NIH Public Access
Author Manuscript
Int J Pediatr Otorhinolaryngol. Author manuscript; available in PMC 2015 September 01.

Published in final edited form as:
Int J Pediatr Otorhinolaryngol. 2014 September ; 78(9): 1517–1521. doi:10.1016/j.ijporl.2014.06.024.

Pneumococcal PspA and PspC Proteins: Potential Vaccine Candidates for Experimental Otitis Media

Patricia A Schachern, BS[a], **Vladimir Tsuprun, PhD**[a], **Patricia Ferrieri, MD**[b,c], **David E Briles, PhD**[d], **Sarah Goetz, BA**[b], **Sebahattin Cureoglu, MD**[a], **Michael M Paparella, MD**[a], and **Steven Juhn, MD**[a]

[a]Department of Otolaryngology, University of Minnesota, Minneapolis, MN

[b]Department of Pediatrics, University of Minnesota, Minneapolis, MN

[c]Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN

[d]Department of Microbiology, University of Alabama at Birmingham, AL

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 ($PspA^-$ and $PspC^-$) and double ($PspA^-/PspC^-$) mutants of pneumococcal PspA and PspC proteins in the chinchilla middle ear.

Methods—Bullae of 24 chinchillas were inoculated with 0.5 ml of 10^6 colony forming units (CFUs)/ml bacteria: 6 with wild-type D39 strain; 6 with $PspA^-$; 6 with $PspC^-$; and 6 with $PspA^-/PspC^-$ 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 hours after inoculation.

Results—At 48 hours, CFUs in middle ears were increased for wild-type and $PspC^-$ strains compared to inoculum levels; however, they were significantly less for the group inoculated with the $PspC^-$ strain compared to wild-type strain. No bacteria were detected in the $PspA^-$ and $PspA^-/PspC^-$ groups. The number of inflammatory cells in the RWM was significantly higher in wild-

© 2014 Elsevier Ireland Ltd. All rights reserved.

Correspondence: Vladimir Tsuprun, Department of Otolaryngology, University of Minnesota, 2001 6th Street SE, Lions Research Building, Room 226, Minneapolis, MN 55455, tsupr001@umn.edu, Phone: 612-625-0512.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

type compared to the PspA$^-$, PspC$^-$, and PspA$^-$/PspC$^-$ 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$^-$ strain were similar to the wild-type strain. The single PspA$^-$ and double PspA$^-$ /PspC$^-$ mutants were highly attenuated in the ear. Bacterial clearance of the PspA$^-$ /PspC$^-$ 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.

Keywords

Otitis media with effusion; *Streptococcus pneumoniae*; PspA$^-$; PspC$^-$; PspA$^-$/PspC$^-$; chinchilla

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 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$^-$ and PspC$^-$) and double (PspA$^-$/PspC$^-$) mutant strains of pneumococcal PspA and PspC proteins in the chinchilla ear.

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

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

0.5 percent yeast extract (BD Diagnostics, Sparks, Maryland), plated on sheep blood agar (SBA) plates, and stored in 10% glycerin solution at - 80°C. Isogenic mutants $PspA^{-}$, $PspC^{-}$, and $PspA^{-}/PspC^{-}$ were grown on SBA plates and in THB containing 0.5% yeast extract, with 0.3 μg/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 2.3×10^6 CFU/ml wild-type; 6 with 1.6×10^6 CFU/ml $PspA^{-}$; 6 with 3.5×10^6 CFU/ml $PspC^{-}$ and 6 with 1.4×10^6 CFU/ml $PspA^{-}/PspC^{-}$. 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 ethaline diamine trichloracetic acid, and embedded in epoxy resin. Sections of round window membrane were cut at a thickness of 1 μm 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 1,000x. Counts were made manually in a blinded fashion using a 10 × 10 unit eye piece grid calibrated in units of 0.16 μm. The images are then printed on 8 × 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 0.05.

RESULTS

Bacterial counts in middle ear effusions were highly increased for the wild-type (Wt) and $PspC^{-}$ strains at 48 hours compared to the initial inoculum level. No bacteria were detected in animal groups inoculated with the $PspA^{-}$ and $PspA^{-}/PspC^{-}$ strains (Fig. 1). Significant differences in bacterial CFUs were seen between the Wt and the $PspC^{-}$ groups ($P < 0.001$) and the Wt compared to $PspA^{-}$ and $PspA^{-}/PspC^{-}$ 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

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

[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$^-$ (P = 0.037), PspA$^-$ (P = 0.021), and PspA$^-$/PspC$^-$ (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$^-$ group (Fig. 3b), the number of inflammatory cells was less than in the Wt, but greater than in groups inoculated with the PspA$^-$ (Fig 3.c) and PspA$^-$/PspC$^-$ (Fig. 3d) mutants.

