ClyJ Is a Novel Pneumococcal Chimeric Lysin with a Cysteine- and Histidine-Dependent Amidohydrolase/Peptidase Catalytic Domain

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ABSTRACT *Streptococcus pneumoniae* is one of the leading pathogens that cause a variety of mucosal and invasive infections. With the increased emergence of multi-drug-resistant *S. pneumoniae*, new antimicrobials with mechanisms of action different from conventional antibiotics are urgently needed. In this study, we identified a putative lysin (gp20) encoded by the *Streptococcus* phage SPSL1 using the LytA autolysin as a template. Molecular dissection of gp20 revealed a binding domain (GPB) containing choline-binding repeats (CBRs) that are high specificity for *S. pneumoniae*. By fusing GPB to the CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) catalytic domain of the PlyC lysin, we constructed a novel chimeric lysin, ClyJ, with improved activity to the pneumococcal Cpl-1 lysin. No resistance was observed in *S. pneumoniae* strains after exposure to incrementally doubling concentrations of ClyJ for 8 continuous days *in vitro*. In a mouse bacteremia model using penicillin G as a control, a single intraperitoneal injection of ClyJ improved the survival rate of lethal *S. pneumoniae*-infected mice in a dose-dependent manner. Given its high lytic activity and safety profile, ClyJ may represent a promising alternative to combat pneumococcal infections.

KEYWORDS CHAP domain, antibiotic resistance, bacteriophage, chimeric lysin, endolysin, lysin

Streptococcus pneumoniae is an encapsulated Gram-positive bacterium usually found in the nasopharynx of healthy humans as part of the normal flora (1). However, in certain instances it can cause a variety of invasive diseases such as sepsis, community-acquired pneumonia, and meningitis, as well as mucosal infections such as acute otitis media and rhinosinusitis (2). As a challenging human pathogen that causes high morbidity and mortality in young children and immunocompromised patients (3), *S. pneumoniae* remains a critical target for new drug development. However, in recent years few new antibiotics have been approved by the U.S. Food and Drug Administration, and yet the emergence of antimicrobial-resistant bacteria continues to increase (4), which has led to a worldwide health care crisis, necessitating an urgent need for novel antibiotic alternatives (5).

Bacteriophage endolysins (also called lysins) have been increasingly recognized as a promising approach to combat multidrug-resistant pathogens. These enzymes are encoded by the genomes of bacteriophage and exhibit high species-specific degrading

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activity against the bacterial peptidoglycan. To fully perform their function, lysins usually contain two distinguished domains; an N-terminal catalytic domain (CD) and a C-terminal cell wall binding domain (CBD) (6). The CBD binds to a specific substrate (usually carbohydrate) found in the cell wall of a target bacterium (6, 7), promoting access of the CD to its substrate (8, 9). Adding purified lysins exogenously to susceptible Gram-positive bacteria can cause rapid cell lysis. Due to their high specificity and robust lytic activity, lysins offer an alternative antimicrobial approach to conventional antibiotics (10, 11). Currently, several lysins targeting *Staphylococcus aureus* have progressed to human clinical trials, such as the natural lysin CF-301 (<https://clinicaltrials.gov/ct2/show/NCT03163446#wrapper>) and the chimeric lysin P128 (<https://www.clinicaltrials.gov/ct2/show/NCT01746654?term=NCT01746654&rank=1>). Protein engineering strategies can be applied to naturally occurring lysins in order to achieve improved lytic activity (12), enhanced solubility and stability (13, 14), or an extended lytic spectrum (15). Furthermore, lysins can be engineered to breach the bacterial outer membrane (16), extending the utility of the lysin approach to target Gram-negative pathogens (17).

Several naturally occurring lysins have been reported to be active against *S. pneumoniae* in animal colonization and infection models. Among the most studied are Pal (18) and Cpl-1 (19), each possessing a CBD with six choline-binding repeats (CBRs) that are known to bind teichoic acids unique to the pneumococcal surface. The Cpl-1 lysin is encoded by the virulent pneumococcal bacteriophage Cp-1 and has been reported to be the most active natural pneumococcal lysin to date (20). In contrast, the Cpl-7 lysin, encoded by the pneumococcal phage Cp-7, has a CBD with three identical tandem repeats (CW_7 repeats) that are unrelated to choline-targeting motifs, but nonetheless specifically target the pneumococcal surface (21). Based on the CW_7 repeats, chimeric lysins engineered by domain swapping between Cpl-7 and other lysins demonstrated increased bactericidal activities *in vitro* and in colonization models (20, 22).

