Novel Immunoprotective Proteins of *Streptococcus pneumoniae* Identified by Opsonophagocytosis Killing Screen

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ABSTRACT The success of polysaccharide conjugate vaccines represents a major advance in the prevention of pneumococcal disease, but the power of these vaccines is limited by partial spectrum of coverage and high cost. Vaccines using immunoprotective proteins are a promising alternative type of pneumococcal vaccines. In this study, we constructed a library of antisera against conserved pneumococcal proteins predicted to be associated with cell surface or virulence using a combination of bioinformatic prediction and immunization of rabbits with recombinant proteins. Screening of the library by an opsonophagocytosis killing (OPK) assay identified the OPK-positive antisera, which represented 15 (OPK-positive) proteins. Further tests showed that virtually all of these OPK-positive antisera conferred passive protection against lethal infection of virulent pneumococci. More importantly, immunization with recombinant forms of three OPK-positive proteins (SP148, PBP2b, and ScpB), alone or in combination, conferred significant protection against lethal challenge of pneumococcal strains representing capsular serotypes 3, 4, and 6A in a mouse sepsis model. To our best knowledge, this work represents the first example in which novel vaccine candidates are successfully identified by the OPK screening. Our data have also provided further confirmation that the OPK activity may serve as a reliable *in vitro* surrogate for evaluating vaccine efficacy of pneumococcal proteins.

KEYWORDS *Streptococcus pneumoniae*, protein antigen, antiserum, opsonophagocytosis killing, immunoprotection, vaccine

Streptococcus pneumoniae (pneumococcus) is a common cause of community-acquired pneumonia, meningitis, sepsis, and otitis media (1–3). Young children, elderly people, and individuals with immunodeficiency are the most susceptible to *S. pneumoniae* infections (4). Virtually all clinical isolates of *S. pneumoniae* are enveloped by a polysaccharide capsule, which prevents bacterial cell aggregation and helps evade phagocytic killing (5). However, pneumococcal capsule is highly variable in chemical structure and immunological properties among strains. More than 90 capsular serotypes have been identified in *S. pneumoniae* (6), which represents the most variable capsule of microorganisms known to date (7).

The capsular polysaccharides (CPS) of *S. pneumoniae* are immunoprotective and serve as the immunogens in current vaccines (8). These vaccines contain either CPS alone from multiple serotypes (e.g., pneumococcal polysaccharide vaccine [PPV]) or CPS from a subset of the serotypes chemically conjugated to a protein carrier (e.g., pneumococcal conjugate vaccine [PCV]). The broadest PPV and PCV vaccines include 23 (e.g., Pneumovax) and 13 (e.g., Prevnar 13) serotypes, respectively. While the CPS-based vaccines have substantially reduced invasive pneumococcal disease (IPD) in the countries where the vaccines are used (9), their impact is in part hampered by limited coverage of pathogen serotypes. A recent carriage study in children revealed that, after

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introduction of PCVs, many new isolates with non-PCV13 capsular types have emerged (e.g., types 22F, 12F, 33F, 24F, 15C, 15B, 23B, 10A, and 38) (10). The nonvaccine types also cause a considerable proportion of IPD in children across many parts of the globe (11). Furthermore, nonencapsulated pneumococci (NESp) have been frequently isolated in clinical cases although the NESp isolates were mostly identified in nasal carriage and non-IPD episodes (12). Some of the NESp strains carry surface proteins in the capsule locus (13, 14). The loss of capsule allows the nonencapsulated strains to evade vaccine immunity targeting the CPS. Last, the polysaccharide vaccines are associated with multiple complex steps in the manufacturing process and thus high cost (15). As a result, the CPS-based vaccines have not been widely applied in many developing countries where most cases of pneumococcal disease occur without subsidies from the developed world (16, 17).

It has been well known that humans acquire immunity to pneumococcal infection after exposure to *S. pneumoniae* (1). This acquired natural immunity depends on antibody response to protein antigens (18). A number of pneumococcal proteins have been identified as immunoprotective antigens in previous studies (19), including those identified in several large-scale screens (20–22). Numerous conserved proteins have been shown to confer protection in animal models against lethal infection by multiple serotypes of virulent *S. pneumoniae* strains. These protection-eliciting proteins include pneumococcal surface protein A (PspA) (23, 24), peptidoglycan hydrolase PcsB (20), autolysin LytA (21, 25), and endo-beta-*N*-acetylglucosaminidase LytB (21, 26). Immunization with certain combinations of pneumococcal proteins proves to be safe in humans (19, 27–29). These lines of evidence strongly suggest that protein-based vaccines with broad protection efficacy can serve as an alternative, or complement, to the polysaccharide-containing vaccines. However, there is not yet an approved protein-based vaccine for the control of pneumococcal disease.

Antibody-mediated opsonophagocytosis is the major protection mechanism of the CPS-based vaccines (30–32). This principle allows the vaccine efficacy in the immunized individuals to be quantitatively assessed by antibody response using the opsonophagocytosis killing (OPK) assay (33, 34). Previous studies revealed that antibodies against pneumococcal proteins also promote bacterial clearance from the blood through opsonophagocytosis in a complement- and phagocyte-dependent manner (20, 24, 26, 35). Daniels et al. have recently described a modified version of the OPK assay to evaluate the level of protective antibody against PspA by increasing the incubation time (36). In this study, we screened a library of rabbit antisera against pneumococcal proteins for promoting opsonophagocytosis killing of pneumococci using a modified OPK assay. This test identified 15 OPK-positive proteins. Further characterization of the four OPK-positive proteins validated their capacity of inducing serotype-independent protection against lethal infection of virulent *S. pneumoniae* in a sepsis mouse model.

