



## Pneumococcal PspA and PspC proteins: Potential vaccine candidates for experimental otitis media



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### ABSTRACT

**Objective:** Otitis media is the most commonly diagnosed disease in ambulatory care and *Streptococcus pneumoniae* continues to be the most common bacterial agent. Bacterial resistance to antibiotics underscores the need for better vaccines. Current pneumococcal conjugate vaccines are modestly protective against otitis media; however, limited serotype coverage and serotype replacement have led to the investigation of pneumococcal proteins as potential vaccine candidates. Two proteins, pneumococcal surface proteins A (PspA) and C (PspC) are important virulence factors, expressed by virtually all strains. Although a number of pneumococcal proteins have been investigated in other infection sites, these proteins can have diverse organ-specific effects. In this study, we investigated the viability and virulence of single (PspA<sup>-</sup> and PspC<sup>-</sup>) and double (PspA<sup>-</sup>/PspC<sup>-</sup>) mutants of pneumococcal PspA and PspC proteins in the chinchilla middle ear.

**Methods:** Bullae of 24 chinchillas were inoculated with 0.5 ml of 10<sup>6</sup> colony forming units (CFUs)/ml bacteria: 6 with wild-type D39 strain; 6 with PspA<sup>-</sup>; 6 with PspC<sup>-</sup>; and 6 with PspA<sup>-</sup>/PspC<sup>-</sup> isogenic mutant strains. Bacterial CFU levels in middle ear effusions and light microscopic analysis of the number of inflammatory cells in the round window membrane (RWM) were compared 48 h after inoculation. **Results:** At 48 h, CFUs in middle ears were increased for wild-type and PspC<sup>-</sup> strains compared to inoculum levels; however, they were significantly less for the group inoculated with the PspA<sup>-</sup> strain compared to wild-type strain. No bacteria were detected in the PspA<sup>-</sup> and PspA<sup>-</sup>/PspC<sup>-</sup> groups. The number of inflammatory cells in the RWM was significantly higher in wild-type compared to the PspA<sup>-</sup>, PspC<sup>-</sup>, and PspA<sup>-</sup>/PspC<sup>-</sup> groups. No significant difference in number of inflammatory cells was observed between any pairs of groups inoculated with mutant strains.

**Conclusion:** Viability and virulence of the PspC<sup>-</sup> strain were similar to the wild-type strain. The single PspA<sup>-</sup> and double PspA<sup>-</sup>/PspC<sup>-</sup> mutants were highly attenuated in the ear. Bacterial clearance of the PspA<sup>-</sup>/PspC<sup>-</sup> double mutant was indistinguishable from that of the PspA mutant. These studies provide no reason to exclude PspC from a multi-component protein vaccine containing PspA.

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### 1. Introduction

Otitis media is the most frequently diagnosed disease in childhood and the most common reason for antimicrobial treatment in children under 6 years of age, and *Streptococcus*

*pneumoniae* (*S. pneumoniae*) is the most commonly reported bacterial agent, occurring in 30–50% of patients with otitis media [1]. Pneumococcal resistance to antibiotics has underscored the need for better vaccines. Seven-valent pneumococcal conjugate vaccine (PCV7) introduced in 2000 was shown to provide serotype-specific protection, but increased antimicrobial resistance emerged among non-vaccine serotypes in pediatric populations [2]. The PCV13 vaccine that was licensed in 2010 is promising in protection against these serotypes [3]. The long-term impact on replacement of pneumococcal and other bacterial strains is not well known; however, even before PCV13 was put into use, 50% of

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isolates from the middle ear were of capsular types not covered by PCV13 [4]. An alternative to the current pneumococcal conjugate vaccines is the use of pneumococcal proteins that penetrate the bacterial capsule. Two of these surface proteins, pneumococcal surface proteins A (PspA) and C (PspC), are important virulence factors, expressed by the majority of pneumococcal strains. They have been shown to elicit a significant level of protection in animal models against systemic challenge with one or more serotypes [5]. PspA interferes with complement deposition on pneumococci, reducing opsonization and clearance of bacteria by the host immune system [6]. It can also bind to apolactoferrin to prevent apolactoferrin-mediated killing of pneumococci [7]. PspC (also known as CbpA and SpsA) interferes with complement activity by binding complement factor H [8]. It binds secretory IgA [9] and acts as an adhesion [10]. It can serve as an immunogen and elicit protection against nasopharyngeal colonization [9,11].

