**Streptococcus pneumoniae** Surface Protein PcpA Elicits Protection against Lung Infection and Fatal Sepsis

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Received 13 August 2007/Returned for modification 19 September 2007/Accepted 26 March 2008

Previous studies have suggested that pneumococcal choline binding protein A (PcpA) is important for the full virulence of *Streptococcus pneumoniae*, and its amino acid sequence suggests that it may play a role in cellular adherence. PcpA is under the control of a manganese-dependent regulator and is only expressed at low manganese concentrations, similar to those found in the blood and lungs. PcpA expression is repressed under high manganese concentrations, similar to those found in secretions. In this study, we have demonstrated that PcpA elicits statistically significant protection in murine models of pneumonia and sepsis. In the model of pneumonia with each of four challenge strains, statistically fewer *S. pneumoniae* cells were recovered from the lungs of mice immunized with PcpA and alum versus mice immunized with alum only. The immunizations reduced the median CFU by 4- to 400-fold (average of 28-fold). In the model of sepsis using strain TIGR4, PcpA expression resulted in shorter times to become moribund and subcutaneous immunization with PcpA increased survival times of mice infected with wild-type PcpA-expressing pneumococci.

*Streptococcus pneumoniae* is a gram-positive facultative anaerobe that is a major cause of morbidity and mortality worldwide. It is often the cause of bacterial pneumonia, otitis media, meningitis, and septicemia. The people at the highest risk of developing pneumococcal pneumonia are those with weakened or compromised immune systems, such as the elderly, infants, cancer patients, AIDS patients, and postoperative patients (13, 14, 38, 60). *S. pneumoniae* is estimated to cause more than 1 million deaths in children each year worldwide (38).

Although advances in drug therapies and vaccines have brought about a significant reduction in the incidence of pneumococcal disease, *S. pneumoniae* continues to be a threat to its target population (12, 13, 33). A 7-valent polysaccharide-protein conjugate vaccine is currently being used in children. This vaccine contains the seven most common capsular serotypes associated with pneumococcal disease in children in the United States. In children, the vaccine is highly immunogenic and effective against invasive disease, but it is less effective against otitis media. Unfortunately, the 7-valent polysaccharide-protein conjugate vaccine is too expensive for use in most developing countries, where most of the pneumococcal deaths occur.

Of even greater concern, however, is the increased incidence of colonization and disease caused by non-vaccine-type pneumococci (3, 54, 57), which had been predicted by prior studies using mathematical models (29, 30). It has been observed that in a Native American population in Alaska, the continued use of the 7-valent vaccine has resulted in almost complete replacement disease caused by vaccine types not present in the 7-valent vaccine (54). Continued increases in the magnitude of this serotype shift could result in a major reduction in the effectiveness of the 7-valent conjugate vaccine worldwide (22).

In addition to the concern about serotype shift, attention should be given to the effect that a reduction in nasopharyngeal colonization with *S. pneumoniae* could have on other pathogens that colonize the nasopharynx. It has also been reported that colonization with *Staphylococcus aureus* of healthy children is inversely correlated with colonization with vaccine-type *S. pneumoniae* (3, 31, 44, 57). This negative correlation is not seen between *S. aureus* and non-vaccine-type *S. pneumoniae*. These observations have been interpreted to suggest a direct competition between colonization with vaccine-type pneumococci and *S. aureus*, which could explain the increased incidence of *S. aureus* colonization after vaccination with the 7-valent conjugate vaccine.

If the above findings illustrating competition in colonization between pneumococci and *S. aureus* are supported by additional studies of colonization and disease with *S. aureus*, then it may be important to develop a pneumococcal vaccine antigen that will confer protection against invasive disease but not against pneumococcal colonization. Although such a vaccine would not be expected to elicit herd immunity against pneumococci, it should have the added advantage of not selecting for variant pneumococci that lack the vaccine antigen being used for immunization.