LytA is the major autolysin of *S. pneumoniae* essential for bacterial survival and cell wall synthesis (23). Recent structural elucidation of the full-length LytA and its complex with large peptidoglycan fragments reveals new insights into its function and lysis mechanism (24, 25), providing new clues for developing chimeric lysins against *S. pneumoniae*. PlyC, a multimeric streptococcal lysin composed of one PlyCA subunit and eight subunits of PlyCB, is specific for groups A, C, and E streptococci (26–28). Our previous study reported a chimeric lysin, ClyR, containing the CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) domain from PlyCA and the CBD from the PlySs2 lysin (14). Although its CBD does not contain any known modules specific for *S. pneumoniae* (29), ClyR displayed an extended streptococcal host range, including *S. pneumoniae* (14), suggesting that the CHAP domain of PlyCA is active against *S. pneumoniae*.

In the present study, we identified a new lysin encoded by the *Streptococcus* phage SP5L1 (Gene ID 24725347, termed gp20 here) using SWISS-MODEL with LytA as the template molecule (30, 31). Molecular dissection of gp20 was performed and an N-acetylmuramic acid L-alanine amidase CD was identified (here known as GPC), as well as a CBD with six choline binding repeats (here known as GPB). We further created a novel chimeric lysin, ClyJ, by fusing the CHAP domain from PlyCA with the newly identified GPB from gp20 and confirmed the bactericidal activity of ClyJ against *S. pneumoniae* *in vitro* and in a mouse bacteremia model.

RESULTS

GPB is highly specific to *S. pneumoniae*. By using the major autolysin of *S. pneumoniae*, LytA, as the template, we identified a new putative lysin (gp20) in the genome of the *Streptococcus* phage SP5L1 that shares 78% similarity with LytA (Fig. S1). Further analysis based on the amino acids of gp20 revealed that its catalytic domain (GPC) belongs to the PGRP superfamily and its binding domain (GPB) is composed of six typical CW_binding_1 repeats, also known as a choline binding module (CBM) that specifically recognizes and binds choline residues on the cell wall (Fig. S2).

FIG 1 Molecular dissection of gp20. (a and b) Lytic activities of gp20 and GPC against *S. pneumoniae* strains in comparison with Cpl-1 lysin. *S. pneumoniae* NS26 (a) and *S. pneumoniae* NS63 (b) cells are treated with an equal molar amount of each lysin and monitored by a microplate reader at OD₆₀₀ for 60 min. (c) Binding specificity of EGPB. Various streptococcal strains, including *S. pneumoniae* (*Spn*), *S. agalactiae* (*Sag*), *S. dysgalactiae* (*Sdy*), *S. pyogenes* (*SPy*), and *S. mutans* (*Smu*), were incubated with an equal concentration of EGPB or EGFP protein for 60 min at 37°C. After three washes with phosphate-buffered saline (PBS), the bacteria were imaged by a Nikon fluorescence microscope (TS2-FL). Scale bar, 5 μ m.

As an attempt to assess the lytic activity of gp20, we purified the full-length lysin, as well as its catalytic domain from *Escherichia coli* (Fig. S3), and tested their activities against two clinical *S. pneumoniae* strains. Results showed that the lytic activity of the full-length lysin, gp20, is very weak in comparison to that of Cpl-1, while the single catalytic domain, GPC, has negligible activity (Fig. 1a and b). Because the CBD of gp20 (GPB) has a typical CBM similar to that of LytA (Fig. S1), we hypothesized that it should maintain a specific binding ability for *S. pneumoniae*. To visualize this, we fused enhanced green fluorescent protein (EGFP) with GPB and purified the recombinant protein, EGPB, from *E. coli* (Fig. S3). Fluorescence microscopy analysis showed that EGPB binds to the cell surface of both *S. pneumoniae* strains, but not other streptococcal strains tested (Fig. 1c), suggesting a specific recognition of *S. pneumoniae*.

