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BACTERIAL INFECTIONS

Targeted Transcriptomic Screen of Pneumococcal Genes Expressed during Murine and Human Infection

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ABSTRACT The advent of pneumococcal conjugate vaccines led to the near disappearance of most of the included serotypes in high-income settings but also the rise of nonvaccine-type colonization and disease. Alternative strategies, using genetically conserved proteins as antigens, have been evaluated preclinically and clinically for years, so far unsuccessfully. One possible explanation for the failure of these efforts is that the choice of antigens may not have been sufficiently guided by an understanding of the gene expression pattern in the context of infection. Here, we present a targeted transcriptomic analysis of 160 pneumococcal genes encoding bacterial surface-exposed proteins in mouse models, human colonization, and human meningitis. We present the overlap of these different transcriptomic profiles. We identify two bacterial genes that are highly expressed in the context of mouse and human infection: SP_0282, an IID component of a mannose phosphotransferase system (PTS), and SP_1739, encoding RNase Y. We show that these two proteins can confer protection against pneumococcal nasopharyngeal colonization and intraperitoneal challenge in a murine model and generate opsonophagocytic antibodies. This study emphasizes and confirms the importance of studies of pneumococcal gene expression of bacterial surface proteins during human infection and colonization and may pave the way for the selection of a protein-based vaccine candidate.

KEYWORDS colonization, invasive disease, pneumococcus, transcriptome

The World Health Organization (WHO) estimates that *Streptococcus pneumoniae* kills over 300,000 children under 5 years old worldwide every year, mostly in developing countries (1). Pneumococcal conjugate vaccines have nearly eradicated the included serotypes in high-income countries that have instituted infant immunization programs (2–7). The success of these vaccines has been somewhat mitigated by the rise in nonvaccine serotypes, which have become more common causes of invasive disease in both infants and the older adult population in high-income countries (8). In response, several vaccine manufacturers are developing newer pneumococcal conjugate vaccines, which include greater numbers of serotypes (9–12). But even this strategy may not suffice because of the diversity of serotypes (13) and the lack of efficacy against serotype 3 (14).

These considerations highlight the need to study the feasibility of new types of capsule-independent vaccines such as protein-based vaccines (15). Ideally, a protein-based

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vaccine could provide broad, serotype-independent protection against mucosal and invasive pneumococcal disease by virtue of containing antigens that are expressed on most circulating clinical isolates in the context of human infection (16, 17). However, this is not how most pneumococcal antigens that have been included in vaccine studies have been selected. Instead, most protein antigens have been chosen on the basis of the ability to confer protection in mouse models of disease or colonization (17). It is unknown whether these antigens are well expressed during human colonization or human infection. Therefore, in this study, we decided to evaluate the gene expression of a number of likely surface-exposed antigens during human infection or colonization and compare their expression to that observed in mouse models of disease and colonization. To circumvent the problem of limited amounts of bacterial RNA from clinical samples, we used the NanoString platform (18, 19). The NanoString technique counts the specific RNA transcripts with no need for preliminary RNA amplification, avoiding the generation of biases in RNA ratios induced by other methods (20). Here, we were able to identify highly expressed pneumococcal genes during human colonization and human cases of meningitis and compare the relative ranking of expression to that observed in mouse models. We identified two genes, SP_0282, a mannose-specific phosphotransferase system (PTS) component IID protein, and SP_1739, a protein potentially involved in RNA degradation, that are highly expressed in every model evaluated. Antibodies directed against these 2 proteins encoded by these genes were able to induce opsonophagocytic killing; immunization with antigen SP_0282 reduced nasopharyngeal colonization and, combined with SP_1739, conferred protection against invasive disease in mice.

RESULTS

Generation of a NanoString code set for pneumococcal genes. We analyzed how well primers matched the different strains by evaluating the readouts from control cultures of serotype 4, serotype 1 from Malawi, and serotype 6 grown in broth. Any gene with no significant reads for one (or more) of the three serotypes was discarded from the study. Ultimately, we were able to analyze 4 genes coding for choline-binding proteins, 40 genes coding for proteins with a signal peptide, 92 genes coding for transmembrane proteins, and 23 genes coding for transmembrane proteins with a signal peptide, which were included in the code set.

Mouse model RNA counts. Colonization data were collected from 2 separate pools of 10 mice after 1 day of colonization and from 3 separate pools of 10 mice after either 3 or 7 days of colonization. After normalization, we generated a list of the 30 most commonly expressed genes across the 3 collection days of colonization as shown in Fig. 1A to C. A Venn diagram shows the 30 most highly expressed genes under every condition (Fig. 1D). For the lung infection model, we recognized the possibility that some of the bacteria recovered from bronchoalveolar lavage (BAL) fluid could be the result of intercurrent bacteremia. With this concern in mind, we performed a lung infection model where the CFU in BAL fluid and peripheral blood samples following infection were monitored over time, to select a time point when the concentration of bacteria in the lung greatly exceeded that in the blood at the same time. BALs were performed at 9 h postinfection due to the relatively high ratio of bacterial CFU found in the lung to those found in the blood, keeping potential contamination from the bloodstream to a minimum (Fig. 2A). The list of the 30 most highly expressed genes in bronchoalveolar lavage fluid samples represents the results combined from 3 pools of 10 mice (Fig. 2B). Blood samples were collected 2 days after lung infection, and the list of the 30 most highly expressed genes represents the pool of data for 10 individual mice (Fig. 2C). A Venn diagram shows that genes SP_1739, SP_1891, SP_2216, SP_0366, SP_1500, SP_0107, SP_0757, SP_0845, SP_1032, SP_0348, SP_1400, SP_2203, SP_0282, SP_0807, and SP_1650 are consistently highly expressed in all 3 mouse models studied (Fig. 2D). Of note, the total RNA concentration in each sample did not predict a successful NanoString run, likely due to the fact that the majority of the RNA in the sample is host derived.