Pneumococcal choline-binding protein A (PcpA) (20, 50) is a potential vaccine candidate which is unlikely to elicit protection against colonization. PcpA is transcribed as a 2.3-kb monocistronic transcript encoding a putative 79-kDa protein containing a C-terminal choline-binding domain. PcpA is quite distinct, however, from a similarly named pneumococcal choline-binding protein referred to as both PspC and CbpA (11). PcpA contains several leucine-rich repeats (LRRs) in its N-terminal half (50). The protein is under the control of the manganese-dependent regulator *psaR*. RNA slot-blot analysis has revealed that an in vitro manganese concentration of 50 μM resulted in repression of *pepA* expression and that a con-
centration of 0.1 μM or lower resulted in full expression of pcpA (25). PcpA mutants were identified as having reduced virulence in murine models of sepsis and pneumonia in a large-scale signature-tagged mutagenesis study in which muta-
tants were forced to compete with wild-type bacteria during infections. In that study, mice were infected with serotype 4 S. pneumoniae by aspiration into the lungs (pneumonia model) and intraperitoneal injection (sepsis model) (20).

LRRs are present in over 2,000 proteins found in viruses, bacteria, archaea, and eukaryotes. These proteins have been shown to participate in a wide variety of biological functions, including plant and animal immune responses, apoptosis, cell adhesion, signal transduction, DNA repair, DNA recombination and transcription, and RNA processing (4, 27). The variable lymphocyte receptors of agnathans (jawless fish) are as-
sembled through the recombination of LRR modular units (42). Eukaryotic Toll-like receptors also employ LRR in their recognition of pathogen-associated molecular patterns (42, 43, 47).

The LRR motif of LRR proteins participates in a common structural unit consisting of alpha helices and beta strands joined by loops, formed by the LRR units. This arrangement results in a “horseshoe-shaped” molecule, which provides a versatile scaffold for protein-protein interactions. There are several subfamilies of bacterial LRR proteins. PcpA is a member of the LRR<sub><i>T</i></sub> subfamily. This subfamily was originally identified in Treponema pallidum. Most of the proteins in this subfamily have been found associated with bacterial cell surfaces (24, 27, 45). Most of the bacterial LRR<sub><i>T</i></sub> proteins that have been studied appear to play a role in surface adherence or aggregation (23, 24, 45, 52, 59).

Here we report the partial characterization of the <i>S. pneumoniae</i> LRR protein PcpA, the prevalence of the pcpA gene in strains of <i>S. pneumoniae</i>, the requirement for low manganese to permit PepA expression in diverse pneumococci, the role that PcpA plays in the virulence of <i>S. pneumoniae</i>, and the protective effects of immunity to PepA. Since PepA is not required for colonization and is not expected to be expressed by pneumococci bathed in nasal mucosal secretions, immunity to it should not protect against colonization and, thus, may be able to protect against invasive disease without significantly enhancing the evolution of pneumococci to avoid the anti-
PcpA vaccine.

**Materials and Methods**

**Bacterial strains, medium, and growth conditions.** <i>S. pneumoniae</i> strains TIGR4 and EF3030 and their derivatives were used in this study. Unless other-
wise indicated, pneumococci were grown at 37°C in Todd-Hewitt broth with 0.5% yeast extract (THY) or on blood agar plates (Becton Dickinson, Sparks, MD). When appropriate, erythromycin was added to the medium at a concentra-
tion of 0.3 μg/ml. Clinical isolates of <i>S. pneumoniae</i> (Table 1) and isolates of major clonal groups (Table 2) were used.

