Infection with Conditionally Virulent *Streptococcus pneumoniae* Δ*pab* Strains Induces Antibody to Conserved Protein Antigens but Does Not Protect against Systemic Infection with Heterologous Strains[▽]

Suneeta Chimalapati,[1] Jonathan Cohen,[1] Emilie Camberlein,[1] Claire Durmort,[2] Helen Baxendale,[3] Corné de Vogel,[4] Alex van Belkum,[4] and Jeremy S. Brown[1]*

Centre for Respiratory Research, Department of Medicine, Royal Free and University College Medical School, Rayne Institute, London WC1E 6JF, United Kingdom[1]; CEA, Grenoble 1, Institut de Biologie Structurale Jean-Pierre Ebel, Grenoble, 41 Rue Jules Horowitz, 38027, Grenoble Cedex 1, France[2]; Papworth Hospital NHS Foundation Trust, Papworth Everard, Cambridge CB23 3RE, United Kingdom[3]; and Department of Medical Microbiology and Infectious Diseases, Erasmus Medical Centre, Rotterdam, The Netherlands[4]

Avirulent strains of a bacterial pathogen could be useful tools for investigating immunological responses to infection and potentially effective vaccines. We have therefore constructed an auxotrophic TIGR4 Δ*pab* strain of *Streptococcus pneumoniae* by deleting the *pabB* gene Sp_0665. The TIGR4 Δ*pab* strain grew well in complete medium but was unable to grow in serum unless it was supplemented with *para*-aminobenzoic acid (PABA). The TIGR4 Δ*pab* strain was markedly attenuated in virulence in mouse models of *S. pneumoniae* nasopharyngeal colonization, pneumonia, and sepsis. Supplementing mouse drinking water with PABA largely restored the virulence of TIGR4 Δ*pab*. An additional Δ*pab* strain constructed in the D39 capsular serotype 2 background was also avirulent in a sepsis model. Systemic inoculation of mice with TIGR4 Δ*pab* induced antibody responses to *S. pneumoniae* protein antigens, including PpmA, PsaA, pneumolysin, and CbpD, but not capsular polysaccharide. Flow cytometry demonstrated that IgG in sera from TIGR4 Δ*pab*-vaccinated mice bound to the surface of TIGR4 and D39 bacteria but not to a capsular serotype 3 strain, strain 0100993. Mice vaccinated with the TIGR4 Δ*pab* or D39 Δ*pab* strain by intraperitoneal inoculation were protected from developing septicemia when challenged with the homologous *S. pneumoniae* strain. Vaccination with the TIGR4 Δ*pab* strain provided only weak or no protection against heterologous challenge with the D39 or 0100993 strain but did strongly protect against a TIGR4 capsular-switch strain expressing a serotype 2 capsule. The failure of cross-protection after systemic vaccination with Δ*pab* bacteria suggests that parenteral administration of a live attenuated vaccine is not an attractive approach for preventing *S. pneumoniae* infection.

Streptococcus pneumoniae is a common cause of pneumonia, meningitis, and septicemia both in adults and in children. It is estimated to cause over 800,000 deaths in children under 5 years of age worldwide, 11% of all mortality in this age group (29). Although precise data are lacking, *S. pneumoniae* is also an important cause of adult morbidity and mortality. Prevention of *S. pneumoniae* infections is therefore an important public health priority, which the recent introduction of the conjugated capsular polysaccharide antigen vaccine has partially addressed (20). This vaccine provides effective prevention against colonization and invasive infection caused by strains of *S. pneumoniae* expressing capsular serotypes included in the vaccine. However, at present this vaccine has generally been used in children and not adults (14), and because it protects only against selected *S. pneumoniae* strains, its routine use has had a profound effect on *S. pneumoniae* ecology, with an increasing incidence of previously uncommon

strains as colonizers and as causes of invasive disease (25). The conjugated vaccine is also very expensive and is complex in design, making its introduction to the low-income countries in which the burden of *S. pneumoniae* infections is the highest more difficult (22, 32). Routine vaccination of adults uses a nonconjugated capsular antigen vaccine which, although it has broader coverage against 23 of the 91 capsular serotypes, has weak efficacy against pneumonia, the commonest severe manifestation of *S. pneumoniae* infection (12).

As a consequence of these difficulties with the existing conjugated and nonconjugated *S. pneumoniae* vaccines, other approaches have been suggested for an *S. pneumoniae* vaccine. These include vaccines using protein antigens, killed whole-cell *S. pneumoniae*, or attenuated live *S. pneumoniae* cells (22, 32). Given the ability of bacteria to acquire new genetic traits or undergo mutation which could revert attenuated bacteria to virulence, there are important questions about safety that make live attenuated vaccines against bacterial infections a relatively unattractive option. Furthermore, there are important practical difficulties about preservation and transport of a live attenuated vaccine compared to non-live attenuated vaccines. As a consequence, only a limited number of live bacterial vaccines have been used in practice, including the *Mycobacterium bovis* BCG vaccine for prevention of tuberculosis (43) and

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more recently developed vaccines for prevention of typhoid (11). However, the wide range of antigens and pathogen-associated molecular patterns (PAMPs) present in a live attenuated vaccine (26) suggests that the immune responses that they induce are likely to be very powerful and may also more closely mimic those obtained after natural infection than immunity to a subcomponent or dead bacterial vaccine. This may provide a mechanism for inducing protective immunity under circumstances where other types of vaccines may not or for creating highly protective sera that could be used for passive immunization during outbreaks of *S. pneumoniae* infection or to assist with the treatment of antibiotic-resistant strains. In addition, the wide range of different patient populations that are susceptible to serious *S. pneumoniae* infections suggests that there is a need for a range of different preventative strategies. Hence, a live vaccine could be potentially useful against *S. pneumoniae*, and there are published data on possible live attenuated vaccine candidates. Roche et al. used live unencapsulated attenuated *S. pneumoniae* strains to vaccinate mice via a colonization model and showed effective systemic and mucosal protection (32), and Richards et al. demonstrated that prior nasopharyngeal colonization with a pneumolysin-negative *S. pneumoniae* mutant resulted in significant serotype cross-protection against invasive pneumococcal disease (31).

To develop a safer potential vaccine, a strain containing two or more mutations profoundly affecting virulence would be beneficial, and there is a need to identify mutations that can effectively attenuate the virulence of *S. pneumoniae*, preferably without markedly altering the surface properties of the bacteria to preserve immune stimulation and potential antigenic targets. An auxotrophic mutation affecting *in vivo* growth but dispensable for growth under at least some laboratory culture conditions would fulfill these criteria, and the folate biosynthetic pathway is an attractive target to achieve this (3). Folate is synthesized by microorganisms from chorismate via the intermediate *para*-aminobenzoic acid (PABA), and disruption or deletion of the gene encoding the enzyme converting chorismate to PABA, PABA synthetase, in Gram-negative bacteria or the fungal pathogen *Aspergillus fumigatus* creates PABA auxotrophs. These PABA auxotrophs are totally attenuated in virulence due to the low levels of PABA present in mammalian systems (5), and a *Salmonella enterica* serovar Typhi PABA auxotroph has been used as a live attenuated vaccine (36). A signature-tagged mutagenesis screen for *S. pneumoniae* virulence determinants identified that a mutant with disruption of the *pabB* gene was attenuated for virulence in mouse models of colonization, sepsis, and pneumonia (9). These data suggest that deletion of *pabB* would result in a live attenuated strain of *S. pneumoniae* with conditional virulence that could be a useful tool for investigating disease pathogenesis and potentially contribute to a live attenuated vaccine.

We have therefore created an *S. pneumoniae* strain containing a deletion of the gene encoding PABA synthetase (*pabB*, Sp_0665 in the strain TIGR4 genome) and characterized its virulence and the potential efficacy of this mutant strain at inducing protective immune responses using murine models of *S. pneumoniae* infection.