RESULTS

Generation of an antibody library for pneumococcal proteins. In the context of the limitations associated with the current polysaccharide vaccines, we conducted a genome-wide screen of pneumococcal virulence factors and surface-exposed proteins for immunoprotective proteins by a combination of bioinformatic prediction and OPK tests. Using the complete genomes of 39 pneumococcal strains, we first conducted bioinformatic mining of highly conserved pneumococcal proteins that are potentially associated with the cell surface or that have been implicated as virulence factors in literature using the Vaccine Investigation and Online Information Network (VIOLIN)/Vaxign reverse vaccinology pipeline (37). The lack of human homologs was another consideration in the Vaxign computation. This search identified a list of 220 genes encoding highly conserved proteins. Cloning of these genes in a pGEX-2T plasmid yielded protein expression constructs for 148 genes, each of which yielded soluble glutathione *S*-transferase (GST) fusion proteins, as detected by SDS-PAGE after induction with isopropyl- β -D-thiogalactopyranoside (IPTG) (data not shown). To generate antiserum to each protein, the proteins were purified by affinity chromatography to

FIG 1 OPK screening of an antiserum library for opsonophagocytosis killing of pneumococci. (A) Opsonophagocytosis killing of pneumococci by selected antisera. Pneumococci (serotype 19F strain ST556; 1,000 CFU) were incubated with differentiated HL-60 cells in the presence of complement and serially diluted heat-inactivated antiserum of pneumococcal proteins (solid line). A pool of 10 nonimmune serum samples (dashed line) was used as a negative control. After incubation, CFU (live bacteria) were quantified to estimate the number of killed bacteria in the reaction mixture at each dilution of the antiserum. The horizontal dashed lines indicate 50% killing of the bacteria. (B) The number of unique proteins represented by the antisera at each level of the OPK activity. The antiserum library representing 119 pneumococcal proteins was divided into four groups according to the dilution at which the serum showed 50% killing in the OPK screen. The number of the proteins represented by each group is shown at the top of each bar. When antiserum samples from multiple rabbits represented a single protein, only the sample with the highest OPK activity was included.

individually immunize rabbits (at least two animals/protein). Further immunoblotting detection yielded 186 antisera that reacted with 119 unique pneumococcal proteins (see Table S2 in the supplemental material; also data not shown). This list included several proteins that have been shown to be vaccine candidates, such as PspA (23, 24), PcsB (20), LytA (21, 25), and LytB (21, 26).

Identification of OPK-positive proteins. It has been demonstrated that antibodies specific for immunoprotective proteins are also able to mediate opsonophagocytosis killing of pneumococci, such as PcsB and PspA (20, 36). To facilitate identification of novel immunoprotective proteins, we screened the antiserum library for enhancing opsonophagocytosis killing of pneumococci by an OPK assay to detect protective antibodies for PspA (36). This screen was performed with the 186 antisera that were immunoreactive to their corresponding proteins. To minimize the impact of anti-pneumococcus activity in the normal rabbit serum, a mixture of nonimmune sera collected from 10 rabbits before immunization was pooled and used as a nonimmune serum or negative control for this screen. As represented in Fig. 1A, in the OPK assay

TABLE 1 OPK-positive antisera and corresponding immunogens

Immunogen ^a	TIGR4 ID ^b	Antiserum ID ^c	Description
AsnA	SP1970	AB436	Asparagine synthetase
CodY	SP1584	AB295	GTP-sensing transcriptional repressor
Eno	SP1128	AB275	Enolase
LytA	SP1937	AB291	Autolysin
LytB	SP965	AB414	Endo-beta- <i>N</i> -acetylglucosaminidase
MvaK2	SP383	AB479	Phosphomevalonate kinase
PcsB	SP2216	AB270	PcsB protein required for cell separation
PBP2b	SP1673	AB267	Penicillin-binding protein 2B
ProWX	SP1860	AB183	Putative transporter permease
PspA	SP117	AB557	Pneumococcal surface protein A
ScpB	SP1875	AB514	Putative segregation and condensation protein B
SP148	SP148	AB281	ABC transporter substrate-binding protein
SP285	SP285	AB279	Zinc-containing alcohol dehydrogenase
SP7	SP7	AB505	Small heat shock protein
SpxB	SP730	AB160	Pyruvate oxidase

^aImmunogen proteins are labeled with either their gene names or numbers in the TIGR4 genome (GenBank accession number [AE005672](#)).

^bGene identification (ID) is listed as in the TIGR4 genome.

^cAntisera are labeled with their identification (ID) numbers in our antibody collection; only a single antiserum is listed for each protein when more than one OPK-positive sample was identified.

the percentage of killed bacteria decreased following serial dilutions of antiserum. The antisera against PspA and PcsB achieved killing of $\geq 50\%$ pneumococci at dilutions of 1:108 and 1:36, respectively. We thus used a dilution of 1:36 as a cutoff value for the OPK-positive antiserum samples.

This screen yielded a total of 17 opsonophagocytosis killing (OPK)-positive antisera (Fig. 1A and Table S2). These sera and their corresponding immunogens are referred to as OPK-positive antisera and proteins, respectively. These OPK-positive antisera represented 15 pneumococcal proteins (Table 1), including PcsB and PspA, two known vaccine candidates (20, 36). The remaining antisera reached the 50% bacterial killing level at relatively lower dilutions, which represented 33 proteins with effective dilutions between 1:12 and 1:36, 63 with dilutions between 1:4 and 1:12, and 8 with dilutions below 1:4 (Fig. 1B and Table S2). Eight of the 15 OPK-positive proteins identified by this screen are known or predicted to be associated with cell wall or membrane, including PspA (23), PcsB (20), LytA (21, 25), LytB (21, 26), enolase, penicillin-binding protein 2b (PBP2b), the putative transporter permease ProWX, and the putative transporter substrate-binding lipoprotein SP148. The other seven proteins do not have obvious signal sequence for secretion or transmembrane segments and thus appear to be cytoplasmic proteins (i.e., the asparagine synthetase AsnA, the transcriptional regulator CodY, the phosphomevalonate kinase MvaK2, the putative segregation and condensation protein ScpB, the pyruvate oxidase SpxB, the small heat shock protein SP7, and the alcohol dehydrogenase SP285).