The clinical strains used in these studies were isolated within the last 25 years. Laboratory passage has been minimized by storing the bacteria in 10% glycerol (24, 47). When appropriate, erythromycin was added to the medium at a concentra-
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**Cloning of plasmids.** The primers used in this study are summarized in Table 1. The LRR motif of LRR proteins participates in a common structural unit consisting of alpha helices and beta strands joined by loops, forming a versatile scaffold for protein-protein interactions. There are several subfamilies of bacterial LRR proteins. PcpA is a member of the LRR<sub><i>T</i></sub> subfamily. This subfamily was originally identified in Treponema pallidum. Most of the proteins in this subfamily have been found associated with bacterial cell surfaces (24, 27, 45). Most of the bacterial LRR<sub><i>T</i></sub> proteins that have been studied appear to play a role in surface adherence or aggregation (23, 24, 45, 52, 59).

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terminal fragment of \textit{pcpA} in strain TIGR4. The PCR products were then separated on a 0.8% Tris-acetate-buffered agarose gel, stained with ethidium bromide, and examined to determine their size.

\textbf{S. pneumoniae cell fractionation.} Protoplasts were produced by the method described by Yother and White (63), with slight modification. Log-phase cells, grown in low-manganese medium, were pelleted and washed in phosphate-buffered saline (PBS). The cells were then resuspended in 0.5 ml of 2% choline chloride, and the tube was inverted several times. The cells were then pelleted, and the supernatant was drawn off and stored at 4°C for 30 min. The cells were then washed twice with PBS and resuspended in PBS with 1% bovine serum albumin (PBSB), and incubated at room temperature for 20 min. Cells were pelleted and resuspended in PBSB or anti-PcpA serum diluted 1:100 in PBSB and incubated at 37°C for 30 min. Incubation was followed by two washes with PBS. Cells were then incubated with goat anti-rabbit immunoglobulin G (IgG; heavy and light chains [H+L])-fluorescein isothiocyanate (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted in PBSB at 4°C for 30 min. The cells were then washed twice with PBS and resuspended in 4% formaldehyde in PBS containing 0.01 mM of the lipophilic membrane dye trimethylamino-diphenylhexatriene (TMA-DPH; Invitrogen). Bacterial cells were then inspected by epifluorescence using the Olympus IX 70 microscope.

\begin{table}[h]
\centering
\caption{S. pneumoniae strains of major clonal groups}
\begin{tabular}{|l|l|l|l|l|}
\hline
Strain & Capsular type & Origin & Characteristics & Reference \\
\hline
MA-14 & 14 & United Kingdom & Worldwide Erm\(^{\text{r}}\) clone; MLST sequence type 9 & 17 \\
MB-23F & 23F & United Kingdom & Unknown disease; MLST sequence type 81 & 17 \\
MC-6B & 6B & Spain & Unknown disease; MLST sequence type 90 & 18, 19 \\
MD-6B & 6B & Alaska & Unknown disease; MLST sequence type 138 & 48 \\
ME-19 & 19 & Tennessee & Carriage clone; MLST sequence type 236 & 48 \\
MF-6A & 6A & Tennessee & Carriage clone; unknown MLST sequence type & 48 \\
MG-1 & 1 & United Kingdom & Major invasive clone; MLST sequence type 227 & 17 \\
MI-7F & 7F & Norway & Major invasive clone; MLST sequence type 191 & 17 \\
MJ-35 & 35 & Tennessee & Carriage clone; MLST sequence type 65 & 48 \\
MK-22 & 22 & Tennessee & Major invasive clone; unknown MLST sequence type & 48 \\
ML-11 & 11 & Tennessee & Carriage clone; MLST sequence type 62 & 48 \\
MM-14 & 14 & Tennessee & Major invasive clone; MLST sequence type 124 & 48 \\
MN-23F & 23 & Tennessee & Carriage clone; MLST sequence type 37 & 48 \\
\hline
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\end{table}

