetical protein (35.4 kDa) correlated with the size of protein 4 (approximately 35 kDa) deduced from the protein gel. This protein has been described before and designated putative proteinase maturation protein A (PpmA) due to its homology to proteinase maturation proteins (PrtM) of lactic acid bacteria (35a).

PpmA is located at the surface of *S. pneumoniae*. Since the three proteins PspA, AmiA, and PpmA are able to elicit opsonophagocytically active antibodies, they are presumed to be surface associated. The monospecificity of PpmA rabbit serum was confirmed by Western blot analysis. PpmA serum recognized a single protein band with the correct molecular size (35 kDa) in whole-cell lysates (Fig. 5). We performed indirect

TABLE 2. Partial amino acid sequences of surface proteins 2, 3, and 4 identified by amino acid sequence analysis[a]

Protein	Name	Start-end positions	Sequence
2	PspA	1–21	EEAPVAXQXKAEKDYDAAVXK
3	AmiA	40–49	1. VYTADPETLD
		148–159	2. DYLSGTSTDFST
		387–394	3. VAAQLPAY
4	PpmA	51–59	1. PSAQQVLLN
		64–66	2. QVF
		74–83	3. LDDQEVDDTL
		120–123	4. LAVQ
		125–137	5. VAEAELTDEAYQQ
		142–146	6. YTPDV
		161–165	7. EVLEQ
		195–198	8. FDSA
		221–235	9. VLTATGTQAYSSQYY
		248–251	10. NLDD
		260–264	11. LLTQQ
		268–277	12. STFVQSLLGK

[a] It is not possible to distinguish between Ile and Leu, Gln and Lys, or Phe and oxidized Met by mass spectrometry.

immunoelectron microscopy to identify the subcellular location of PpmA. For this purpose, anti-PpmA serum was used. Rabbit serum raised against PspA and normal rabbit serum were used as positive and negative controls, respectively. Immunoelectron microscopy demonstrated that both PpmA and PspA antibodies bound to the surface of pneumococci (Fig. 6).

PpmA is able to elicit species-specific opsonophagocytic antibodies that are cross-reactive against various pneumococcal strains. The PpmA antibodies were shown to facilitate phagocytosis of eight genetically distinct pneumococcal strains representing serotypes 4, 6A, 9V, 14, 18C, 19F, and 23F. Preimmune rabbit serum was only opsonophagocytically active when the unencapsulated variant of strain 19F was used. The opsonophagocytic activity of the PpmA antibodies was very low when *S. bovis* and *E. faecalis* were used (Fig. 7).
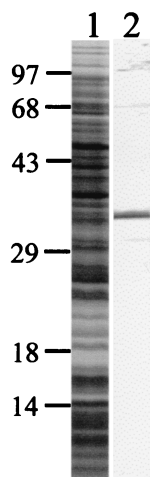

FIG. 5. Specificity of PpmA rabbit serum. Whole-cell lysates of *S. pneumoniae* strain FT231 were separated by one-dimensional protein gel electrophoresis, stained with CBB (lane 1), and analyzed by Western blotting with PpmA serum (lane 2). The positions of the size markers are indicated (in kilodaltons).

ppmA **is conserved among pneumococcal strains.** DNA sequence analysis of *ppmA* from 16 pneumococcal strains representing 15 distinct genotypes and 13 serotypes, and including the seven serotypes that cause most of the infections in young children, revealed limited genetic variation. The variation in *ppmA* was randomly distributed, and most of the point mutations were synonymous. Compared to the TIGR genome sequence, we found variability in six nucleotides: A33G ($n = 1$), T81A ($n = 10$), T81G ($n = 2$), C87T ($n = 1$), T114C ($n = 13$), G146A ($n = 15$), T339C ($n = 3$) and G818A ($n = 1$). Except for G146A and G818A, none of the point mutations resulted in an amino acid substitution. Mutation G146A results in a Ser49Asp substitution, and mutation G818A results in a Ser272Asp substitution.

