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Enzymatic Hydrolysis of Pneumococcal Capsular Polysaccharide Renders the Bacterium Vulnerable to Host Defense

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ABSTRACT Despite a century of investigation, *Streptococcus pneumoniae* remains a major human pathogen, causing a number of diseases, such as pneumonia, meningitis, and otitis media. Like many encapsulated pathogens, the capsular polysaccharide (CPS) of *S. pneumoniae* is a critical component for colonization and virulence in mammalian hosts. This study aimed to evaluate the protective role of a glycoside hydrolase, Pn3Pase, targeting the CPS of type 3 *S. pneumoniae*, which is one of the most virulent serotypes. We have assessed the ability of Pn3Pase to degrade the capsule on a live type 3 strain. Through *in vitro* assays, we observed that Pn3Pase treatment increases the bacterium's susceptibility to phagocytosis by macrophages and complement-mediated killing by neutrophils. We have demonstrated that *in vivo* Pn3Pase treatment reduces nasopharyngeal colonization and protects mice from sepsis caused by type 3 *S. pneumoniae*. Due to the increasing shifts in serotype distribution, the rise in drug-resistant strains, and poor immune responses to vaccine-included serotypes, it is necessary to investigate approaches to combat pneumococcal infections. This study evaluates the interaction of pneumococcal CPS with the host at molecular, cellular, and systemic levels and offers an alternative therapeutic approach for diseases caused by *S. pneumoniae* through enzymatic hydrolysis of the CPS.

KEYWORDS *Streptococcus pneumoniae*, capsular polysaccharide, colonization, complement, glycoside hydrolase, invasive pneumococcal diseases, serotype 3

Streptococcus pneumoniae, the causal agent of pneumonia, meningitis, and otitis media, remains a major threat to human health. This bacterium can stably colonize the human nasopharynx as a part of the normal commensal microflora (1–3). Colonization is the primary mode of transmission and a key step in the initiation of disease, despite asymptomatic carriage (4, 5). A critical component for survival within the host and full pathogenicity of most *S. pneumoniae* strains is the capsular polysaccharide (CPS) (6, 7). The CPS is a large and distinct polysaccharide structure coating the entire surface of the bacterium. The capsule helps *S. pneumoniae* evade the host immune system through resisting or inhibiting its phagocytosis by host macrophages while also limiting mucus-mediated clearance (8–11). *S. pneumoniae* has over 90 unique capsular serotypes, each differing in monosaccharide composition and linkage, as well as other modifications, such as acetylation (12). The requirement of the CPS in bacterial virulence, surface accessibility, and antigenicity has made it a target in vaccination studies for over 100 years (12–16). Great strides have been made in increasing the immunogenicity and efficacy of pneumococcal vaccines that utilize the CPS by conjugation to a protein carrier (17, 18). Current pneumococcal vaccines aim to provide serotype-specific protection for some of the most relevant clinical serotypes (12, 13). The use of the 7- and 13-valent pneumococcal conjugate vaccines PCV7 and PCV13 (Prevnar;

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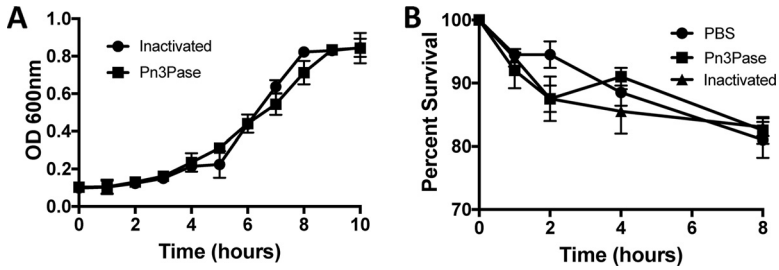

FIG 1 Effects of Pn3Pase treatment on type 3 *S. pneumoniae* viability. (A) Growth curve of WU2 in THY broth in the presence of 100 μg/ml Pn3Pase following the OD_{600}. (B) WU2 survival in PBS in the presence of 100 μg/ml active or heat-inactivated Pn3Pase.

Pfizer) has been a major success, reducing invasive pneumococcal disease (IPD) rates significantly in both vaccinated and unvaccinated populations (15, 19–22).

While the conjugate vaccines have been effective for preventing carriage and IPD caused by most included serotypes, the exception has been serotype 3. The pneumococcal type 3 polysaccharide (Pn3P) component of the current PCV13 induces variable immune responses to *S. pneumoniae* serotype 3 (23–25). Pn3P is a linear polymer of -3)βGlcA(1–4)βGlc(1- disaccharide repeating units with an average molecular weight of $>$400 kDa (26, 27). It was noted that significantly higher serum titers are required for serotype 3 in comparison with other serotypes for opsonophagocytic killing of *S. pneumoniae* (12, 28, 29). Numerous animal model and epidemiology studies have associated type 3 strains with increased virulence and risk of death compared to other pneumococcal serotypes (30–32). A recent case report demonstrated a fatal case of IPD caused by serotype 3, highlighting increased complications and generally poor outcomes associated with this invasive serotype (33). In addition, recent data indicate that *S. pneumoniae* strains are resistant to one or more antibiotics in 30% of IPD cases (34). The Centers for Disease Control and Prevention predict a rise in antibiotic resistance features of *S. pneumoniae* (35, 36).

The inability of vaccination to provide adequate protection against one of the most aggressive serotypes of this major human pathogen necessitates the urgent exploration of alternative approaches for type 3 pneumococcal infections. This, along with a rise in the prevalence of antibiotic-resistant strains (37, 38), led us to revisit early studies by Avery and Dubos, who discovered a soil-dwelling bacterium producing an enzyme that hydrolyzes Pn3P (39–41). Previously, we identified this bacterium as a *Paenibacillus* species, cloned its type 3-specific glycosyl hydrolase, Pn3Pase, and characterized its degradation products (26, 42). In light of the continued prevalence and severity of serotype 3 *S. pneumoniae*, we postulated the potential use of this purified protein as a therapeutic agent for hypervirulent serotype 3 infections. Here, we investigated the ability of Pn3Pase to degrade the capsule on a live virulent type 3 *S. pneumoniae* strain and therefore render the bacterium susceptible to host immune clearance.