TABLE 1. Strains and primers used in this study

Name	Description or sequence (source or reference)
Strains	
TIGR4	*S. pneumoniae* serotype 4 clinical isolate
D39	*S. pneumoniae* serotype 2 clinical isolate
D39DΔ	Unencapsulated D39 due to deletion of *cpsD* (27)
0100993	*S. pneumoniae* serotype 3 clinical isolate
0100993 Δ*cps*	Unencapsulated 0100993 due to replacement of the *cps* locus with kanamycin resistance cassette (unpublished strain); Km^{ra}
T4-4+4	TIGR4 capsular-switch strain reexpressing serotype 4 (40)
T4-4+2	TIGR4 capsular-switch strain expressing serotype 2 (40)
T4-4+3	TIGR4 capsular-switch strain expressing serotype 3 (40)
TIGR4 Δ*pab*	TIGR4 with in-frame deletion of Sp_0665; Km^r (this study)
D39 Δ*pab*	D39 with in-frame deletion of Sp_0665; Km^r (this study)
Primers	
Sp0664F	GGACACCGTGAAGGTACTGATG
KmSp0664R	ATCTGCCTCCTCATCCTCTTTTATT TAAAGATTGAAGTTTTAA
KmSp0666F	CGCTAGATAGGGGTCCCGAGCAT GAAATTAATATTTTTGCATGG
Sp0667R	GGTTCTCCCTCGTGAATATCCG
KmF	AAGAGGATGAGGAGGCAGAT
KmR	GCTCGGGACCCCTATCTA
Sp0665RTF	CTTGAACAGGCCAGTTGGTT
Sp0665RTR	ACAAGGTCCACATCCTCTCG
Sp0666RTF(3)	TCAGCACCTAGCTCAAGAGACA
Sp0666RTR(3)	CTCCTCAGAAACTCCGACCA
Sp0667RTF	GAGGGAGAACCTGGTTATTCTG
Sp0667RTR	TCAGCTTTCAGGTAATAGCTTCC
Sp0668RTF	TGTTGGTGGTGGTATTGTCG
Sp0668RTR	TGACAATCCCTGTTGCTGAA
Sp0669RTF(3)	TTGGAAACCAATCCTTGGAA
Sp0669RTR(3)	TGCAAAGCCACATACTGCAT

^{a} Km^r, kanamycin resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *S. pneumoniae* strains used in this work are listed in Table 1. The mutant strain was constructed with TIGR4 capsular serotype 4 and D39 capsular serotype 2 clinical isolates. Additional experiments used the capsular serotype 3 clinical isolate 0100993 (19) and TIGR4 strains expressing capsular serotypes 2 and 3 (kind gifts from M. Lipsitch) (37, 40). *S. pneumoniae* strains were cultured at 37°C in 5% CO_2 on Columbia agar (Oxoid) supplemented with 5% horse blood (TCS Biosciences) or in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). The antibiotic kanamycin (500 µg ml^{-1}) was added to blood agar plates where appropriate. Growth of strains was compared in broth culture by measuring the optical density at 580 nm (OD$_{580}$) at hourly intervals. Working stocks of bacterial cultures in THY (OD$_{580}$, 0.3 to 0.4) were stored at −80°C with 10% glycerol.

Construction of the Sp_0665 deletion mutant. For the in-frame deletion of Sp_0665, a construct was created with the following 3 fragments. Two fragments consisting of 989 bp of flanking DNA 5′ to the Sp_0665 ATG (primers Sp0664F and KmSp0664R) and 818 bp of flanking DNA 3′ to the Sp_0665 open reading frame (ORF; starting from the ATG of the overlapping Sp_0666 ORF; primers KmSp0666F and Sp0667R) with 3′ and 5′ linkers complementary to the 5′ and 3′ portions of the *kan* cassette (*aphIII* gene), respectively, were amplified by PCR from TIGR4 genomic DNA. A third fragment consisting of the *kan* cassette was amplified from nonencapsulated *S. pneumoniae* TIGR4 mutant strain genomic DNA (30) (a kind gift from Tim Mitchell, University of Glasgow) with the primers KmF and KmR and fused with the flanking fragments of the Sp_0665 gene by overlap extension PCR (16). Primers used for the overlap extension PCR

TABLE 2. BLAST alignments of Sp_0665 amino acid sequence to other organisms

Organism	Gene no. or name	Gene description	Size (no. of amino acids)	% identity/% similarity[a]
S. pyogenes	pabB	Putative PABA synthase	585	56/73 (573)
Streptococcus equi	SEQ2030	Chorismate binding enzyme	578	54/73 (574)
Listeria monocytogenes	LmonH_020100001050	PABA synthase	568	41/57 (566)
Lactococcus lactis	pabB	PABA synthase Component I	628	32/48 (484)
Bacillus subtilis	pabB	PABA synthase	470	39/60 (257)
Staphylococcus aureus	SAB0663	PABA synthase component I	386	48/64 (258)
E. coli	ECP_1755	PABA synthase component I	453	38/57 (275)

[a] Numbers in parentheses indicate the length (number) of amino acids compared.

are shown in Table 1. The construct was transformed into *S. pneumoniae* by homologous recombination and allelic replacement using competence-stimulating peptide (CSP-2; a kind gift from Don Morrison, University of Illinois) and standard protocols (10).

DNA, RNA extraction, and RT-PCR. Genomic DNA and total RNA were isolated from *S. pneumoniae* strains using a Wizard genomic DNA isolation kit and SV total RNA isolation system (Promega), respectively, following the manufacturer's instructions, except that cells were incubated with 0.1% deoxycholic acid (Sigma) at 37°C for 10 min before extraction. RNasin (0.5%; Promega) was added to extracted RNA to prevent it from degradation. cDNA was derived and amplified from RNA using an Access reverse transcription-PCR (RT-PCR) system (Promega) and target-specific primers. Primers used for the transcriptional analysis of the Sp_0665-0669 operon are described in Table 1. The National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast) was used for DNA and protein BLAST searches.

Flow cytometry assays for C3b/iC3b deposition and neutrophil phagocytosis. To assess the effect of *pabB* deletion on the complement deposition on *S. pneumoniae* and on the interaction with phagocytes, flow cytometry assays were performed according to previously described methods (13). For C3b/iC3b deposition, 2×10^6 CFU of bacterial pellets, human serum (1:4 dilution in PBS), and fluorescein isothiocyanate-conjugated polyclonal goat anti-human C3 antibody (1:300; ICN Cappel, Aurora, OH) were used. The proportion of bacteria positive for C3b/iC3b and mean fluorescence intensity (MFI) were obtained using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), collecting data from at least 20,000 bacteria. For the opsonophagocytosis assay, the proportion of freshly extracted human neutrophils associated with 5,6-carboxyfluorescein-succinimidyl ester (FAM-SE; Molecular Probes, Eugene, OR)-labeled fluorescent bacteria (1×10^6 CFU) was measured by flow cytometry after opsonization with 1/8 and 1/4 dilutions of commercially available baby rabbit serum (S7764; Sigma) and at a multiplicity of infection of 10.

ELISA. Antibody levels in mouse immune sera were measured by standard whole-cell enzyme-linked immunosorbent assays (ELISAs) or by an anti-capsular polysaccharide/cell wall polysaccharide protocol as described previously (41). ELISA plates (Nunc Maxisorp) were coated with *S. pneumoniae* bacterial suspension at 50 μl/well for whole-cell ELISAs or with *S. pneumoniae* capsular polysaccharide antigen at 100 μl/well (10 μg/ml). For the anti-capsular polysaccharide ELISA, serum samples were preincubated in buffer containing both cell wall polysaccharide and 22F polysaccharide for 30 min at room temperature prior to addition to the ELISA plates to increase the specificity of captured and measured antibody. The plates were developed using monoclonal goat anti-mouse IgG heavy-chain alkaline phosphatase conjugate (Sigma), and antibody levels were assessed by measuring ODs at different serum dilutions.

IgG binding to live *S. pneumoniae* TIGR4 and immunoblots. Flow cytometry assays of IgG deposition on the surface of *S. pneumoniae* were performed using a previously described protocol of Jomaa et al. (15). Bacterial pellets containing 5×10^6 CFU, pooled mouse serum (1:5 dilution in phosphate-buffered saline [PBS]), and a 1:50 dilution of phycoerythrin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) were used for the assay. Results are presented as the percentage of bacteria positive for IgG binding. For the immunoblots, whole-cell lysates (16) of *S. pneumoniae* were separated on SDS-polyacrylamide gels using 4 to 12% bis-Tris gels (Invitrogen, United Kingdom), blotted onto nitrocellulose membranes, and probed with pooled mouse sera (1:100 dilution) using conventional techniques.

Luminex bead assay. Antibody responses to multiple protein antigens were measured using a multiplex flow cytometry Luminex assay based on *S. pneumoniae* proteins conjugated to xMAP beads (35). Recombinant TIGR4-, D39-, or serotype 23 strain-derived proteins were conjugated to xMAP beads (Lu-minex) as described previously (35). Combined beads (3,000 per antigen) were incubated with 10% or 1% serum in PBS–1% bovine serum albumin and then with goat anti-mouse IgG-phycoerythrin (Jackson ImmunoResearch). IgG binding was subsequently assessed using a Bioplex instrument (Bio-Rad Labs) and Bio-Plex Manager software. Data are presented as $\log_{10}$ MFIs of IgG binding to each bead type, after subtraction of the results for blank beads as well as the MFI for that antigen for beads incubated in control serum obtained from PBS-treated mice.