ClyJ is active against *S. pneumoniae*. Based on the specificity of GPB to *S. pneumoniae*, we created a novel chimeric lysin, ClyJ, by fusing GPB with the CHAP domain of PlyCA (Fig. 2a). We purified this chimera from *E. coli* (Fig. S4) and tested its activity against *S. pneumoniae* in comparison to Cpl-1, the best natural lysin against *S. pneumoniae*, and ClyR, another chimeric lysin that shares the same CHAP domain with ClyJ (Fig. 2a). Results showed that ClyJ displays higher lytic activities than either Cpl-1 or ClyR against *S. pneumoniae* strains NS26 and NS63 (Fig. 2b). Accordingly, a greater decrease in viable cell number of *S. pneumoniae* NS26 was observed in the ClyJ-treated group compared to groups treated with an equal molar amount of ClyR or Cpl-1 (Fig. 2c). A dose-dependent comparison also confirmed that the lytic activity of ClyJ is significantly higher than that of Cpl-1 (Fig. 2d). Meanwhile, an obvious synergism was observed between ClyJ and Cpl-1. For instance, under an equal molar concentration of 2.76 μ M, Cpl-1 and ClyJ alone caused a reduction of 2.3 and 4.2 logs, respectively, while,

FIG 2 Overall features of ClyJ. (a) Schematic representation of ClyJ and closely related lysins. Conserved domains were analyzed by SMART and are depicted by colors. The CHAP and CBM domain of ClyJ is linked by GS amino acids. CHAP, cysteine, histidine-dependent amidohydrolases/peptidases; PlyCA CHAP, the CHAP domain from PlyCA (amino acids 314 to 465); PlyCA GyH, the glycosyl hydrolase domain from PlyCA (amino acids 1 to 205); PlySs2 SH3b, the SH3b domain from PlySs2 lysin (amino acids 147 to 245); CBM, choline binding module. (b) Lytic activity of ClyJ against *S. pneumoniae* NS26 and NS63 compared to that of Cpl-1 and ClyR at the same concentration (25 μg/ml). (c) Log killing activity of ClyJ against *S. pneumoniae* NS26 compared to that of Cpl-1 and ClyR at the same molar concentration (0.69 μM). (d) Synergism between ClyJ and Cpl-1. *S. pneumoniae* NS26 was treated with an equal molar concentration (0, 0.69, 1.38, and 2.76 μM) of either Cpl-1, ClyJ, or an equal combination of Cpl-1 and ClyJ for 30 min. The viable cells were detected by dilution and plating on THY agar. The result was analyzed by two-tailed Student *t* test. The data are shown as means ± the standard deviations (***, *P* < 0.001).

a reduction of 6.3 logs was achieved in groups treated with an equal (1.38 μM each) combination of Cpl-1 and ClyJ (Fig. 2d). Moreover, an MIC assay showed that *S. pneumoniae* NS26 and NS63 are more sensitive to ClyJ than to Cpl-1 (Table S1), specifically, the MIC of Cpl-1 against either strain is about 2-fold higher than that of ClyJ.

Expanded host-range testing was conducted on an additional 13 *S. pneumoniae* strains representing multiple serovars, as well as *S. mutans*, *S. gordonii*, *S. mitis*, *S. rattus*, *S. salivarius*, *S. parasanguis*, *S. intermedius*, and several representative bacilli. It was revealed that ClyJ was active against all *S. pneumoniae* strains tested, similar to Cpl-1, but had no activity against other streptococcus or bacillus species (Fig. 3), suggesting the host range of ClyJ is highly pneumococcus specific.

Characteristics of ClyJ. Because the initial data showed high lytic activity for ClyJ, we further ascertained its biochemical properties. First, we examined the time- and

FIG 3 Host range of ClyJ. The susceptibility of various strains to ClyJ was tested in a turbidity assay in comparison to the Cpl-1 lysin. Each strain was treated with 25 $\mu\text{g/ml}$ ClyJ or Cpl-1 for 20 min, and the decrease in the OD_{600} was recorded by a microplate reader. The data are shown as means $\pm$ the standard deviations.

