PsrP, a Protective Pneumococcal Antigen, Is Highly Prevalent in Children with Pneumonia and Is Strongly Associated with Clonal Type\textsuperscript{7}

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Invasive pneumococcal disease (IPD) is a major health problem worldwide. Due to ongoing serotype replacement, current efforts are focused in an attempt to identify the pneumococcal antigens that could be used in a next-generation multivalent protein vaccine. The objective of our study was to use real-time PCR to determine the distribution and clonal type variability of PsrP, a protective pneumococcal antigen, among pneumococcal isolates from children with IPD or healthy nasopharyngeal carriers. \textit{psrP} was detected in 52.4\% of the 441 strains tested. While no differences were determined when the prevalence of \textit{psrP} in colonizing strains (\(n=89\)) versus that in all invasive strains (\(n=352\)) was compared, a strong trend was observed when the prevalence of \textit{psrP} in all pneumonia isolates (\(n=209\)) and colonizing isolates (\(P=0.067\)) was compared, and a significant difference was observed when the prevalence in all pneumonia isolates and those causing bacteremia (\(n=76\)) was compared (\(P=0.001\)). An age-dependent distribution of \textit{psrP} was also observed, with the incidence of \textit{psrP} being the greatest in strains isolated from children >2 years of age (\(P=0.02\)). Strikingly, the presence of \textit{psrP} within a serotype was highly dependent on the clonotype, with all isolates of invasive clones such as clonal complex 306 carrying \textit{psrP} (\(n=88\)), whereas for sequence type 304, only 1 of 19 isolates carried \textit{psrP}; moreover, this was inversely correlated with antibiotic susceptibility. This finding suggests that inclusion of \textit{psrP} in a vaccine formulation would not target resistant strains. We conclude that \textit{psrP} is highly prevalent in strains that cause IPD, but is most prevalent in strains isolated from older children with pneumonia. These data support the potential use of \textit{PsrP} as one component in a multivalent protein-based vaccine.

Invasive pneumococcal disease (IPD), defined herein as the isolation of \textit{Streptococcus pneumoniae} from normally sterile sites during a clinical syndrome of infection such as bacteremia/sepsis, pneumonia, or meningitis, is an important health problem worldwide. In the year 2000, it is estimated that there were 11 million to 18 million episodes/cases of IPD and 0.7 million to 1 million deaths in children younger than 5 years of age as a result (17). \textit{Streptococcus pneumoniae} is a Gram-positive commensal that colonizes the nasopharynx of healthy children and, less frequently, adults. From the upper respiratory tract, the bacteria can be aspirated into the lungs and can translocate through mucosal cell barriers to the bloodstream and lead to development of IPD (18). This primarily occurs in young children, elderly individuals, and those who are immunocompromised.

The ability of \textit{S. pneumoniae} to cause IPD is dependent on the presence of a polysaccharide capsule that prevents phagocytosis (1). At least 92 chemically and immunologically distinct capsular types (i.e., serotypes) can be produced by the pneumococcus, with certain serotypes more frequently being associated with invasive disease (23). Importantly, while the capsule is requisite for IPD, it is insufficient alone to confer virulence; and an assortment of additional determinants such as adhesins, proteases, toxins, transport systems, and enzymes that modify the extracellular milieu are also required (25). This requirement for noncapsular virulence determinants is proven by human epidemiological studies that show that invasive and noninvvasive clonotypes exist within the most invasive serotypes, comparative genomic analyses that find an unequal distribution of noncapsular genes between invasive and noninvasive isolates within the same serotype, and scores of studies that show that deletion of noncapsular genes impact pneumococcal virulence in animal models of pneumonia, sepsis, and meningitis (7, 11, 19, 22).

One recently identified pneumococcal virulence determinant is the pneumococcal serine-rich repeat protein (PsrP), a lung cell and intraspecies bacterial adhesin that is encoded within the 37-kb pathogenicity island called \textit{psrP-secY2A2} (16). PsrP is an extremely large glycosylated cell surface protein that belongs to the serine-rich repeat protein (SRRP) family of Gram-positive bacteria (22). For the pneumococcus, the presence of PsrP has been positively correlated with strains that cause human disease, and PsrP has been shown to mediate adhesion to keratin 10 on lung cells and to mediate the formation of bacterial aggregates in the nasopharynges and lungs of infected mice (21, 22). Antibodies against PsrP neutralize...

