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Construction and Immunological Characterization of a Novel Nontoxic Protective Pneumolysin Mutant for Use in Future Pneumococcal Vaccines

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Pneumolysin, the pore-forming toxin produced by *Streptococcus pneumoniae*, may have an application as an immunogenic carrier protein in future pneumococcal conjugate vaccines. Most of the 90 *S. pneumoniae* serotypes identified produce pneumolysin; therefore, this protein may confer non-serotype-specific protection against pneumococcal infections such as pneumonia, meningitis, and otitis media. However, as pneumolysin is highly toxic, a nontoxic form of pneumolysin would be a more desirable starting point in terms of vaccine production. Previous pneumolysin mutants have reduced activity but retain residual toxicity. We have found a single amino acid deletion that blocks pore formation, resulting in a form of pneumolysin that is unable to form large oligomeric ring structures. This mutant is nontoxic at concentrations greater than 1,000 times that of the native toxin. We have demonstrated that this mutant is as immunogenic as native pneumolysin without the associated effects such as production of the inflammatory mediators interleukin-6 and cytokine-induced neutrophil chemoattractant KC, damage to lung integrity, and hypothermia in mice. Vaccination with this mutant protects mice from challenge with *S. pneumoniae*. Incorporation of this mutant pneumolysin into current pneumococcal vaccines may increase their efficacy.

Streptococcus pneumoniae remains an important human pathogen that causes fatal infections, such as bacterial pneumonia and meningitis, and the less severe, but common, childhood middle ear infection, acute otitis media. The heptavalent polysaccharide conjugate vaccine (7PCV) is currently the most effective conjugate vaccine available for protection against *S. pneumoniae* infection (5, 29). The protein used for conjugation of the seven capsular polysaccharides is a diphtheria toxoid, CRM_{197}, conferring an increased immune response to the capsule polysaccharides in infants (5, 35). However, as only 7 out of a possible 90 (15) pneumococcal serotypes are covered in 7PCV (serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F), protection from this vaccine is somewhat limited and varies globally with pneumococcal serotype prevalence (14, 44). For example, the serotypes included in 7PCV cover almost 90% of invasive pneumococcal disease-causing serotypes in North America and Canada but cover <60% of the predominant serotypes in Asia, where serotypes 1 and 5 are the predominant cause of invasive pneumococcal disease (14).

Studies of children vaccinated with 7PCV in Finland have revealed a 57% decrease in otitis media caused by the seven vaccine serotypes. However, this was found to coincide with a 34% increase in otitis media from nonvaccine serotypes (19). This promoted selection for nonvaccine serotypes is a major shortcoming of current pneumococcal vaccines, with an increase in serotype replacement reported by countries where 7PCV has been introduced (24). Alternatives to serotype-specific vaccination are currently being investigated, with efforts focused on using a protective species-common pneumococcal protein such as pneumolysin.

Pneumolysin (Ply) is produced by virtually all invasive strains of *S. pneumoniae* (17) and has been shown to be immunogenic (33). Ply could be used alone (1, 33) or as a carrier protein to the polysaccharides in current vaccine preparations (21, 26, 34), conferring increased protection against pneumococcal disease. Ply belongs to the cholesterol-dependent cytolysin (CDC) family of toxins that bind to the cholesterol of eukaryotic membranes. Following membrane binding, the CDC monomers oligomerize into ring-shaped structures of 30 to 50 monomers and insert into the host cell membrane to create pores (10, 11, 30), a problem in terms of incorporating Ply into new vaccines. Immunogenic Ply mutants (1, 34) with reduced cytotoxicity have previously been constructed (2, 6); however, these mutants retain the ability to form pores in host cell membranes. One Ply mutant extensively researched and commonly referred to as the PdB toxoid has a tryptophan-to-phenylalanine substitution at position 433. This mutant forms large pores in host cell membranes (20) and retains 0.1 to 1% hemolytic activity compared with wild-type (WT) Ply (20, 32). Another Ply mutant with negligible activity was reported by Michon et al. (26), but the location of the mutation was not identified and protection against pneumococcal disease was not proven. In this paper, we describe the construction of a series of mutations in Ply in a region implied to have a role in oligomerization (9a). The resulting mutants had no hemolytic activity. All mutants were recognized by monoclonal antibody Ply4, an antibody that recognizes an important antigenic region of Ply and can block oligomerization (9a). One single-amino-

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TABLE 1. Primers used for site-directed mutagenesis

Primer[a]	Sequence	Amino acid change
Δ1 fwd	5′-CGATTTGTTGGCTAAGCAAGATTATGGTCAGG-3′	ΔW134H135
Δ1 rev	5′-CCTGACCATAATCTTGCTTAGCCAACAAATCG-3′	
Δ2 fwd	5′-GTTGGCTAAGTGGCATTATGGTCAGGTCAATAATGTCCC-3′	ΔQ136D137
Δ2 rev	5′-GGGACATTATTGACCTGACCATAATGCCACTTAGCCAAC-3′	
Δ3 fwd	5′-GGCTAAGTGGCATCAAGATCAGGTCAATAATGTCCC-3′	ΔY138G139
Δ3 rev	5′-GGGACATTATTGACCTGATCTTGATGCCACTTAGCC-3′	
Δ4 fwd	5′-GGCATCAAGATTATGGTAATAATGTCCCAGCTAG-3′	ΔQ140V141
Δ4 rev	5′-CTAGCTGGGACATTATTACCATAATCTTGATGCC-3′	
Δ5 fwd	5′-GGTCAGGTCAATAATGCTAGAATGCAGTATG-3′	ΔV144P145
Δ5 rev	5′-CATACTGCATTCTAGCATTATTGACCTGACC-3′	
Δ6 fwd	5′-GGTCAATAATGTCCCAATGCAGTATGAAAAAATAACGGCTC-3′	ΔA146R147
Δ6 rev	5′-GAGCCGTTATTTTTTCATACTGCATTGGGACATTATTGACC-3′	
Δ7 fwd	5′-GGTCAATAATGTCCCAGCTAGATATGAAAAAATAACGGCTC-3′	ΔM148Q149
Δ7 rev	5′-GAGCCGTTATTTTTTCATATCTAGCTGGGACATTATTGACC-3′	
Δ8 fwd	5′-GTCCCAGCTAGAATGCAGAAAATAACGGCTCACAGC-3′	ΔY150E151
Δ8 rev	5′-GCTGTGAGCCGTTATTTTCTGCATTCTAGCTGGGAC-3′	
ΔA146 fwd	5′-GGTCAGGTCAATAATGTCCCAAGAATGCAGTATGAAAAAATAAC-3′	ΔA146
ΔA146 rev	5′-GAGCCGTTATCATACTGCATTCTTGGGACATTATTGACCTGACC-3′	