Animal models of infection. All animal experiments conformed to institutional and governmental guidelines and regulations. Outbred CD1 female white mice (Charles Rivers Breeders) weighing approximately 25 g were used for animal infection experiments. Aliquots of −80°C stocks of bacteria grown in THY (i.e., grown in the presence of PABA) were used for challenge and vaccination experiments. Mice were anesthetized by inhalation of halothane (Zeneca) and inoculated intranasally (i.n.) with 5×10^6 CFU in an inoculum volume of 50 μl (pneumonia model and vaccination via the respiratory tract) or by intraperitoneal (i.p.) inoculation of 5×10^4, 5×10^5, or 5×10^6 CFU in an inoculum volume of 100 μl (sepsis model and systemic vaccination). To compare the course of disease between the Δ*pab* and wild-type strains, groups of mice inoculated with *S. pneumoniae* strains were closely observed over the next 14 days. Mice were killed by humane methods when they exhibited the following signs of disease: hunched posture, poor mobility, weight loss, coughing, and tachypnea. Mixed-infection experiments were used to calculate competitive indexes (CIs; the ratio of mutant strain to wild-type strain recovered from the mice divided by the ratio of mutant strain to wild-type strain in the inoculum), using 7.5×10^3 CFU each of the wild-type and mutant strains in the sepsis model and groups of 4 or 6 mice (16). Mice were killed after 24 h, and spleens were recovered and homogenized in 0.5 ml of 0.9% saline, before plating dilutions on plain and kanamycin-containing blood agar plates for calculation of the CI. A CI of less than 1 indicates that the mutant strain is attenuated in virulence compared to the wild-type strain, and the lower that the CI is, the more attenuated the mutant strain is.

Statistical analysis. All the *in vitro* growth curves were performed in triplicate and are represented as means and standard deviations (SDs). Results of growth curves and binding assays were analyzed using two-tailed *t* tests and analysis of variance (ANOVA) with *post hoc* tests. The numbers of target organ CFU were compared between strains using the Mann-Whitney U test, and the survival of infected mice was compared using the log rank test.

RESULTS

Construction of Δ*pab* S. pneumoniae strain. Searches of the *S. pneumoniae* TIGR4 genome identified a single gene, Sp_0665, which encodes a product with 38% identity and 57% similarity to the *Escherichia coli* PabB synthase (Table 2). Sp_0665 was the first gene in a putative 5-gene operon that also contained genes encoding a putative pyrimidine utilization protein (Sp_0666), pneumococcal surface protein (Sp_0667), glucokinase (Sp_0668), and thymidylate synthase (Sp_0668) (Fig. 1). To construct a PABA auxotrophic mutant strain, the TIGR4 strain was transformed with a construct made by overlap extension PCR in which Sp_0665 was replaced with the kanamycin resistance cassette (*kan*) in frame. The identities of
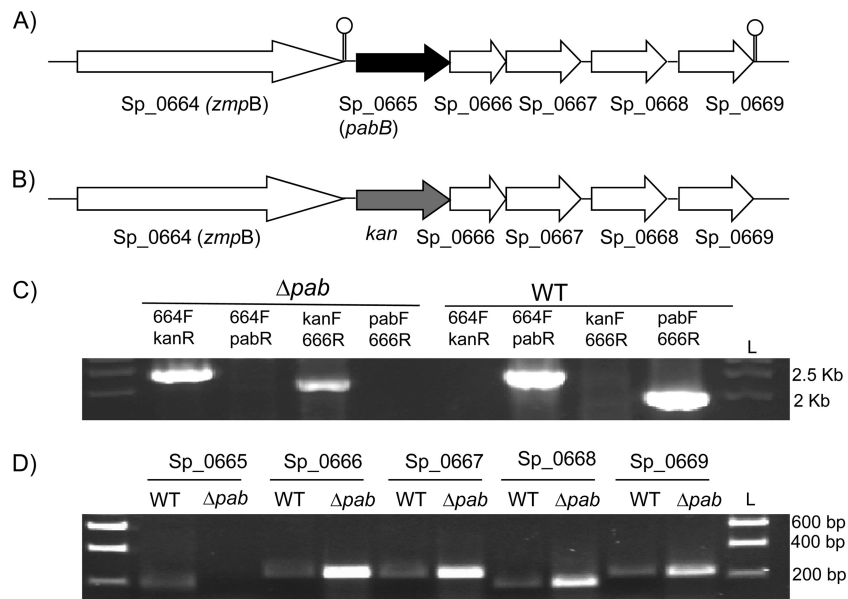
FIG. 1. Construction of Δ*pab* strains. (A) Schematic of the Sp_0665-0669 locus with the TIGR4 genome gene number and the assigned gene names in parentheses, if available. Arrows indicate transcriptional direction, lollipop structures indicate transcriptional termination, and the putative *pabB* gene is shaded black. (B) Structure of the Sp_0665-0669 locus in the Δ*pab* mutant strain derived from the wild-type TIGR4 strain, showing replacement of Sp_0665 with an in-frame copy of *kan*, which is shaded gray. (C) Ethidium bromide-stained agarose gels showing PCR analysis of the TIGR4 Δ*pab* strain confirming replacement of Sp_0665 with *kan*. The primer pairs (Table 1) used for each reaction are given above each lane. Lane L, DNA ladder size marker, with sizes listed on the right. (D) Ethidium bromide-stained agarose gels of products of RT-PCRs using internal primers for each gene within the putative Sp_0665-0669 operon (primers are listed in Table 1), confirming the nonpolar deletion of Sp_0665 in the Δ*pab* strain. Reaction mixtures not containing reverse transcriptase gave no products (data not shown). Lane L, DNA ladder size marker, with sizes listed on the right. WT, wild type.

the resulting TIGR4 Δ*pab* mutant strains were confirmed by PCR (Fig. 1C) and sequencing. RT-PCR demonstrated that the mutation was nonpolar with continued transcription of the remaining genes in this putative operon (Fig. 1D). The stability of the mutant strain was confirmed by growing it in THY without kanamycin for two consecutive growth cycles and then plating it onto blood agar plates with and without kanamycin. All bacteria remained kanamycin resistant.

***In vitro* characterization of Δ*pab* *S. pneumoniae* strain.** The growth of the TIGR4 Δ*pab* strain in the complete medium THY was similar to the growth of the parental TIGR4 strain (Fig. 2A). However, in human serum, in which there are only negligible concentrations of PABA, the TIGR4 Δ*pab* strain had markedly impaired growth compared to TIGR4. Over 6 h, the numbers of TIGR4 Δ*pab* CFU fell to 14.5% (SD, 2.86%) of the inoculating dose, whereas the numbers of TIGR4 CFU increased by 254% (SD, 22.5%). Supplementation of the serum with 2 mg/ml PABA partially reversed the survival defect of the TIGR4 Δ*pab* strain, with the numbers of CFU increasing by 136% (SD, 14.3%) after 6 h. Hence, the TIGR4 Δ*pab* strain had markedly attenuated growth under physiological conditions representative of those found during infection, and this is related to PABA auxotrophy. To assess whether interactions with important components of the host immune response to *S. pneumoniae* had been affected in the TIGR4 Δ*pab* strain, the susceptibility of this strain to opsonization with complement components C3b/iC3b and to neutrophil phagocytosis was assessed using established flow cytometry assays (13). The TIGR4 Δ*pab* strain had similar levels of C3b/iC3b deposition on its surface as the TIGR4 strain (fluorescence

intensities [FIs], 4,373 [SD, 148] and 5,566 [SD, 222], respectively). Furthermore, there were no biologically significant differences in the degree of neutrophil association (a measure of phagocytosis) between the TIGR4 Δ*pab* strain (FI, 603 [SD, 22]) and the TIGR4 strain (FI, 559 [SD, 24]).