dose-dependent lytic activity of ClyJ against *S. pneumoniae* NS26 and found 25 $\mu\text{g/ml}$ ClyJ caused $\sim 3 \log_{10}$ decrease in viable *S. pneumoniae* cells after 60 min, with a reduction of $>1 \log_{10}$ noted within the first minute (Fig. 4a). Notably, treatment with 100 $\mu\text{g/ml}$ ClyJ caused a reduction of $>4 \log_{10}$ within 60 min (Fig. 4a). Next, we tested the influence of various environment factors on the lytic activity of ClyJ, including pH, EDTA, NaCl, and temperature. Results showed that ClyJ is active in a pH range of 5 to 9, with an optimum pH of 7 (Fig. S5a). EDTA displayed little effect on the activity of ClyJ, with over 90% activity remaining under EDTA concentrations up to 500 μM (Fig. S5b). Likewise, NaCl had little effect on the lytic activity of ClyJ (Fig. S5c). A thermal stability test showed that while ClyJ is stable at room temperature, displaying 97% activity after being stored at 25°C for 60 min (Fig. S5d), activity dropped to 66.7% after being stored at 37°C for 60 min, and activity was nearly abolished after 60 min at 42°C (Fig. S5d). Choline enhanced the thermal stability of ClyJ, with 97.8 and 46.3% activity remained after being incubated at 37 and 40°C for 1 h in the presence of choline, respectively (Fig. S5d), suggesting the enzymes are stabilized once bound to the choline-containing pneumococcal surface. More importantly, direct examination of the lytic activity of ClyJ at various temperatures showed minimal loss of lytic activity up to temperature of 45°C (Fig. S5e), presumably due to the rapid lytic nature of these enzymes. It has been demonstrated that CBM-containing lysins, such as Cpl-1 and LytA, dimerize upon binding choline present in the wall teichoic acids of pneumococci, and this monomer-to-dimer switch is necessary for cell wall hydrolytic activity of these enzymes (32, 33). Therefore, we further investigated the impact of choline on ClyJ activity, hypothesizing low levels may activate the enzyme, but high levels may inhibit the enzyme from binding to its cellular receptor. Results demonstrated that low concentrations of choline ($<20 \text{ mM}$) improved the lytic activity of ClyJ on *S. pneumoniae* NS26, but the activity gradually dropped as the choline concentration increased (Fig. 4b). Under the same conditions, Cpl-1 displayed less activity stimulation at low choline concentrations and a more dramatic decrease in activity at higher choline concentrations. However, both enzymes displayed very similar responses to choline concentration, i.e., initial

FIG 4 Biochemical characteristics of ClyJ. (a) Time- and dose-dependent killing capacity of ClyJ. *S. pneumoniae* NS26 was treated with 25 µg/ml ClyJ for 0, 1, 5, 15, 30, or 60 min (black lines) or treated with 0, 2.5, 5, 10, 25, 50, and 100 µg/ml ClyJ for 60 min (blue lines) before dilution and plating on THY agar. (b) Relative activities of ClyJ and Cpl-1 against *S. pneumoniae* NS26 in the presence of various concentrations of choline. (c) Morphology of *S. pneumoniae* NS26 after exposure to 100 µg/ml ClyJ for 0, 30, and 60 s, as observed by transmission electron microscopy. Scale bar, 2 µm.

increase and subsequent decrease in lytic activity, when tested with *S. pneumoniae* NS63 (Fig. S5f). These observations are also consistent with previously published choline response curves of LytA (34, 35).

The rapid lytic activity of ClyJ against *S. pneumoniae* was also confirmed by transmission electron microscopy thin-section imaging (Fig. 4c), which showed cells beginning to lyse and lose their cytoplasmic contents after exposure to ClyJ for 30 s, with lysis almost complete by 60 s (Fig. 4c).

No resistance development to ClyJ. It is well known that bacterial pathogens can easily develop tolerance or resistance to the immediate life-or-death pressure placed by selected antibiotics. However, because the killing mechanism of lysins is different from that of antibiotics, we assessed the rate of resistance development against ClyJ for *S. pneumoniae*. First, we examined the susceptibility of *S. pneumoniae* NS26 and NS63 to ClyJ and penicillin G using the broth microdilution method (Table S1). Next, we cultured these two strains under incrementally doubling concentrations of ClyJ or penicillin G (from 1/32× to 4× the initial MIC) and checked their susceptibility daily for 8 days. The results showed that both strains acquire resistance, defined as a 4-fold increase over the original MIC, to penicillin G, with 8- and 16-fold increases in MIC after 8 days of exposure (Fig. 5). In contrast, no resistance to ClyJ was observed under identical conditions.