In this study, we investigated the viability and virulence of single (PspA<sup>-</sup> and PspC<sup>-</sup>) and double (PspA<sup>-</sup>/PspC<sup>-</sup>) mutant strains of pneumococcal PspA and PspC proteins in the chinchilla ear.

## 2. Methods

We chose *S. pneumoniae* D39 Serotype 2 (NCTC 7466) as our wild-type strain. It has been studied in many pneumococcal infectious diseases, including otitis media. In addition *S. pneumoniae* D39 has single and double mutants of pneumococcal surface proteins, which may be important antigens for future protein-based vaccine design. *S. pneumoniae* serotype 2 strain D39, the wild-type parent strain was grown in Todd Hewitt Broth (THB) containing 0.5% yeast extract (BD Diagnostics, Sparks, Maryland), plated on sheep blood agar (SBA) plates, and stored in 10% glycerin solution at  $-80^{\circ}\text{C}$ . Isogenic mutants PspA<sup>-</sup>, PspC<sup>-</sup>, and PspA<sup>-</sup>/PspC<sup>-</sup> were grown on SBA plates and in THB containing 0.5% yeast extract, with 0.3  $\mu\text{g}/\text{ml}$  erythromycin. Bacteria were grown until log phase, optical densities were measured at 660 nm and bacteria diluted in phosphate buffered saline. Ten-fold dilutions were plated, and viable cells counted to confirm the actual concentration. The Institutional Animal Care and Use Committee of the University of Minnesota, Minneapolis approved care and use of animals. Twenty-four chinchillas were anesthetized with 0.25 ml of ketamine hydrochloride (100 mg/kg)/acepromazine maleate (10 mg/kg) and middle ears inoculated bilaterally with 0.5 ml of bacteria: 6 chinchillas with  $2.3 \times 10^6$  CFU/ml wild-type; 6 with  $1.6 \times 10^6$  CFU/ml PspA<sup>-</sup>; 6 with  $3.5 \times 10^6$  CFU/ml PspC<sup>-</sup>; and 6 with  $1.4 \times 10^6$  CFU/ml PspA<sup>-</sup>/PspC<sup>-</sup>. In an effort to conserve the number of animals used in our experiments, we did not include a saline control group. We have previously utilized this group in other studies and have found it to have no functional or histopathological changes [12,13]. Selection of intrabullar inoculation and CFU levels was based on our previous studies that resulted in otitis media in the majority of ears [13–15].

Forty-eight hours after inoculation, animals were killed by overdose of anesthesia, decapitated, their bullae removed, and middle ear effusions (MEE) harvested for bacterial CFU counts. MEEs were serially diluted in PBS, plated neat and 5 serial dilutions down and bacteria manually counted to calculate CFUs per ml. Cochleae were fixed in 2% glutaraldehyde, decalcified in ethylenediamine tetraacetic acid, and embedded in epoxy resin. Sections of round window membrane were cut at a thickness of 1  $\mu\text{m}$  and stained with toluidine blue. Round window membranes (RWMs) were bisected and one side randomly selected for histological evaluation. Digital Images of the RWMs were taken at the center and 1 mm to the right and left of center at a magnification of 1000 $\times$ . Counts were made manually in a blinded fashion using a 10  $\times$  10 unit eye piece grid calibrated in units of 0.16  $\mu\text{m}$ . The images are then printed on 8  $\times$  10 photo paper for