RESULTS

Pn3Pase removes capsule from growing type 3 *S. pneumoniae*. The encapsulated type 3 WU2 strain was cultured for 10 h with the recombinant enzyme added to the growth medium, and bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) to assess effects of Pn3Pase treatment on growing type 3 *S. pneumoniae* cells. Pn3Pase treatment of cells demonstrated no adverse growth or cytotoxic effects on the bacteria (Fig. 1A). In the same experiment, we obtained comparable CFU values for both enzyme-treated and nontreated groups at 2-h intervals (data not shown). To assess the direct impact of enzyme treatment on bacterial survival, a log-phase culture was isolated and suspended in nutrient-free buffer with active or heat-inactivated Pn3Pase. Enzyme-treated cells showed comparable counts to inactivated and phosphate-buffered saline (PBS) controls over the 8-h time course (Fig. 1B).

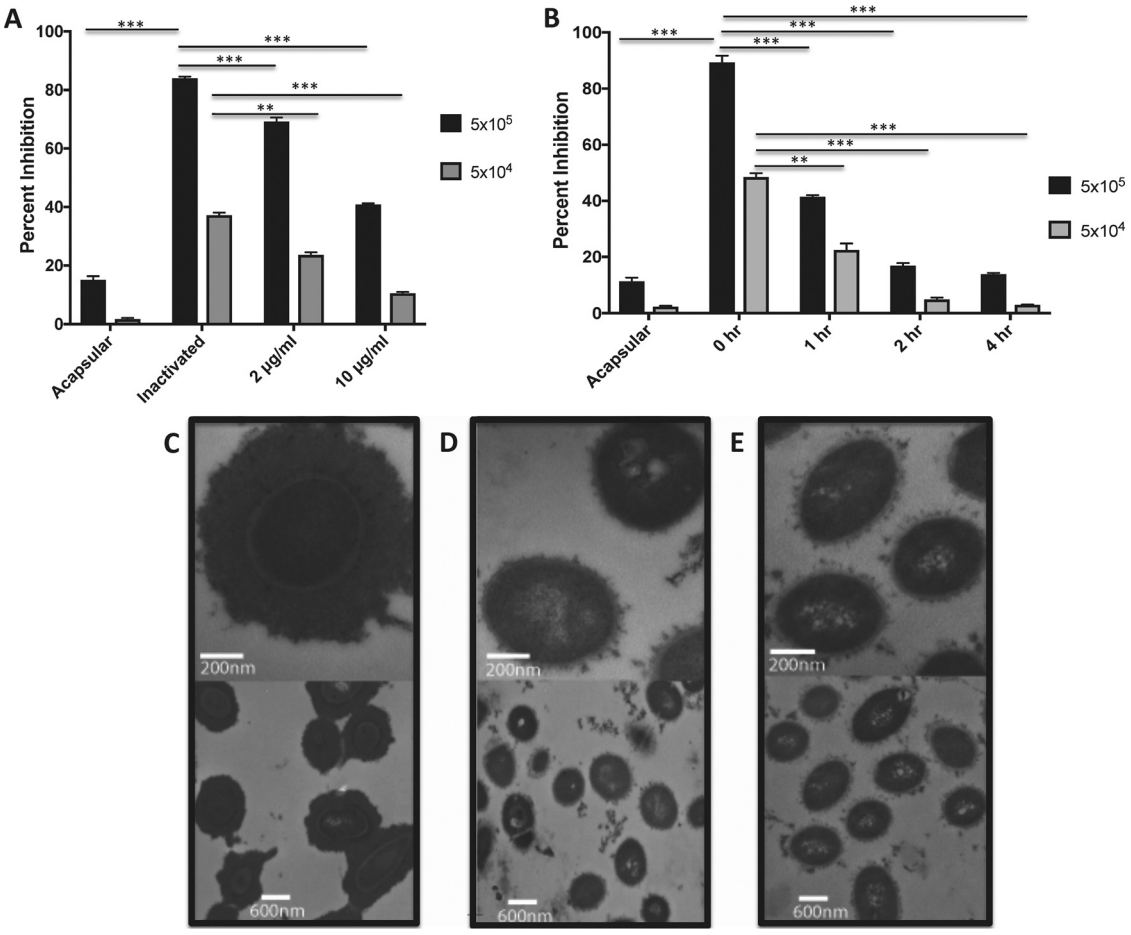

FIG 2 Depleting the capsule on live type 3 *S. pneumoniae* by Pn3Pase treatment. (A and B) Competition ELISA in which cells of acapsular WU2, WU2 treated with heat-inactivated Pn3Pase, or WU2 treated with Pn3Pase at two different concentrations (2 or 10 μg/ml) were used to compete for Pn3P-specific antibody binding to a Pn3P-coated ELISA plate. Data are presented as percentage of inhibition of antibody binding. Statistical significance was determined with the two-tailed Student's *t* test. **, $P < 0.01$; ***, $P < 0.001$. Transmission electron microscopy images of WU2 mock treated with 2 μg/ml heat-inactivated Pn3Pase (C), acapsular WU2 strain (D), and 2 μg/ml active-Pn3Pase treated WU2 (E). Bottom panels in panels C to E are imaged at 15,000× direct magnification, and top panels are imaged at 10,000× direct magnification.