DISCUSSION

The pneumococcal conjugate vaccines have been fairly successful providing serotype-specific protection, but increased antimicrobial resistance emerged among non-vaccine serotypes in pediatric populations [2,4]. One way to improve pneumococcal vaccines may be immunization with protection-eliciting, cross-reactive proteins of pneumococci, based on antigens conserved across different pneumococcal strains. Due to the genetic diversity of pneumococcus and its propensity for transformation, combinations of several protein antigens have been proposed [17]. We selected two pneumococcal surface proteins, pneumococcal surface protein A (PspA) and C (PspC) for our study. Both are highly conserved across strains [9,18] and immunogenic [5,19].

PspA inhibits complement activation and binds with apolactoferrin, preventing host killing [7]. PspA has been divided into 3 families [20]. In a study of PspA isolates of children with acute otitis media, 97% of the isolates belonged to families 1 and 2 [21]. The strain used in our study had PspA that was from family 1 clade 2 and is likely to be cross-protective against pneumococcal strains of this family and clade. PspC is similar in overall structure to PspA, but only cross-reacts with it in their shared proline rich domains [22].

PspC is a fundamental determinant in mucosal colonization [23]. Furthermore, immunization with purified PspC has been shown to protect against sepsis and is apparently mediated by antibodies that cross-react with PspA [11]. PspC is preferentially expressed in the transparent phenotype of *S. pneumoniae*, more common in the nasopharynx [24]. Immunization with PspC has been shown to elicit systemic mucosal antibody and reduce the level of nasopharyngeal carriage [25]. Thus, PspC may be a reasonable choice as an additional pneumococcal antigen to combine with PspA for vaccine design against otitis media.

In our study, CFU levels of bacteria in middle ear effusions at 48 hours post-inoculation were significantly increased over their initial inoculum in the wild-type and the PspC$^-$ groups. When the *pspC* mutation was combined with the *pspA* mutation, however, the double PspA$^-$/PspC$^-$ mutant showed the same decrease in bacterial viability as the single PspA$^-$ mutant. Our results on viability and virulence of PspA $^-$ and PspC $^-$ mutants in the ear were similar to those reported by Ogunniyi et al [25] for nasopharyngeal carriage and

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

pneumonia, where a PspC $^{-}$ strain behaved like the wild-type strain, but the absence of both PspA and PspC proteins accelerated clearance of bacteria [23].

The mutation of *pspA* gene in combination with mutations of other genes of pneumococcal proteins, however, does not always lead to a decrease in virulence. In a previous study in the ear, we found the PspA-deficient mutant was less viable and virulent than the pneumonlysin-deficient mutant (Ply^{-}) or the PspA^{-}/Ply^{-} double mutant [26]. In a study in the lung, however, virulence of the double PspA^{-}/Ply^{-} mutant was highly attenuated, but the triple PspA^{-}/PspC^{-}/Ply^{-} mutant was more virulent than its single or double mutant counterparts [25].

It has become increasingly clear that the proteins of *S. pneumoniae* have diverse strain, capsular serotype [27], and organ [26] specific responses. In this study, we used *S. pneumoniae* D39, serotype 2 strain; however, the protection against other strains from diverse pneumococcal infections requires consideration of the diversity of pneumococcal PspA and PspC proteins. In a murine model of pneumonia, effect of PspC mutation was shown to be strain-dependent, with reduced virulence observed for a PspC^{-} strain of serotype 4 and no effect in serotypes 2, 3, and 19F [28].

The organ-specific effects in viability and virulence mediated by these pneumococcal proteins may in part be related to systemic versus mucosal immunity in the host defense response of different tissues. The middle ear mounts both a mucosal and systemic immune response, and studies have shown that resistance to bacterial carriage is dependent largely on mucosal rather than systemic immunity [29], suggesting the need for an intranasal route of immunization. Comparing systemic and mucosal immunization for the prevention of otitis media is an important part of our future studies. Balachandran et al [23] showed that PspC is a mucosal immunogen that can elicit protection against nasopharyngeal colonization and suggested that it was an excellent candidate for a mucosal pneumococcal vaccine. A mutation in *pspC* does not increase bacterial clearance in the ear. If PspC is found to be effective for protection in sites other than the ear, then our data would suggest that its combination with PspA would still provide clearance of bacteria in the ear. Immunization studies are necessary, to determine the effects of multiple families of the PspA protein and its combination with other pneumococcal surface proteins.

Acknowledgments

This work was supported in part by: NIH/NIDCD R01 DC006452, NIH/NIDCD 3U24 DC011968, NIH/NIAID R01 A1021548, The International Hearing Foundation, The Starkey Foundation, and 5 M Lions International. The authors would like to thank Monika Schachern for her technical assistance in experiments. The double mutant (PspA^{-}/PspC^{-}) used here was originally produced in the lab of James Paton. PspA has been patented for use in vaccines by the University of Alabama. Because Dr. Briles is listed as an inventor on those patents, he may receive royalties if a PspA-containing vaccine is licensed.