\begin{table}[h]
\centering
\caption{Recombinant bacterial strains, plasmids, and PCR primers used in this study}
\begin{tabular}{|l|l|l|l|}
\hline
Strains & Source or reference \\
\hline
\textit{S. pneumoniae} & & \\
TIGR4\textit{psaR} (JEN7) & This study & \\
TIGR4\textit{pcpA} (JEN11) & 25 & \\
EF3030\textit{pcpA} (JEN18) & 25 & \\
EF3030\textit{pcpA}\(^{\text{T}}\) (NAT1) & JEN18 transformed to wild type & This study & \\
\textit{E. coli} & & \\
TOP10 & General cloning strain & Invitrogen & \\
Rosetta (DE3)pLysS & Expression strain & Novagen & \\
\hline
\text{Plasmids} & & \\
pCR2.1 & 3.9 kb; Amp\(^{\text{r}}\) Kan\(^{\text{r}}\) & Invitrogen & \\
pCR4 & 3.9 kb; Amp\(^{\text{r}}\) Kan\(^{\text{r}}\) & Invitrogen & \\
pET-20b & 3.7 kb; Amp\(^{\text{r}}\), C-terminal His tag & Novagen & \\
pDG-1 & pCR4 with \textit{pcpA} fragment; Amp\(^{\text{r}}\) & This study & \\
pJM-1 & pET-20b with \textit{pcpA} fragment; Amp\(^{\text{r}}\) & This study & \\
pJJ035 & pCR2.1 with 412-bp internal \textit{pcpA} fragment; Amp\(^{\text{r}}\) & This study & \\
\hline
\text{Primers} & & \\
DTG-16 & cgaggatcATATGTTCCCTAATGAACC; \textit{pcpA} forward & This study & \\
DTG-12 & gcctcgagTTCCTTTAATGAACTGCACTTGAACCGCCACCTTTAGGAAGGAGGAC; \textit{pcpA} reverse & This study & \\
JW128 & AACTGTCAAGTGGTAATGG; \textit{pcpA} forward & 25 & \\
JW129 & TGAACTGGAGGAAAAGGTTAGC; \textit{pcpA} reverse & 25 & \\
BG1 & ATGAAAAAACTACAATATTATATACCACTCAATGCG; \textit{pcpA} forward & This study & \\
BG2 & CCATACACATTTGTCTTAAAACCCCAACCAACTAC; \textit{pcpA} reverse & This study & \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Primers were based on the complete genome sequence of \textit{S. pneumoniae} TIGR4 (60). Lowercase letters in the primer sequences denote mismatches used to create restriction endonuclease sites. All sequences are expressed 5‘ to 3‘.
Western blotting. Bacterial cultures were grown in high- or low-manganese medium to mid-log phase (OD600 of 0.6). Equivalent amounts of each strain were washed twice with PBS, resuspended in PBS with SDS-PAGE sample buffer, and boiled for 5 min. Samples and a prestained protein standard (Invivogen) were loaded onto a NuPAGE 10% Bis-Tris gel (Invivogen) and separated by electrophoresis in MES (morpholinethanesulfonic acid)-SDS running buffer (Invivogen) in accordance with the manufacturer’s instructions. The proteins were then transferred to a nitrocellulose membrane with the Trans-Blot SD semidyed transfer cell (Bio-Rad, Hercules, CA). The blot was probed with anti-PcpA polyclonal antibody diluted 1:1,000 in PBST. Goat anti-rabbit IgG (H+L)-alkaline phosphatase and streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Inc., Birmingham, AL) were used as the secondary antibody. Colorimetric detection was performed with Sigma Fast nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) tablets (Sigma Aldrich). Colorimetric detection was performed with Sigma Fast nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) tablets (Sigma Aldrich). Conjugates on nitrocellulose and probed with an rPcpA polyclonal antiserum. Lane 1, TIGR4 pcpA (pcpA constitutive mutant); lane 3, D1091B; lane 4, EF5668; lane 5, BG10752; lane 6, V175; lane 7, LS2013; lane 8, BG12730; lane 9, TJ0893. When pneumomine rabbit serum was used in place of immune rabbit sera, the PcpA bands were not detected (data not shown). When the same strains were cultured in Todd-Hewitt broth (high manganese) none of them produced detectable PcpA (data not shown).