ClyJ protects mice from lethal *S. pneumoniae* infection. The *in vivo* efficacy of ClyJ was evaluated in a systemic *S. pneumoniae* infection model. First, we assessed the virulence of *S. pneumoniae* NS26 and found that a single intraperitoneal injection of 2.68×10^7 CFU/mouse causes 100% death within 24 h (Fig. 6a). Next, we examined the protective efficacy of a single dose of ClyJ administered 1 h after the infection with 2.68×10^7 CFU/mouse *S. pneumoniae* NS26. As shown in Fig. 6b, a large amount of bacteria could be detected in the blood (1.5×10^6 CFU/ml) and organs (2.4×10^6 CFU/g tissue, 1.4×10^6 CFU/g tissue, and 2.2×10^6 CFU/g tissue in kidney, liver, and

FIG 5 *In vitro* resistance development assay. *S. pneumoniae* strains were exposed to $1/32\times$ to $4\times$ the initial MIC of ClyJ and penicillin G over 8 days, and the daily MICs of ClyJ and penicillin G were examined and compared to the initial MIC for each strain.

lung, respectively) in infected mice at 1 h postinfection, suggesting rapid migration and dissemination from the site of intraperitoneal injection. Administration of penicillin G at 1 h postinfection showed 40% (4/10) protective efficacy (Fig. 6c), while a dose-dependent protection was observed in ClyJ-treated groups, with 90% (9/10) of the mice surviving 10 days in the group that received 0.3 mg/mouse and 100% (10/10) protection in the group that received 0.4 mg/mouse (Fig. 6c). Moreover, a single high dose of ClyJ (1 mg/mouse) did not cause a noticeable change in health or affect the survival rate of mice (Fig. 6c). At 3 h after bacterial challenge with a lethal dose of *S. pneumoniae* NS26, the kidney and liver maintained high pneumococcal loads, i.e., 2.2×10^6 CFU/g tissue and 1.3×10^6 CFU/g tissue, respectively (Fig. 6b). Compared to the number of bacteria detected at 1 h postinfection, the bacterial burden 3 h postinfection significantly decreased to an average of 6.8×10^5 CFU/ml in the blood and increased significantly to 5.3×10^6 CFU/g tissue in the lung, consistent with dissemination of the infection to sensitive organs (Fig. 6b). Protective efficacies administered 3 h postinfection dropped to 20% (2/10) and 50% (5/10) with single doses of 0.4 and 0.8 mg/mouse of ClyJ, respectively (Fig. 6d), presumable due to the extent of bacteremia. Notably, none (0/10) of the mice given 0.25 mg/mouse of penicillin G at 3 h postinfection survived (Fig. 6d).

To further confirm the safety of ClyJ, we scanned the hematological parameters and cytokine expression levels in mice receiving a single high therapeutic dose of ClyJ (0.4 mg/mouse, $n = 5$). Compared with the PBS-treated group, no significant changes of hematological parameters were observed in the ClyJ-treated group, whereas a higher content of monocytes was observed in the lipopolysaccharide (LPS)-treated group (from 4.66 to 11.58%, Table S2). Although a slight increase in the MCHC (mean corpuscular hemoglobin concentration) was observed in the ClyJ-treated group, it is still within the normal range of 30 to 38 g/dl for mouse serum (36). Meanwhile, no significant difference was observed in expression levels of IL-1 β (Fig. 6e) or IL-6 (Fig. 6f) in sera from groups treated with ClyJ and PBS, suggesting that no significant inflammatory response is evoked by ClyJ under the present experimental conditions.

Cytotoxicity of ClyJ. The SRB assay showed that FaDu and HLMEC cell lines remained healthy at ClyJ concentrations less than 250 μ g/ml (Fig. 7a) but had increasing cytotoxicity at ClyJ concentrations of >250 μ g/ml. Furthermore, no hemolysis was observed in human red blood cells (RBCs) exposed to high concentrations of ClyJ up to 500 μ g/ml (Fig. 7b). In addition, the overall analysis of gene expression profiles in FaDu cells cocultured with ClyJ (50 μ g/ml) revealed no significant differences compared to these cells cocultured with albumin (Fig. S6).