Passive protection of the OPK-positive antiserum against lethal pneumococcal infection. We next tested the OPK-positive antibodies for their capacity for passive protection against pneumococcal lethal infection in a mouse sepsis model. ST870, a virulent serotype 6A strain of *S. pneumoniae*, was used in the challenge experiments (see Materials and Methods for details). Each of the OPK-positive antisera or nonimmune sera was heat inactivated and premixed with 100 CFU of ST870 to a final concentration of 10% (vol/vol) and used for intraperitoneal (i.p.) infection. While all of the mice in the nonimmune serum group succumbed to the infection within 3 days, the antisera representing 14 of the 15 OPK-positive proteins showed significant protective activity against lethal infection (Table 2). All of the mice survived the otherwise lethal infection when they simultaneously received the antisera for PspA, PcsB, LytB, MvaK2, SpxB, PBP2b, and SP285. Partial protection was observed with the antisera for seven other proteins (80% survival for Eno, LytA, SP7, SP148 and ProWX; 60% survival for CodY and ScpB). The antiserum of AsnA did not show any protective efficacy. As a control, the rabbit antiserum raised with PCV7 also showed full protection, which was likely due to

TABLE 2 Passive protection of antisera against lethal infection by ST870

Immunogen	Antiserum ID	Survival on day 14 (%)	<i>P</i> value ^a
AsnA	AB436	0	0.3161
CodY	AB295	60	0.0105
Eno	AB275	80	<0.0001
LytA	AB291	80	<0.0001
LytB	AB414	100	<0.0001
MvaK2	AB479	100	<0.0001
PBP2b	AB267	100	<0.0001
PcsB	AB270	100	<0.0001
ProWX	AB183	80	0.0012
PspA	AB557	100	<0.0001
ScpB	AB514	60	<0.0001
SP148	AB281	80	<0.0001
SP285	AB279	100	<0.0001
SP7	AB505	80	<0.0001
SpxB	AB160	100	<0.0001
PCV7	AB525	100	<0.0001
	Nonimmune	0	

^aStatistical comparison was determined by a log rank (Mantel-Cox) test.

the cross-protection against infection of serotype 6A pneumococci by the serotype 6B CPS in the vaccine.

We next validated the passive protection regimen by separate inoculation of the antiserum and pathogen. Four of the antisera conferring passive protection (targeting PBP2b, SP148, ScpB, and SpxB), along with the anti-PspA serum (positive control), were individually inoculated into BALB/c mice by intraperitoneal injection 1 h prior to infection with ST870. Consistent with the result of the initial trial (Table 2), separate inoculation of the antiserum and pathogen also led to significant protection of the mice against lethal infection of the pneumococci. Except for the anti-SpxB serum (100% survival) (Fig. 2), partial protection was observed with the mice pretreated with the antisera for PBP2b (70% survival), SP148 (50% survival), ScpB (60% survival), and PspA (90% survival). The relatively lower rates of protection shown in Fig. 2 might be due to higher levels of opsonization *in vitro* in the initial trial with the premixture of the antiserum and pathogen. We also attempted to test the potential antigen-independent

FIG 2 Passive protection of the selected rabbit antisera against lethal infection by ST870. BALB/c mice were i.p. inoculated with 10 μ l of rabbit antiserum against SP148 (A), PBP2b (B), ScpB (C), and PspA (D) 1 h prior to i.p. infection with serotype 6A strain ST870 (100 CFU). Nonimmune serum (dashed line) was used as a negative control. The results from at least two independent experiments were combined for statistical comparison using a log rank (Mantel-Cox) test ($n = 10$ to 11). **, $P < 0.01$; ***, $P < 0.001$.

FIG 3 Immunoprotection of selected OPK-positive proteins against lethal infection by ST870. BALB/c mice were subcutaneously immunized with adjuvant-emulsified SP148 (A), PBP2b (B), ScpB (C), SpxB (D), PspA (E), or PcsB (F). The control group (dashed line) received adjuvant alone ($n = 26$). After the final boost immunization, the mice were i.p. challenged with a lethal dose of ST870 (100 CFU/mouse). Survival was monitored for 2 weeks postinfection. The results from at least two independent experiments were combined for statistical comparison between the immunized and control groups using a log rank (Mantel-Cox) test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The number of mice used in each group is described in Table 3.

detrimental effect of the antiserum on pneumococcal survival by performing a passive protection assay with pneumococcal mutants lacking the target proteins. The rationale was that if the protection of an antiserum is due to its nonspecific toxicity toward the pneumococcus, removing the corresponding target antigen should not affect the protection activity. Unfortunately, some of the target genes (e.g., PBP2b) could not be deleted, while the mutants of the other genes (e.g., SP148, ScpB, and SpxB) lost virulence in the septic mouse model. Nevertheless, these results revealed a strong association between the OPK activity and *in vivo* protection of antibodies against pneumococcal proteins, supporting the reliability of the OPK assay as an *in vitro* surrogate for evaluation of immunoprotection by pneumococcal protein antigens.

Vaccine efficacy of the OPK-positive proteins against lethal pneumococcal infection. To determine the immunoprotection efficacy of the OPK-positive proteins, we performed immunization in BALB/c mice with recombinant forms of ScpB, PBP2b, SP148, and SpxB in the sepsis model, using 4 of the 15 OPK-positive proteins. After i.p. challenge with the lethal dose of strain ST870, the mice immunized with each of the four proteins displayed significant immunoprotection against lethal infection. The mice immunized with ScpB showed the highest survival rate (50.0%; $n = 10$, $P = 0.0008$) and the longest mean survival time (8.9 days) (Fig. 3A and Table 3). The SP148 group displayed an intermediate level of protection (survival rate, 27.2%; mean survival time, 7.7 days; $n = 11$, $P = 0.0023$) (Fig. 3B and Table 3). Immunization with PBP2b led to a similar level of protection (survival rate, 33.3%; mean survival time, 6.7 days; $n = 15$, $P = 0.0089$) (Fig. 3C and Table 3). SpxB showed the least protection (survival rate, 13.3%; mean survival time, 5.9 days; $n = 15$, $P = 0.0155$) (Fig. 3D and Table 3). PspA (Fig. 3E) ($n = 20$) and PcsB (Fig. 3F) ($n = 10$), two known immunoprotective pneumococcal proteins (20, 36), displayed the same levels of protective efficacy (40% survival). In sharp contrast, all of the mice receiving just the adjuvant succumbed to the lethal infection, with a mean survival time of 3.4 days (Table 3). This trial revealed that ScpB, PBP2b, SP148, and SpxB are able to elicit significant levels of protective immunity against lethal infection of virulent type 6A pneumococci.