PpmA plays a role in the pathogenesis of pneumococcal infections in vivo. A *ppmA* knockout mutant of strain D39 was generated by insertion mutagenesis. Interruption of *ppmA* in erythromycin-resistant transformants was confirmed by PCR analysis (data not shown). In addition, absence of PpmA expression was demonstrated by Western blot analysis (data not shown). To confirm that the *ppmA* mutation did not affect the in vitro growth rate, both the mutant and the parent strain were grown overnight on blood agar, inoculated into THY broth, and incubated at 37°C for 8 h. During this period, there was no significant difference in growth rate between the *ppmA*-deficient strain and wild-type D39, as judged from the optical density of the culture (data not shown). To determine the effect of inactivation of *ppmA* on virulence, mice were challenged via the intranasal route with strain D39 and the *ppmA* mutant, respectively. Mice challenged with the *ppmA* mutant survived significantly longer than mice challenged with the parent strain D39 ($P = 0.023$); median survival times were 51.5 and 33.0 h, respectively (Fig. 8). These data demonstrate that pneumococcal *ppmA* deficiency results in an extended survival time for mice during infection.

DISCUSSION

Various pneumococcal proteins are displayed on the cell surface. These proteins have a wide range of functions, including adherence to host tissues, binding to specific immune system components, protein processing, nutrient acquisition, and uptake of DNA from the environment. Immunization with several pneumococcal surface proteins has been shown to confer protection against pneumococcal infection in animal models. These include PspA (45), PsaA (44), autolysin (9), and neuraminidase (28). To identify novel protection-eliciting pneumococcal proteins, we isolated a pool of SB14-soluble, potentially surface-associated proteins of *S. pneumoniae* strain FT231. This protein fraction was able to induce antibodies that facilitated phagocytosis in vitro. Phagocytosis is a major defense mechanism against pneumococci, and the induction of opsonophagocytic antibodies is presumed to correlate with in vivo protection against *S. pneumoniae* infection (4). Among the proteins, at least three surface-associated proteins contributed to the in vitro opsonophagocytic activity.