RNA counts from clinical samples. We were able to obtain successful NanoString reads when the total bacterial density from the nasal cultures of children was 10^5 CFU

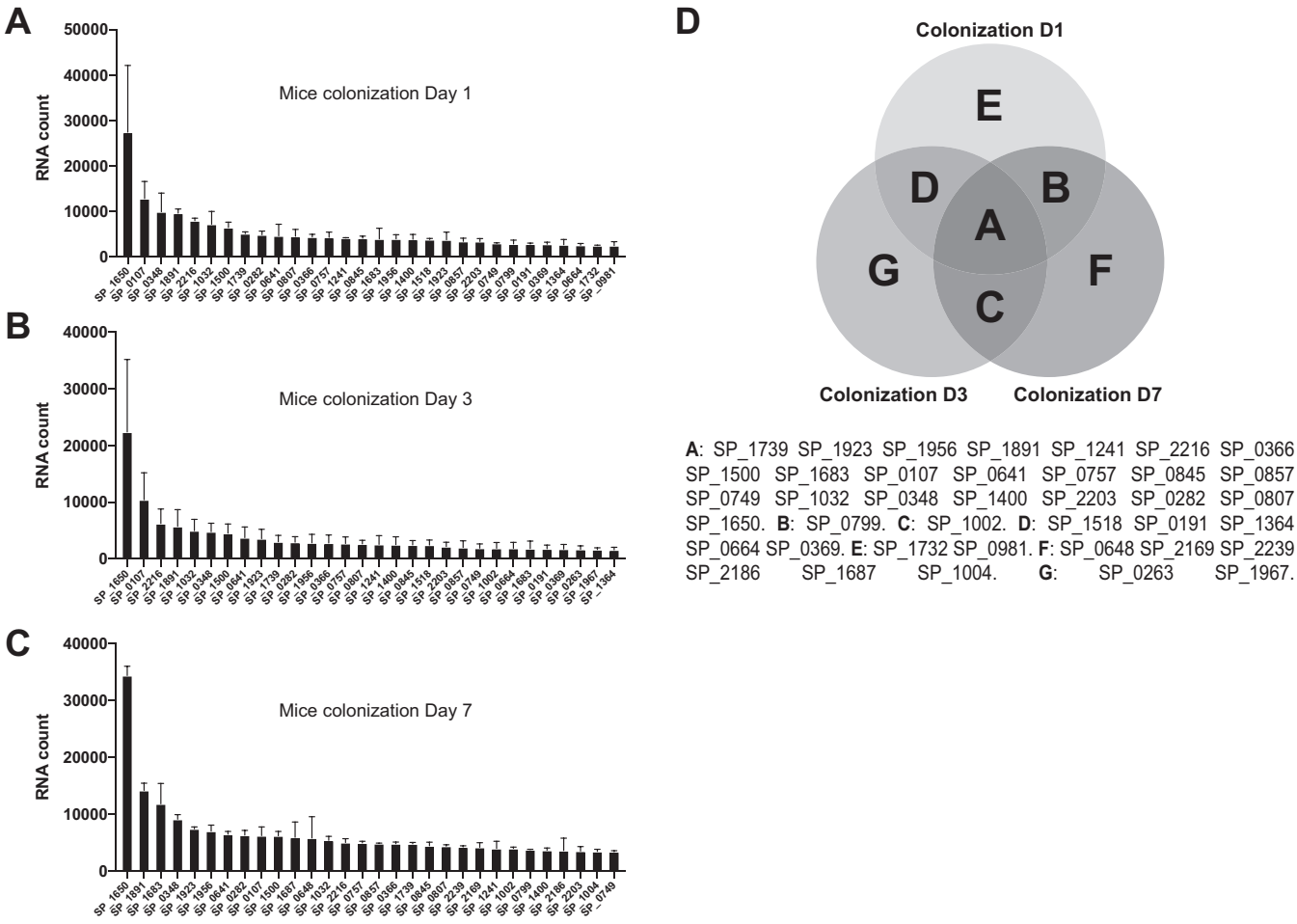


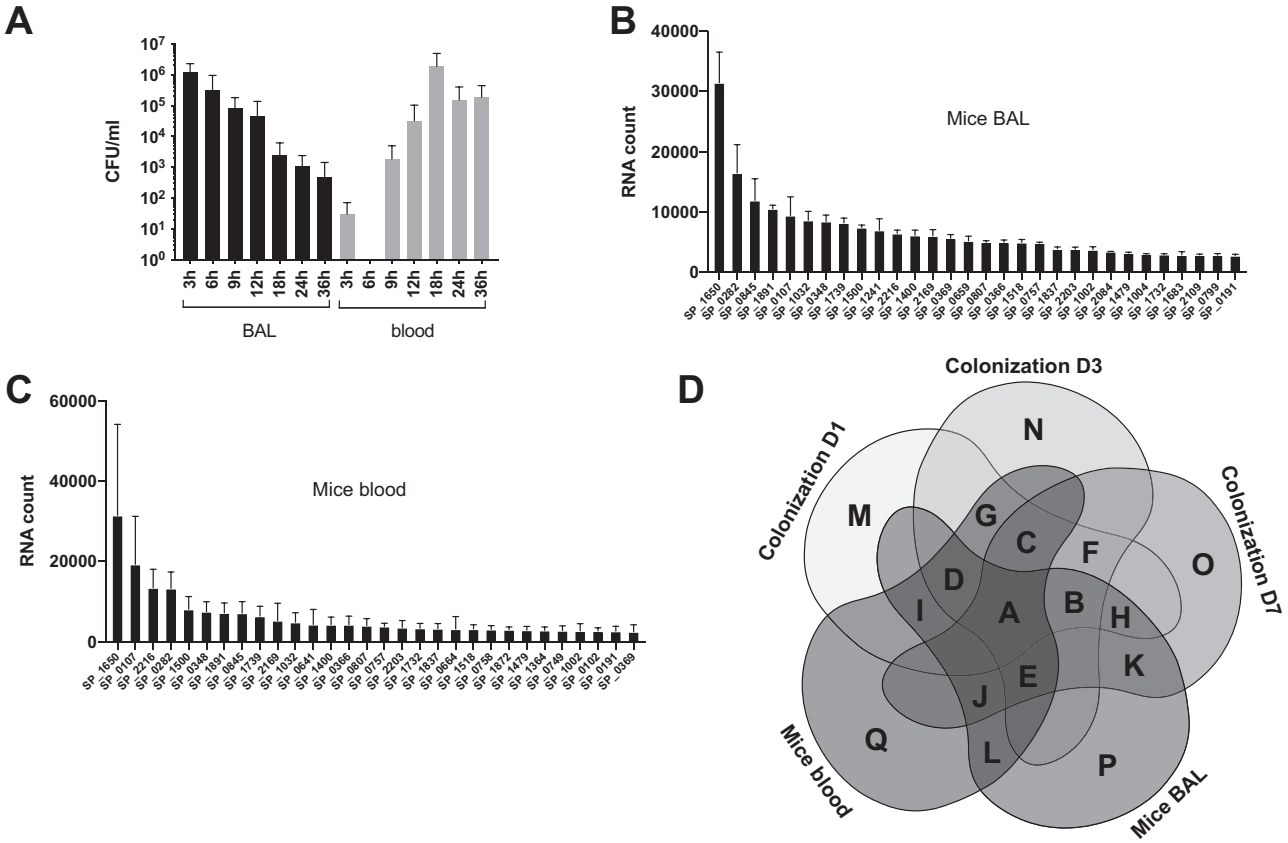
FIG 1 NanoString data representing highly expressed bacterial genes in a mouse colonization model. (A to C) The 30 most highly expressed pneumococcal genes from bacteria recovered on day 1 (A), day 3 (B), and day 7 (C) after initial colonization. (D) Venn diagram representing the most highly transcribed shared pneumococcal genes at all three time points. D1, D3, and D7 represent sample collection on days 1, 3, and 7, respectively.