[a] fwd, forward; rev, reverse.

acid-deletion Ply mutant, ΔA146 Ply, was further characterized and demonstrated to be unable to form pores in cell membranes or stimulate the in vivo inflammatory effects associated with native Ply treatment. Mice vaccinated with ΔA146 Ply plus alum have high titers of neutralizing anti-Ply immunoglobulin G (IgG) and are protected from *S. pneumoniae* infection significantly longer than mice given alum alone. These Ply toxoids may be suitable candidates for inclusion into the next generation of pneumococcal vaccines.

MATERIALS AND METHODS

Site-directed mutagenesis. Eight double-amino-acid deletions (ΔW134H135, ΔQ136D137, ΔY138G139, ΔQ140V141, ΔV144P145, ΔA146R147, ΔM148Q149, and ΔY150E151) and a single (A146) deletion were created using a QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam Zuidoost, The Netherlands). The template plasmid was the high-expression vector pKK233-2 (Clontech Laboratories Inc., Palo Alto, CA) in which Ply was previously inserted (34). Primers (Sigma-Genosys Ltd., Haverhill, United Kingdom) designed to delete the relevant amino acids are shown in Table 1.

Protein expression and purification. WT and mutant Ply were expressed in *Escherichia coli* XL1 cells (Stratagene) in HySoy J medium (Sigma-Aldrich, Dorset, United Kingdom) and purified by hydrophobic interaction chromatography as described previously (27). Eluted fractions were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie stained using standard protocols (22). Fractions containing >98% pure Ply were pooled and dialyzed overnight in phosphate buffer (0.2 g/liter KCl, 0.24 g/liter KH_2PO_4, 1.44 g/liter Na_2HPO_4; Sigma-Aldrich) prior to concentration using Amicon Ultra-15 centrifugal filter tubes (Millipore, Watford, United Kingdom). The concentrated sample was then further purified by anion exchange with Poros HQ20 Micron chromatography media using the BioCAD 700E workstation (Applied Biosystems Ltd., Warrington, United Kingdom) and introducing a 0 to 1 M NaCl gradient. The hemolytic activity of purified protein was assessed by a hemolysis assay (47) using a 2% (vol/vol) horse red blood cell (E & O Laboratories, Bonnybridge, United Kingdom) solution in 1× phosphate-buffered saline (PBS) (Oxoid, Basingstoke, United Kingdom) to give a specific activity for WT Ply of 10^5 to 10^6 hemolytic units/mg. ΔA146R147 Ply and ΔA146 Ply have zero specific activity. Lipopolysaccharide content of purified toxin was determined using the *Limulus* amebocyte lysate kinetic-QCL kit (Cambrex, Nottingham, United Kingdom) and run according to the manufacturer's instructions. All purified proteins had <0.6 endotoxin units per μg protein. Ply was detected using standard Western blotting techniques; briefly, samples were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred onto Hybond-C nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, United Kingdom) at 80 V for 90 min. Blots were incubated with polyclonal rabbit anti-Ply serum (27) or monoclonal Ply4 mouse anti-Ply serum (9a) and then incubated with the relevant horseradish peroxidase-linked antibody (Amersham Biosciences).

Quantitative hemolytic assay. Serial dilutions of toxin were prepared in 96-well plates with U-shaped bottoms using 1× PBS. An equal volume of 2% (vol/vol) human red blood cell suspension (Blood Transfusion Service, Scotland) was added to each dilution and incubated at 37°C for 30 min. Ammonium (0.4%) was used to give 100% hemolysis. Plates were then centrifuged for 1 min at 1,000 × *g*, the supernatant was carefully transferred to 96-well flat-bottomed plates, and the absorbance was read at 540 nm.

L929 cytotoxicity assay. L929 murine fibroblasts (European Collection of Cell Cultures no. 85011425; Wiltshire, United Kingdom) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomyein (5 U/ml penicillin G sodium–5 μg/ml streptomycin sulfate and 2 mM L-glutamine; all Invitrogen, Paisley, United Kingdom). Cells were seeded at 3.5×10^5 cells per ml in 200-μl aliquots to flat-bottomed 96-well tissue culture plates and incubated for 24 h at 37°C with 5% CO_2. The cells were treated with serial dilutions of recombinant Ply or derivatives for 24 h, then supernatant was removed and cell viability assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). Absorbance was read at 540 nm.