Virulence of Δ*pab* *S. pneumoniae* strains. The *in vitro* growth data suggested that the Δ*pab* strains will be attenuated during infection, and this was investigated using mouse models of sepsis (i.p. inoculation), pneumonia (i.n. inoculation), and nasopharyngeal colonization (small-volume i.n. inoculation under light anesthesia). In the sepsis model, all mice inoculated with the TIGR4 strain (5×10^4 CFU per mouse) developed fatal disease within 4 days, whereas none of the mice inoculated with the TIGR4 Δ*pab* strain developed fatal disease (Fig. 3A). Similar results were obtained with the pneumonia model (5×10^6 CFU per mouse), with over 50% of mice inoculated with TIGR4 developing fatal disease within 9 days, whereas all the mice inoculated with the TIGR4 Δ*pab* strain survived (Fig. 3B). In the nasopharyngeal colonization model, by 2 days after inoculation, no TIGR4 Δ*pab* bacteria were found in the nasal washes, whereas significant numbers of the wild-type strain persisted (Table 3). To assess whether this loss of virulence was related to PABA auxotrophy, additional experiments were performed in which mice infected with the TIGR4 Δ*pab* strain had PABA added to their drinking water (1 mg/ml). After infection, mice were culled at 24 h (i.p. inoculation, 7×10^3 CFU/ mouse) or 48 h (i.n. inoculation, 5×10^6 CFU/mouse), and the numbers of bacterial CFU in target organs were calculated by plating serial dilutions on plain and antibiotic-containing blood plates. No TIGR4 Δ*pab* strain bacteria were recovered in ei-
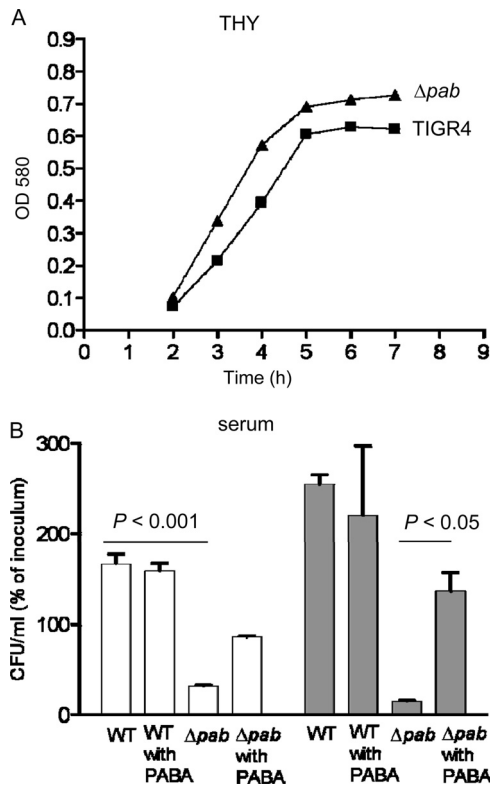
FIG. 2. *In vitro* characterization of the TIGR4 Δ*pab* strain. (A) Growth of wild-type TIGR4 and TIGR4 Δ*pab* strains in THY broth measured by OD_{580}. (B) Numbers of bacterial CFU for the wild-type and the Δ*pab* strains after incubation in human serum with and without supplementation of PABA (2 mg/ml). Results are expressed as a percentage of the inoculating CFU number, and error bars represent SDs. Clear columns, results after 3 h; gray columns, results after 6 h. For the results for the TIGR4 Δ*pab* strain without PABA supplementation versus the wild-type strain, *P* was <0.001, and for the results for the TIGR4 Δ*pab* strain without PABA supplementation versus the TIGR4 Δ*pab* strain with PABA supplementation, *P* was <0.05 (ANOVA with *post hoc* tests).

FIG. 3. Time course of development of fatal infection for mice inoculated with the TIGR4 Δ*pab* or D39 Δ*pab* strain. (A) Results for mice inoculated i.p. with 5×10^4 CFU of the TIGR4 or TIGR4 Δ*pab* strain; (B) results for mice inoculated i.n. with 5×10^6 CFU of the TIGR4 or TIGR4 Δ*pab* strain; (C) results for mice inoculated i.p. with 5×10^4 CFU of the D39 strain (triangles), D39 Δ*pab* strain (open circles), or D39 Δ*pab* strain with PABA supplementation to the water (squares) or 5×10^6 CFU of the D39 Δ*pab* strain (black circles). *P* values were obtained using log rank tests, and for panel C, *P* was <0.001 for the D39 strain or D39 Δ*pab* strain with PABA supplementation versus both CFU doses for the D39 Δ*pab* strains.

ther model from mice without PABA supplementation, whereas similar numbers of CFU of the Δ*pab* strain were found in the spleens and lungs of mice supplemented with PABA and those of mice infected with the TIGR4 strain (Table 3).

To further confirm that the Δ*pab* mutation has marked effects on virulence, the D39 strain was transformed with the Sp_0665 deletion construct to obtain a D39 Δ*pab* strain. The virulences of the D39 Δ*pab* and wild-type D39 strains were compared in mouse models of sepsis. The D39 Δ*pab* strain was severely attenuated in virulence, with no mice inoculated i.p. with 5×10^4 CFU of the D39 Δ*pab* strain developing fatal disease but 100% of mice inoculated with the wild-type D39 strain or with the D39 Δ*pab* strain and given PABA supplementation in the drinking water developing fatal disease (Fig. 3C). The D39 Δ*pab* strain did cause slowly progressive infection in a proportion of mice when inoculated i.p. at a higher dose of 5×10^6 CFU (Fig. 3C), with only kanamycin-resistant bacteria being recovered from the spleens of dead mice, suggesting that fatal infection was not due to mutant reversion. Overall, the data show that deletion of *pabB* caused a marked
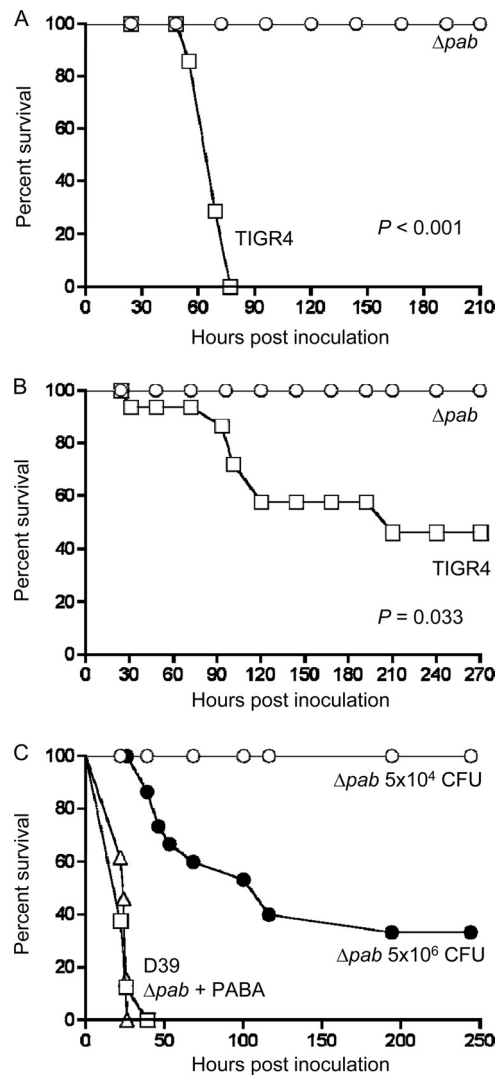
attenuation in *S. pneumoniae* virulence that can be conditionally restored by addition of PABA to the drinking water.

The results of the growth-in-serum experiments showed that PABA supplementation only partially restored the growth of the Δ*pab* strain. Hence, to accurately assess whether virulence of the Δ*pab* strain is also only partially restored by PABA supplementation, mixed-infection experiments were performed to obtain a CI for the TIGR4 Δ*pab* strain versus the TIGR4 strain with or without PABA supplementation of the mouse drinking water. In the absence of PABA supplementation, no TIGR4 Δ*pab* strain CFU was recovered from mice inoculated intraperitoneally (*n* = 8) with a 50/50 mix of 7.5×10^3 CFU of the TIGR4 Δ*pab* and

TABLE 3. Virulence of Δpab mutant[a]

Route of inoculation	Target organ	No. of CFU of *S. pneumoniae* strain[d]:			*P* value[e]	
		TIGR4	TIGR4 Δpab[f]	TIGR4 Δpab + PABA	TIGR4 vs TIGR4 Δpab	TIGR4 Δpab vs TIGR4 Δpab + PABA
i.p.	Spleen[b]	5.8 (0.2)	0	4.5 (0.25)	<0.01	<0.05
i.n.	Lung[c]	4.3 (0.5)	0	3.1 (0.8)	<0.05	<0.05
i.n.	Spleen[c]	1.5 (0.2)	0	1.7 (0.5)	<0.05	<0.05
NP	Nasal washes[c]	4.79 (0.36)	0	ND[g]	<0.05	

[a] Virulence was assessed from the numbers of $\log_{10}$ CFU/ml recovered from target organs in mouse models of sepsis (i.p. inoculation), pneumonia (i.n. inoculation), or nasopharyngeal colonization (NP) ($n = 4$ or 5 mice per group) with and without supplementation of the drinking water with PABA (1 mg/ml).
[b] Twenty-four hours postinoculation of 7×10^3 CFU/mouse.
[c] Forty-eight hours postinoculation of 5×10^6 CFU/mouse.
[d] Data show $\log_{10}$ values of the mean (standard error of the mean) numbers of *S. pneumoniae* CFU recovered from spleen or lung homogenates and nasal washes.
[e] *P* values were calculated using the Kruskal-Wallis test with *post hoc* tests or the Mann-Whitney U test.
[f] No colonies were recovered after plating neat organ preparations.
[g] ND, not done.