or higher. The list of the 30 most highly expressed genes in colonized pediatric samples (nasopharyngeal samples [NPSs]) was generated from a pool of 9 swabs, each from children who were naturally colonized with a pneumococcal strain. Some strains recovered were typeable (serotypes 15, 23, and 11), whereas two were nontypeable but reacted with antisera for group E and group I, respectively (Fig. 3A).

In a subanalysis, we also evaluated nasal curettage samples collected from the inferior turbinate prior to NPS collection. We were able to analyze 5 that we compared to their respective nasal cultures; the only gene that had a >3-fold difference in expression between the two methods of sampling was SP_0945, a lipoprotein. While limited by small numbers, these data suggest that the bacteria collected from NPSs versus more anterior nasal scrapings were likely in similar transcriptomic states.

We also analyzed the results of RNA expression from cerebrospinal fluid (CSF) samples. The list of the 30 most highly expressed genes in cerebrospinal fluid samples represents a pool of data collected from 16 patients, of whom 12 died from pneumococcal meningitis and the remaining patients survived (Fig. 3B). A Venn diagram analysis shows that bacterial genes SP_0107, SP_0282, SP_0641, SP_0648, SP_0757, SP_0758, SP_0799, SP_1032, SP_1650, SP_1683, SP_1732, SP_1739, SP_1837, SP_1891, SP_1923, SP_2136, SP_2186, SP_2216, and SP_2239 are consistently highly expressed in NPS and CSF samples.

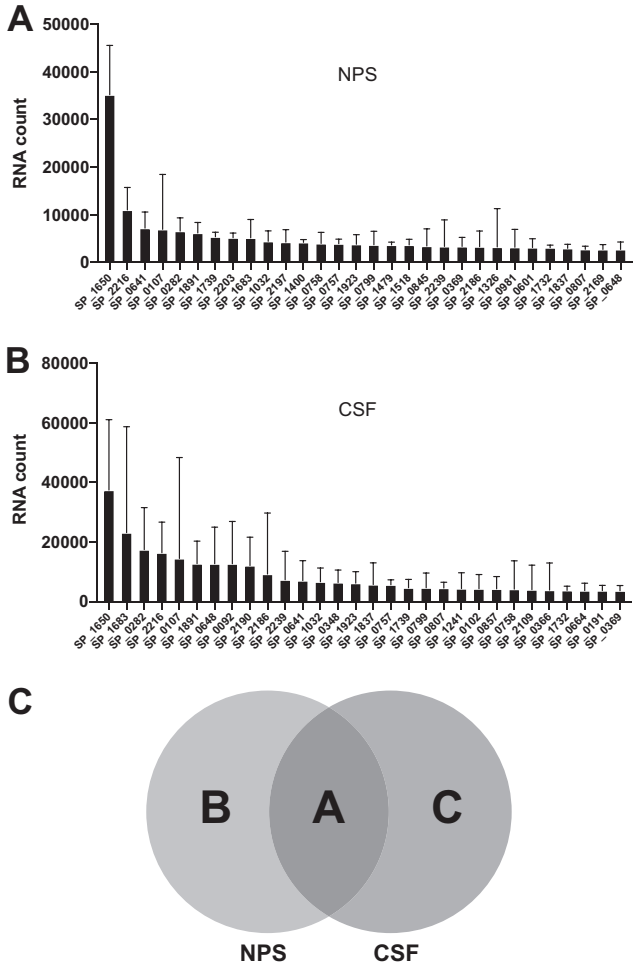
Correlations. Spearman coefficient correlation (ρ) factors were determined between models and control cultures in Todd-Hewitt broth supplemented with yeast extract (THY) (Fig. 4). When the whole data sets were compared, there was generally an excellent



A: SP_1739 SP_1891 SP_2216 SP_0366 SP_1500 SP_0107 SP_0757 SP_0845 SP_1032 SP_0348 SP_1400 SP_2203 SP_0282 SP_0807 SP_1650. **B**: SP_1241 SP_1683. **C**: SP_0641 SP_0749. **D**:SP_1518 SP_0191 SP_0369. **E**: SP_1002. **F**: SP_1923 SP_1956 SP_0857. **G**: SP_1364 SP_0664. **H**: SP_0799. **I**: SP_1732. **J**: SP_2169. **K**: SP_1004. **L**: SP_1479 SP_1837. **M**: SP_0981. **N**: SP_0263 SP_1967. **O**: SP_0648 SP_2239 SP_2186 SP_1687. **P**: SP_2109 SP_2084 SP_0659. **Q**: SP_1872 SP_0102 SP_0758.