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bacterial adhesion to cells in vitro and inhibit biofilm formation (20, 21). Furthermore, passive immunization of mice with PsrP antisera or active immunization with recombinant protein vaccinated mice against pneumococcal challenge (20). Thus, PsrP is an important virulence factor by which S. pneumoniae is able to cause IPD and is potentially a vaccine candidate.

At this time, considerable resources are being spent in an attempt to identify the pneumococcal antigens that would be used in a next-generation multivalent protein vaccine designed against the pneumococcus. The advantage of such a vaccine is that it would have a lower cost and potentially expanded global coverage compared with the cost and coverage of existing conjugate vaccines. It is generally accepted that multiple antigens will be necessary due to the fact that not all protein determinants are conserved or found within all pneumococcal strains and on their own are not able to confer sufficient protection. To this end, knowledge of the real prevalence of a protein in different clones and serotypes of Streptococcus pneumoniae is necessary to consider any protein as a candidate vaccine antigen. Therefore, the objective of our study was to determine the distribution and clonal type variability of PsrP among pneumococcal isolates from children with IPD or healthy nasopharyngeal carriers.

MATERIALS AND METHODS

Strain collection. We analyzed all invasive pneumococcal isolates collected at the Molecular Microbiology Department of the University Hospital Sant Joan de Deu, Barcelona, Spain, from January 2004 to November 2009 (n = 358). We have also included 89 strains isolated from the nasopharynges of healthy children during 2004 and 2005 (n = 89). A detailed description of our institution and the geographic area was reported elsewhere (13). IPD was defined as the presence of clinical findings of infection, including pneumonia, together with isolation of S. pneumoniae in blood, cerebrospinal fluid, or any other normally sterile fluid. The clinical syndrome was classified according to the International Classification of Disease, ninth revision (ICD-9), specific for diseases caused by S. pneumoniae, including sepsis, occult bacteremia, meningitis, pneumonia, parapneumonic empyema, peritonitis, arthritis, and endophthalmitis.

Serotyping and antimicrobial susceptibility. All isolates were serotyped by the Quellung reaction at the National Pneumococcus Reference Centre (Majadahonda, Madrid, Spain). In addition, all isolates identified during 2009 were also tested in our laboratory with a rapid specific real-time PCR of the main invasive serotypes according to the methods described for a published assay (24). The agar diffusion technique was used to determine the MICs of penicillin and other antibiotics; antibiotic susceptibility was defined according to the 2008 meningeal breakpoints of the Clinical and Laboratory Standards Institute (formerly NCCLS) (14). Isolates with intermediate or high-level resistance were defined as nonsusceptible.

MLST. Genetic characterization was performed using multilocus sequence typing (MLST). In brief, internal fragments of the arsE, gdh, gki, recP, spa, xpt, and ddl genes were amplified by PCR from chromosomal DNA of pneumococci using the primer pairs described by Enright and Spratt (5). The sequences of both DNA strands were obtained by use of an ABI 3730xl DNA analyzer (Applied Biosystems). The sequences at each of the seven loci were then compared with the sequences of all of the known alleles at that locus. Sequences that were 100% identical to any known allele were assigned new allele numbers. The assignment of alleles at each locus was carried out using the software at the pneumococcal web page (www.mlst.net). The alleles at each of the seven loci define the allelic profile of each isolate and their sequence type (ST). Allelic profiles are shown as the combination of 7 alleles in the order arsE, gdh, gki, recP, spa, xpt, and ddl. A clone is defined as a group of isolates with identical allelic profiles or STs.

OC. Isolates with genotypes with allelic profiles that differ at only one of the seven loci were called single-locus variants (SLVs). SLVs are sufficiently related to be considered members of a cluster of closely related genotypes, referred to as a clonal complex (CC). Analysis of sequence types and assignment to a clonal complex was performed with the eBURST (based upon related sequence types) program. This program compares a data set of sequence types and groups them into related genotypes and clonal complexes (6).