TIGR4 strains, giving a CI of less than 0.0002 (SD, 0.0001). In contrast, in mice supplemented with PABA in the drinking water (1 mg/ml, $n = 4$), approximately 1 in 1,000 recovered bacteria was the TIGR4 Δpab strain, giving a CI of 0.008 (SD, 0.006). These results show that even though PABA supplementation restores virulence to the TIGR4 Δpab strain, this strain is still attenuated in virulence compared to the wild-type strain during mixed infections.
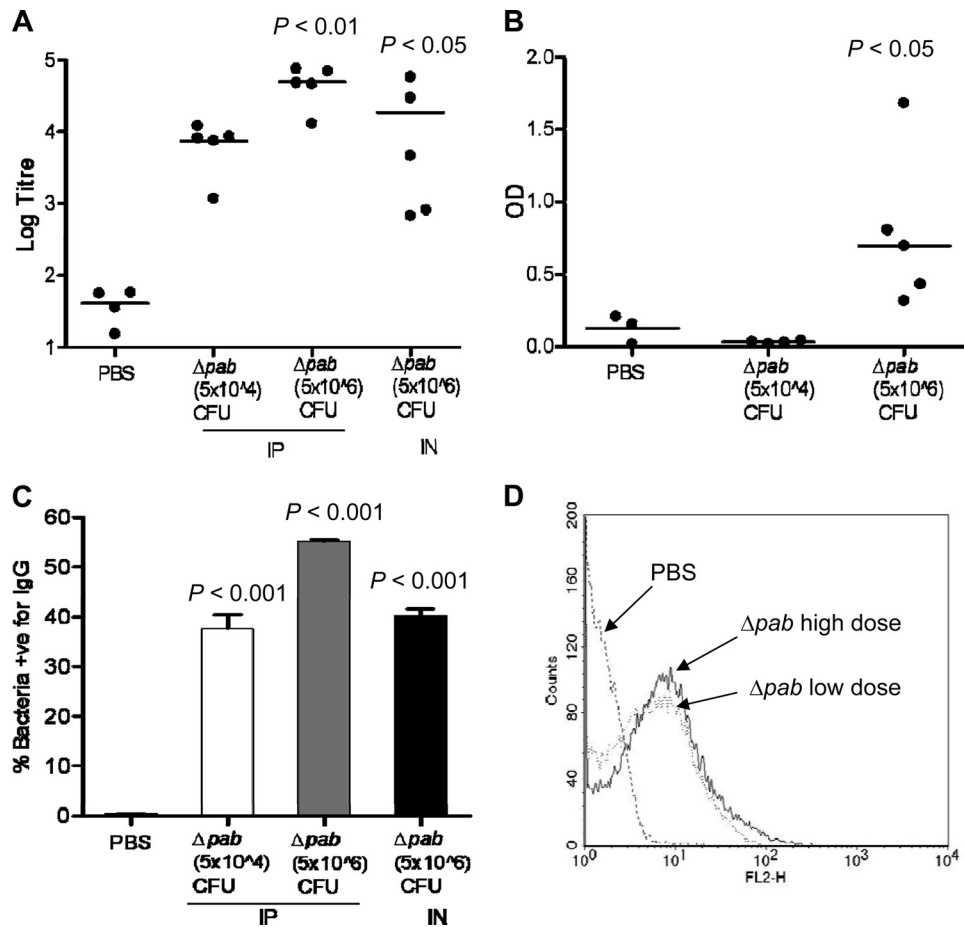

FIG. 4. Antibody responses in serum recovered from mice 4 weeks after vaccination with 5×10^4 or 5×10^6 CFU of the TIGR4 Δpab strain. Control mice were inoculated with PBS only. (A) Whole-cell ELISA IgG titers against the TIGR4 strain; (B) IgG to cell wall polysaccharide expressed as the OD obtained using a 1-in-20 dilution of serum. (A and B) Each data point represents results from an individual mouse, and the bars represent median values. *P* values were obtained with Kruskal-Wallis tests with *post hoc* analysis. (C) Mean proportion of TIGR4 bacteria positive for IgG when assessed by flow cytometry after incubation in sera from mice inoculated with TIGR4 Δpab at 5×10^4 CFU i.p. (clear column), TIGR4 Δpab at 5×10^6 CFU i.p. (gray column), or TIGR4 Δpab at 5×10^6 CFU i.n. (black column). *P* values were obtained using ANOVA tests with *post hoc* analysis. (D) Example of a flow cytometry histogram for IgG binding to the TIGR4 strain in sera from mice inoculated i.p. with the TIGR4 Δpab strain (dotted line, 5×10^4 CFU; solid line, 5×10^6 CFU) or PBS (dashed line).
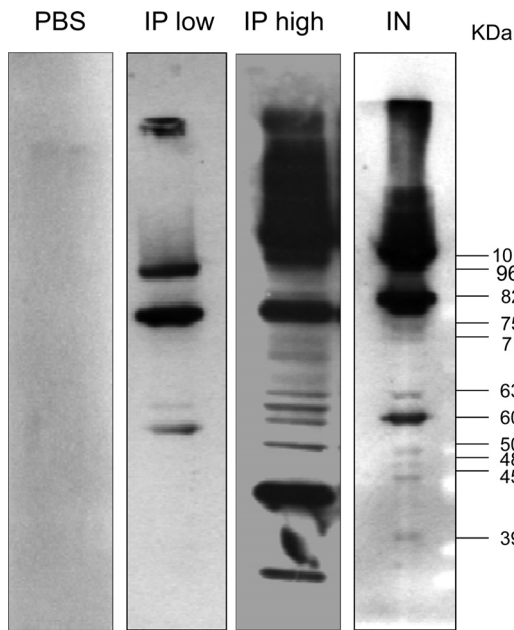
FIG. 5. Immunoblots of wild-type TIGR4 whole-cell lysates probed with 1% serum from mice inoculated with PBS, TIGR4 Δpab at 5×10^4 CFU i.p. (IP low), TIGR4 Δpab at 5×10^6 CFU i.p. (IP high), or TIGR4 Δpab at 5×10^6 CFU i.n. Estimated molecular masses of the proteins detected are indicated to the side of the protein bands.

Vaccination with the TIGR4 Δpab strain induces antibodies to *S. pneumoniae*. To investigate whether vaccination with the TIGR4 Δpab strain induces protective immunity against *S. pneumoniae*, groups of 5 mice were vaccinated by either the i.p. (5×10^4 CFU or 5×10^6 CFU of TIGR4 Δpab/mouse) or i.n. (5×10^6 CFU of TIGR4 Δpab/mouse in a 50-μl volume while the mice were under deep anesthesia, ensuring that bacteria are aspirated into the lungs) route, and antibody responses were assessed by ELISAs, immunoblotting, and flow cytometry with sera recovered from mice 4 weeks later. Whole-cell ELISAs for the TIGR4 strain demonstrated significant antibody levels in sera from mice inoculated with the TIGR4 Δpab strain by both the i.p. and i.n. routes (Fig. 4A). In contrast, no significant antibody response to the capsular serotype 4 antigen was detected (data not shown), and only sera from mice inoculated i.p. with the higher dose of the TIGR4 Δpab strain had significant but low antibody responses to cell wall-associated polysaccharide (Fig. 4B). Flow cytometry analysis showed high levels of IgG binding to live TIGR4 bacteria in sera obtained from mice inoculated either i.p. or i.n. with the TIGR4 Δpab strain (Fig. 4C and D). These data suggest that the antibody responses mainly recognized protein antigens, and immunoblots of TIGR4 whole-cell lysates demonstrated that IgG in serum from mice inoculated with the TIGR4 Δpab strain recognized several protein antigens (Fig. 5). Immunoblots probed with naïve serum showed only minimal binding to *S. pneumoniae* proteins. Two distinct protein bands with molecular masses of 96 kDa and 82 kDa were present in all three immunoblots probed with immune sera from mice inoculated i.p. (both low and high doses) and i.n. (Fig. 5). Several other proteins were also recognized, with molecular masses ranging from 103 to 32 kDa in the blot treated with immune sera from

mice inoculated i.p. with the high dose and from 101 to 39 kDa in the blot treated with immune sera from mice inoculated i.n.