RESULTS

pcpA is present in clinically relevant strains of S. pneumoniae. The presence of pcpA was detected by PCR, with primers (BGP1 and BGP2) spanning the LRR region of the pcpA (Table 3) (data not shown). Each of the 25 strains examined (Tables 1 and 2) yielded a roughly 1,500-bp fragment. Eleven of these strains (Table 1) were isolated from patients within the last 25 years and are representative of the capsular types covered by the 7-valent conjugate vaccine, and one strain, R6, is a rough mutant of capsular type 2 strain D39.

The remaining 13 strains are S. pneumoniae strains (Table 2) that were collected as part of the Genome Diversity Project (http://genome.microbio.uab.edu/strep/info/), which includes strains chosen to span the breadth of diversity in S. pneumoniae. These 12 strains were selected to be highly divergent to each other based on multilocus sequence typing (MLST) data (17). These strains also represent 12 different capsule types from different regions of the world. Four of the strains were from patients with serious invasive disease, five were from asymptomatic carriage, two strains had a disease/colonization status unknown, and one strain was from a worldwide antibiotic-resistant clone (48).

To test for the expression of PcpA in all of the strains, they were grown in low-manganese medium (≤0.1 μM) (19, 25). Total cellular protein samples were prepared from mid-log-phase cells cultured in the low-manganese medium. All of the strains listed in Tables 1 and 2 were examined, but only those representing capsular types included in the 7-valent vaccine are depicted (Fig. 1). Total cellular protein samples were separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed with anti-PcpA polyclonal antiserum. The blot identified a single band in lanes 2, 7, 8, and 9 which appeared to be doublets (suggested partial degradation or possibly differential glycosylation of PcpA) in lanes 3, 4, 5, and 6 (Fig. 1). There was no band identified in the pcpA-inactivated mutant TIGR4 pcpA. The PcpA bands all migrate at approximately 62 kDa, but there was some slight variation in the size of the native proteins. Comparison of the amino acid sequences of available genomes shows variation in the number of choline-binding repeats, as well as other minor differences.

Total cellular protein samples for the same strains were also prepared from all 25 strains by growing them in a high-manganese medium, but no bands were identified with the anti-PcpA antiserum (data not shown). The PCR analysis in combination with the Western blot data demonstrated that pcpA is present in all 25 of the S. pneumoniae strains listed in Tables 1 and 2.

PcpA is present on S. pneumoniae grown under low-manganese conditions. Prior studies from our laboratory showed that, through the action of the regulator PsaR, manganese can repress the transcription of the pcpA gene (25). The present study showed that the manganese concentration directly affected the presence of PcpA on the surface of S. pneumoniae and that surface PcpA was accessible to antibody even on encapsulated pneumococci.

Cell fractionation was performed to determine if PcpA was present...
associated with the cell wall or cell membrane/cytosol of *S. pneumoniae*. Western blot analysis of these cellular fractions revealed that PcpA was present predominantly in the cell wall of *S. pneumoniae* grown in low- but not high-manganese medium (data not shown). A small fraction of the PcpA was found to be associated with the cell membrane/cytosol, probably representing PcpA that had yet to be exported to the surface of the bacteria.

In addition to the cell fractionation, log-phase cells from wild-type *S. pneumoniae* strain TIGR4 were grown in high- or low-manganese medium and then stained with anti-PcpA polyclonal antiserum followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Ig. The labeled bacteria were then examined by immunofluorescence microscopy. The antibodies to PcpA were able to stain the bacteria grown under low-manganese growth conditions but not those grown in high manganese concentrations (Fig. 2).