Extraction of DNA. Genomic DNA was extracted using Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA). Four to 5 CFU/ml was suspended in 100 μl of phosphate-buffered saline (PBS) buffer; 50 μl was transferred to a new microcentrifuge tube and vigorously vortexed with 150 μl of 20% (wt/vol) Chelex-100 in PBS. The bacterium/resin suspensions were incubated for 20 min at 56°C, followed by a 10-min incubation at 99°C. After cooling and centrifugation of the suspensions, the supernatant was used as a template in real-time PCR experiments. Free water and genomic DNA from the PsrP-carrying TIGR4 and an isogenic mutant deficient in psrP (T4 ΔpsrP) were used as positive and negative controls, respectively (16).

Real-time PCR assay. We analyzed the nucleotide sequence of psrP in TIGR4 and all other publically available S. pneumoniae genomes available through the United States National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) for primers and probe design. The primers and probe selected were as follows: the forward primer was 5'-CTTTATACATTACCCCTT ACGCTGCTA, the reverse primer was 3'-CTGAGAGGATCAGTCTAGTG TAAAGTG, and the probe was FAM-CTGGTCGCTGAGT (where FAM is 6-carboxyfluorescein; the quencher was the minor groove binder [MGB] moiety). These primers identified a conserved region within the basic region domain of psrP (16).

The reaction volume of 25 μl contained 5 μl of DNA extract from samples or controls and 12.5 μl 2× TaqMan universal master mix (Applied Biosystems), which includes dUTP and uracil- N-glycosylase; each primer was used at a final concentration of 300 nM. The TaqMan probe was used at a final concentration of 200 nM. Amplification was done under universal amplification conditions: incubation for 2 min at 50°C (uracil-N-glycosylase digestion), 10 min denaturation at 95°C, and 45 cycles of a two-step amplification (15 s at 95°C, 60 s at 60°C). Amplification data were analyzed by SDS software (Applied Biosystems). The reporter dye signal was measured relative to the internal reference dye (carboxy-X-rhodamine) signal to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. The cycle threshold (Ct) value was defined as the cycle at which the reporting dye fluorescence first exceeds the calculated background level.

Statistical analysis. Statistical analysis was performed with the PASW software package (version 17.0). Continuous variables were compared using the t test (for approximately normally distributed data) or the Mann-Whitney U test (for skewed data) and were described as mean values and standard deviations or the median and interquartile ranges (IQRs; 25 to 75th percentiles), according to the presence of a normal distribution. The chi-square test or Fisher’s exact test (two-tailed) was used to compare categorical variables. Comparison between groups was performed by the Kruskal-Wallis test. Statistical significance was set at a P value of <0.05.

RESULTS

Prevalence of psrP in clinical isolates from healthy carriers and individuals with IPD. Of the 358 invasive pneumococcal isolates in our library, 6 of them were not viable and were therefore excluded from the study. As such, we studied a total of 352 invasive pneumococcal isolates and 89 nasopharyngeal pneumococcal isolates (total of 441 strains).

Table 1 shows the prevalence of psrP in pneumococcal isolates according to clinical diagnosis, serotype, and clonal type. Overall, we detected psrP in 231 (52.4%) of pneumococcal strains tested. No significant differences were found when the prevalence of psrP in colonizing strains (43 of 89 isolates; 48.3%) was compared with that in all invasive strains (188 of 352 isolates; 53.4%) (P = 0.4). However, a strong trend was observed when the prevalence of psrP in all pneumonia isolates (125 of 209; 59.8%) was compared with that in colonizing isolates (P = 0.067). The lowest prevalence of psrP was found in strains isolated from children with bacteremia (29 of 76 strains; 38.2%). In a breakdown of those causing pneumonia, psrP was detected in 62 of 104 (59.6%) isolates causing uncomplicated pneumonia (versus colonizing strains, P = 0.1) and 63 of 105 (60%) of isolates from individuals with para-
TABLE 1. Prevalence of positive *psr*P in pneumococcal isolates in children with IPD and healthy carriers by clinical diagnosis, serotype, and clonal type