The bacterial cells grown in the presence of Pn3Pase were then examined by competition enzyme-linked immunosorbent assay (ELISA) to determine if the enzyme led to efficient capsule removal in the growing cultures. After treatment, fixed whole cells at two different concentrations were used to compete for Pn3P-specific antibody binding to the Pn3P-coated plate. The acapsular WU2 mutant strain (JD908) showed minimal to no inhibition of antibody binding due to its lack of capsule. Heat-inactivated Pn3Pase-treated cells demonstrated the highest percentage of inhibition as cell surfaces should be fully decorated with CPS. With active Pn3Pase treatment, inhibition of antibody binding begins to decrease in a Pn3Pase concentration-dependent manner (Fig. 2A), suggesting that the enzyme strips the capsule from live, growing type 3 *S. pneumoniae*.

A time course experiment was performed in which the bacterial cells were grown to mid-log phase, suspended in PBS, and treated with Pn3Pase for 0, 1, 2, or 4 h. The Pn3Pase dose was lowered to 1 μg/ml for this assay, given that the WU2 strain should not be actively growing and therefore should not be producing a substantial amount of new CPS under these conditions. At each time point, the treated cells were fixed and examined by competition ELISA as described above. The acapsular WU2 mutant strain (JD908) again showed minimal to no inhibition due to its lack of capsule. Untreated cells exhibited the highest percentage of inhibition, indicative of a cell surface fully

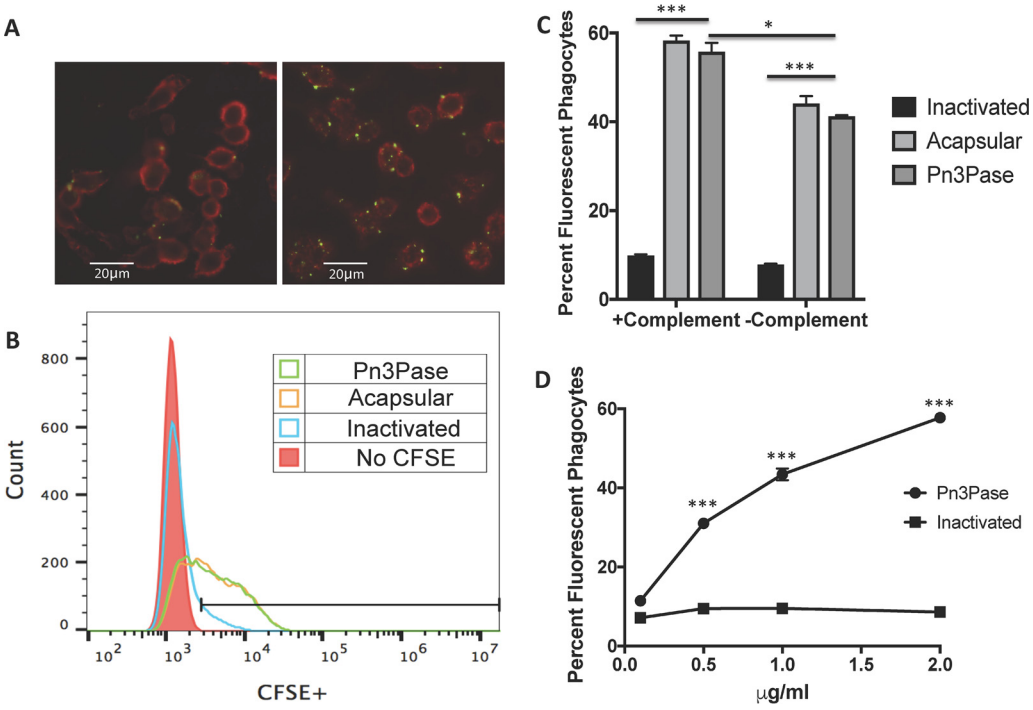

FIG 3 Macrophage uptake of type 3 *S. pneumoniae* treated with Pn3Pase. (A) RAW 264.7 macrophages containing fluorescent streptococci following heat-inactivated (left) or active (right) Pn3Pase treatment. Streptococci labeled with CFSE are in green, and macrophages labeled with biotinylated wheat germ agglutinin (WGA) or streptavidin-APC are in red. (B) Histogram of the flow cytometry analysis of fluorescent phagocytes demonstrating increased fluorescence intensity for the acapsular control and Pn3Pase-treated WU2 strain. (C) Influence of Pn3Pase treatment and complement on macrophage uptake of CFSE-labeled *S. pneumoniae* quantified by flow cytometry. (D) Pn3Pase dose-dependent effect on the percentage of fluorescent phagocytes. Statistical significance was determined with the two-tailed Student's *t* test. ***, $P < 0.001$; *, $P < 0.05$.

decorated with CPS. With increasing Pn3Pase incubation time, inhibition of antibody binding begins to decrease significantly, appearing essentially acapsular after 2- or 4-h treatments (Fig. 2B).

The Pn3Pase-treated cells were visualized by transmission electron microscopy and compared to the cells treated with heat-inactivated Pn3Pase. The heat-inactivated enzyme-treated cells displayed a thick, complete CPS coat across their surface (Fig. 2C), undoubtedly distinct from the acapsular mutant (Fig. 2D). The capsule layer of the enzyme-treated cells exhibited little to no capsule (Fig. 2E), appearing acapsular.