FIG 2 NanoString data representing the most highly expressed bacterial genes from bacteria recovered from the bronchoalveolar lavage (BAL) fluid in a mouse pneumococcal pneumonia or a septicemia model (blood). (A) Infected mice were euthanized at different time points postinoculation. BAL fluid and blood were collected from each mouse. CFU are reported for each condition tested. Each bar represents data from pools of 5 mice. (B) The 30 most highly expressed genes from pneumococci recovered from mouse BAL fluid at 9 h postinfection. (C) The 30 most highly expressed genes from pneumococci recovered from blood collected 2 days after lung infection. (D) Venn diagram representing the most highly transcribed shared pneumococcal genes under all five conditions (three colonization time points, blood, and BAL fluid). D1, D3, and D7 represent sample collection on days 1, 3, and 7, respectively.

correlation across most transcriptomic profiles, with the average correlation coefficient being 0.76 (with a range of 0.56 to 0.97). However, as we are mostly interested in identifying those genes that are highly expressed, we also performed an analysis where only the top quartile was evaluated, in which case subtle differences emerge across the different models. First, it is noteworthy that there is a poor correlation between samples obtained from *in vitro* cultures and any of the other samples, highlighting that the mRNA expression evaluated under culture conditions is not very representative of *in vivo* conditions and confirming the importance of evaluating RNA patterns without an *in vitro* growth step. In the mouse colonization model, a strong correlation ($\rho > 0.7$) is seen across days 1, 3, and 7; however, there is a slightly lower correlation over time in the mouse colonization model, potentially suggesting adaptation of the bacterial transcriptome to the mouse nasal mucosal environment. The same appears to be the case when colonization and BAL fluid or blood samples are compared: the correlation coefficient declines with subsequent time points in colonized mice. Samples obtained from the BAL fluid and blood of mice are strongly correlated (correlation coefficient [ρ] = 0.72).



A: SP_1739 SP_0648 SP_1923 SP_0799 SP_1891 SP_2216 SP_1683 SP_2239 SP_0107 SP_0641 SP_0757 SP_1032 SP_2186 SP_1837 SP_0282 SP_0807 SP_1732 SP_1650 SP_0758. **B**: SP_2169 SP_1326 SP_1518 SP_1479 SP_0369 SP_0845 SP_0601 SP_2203 SP_1400 SP_2197 SP_0981. **C**: SP_2109 SP_0191 SP_1241 SP_0366 SP_0092 SP_0664 SP_2190 SP_0857 SP_0348 SP_0102 SP_0659.

FIG 3 NanoString data representing the most highly expressed bacterial genes during natural pediatric colonization (NPS) or human meningitis (CSF). (A) The 30 most highly expressed genes from pneumococci recovered from children naturally colonized with pneumococcal strains. (B) The 30 most highly expressed genes from pneumococci recovered from human meningitis cases. (C) Venn diagram representing the shared highly transcribed pneumococcal genes under the two conditions (NPS and CSF).

The correlation between pediatric and mouse colonization is more modest and also appears to decrease with the duration of colonization in the mouse. There is only a moderate correlation (0.52) of the top-quartile expression patterns across clinical NPS or CSF samples.

Evaluation of protein vaccine candidate gene expression in mouse and human samples. Only 9 genes happened to be consistently highly expressed (top quartile) among the top 30 genes when all models were taken into consideration: SP_0107, SP_0282, SP_0757, SP_0807, SP_1032, SP_1650, SP_1739, SP_1891, and SP_2216 (Fig. 5A). We selected two protein candidates, SP_0282 and SP_1739, for further analysis, as their genes were among the 30 most highly expressed in each of the models studied; additionally, to our knowledge, neither of these antigens had been studied as a protective antigen previously. Both proteins showed strong expression by Western blotting using a collection of pneumococcal strains, including the ones that we used in

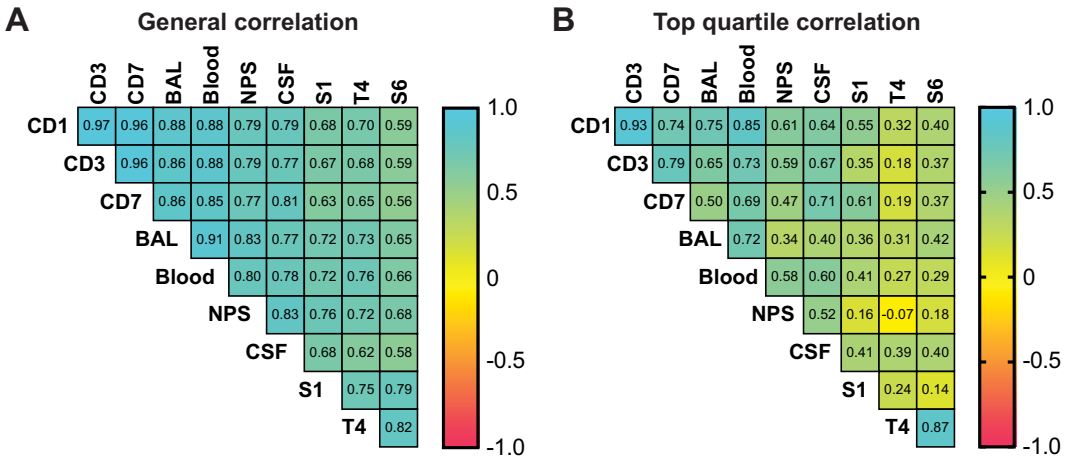


FIG 4 Evaluation of the correlation between mouse and human data. (A) Table indicating the Pearson correlation coefficients between mouse and human data. (B) Table indicating the Pearson correlation coefficients between the results obtained in mice and humans taking into consideration only the top quartile of the most highly expressed bacterial genes under each condition. CD1, CD3, and CD7, colonization days 1, 3, and 7, respectively; BAL, bronchoalveolar lavage fluid from mice in the pneumonia model; Blood, blood samples from mice in the septicemia model; NPS, natural pneumococcal sample, collected from naturally colonized children; CSF, cerebrospinal fluid sample from patients with pneumococcal meningitis; S1, T4, and S6, RNA samples of strains of serotype 1, TIGR4 (type 4), and 6B grown in Todd-Hewitt broth, respectively.

subsequent animal studies (Fig. 5B). We immunized mice for protection studies and rabbits for serum generation. Following bacterial nasal challenge, those immunized with the positive-control whole-cell vaccine (WCV) (consisting of the killed whole-cell pneumococcal antigen and cholera toxin [CT] [21]) were almost completely protected compared to mice that received CT alone. Mice intranasally immunized with SP_0282 and CT showed a statistically significant, nearly 2-log decrease in bacterial CFU counts compared to those in mice that received CT alone (Fig. 5D), a result that is consistent with the strong interleukin-17 (IL-17) response induced against SP_0282 immunization (Fig. 5C). Immunization with SP_1739 and CT did not provide significant protection against colonization.