| Subject group and clinical diagnosis | No. of strains *psr*P positive/total no. of strains (%) | Serotype | No. of strains *psr*P positive/total no. of strains (%) | Clonal type | No. of strains *psr*P positive/total no. of strains (%) |
|-------------------------------------|-------------------------------------------------------|----------|--------------------------------------------------------|------------|--------------------------------------------------------|
|                                     |                                                       | Main serotype |                                                       | Main clonal type |                                                       |
|                                     |                                                       |                      |                                                       |                      |                                                       |
| IPD patients                        | 188/352 (53.4)                                        | 1                    | 84/104 (80.8)                                           | CC306       | 82/82 (100)                                            |
| Pneumonia                           | 125/209 (59.8)                                        | 19A                  | 15/36 (41.7)                                            | ST304       | 1/18 (5.5)                                             |
|                                     |                                                       | 5                    | 15/20 (75)                                              | CC2013      | 2/9 (22.2)                                             |
|                                     |                                                       | 7F                   | 2/13 (15.3)                                             | ST1201      | 5/5 (100)                                              |
|                                     |                                                       | Others               | 9/36 (25)                                               | ST191       | 2/12 (16.6)                                            |
|                                     |                                                       |                      |                                                       |                      |                                                       |
| Bacteremia                          | 29/76 (38.2)                                          | 19A                  | 12/20 (60)                                              | ST1201      | 7/7 (100)                                              |
|                                     |                                                       | 7F                   | 0/9 (0)                                                 | ST320       | 0/4 (0)                                                |
|                                     |                                                       | 14                  | 0/5 (0)                                                 | ST191       | 0/9 (0)                                                |
|                                     |                                                       | 19F                  | 3/5 (60)                                                | CC156       | 0/2 (0)                                                |
|                                     |                                                       | Others               | 14/37 (37.8)                                            | CC177       | 2/2 (100)                                              |
|                                     |                                                       |                      |                                                       |                      |                                                       |
| Meningitis                          | 25/49 (51)                                            | 19A                  | 5/8 (62.5)                                              | CC199       | 2/2 (100)                                              |
|                                     |                                                       | 19F                  | 4/5 (80)                                                | CC2013      | 0/2 (0)                                                |
|                                     |                                                       | 6A                   | 2/4 (50)                                                | CC177       | 2/2 (100)                                              |
|                                     |                                                       | 7F                   | 1/3 (33.3)                                              | ST1692      | 1/2 (50)                                               |
|                                     |                                                       | Others               | 13/29 (44.8)                                            | ST191       | 1/3 (33.3)                                              |
|                                     |                                                       |                      |                                                       |                      |                                                       |
| Other clinical syndromes^d^         | 9/18 (50)                                             | 19A                  | 2/4 (50)                                                | CC2013      | 0/1 (0)                                                |
|                                     |                                                       | 1                    | 2/2 (100)                                               | ST320       | 0/1 (0)                                                |
|                                     |                                                       | 19F                  | 1/2 (50)                                                | ST2948      | 1/1 (100)                                              |
|                                     |                                                       | 23F                  | 0/2 (0)                                                 | ST109       | 0/1 (0)                                                |
|                                     |                                                       | Others               | 4/8 (50)                                                |                      |                                                       |
|                                     |                                                       |                      |                                                       |                      |                                                       |
| Healthy carriers                    | 43/89 (48.3)                                          | 19A                  | 6/9 (66.6)                                              | CC199       | 2/2 (100)                                              |
|                                     |                                                       | 6A                   | 6/9 (66.6)                                              | CC202       | 1/2 (50)                                               |
|                                     |                                                       | 19F                  | 5/7 (71.4)                                              | CC177       | 2/2 (100)                                              |
|                                     |                                                       | 15B                  | 4/6 (66.7)                                              | ST101       | 1/2 (50)                                               |
|                                     |                                                       | 23B                  | 1/6 (16.7)                                              | ST2372      | 0/1 (0)                                                |
|                                     |                                                       | 6B                   | 2/5 (40)                                                | ST386       | 1/1 (100)                                              |
|                                     |                                                       | 9V                   | 1/5 (20)                                                | CC156       | 0/1 (0)                                                |
|                                     |                                                       | Others               | 18/42 (42.8)                                            |                      |                                                       |

^a Other serotypes in pneumonia: S14 (n = 9); S3 (n = 6); S24F/B (n = 6); S6A (n = 3); S6B (n = 3); S9V (n = 3); and S10A, S15B, S18C, S2, S38, and S4 (n = 1 each).