Pn3Pase treatment of type 3 *S. pneumoniae* allows phagocytic cell uptake and killing. A major virulence mechanism of the CPS is to provide the bacterium the ability to resist phagocytosis by host phagocytic cells (1, 6). To investigate the effect of Pn3Pase treatment on uptake by macrophages *in vitro*, we stained mid-log-phase bacterial cultures with carboxyfluorescein succinimidyl ester (CFSE) and then treated them with Pn3Pase followed by incubation with RAW 264.7 macrophages. Macrophages were then washed extensively and imaged by fluorescence microscopy (Fig. 3A). The extent of bacterial uptake by macrophages was quantified by flow cytometry (Fig. 3B). Bacteria treated with the enzyme were taken up by macrophages significantly more than the encapsulated strain. We then determined the percentage of fluorescent macrophages, representing the phagocytosis of fluorescent bacteria (Fig. 3C and D). The encapsulated type 3 strain incubated with heat-inactivated Pn3Pase had minimal fluorescently labeled phagocytes, whereas Pn3Pase-treated bacteria were as efficiently taken up by the macrophages as the acapsular mutant strain. Uptake was partially dependent on complement, as evidenced by higher bacterial uptake in the presence of complement (Fig. 3C). Pn3Pase treatment rendered the bacteria more susceptible to phagocytic engulfment by RAW macrophages in a dose-dependent

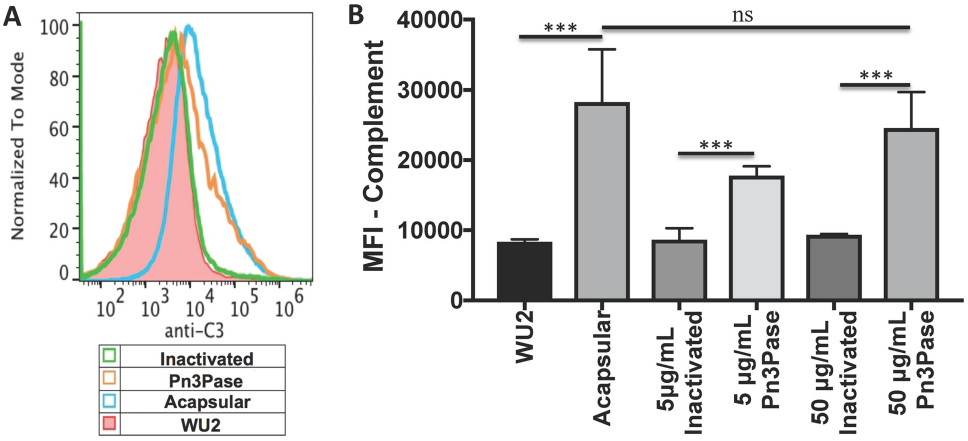

FIG 4 Effect of Pn3Pase treatment on complement deposition on *S. pneumoniae* surface. Shown are the results from analysis of mouse complement deposition on Pn3Pase-treated or untreated type 3 *S. pneumoniae* by flow cytometry. The histogram (A) and quantification by mean fluorescent intensity (MFI) (B) of FITC-A were calculated from gating of Hoechst-positive cells. Statistical significance was determined with the two-tailed Student's *t* test. ***, $P < 0.001$; ns, not significant.

manner, while even high doses of the inactivated enzyme had no significant effect on bacterial uptake (Fig. 3D).

Complement deposition on the pneumococcal surface is an important mechanism aiding in efficient phagocytosis and clearance (10, 11). Since CPS provides complement evasive properties to the bacterium (10, 11), we investigated the effect of capsule removal by Pn3Pase treatment on C3b deposition on the bacterial surface. The encapsulated type 3 WU2 strain was treated with active or inactive Pn3Pase. The untreated type 3 strain and the acapsular mutant were included as additional controls. Bacteria were then incubated with normal mouse serum, washed, and stained with fluorescein isothiocyanate (FITC)-conjugated antibody to mouse complement. Fixed samples were then analyzed by flow cytometry. In a dose-dependent manner, Pn3Pase treatment increased the deposition of complement on the bacterial surface, reaching the levels of deposition on the acapsular strain. Complement had minimal binding to the bacteria either untreated or treated with inactivated Pn3Pase (Fig. 4).

The standard *in vitro* assay to assess phagocytic killing of *S. pneumoniae* is an opsonophagocytosis assay (OPA) in which HL-60 cells are differentiated into neutrophils to engulf and clear antibody-opsonized bacteria. This method has been widely used to measure the quality of antibody responses in numerous vaccine studies (43–48). Neutrophils are one of the most important components of innate immunity against pathogenic bacteria in the lungs (49–51). While the focus of this study is not on humoral immune responses to *S. pneumoniae*, we have used a modified OPA to evaluate complement-mediated neutrophil killing of type 3 *S. pneumoniae* treated with Pn3Pase *in vitro*. Encapsulated type 3 *S. pneumoniae* was incubated with active or heat-inactivated Pn3Pase. Differentiated HL-60 cells were preincubated with active or heat-inactivated complement. The mixture of HL-60 cells and complement was added to the bacteria and incubated at 37°C for 1 h. To quantify the surviving bacteria in the experimental groups, the reaction mixtures were plated and CFU were counted the next day. Percentage of survival was calculated as each duplicate reaction normalized to mean values obtained from reactions without neutrophils, which served as control samples (100% survival). The enzyme-untreated, encapsulated type 3 strain was able to escape neutrophil killing to show maximum survival, while the acapsular mutant strain was reduced to nearly 40% viability upon incubation with active complement and neutrophils (Fig. 5). Pn3Pase treatment to remove the capsule rendered the type 3 strain susceptible to complement-dependent neutrophil killing similar to the acapsular strain (Fig. 5).

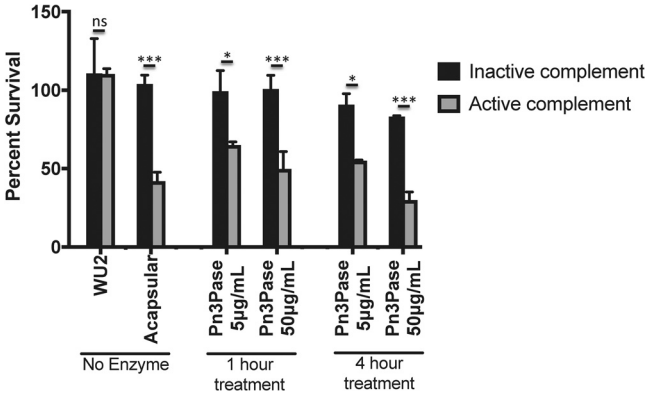

FIG 5 Effect of Pn3Pase treatment on complement-mediated killing by neutrophils. Shown is the complement-mediated killing capacity of differentiated HL-60 cells on Pn3Pase-treated *S. pneumoniae*. The percentage of survival was calculated as each duplicate reaction normalized to mean values obtained for control samples (where reactions without HL60 cells represent 100% survival). Statistical significance was determined with the two-tailed Student's *t* test. ***, $P < 0.001$; *, $P < 0.05$; ns, not significant.