Electron microscopy. Erythrocyte ghosts were prepared from a 2% (vol/vol) horse blood suspension by repeated washing with distilled water. Two hundred microliters of erythrocyte ghost suspension was incubated with an equal volume of either 0.2 mg/ml WT Ply, PdB, ΔA146 Ply, or PBS at 37°C for 30 min and then centrifuged at maximum speed using a bench-top centrifuge to pellet the membranes. Membranes were washed with distilled H_2O (dH_2O) × 6 and resuspended in 50 μl dH_2O. Five microliters of suspension was fixed onto carbon-coated grids and negatively stained with NanoVan (Nanoprobes, Yaphank, NY). Magnification was ×25,000 using an LEO 912 energy filter transmission electron microscope.

In vivo analysis with purified ΔA146 Ply. All in vivo experiments were carried out in accordance with the 1986 United Kingdom Animals Act (Scientific Procedures). As ΔA146R147 Ply and ΔA146 Ply had the same properties in vitro, only the single-amino-acid deletion ΔA146 Ply was used for in vivo analysis. The PdB mutant was not assessed in vivo, as this has been well documented by many groups. Six-week-old female BALB/c mice (Harlan, Bichester, United Kingdom) were implanted with telemetry chips as previously described (18) in order to record core body temperature (Tc). After a 2-week recovery from surgery, mice were lightly anesthetized with 2% halothane-1.5% oxygen (1.5 liter/min) (AstraZeneca, Macclesfield, United Kingdom) and treated intranasally (i.n.) with purified WT Ply or ΔA146 Ply at 1 μg/dose in 50 μl of saline with a saline-only control group (n = 12 to 22). Immediately after treatment, mice were placed in cages on individual receiver boxes to allow recording of Tc. Mice were monitored for 24 h, with Tc readings collected every 30 min. Data were collected using Vital View software (Mini Mitter, Oregon). Serum, bronchoalveolar lavage fluid (BALF), and lung tissue samples were recovered at 24 h from six mice per group and processed as previously described (18). Brains were removed and processed

in the same way as for lungs. Multiplex bead immunoassays (Biosource, Nivelles, Belgium) were used according to the manufacturer's instructions in conjunction with the Luminex 100 system (Bio-Rad, Hertfordshire, United Kingdom) to measure inflammatory mediators produced in response to Ply. Total protein levels in the BALF were measured 24 h post-Ply treatment using a standard Bradford's assay (Sigma-Aldrich) (7).

Immunization of BALB/c mice with ΔA146 Ply. Six-week-old female BALB/c mice were each subcutaneously administered a 100-μl dose of either 20 μg WT Ply plus 100 μg alum (Wyeth, Pearl River, NY), 20 μg ΔA146 Ply plus 100 μg alum, sterile NaCl (0.9% NaCl; Baxter International Inc., Deerfield, IL) plus 100 μg alum, or NaCl only ($n = 8$ per group). Mice were boosted on days 10, 28, and 44. Serum was collected from the lateral tail vein prior to immunization, each day before boosting, and 1 month after the last boost (day 76) and analyzed for anti-Ply IgG by enzyme-linked immunosorbent assay (9a) using 5 μg/ml Ply to coat the plates and horseradish peroxidase-labeled anti-mouse IgG as the detection antibody (Amersham). The ability of anti-Ply antibodies to block the hemolytic action of Ply on erythrocytes was assessed using a neutralizing assay described by Billington et al. (4). The neutralizing ability of the antibodies in the serum from immunized mice was calculated as the reciprocal of the titer at which complete inactivation of 3 hemolytic units of Ply was observed.

Challenge of vaccinated mice. Standard inocula of TIGR4 *S. pneumoniae* (ATCC BAA-334) were prepared and stored as previously described (1). Immunized BALB/c mice were challenged intraperitoneally (i.p.) with 10^2 CFU/100 μl doses of TIGR4 *S. pneumoniae* 1 month after the final boost ($n = 8$ per group). Eighteen hours postchallenge, blood was taken from the lateral tail vein of each mouse to assess the levels of bacteremia in CFU/ml (18). Mice were monitored for survival following challenge, where the endpoint was the time at which mice were deemed lethargic.

Statistical analysis. Inflammatory mediator levels, total protein in BALF, and survival times are expressed as the median (with minimum-to-maximum range or median absolute deviation) with nonparametric analysis by Mann-Whitney U test, where a P value of <0.05 is considered statistically significant (GraphPad Software Inc., San Diego, CA). Anti-Ply IgG levels and bacterial loads are expressed as the means ± standard errors of the means. Bacterial loads in the blood were analyzed with an unpaired t test.

RESULTS

Eight double-amino-acid deletions were constructed within the Ply protein. Four were found to have no detectable hemolytic activity (ΔV144P145, ΔA146R147, ΔM148Q149, and ΔY150E151). A single-amino-acid deletion was also constructed, ΔA146 Ply, which was also found to be nonhemolytic. All mutants were detected in immunoblots with both rabbit polyclonal anti-Ply antibody (raised in our laboratory) and mouse monoclonal Ply4 antibody (kindly provided by Juan de los Toyos, Oviedo, Spain). ΔA146R147 Ply and ΔA146 Ply were selected for purification and further characterization. During protein purification, ΔA146R147 Ply and ΔA146 Ply were retained on the hydrophobic interaction chromatography column for longer than native Ply. This improved protein yield and purity. In general, the mutants were easier to purify than wild-type Ply and could be concentrated to at least 3 mg/ml without the aggregation that often occurs during wild-type Ply purification. The inclusion of an anion exchange step removed residual contaminating proteins and also reduced endotoxin levels 100-fold.

ΔA146R147 Ply and ΔA146 Ply do not lyse erythrocytes or nucleated cells. Purified ΔA146R147 Ply and ΔA146 Ply were found to be nonhemolytic to human erythrocytes at concentrations 10,000 times greater than lytic concentrations of WT Ply (Fig. 1a). PdB (the W433F Ply mutant) was 100-fold less hemolytic than WT Ply but was still lytic at 1 μg/ml. ΔA146 Ply was concentrated to 3 mg/ml and was still not lytic to human erythrocytes. ΔA146R147 Ply and ΔA146 Ply also had no hemolytic activity on horse or sheep erythrocytes.