TABLE 3 Vaccine efficacy of the three proteins in the mouse sepsis model^a

Immunogen	Serotype 6A (ST870)			Serotype 3 (TH2891)			Serotype 4 (TIGR4)		
	Survival rate ^b	Mean survival (avg no. of days)	P value ^c	Survival rate	Mean survival (avg no. of days)	P value	Survival rate	Mean survival (avg no. of days)	P value
ScpB	5/10 (50)	8.9	0.0008	4/14 (28.6)	6.4	0.0268	4/12 (33.3)	6.9	0.0491
PBP2b	5/15 (33.3)	6.7	0.0089	4/11 (36.4)	7.0	0.0243	4/10 (40)	7.7	0.0226
SP148	3/11 (27.2)	7.7	0.0023	7/13 (53.9)	9.1	0.0007	7/12 (58.3)	9.3	0.0150
SpxB	2/15 (13.3)	5.9	0.0155	NT ^d			NT		
ScpB + PBP2b + SP148	7/16 (43.8)	8.4	0.0118	7/11 (63.6)	10.0	0.0009	8/13 (61.5)	9.8	0.0048
PcsB	4/10 (40)	7.9	0.0044	NT			NT		
PspA	8/20 (40)	7.5	0.0016	8/13 (61.5)	9.7	0.0012	5/12 (41.7)	7.7	0.0339
Adjuvant only	0/26	3.4		0/11	2.8		1/11	3.7	

^aThe results shown in Fig. 3, 5, and 6 are summarized here.^bData are number of surviving mice/number of mice inoculated (percent).^cBy a log rank (Mantel-Cox) test.^dNT, not tested.

Vaccine efficacy of ScpB, PBP2b, and SP148 against pneumococcal nasal carriage and lung infection. We further determined the protective efficacy of PBP2b, ScpB, and SP148 in a mouse model of pneumococcal nasal carriage and acute pneumonia. Three weeks after the last immunization, mice were intranasally infected with strain Xen-11 and sacrificed to assess bacterial burden levels in the nasopharynx and lungs at 2 days postinfection. Compared with the bacterial level in the adjuvant control (8.9×10^4 CFU), the median CFU levels in the PBP2b, ScpB, and PspA (positive control) groups were modestly reduced to 5.9×10^4 ($P = 0.0287$), 3.4×10^4 ($P = 0.0117$), and 4.9×10^4 ($P = 0.0272$) CFU, respectively (Fig. 4A). The results for mice immunized with recombinant SP148 (median CFU/ml, 8.1×10^4 ; $P = 0.1803$) and SpxB (median CFU/ml, 1.3×10^5) did not show a significant difference from those for mice immunized with the adjuvant control.

We also compared the bacterial burdens in the lungs of the same groups of mice (described above). In contrast to the median CFU level in the lungs of the adjuvant group (1.2×10^4 CFU), a significant reduction in bacterial burden was observed in the lungs of the mice immunized with recombinant SP148 (median CFU count, 330; $P = 0.0002$), ScpB (median CFU count, 210; $P = 0.0004$), and PspA (median CFU count, 210; $P < 0.0001$) (Fig. 4B). The median value for the PBP2b group (median CFU count, 380) was lower than that of the control groups, but the difference was not statistically significant ($P = 0.0669$). The SpxB-immunized mice showed a level of bacterial burden in the lungs similar to that of the negative control (median CFU count, 7.8×10^3 ; $P = 0.7786$).

FIG 4 Impact of protein immunization on pneumococcal nasopharyngeal carriage and lung infection. Mice ($n = 11$ to 17/antigen) were subcutaneously immunized with SP148, PBP2b, ScpB, SpxB, or PspA as described in the legend of Fig. 3 and intranasally infected with 5×10^6 CFU of serotype 19F strain Xen-11. Mice were sacrificed 2 days postinfection to assess pneumococcal colonization (A) and bacterial burden levels in the lungs (B). The horizontal line indicates the median CFU value. Statistical comparison was performed between experimental and control groups using a Mann-Whitney test. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

Broad protection of ScpB, PBP2b, and SP148 against types 3 and 4 pneumococci. Based on the protective immunity induced by ScpB, PBP2b, and SP148 against pneumococcal airway infection and septic disease, we investigated conservation levels of these proteins across pneumococcal strains/serotypes. As exemplified in Fig. S1, the ScpB sequence (189 amino acids) is identical among pneumococcal strains covered by the PCV13 vaccine and all other strains tested thus far (data not shown). There are only three polymorphisms in the entire sequence of SP148 (276 amino acids) (Fig. S1). PBP2b is the most polymorphic among the three proteins. As illustrated in Fig. S1, there are a total of 18 mutations in the amino acid sequence of the PBP2b proteins (680 amino acids) among the 13 strains, 16 of which occur only in the penicillin-resistant strain ST556. Many of the PBP2b mutations in ST556 are involved in pneumococcal resistance to penicillin and other β -lactam antibiotics (38). In summary, ScpB, PBP2b, and SP148 are highly conserved among pneumococcal strains.

To determine whether ScpB, PBP2b, and SP148 could provide protection against lethal infection of virulent pneumococci beyond serotype 6A, we next tested the immunoprotection with serotype 3 strain TH2891, for which the 50% lethal dose (LD_{50}) was approximately 10 CFU in our preliminary test (data not shown). Each of the BALB/c mice preimmunized with the immunogens was challenged with 100 CFU of TH2891. While i.p. challenge with TH2891 led to the full mortality of the sham immunization group, the animals immunized with ScpB (survival rate, 28.6%; $P = 0.0268$), PBP2b (survival rate, 36.4%; $P = 0.0243$), and SP148 (survival rate, 53.9%; $P = 0.0007$) showed significant protection (Fig. 5A and Table 3). The mean survival times of the groups immunized with ScpB (6.4 days), PBP2b (7.0 days), and SP148 (9.1 days) corroborate their survival rates. PspA prevented 61.5% ($P = 0.0012$) of the immunized mice from lethality. This trial showed that ScpB, PBP2b, and SP148 are able to elicit significant immunity against lethal infection by virulent type 3 pneumococci.