Pn3Pase limits nasopharyngeal colonization. To investigate the enzyme's protective abilities *in vivo*, we first performed an intranasal colonization experiment with BALB/c mice. Nasal colonization by *S. pneumoniae* is essential for transition to invasive pneumococcal disease (4, 5). It is established that the capsule of this strain is required for intranasal colonization (6). Therefore, we used the nasal colonization model to assess the ability of Pn3Pase to reduce bacterial colonization in the nasopharynx through removal of the capsule of the colonizing type 3 strain. We first confirmed that the acapsular mutant, JD908, failed to colonize the nasopharynx (data not shown). Groups of mice were then intranasally inoculated with 10^6 log-phase wild-type (wt) encapsulated bacteria in 10 μl PBS. All inocula were chased with either Pn3Pase or buffer control. Groups were dosed with the enzyme at day 0, days 0 and 3, or days 0, 3, and 7 to assess the effects of multiple administrations. Mice were euthanized, and bacterial load was quantified on day 10. Nasal lavage fluid was obtained, serially diluted, and plated to enumerate the bacterial load. Vehicle control-treated mice were colonized with significantly higher bacterial loads than mice treated with only a single dose of Pn3Pase. Administration of two or three doses of Pn3Pase made the majority of the animal lavage fluid void of any viable bacterial colonies (Fig. 6A). Lung homogenates and serum samples showed no evidence of a bacterial burden (data not shown). Signature proinflammatory cytokine levels were measured in the nasal lavage fluid by ELISA (52). Vehicle-treated mice had significantly increased levels of the cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) compared to Pn3Pase-treated animals, a reflection of a continued host inflammatory response to the bacterial burden in this group (Fig. 6B and C) (52). A significant reduction in IL-6 and TNF-α was observed in most animals even after a single dose of Pn3Pase on day 0. The mouse with a higher bacterial load in the single-dose group contributed to the increased cytokine levels in this group.

Pn3Pase protects mice from lethal challenge. To further assess Pn3Pase for its protective abilities and to evaluate the utility of Pn3Pase as a therapeutic agent, we employed an intraperitoneal (i.p.) sepsis model (53, 54). Groups of mice were infected with 5×10^3 CFU of the log-phase WU2 type 3 strain of *S. pneumoniae*. We assessed the effect of a single dose of 5 μg or 0.5 μg, administered at time 0, 12, or 24 h postinfection. Control groups treated with heat-inactivated enzyme died within 48 h of infection. Regardless of the enzyme dose or the timing of the administration, all treated groups displayed no signs of illness and experienced full protection from the i.p. challenge (Fig. 7).

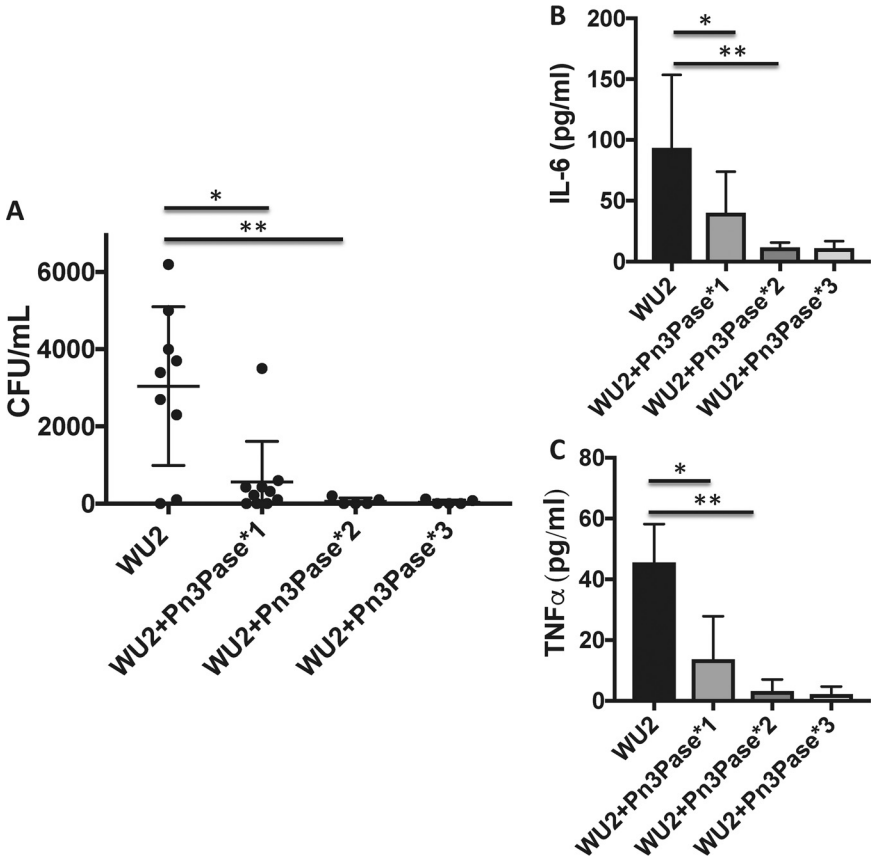

FIG 6 Intranasal colonization with type 3 *S. pneumoniae*. Shown is the ability of Pn3Pase treatment to reduce *S. pneumoniae* colonization in the nasopharynx of BALB/c mice. (A) Groups of mice were intranasally inoculated with 10^6 log-phase bacteria in 10 μl PBS. All inocula were chased with either 50 μg of Pn3Pase or buffer control (10 μl). Groups were dosed with the enzyme at either day 0 (WU2+Pn3Pase*1), days 0 and 3 (WU2+Pn3Pase*2), or days 0, 3, and 7 (WU2+Pn3Pase*3). Bacterial load was quantified on day 10. Serial dilutions of nasal lavage fluid were plated in duplicate to determine CFU values. (B and C) IL-6 (B) and TNF-α (C) concentrations in nasal lavage fluid were determined by ELISA. Statistical significance was determined with the two-tailed Student's *t* test *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