Multiplex opsonophagocytosis assays (MOPAs) were then performed using rabbit pre- and postimmunization sera for SP_0282 and SP_1739 proteins, using strains of pneumococcal serotype 4, 6B, 14, 35 and 3 (Fig. 5E). The serum from SP_1739-immunized rabbits killed pneumococci of most serotypes, although the effect on the serotype 4 strain (TIGR4) was more modest. No effect was observed against the serotype 3 strain (WU2) for any of the antisera under the conditions tested.

Mice were then subcutaneously immunized with either or both proteins. Specific antibody responses were observed against both single proteins by an enzyme-linked immunosorbent assay (ELISA) (Fig. 5F). Of interest, a reduced antibody response to each protein was noted when a mixture of both proteins was used as the immunogen; despite this, mice immunized with both antigens (SP_0282 and SP_1739) showed statistically significant protection against pneumococcal type 3 intraperitoneal (i.p.) challenge, which was not noted with either antigen alone.

DISCUSSION

Despite decades of research and highly supportive preclinical data, the development of a universal, protein-based pneumococcal vaccine has yet to produce a viable and effective vaccine candidate (15). There are, of course, many possible reasons for this disappointing reality. Certainly, the success of conjugate vaccines and the gradual reduction in the cost of pneumococcal vaccines may have dampened the enthusiasm for the search for alternatives. To complicate matters further, when candidates were

A

SP_0107	LysM domain protein	(39 and 40)
SP_0282	mannose PTS system EIID component	(41 and 42)
SP_0757	FtsX cell division transport system permease protein	(43)
SP_0807	septation ring formation regulator	(44)
SP_1032	iron complex transport system substrate-binding protein	(43 and 45)
SP_1650	PsaA manganese/zinc transport system substrate-binding protein	(46 and 47)
SP_1739	ribonuclease Y	(37)
SP_1891	AmiA, oligopeptide ABC transporter	(39)
SP_2216	usp45, peptidoglycan DL-endopeptidase CwlO	(39 and 48)