The pneumococcal proteins were selected for hydrophobicity, immunogenic characteristics, and the ability to induce opsonophagocytic antibodies. One of the proteins was the previously characterized protein PspA. PspA inhibits complement activation and is proposed to exert a virulence function by recruitment of the alternative complement pathway, thereby reducing the effectiveness of complement receptor-mediated pathways of clearance (48). In addition, PspA functions as a lactoferrin-binding protein and is suggested to be involved in iron uptake and thus to contribute to pneumococcal growth
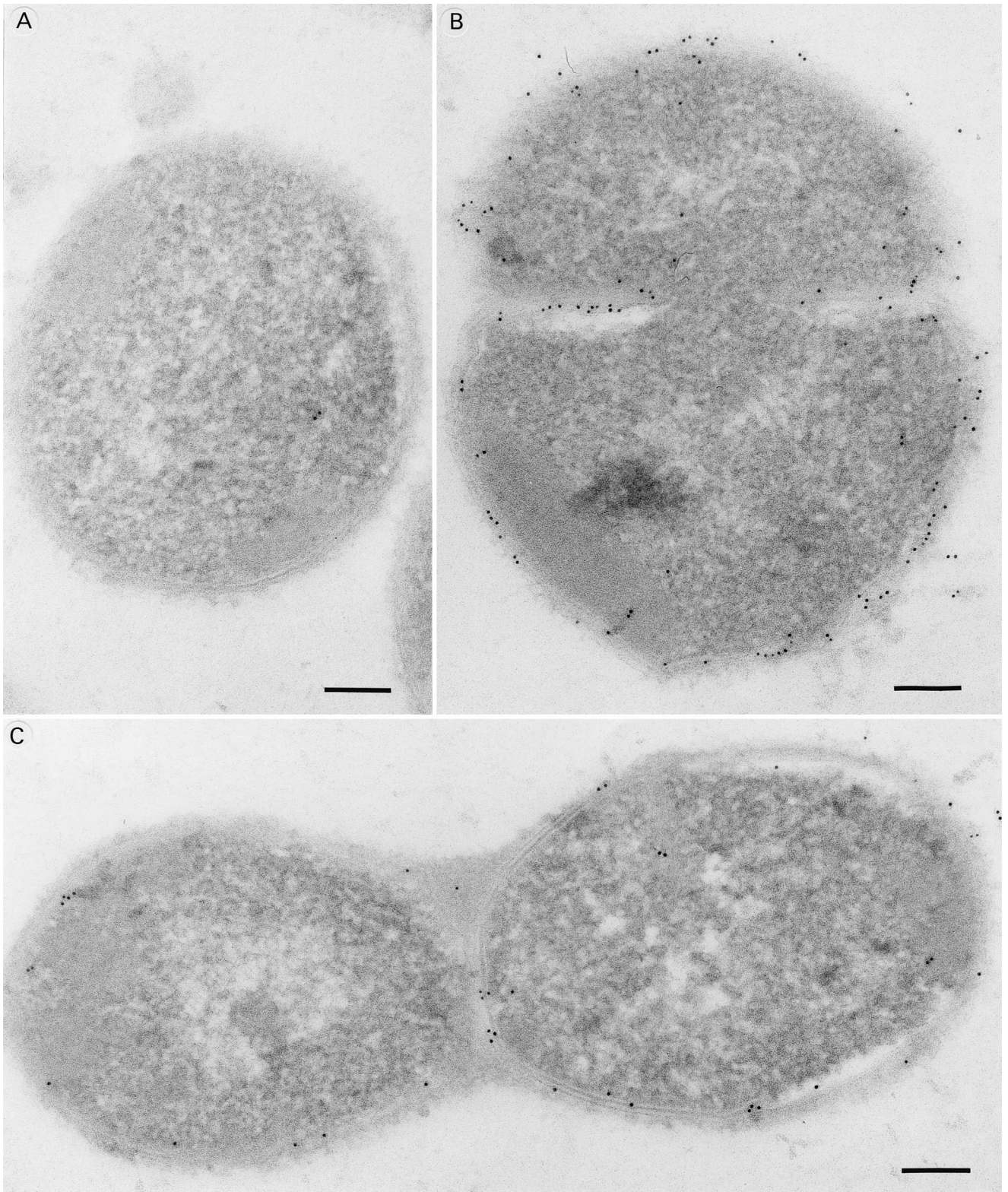
FIG. 6. Cellular localization of PpmA (B) and PspA (C) demonstrated by immunoelectron microscopy. Normal rabbit serum was used as negative control (A). Electron-dense immunogold particles are located mainly on the bacterial surface. Magnification, ×1,125,000. Bar, 100 nm.
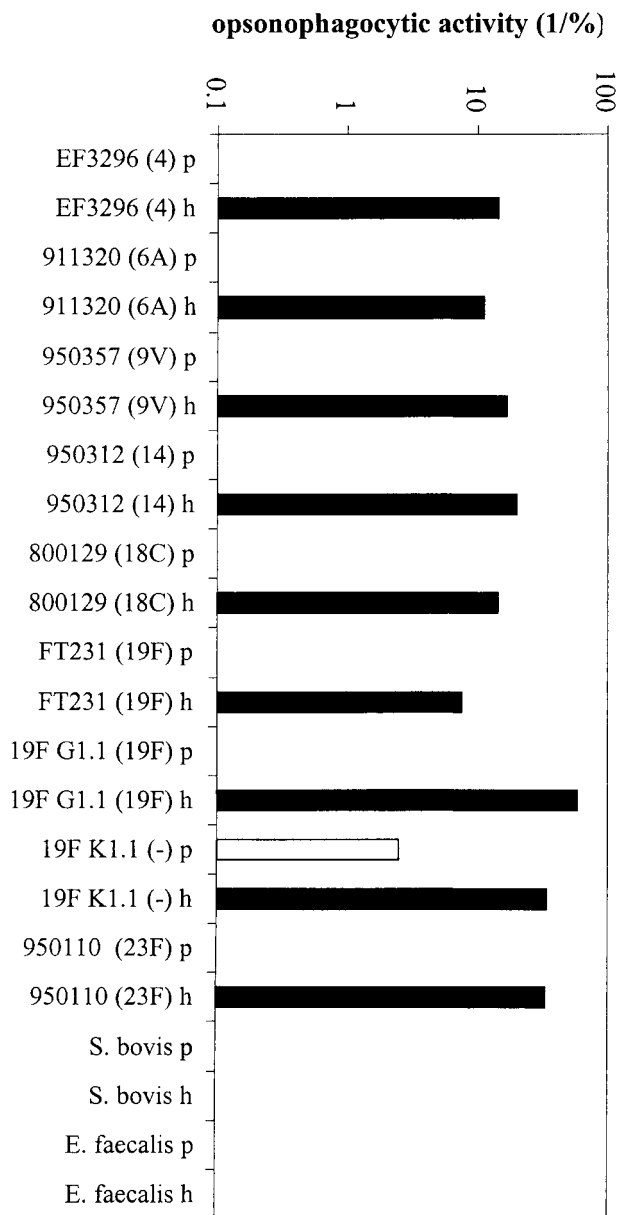
opsonophagocytic activity (1/%)