This study aimed to evaluate the protective role of a carbohydrate-degrading enzyme (glycoside hydrolase), Pn3Pase, targeting the CPS of the pathogenic bacterium serotype 3 *S. pneumoniae*. Invasive pneumococcal diseases (IPDs) caused by *S. pneu-*

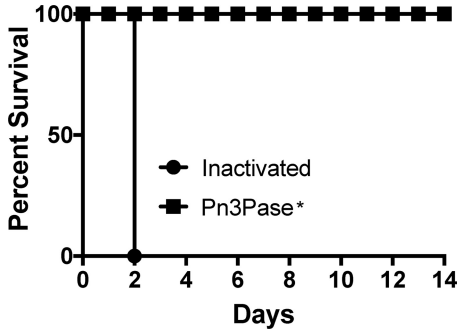

FIG 7 Protective ability of Pn3Pase. Shown are the results from assessment of the ability of Pn3Pase to protect BALB/c mice from lethal challenge. Groups of mice were infected through intraperitoneal administration of 5×10^3 log-phase virulent type 3 *S. pneumoniae* cells. The inactivated group is heat-inactivated Pn3Pase. "Pn3Pase*" indicates the results shown represent the effect of a single dose of 5 μg or 0.5 μg administered at time 0, 12, or 24 h postinfection for $n = 5$ mice per group.

moniae have been a major threat to human health, with alarming mortality rates. Despite a global vaccination program and the use of antibiotics, *S. pneumoniae* remains among the deadliest infectious agents worldwide. Pneumococcal vaccines are made empirically and are variably/poorly immunogenic, especially among elderly and immunocompromised individuals. Widespread use of antibiotics against IPDs has led to the spread of drug-resistant pneumococcal strains (34, 35). This study offers an alternative targeted therapeutic approach to the shortcomings of the incumbent vaccine and antibiotic solutions to IPDs.

First we demonstrated that Pn3Pase could efficiently remove the capsule from live pneumococci without having bactericidal effects on the cells. Through *in vitro* assays, we observed that Pn3Pase treatment increases the bacterium's susceptibility to phagocytosis by a macrophage cell line. These results were promising since the capsule is a major host immune evasion component that allows *S. pneumoniae* to resist engulfment by host phagocytes (9). We then concluded that enzyme treatment significantly increased complement-mediated killing by the neutrophils. A single dose of Pn3Pase reduced murine nasopharyngeal colonization by type 3 *S. pneumoniae* significantly, indicating that the enzyme may function as a prophylactic measure to control colonization by this serotype in at-risk populations. Finally, an intraperitoneal challenge was performed to assess the protective capacity of Pn3Pase in a sepsis model. Notably, a single low dose of 0.5 μg administered 24 h after infection was able to protect 100% of the challenged mice from the bacterial challenge, while control treated animals did not survive longer than 48 h. The robust protective capacity of Pn3Pase in this model demonstrates the enzymatic activity is sufficient within the host to effectively degrade the capsule even at low doses.

Given that Pn3Pase has therapeutic potential for pneumococcal infections, practical issues pertaining to the application of the enzyme such as immunogenicity, administration routes, and substrate specificity will need to be addressed (55). Our preliminary assessment of antibody titers generated against Pn3Pase in the challenge experiments observed no IgM or IgG response generated against the effective dose of the enzyme. Future studies will evaluate humoral and cellular immune responses to Pn3Pase and investigate alternative routes of administration, such as intravenous for a bacteremia model or aerosolized spray for the pneumonia model. A useful example of enzyme delivery to the respiratory tract by aerosol spray is the recombinant human DNase known as Pulmozyme, used to relieve airway obstruction by secreted DNA in cystic fibrosis patients (56). Another potential problem with the use of Pn3Pase as a therapeutic agent is its activity on host glycans. Relaxed substrate specificity of Pn3Pase on mammalian glycans structurally similar to Pn3P will need to be assessed.

In addition to the high potential of Pn3Pase as a therapeutic enzyme, this glycosyl hydrolase has unique properties from a structure/function point of view in that it does not fall into a currently established glycosyl hydrolase carbohydrate active enzyme (CAZY) family (57). Further examination of structural properties of this protein may lead to the discovery of structurally similar enzymes with activities toward other unique bacterial CPSs. Future investigations will explore the existence of enzymes for use against other prevalent pneumococcal serotypes and other encapsulated pathogenic bacteria. Based on earlier studies, the native species expressing Pn3Pase has the capacity to degrade two additional pneumococcal CPSs (58, 59). While this Pn3Pase-expressing *Paenibacillus* species was isolated from the soil (26, 42, 59), it is befitting to question why this species would evolve to possess such enzymes that are capable of degrading capsules of a human pathogen. Whether these are the natural substrates for the enzymes, indicating a coevolutionary relationship, or whether other soil-dwelling microbes or plants express similar glycan residues and linkages remains to be explored.