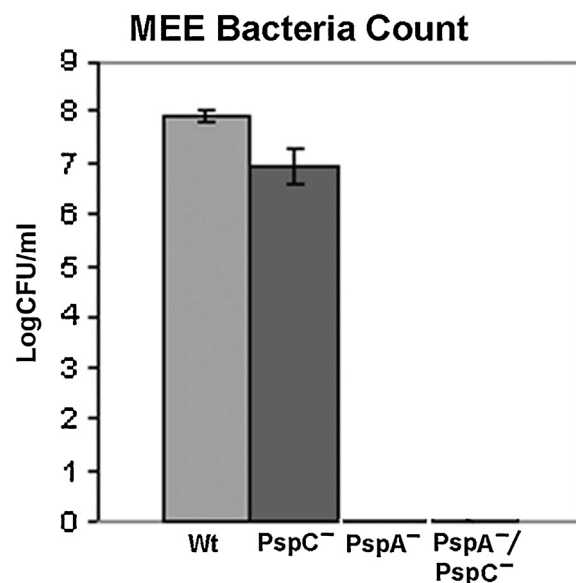
further analysis. Analysis of the RWM included the number of inflammatory cells (polymorphonuclear and mononuclear) per area. The measurements of the three selected areas of RWM were averaged for each animal, and averages were used for statistical analysis to compare all animal groups infected with the wild-type or its isogenic mutant strains. Differences between groups were analyzed with one-way analysis of variance (ANOVA) using SPSS v21 software (SPSS Inc Chicago, IL). Differences were considered to be significant if  $P \leq 0.05$ .

## 3. Results

Bacterial counts in middle ear effusions were highly increased for the wild-type (Wt) and PspC<sup>-</sup> strains at 48 h compared to the initial inoculum level. No bacteria were detected in animal groups inoculated with the PspA<sup>-</sup> and PspA<sup>-</sup>/PspC<sup>-</sup> strains (Fig. 1). Significant differences in bacterial CFUs were seen between the Wt and the PspC<sup>-</sup> groups ( $P < 0.001$ ) and the Wt compared to PspA<sup>-</sup> and PspA<sup>-</sup>/PspC<sup>-</sup> groups ( $P < 0.001$ ). We selected the round window membrane for analysis, because it is the only soft tissue barrier between the middle and inner ears. It is composed of 3 layers: an outer layer that faces the middle ear cavity and is continuous with the middle ear mucosa, a middle layer composed of collagen and elastin, and an inner layer of mesothelial cells that is continuous with the lining of the scala tympani [16].

Light microscopic analysis of the number of inflammatory cells in the RWM (Fig. 2) showed significant differences in the number of inflammatory cells in the wild-type compared to the PspC<sup>-</sup> ( $P = 0.037$ ), PspA<sup>-</sup> ( $P = 0.021$ ), and PspA<sup>-</sup>/PspC<sup>-</sup> ( $P = 0.018$ ) groups. No significant difference in the number of inflammatory cells was observed among any of the mutant groups.

Because there was variability among animals within each group, Fig. 3a–d represents the most severe pathology of the RWM and adjacent scala tympani in each group. The RWMs of chinchillas inoculated with the Wt strain (Fig. 3a) had the greatest number of inflammatory cells. In the PspC<sup>-</sup> group (Fig. 3b), the number of inflammatory cells was less than in the Wt, but greater than in



**Fig. 1.** At 48 h after inoculation bacterial counts of middle ear effusions (MEE) were greater than the inoculum levels in the wild-type (Wt) and the PspC<sup>-</sup> groups but bacteria were not detectable in the PspA<sup>-</sup> and PspA<sup>-</sup>/PspC<sup>-</sup> groups. CFUs were significantly lower ( $P < 0.001$ ) for the PspC<sup>-</sup>, PspA<sup>-</sup>, and PspA<sup>-</sup>/PspC<sup>-</sup> mutants compared to the Wt strain and for the PspA<sup>-</sup>, and PspA<sup>-</sup>/PspC<sup>-</sup> mutants compared to the PspC<sup>-</sup> single mutant.

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