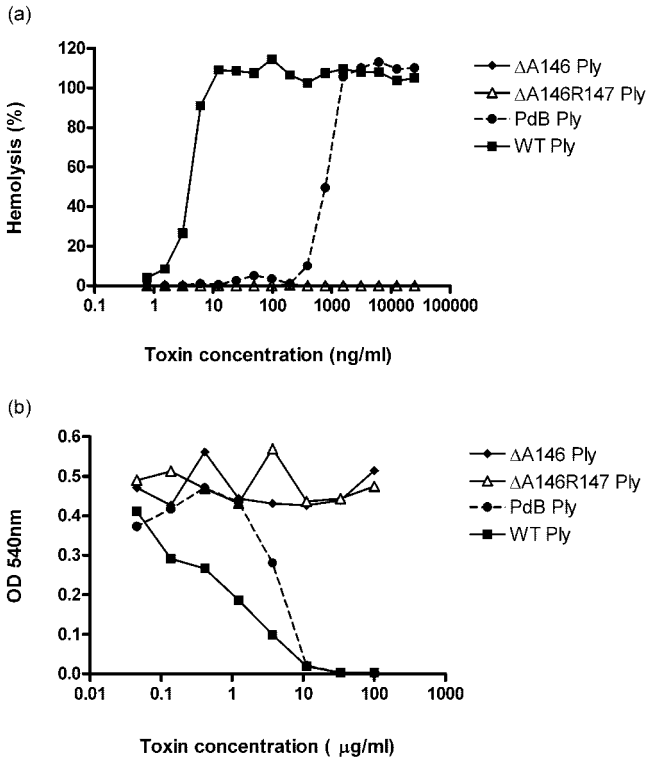

FIG. 1. (a) Ply mutants ΔA146R147 Ply and ΔA146 Ply were compared with WT Ply and PdB in a quantitative hemolytic assay. Following incubations of known concentrations of toxin with a 2% human erythrocyte suspension, supernatants were collected and the hemoglobin released from lysed erythrocytes was measured at an optical density of 540 nm. There was a 100-fold decrease in the hemolytic activity of PdB compared with that of WT Ply, whereas ΔA146R147 Ply and ΔA146 Ply were not hemolytic at greater than 1,000 times the lytic concentration of WT Ply. (b) Cytotoxicity of ΔA146R147 Ply and ΔA146 Ply to nucleated cells compared with that of WT Ply and PdB was assessed with L929 murine fibroblasts. Cells were incubated with serial dilutions of Ply or derivatives, and MTT was used to assess viability of the cells. WT Ply and PdB were found to be cytotoxic to the fibroblasts at concentrations less than 10 μg/ml, whereas ΔA146R147 Ply and ΔA146 Ply were not cytotoxic at 100 μg/ml. OD 540nm, optical density at 540 nm.

L929 murine fibroblasts were used to assess the toxicities of ΔA146R147 Ply and ΔA146 Ply to nucleated cells compared with those of WT Ply and PdB. An MTT dye was used, where mitochondria in live cells convert the yellow dye to a purple precipitate, thereby giving a high absorbance at 540 nm, compared with dead cells, where the dye remains yellow. At concentrations of 100 μg/ml, ΔA146R147 Ply and ΔA146 Ply were not toxic to fibroblasts, whereas <0.5 μg/ml of WT Ply and 5 μg/ml of PdB were cytotoxic to the fibroblasts (Fig. 1b). RBL-2H3 mast cells, previously shown to degranulate when treated with streptolysin O (45), did not degranulate when treated with ΔA146 Ply at concentrations 1,000 times the cytotoxic levels of WT Ply (data not shown). The hemolytic assay and the cytotoxicity assay have similar levels of sensitivity in regard to the toxicity of WT Ply and PdB. Neither assay detected cell damage induced by incubation with ΔA146R147 Ply or ΔA146 Ply. ΔA146 Ply was selected for further analysis on the basis that a single-amino-acid deletion was more likely to retain structural integrity.