The results presented here indicate that enzymatic hydrolysis of the CPS may be a valid alternative or complementary therapeutic approach for diseases caused by *S. pneumoniae* and potentially other important encapsulated pathogens, such as *Neisseria meningitidis* and methicillin-resistant *Staphylococcus aureus* (MRSA). In summary, this study serves as the first comprehensive evaluation of the protective role of a glycoside

hydrolase, Pn3Pase, debilitating an otherwise lethal bacterial pathogen through targeting its capsular polysaccharide.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Streptococcus pneumoniae* type 3 (WU2 strain) and its acapsular derivative (JD908) (60, 61), generous gifts from Moon Nahm (University of Alabama at Birmingham), were cultured aerobically without shaking at 37°C on tryptic soy agar with 5% sheep blood (TSAB) or in Todd-Hewitt broth plus 0.5% yeast extract (THY) (BD Biosciences).

Mice. Eight-week-old female BALB/c mice were obtained from Taconic Biosciences (Hudson, NY) and housed in the Central Animal Facility at the University of Georgia. Mice were kept in microisolator cages and handled under biosafety level 2 (BSL2) hoods.

Production of recombinant Pn3Pase. Pn3Pase was produced as described previously with minor modifications (26). Briefly, BL21(DE3) cells transformed with the pET-DEST42-Pn3Pase plasmid were grown in Terrific broth supplemented with 100 μg/ml ampicillin at 37°C, and cell density was monitored by absorbance at 600 nm. Once the OD_{600} reached 1.0, the cells were transferred to 18°C. Protein expression was induced by the addition of IPTG (isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 1 mM, and the cell culture was allowed to incubate for 18 h. Cells were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS [pH 7.2]), and lysed by pressure lysis. The lysate was clarified by centrifugation at 17,000 $\times$ *g* for 1 h at 4°C and passed through a 0.45-μm syringe filter. Recombinant Pn3Pase was purified by Ni^{2+}-nitrilotriacetic acid (NTA) resin at 4°C, eluted with 300 mM imidazole, and buffer exchanged into PBS (pH 7.2). The protein concentration was determined by the bicinchoninic acid assay according to the instructions of the manufacturer. Purity was evaluated by Coomassie staining.

Pn3Pase treatment of type 3 *S. pneumoniae*. Fresh *Streptococcus pneumoniae* type 3 (WU2) and acapsular derivative (JD908) colonies on TSAB plates were inoculated to an OD_{600} of 0.1 in THY broth and cultured as described above. WU2 growth in the presence of 100 μg/ml Pn3Pase was monitored over the course of 10 h by measuring OD_{600}. WU2 was grown in the presence of 2, 10, or 10 μg/ml heat-inactivated Pn3Pase for 6 h, serially diluted, and plated to determine CFU. Cultures were harvested by centrifugation, washed in PBS, fixed in 2% paraformaldehyde for 20 min on ice, washed once more, and suspended in 1 ml PBS. For the time course experiment, WU2 and the acapsular strain were grown to the mid-log phase (OD_{600} of 0.6), harvested by centrifugation, washed in PBS, and then suspended in 1 ml PBS. Then 1 μg/ml Pn3Pase was added, and the mixture was incubated at 37°C for 1, 2, or 4 h. Treated cells were serially diluted and plated to determine CFU, fixed in 2% paraformaldehyde for 20 min on ice, washed once more, and suspended in 1 ml PBS.

Competition ELISA. ELISA plates (96 well; Nunc) were coated with 5 μg/ml Pn3P in 0.1 M carbonate buffer (pH 9.0) overnight at room temperature. Plates were washed 4 times with PBS plus 0.1% Tween 20 (PBS-T) using a Biotek 405/LS microplate washer. After 1 h of blocking at room temperature with 1% BSA in PBS, microplate wells were incubated for 2 h at room temperature with fixed, treated cells that were preincubated for 30 min with Pn3P-specific antiserum in PBS-T. Plates were washed and then incubated for 2 h at room temperature with a 1:2,000 dilution of goat anti-mouse IgG-alkaline phosphatase (AP) (Southern Biotech 1030-04) in PBS-T. After washing, plates were incubated for ~30 min at 37°C with 2 mg/ml phosphatase substrate (Sigma S0942) in 1 M Tris–0.3 mM $MgCl_2$. Absorbance at 405 nm was measured on a Biotek synergy H1 microplate reader. The percentage of inhibition of antibody binding was calculated as [(uninhibited at OD_{405} − inhibited at OD_{405})/uninhibited at OD_{405}] $\times$ 100.

Electron microscopy. Electron microscopy was performed by the Georgia Electron Microscopy core facility at the University of Georgia according to a modified method by Hammerschmidt et al. (62). Treated cells were fixed in a mixture of 2% glutaraldehyde, 2% paraformaldehyde, 0.075 M lysine-acetate, and 0.075% ruthenium red in PBS buffer for 1 h on ice and then rinsed 2$\times$ with buffer containing 0.15% ruthenium red for 15 min per rinse. Cells were then fixed in 1% osmium tetroxide in buffer containing 0.15% ruthenium red for 1 h at room temperature followed by two rinses in buffer containing 0.15% ruthenium red for 15 min per rinse. The pellet was dehydrated in a graded ethanol series (30, 50, 75, 95, 100, and 100%) and two changes in 100% acetone for 15 min each step. The pellet was then infiltrated with 25% Spurr's resin and 75% acetone for 2 h followed by sequential infiltration with 50% Spurr's resin and 50% acetone, 75% Spurr's resin and 25% acetone, and 100% Spurr's resin and then polymerized in a 70°C oven for 24 h. Samples were sectioned at 60 nm with a Diatome diamond knife and picked up on slot grids. Grids were poststained on drops of uranyl acetate and lead citrate for 5 min each and rinsed with H_2O for 30 s between stains. Samples were scoped using a JEOL JEM 1011 transmission electron microscope (JEOL USA, Peabody, MA) operated at 80 kV.