^b Other serotypes in bacteremia: S1 (n = 4); S3 (n = 4); S5 (n = 4); 23B (n = 3); S38 (n = 3); S6A (n = 3); S10A (n = 2); S15B (n = 2); S23F (n = 2); S34 (n = 2); S12F, S18C, S21, S22F, S24F, S27, S35B, and S4 (n = 1 each).

^c Other serotypes in meningitis: S15C, S18C, S22, S23B, S23F, S3, S5, S6BS, and 24F (n = 2 each) and S1, S10A, S12F, S13, S14, S15A, S16, S16F, S27, S31, and S9N (n = 1 each).

^d Other clinical syndromes: arthritis (n = 11), appendicitis (n = 5), pericarditis (n = 1), and peritonitis (n = 1).

^e Other serotypes in other clinical syndromes: S14, S28, S35B, S38, S4, S5, S6A, and S7F (n = 1 each).

^f Other serotypes in healthy carriers: S21 (n = 4); S23F (n = 4); S10A (n = 3); S15A (n = 3); S23A (n = 3); S3 (n = 3); S1 (n = 2); S15C (n = 2); S24 (n = 2); S29 (n = 2); S35B (n = 2); S37 (n = 2); and S10, S11, S16, S17, S28, S31, S38, S37, 7F, and 9N (n = 1 each).

Pneumococcal empyema (versus colonizing strains, *P* = 0.19). Thus, consistent with its role as a lung cell adhesin, *psr*P was more frequently present in pneumonia isolates than in colonizing isolates, albeit not to a significant level, and was present at a significantly higher rate in pneumonia isolates than in those causing bacteremia (*P* = 0.001). Surprisingly, we also observed an age-dependent distribution for *psr*P in clinical isolates from children with IPD. *psr*P was detected in 76 of 175 (46.1%) strains isolated from children with IPD less than 24 months old, while in older children, this rate was significantly higher: 112 of 187 (59.9%) (*P* = 0.01). Specifically, the prevalence of *psr*P in pneumonia isolates from children less than 24 months was 49.3% (36 of 73 strains), and that in isolates from older children was 65.4% (89 of 136 strains) (*P* = 0.02). Moreover, for isolates from older children, this 65.4% prevalence of *psr*P was significantly higher than that for nonpneumonia IPD isolates (56 of 116; 48.3%; *P* = 0.006) and that for colonizing isolates (33 of 65; 50.8%; *P* = 0.04).
Prevalence of *psrP* according to serotype of isolates. A total of 37 different serotypes were detected among the isolates causing IPD and 29 were detected among those from carriers. Significant differences in the prevalence of *psrP* were observed according to the serotype of the strains (*P* < 0.0001). Figure 1 show the distribution of *psrP* according to serotypes with more than 5 isolates. *psrP* was observed at very high frequencies in some epidemic serotypes, such as serotype 1 (91 of 113 isolates; 80.5%) and serotype 5 (22 of 27 isolates; 81.5%). In contrast, positivity for *psrP* was rarely detected in other serotypes, such as serotype 3 (2 of 15 isolates; 13.3%), serotype 7F (4 of 27 isolates; 14.8%), and serotype 14 (1 of 16 strains; 6.3%). The prevalence of *psrP* in serotype 19A, which is an emergent serotype in the geographic area where the isolates for this study were collected (12), was 51.9% (40 of 77 strains).