DISCUSSION

The increased emergence of multidrug-resistant pathogens has posed a challenging medical threat to the public health worldwide (37), which has renewed interest in

FIG 6 ClyJ protects mice from lethal *S. pneumoniae* infection. (a) Virulence of *S. pneumoniae* NS26 *in vivo*. Mice were intraperitoneally injected with different concentrations of *S. pneumoniae* NS26, and the survival rate of each group ($n = 5$ each) was recorded for 5 days. (b) Bacterial burden in blood and organs at 1 and 3 h after inoculation of a lethal dose of *S. pneumoniae* NS26. The numbers of bacteria in the blood and lungs were compared between groups euthanized at 1 and 3 h postinfection and analyzed by a two-tailed Student *t* test. The data are shown as means $\pm$ the standard deviations (*, $P < 0.05$; ***, $P < 0.001$). (c and d) Protective efficacy of ClyJ. Female BALB/c mice were infected with 2.68×10^7 CFU/mouse *S. pneumoniae* NS26 and divided into eight groups randomly after infection. Three groups ($n = 10$) intraperitoneally received a single dose of 0.3 mg/mouse ClyJ, 0.4 mg/mouse ClyJ, or 0.25 mg/mouse penicillin G at 1 h postinfection, respectively (c). Three other groups ($n = 10$) intraperitoneally received a single dose of 0.4 mg/mouse ClyJ, 0.8 mg/mouse ClyJ, or 0.25 mg/mouse penicillin G at 3 h postinfection, respectively (d). The final two groups ($n = 10$) were injected with PBS and served as controls. Another group ($n = 5$) without *S. pneumoniae* infection was intraperitoneally administered a single high dose of 1 mg/mouse ClyJ to assess its toxicity (c). The survival rates in all groups were recorded for 10 days. (e and f) Inflammatory cytokines in mouse blood. The mice were intraperitoneally injected with one ClyJ dose of 0.4 mg/mouse ($n = 5$), 3,000 EU/mouse LPS ($n = 5$), or PBS ($n = 5$); 6 h later, the expression levels of IL-1 β (e) and IL-6 (f) in each group were detected by ELISA. The expression level in the ClyJ-treated group was compared to that of the PBS- and LPS-treated controls by two-tailed Student *t* test. The data are shown as a minimum-to-maximum boxplot, where the box represents the standard deviations, and the medians are marked by a bar. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$.

alternative therapies such as phage-derived lysin therapy (10). As an attempt to develop novel antimicrobials against *S. pneumoniae*, the leading pathogen responsible for bacterial pneumonia in young children and the elderly (1), we identified a new lysin, gp20, encoded by the *Streptococcus* phage SPSL1. In the present study, we dissected the functional domains of gp20 and created a novel chimeric lysin, ClyJ, by fusing the pneumococcus-specific binding domain from gp20 to the CHAP catalytic domain derived from the PlyC lysin. The bactericidal activity of ClyJ against *S. pneumoniae* was further assessed both *in vitro* and in a mouse bacteremia model.

Interestingly, CHAP domains are usually found in staphylococcal phage lysins (38), while pneumococcal phage lysins typically consist of a lysozyme catalytic domain belonging to the GH-25 superfamily, as is the case in Cpl-1 (39) and Cpl-7 (21), or an amidase catalytic domain as seen in Pal (40) and LytA (25). Moreover, the lysozyme

FIG 7 Effects of ClyJ on viability of human cell lines. (a) Relative viabilities of FaDu and HLMEC cells exposed to various concentrations of ClyJ. Cells were cocultured with a series of concentrations of ClyJ (0, 62.5, 125, 250, and 500 µg/ml) for 48 h, and the relative viabilities of cells after each treatment were determined by an SRB assay. (b) Hemolysis effect of ClyJ on RBCs. Cells were incubated with different concentrations of ClyJ (62.5, 125, 250, and 500 µg/ml) for 60 min at 37°C, and the degree of hemolysis was estimated by measuring the absorbance at 540 nm. Groups treated with endotoxin-free water and PBS with 10 mM glucose served as positive controls (PC).

catalytic domain from Cpl-1 or Cpl-7 (20, 22) and the amidase catalytic domain from Pal (41) have been used as potential donors in construction of chimeric lysins targeting *S. pneumoniae*. Therefore, ClyJ is the first chimeric lysin specific for *S. pneumoniae* that contains a CHAP domain.