These results showed that PcpA is present on the surface of wild-type *S. pneumoniae* cultured under low-manganese conditions in vitro and suggested that PcpA is probably expressed in bacteria infecting low-manganese sites inside the host, such as the lungs and blood. Whether PcpA is exposed well enough to facilitate necessary PcpA-ligand interactions between the bacterium and the host epithelium during infection is not known. Whether regulation of PcpA production by manganese is generalizable to most pneumococci is not yet established and may be controversial (21, 31).

**Immunization with rPcpA elicits antibody and provides protection against lung and systemic infection but does not significantly affect nasopharyngeal colonization.** Prior to challenge with *S. pneumoniae*, the mice were immunized with rPcpA mixed with aluminum hydroxide or received aluminum hydroxide alone. Total Ig (H+L) was quantified for both groups of mice by enzyme-linked immunosorbent assay (9). The geometric mean (± standard error of the mean) level of antibody specific for PcpA in the serum of the immunized mice was 0.465 ± 0.119 µg/ml, versus a mean of 0.002 ± 0.002 µg/ml for mice receiving the adjuvant alone. This indicated that the immunization successfully elicited an immune response to rPcpA.

To determine if the immunization protected mice from pneumonia, the immunized and alum-only mice were lightly anesthetized and inoculated in the nares with 5 × 10⁶ CFU of either wild-type strain. However, as expected (25), there were significant differences in the numbers of CFU recovered from mice in the colonization model above (Fig. 5).

Because of the possibility that the EF3030 strain carrying the pcpA mutation may have had additional mutations that caused the observed effect on virulence, we investigated this issue further. The wild-type *pcpA* gene was reintegrated, replacing the EF3030 *pcpA* mutation. CFU in colonization and pneumonia models for the wild-type parent, the EF3030 *pcpA* mutant, and the repaired strain were then compared. In colonization, no significant difference was found between the numbers of bacteria recovered from the nasal washes of mice inoculated with either EF3030 or EF3030 pcpA (Fig. 5).
We have thus observed that neither the absence of an intact pcpA gene nor the presence of an immune response in the host elicited by immunization with rPcpA resulted in a reduction in the numbers of pneumococci that were recovered in the nasal washes of mice. These observations were consistent with the fact that the manganese concentration in the rat and human nasopharynx (>36 μM) is high enough to suppress pepA transcription (15, 26, 51, 58). Under these conditions, pepA tran-

FIG. 2. Surface exposure of PcpA on S. pneumoniae strain TIGR4. TIGR4 was cultured in (A) high- or (B) low-manganese medium until mid-log phase. The bacteria were incubated with anti-PcpA rabbit serum, followed by incubation with FITC-conjugated anti-rabbit Ig antibodies. The cells were next fixed in 4% formaldehyde containing the membrane dye TMA-DPH. Blue fluorescence indicates staining with TMA-DPH, and green fluorescence indicates staining with anti-PcpA antibodies. (C) Bacterial culture in high-manganese medium, incubated with FITC-conjugated antibodies to rabbit Ig, without any rabbit anti-PcpA.
scription would be repressed by PsAR (25) in the nasopharynx. Thus, immunity to PcpA would be expected to have little effect on bacteria in this host site. However, the lack of an effect of subcutaneous immunization with PcpA on colonization would not, by itself, rule out the possibility that mucosal immunization may lead to protection, since intranasal immunization has been more effective than subcutaneous immunization using another pneumococcal antigen (61).