References

1. Grevers G. Challenges in reducing the burden of otitis media disease: An ENT perspective on improving management and prospects for prevention. Int J Pediatric Otorhinolaryng. 2010; 74:572–577.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

2. Farrell DJ, Klugman KP, Pichichero M. Increased antimicrobial resistance among nonvaccine serotypes of *Streptococcus pneumoniae* in the pediatric population after the introduction of 7-valent pneumococcal vaccine in the United States. Pediatr Infect Dis J. 2007; 26:123–128. [PubMed: 17259873]

3. Zhao AS, Boyle S, Butrymowicz A, Engle RD, Roberts JM, Mouzakes J. Impact of 13-valent pneumococcal conjugate vaccine on otitis media bacteriology. Int J Pediatr Otorhinolaryngol. 2014; 78:499–503. [PubMed: 24461461]

4. Croney CM, Nahm MH, Juhn SK, Briles DE, Crain MJ. Invasive and noninvasive *Streptococcus pneumoniae* capsule and surface protein diversity following the use of a conjugate vaccine. Clin Vaccine Immunol. 2013; 20:1711–1718. [PubMed: 24006139]

5. Palaniappan R, Singh S, Singh UP, Sakthivel SKK, Ades EW, Briles DE, et al. Differential PsaA-, PspA-, PspC-, and PdB-specific immune responses in a mouse model of pneumococcal carriage. Infect Immun. 2005; 73(2):1006–1013. [PubMed: 15664944]

6. Ren B, Szalai AJ, Thomas O, Hollingshead SK, Briles DE. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of *Streptococcus pneumoniae*. Infect Immun. 2003; 71(1):75–85. [PubMed: 12496151]

7. Shaper M, Hollingshead SK, Benjamin WH Jr, Briles DE. PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin. Infect Immun. 2004; 72(9):5031–5040. [PubMed: 15321996]

8. Janulczyk R, Iannelli F, Sjoholm AG, Pozzi G, Bjorck L. Hic, a novel surface protein of *Streptococcus pneumoniae* that interferes with complement function. J Biol Chem. 2000; 275(47): 37257–37263. [PubMed: 10967103]

9. Hammerschmidt S, Talay SR, Brandtzaeg P, Chhatwal GS. SpsA, a novel pneumococcal surface protein with specific binding to secretory Immunoglobulin A and secretory component. Mol Microbiol. 1997; 25(6):1113–1124. [PubMed: 9350867]

10. Luo R, Mann B, Lewis WS, Rowe A, Heath R, Stewart ML, et al. Solution structure of choline binding protein A, the major adhesin of *Streptococcus pneumoniae.* Embo J. 2005; 24(1):34–43. [PubMed: 15616594]

11. Brooks-Walter A, Briles DE, Hollingshead SK. The *pspC* gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. Infect Immun. 1999; 67(12):6533–6542. [PubMed: 10569772]

12. Schachern PA, Paparella MM, Goycoolea M, Goldberg B, Schlievert P. The round window membrane following application of staphylococcal exotoxin: an electron microscopic study. Laryngoscope. 1981; 91(12):2007–2017. [PubMed: 7321721]

13. Tsuprun V, Cureoglu S, Schachern PA, Ferrieri P, Briles DE, Paparella MM, et al. Role of pneumococcal proteins in sensorineural hearing loss due to otitis media. Otol Neurotol. 2008; 29(8):1056–1060. [PubMed: 18833010]

14. Schachern P, Tsuprun V, Cureoglu S, Ferrieri P, Briles D, Paparella M, et al. The round window membrane in otitis media: effect of pneumococcal proteins. Arch Otolaryngol Head Neck Surg. 2008; 134(6):658–662. [PubMed: 18559736]

15. Schachern PA, Tsuprun V, Cureoglu S, Ferrieri P, Briles DE, Paparella MM, et al. Virulence of pneumococcal proteins on the inner ear. Arch Otolaryngol Head Neck Surg. 2009; 135(7):657–661. [PubMed: 19620586]

16. Schachern PA, Paparella MM, Duvall AJ 3rd, Choo YB. The human round window membrane. An electron microscopic study. Arch Otolaryngol. 1984; 110(1):15–21. [PubMed: 6689900]

17. Ogunniyi AD, Grabowicz M, Briles DE, Cook J, Paton JC. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. Infect Immun. 2007; 75(1):350–357. [PubMed: 17088353]

18. Crain MJ, Waltman WD II, Turner JS, Yother J, Talkington DF, McDaniel LS, et al. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of Streptococcus pneumoniae. Infect Immun. 1990; 58(10): 3293–3299. [PubMed: 1698178]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

19. Dave S, Brooks-Walter A, Pangburn MK, McDaniel LS. PspC, a pneumococcal surface protein, binds human factor H. Infect Immun. 2001; 69(5):3435–3437. [PubMed: 11292770]

20. Hollingshead SK, Becker R, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun. 2000; 68(10):5889–5900. [PubMed: 10992499]