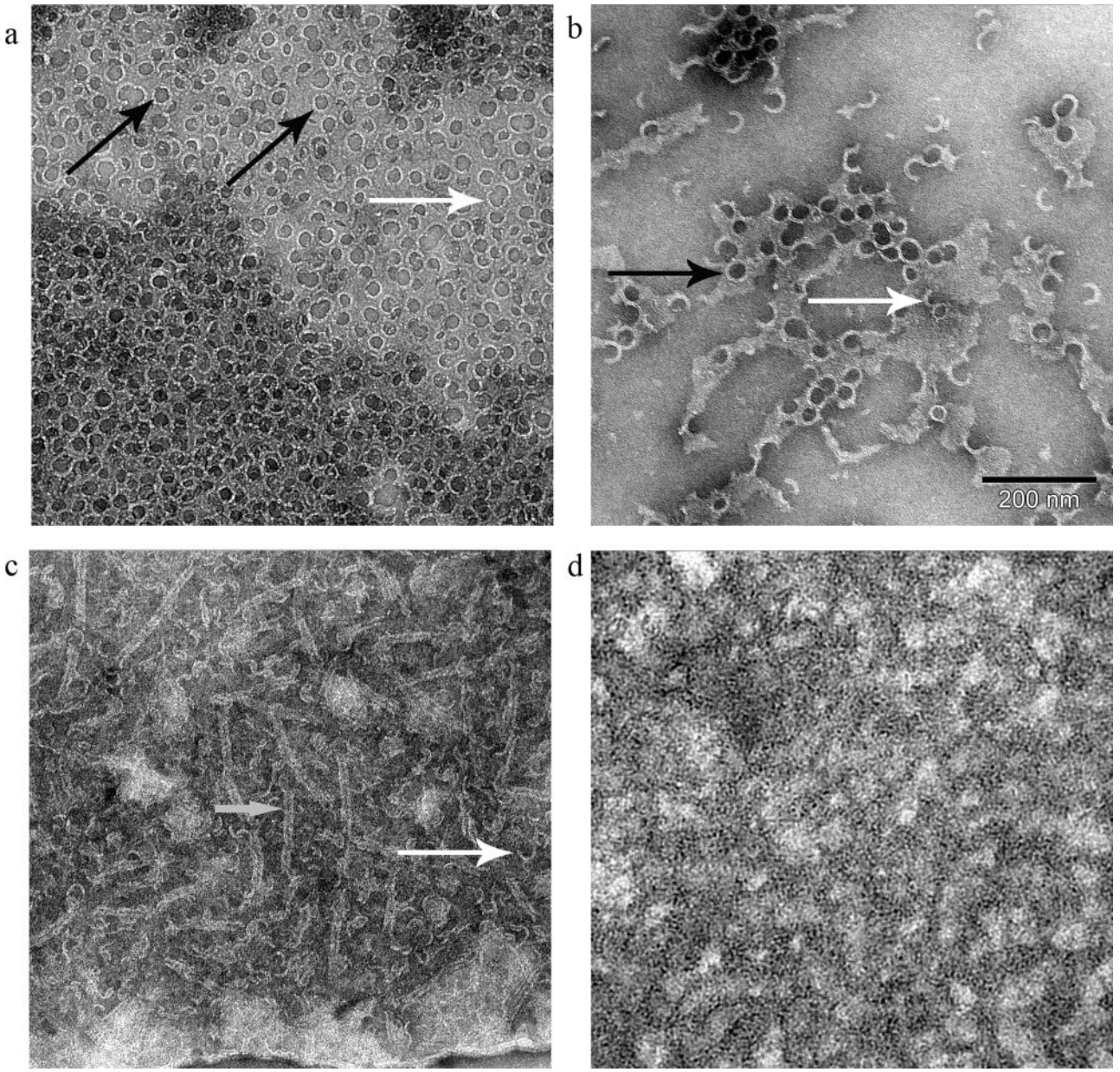

FIG. 2. Electron micrographs of negatively stained horse erythrocyte membranes treated with (a) 0.2 mg/ml WT Ply, (b) 0.2 mg/ml PdB, (c) 0.2 mg/ml ΔA146 Ply, and (d) PBS. Black horizontal scale bar represents 200 nm; magnification is ×25,000. White arrows highlight arcs, black arrows highlight pores, and the gray arrow highlights a rod structure.

ΔA146 Ply does not form pores. Pores were readily visualized by electron microscopy of erythrocyte membranes treated with WT Ply (Fig. 2a) and PdB (Fig. 2b) but not membranes treated with ΔA146 Ply (Fig. 2c). Instead, arcs and rodlike structures were observed on ΔA146 Ply-treated membranes that were not present on untreated membranes (Fig. 2d).

In vivo treatment with ΔA146 Ply did not result in the symptoms observed following WT Ply treatment. BALB/c mice treated i.n. with 1 μg ΔA146 Ply recovered from anesthesia quicker than mice receiving any dose of WT Ply from 0.1 μg to 1 μg. In the initial hours posttreatment, the behavior of ΔA146 Ply-treated mice was similar to that of the saline control group but WT Ply-treated mice exhibited piloerection, labored breathing, and a hunched stance over a 6-h period, recovering within the 24-h time scale. When a 1-μg WT Ply dose was administered i.n. to six MF-1 mice, three mice reached a moribund state within 6 h, whereas i.n. administration of 10 μg of ΔA146 Ply had no adverse effects; higher doses were not investigated. A dose of 5 μg/ml PdB resulted in 100% mortality in MF-1 mice (our unpublished results). BALB/c mice were used for further investigation with Ply treatment, as this allowed the Tc in response to Ply to be correlated with our previously published observation of Tc in response to *S. pneumoniae* infection (18).

Hypothermia was not observed in mice treated with ΔA146 Ply. BALB/c mice treated i.n. with 1 μg ΔA146 Ply did not