Identification of antigen targets for serum antibodies from Δpab-vaccinated mice. xMAP beads conjugated with 16 recombinant pneumococcal proteins and a Luminex bead assay were used to identify possible pneumococcal proteins recognized by serum IgG from TIGR4 Δpab-vaccinated mice. The number of antigens recognized and/or the strength of the antibody response varied between the three vaccination conditions, with immune sera from mice vaccinated i.p. with a high dose (5×10^6 CFU) showing the strongest antibody response, sera from mice vaccinated with a low dose (5×10^4 CFU) i.p. showing the weakest, and sera from mice vaccinated i.n. into the lungs having an intermediate response (Fig. 6). IgG from the high-dose i.p. and the i.n. vaccinated mice recognized most (i.n.) or all (i.p.) 16 of the tested antigens. PpmA, Ply, and CbpD were dominant antigens for both high-dose i.p. and i.n. vaccinated mice, with sera from high-dose i.p. vaccinated mice also having high levels of IgG against PsaA, PspA, pilus A, and IgA1. Sera from low-dose i.p. vaccinated mice recognized only six antigens: PpmA, PsaA, PsaD, Ply, Eno, and CbpD (Fig. 6). These results demonstrate that vaccination with the TIGR4 Δpab strain induced an effective antibody response to *S. pneumoniae* and suggest that these antibodies mainly recognized TIGR4 protein antigens rather than capsule or cell wall polysaccharide.

Vaccination with the Δpab strain is strongly protective against infection with homologous *S. pneumoniae* strains. To investigate whether vaccination with the Δpab strain protects against subsequent *S. pneumoniae* infection, groups of 5 mice vaccinated i.p. with a single dose of 5×10^4 CFU of the TIGR4 Δpab strain were 4 weeks later inoculated i.p. with 10^3 CFU of the TIGR4 strain. Mice were culled after 24 h, and the numbers of bacterial CFU in the spleen were calculated by plating serial dilutions. Although a median of 4.2 $\log_{10}$ bacteria were recovered from control mice (vaccinated with PBS), no bacteria were recovered from mice that had previously been vaccinated with the TIGR4 Δpab strain (Table 4). Similarly, vaccination by i.n. inoculation of 5×10^6 CFU of the TIGR4 Δpab strain into the lungs while the mice were under deep anesthesia protected against subsequent i.n. challenge with 5×10^6 CFU of the TIGR4 strain, with a median $\log_{10}$ number of CFU of 0 in spleens versus 1.9 for control mice (Table 4). There were also lower numbers of CFU in the lungs, although this result was not statistically significant (Table 4). Hence, both i.p. and i.n. vaccination with the TIGR4 Δpab strain protects against subsequent challenge via the same inoculation route with fully virulent TIGR4 bacteria. Mice vaccinated i.p. with the D39 Δpab strain were also protected against subsequent infection with the D39 strain, with partial protection after vaccination with 5×10^4 CFU and complete protection after vaccination with 5×10^6 CFU detected (Table 4).

Vaccination with the TIGR4 Δpab strain provides minimal or no protection against systemic infection with the D39 or 0100993 *S. pneumoniae* strain. To investigate whether vaccination with Δpab strains protects against heterologous infection, mice inoculated i.p. with 5×10^4 CFU of the TIGR4 Δpab or D39 Δpab strain were challenged with the D39 and 0100993 strains. Prior vaccination with TIGR4 Δpab caused a trend toward lower numbers of bacterial CFU in spleen and blood
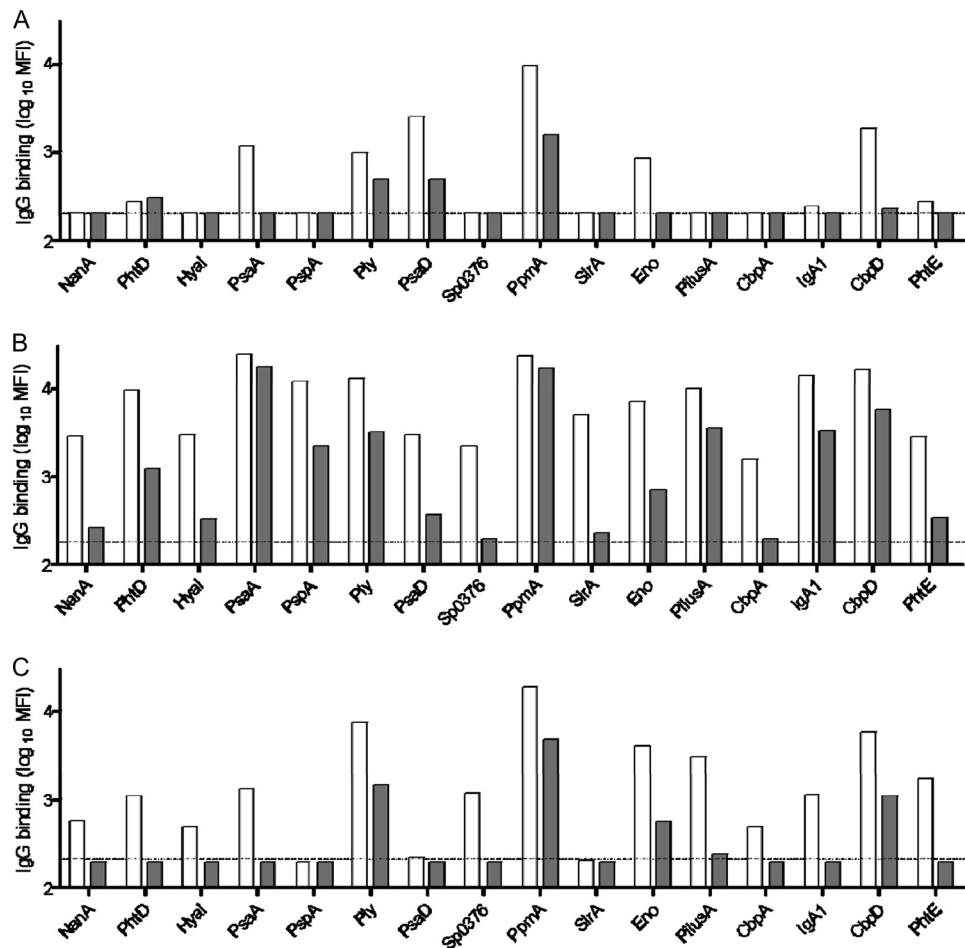
FIG. 6. Results of Luminex IgG binding to various *S. pneumoniae* cell surface antigens on xMAP beads in immune sera from mice inoculated with TIGR4 Δ*pab* at 5×10^4 CFU i.p. (A), 5×10^6 CFU i.p. (B), or 5×10^6 CFU i.n. (C) assayed by flow cytometry. Clear columns, results obtained with 10% serum; gray columns, results obtained with 1% serum. Pooled sera were assayed in duplicate at serial dilutions, and results are presented as the $\log_{10}$ mean MFI at each dilution. Results are presented after subtracting the background levels in the serum from naïve mice inoculated with PBS and the background levels from blank protein beads.

when mice were challenged with the D39 strain, but this was not statistically significant (Table 4). Vaccination with either the TIGR4 Δ*pab* or D39 Δ*pab* strain did not protect against infection with the 0100993 strain (Table 4). Flow cytometry demonstrated that after incubation in sera from TIGR4 Δ*pab*-vaccinated mice, there was significant IgG binding to live D39 bacteria but only minimal IgG binding to strain 01000993 bacteria, even unencapsulated strains (Fig. 7A to C). To assess whether protection could be induced by higher-dose vaccination, mice were vaccinated with 5×10^5 CFU of the TIGR4 Δ*pab* strain twice 10 days apart before challenge 4 weeks later with the TIGR4, D39, and 0100993 strains. Despite complete protection against the homologous challenge with the TIGR4 strain, vaccination with the TIGR4 Δ*pab* strain caused a reduction in the numbers of CFU in blood only when challenge was with the D39 strain and no protection against the 0100993 strain (Fig. 7D). To identify whether the different capsular serotypes prevented protection against heterologous strains after vaccination with TIGR4 Δ*pab*, mice were also challenged with TIGR4 capsular-switch strains expressing the serotype 2 or 3 capsule (37, 40). The TIGR4 serotype 3 capsular-switch

strain was unable to establish infection in the PBS-treated mice after i.p. inoculation, preventing assessment of the role of the serotype 3 capsule (data not shown). The TIGR4 serotype 2 capsular-switch strain established significant infection in the PBS-treated mice but was unable to establish infection in TIGR4 Δ*pab*-vaccinated mice (Fig. 7D). Hence, failure of vaccination with the TIGR4 Δ*pab* strain to protect against the D39 strain was not due to the different capsular serotype but was dependent on strain background.