21. Melin MM, Hollingshead SK, Briles DE, Hanage WP, Lahdenkari M, Kaijalainen T, et al. Distribution of pneumococcal surface protein A families 1 and 2 among *Streptococcus pneumoniae* isolates from children in Finland who had acute otitis media or were nasopharyngeal carriers. Clin Vaccine Immunol. 2008; 15(10):1555–1563. [PubMed: 18753340]

22. Daniels CC, Coan P, King J, Hale J, Benton KA, Briles DE, et al. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. Infect Immun. 2010; 78(5): 2163–2172. [PubMed: 20194601]

23. Balachandran P, Brooks-Walter A, Virolainen-Julkunen A, Hollingshead SK, Briles DE. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. Infect Immun. 2002; 70(5):2526–2534. [PubMed: 11953392]

24. Weiser JN, Austrian R, Sreenivasan PK, Masure HR. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect Immun. 1994; 62(6):2582–2589. [PubMed: 8188381]

25. Ogunniyi AD, LeMessurier KS, Graham RMA, Watt JM, Briles DE, Stroeher UH, et al. Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of *Streptococcus pneumoniae* D39 in a mouse model. Infect Immun. 2007; 75(4):1843–1851. [PubMed: 17261599]

26. Schachern PA, Tsuprun V, Goetz S, Cureoglu S, Juhn SK, Briles DE, et al. Viability and virulence of pneumolysin, pneumococcal surface protein A, and pneumolysin/pneumococcal surface protein A mutants in the ear. JAMA Otolaryngol Head Neck Surg. 2013; 139(9):937–943. [PubMed: 24051749]

27. Yuste J, Khandavilli S, Ansari N, Muttardi K, Ismail L, Hyams C, et al. The effects of PspC on complement-mediated immunity to *Streptococcus pneumoniae* vary with strain background and capsular serotype. Infect Immun. 2010; 78(1):283–292. [PubMed: 19884335]

28. Kerr AR, Paterson GK, McCluskey J, Iannelli F, Oggioni MR, Pozzi G, et al. The contribution of PspC to pneumococcal virulence varies between strains and is accomplished by both complement evasion and complement-independent mechanisms. Infect Immun. 2006; 74(9):5319–5324. [PubMed: 16926426]

29. Wu HY, Nahm MH, Guo Y, Russell MW, Briles DE. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. J Infect Dis. 1997; 175(4):839–846. [PubMed: 9086139]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