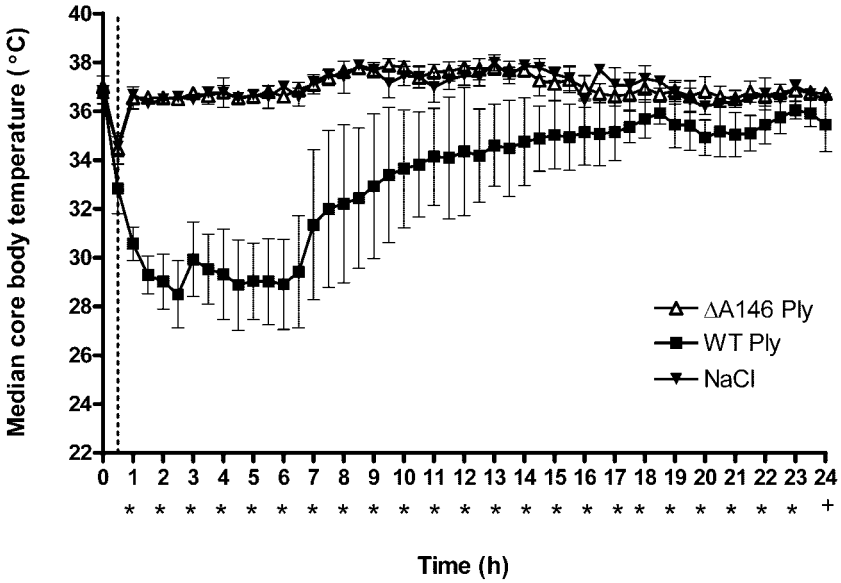

FIG. 3. Median (± MAD) Tc of BALB/c mice over 24 h following i.n. treatment with 1 μg of purified WT Ply, ΔA146 Ply, or NaCl. $n = 12$ to 22. When the WT Ply group is compared with either ΔA146 Ply or NaCl treatment: *, $P < 0.01$; +, $P < 0.05$. An initial drop in Tc was observed in all groups due to time under anesthetic; however, both the ΔA146 Ply- and the NaCl-treated groups recovered immediately, whereas the Tc of WT Ply-treated mice dropped significantly lower and continued to drop to 28°C within 6 h of treatment (*, $P < 0.01$). The WT Ply Tc remained below 30°C for 7 h and gradually increased by ~0.6°C/h for 11 h, where the Tc stabilized at 35°C within 24 h posttreatment; however, this Tc was still significantly lower than the ΔA146 Ply-treated group (+, $P < 0.05$) and pretreatment Tc was not reached within 24 h by the WT Ply-treated group. The dashed vertical line represents the time of treatment.

exhibit the hypothermic response observed in mice treated with 1 μg WT Ply (Fig. 3). The Tc of all treated groups dropped initially by approximately 1°C for the ΔA146 Ply- and NaCl-treated groups, whereas the Tc of the WT Ply-treated group significantly dropped by 2°C ($P < 0.01$). Following this initial drop in Tc, the ΔA146 Ply- and NaCl-treated groups recovered to their pretreatment Tc, whereas the median Tc of the WT Ply-treated mice continued to decrease to 28°C within 6 h posttreatment. From treatment to the 24-h endpoint, the Tc of the WT Ply-treated group was statistically significantly lower than the ΔA146 Ply- and NaCl-treated groups. After 6 h, the Tc of WT Ply-treated mice increased to 35°C; however, the pretreatment median Tc of 37°C was not obtained within 24 h.

ΔA146 Ply does not stimulate the inflammatory responses observed with wild-type Ply treatment. Blood, brain, BALF, and lung tissue samples were collected at 24 h posttreatment with either 1 μg WT Ply, 1 μg ΔA146 Ply, or NaCl and were assessed for inflammatory cytokines and chemokines. Interleukin-6 (IL-6) and cytokine-induced neutrophil chemoattractant KC levels were significantly higher in the lung tissue and BALF of the WT Ply-treated group than in those of the ΔA146 Ply-treated group ($P < 0.05$) (Table 2). There was a 10-fold IL-6 increase and an 8-fold KC increase in the lung tissue of WT Ply-treated mice compared with that of ΔA146 Ply-treated mice. There was a 35-fold increase in IL-6 levels and a 1.6-fold increase in KC in the BALF of WT Ply-treated mice compared with ΔA146 Ply-treated mice at 24 h posttreatment (Table 2). There was an increase in IL-5 in the BALF of WT Ply-treated mice (data not shown). The following cytokines were also assessed, but levels were low in all 24-h samples, with no difference between treatment groups: IL-1β, IL-2, IL-4, IL-10, IL-12, gamma interferon, tumor necrosis factor alpha, and granulocyte-macrophage colony-stimulating factor. No significant levels of mediators were found in the blood or brain tissue from any treatment group.

ΔA146 Ply does not damage lung integrity. Total protein levels were measured in the BALF at 24 h posttreatment with Ply to assess damage to lung integrity (18). Intranasal treatment of BALB/c mice with 1 μg WT Ply resulted in a 25-fold increase in total protein levels in the airways, from 0.2 mg/ml

TABLE 2. Levels of IL-6 and KC within the lung tissue and BALF of BALB/c mice[a]

Treatment	Median (minimum–maximum) level of:				
	IL-6 (pg/ml)		KC (pg/ml)		Total protein (mg/ml)
	Lung tissue	BALF	Lung tissue	BALF	BALF
1 μg WT Ply	105 (19–325)*	250 (24–1,718)**	353 (87–1,117)*	40 (29–331)*	5 (0.4–6)**
1 μg ΔA146 Ply	9 (6–85)	7 (5–8)	40 (31–172)	25 (17–32)	0.2 (0.2–0.3)

[a] Results following intranasal treatment are shown. $n = 6$. Significant increases in WT Ply-treated group versus ΔA146 Ply-treated group are marked with asterisks: *, $P < 0.05$; **, $P < 0.01$.