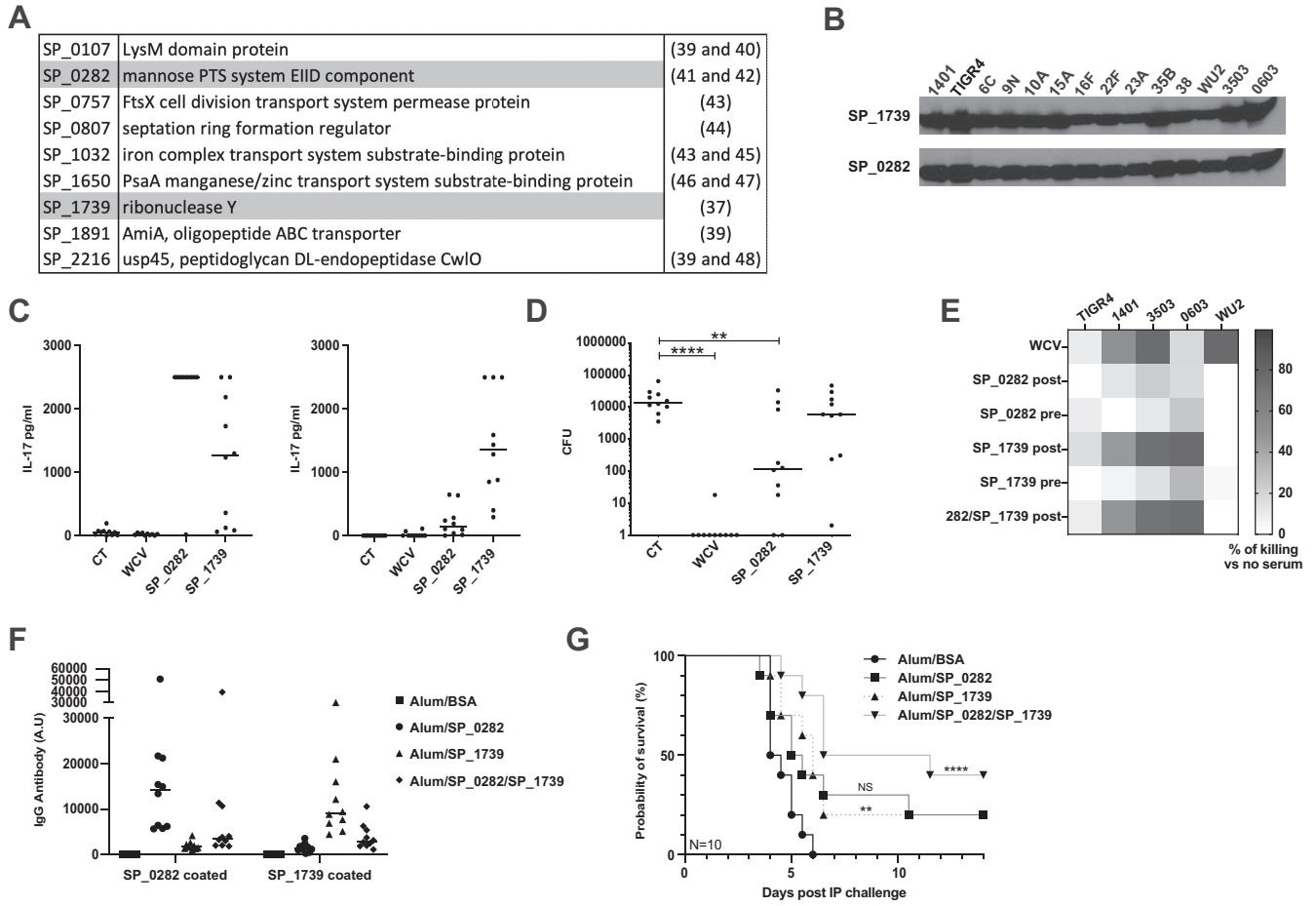


FIG 5 (A) List of the genes most highly expressed (top quartile) in all evaluated models (33). (B) Western blots targeting the SP_1739 and SP_0282 proteins. (C) IL-17 concentrations in the supernatants obtained from blood samples from immunized mice that were stimulated *ex vivo* with SP_0282 (left) or SP_1739 (right) protein. (D) Mice were immunized intranasally with SP_0282 and SP_1739 (10 μg of each protein and 1 μg of cholera toxin) twice (1 week apart) and challenged 4 weeks after the last immunization with a serotype 6B clinical strain. SP_0282 conferred statistically significant protection against nasal colonization. Plotted are the bacterial CFU recovered following retrograde nasopharyngeal washes. (E) Pneumococcal strains of serotypes 4, 14, 35, 0603, and 3 were used in a modified opsonophagocytosis assay (MOPA) with pre- and postimmunization rabbit serum targeting SP_0282 and SP_1739 antigens. Plotted are percentages of bacteria recovered following MOPAs in immune sera versus nonimmune sera. WCV is used as a positive control. (F) Specific protein antibody ELISA performed on sera collected from subcutaneously immunized mice. The ELISA plate was coated with SP_0282 protein (1 μg/mL) (left) or SP_1739 protein (1 μg/mL) (right). Sera were adsorbed with a nonspecific His-tagged protein purified in the same way as for SP_0282 and SP_1739. A.U, arbitrary units. (G) Mice were challenged with 10^3 CFU of the WU2AR strain by intraperitoneal (IP) challenge and monitored for 14 days for illness onset or death. Mice that received the mixture of proteins (SP_0282 and SP_1739) were significantly protected compared to the adjuvant-alone group ($P = 0.0003$ by a Gehan-Breslow-Wilcoxon test). Statistical analysis was performed using an unpaired nonparametric Mann-Whitney test. NS, not significant; *, $P < 0.05$; ****, $P < 0.0001$. BSA, bovine serum albumin.

tested for evidence of a clinical impact in a pediatric trial, the results were largely disappointing, with no evidence of efficacy (22, 23).

A potential explanation for these failures may be that the selection of the antigen candidates has been dictated mostly by preclinical studies performed in mouse models. There are many reasons why mouse models may not be reflective of human disease; here, we evaluate whether there are differences in protein antigen expression between mouse and human infection, which may explain these results.

There have been several studies of the pneumococcal transcriptome with bacteria grown under different *in vitro* conditions (24–30). To our knowledge, ours is the first direct study of the pneumococcal transcriptome in human colonization and meningitis. The main technical challenge in these studies is the relatively small amount of RNA and the need to avoid growing the organism *in vitro*, which will distort the transcriptome, as shown here. The NanoString platform, which we have recently used for the evaluation of the *Staphylococcus aureus* transcriptome under different conditions (31),

allows the quantitation of RNA transcripts from relatively low concentrations of RNA. This platform allowed us to evaluate the transcriptomic profiles of 160 genes, selected for their (presumed) surface location and conservation across the pneumococcal genome. Overall, we were able to obtain 75% successful reads for nasal sampling and 52% with CSF samples from human subjects.

In this study, we compared the RNA expression profiles in mouse and clinical samples. When we focused on the top quartile of expressed genes, we found that mouse *in vivo* samples were not highly correlated with samples grown *in vitro*, confirming the need to avoid a step in which isolates obtained from clinical specimens are grown in media prior to RNA harvest. Additionally, we found that in general, samples obtained from mouse colonization, BAL fluid, and blood specimens were strongly correlated. This correlation is higher for day 1 than for day 7 data, suggesting bacterial adaptation to different environments over time. At the same time, due to our need to collect BAL fluid relatively early postinfection (to avoid bacterial contamination coming from the blood), we cannot determine the transcriptomic profile of pneumococci in the lungs over time. It is also notable that the invasive models in mice did not correlate as well with CSF data obtained from humans with pneumococcal meningitis.

Perhaps most importantly, there was little correlation between mouse and human clinical samples. In particular, the genes that were highly expressed in the invasive mouse models did not correlate well with human CSF data, raising questions with respect to the mouse model to screen for potential protein vaccine candidates. For vaccine development, an optimal goal would be to identify a gene that is highly expressed in isolates obtained from relevant human samples and is also relevant in an animal model such that protection by a vaccine consisting of the gene product can be tested. For this reason, we chose to focus our studies on candidates that demonstrated strong expression in both mouse and human infection.

Using the results of our study, we generated a list of candidate genes that are highly expressed across these mouse models and human samples, which will be tested in our mouse models of immunization and opsonophagocytosis assays in the future. In the present study, we focused on the SP_0282 and SP_1739 proteins. SP_0282 is part of a PTS, which was shown to have increased expression after contact with THP-1 cells, and is a virulence factor in pneumococcal meningitis (30, 32). SP_1739 is a protein of hypothetical function that may play a role in biofilm formation (33) and appears to be a target of human T cell responses (34). The SP_0282 protein was able to confer immune protection against pneumococcal intranasal colonization in mice, and the combination of both antigens was able to confer protection against intraperitoneal challenge with a type 3 strain in mice. SP_1739 rabbit antiserum was also able to induce strong opsonophagocytic killing capacities against three different serotypes (serotypes 14, 35, and 0603) and moderate killing against TIGR4. Despite the absence of opsonophagocytosis against WU2, it is interesting that mice immunized with the combination of both proteins had significant protection against WU2AR strain i.p. challenge. The discrepancy in these results could be due to the difference in the states of the transcriptomes and/or specifically capsule production between bacteria grown in media for MOPAs and bacteria during mouse challenge.