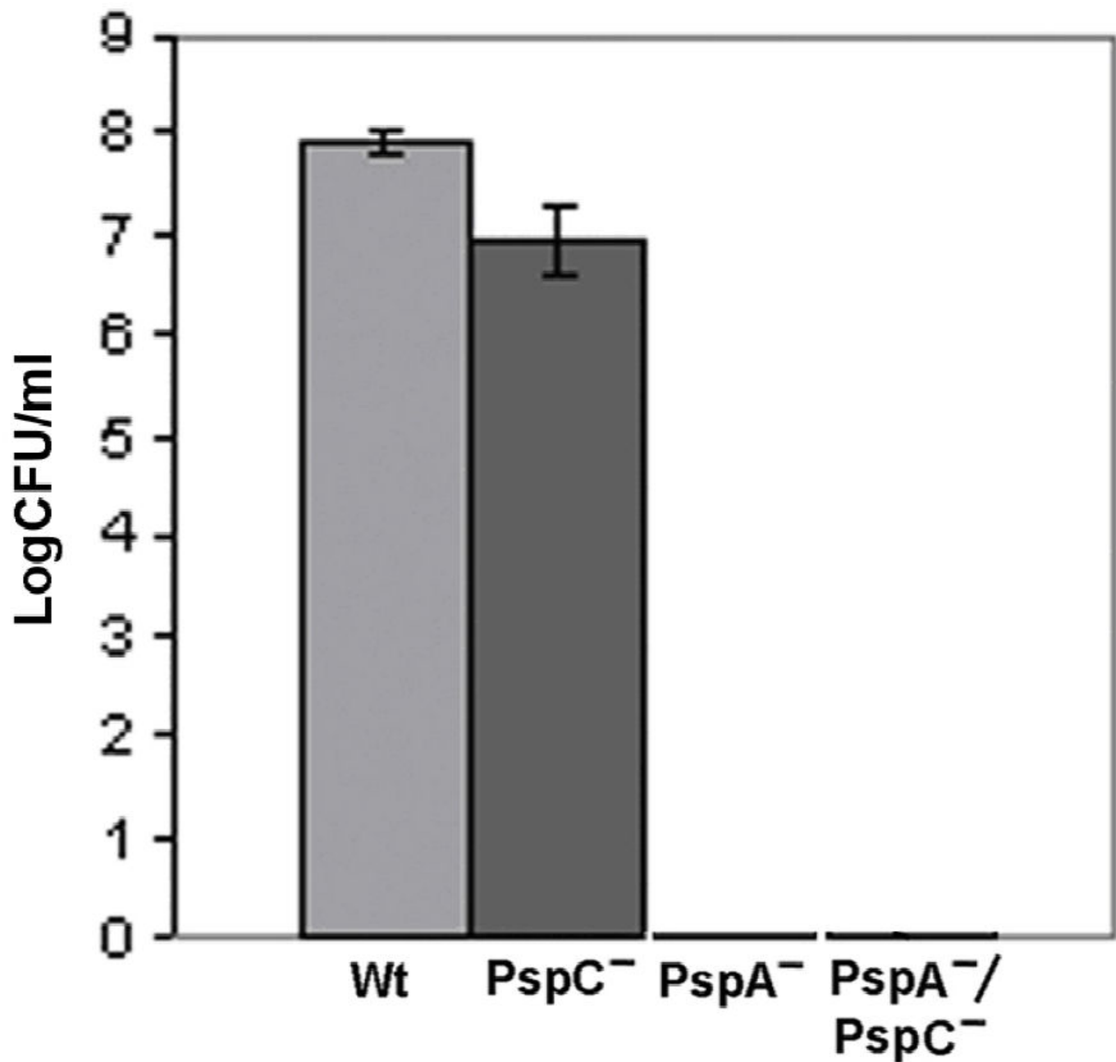


Figure 1.

At 48 hours after inoculation bacterial counts of middle ear effusions (MEE) were greater than the inoculum levels in the wild-type (WT) and the PspC $^-$ deficient mutant, but bacteria were not detectable in the PspA$^-$ and PspA$^-$/PspC$^-$ deficient mutants. CFUs were significantly lower ($P < 0.001$) for the PspC$^-$, PspA$^-$, and PspA$^-$/PspC$^-$ mutants compared to the WT strain and for the PspA$^-$, and PspA$^-$/PspC$^-$ mutants compared to the PspC $^-$ single mutant.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