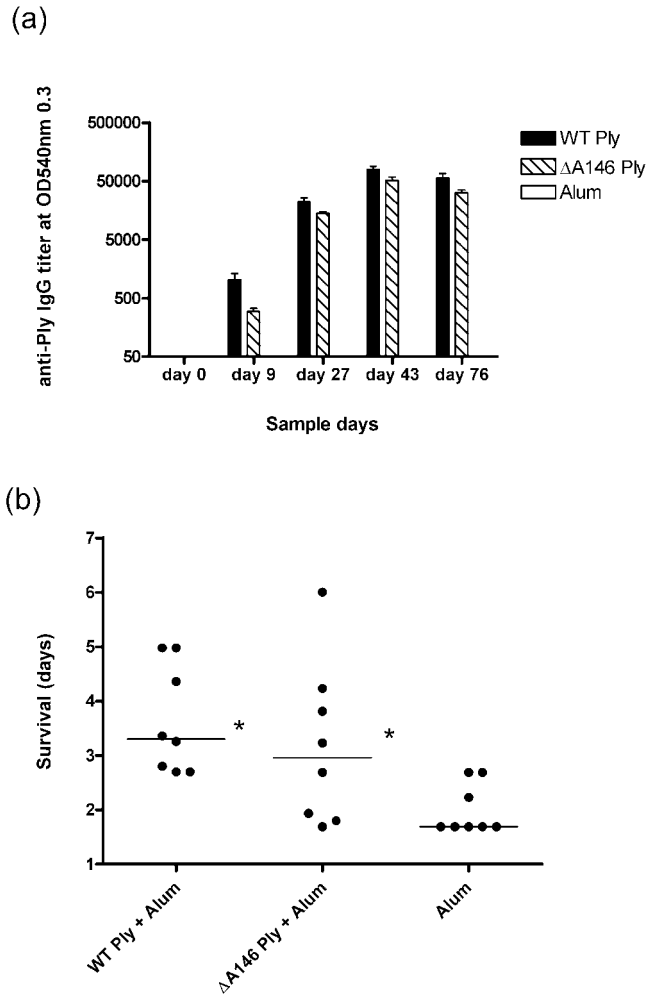

FIG. 4. (a) BALB/c mice were given an initial subcutaneous injection and three boosts of either WT Ply plus alum, ΔA146 Ply plus alum, or alum alone. Serum was collected before vaccination (day 0) and before each boost (days 9, 27, and 43) and on day 76 of the vaccination protocol and analyzed for anti-Ply IgG antibodies by enzyme-linked immunosorbent assay (7 days prior to challenge with TIGR4). The WT Ply-plus-alum- and ΔA146 Ply-plus-alum-immunized groups had high anti-Ply IgG titers from day 27 onwards, whereas anti-Ply IgG was not detected in serum from the alum-vaccinated control group. The bars represent the mean anti-Ply IgG titers for each group ± standard error of the mean at an optical density at 450 nm of 0.3 ($n = 8$). (b) Survival time (in days) of vaccinated BALB/c mice following i.p. challenge with 10^2 CFU TIGR4 *S. pneumoniae*. The WT Ply-plus-alum- and ΔA146 Ply-plus-alum-vaccinated groups survived significantly longer than the alum-only control group. Each spot represents the survival time of an individual mouse; horizontal bars represent the median survival time for each group ($n = 8$). *, $P < 0.05$ compared with alum alone.

to 5 mg/ml, compared with mice treated with 1 μg ΔA146 Ply (Table 2) ($P < 0.01$). We did not observe increases in total protein levels for ΔA146 Ply-treated mice compared with lavage fluid samples from healthy mice. MF-1 mice treated i.n. with 0.25 μg WT Ply were found to have 10 times the level of total protein in their BALF (1 mg/ml total protein) compared with MF-1 mice treated with four times as much ΔA146 Ply (0.1 mg/ml total protein) ($P < 0.01$; $n = 6$) (data not shown).

ΔA146 Ply has similar immunogenic properties to WT Ply. Anti-Ply IgG antibody levels in day 43 and day 76 sera from the vaccination protocol were high in the groups vaccinated with ΔA146 Ply plus alum and WT Ply plus alum but not in the control mice that were vaccinated with alum only (Fig. 4a) or NaCl (data not shown). The anti-Ply IgG antibodies in day 76 sera from the WT Ply-plus-alum and ΔA146 Ply-plus-alum groups were also found to neutralize Ply's hemolytic activity to a neutralizing titer of 3,200 to 12,800 in comparison with background levels of 800 to 1,600 for the alum-only day 76 serum.

Following vaccination, mice were challenged i.p. with 10^2 CFU TIGR4 *S. pneumoniae*. This dose was chosen as it was confirmed to be lethal to nonimmunized mice, with all mice reaching a moribund state around 48 h (our unpublished results). Eighteen hours following infection, the bacterial loads of all vaccinated groups were within the range of 10^5 to 10^6 CFU/ml, with no significant difference between Ply-vaccinated groups and control groups. However, mice immunized with WT Ply plus alum or ΔA146 Ply plus alum survived the infection significantly longer than the alum-only control group (median survival times of 3.3, 3.0, and 1.7 days, respectively; $P < 0.05$) (Fig. 4b) and the NaCl-only group (median survival time of 1.7 days) (data not shown). Immunization with ΔA146 Ply was as protective as WT Ply ($P = 0.279$). One mouse from the ΔA146 Ply-plus-alum group, which had a blood bacterial load of 2×10^5 CFU/ml TIGR4 at 18 h postinfection, cleared the infection within 6 days.

DISCUSSION

We have constructed and characterized nontoxic forms of Ply that are immunogenic and can be administered at significantly higher doses than native Ply without any detrimental effect. All of the nontoxic Ply mutants we have constructed were recognized by monoclonal antibody (MAb) Ply4 and their mutations are within the region proposed to be involved in oligomerization (9a). Recently, pretreatment with MAb Ply4 has been shown to passively protect mice from subsequent wild-type Ply treatment and *S. pneumoniae* infection (9). This may be a result of MAb Ply4 blocking Ply's ability to oligomerize in vivo, a property that has already been demonstrated in vitro (9a). It is important to note that MAb Ply4's recognition of these mutants shows that we have retained an essential epitope, previously shown to be highly antigenic by epitope scanning, that is recognized by both human convalescent-phase sera and rabbit hyperimmune sera (41).

In vitro characterization of the double-amino-acid deletion ΔA146R147 Ply and the single-amino-acid deletion ΔA146 Ply revealed that they were not cytotoxic to erythrocytes or nucleated cells, unlike native Ply or the PdB mutant. Erythrocytes can be protected from WT Ply lysis by preincubation with ΔA146R147 Ply or ΔA146 Ply (data not shown); this has been previously shown with preincubation of membranes with domain 4 of Ply (2). Such protection of cells is likely to be a result of the saturation of the membrane binding sites by the nonlytic mutant, thereby preventing the native toxin from binding to and damaging the host cell membrane.