The immunoprotection of ScpB, PBP2b, and SP148 was further evaluated with the virulent type 4 strain TIGR4 in the same manner. TIGR4 had an LD_{50} value of approximately 100 CFU in our preliminary trial (data not shown). Following i.p. inoculation (1,000 CFU/mouse), virtually all of the mice in the adjuvant group (10/11) succumbed to the infection (Fig. 5B). In contrast, the mice immunized with ScpB, PBP2b, and SP148 showed significantly higher survival rates of 33.3% ($P = 0.0491$), 40.0% ($P = 0.0243$), and 58.3% ($P = 0.0150$), respectively (Fig. 5B and Table 3). This result was consistent with the mean survival times of the mice immunized with ScpB (6.9 days), PBP2b (7.7 days), and SP148 (9.3 days). A similar level of immunoprotection (survival rate, 41.7%; mean survival time, 7.7 days; $P = 0.0339$) was observed with the PspA-immunized group (Fig. 5B and Table 3). This result indicated that immunization with each of ScpB, PBP2b, and SP148 is able to protect mice against lethal infection by virulent type 4 pneumococci. Together, the immunoprotection experiments revealed significant protective efficacy of ScpB, PBP2b, and SP148 against the pneumococci of three different capsular serotypes.

Immunoprotection of a combination of three proteins against pneumococcal disease. To test whether the combination of ScpB, PBP2b, and SP148 confers a higher level of immunoprotection, purified recombinant proteins of the three proteins (15 μ g in total, 5 μ g for each protein) were proportionally pooled and used for immunization of BALB/c mice as described above. An enzyme-linked immunosorbent assay (ELISA) revealed that each of the three composite proteins elicited equivalent levels of antibody response (Fig. 6A). We first tested the impact of the protein mixture on nasal carriage and lung infection of *S. pneumoniae*. At 2 days post-intranasal infection with strain Xen-11, the vaccinated mice displayed a level of bacterial burden at the nasopharynx similar to that of the adjuvant control (Fig. 6B). In contrast, a significant reduction in bacterial burden in lungs (median CFU count, 125) was observed compared with the level in the adjuvant group (median CFU count, 1.2×10^4) ($P = 0.001$) (Fig. 6C). This result indicated that subcutaneous immunization with the protein mixture does not inhibit nasal carriage of *S. pneumoniae* but confers significant protection against pneumococcal proliferation in the lungs.

FIG 5 Immunoprotection against lethal infection by serotype 3 and 4 pneumococcal strains. Immunization and challenge experiments were carried out and analyzed as described in the legend of Fig. 3, with the exception that challenge was with TH2891 (type 3; 100 CFU/mouse) (A) or TIGR4 (type 4; 1,000 CFU/mouse) (B). The results from at least two independent experiments were combined for statistical comparison between the immunized and control groups using a log rank (Mantel-Cox) test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The number of mice used in each group is indicated in Table 3.

We next tested the vaccine efficacy of the three-protein mixture with strains ST870, TH2891, and TIGR4 in a sepsis model. Compared with the mice in the adjuvant control, those immunized with the mixture of ScpB, PBP2b, and SP148 showed significant protection (survival rate, 43.8%; mean survival time, 8.4 days; $P = 0.0118$) (Fig. 6D and Table 3) against ST870. Similar challenge experiments with strains TH2891 (Fig. 6E and Table 3) and TIGR4 (Fig. 6F and Table 3) also showed significantly higher survival rates in the groups immunized with the protein mixture. The survival rates and mean survival times were 63.6% and 10.0 days for the TH2891 group ($P = 0.0009$) and 61.5% and 9.8 days for the TIGR4 group ($P = 0.0048$).

In short, immunization with the mixture of ScpB, PBP2b, and SP148 resulted in a survival rate of 56.3%. Similar analysis revealed lower levels of survival in the mice immunized individually with ScpB (37.3%), PBP2b (36.6%), SP148 (46.5%), or PspA (47.7%). This result strongly suggests that vaccine efficacy of the pneumococcal pro-

FIG 6 Vaccine efficacy of combined antigens. Mice were immunized with a mixture of SP148, PBP2b, and ScpB as described in the legend of Fig. 3. Antibody titer specific for each antigen was assessed by ELISA at 1 week after the final immunization (A). Two weeks after the final immunization, each mouse was intranasally infected with strain Xen-11 to assess the impact of immunization on nasal colonization (B) and lung infection (C) as described in the legend of Fig. 4. Immunoprotection experiments were conducted with ST870 (D), TH2891 (E), or TIGR4 (F) as in described in the legends of Fig. 3 and 4. The results from at least two independent experiments were combined for statistical comparison between the immunized and control groups using a log rank (Mantel-Cox) test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. The number of mice used in each group is indicated in Table 3.

teins can be improved by combining multiple antigens with relatively lower levels of immunoprotection. However, ascertaining the differences between these antigens alone or in combination in terms of the level of vaccine efficacy requires direct comparison in the same experiments.

Surface staining of pneumococci by antigen-specific immune serum. We finally tested potential surface exposure of ScpB, PBP2b, and SP148 in the intact pneumococcal cells by treatment with antigen-specific immune serum. Compared with the pneumococci treated with the nonimmune serum, the cells stained with the antigen immune serum against ScpB, PBP2b, or SP148 showed a significant increase in the level of fluorescence as detected by flow cytometry (Fig. 7), suggesting that these proteins are associated with the surface of pneumococci. Consistently, a significant shift in bacterial fluorescence was observed with the pneumococci treated with the immune serum against PspA, a known surface-exposed protein (39). It is worth of noting that TH2891, ST870, and Xen-11 were stained to relatively higher levels of fluorescence with the anti-PspA than TIGR4 and ST556. This observation agrees with the fact that the *pspA* alleles of strain D39 and the former strains belong to the family 1 category, but the alleles of TIGR4 and ST556 fall into another *pspA* family (data not shown). This result strongly suggests that ScpB, PBP2b, and SP148 are associated with the pneumococcal surface.