In conclusion, we believe that our work represents a potential new strategy to identify pneumococcal antigens with relatively high expression levels under different conditions, which may better predict their efficacy as vaccine antigens. Our results with SP_0282 and SP_1739 suggest that this type of screening may indeed reveal additional antigens worthy of consideration in a broad, serotype-independent, protein-based vaccine.

MATERIALS AND METHODS

Bacterial strains. Strains TIGR4 (serotype 4); 0603 (serotype 6B); 1401 (serotype 14); 6C, 9N, 10A, 15A, 16F, 22F, 23A, 35B, 38, and WU2 (serotype 3); and 3503 (serotype 35) were used for *in vitro* and *in vivo* experiments. For invasive disease experiments in mice, we used strain WU2AR, a mutant strain of WU2 in which a histidine tag sequence is integrated into the pneumolysin gene; this strain is highly

virulent in mice but with a delay in the time to illness compared to the wild-type WU2 strain. For challenge studies, the strains were chosen based on their ability to cause invasive disease (TIGR4 and WU2AR) or colonization (0603) in mice. Clinical isolates were obtained from individuals in Blantyre, Malawi, who presented with pneumococcal meningitis and from children from Liverpool, United Kingdom, in whom pneumococcal colonization had been detected. Pneumococci were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) or on tryptic soy agar (TSA) plates with 5% sheep's blood. *Escherichia coli* strains were grown in LB medium supplemented with ampicillin at 100 μg/mL as needed. A killed pneumococcal whole-cell vaccine (WCV) was used as a positive control for modified opsonophagocytic killing assays and mouse immunization, as described previously (35).

Animal models. Four- to six-week-old female C57BL/6J mice from Jackson Laboratories (Bar Harbor, ME) were used in animal experiments.

Mouse immunization. (i) Intranasal immunization. Four- to six-week-old female C57BL/6J mice were immunized twice, 1 week apart, with 10 μg of protein and 1 μg of cholera toxin (CT) as an adjuvant. At 3 weeks postimmunization, retro-orbital blood draws were performed to obtain whole blood for stimulation and IL-17A ELISAs. At 4 weeks postimmunization, mice were intranasally inoculated with 10^7 CFU of a pneumococcal strain of serotype 6 (0603). Finally, at 5 weeks postimmunization, mice were euthanized, and nasopharyngeal lavage fluid samples through the trachea were obtained to evaluate the remaining CFU counts. As a positive control, mice were immunized with a killed WCV adjuvanted with cholera toxin as previously described (21).

(ii) Subcutaneous immunizations. Four- to six-week-old female C57BL/6J mice were immunized subcutaneously three times 2 weeks apart with 200 μL containing alum with the protein antigen (10 μg of each protein/mouse). Two weeks after the last immunization, blood was obtained for measurement of serum antibody titers by an ELISA.

Challenge models. (i) Nasopharyngeal colonization. A total of 10^5 CFU of live pneumococci was inoculated onto the nares of gently restrained, unanesthetized mice. On day 1, 3, or 7 postinoculation, animals were humanely euthanized, and nasopharyngeal secretions were recovered by retrograde instillation of Tri reagent (MRC) in the transected trachea. Pools of 10 nasal secretion samples were assembled before RNA extraction.

(ii) Intraperitoneal challenge. A total of 10^3 CFU of live WU2AR was injected intraperitoneally. Mice were monitored for survival twice daily for 14 days; any ill-appearing animal was humanely euthanized.

Isolation of bacteria from lungs. Mice were lightly anesthetized with isoflurane and then nasally inoculated with 50 μL of phosphate-buffered saline (PBS) containing 10^7 CFU of pneumococci. Animals were humanely euthanized at 9 h postinfection. Bronchoalveolar lavages (BALs) were performed using a 1:1 mix of RNA Protect bacteria (Qiagen) and 1$\times$ PBS before RNA extraction. Pools of 10 BAL fluid samples were assembled before RNA extraction.

Isolation of bacteria from blood. Mice that were infected as described above were killed at 2 days postinfection. Heart punctures were performed for blood collection. Blood was immediately mixed with Tri reagent before RNA collection. All animal work was performed in accordance with NIH guidelines and approved by the IACUC at Boston Children's Hospital.

Isolation of bacteria from colonized children. Nasopharyngeal swab samples from a previously described pediatric cohort (36) were analyzed for bacterial RNA expression. This study was approved by the National Health Service Research and Ethics Committee (17/NW/0663). Informed consent from a parent was obtained, and the study complied with relevant regulatory standards (Human Tissue Act, 2004). In short, samples were collected from hospitalized children aged 1 to 5 years, directly after the onset of general anesthesia but prior to the start of their planned procedure (e.g., dental extraction or plastic surgery). For the detection of pneumococcal colonization, a nasopharyngeal swab was collected in medium containing skim milk, tryptone, glucose, and glycerin (STGG), and classical culture was performed to assess colonization. A second nasopharyngeal swab was collected in 1 mL RNA Protect (Qiagen) and frozen at -80°C. Nasal curettage samples were also collected from the inferior turbinate using two Rhino-Pro curettes (Arlington Scientific) and collected in 0.5 mL RNA Protect (Qiagen). RNA was extracted using the Split RNA extraction kit (Lexogen), according to the manufacturer's protocol. RNA quantity and quality were analyzed using the Qubit RNA high sensitivity (HS) assay kit (Thermo Fisher) and a Nanodrop spectrometer (Thermo Fisher). RNA was frozen at -80°C until analysis.