PcpA and immunity to PcpA affect virulence in the murine model of systemic infection. To evaluate the ability of immunity to PcpA to protect against sepsis, CBA/N mice were first subcutaneously immunized with PcpA in aluminum hydroxide or with aluminum hydroxide alone as a control and then challenged with \textit{S. pneumoniae} capsular type 4 strain TIGR4. This strain was used rather than EF3030 since TIGR4 can much more readily cause bacteremia and sepsis in mice (25, 49). The immunized animals were challenged intravenously with 300 CFU of \textit{S. pneumoniae} TIGR4. Survival was monitored for 21 days. Mice receiving rPcpA immunizations had a median time to become moribund that was extended by 43.5 h compared with mice receiving adjuvant alone (Fig. 6). Twenty-six percent of mice immunized with rPcpA lived, while no mice immunized with aluminum hydroxide alone lived; this difference in survival rates was statistically significant ($P = 0.007$). Inactivation of \textit{pcpA} reduces the virulence of pneumococci following intravenous inoculation. It was previously reported that inactivation of \textit{pcpA} results in reduced virulence in the murine model of pneumonia (25) and in a lung-sepsis model (20). In the present studies, the effect of \textit{pcpA} inactivation on systemic infection following intravenous challenge was examined by infecting naive mice with 300 CFU of either TIGR4 or its \textit{pcpA}-inactivated mutant, TIGR4 \textit{pcpA}. The median time for mice infected with TIGR4 \textit{pcpA} to become moribund was extended by 31.5 h ($P = 0.0299$) compared with those infected with wild-type bacteria (Fig. 7). This demonstrated that PcpA plays at least a small role in the ability of \textit{S. pneumoniae} to cause systemic diseases.

**FIG. 3.** Protection conferred by rPcpA immunization in a murine model of pneumonia. CBA/N mice were subcutaneously immunized with rPcpA adsorbed to aluminum hydroxide or with aluminum hydroxide alone. The mice were challenged intranasally under light anesthesia with $5 \times 10^6$ CFU of EF3030. After the mice were sacrificed 7 days postinfection, bacterial counts were determined from lung homogenates (A) and nasal washes (B). The horizontal line denotes median log$_{10}$ CFU. ***, $P = 0.0019$, Mann-Whitney test.

**FIG. 4.** Protection conferred against other \textit{S. pneumoniae} capsular serotypes by rPcpA immunization in a murine model of pneumonia. Mice were challenged with the following strains: (A) TJ0893, serotype 14 (**, $P = 0.0209$); (B) L82016X, serotype 6B (**, $P = 0.0193$); and (C) EF9303, serotype 23F (**, $P = 0.0388$) (Mann-Whitney test). The horizontal line denotes median log$_{10}$ CFU.

**FIG. 5.** Effect of \textit{pcpA} inactivation on intranasal colonization of \textit{S. pneumoniae}. The mice were challenged intranasally with $10^6$ CFU of EF3030 or EF3030 \textit{pcpA}. After the mice were sacrificed 7 days postinfection, bacterial counts were determined from nasal washes. The horizontal line denotes median log$_{10}$ CFU/nose.
DISCUSSION

The *S. pneumoniae* LRR protein PcpA was found to be an important virulence determinant in pneumococcal lung infections. PcpA also appears to play a role in sepsis. The original evidence that PcpA may be required for virulence came from Hava and Camilli, who used a genetic screen to identify genes in which mutations impacted the virulence of a highly invasive strain (20) when bacteria are aspirated into the lungs. Since they monitored the effects of the mutation on the numbers of CFU in the lungs of the infected mice (20), their data suggest that pcpA could play a role in lung infection. The studies of Hava and Camilli used competition between mutant and wild-type pneumococci, which, for many genes, can be an extremely sensitive measure of relative virulence. On the other hand, it often magnifies the difference seen with pure cultures in vivo. We have confirmed the findings of Hava and Camilli with regard to the effect of PcpA on lung infection by using an aspiration pneumonia model that employs a challenge strain that causes focal pneumonia and does not invade the blood (25). Our present and past (25) studies thus clearly demonstrate that PcpA is able to play an important role in the lungs. The prior studies of Hava and Camilli, however, did not determine whether or not immunization with rPcpA might be protective, nor did they examine the percentage of strains of *S. pneumoniae* that express the protein.