DISCUSSION

Pneumococcal disease remains as a major threat to public health globally although the CPS-based pneumococcal vaccines have been marketed for many years. We believe that, for further reduction of pneumococcal disease burden, immunization with conserved immunoprotective bacterial proteins represents a viable alternative to replace or complement the current CPS-based vaccines. In this study, we identified the novel immunoprotective antigens of *S. pneumoniae* (e.g., ScpB, PBP2b, and SP148) by a combination of bioinformatic prediction, *in vitro* OPK screening, and *in vivo* immunoprotection validation. To the best of our knowledge, this is the first study to use an opsonophagocytosis screen to systematically identify vaccine candidates. These immu-

FIG 7 Surface staining of pneumococci with antigen-specific immune sera. The intact cells of five strains used in this work were treated with the nonimmune rabbit serum (dashed lines) and the rabbit antiserum (solid lines) against ScpB, SP148, PBP2b, and PspA, followed by incubation with FITC-labeled secondary antibody and detection of bacterial fluorescence by flow cytometry.

noprotective proteins will upgrade our capability of developing a protein-based vaccine with broad coverage of all pneumococcal serotypes.

Our results revealed that ScpB is a novel vaccine candidate with broad immunoprotective efficacy. Immunization of mice with ScpB conferred significant protection

against lung infection and septic death caused by serotype 6A pneumococci. ScpB displayed the highest protection against lethal challenge by the type 6A strain ST870 (50% protection) among the four OPK-positive proteins, which is at least as potent as PspA and PcsB (each with a 40% protection), two known vaccine candidates. This immunoprotection was confirmed with the type 3 strain TH2891 and type 4 strain TIGR4 although the efficacy levels were lower than those with ST870. It is intriguing that ScpB showed substantial variation in immunoprotection against systemic infection of three different pneumococcal strains because the amino acid sequences of the ScpB proteins is identical among the three challenge strains. This difference cannot be explained by potential diversity in antigen availability for antibody interaction because the flow cytometry test did not reveal a substantial difference in antibody staining patterns of the three challenge strains.

ScpB is annotated as a segregation and condensation protein in the pneumococcal genome (40) because it shares 37.3% protein sequence identity with the segregation and condensation protein ScpB of *Bacillus subtilis*. ScpB and ScpA form a complex with the structural maintenance of chromosome protein (SMC) and contribute to chromosome segregation by compacting and organizing the chromosomal DNA (41, 42). While the functional role and subcellular localization of ScpB in *S. pneumoniae* remain to be defined, sequence homology with the *Bacillus* ScpB protein suggests that pneumococcal ScpB functions in the cytoplasm. However, this prediction cannot explain how pneumococcal ScpB is associated with opsonophagocytosis of the bacterium, passive protection, immunoprotection, and antibody staining. This situation is reminiscent of that of other cytoplasmic proteins in *S. pneumoniae*, which are detected in the cell wall fraction. Pneumolysin (43), α -enolase (44), glutamyl tRNA synthetase (45), and 6-phosphogluconate dehydrogenase (46) are associated with the pneumococcal surface and protective immune responses in mice. Olaya-Abril et al. report that *S. pneumoniae* produces membrane-derived extracellular vesicles (EVs) that contain membrane-bound and cytoplasmic proteins; immunization with the purified EVs confers significant protection of mice from lethal infection of *S. pneumoniae* (47). It is possible that ScpB and other cytoplasmic proteins gain access to the extracellular milieu through the EV route.

PBP2b represents the first penicillin-binding protein (PBP) with broad immunoprotective capacity against pneumococcal infection among the six penicillin-binding proteins (PBPs) in *S. pneumoniae*. The immunity elicited by PBP2b provided partial but significant protection of mice from septic death caused by three serotypes of virulent pneumococci. In contrast to the large variability of ScpB and SP148 in immunoprotection among different challenge strains, PBP2b conferred similar levels of protection against lethal infection with ST870 (33.3%), TH2891 (36.4%), and TIGR4 (40.0%). PBP2b anchors to the cell membrane through a hydrophobic sequence at its amino terminus (48) and functions as a membrane-bound extracellular transpeptidase cross-linking the peptidoglycan layers. The immunoprotection elicited by PBP2b in the sepsis model might be caused by enhancing antibody-mediated opsonophagocytosis and/or disturbing the function of PBP2b in cell wall synthesis. As an essential protein for pneumococcal survival (49), PBP2b is highly conserved in *S. pneumoniae*, with the exception of polymorphisms in certain amino acids of the protein (see Fig. S1 in the supplemental material).

To the best of our knowledge, this is the first report that SP148 confers immunoprotection against lung infection and lethal disease of pneumococci. Among the OPK-positive proteins tested, SP148 showed the highest immunoprotection efficacy, with an average of 46.5% protection against lethal infection of three pneumococcal strains/serotypes when the proteins were individually inoculated. Moffitt et al. showed that SP148 is a lipoprotein (50), which is recognized by IL-17A-secreting CD4⁺ T (T_H17) cells; intranasal immunization with SP148 significantly reduces nasopharyngeal colonization of *S. pneumoniae* in mice (22). Although the function of SP148 is currently uncharacterized, it is annotated as a periplasmic substrate-binding protein of a putative ATP-binding cassette (ABC) transporter in the pneumococcal genome (40). Since SP148 is predicted to be tethered to the outer

leaflet of the cell membrane via its lipid tail at the amino (N) terminus, it should be accessible for anti-pneumococcal antibody, in addition to its potential role in T_H17 cell activation (50). Interaction of antibodies with SP148 may enhance bacterial clearance by promoting opsonophagocytosis of pneumococci and/or impair uptake of the SP148's substrate by the ABC transporter. A similar mode of action can explain the immunoprotective effects of other pneumococcal substrate-binding proteins, such as PsaA, PiuA, PiaA, PotD, and Pit1 (19, 51). As exemplified in Fig. S1, SP148 is highly conserved among sequenced *S. pneumoniae* strains. A recent survey identified the SP148 gene in all of the 445 serotype 1 strains tested (52).

In sharp contrast to significant immunoprotection in the sepsis model, immunization with ScpB, SP148, and PBP2b, along with PspA, yielded marginal or no impact on pneumococcal colonization in the nasopharynx of mice. This could be caused by inadequate induction of mucosal immunity against colonizing pneumococci by the subcutaneous immunization route employed in this work. This hypothesis explains the previous observations that SP148 confers significant immunoprotection against pneumococcal colonization of mice (22, 50). Moffitt et al. introduced purified recombinant SP148 by intranasal inoculation (22, 50), which tends to induce mucosal immunity (53). Accordingly, intranasal immunization with recombinant PspA induces protective immunity against nasopharyngeal colonization (54) and septic infection (55). In addition, a lipidated form of SP148 induces more effective immunity against pneumococcal colonization than the unlipidated form in a TLR2-dependent manner when the antigens are inoculated via the subcutaneous route (50). These lines of evidence suggest that future modifications in immunization route and antigen form are needed for improvement of immunoprotective efficacy of the OPK-positive proteins.