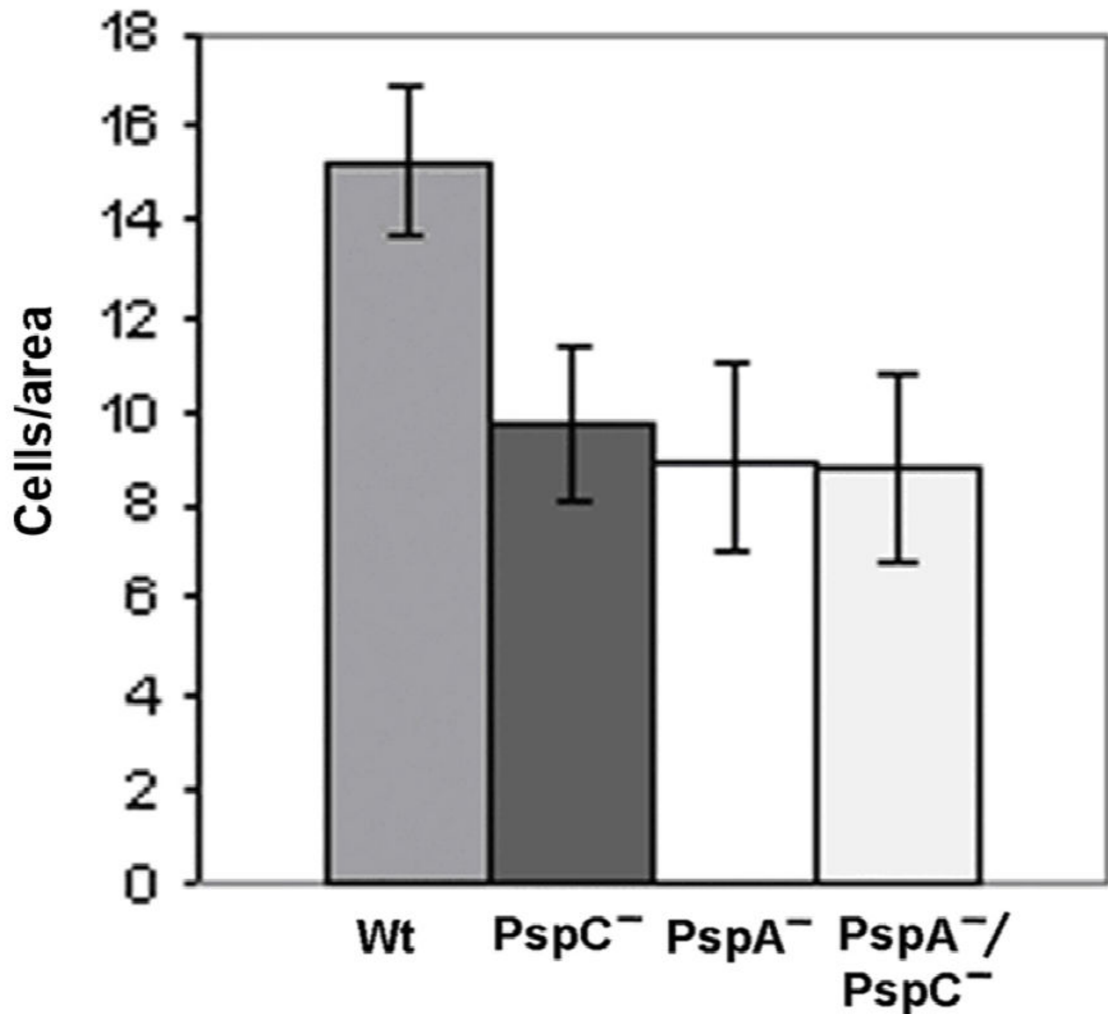
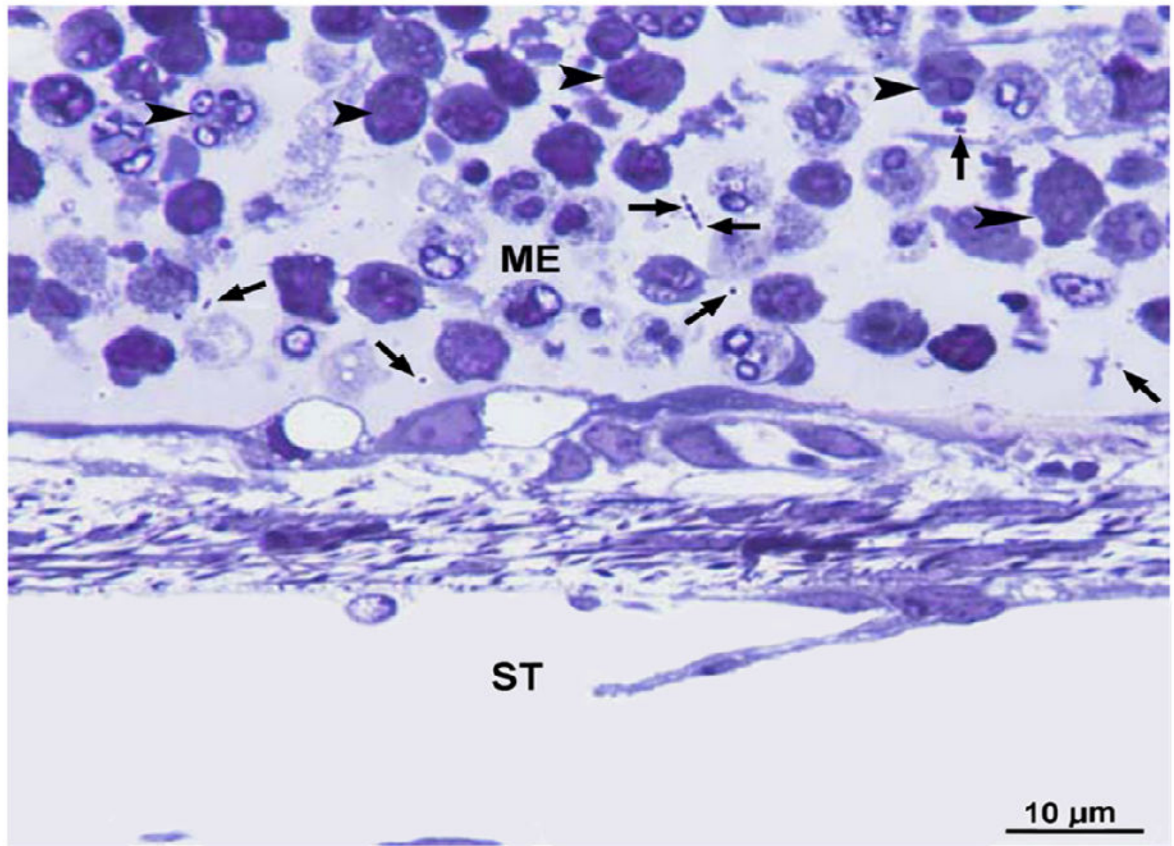


Figure 2.
Light microscopic analysis of the round window membrane (RWM) 48 hours after inoculation showed a significant difference in the number of inflammatory cells/area between the wild-type (Wt) and the PspC⁻ ($P = 0.037$), PspA⁻ ($P = 0.021$), and PspA⁻/PspC⁻ ($P = 0.018$) groups.