Importantly, the prevalence of *psrP* in serotypes included in the 7-valent conjugate vaccine (PCV-7; 26 of 70 strains; 37.1%) was significantly lower than that in nonvaccine serotypes (205 of 371; 55.3%) (*P* = 0.006). In contrast, in the context of the newly approved 10-valent pneumococcal conjugate vaccine (PCV-10) and 13-valent pneumococcal conjugate vaccine (PCV-13), the difference between vaccine and nonvaccine strains was switched. For PCV-10, a higher rate of positivity for *psrP* in the vaccine serotypes was observed: 143 of 237 (60.3%) among PCV-10 serotypes versus 88 of 204 (43.1%) among non-PCV-10 serotypes (*P* < 0.0001). For PCV-13, *psrP* was detected in 195 of 349 (55.9%) strains of PCV-13 serotypes and 36 of 92 (39.1%) strains of non-PCV-13 serotypes (*P* = 0.004).

Prevalence of *psrP* according to antibiotic susceptibility of strains. Antibiotic susceptibility information was obtained for 432 of 441 total isolates (98%). In general, a positive correlation between the presence of *psrP* and antibiotic susceptibility was observed. The prevalence of *psrP* in penicillin-susceptible strains from children with IPD was 64.5% (171 of 265 strains), whereas it was only 15.2% (12 of 79 strains) in nonsusceptible strains (*P* < 0.0001). The same pattern was observed for cefotaxime, tetracycline, and erythromycin (Table 2). Strikingly, *psrP* was absent in almost all strains with high-level resistance to penicillin or cefotaxime: only 2 (10%) of 20 strains with penicillin MICs of ≥2 μg/ml and none of 6 strains with cefotaxime MICs of ≥2 μg/ml were positive for *psrP*. No significant differences in *psrP* prevalence were found according to the susceptibilities of nasopharyngeal strains in healthy carriers.

Prevalence of *psrP* according to clonal type. Sequence and clonal type analyses were performed for 372 of 441 strains (84% of the collection). A total of 94 different sequence types were found, and these were grouped into 17 clonal complexes and 52 SLVs. The overall prevalence of *psrP* in this collection was 54.8% (204 of 372).

A significant difference in the prevalence of *psrP* was observed according to clonal type (*P* < 0.0001). Moreover, the presence or absence of *psrP* was closely related to specific genotypes but not to specific serotypes. Figure 2 shows the relative frequency of *psrP* among genotypes with more than 5 isolates. In brief, *psrP* was present in all 89 isolates (100%) belonging to CC306 (all of them serotype 1) and, in contrast, was absent in 18 of 19 isolates (5.3%) of serotype 1 belonging to ST304. Similarly, all 15 isolates belonging to ST1201 and all 11 strains belonging to CC199 (all of them serotype 19A) were positive for *psrP*. In contrast, 100% of 9 strains of serotype 19A belonging to multiresistant clone ST320 and 87% of 23 strains belonging to multiresistant clone CC230 (16 serotype 19A isolates and 7 serogroup 24 isolates) were negative for *psrP*. Other clones with a high prevalence of *psrP* were CC177 (7 of 7 isolates) and CC289 (21 of 26 isolates).
### DISCUSSION

The current conjugate pneumococcal vaccines, in which capsular polysaccharides are bound to either diphtheria or tetanus toxoid, are immunogenic and efficacious in children and prevent disease caused by the serotypes whose capsule types are in the vaccine (2, 4, 26). However, as these vaccines do not cover the full spectrum of invasive pneumococcal serotypes, temporal and geographic changes in serotype frequency associated with IPD exist. The pneumococcus is able to replace its capsule type through natural transformation, and children remain at risk of infection by nonvaccine serotypes. Moreover, the possibility of serotype shift, where the nonvaccine serotypes acquire an ecological advantage for increased carriage prevalence and, concomitantly, disease, remains real (8).

A possible solution to this problem is either replacement of the carrier toxoid with a conserved and highly antigenic single pneumococcal protein, thus providing serotype-independent protection, or alternatively, if a single antigen is insufficient, creation of a multivalent protein vaccine that eschews the capsular polysaccharide entirely. At this time, it is not clear which approach is best or which pneumococcal protein(s) should be included in any revised vaccine formulations. To address these issues, detailed molecular epidemiology is required to assess the frequency and distribution of various pneumococcal determinants in invasive clinical isolates. This is the first large study analyzing the prevalence of psrP in pneumococcal isolates from children with IPD and healthy nasopharyngeal carriers. The results of our study are in agreement with published data regarding the function of this new pneumococcal virulence factor and provide clues to the forces responsible for the spread of the pathogenicity island encoding PsrP, psrP-secY2A2, among different serotypes and clones.