DISCUSSION

As well as being a central branch point in the biosynthesis of aromatic amino acids such as tryptophan, tyrosine, and phenylalanine, chorismate is necessary for the synthesis of PABA and therefore for folic acid, an essential cofactor for the biosyntheses of purines, pyrimidines, etc. (23, 38). Many bacteria and plants have the ability to synthesize PABA, but higher organisms do not (3, 34), which makes the PABA biosynthetic pathway an attractive target for new vaccination and therapeutic strategies (18, 36). Genome-wide searches with *S. pneu-*

TABLE 4. Protective effect of vaccination with TIGR4 Δ*pab* or D39 Δ*pab* strain or PBS for mice subsequently challenged[a]

Vaccination strain	No. of CFU and vaccination route	Challenge strain and route	Target organ	Median (IQR) $\log_{10}$ CFU/ml[b]		P value[c]
				PBS control	Δ*pab* strain	
TIGR4 Δ*pab*	5×10^4 i.p.	TIGR4 i.p.	Spleen	4.16 (3.65–4.60)	0[d]	<0.001
	5×10^4 i.p.	D39 i.p.	Spleen	6.38 (6.34–6.38)	5.46 (1.69–6.36)	0.191
			Blood	7.96 (7.93–7.99)	5.92 (2.34–7.49)	0.191
	5×10^4 i.p.	0100993 i.p.	Spleen	5.04 (3.73–5.90)	5.08 (3.90–5.63)	0.84
			Blood	5.60 (5.06–6.11)	5.42 (5.14–6.36)	0.84
	5×10^6 i.n.	TIGR4 i.n.	Lung	4.04 (3.24–4.85)	1.30 (0–4.80)[e]	0.125
			Spleen	1.88 (1.18–3.18)	0 (0–1.3)[e]	0.017
D39 Δ*pab*	5×10^4 i.p.	D39 i.p.	Spleen	7.83 (4.92–7.99)	0 (0–3.36)	0.0159
			Blood	8.57 (3.83–8.57)	0 (0–4.07)	0.095
	5×10^4 i.p.	0100993 i.p.	Spleen	5.04 (3.73–5.90)	5.08 (2.24–6.05)	0.90
			Blood	5.60 (5.06–6.11)	5.41 (4.03–6.71)	0.29
	5×10^6 i.p.	D39 i.p.	Spleen	6.05 (4.11–7.12)	0[d]	<0.001
			Blood	6.76 (3.31–8.14)	0[d]	<0.001

[a] The mice were challenged by i.p. inoculation with 1×10^3 or i.n. inoculation with 5×10^6 TIGR4, D39, or 0100993 *S. pneumoniae* CFU.

[b] Data show $\log_{10}$ values of the median (interquartile ratio [IQR]) numbers of *S. pneumoniae* CFU recovered from blood, spleen, or lung homogenates ($n = 5$ or 6) at 24 h (i.p. route) or 48 h (i.n. route) after challenge.

[c] P values refer to the comparison of the results for PBS and Δ*pab* strain-vaccinated mice (Mann-Whitney U tests).

[d] No colonies were recovered from any mice after plating neat organ preparations.

[e] Data in parentheses are minimum and maximum values and not interquartile ratios.

moniae TIGR4 and the BLAST program revealed 2 genes whose products have high degrees of similarity to PabB and PabA of *E. coli*, components I and II of PABA synthase (also called 4-amino-4-deoxychorismate [ADC] synthase), respectively, which catalyze the formation of ADC from chorismate in the presence of glutamine (42). In bacteria, PABA biosynthesis is generally believed to proceed via a two-step process requiring the presence of two enzymes, ADC synthase and ADC lyase (3, 28, 34, 39, 42). ADC lyase is encoded by the gene *pabC* and cleaves ADC to generate PABA and pyruvate (8). However, we found no match to *pabC* in the *S. pneumoniae* genome using BLAST searches. Wegkamp et al. reported a *pabBC* fusion gene in *Lactococcus lactis*, *Streptococcus pyogenes*, and *Helicobacter pylori* (39) with homology to both *pabB* and *pabC* of *E. coli*. The high homology (56% identity and 73% similarity over 573 amino acid residues) of the *S. pyogenes* *pabBC* fusion gene to the annotated *pabB* of *S. pneumoniae* TIGR4 suggests that a *pabBC* fusion gene is also likely to be present in *S. pneumoniae*. Whether this gene encodes a single enzyme which synthesizes PABA in one step is not known. We created Δ*pab* deletion mutants in the TIGR4 and D39 strain backgrounds by replacing the annotated *pabB* gene with a kanamycin resistance marker in frame. *In vitro* growth experiments in serum revealed a profound lack of growth of the TIGR4 Δ*pab* strain that was partially restored by supplementing with external PABA, confirming that deletion of Sp_0665 created PABA-dependent auxotrophic mutant strains. However, in the nutrient-rich undefined medium THY, growth of the Δ*pab* strains was normal. Hava and Camilli have previously identified that disruption of *pabB* affects *S. pneumoniae* virulence (9), and consistent with these data, we found that both the TIGR4 Δ*pab* and D39 Δ*pab* strains were severely but not completely attenuated in virulence in mouse models of disease. Inoculation with a lower dose of Δ*pab* bacteria failed to cause disease, although a higher dose, given i.p., of the D39 Δ*pab* strain caused delayed but fatal infection in a proportion of mice. This perhaps reflects slow bacterial growth due to direct

acquisition of some folate from the host or low PABA availability from the diet. Strikingly, PABA supplementation of the mouse drinking water restored the virulence of both the TIGR4 Δ*pab* and D39 Δ*pab* strains, with the numbers of bacterial CFU in target organs and rates of development of fatal infection being similar to those for the wild-type strains. PABA auxotrophic mutations causing a similar phenotype have previously been reported in Gram-negative bacterial pathogens, such as *E. coli* (8, 17), *Salmonella enterica* serovar Typhi (36), and *Shigella flexneri* (6), and in the fungal pathogen *A. fumigatus* (5). Using highly sensitive mixed-infection experiments, the virulence of the TIGR4 Δ*pab* strain was still attenuated compared to that of the wild-type TIGR4 strain when mouse drinking water was supplemented with PABA. This may be because even *in vitro* exogenous complementation with PABA did not fully restore growth of the TIGR4 Δ*pab* strain in serum to wild-type levels, perhaps due to unexpected effects of the mutation unrelated to loss of *pabB*.

The PABA-dependent conditional virulence of the Δ*pab* mutant makes it an attractive tool for studying host-pathogen interactions. For example, the inability of this strain to undergo significant replication within the host while retaining normal interactions with complement and neutrophils suggests that it could be used to assess the rate of clearance of *S. pneumoniae* from different sites of infection and under different host conditions. Furthermore, studying the effects of specific mutations on the host inflammatory response is often confounded by large differentials in numbers of bacterial CFU *in vivo* between the wild-type control and the mutant strains under investigation, which themselves create differences in inflammatory responses. This problem could be averted by using a Δ*pab* *S. pneumoniae* strain background, preventing replication of both the mutant strain under investigation and the control strain so that differences in the inflammatory response are solely due to the effects of the mutation.