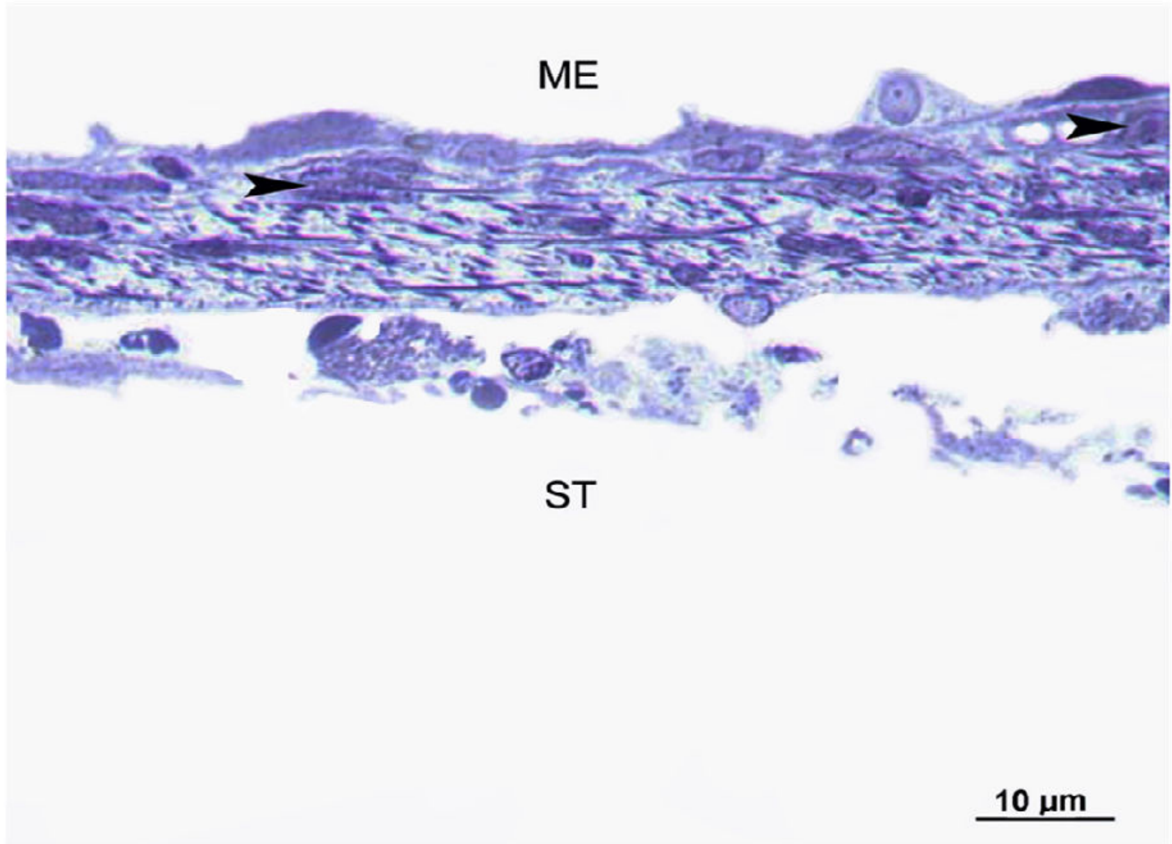
NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript



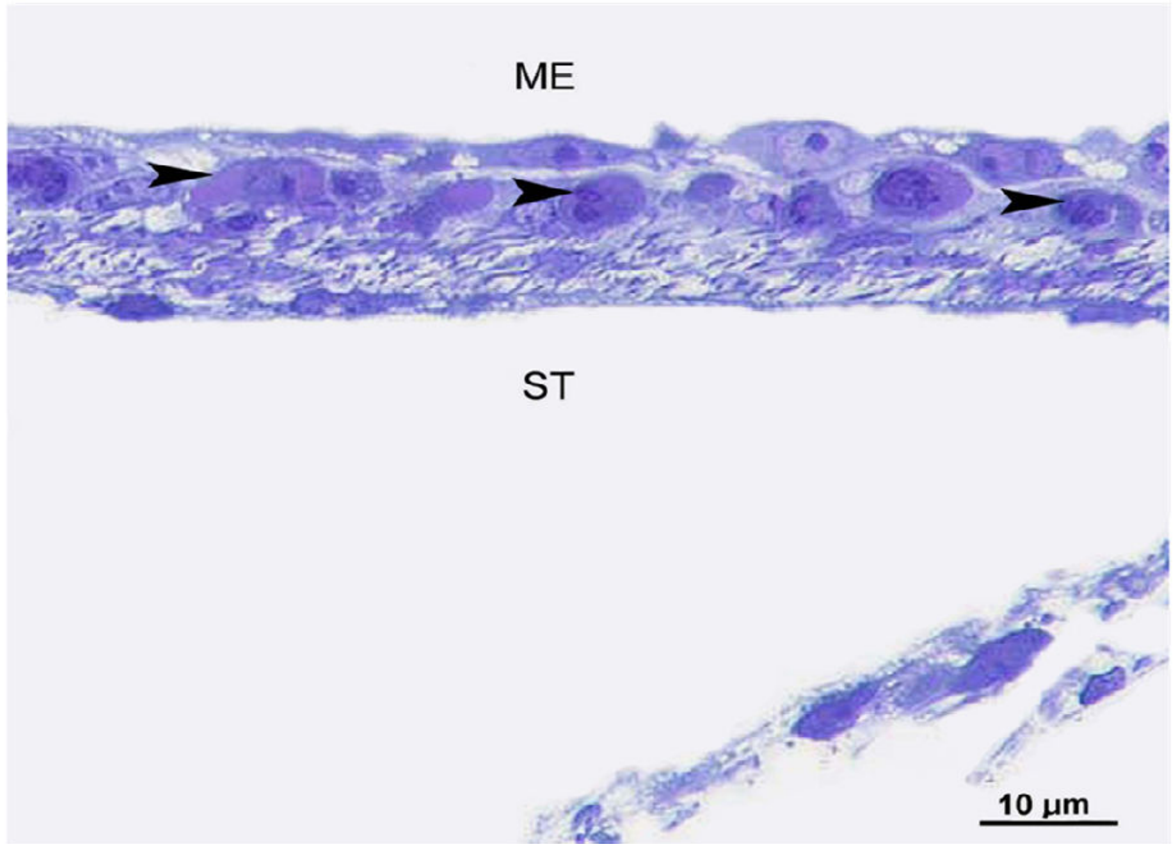
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript



NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript



NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

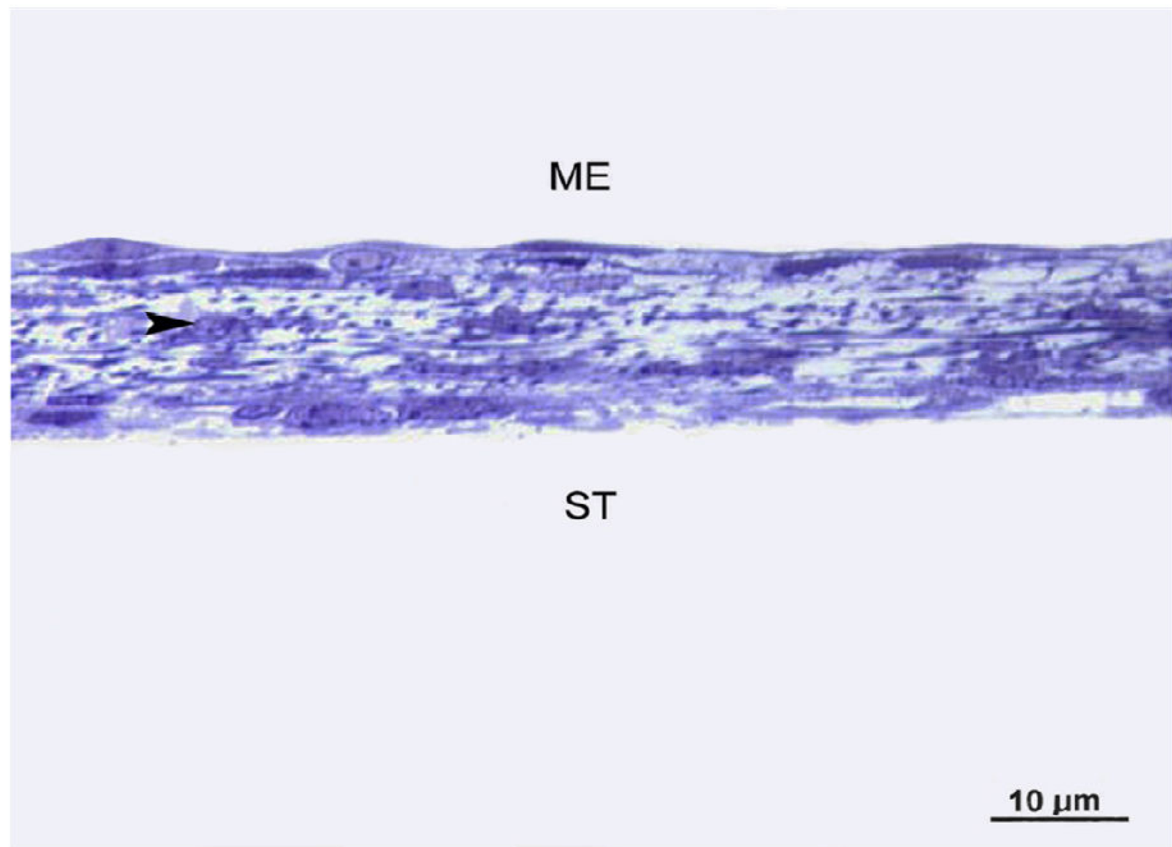


Figure 3.
This figure represents the most severe pathology of the round window membrane (RWM) in each group, at 48 hours after inoculation. Original magnification 1,000 X. Staining with toluidine blue. ME indicates middle ear. ST indicates scala tympani.

a) Ear inoculated with wild-type bacteria. There are bacteria (arrows) surrounded by numerous inflammatory cells (arrowheads) in the ME and epithelial vacuolization and subepithelial edema.

b) Ear inoculated with the PspA$^-$ strain. Arrowheads indicate inflammatory cells.

c) Ear inoculated with the PspC$^-$ strain. There is vacuolization of the epithelia facing the ME and inflammatory infiltration (arrowheads) of the RWM.

d) Ear inoculated with the PspA$^-$/PspC$^-$ strain. Arrowheads indicate inflammatory cells.