The increased frequency of the gene encoding PsrP in clinical isolates from individuals with pneumonia compared with the frequency in those isolated from the nasopharynges of healthy carriers or children with bacteremia is consistent with published findings showing that PsrP is exclusively a lung cell adhesin and that it does not play a role in the nasopharynx during colonization or in the bloodstream in an intraperitoneal model of sepsis. These data also suggest that PsrP alone would protect against only 60% of strains that are capable of causing pneumonia. Thus, these findings indicate that, at best, PsrP could be a single component of a multicomponent vaccine formulation. The inclusion of a second or third protein that protects against bacteremia and whose coverage helps to cover the ∼40% of invasive isolates that lack PsrP would be required.

Given that psrP was found to be predominantly associated with antimicrobial-susceptible isolates, it can be inferred that its inclusion within any vaccine would not serve as a mechanism to decrease the incidence of existing multidrug-resistant pneumococcal isolates. It also suggests that the extensive use of antimicrobials within the community is not responsible for promoting the preponderance of clonotypes that carry psrP. Counter to the latter view, we have recently shown that PsrP mediates the formation of bacterial aggregates within the lungs and the formation of more dense biofilms in vitro (20). As psrP is predominantly found in antimicrobial-susceptible isolates and bacterial aggregates and biofilms are considered to be more resistant to antimicrobials, it is a distinct possibility that in the absence of a dedicated antimicrobial resistance mechanism, PsrP confers resistance to susceptible isolates in vivo through enhanced biofilm formation. Thus, antimicrobial pressures may be serving to maintain psrP within susceptible clonotypes. To test this hypothesis, ongoing experiments are testing the resistance of these PsrP-mediated aggregates to antimicrobials.

When we stratified the incidence of psrP in children with pneumonia by age, we found that 65.4% of pneumococcal strains that caused disease in children greater than 2 years of age carry this virulence gene. This rate was significantly higher than the rate found in strains that cause disease in young children. One possible explanation for this may be the serotype distribution of psrP. psrP was found to be predominant in serotypes not covered by the 7-valent conjugate vaccine. In the prevaccine era, the 7 serotypes included in the vaccine were the full spectrum of invasive pneumococcal serotypes, temporal and geographic changes in serotype frequency associated with IPD exist. The pneumococcus is able to replace its capsule type through natural transformation, and children remain at risk of infection by nonvaccine serotypes. Moreover, the possibility of serotype shift, where the nonvaccine serotypes acquire an ecological advantage for increased carriage prevalence and, concomitantly, disease, remains real (8).

A possible solution to this problem is either replacement of the carrier toxoid with a conserved and highly antigenic single pneumococcal protein, thus providing serotype-independent

### TABLE 2. Prevalence of positivity for psrP according to antimicrobial resistance (meningeal breakpoints) in pneumococcal isolates in children with IPD and healthy carriers