The attenuated virulence of the Δ*pab* TIGR4 *S. pneumoniae* strain allowed us to assess the potential efficacy of systemic
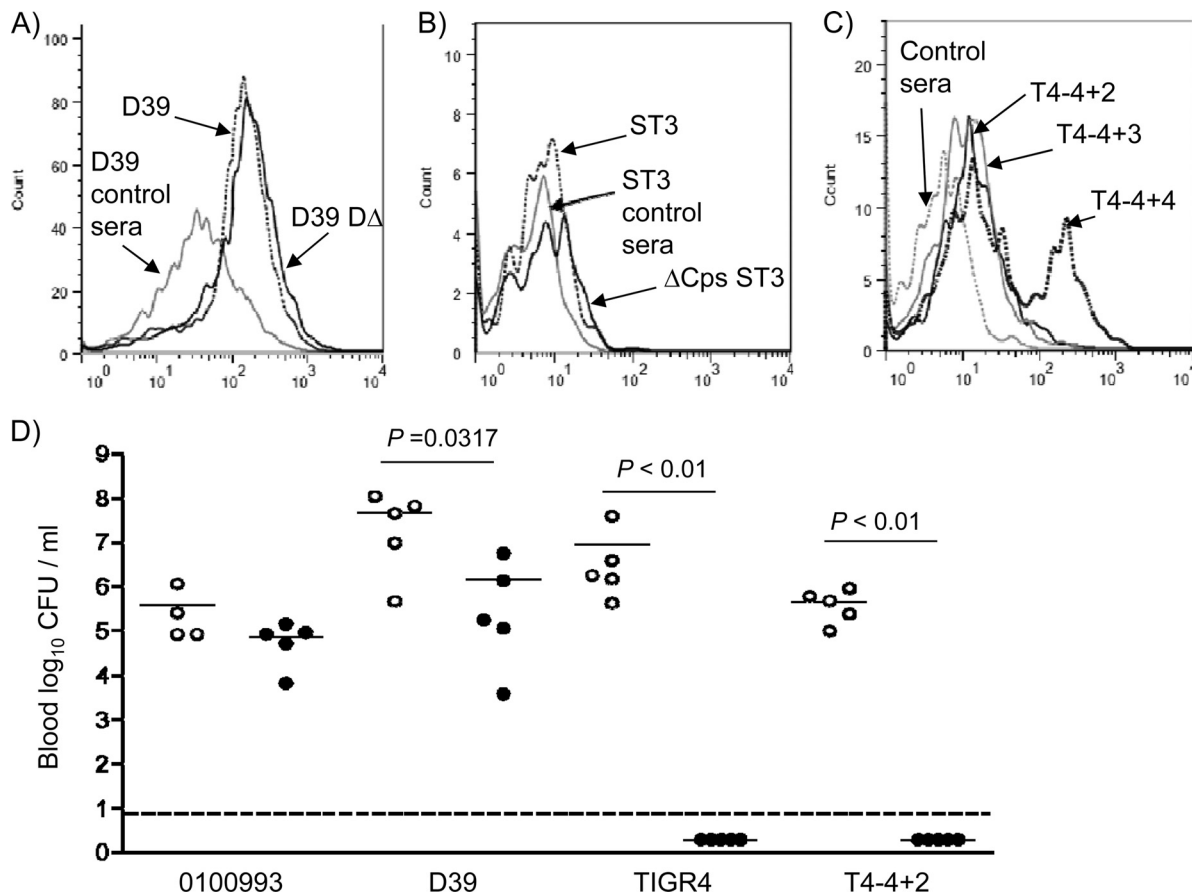
FIG. 7. Protection against heterologous strains after vaccination with TIGR4 Δpab. (A to C) Examples of flow cytometry histograms for IgG binding in serum from mice inoculated i.p. with the TIGR4 Δpab strain (5×10^6 CFU) to the D39 (dotted line) and unencapsulated D39 (D39DΔ; solid line) strains (A), the 0100993 (ST3; dotted line) and unencapsulated 0100993 (ΔCps ST3; solid line) strains (B), and the TIGR4 capsular-switch strains expressing capsular serotypes 4 (T4-4+4), 2 (T4-4+2), and 3 (T4-4+3) or PBS (control sera; dashed line) (C). (D) Log$_{10}$ numbers of bacterial CFU/ml in blood recovered 24 h after challenge with 10^3 CFU of the 0100993, D39, TIGR4, and TIGR4 capsular-switch expressing serotype 2 (T4-4+2) strains. Mice were previously vaccinated i.p. with 5×10^5 CFU of the TIGR4 Δpab strain (black circles) or with PBS as controls (clear circles) twice, 10 days apart, with the last dose given 4 weeks before challenge. Each symbol represents the result from one mouse, and the P values were generated using Mann-Whitney U tests. Dotted line, lower limit of detection for bacteria in blood (no colonies were cultured from the blood of TIGR4 Δpab-vaccinated mice inoculated with the TIGR4 or T4-4+2 capsular-switch strain).

administration of a live attenuated vaccine against *S. pneumoniae*. We used i.p. inoculation of the Δpab strain, a standard approach for establishing the potential efficacy of new *S. pneumoniae* vaccine candidates (7, 14, 33). To assess whether the site of vaccination was important, we also investigated the efficacy of i.n. inoculation of the TIGR4 Δpab strain into the lungs. Neither vaccination route is practical in humans, but the results for those routes can provide proof of principle that can be further developed using the subcutaneous, sublingual, or intramuscular vaccination route. Despite rapid clearance when given systemically, the Δpab mutant induced powerful antibody responses against TIGR4 but, surprisingly, not against the capsule or the cell wall polysaccharide. This suggested that most of the antibody response was targeted against protein antigens, and this was confirmed by immunoblots of bacterial whole-cell lysates and the xMAP bead assay measuring IgG binding to multiple protein antigens. The xMAP bead assays contained 16 different antigens (35), and strikingly, after inoculation with the Δpab strain, serum IgG against all these antigens was detectable, although responses to certain antigens

(e.g., PsaA and PpmA) seemed to be dominant. There were only negligible IgG responses to these antigens detectable in sera from controls. The dominant antigens identified from the xMAP bead assay may correspond to the dominant antigens seen on the immunoblots of whole-cell lysates, as PsaA has an estimated molecular mass of 33.7 kDa, and PpmA, CbpD, Ply, and PspA have estimated molecular masses of 35, 50.3, 52.5, and 62 kDa, respectively.

The antigens included in the xMAP bead assays were largely pneumococcal surface proteins, many of which are conserved between strains and known to protect against invasive *S. pneumoniae* infections in animal models when used as protein vaccines (1, 2, 4, 7, 21, 24). However, although vaccination with Δpab strains induced strong protection against subsequent infection with the homologous wild-type *S. pneumoniae* strain, vaccination with the Δpab strain failed, surprisingly, to induce biologically significantly protection against infection with heterologous strains, even after a two-dose vaccination schedule. One potential explanation for a lack of cross-protection could be variable access to target antigens due to differences in cap-

sular properties between serotypes. However, there was strong IgG binding to the D39 strain in sera from TIGR4 Δ*pab*-vaccinated mice, and conversely, although vaccination with the TIGR4 Δ*pab* strongly protected against infection with a capsular-switch TIGR4 strain expressing a serotype 2 capsule, there was reduced IgG binding to this strain compared to that to the TIGR4 strain. These data demonstrate that serotype-dependent effects on access to subcapsular antigens do not explain failure of protection against heterologous strains. Instead, our results indicate that although after vaccination with the TIGR4 Δ*pab* strain there are antibody responses to a range of antigens, perhaps antibody responses to only select antigens are functionally important and actually mediate protection. Protective efficacy could then vary between strains if these important antigens are present in only a subset of strains (e.g., pilus A), show allelic variation (e.g., PspA), or perhaps vary markedly in their expression between strains. Furthermore, if antibody efficacy depends on blocking target protein function (as shown for antibody to PsrP and Ply) (33), then the effects on bacterial virulence may also vary between strains. Alternatively, protection may be mediated by cellular immune responses rather than by the antibody to protein antigens. However, Th17-mediated adaptive immune responses protect against mucosal infection (22, 23, 44) rather than systemic disease, and it is likely that antibody responses have an important role for Δ*pab*-induced systemic immunity. Our results contrast with published data suggesting that nasopharyngeal colonization with *S. pneumoniae* mutant strains can cross-protect against heterologous strains (31, 32). The reasons for these differences are not clear but could be related to the route of inoculation (nasopharyngeal versus systemic), the prolonged duration of exposure to the vaccine strain during colonization compared to rapid clearance after i.p. vaccination, or bacterial/mouse genetic factors. In addition, both Roche et al. (32) and Richards et al. (31) used a pneumonia challenge model, which is less stringent than the sepsis model used in this work and therefore more likely to demonstrate cross-protective immunity. Further investigation of the factors influencing whether antibody responses to live *S. pneumoniae* induce cross-protective responses against heterologous strains is necessary.

To conclude, in this study we have created a PABA auxotrophic mutant *S. pneumoniae* strain that has conditional virulence in mice, which may make this strain a useful tool for investigating host-bacterial interactions. We have used the Δ*pab* strain to assess the potential efficacy of a live attenuated *S. pneumoniae* vaccine. However, despite inducing antibody responses to a number of conserved protein antigens and strong protection against challenge with homologous strains expressing different capsular serotypes, vaccination with the Δ*pab* bacteria failed to significantly protect against infection with heterologous strains. Overall, our data suggest that parenteral administration of a live attenuated vaccine is not an attractive approach for preventing *S. pneumoniae* infections due to the potential difficulty in inducing protection against a broad range of strains.

ACKNOWLEDGMENTS

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Editor: A. Camilli