FIG. 7. Opsonophagocytic cross-reactivity of the monospecific hyperimmune sera raised against PpmA using the heterologous pneumococcal strains EF3296, 911320, 950312, FT231, 19F G1.1, 950110, 19F K1.1, and Rx1, *S. bovis* strain 961008, and *E. faecalis* strain ATCC 29212. The serotype of the pneumococcal strains is indicated in parentheses. Y axis, opsonophagocytic activity, defined as the reciprocal of the serum concentration at which 10% of the human PMNs were positive by phagocytosis of fluorescein isothiocyanate-labeled bacteria. p, preimmune serum; h, hyperimmune serum raised against PpmA.

FIG. 8. Intranasal challenge of mice with *ppmA*-deficient *S. pneumoniae*. Groups of 10 mice were challenged with 10^6 CFU of *S. pneumoniae* strain D39 (solid circles) and its *ppmA*-deficient derivative (open circles). The survival time of each mouse is presented. Bars represent the median survival time for each group.

under iron-limited conditions, i.e., in the human host (21). Surface exposure of PspA has been demonstrated previously (32, 46), and this characteristic was confirmed in this study by immunoelectron microscopy. The immunogenic nature of PspA observed in this study has also been demonstrated in previous studies (32). In line with the ability of PspA-specific antibodies to induce opsonophagocytic activity against strains expressing distinct capsular types, PspA has shown to possess immune protective potential (14, 32) with cross-protection (17, 47).

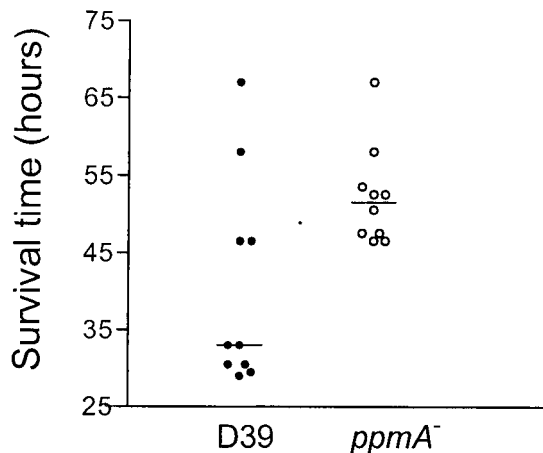The second protein that induced opsonophagocytic antibod-