RNA extraction on mouse samples and human CSF samples. Bacteria were centrifuged and rinsed once in sterile PBS before resuspension in Tri reagent buffer. Bacteria were then lysed 5 times for 30 s using a mini-bead beater from Biospec with acid-washed glass beads of $\leq$106 μm. RNA was extracted using a Tri reagent-chloroform protocol followed by an ethanol precipitation step. The RNA concentration was quantified using a Thermo Scientific Nanodrop 1000 instrument. Patients presenting to the Queen Elizabeth Central Hospital in Blantyre, Malawi, with bacterial meningitis caused by *S. pneumoniae* between 2011 and 2013 were included (Current Controlled Trials registration number ISRCTN96218197) (37). RNA was extracted from blood and CSF using the PAXgene blood miRNA (microRNA) kit (PreAnalytix, Qiagen, USA) according to the manufacturer's instructions, with an additional mechanical disruption step for the CSF samples to disrupt the pneumococcal cell wall at 6,200 rpm for 45 s in the Precellys evolution tissue homogenizer (Bertin Instruments). The extracted RNA was quantified, and the RNA integrity number (RIN) scores were calculated using the RNA Tapestation 4200 (Agilent, USA) and Nanodrop (Thermo Scientific, USA) systems.

Generation of the pneumococcal code set. Two hundred four unique molecular fluorescent barcodes linked to reporter tags were used in this study. The JGI website (https://img.jgi.doe.gov/) was used to generate a NanoString code set specifically including surface-exposed proteins. We selected genes encoding secreted proteins, surface-attached proteins, or transmembrane proteins with presumptive

extracellular domains (to maximize the chance of accessibility to antibodies). Only one gene per operon was selected to avoid crowding the code set. Code set primers were designed based on the TIGR4 genome sequence targeting the most conserved regions of the genes among pneumococcal strains. In total, 4 genes coding for choline-binding proteins, 40 genes coding for proteins with a signal peptide, 91 genes coding for transmembrane proteins, and 25 genes coding for transmembrane proteins with a signal peptide were included in the code set. Primers for these 160 pneumococcal genes were designed using the TIGR4 reference genome sequence (at https://img.jgi.doe.gov/). For the design of the primers, areas of homology with human and murine genes were avoided using *in silico* analysis. The Ncounter XT assay protocol provided by NanoString Technologies was performed to analyze each RNA sample. RNA was hybridized for 24 h at 65°C with primer sets and NanoString Elements reagents (30-μL total volume for each hybridization). Hybridized samples were washed and immobilized onto cartridges (NanoString prep station) that were analyzed (Digital Analyzer) for direct counting of the fluorescent molecular barcodes unique to each primer that attached to the hybridized transcript within each flow cell.

Protein purification. Genes of interest were cloned into the pET21b vector for expression. Bacteria were grown to an optical density at 600 nm of 0.6, and protein expression was induced with 0.25 mM IPTG (isopropyl-β-D-thiogalactopyranoside) overnight at 16°C. Bacteria were harvested and then suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1× Halt protease inhibitor cocktail [Thermo Scientific], 0.1% lysozyme, 10 mM $MgCl_2$, silicone, and DNase) before being sonicated six times for 30 s each. Samples were centrifuged for 30 min at 14,000 rpm in the cold, and the supernatants were collected. Five hundred microliters of Ni-nitrilotriacetic acid (NTA) was added to the samples, and the mixture was incubated for 1 h at 4°C. Samples were loaded onto a column, washed twice with buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole) at pH 8.0, and then eluted in buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole) at pH 8.0. Proteins were further purified through a Superdex S200 column using an Äkta Pure 25 system. Protein purity was analyzed on a NuPage 4 to 12% Bis-Tris gel (Invitrogen), and protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay (Pierce).

Generation and testing of polyclonal antibodies. Purified recombinant His_6-tagged SP_0282 and SP_1739 were used to immunize New Zealand White rabbits three times at 2-week intervals using alum as an adjuvant (Cocalico Biologicals, Inc.). Blood samples were obtained at least 2 weeks following the third immunization.

IL-17A ELISA. Retro-orbital blood draws were performed on anesthetized immunized mice using collection tubes containing lithium heparin (BD Microtainer). For IL-17 ELISAs, 25 μL of blood was incubated for 5 days at 37°C with 225 μL of Dulbecco's modified Eagle's medium (DMEM)–F-12 medium with 10% fetal bovine serum (FBS), containing 11 μg/mL of the specific pneumococcal antigen. The supernatants were then collected by centrifugation, and an IL-17A ELISA was performed according to the instructions provided by the manufacturer (Thermo Fisher).

Protein-specific ELISA for measurement of antibodies in serum. Plates were coated overnight with the protein of interest (1 μg/mL), and heat-inactivated sera were serially diluted. An irrelevant His-tagged protein, purified according to the same protocol as the one used for SP_1739 and SP_0282, was used to adsorb mouse sera at 100 μg/mL, to decrease nonspecific signals.

Multiplex opsonophagocytosis assay. We used a slightly modified multiplex opsonophagocytosis assay (MOPA) as described previously by Bogaert et al. (38). Briefly, specific sera were heat inactivated at 56°C for 30 min and diluted 4 times in Hanks' balanced salt solution [HBSS] and 10% fetal bovine serum [FBS]. Two thousand CFU were added per well to diluted sera, and the mixture as incubated for 30 min at room temperature under agitation. Next, 40 μL of differentiated HL-60 corresponding to 4×10^5 cells and 10 μL of baby rabbit complement (Pel-freeze) were added, and the mixture was incubated for 1 h at 37°C under agitation. After incubation, CFU were determined by serial dilutions on TSA plates.

Statistical analysis. Data were normalized using n Solver software analysis 3.0, against data gathered for 3 housekeeping genes (SP_0668, SP_0806, and SP_1243), either by using the geometric mean or by dividing the result for each sample by the respective median for each sample. The top quartiles for each model were compared using both normalization methods, and the ρ factors obtained using the Spearman rank correlation method are listed in Fig. 4. ρ factor values were very high and showed no appreciable difference between both procedures for data normalization (Fig. 4). Ultimately, normalization against the median for each sample was chosen. Bacterial densities from nasopharyngeal wash specimens or pulmonary cultures were compared using a nonparametric Mann-Whitney U test, using Prism (version 4.0a; GraphPad Software, Inc.). Correlations were calculated using a nonparametric Spearman two-tailed test, using Prism (version 4.0a; GraphPad Software, Inc.).

All clinical studies were approved by the relevant Institutional Review Boards. Animal studies were approved by the Animal Care and Use Committee of Boston Children's Hospital.

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