According to the Ply structural model based on perfringolysin O (Pfo) (39), the CDC produced by *Clostridium perfringens*, the ΔA146 Ply mutation is at the domain 1 and domain 3 interface. This region is packed in an awkward manner, possi-

bly to provide energy for the movement of domain 3 during membrane insertion (46). Domain 3 of Pfo has two transmembrane β-hairpins (TMH1 and TMH2) that unfold upon oligomerization to allow insertion of the oligomer into the lipid bilayer (43). A Y181A substitution in Pfo prevents insertion into the membrane and arrests the oligomer in a "prepore" state on the membrane surface (16). This mutation is immediately upstream of TMH1 and is in the same conserved region as ΔA146 is in Ply. If ΔA146 Ply behaved in the same manner, we would expect to detect prepore structures on the membrane surface. Oligomeric rings (prepores) have not been observed on membranes treated with ΔA146 Ply; instead, arcs (also evident in the WT Ply-treated sample) and long chains were observed by electron microscopy of ΔA146 Ply-treated erythrocytes (Fig. 2c). The properties of the arcs are a subject of much debate (31, 46). The long chains formed on ΔA146 Ply-treated membranes have not been described before. Helical structures have been shown to form with high concentrations of Ply, both in solution and on membranes (12, 13); however, the diameter of these structures is similar to the 30-nm ring structures and not the 10-nm diameter of the chains shown in Fig. 2c. A possible explanation for these long chains is that the self-recognition site has been altered on the ΔA146 Ply monomers, not so much that association is inhibited, but enough to prevent the regulation that must occur in native Ply to give the uniform oligomer ring shape and size of ~50 monomers (28).

Recently, specific sites on domain 3 of Pfo have been identified as being involved in monomer-monomer interaction (36), and these regions are homologous to where the deletions were made in Ply. This region is highly conserved within the CDCs, with the majority possessing the VPARMQYE motif. Indeed, deletions of A177R178 in Pfo, A204R205 in intermedilysin (produced by *Streptococcus intermedius*) and A190R191 in anthrolysin O (produced by *Bacillus anthracis*) give the same nonhemolytic phenotype as ΔA146R147 Ply and ΔA146 Ply (our unpublished results). From the experimental evidence we have produced and detailed analysis of Pfo, we suggest that by deleting the residues within the VPARMQYE region, we have modified a self-recognition site of domain 3 that abolishes the ability of the toxin to form oligomeric rings and insert into the host cell membrane.

We have established that treatment of mice with ΔA146 Ply does not induce the inflammatory effects that are associated with native Ply treatment. When BALB/c mice are treated with WT Ply, there is a localized inflammatory response 24 h after treatment, which is characterized by significant increases in IL-6 and KC levels in lung tissue and BALF. Localized production of IL-6 and KC in response to WT Ply has previously been reported and is likely to be from alveolar macrophages and recruited neutrophils (37). Mice treated with WT Ply had severely damaged lungs in comparison with the healthy lungs retrieved from mice treated with ΔA146 Ply. The large amount of total protein observed in the airways of WT Ply-treated mice is a result of Ply's disruption of tight junctions at the capillary/airway barrier (40), allowing an influx of host serum proteins to flood the lung airways. This damage to the airways has recently been shown to be entirely due to the action of Ply and is independent of recruited leukocytes (25).

Our group has previously shown that BALB/c mice have a profound early hypothermic response to i.n. challenge with D39 *S. pneumoniae* but they are able to clear the infection, whereas CBA/Ca mice do not have the early hypothermic response and subsequently succumb to pneumococcal infection (18). To our knowledge, such a dramatic drop in Tc following WT Ply treatment has not been reported before. We propose that the 12-h hypothermia observed with pneumococcal infection (18) may occur when the bacteria have established within the host and are lysing, resulting in the release of Ply. The immediate hypothermic response we observe may be due to the direct intranasal administration of the toxin, but this may also be a result of the mice being unable to maintain their Tc following treatment with the anesthetic. Further experiments recording the Tc of BALB/c mice infected with the Ply negative strain of *S. pneumoniae*, PLN-A (3), would provide information on whether the hypothermic response found with D39 *S. pneumoniae* infection is due to Ply. Although the mechanisms of thermoregulation are not fully understood, cytokines are known to be involved in regulation/induction of hypothermia (23). However, at 24 h posttreatment, IL-6 and KC were only elevated at the site of Ply administration and were not detected in the blood or brain tissue. Of the 11 mediators measured, none were found systemically at 24 h posttreatment. It may be that the systemic responses occurred earlier to cause the early drop in Tc. We have therefore not identified any inflammatory mediators involved in thermoregulation during exposure to Ply and ultimately *S. pneumoniae* infection.

A similar immunogenic response to ΔA146 Ply and WT Ply demonstrates that although the mutant is not toxic, antibodies are still produced against it. The ability of the raised antibodies to block Ply's lytic action indicates that they are fully functional and recognize an active region of the toxin. Mice vaccinated with ΔA146 Ply or WT Ply were protected from infection significantly longer than animals that were not vaccinated. It may be that the anti-Ply antibodies are enough to inhibit infection to allow anti-capsule antibodies to develop, albeit to a suboptimal level. There was no inflammatory response, hypothermia, or disruption of lungs in mice treated with ΔA146 Ply, correlating with the inability of this mutant to create pores in host cell membranes. We hope to increase the efficacy of current pneumococcal polysaccharide vaccines, proposing the use of ΔA146 Ply (or derivatives) as supplementary carrier proteins. Consequently, construction of similar mutations in other CDCs may hold future applications for use as vaccine candidates to protect against a range of infections, from gas gangrene caused by *C. perfringens* (38) to anthrax from *B. anthracis* (42).

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