ies was AmiA, which is a membrane-bound lipoprotein in *S. pneumoniae* (2) and part of the AmiA-AliAB oligopeptide permease that mediates the uptake of oligopeptides (2, 3). Since *S. pneumoniae* is auxotrophic for valine, leucine, arginine, asparagine, histidine, and glutamine, uptake of oligopeptides is important from a nutritional point of view (43). So far, no data are available on the possible protective abilities of this protein. Our data derived from phagocytosis experiments with AmiA antibodies are the first indications that AmiA may be protective against pneumococcal infections. The contribution of the AmiA-AliAB oligopeptide permease system to pneumococcal virulence is currently under investigation.

The third surface-associated protein which possessed the ability to induce opsonophagocytic antibodies was PpmA. PpmA is a recently identified pneumococcal protein with significant sequence homology to the proteinase maturation protein (PrtM) of lactic acid bacteria (Overweg et al., submitted for publication). Like PrtM from lactic acid bacteria (20), PpmA contains an N-terminal signal sequence, which serves as a label for translocation and cell membrane anchoring. In this study, the surface location of PpmA was confirmed by immunoelectron microscopy. The protein was able to induce antibodies in rabbits with opsonophagocytic activity. Although the affinity of the antibodies is unknown, the higher opsonophagocytic activity of PpmA antibodies compared to PspA and AmiA antibodies indicates the presence of relatively more PpmA molecules at the surface of the pneumococcus. Importantly, the opsonophagocytic activity of the PpmA antibodies was species specific and cross-reactive among heterologous pneumococcal strains. The observed opsonophagocytic activity of preimmune serum when the unencapsulated pneumococcal strain 19G K was used might be due to the absence of the capsule leading to the exposure of epitopes that are recognized by the preimmune serum. The cross-reactivity of the PpmA antibodies was in line with the limited sequence variation of *ppmA*. Like PrtM, PpmA is suggested to function as a membrane-bound isomerase (35a). PrtM is a *trans*-acting protein involved in the processing of precursors of serine protease PrtP into active enzymes (49) and belongs to the family of peptidyl-prolyl *cis/trans* isomerases. These enzymes are thought to assist in protein folding by catalyzing the *cis/trans* isomerization of the peptidyl-prolyl bonds in peptides and proteins (38). How-

ever, the pneumococcal protein(s) that is activated by PpmA is currently unknown. The differential expression of PpmA in phenotypic variants of *S. pneumoniae* indicates that PpmA may play a role in the pathogenesis of pneumococcal infections (35a). In the transparent phenotype that is selected for during nasopharyngeal colonization, PpmA is more prevalent, and therefore the protein may be involved in adherence through maturation of surface components or by the activation of proteases or other secreted proteins. In this study, PpmA was demonstrated to be involved in virulence. Inactivation of *ppmA* significantly reduced the virulence of strain D39 for mice as judged by survival time after intranasal challenge. However, the *ppmA* mutant was not completely avirulent. Like D39 mutants deficient in the production of pneumolysin (12), PspA (10), NanA (10), and LytA (11) that were also reduced in virulence, the *ppmA* mutant was still capable of killing mice in our animal model. The proposed role of PpmA in speeding up the folding reactions (*cis/trans* isomerization of the peptidyl-prolyl bonds) is consistent with the significant but limited reduction in virulence of the mutant strain. The rate of maturation of target proteins is presumed to slow in the absence of PpmA. This will subsequently result in a reduction but not elimination of target proteins that are modified in their biologically active configuration. Another explanation for the limited reduction in virulence of PpmA-deficient mutants might be the presence of other as yet unknown *cis/trans* isomerases that partially substitute for the PpmA activity. We conclude that PpmA contributes to pneumococcal virulence. Based on the surface location of PpmA and its ability to elicit protective species-specific antibodies, we also conclude that PpmA may be an interesting candidate for inclusion in future multicomponent protein vaccines.

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