This work suggests OPK as a valid *in vitro* surrogate for evaluating protection efficiency of the protein-based vaccines. The OPK screen has become a standard *in vitro* method for quantitative assessment of the CPS-based vaccines on the basis of opsonophagocytosis mediated by pneumococcus-specific antibodies (33). However, the reliability of the OPK screen in evaluating the protection efficacy of protein-based pneumococcal vaccines has not been defined. Based on a modified OPK screen for measuring protective antibodies against PspA (36), we identified 15 OPK-positive proteins by screening our library of rabbit antiserum against pneumococcal proteins. Among these proteins, we rediscovered four known protective antigens: PspA, PcsB, LytA, and LytB. Virtually all of the 15 OPK-positive antiserum samples displayed passive protection in the sepsis model. Further characterization of the four OPK-positive proteins (e.g., ScpB, PBP2b, SP148, and SpxB) revealed that these proteins elicit significant immunoprotection against lethal infection of virulent pneumococci. We are currently characterizing additional OPK-positive proteins identified in this work.

There are obvious limitations for the OPK screen of protective antigens. This approach cannot be applied to the proteins for which it is difficult to generate soluble antigens, such as transmembrane proteins or the proteins with low *in vitro* solubility and/or low stability. The OPK screen is not suitable for identifying T cell antigens that promote bacterial clearance because it relies on the opsonic activity of antibody. In addition, the potential antigen-independent effect of the rabbit antisera may contribute to their nonspecific antibacterial toxicity in the passive protection test. Nevertheless, this work demonstrates that this antibody-based approach is suitable for discovery of protective antigens that elicit opsonic antibodies. Our results have strongly suggested that the PspA-based OPK screen can serve as a convenient and reliable surrogate for assessment of pneumococcal protein vaccines in the future.

MATERIALS AND METHODS

Bacterial strains and reagents. *S. pneumoniae* was cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) or plated on Trypticase soy agar (TSA) with 5% sheep blood as described previously (56). Bacterial strains used in this work are described in Table 4. *Escherichia coli* DH5 α and BL21 were used for DNA cloning and protein expression, respectively. All chemicals for bacterial media and enzymes for molecular cloning were purchased from Sigma (Shanghai, China) and New England Biolabs (Beijing, China), respectively, unless stated otherwise.

TABLE 4 Pneumococcal strains used in this study

<i>S. pneumoniae</i> strain	Description	Source and/or reference
D39	Serotype 2, encapsulated	NCTC (73)
ST556	Serotype 19F, encapsulated	74
ST870	Blood isolate, serotype 6A, encapsulated	U.S. CDC ABCs bank ^a
TH2891	Blood isolate, serotype 3, encapsulated	Beijing Children's Hospital
TIGR4	Serotype 4, encapsulated	ATCC (57)
Xen-11	Serotype 19F, encapsulated, a bioluminescence derivative of EF3030	Xenogen (75)

^aCDC Active Bacterial Core surveillance isolate bank.

Bioinformatic prediction. Based on the protein sequences in the annotation of 39 pneumococcal strains publicly available on 1 July 2011, the Vaxign reverse vaccinology pipeline was used to predict the following features: protein secretion signal peptides, cell wall anchoring motifs (e.g., LPXTG and choline-binding domain), transmembrane helices, similarity to host sequences, and sequence conservation among the genomes (37). We also included the proteins that do not display apparent signature sequences for surface exposure but were identified as potential virulence factors in previous high-throughput screens (57–59) because certain virulence factors were shown as immunoprotective antigens that were either without any apparent signature sequences for secretion or cell wall anchoring (e.g., pneumolysin) (60, 61) or were deeply buried beneath the cell wall and polysaccharide capsule (e.g., the PsaA lipoprotein) (62, 63). Only the proteins conserved in all 39 *S. pneumoniae* strains were included for further tests. The proteins with significant homology (20% amino acid identity) with human and mouse proteins were excluded.

Rabbit antiserum production. Antiserum for recombinant pneumococcal proteins was prepared in New Zealand White rabbits as described previously (64). We first established a protein expression library of pneumococcal genes by individually amplifying coding sequences of target genes from genomic DNA of *S. pneumoniae* ST556 and cloned behind the glutathione *S*-transferase (GST) gene in the *AscI*/*NotI* site of plasmid pST2700 (65). The inserts of all recombinant plasmids were confirmed by DNA sequencing. To align with the previous immunization studies on PspA (mostly using the sequence from type 2 strain D39) (39), the expression construct of *pspA* was prepared using the genomic DNA from strain D39 in pST2700. For comparative analysis of our data with previous studies, we adopted the gene identification and annotation information from the genome of strain TIGR4 in the study of Tettelin et al. (40). The plasmids were then used to produce and purify GST fusion proteins with glutathione Sepharose 4 Fast Flow resins (GE Healthcare Bio-Science, Piscataway, NJ) according to the supplier's instructions. Purified proteins were analyzed by SDS-PAGE, dialyzed in phosphate-buffered saline (PBS), quantified by a bicinchoninic acid (BCA) assay kit (Solarbio, Beijing, China), and used to immunize rabbits for antiserum production (≥ 2 rabbits/protein). Briefly, rabbits (1.5 to 2 kg; female) were subcutaneously immunized with three doses of purified protein (0.5 mg in 0.5 ml of PBS) emulsified with 0.5 ml of Freund's adjuvant (complete for the first immunization and incomplete for the second and third immunizations) at an interval of 21 days. Nonimmune serum was collected from ear capillaries before the first immunization, and postimmune serum was collected by heart puncture 9 days after the third immunization.

The immunoreactivity of the antisera against their immunogens was detected by immunoblotting (66). Briefly, purified recombinant proteins were separated by SDS-PAGE and blotted to a polyvinylidene difluoride (PVDF) membrane by electro-transfer, reacted with the corresponding antisera (1:5,000 dilution), and detected by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution). The expression plasmid and resulting representative antiserum for each of the 119 pneumococcal proteins are listed in Table S2 in the supplemental material.