| Subject group, antimicrobial, and MIC | No. of strains psrP positive/total no. of strains (%) | P     |
|-------------------------------------|-----------------------------------------------|-------|
| **Patients with IPD**               |                                               |       |
| Penicillin                          |                                               |       |
| MIC ≈ 0.06 µg/ml                   | 171/265 (64.5)                               | <0.000|
| MIC ≈ 0.12 µg/ml                   | 12/79 (15.2)                                 |       |
| Cefotaxime                          |                                               |       |
| MIC ≈ 0.5 µg/ml                    | 180/307 (58.6)                               | <0.000|
| MIC = 1 µg/ml                      | 3/31 (9.7)                                   |       |
| MIC ≥ 2 µg/ml                      | 0/6 (0)                                      |       |
| Erythromycin                        |                                               |       |
| MIC ≈ 0.25 µg/ml                   | 162/267 (60.7)                               | <0.000|
| MIC = 0.5 µg/ml                    | 0/0                                           |       |
| MIC ≥ 1 µg/ml                      | 21/77 (27.3)                                 |       |
| Tetracycline                        |                                               |       |
| MIC ≈ 2 µg/ml                      | 160/262 (61.1)                               | <0.000|
| MIC = 4 µg/ml                      | 1/1                                           |       |
| MIC ≥ 8 µg/ml                      | 21/78 (26.9)                                 |       |
| **Healthy carriers**                |                                               |       |
| Penicillin                          |                                               |       |
| MIC ≈ 0.06 µg/ml                   | 33/61 (54.1)                                 | 0.10  |
| MIC ≈ 0.12 µg/ml                   | 9/27 (33.3)                                  |       |
| Cefotaxime                          |                                               |       |
| MIC ≈ 0.5 µg/ml                    | 38/76 (50)                                   | 0.49  |
| MIC = 1 µg/ml                      | 3/10 (30)                                    |       |
| MIC ≥ 2 µg/ml                      | 1/2 (50)                                     |       |
| Erythromycin                        |                                               |       |
| MIC ≈ 0.25 µg/ml                   | 30/62 (48.4)                                 | 1     |
| MIC = 0.5 µg/ml                    | 0/0                                           |       |
| MIC ≥ 1 µg/ml                      | 12/26 (46.2)                                 |       |
| Tetracycline                        |                                               |       |
| MIC ≈ 2 µg/ml                      | 34/65 (52.3)                                 | 0.22  |
| MIC = 4 µg/ml                      | 0/0                                           |       |
| MIC ≥ 8 µg/ml                      | 8/23 (34.8)                                  |       |
occupied by these 7 serotypes and is then filled with those from the nonvaccine serotypes at a later age. Studies by Melegaro and colleagues would support this explanation (10). However, in our study, this is not a valid explanation because PCV-7 serotypes were not found to be predominant in either younger children or older children (36 of 189 [19%] in children less than 2 years of age versus 34 of 252 [13.5%] in children ≥2 years of age; \( P = 0.1 \)). Thus, other reasons, which are not yet clear, must explain why strains that carry psrP cause IPD at a later age than those that do not. Importantly, the fact that psrP is found predominantly in the PCV-7 nonvaccine serotypes suggests that its inclusion would expand coverage beyond that of the current vaccine. However, this is less so for the PCV-10 and PCV-13 formulations due to the inclusion of serotypes 1 and 19A, the strains of which have a high frequency of psrP. Of note, the reported prevalence of psrP among PCV-7 serotype strains were isolated in our study. It would therefore be interesting to test a collection of clinical isolates archived prior to the introduction of the vaccine. Such a study would determine if the current 60% incidence of psrP in pneumonia clinical isolates was due to serotype replacement (i.e., positive selection of nonvaccine serotypes with clones that carry psrP) or if psrP has been prevalent all along among serotypes and clones that frequently cause pneumonia.

Finally, we observed the presence of psrP in certain clonotypes and its absence in others. For example, all strains of serotype 1 with CC306 were positive for psrP, while only 1 of 18 strains with ST304 was positive. ST306, together with ST304, ST228, and others, belongs to lineage A of serotype 1, which is the major lineage detected in North America and Europe (3). According to some epidemiological studies ST306 has been related to several outbreaks of invasive pneumococcal disease (9) and the emergence of pleuropneumonia in the vaccine era (15), while ST304 has not. The high prevalence of PsrP, a lung cell bacterial adhesin, in ST306 strains could be associated with this fact. It is not clear why psrP would be present in some isolates but not others; however, this suggests that other pneumococcal virulence determinants compensate for the absence of PsrP. Thus, detailed comparative genomic analyses of invasive clonotypes within the same serotype containing psrP and not containing psrP may be warranted to identify the compensatory determinants that are responsible for disease and, moreover, to determine if their inclusion along with PsrP in a multicomponent vaccine would enhance coverage.

In conclusion, psrP is highly prevalent in our clinical collection and is mainly present in strains isolated from older children with pneumonia. The distribution of psrP seemed to be strongly associated with antimicrobial sensitivity, non-PCV-7 serotypes, and specific clonotypes of pneumococci. These data support the potential use of PsrP as a protective antigen in the design of a next generation of protein-based combination vaccines. However, the data also indicate that additional components that fill the bacteremia and serotype niche not covered by PsrP are required.

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