Phagocytosis. Mid-log-phase WU2 and JD908 (acapsular) cultures were washed and stained with 10 μM carboxyfluorescein succinimidyl ester (CFSE) for 30 min at room temperature. The WU2 strain was concurrently treated with 2 μg/ml of active or heat-inactivated Pn3Pase. Bacterial cell pellets were washed extensively and suspended in 1 ml sterile PBS. A total of 10^7 bacteria were added to a confluent monolayer of murine leukemia virus-transformed macrophage line RAW 264.7 (American Type Culture Collection [ATCC] Manassas, VA) with active or heat-inactivated baby rabbit complement (Pel-Freez) in a 24-well plate and incubated for 1 h at 37°C. Wells were washed 4$\times$ with PBS to remove extracellular bacteria. Macrophages were fixed with 2% paraformaldehyde at 4°C for 15 min and removed from the plate. For microscopy, cells were incubated at room temperature for 30 min with a 1/500 dilution of biotinylated wheat germ agglutinin (Vector Laboratories) in 1% bovine serum albumin followed by a 30-min room temperature incubation with a 1/1,000 dilution of

streptavidin-allophycocyanin (APC [Biolegend]). Cells were imaged with a 40× objective lens. Flow cytometry was performed on a Beckman Cytoflex S cytometer and analyzed by FlowJo. Cells were gated on the macrophage population by scatter plot. A non-CFSE-labeled control served to gate highly fluorescent cell populations. The Pn3Pase dose-dependent experiment was performed as described above with addition of active complement.

Complement deposition. A complement deposition assay was performed as described previously (63). Type 3 WU2 and acapsular (JD908) strains of bacteria were resuspended in 3% BSA in PBS. Aliquots (in duplicate wells on a 96-well round-bottom plate) were stained with Hoechst 33342 and treated with inactivated or functional Pn3Pase at 5 or 50 μg/ml for 1 h at 37°C. Normal mouse serum (1:10 dilution) was added to the samples for 30 min at 37°C. Cells were washed and stained with FITC-conjugated goat antibody to mouse complement (MP BioMedical, Santa Ana, CA) at 4°C for 30 min. Samples were washed with 3% bovine serum albumin (BSA) in PBS and resuspended in 2% paraformaldehyde to fix. Samples were then analyzed by flow cytometry. The mean fluorescent intensity (MFI) of FITC-A was calculated from gating of Hoechst-positive cells.

Modified OPA. An opsonophagocytic killing assay was performed as described previously with modifications (43). Briefly, the type 3 WU2 and acapsular (JD908) strains were incubated in duplicate wells in a 96-well round-bottom plate for 1 or 4 h at 37°C with or without Pn3Pase (inactivated or functional at concentrations of 5 or 50 μg/ml) in opsonization buffer B (OBB: sterile 1× PBS with Ca^{2+}/Mg^{2+}, 0.1% gelatin, and 5% heat-inactivated FetalClone). Cells of the human promyelocytic leukemia cell line HL-60 (ATCC, Manassas, VA) were cultured in RPMI with 10% heat-inactivated FetalClone (HyClone) and 1% L-glutamine. HL-60 cells were differentiated using 0.6% *N*,*N*-dimethylformamide (DMF [Fisher]) for 3 days before performing the OPA assay, harvested, and resuspended in OBB. Active or heat-inactivated (no complement) baby rabbit complement (Pel-Freez) was added to HL-60 cells at a 1:5 final volume. The HL-60–complement mixture was added to the serum/bacteria at 5×10^5 cells/well. (For controls, no HL-60/complement was added; equal volumes of OBB buffer was added instead.) The final reaction mixtures were incubated at 37°C for 1 h. The reactions were stopped by incubating the samples on ice for approximately 20 min. Then 10 μl of each reaction mixture was diluted to a final volume of 50 μl and plated onto blood agar plates in duplicate. Plates were incubated overnight at 30°C under anaerobic conditions and counted the next day. The percentage of survival was calculated as each duplicate reaction normalized to mean values obtained for control samples (with reactions without HL-60 cells representing 100% survival).

Murine intranasal colonization. An intranasal colonization was performed essentially as described by Puchta et al. (64). Mid-log-phase WU2 cultures were washed with sterile PBS and suspended at a concentration of 10^8 or 10^6 CFU/10 μl. Groups of 5 to 10 unanesthetized 8-week-old female BALB/c mice (Taconic) were intranasally inoculated with 10^6 CFU/10 μl as previously described. Mice were either inoculated with 10 μl of PBS as vehicle on days 0, 3, and 7 or treated by administering 50 μg of Pn3Pase in 10 μl PBS on days 0, 0, and 3 or days 0, 3, and 7. Nasal lavage fluid was obtained on day 10 by flushing out the nasopharynx with PBS by insertion of a 25-gauge needle into the trachea to expel 500 μl through the nares. Serial dilutions of the nasal lavage fluid were plated on TSAB to enumerate the CFU. A sandwich ELISA (Biolegend mouse ELISA Max) was performed according to the manufacturer's instructions to determine IL-6 and TNF-α cytokine levels in the lavage fluid.

Murine sepsis challenge. Mid-log-phase WU2 cultures were washed with sterile PBS and suspended at a concentration of 5×10^3 CFU/100 μl. Groups of 4 unanesthetized 8-week-old female BALB/c mice (Taconic) were injected intraperitoneally (i.p.) with 5×10^3 CFU. Control mice were injected i.p. with 5 μg of heat-inactivated Pn3Pase in 100 μl of PBS at time 0 or directly after infection. Treated mice were administered i.p. 0.5 or 5 μg of Pn3Pase in 100 μl PBS at time 0, 12, or 24 h postinfection. Animals were monitored every 12 h.

Ethics statement. All mouse experiments were in compliance with the University of Georgia Institutional Animal Care and Use Committee under the approved animal use protocol 2478 A2016 11-022-Y1-A0. Our animal use protocol adheres to the principles outlined in *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training*, the Animal Welfare Act, the *Guide for the Care and Use of Laboratory Animals*, and the *AVMA Guidelines for the Euthanasia of Animals*.

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