We demonstrated here that *pcpA* is expressed by each of the 25 diverse *S. pneumoniae* strains examined when they are grown under low- but not high-manganese conditions. In addition, we demonstrated that, under conditions of low manganese, PcpA is present on the surface of *S. pneumoniae*. The surface expression of PcpA is in accordance with the expression of other LRR proteins described in group A and group B streptococci. PcpA's potential for use as a vaccine antigen is supported by our observation that prior immunization with rPcpA resulted in the presence of 1/100 as many pneumococci in infected lungs. We also observed a statistically significant effect of immunization with PcpA on the protection against sepsis.

The failure of PcpA to be expressed at high manganese concentrations was consistent with the failure of *pcpA* mutants to affect colonization and thus strongly suggests that immunization with PcpA should not protect against colonization. Thus, taken together our data indicate that immunity to PcpA elicited by systemic immunization may be relatively specific for invasive disease. In two recent papers (21, 31), results for *pcpA* expression appeared to be at variance with that reported previously in our lab (25). Both studies examined capsular type 2 strain D39, which was originally isolated over 100 years ago and has been extensively passaged ever since, yielding many different sublines (28). Although one of the studies examined two additional strains (31), *pcpA* expression was not the predominant focus of either study. Moreover, neither of the two studies took into account the need to control manganese levels (25) when examining *pcpA* expression. Any differences in results between the three studies will hopefully be resolved in future publications.

Although other antigens have been reported to elicit protection against pulmonary infection and sepsis (9, 11, 35, 56), PcpA is the first antigen that may offer stronger protection against pneumonia than against sepsis. The ability of PcpA to protect against lung infection and sepsis but not colonization is similar to what has been observed with PdB (5, 39, 40), a genetic toxoid of pneumolysin, which, like PcpA, fails to elicit protection against colonization (6).

This "tissue site-specific" protection afforded by rPcpA may prove to be of value for vaccine development since it can elicit protection against lung infection without affecting the dynamics of nasopharyngeal colonization. Immunization of children with the 7-valent conjugate vaccine has reduced colonization and disease with the vaccine capsular serotypes, while there has been a replacement in colonization (3, 34, 54) as well as disease (22, 54) with capsular types not present in the vaccine.

A vaccine that protected against pneumonia without affecting colonization might avoid causing serotype replacement and increased replacement disease from organisms such as *S. au-

ACKNOWLEDGMENTS

We are grateful to Jason Johnston, who constructed the mutants used in this work for a previous publication and has generously made ...
they available for use in these studies. We thank Mamie Thomas-Coats, Yvette Hale, and Janice King for assistance with our infection studies as well as for advice and encouragement. We also thank Albert Toussan of the UAB Imaging Facility for help in obtaining the microscopic images used. We acknowledge Flora Gathof for administrative assistance.

David T. Glover was supported by training grant NHLBI T32-HL07553. These studies were also supported by NIH grants AI21548 and AI53749 to David E. Bries and Susan K. Hollingshead, respectively.

These studies involve research and intellectual property of the UAB Research Foundation that has been licensed to and was partially sponsored by Sanofi Pasteur. S. K. Hollingshead and D. E. Bries have been consultants for Sanofi Pasteur.

REFERENCES

1. Balachandran, P., A. Brooks-Walter, A. Virolainen-Julkunen, S. K. Holling-


3. Bogut, D., R. H. Veenhoven, M. Shuijtjer, W. J. W. Wanneet, G. T. Rijkers,

4. Bogaert, D., R. H. Veenhoven, M. Shuijtjer, W. J. W. Wanneet, G. T. Rijkers,

5. Bogaert, D., R. H. Veenhoven, M. Shuijtjer, W. J. W. Wanneet, G. T. Rijkers,


7. Bogaert, D., R. H. Veenhoven, M. Shuijtjer, W. J. W. Wanneet, G. T. Rijkers,

8. Bogaert, D., R. H. Veenhoven, M. Shuijtjer, W. J. W. Wanneet, G. T. Rijkers,


Editor: J. N. Weiser

Infect. Immun.