OPK assay. The OPK activity was detected as previously described (36). HL-60 cells were purchased from ATCC (Manassas, VA), cultured, and differentiated as described previously (30). Complement was collected from naive 3-week-old rabbits. *S. pneumoniae* ST556 bacteria were washed with Ringer's solution and diluted in the assay buffer to 1×10^5 cell/ml. Serum samples were heat inactivated at 56°C for 30 min, serially diluted in 96-well plates in the assay buffer (30 μ l/well), mixed with bacterial suspension (10 μ l/well), and gently mixed for 30 min at room temperature before undiluted baby rabbit complement (10 μ l) was added to the mixture along with 40 μ l of assay buffer containing 400,000 HL-60 cells (400:1 phagocyte-to-bacterium ratio). The resultant mixture was incubated for 45 min at 37°C with gentle shaking. The phagocytosis was ended by cooling the plate on ice for 20 min before 10 μ l of the reaction mixture from each well was spotted on blood agar plates in three replicates for enumeration of colonies. The colonies from control wells without antiserum represented the total number of bacteria after the entire incubation. The antibacterial potency levels of the antisera were represented by their maximal dilutions, at which 50% killing was achieved. Each sample was tested in duplicate at three different times. Anti-PspA and anti-PcsB sera were used as positive controls on the basis of previous studies (20, 36). A mixture of 10 nonimmune sera was used as a negative control.

ELISA. Antibody responses of mice to immunization with recombinant pneumococcal proteins were quantified by ELISA (66). Briefly, target immunogens (1 μ g/well) were coated to each well of 96-well plates (Angle Nunc International, Rochester, NY, USA) by incubation overnight at 4°C. Mouse serum was serially diluted in PBS containing 1% bovine serum albumin and incubated at 37°C for 2 h. The antibody was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (1:10,000 dilution) at 37°C for 1.5 h. Absorbance was read on a BioTek Synergy H1 microplate reader at a wavelength of 490 nm. The ELISA results are presented as the means of the absorbance units from triplicate wells after subtraction of background readings.

Immunoprotection. All infection experiments were performed in BALB/c mice (female, 5 to 6 weeks old) (Vital River, Beijing, China) in compliance with the guidelines of the Institutional Animal Care and Use Committee in Tsinghua University. Passive protection was evaluated in two different ways essentially as described previously (67), except for the use of strain ST870 in the challenge step. Strain ST556 was initially used as a source for gene cloning and the OPK screen, but our subsequent trials revealed that this strain was unable to cause invasive infections in the mouse models for unknown reasons (a key requirement for the challenge experiments). We thus used the virulent strain ST870 (type 6A) for the challenge studies as an alternative. ST870 was originally isolated from a patient with septicemia. In our preliminary experiment, it showed an LD₅₀ value of approximately 10 CFU after i.p. inoculation in BALB/c mice (data not shown). In the initial trials, each antiserum (10 μ l) was premixed with a lethal dose of ST870 (100 CFU in 90 μ l) before being inoculated into each mouse by i.p. inoculation. The nonimmune serum used in the OPK screen was used as a negative control. Some of the sera conferring significant passive protection were subsequently selected for confirmation by i.p. inoculation of each serum (10 μ l diluted in 90 μ l of PBS) 1 h prior to the challenge step with ST870 (100 CFU in 100 μ l of PBS). All mice were monitored for 14 days postinfection for survival.

Immunoprotection against lethal infection of *S. pneumoniae* was assessed in mice as described previously (68). Briefly, each purified pneumococcal protein was thoroughly mixed with aluminum hydroxide (alum) (Pierce, Rockford, IL, USA) and used to subcutaneously immunize mice. Each mouse received 0.1 ml of the antigen-adjuvant mixture containing 5 μ g of protein and 30% adjuvant; the control group received adjuvant only. The same immunization procedure was repeated twice with an interval of 14 days before challenge with a lethal dose of virulent pneumococci (ST870, 100 CFU; TH2891, 100 CFU; or TIGR4, 1,000 CFU; each in 100 μ l of PBS) by i.p. injection 21 days after the third immunization. The LD₅₀ values of TH2891 and TIGR4 in BALB/c mice were approximately 10 and 100 CFU, respectively, after i.p. injection (data not shown). A 3-week interval was used between the final immunization and pathogen inoculation because antibody titer typically reaches the peak level around 21 days after reexposure to the same antigen or boost immunization (69). A serum sample was collected from each mouse by retro-orbital bleeding 24 h prior to infection for evaluation of antibody titer. All immunoprotection experiments were terminated at 14 days postinfection.

Immunoprotection against pneumococcal carriage and lung infection was assessed as described previously (70). Because intranasal inoculation with ST870 frequently resulted in mortality (data not shown), we used strain Xen-11, which has been previously used to assess nasal carriage and lung infection of *S. pneumoniae* (71, 72). The mice preimmunized with recombinant proteins as described above were intranasally infected with 5×10^6 CFU of Xen-11 in 30 μ l of Ringer's solution. Mice were sacrificed 2 days later to collect nasal washes and the lungs. Nasal washes and supernatants of homogenized lungs were serially diluted and plated onto blood agar plates to enumerate CFU.

Flow cytometry. Pneumococci cultured in THY broth to an optical density at 620 nm (OD₆₂₀) of 0.5 were stained with protein-specific antiserum as described previously (20). Briefly, bacterial cells were incubated with rabbit antiserum (1:50 dilution), treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution 1:100) and fixed with 2% paraformaldehyde (PFA) before being analyzed by a FACSCalibur cytometer (Becton Dickinson).

Statistical analysis. Statistical analysis was carried out using GraphPad Prism, version 6.0, software (San Diego, CA). Statistical analysis of the immunoprotection data was conducted using a log rank (Mantel-Cox) test. The data of the colonization and lung infection experiments and ELISA were analyzed using a Mann-Whitney two-sample rank test. A *P* value of less than 0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00423-18>.

SUPPLEMENTAL FILE 1, PDF file, 6.7 MB.

SUPPLEMENTAL FILE 2, PDF file, 6.2 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.2 MB.

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