The binding domain of gp20 is homologous to that of LytA, Pal, and Cpl-1, showing a typical choline binding module (CBM). The increase of ClyJ activity under low choline concentrations suggests that ClyJ may undergo a structural switch from monomer to dimer after binding the choline containing wall teichoic acids on the pneumococcal surface, a structural change that has been observed in LytA (42), Pal (43), and Cpl-1 (44). However, the conformational dynamics of ClyJ and the structural relationship of its CBM with its lytic activity require further studies.

There are several characteristics that warrant further development of ClyJ as an anti-pneumococcal lysin despite numerous manuscripts already devoted to the merits of Cpl-1 for the same purpose. First, ClyJ could overcome the threat posed by lysozyme-resistant *S. pneumoniae* considering the different peptidoglycan bonds targeted by ClyJ and Cpl-1. Many *S. pneumoniae* strains are resistant to lysozyme because of the lack of acetylation of glucosamine in their peptidoglycan (45, 46). It is therefore possible in theory for *S. pneumoniae* to develop resistance to lysins with lysozyme-like enzymatic activities, such as Cpl-1 and Cpl-7, although, to date, there have been no reports of resistance developed to these enzymes. Second, ClyJ has improved activity compared to Cpl-1. The MIC of ClyJ was about 2-fold lower than that of Cpl-1 against two pneumococcal strains tested, indicating that ClyJ is about 2 times more active than Cpl-1. However, ClyJ and Cpl-1 have yet to be benchmarked against each other *in vivo*. Third, ClyJ and Cpl-1 could work synergistically to achieve improved bactericidal efficacy. The CHAP domain of PlyC, which is reported to contain an *N*-acetylmuramoyl-L-alanine amidase activity, has been shown to demonstrate intrinsic synergy with the glycosidase activity of the PlyCA GyH domain (26). In addition, the amidase domain from the Pal lysin has been shown to work synergistically with Cpl-1 both *in vitro* and *in vivo* (47, 48). Taken together, the evidence provided here supports further developmental studies for ClyJ.

One major concern in the development of antimicrobials is their resistance, a key question that has not been fully resolved. In this context, bacteriophage lysins have been reported to possess a very low possibility of developing resistance compared to traditional antibiotics (6). As expected, no resistance was observed in *S. pneumoniae* strains after exposure to incrementally doubling concentrations of ClyJ for 8 continuous days, while high resistance to penicillin G occurred in both *S. pneumoniae* strains. Nonetheless, how these strains respond to ClyJ with longer induction cycles or with more selective pressure, i.e., stable mutants, still needs to be established. In addition,

there exist mutants (i.e., *tacF*) that have significantly reduced choline on the cell surface (49, 50). Although our screen did not detect any of these mutants, in theory such mutants would be resistant to ClyJ and other lysins that contain CBMs, although it should be noted that pneumococcal mutants lacking choline have a significantly diminished virulence capacity (49).

Aside from resistance development, there are other potential hurdles to be taken into consideration for future translational development of ClyJ. First, ClyJ is sensitive to elevated temperatures. A residual activity of 66.7% was observed for ClyJ after being stored at 37°C for 1 h, and activity was almost lost after exposure at 42°C for the same amount of time. The fact that choline enhanced the thermal stability of ClyJ indicates that ClyJ would gain thermal stability upon bound to the *S. pneumoniae* surface. Further, addition of sugars, amino acids, poloxamers, ions, or other excipients are routinely used to stabilize pharmaceutical preparations of lysins (51), but these thermal stabilization tests have yet to be performed for ClyJ. Second, ClyJ shows potential cytotoxicity at high concentrations. Although no hemolysis was observed in RBCs at ClyJ doses up to 500 µg/ml, toxicity to FaDu and HLMEC cells was observed at this concentration. However, this concentration is more than an order of magnitude higher than the actual doses described for the *in vivo* studies. We also did not evaluate the use of ClyJ in combination with conventional antibiotics, an approach that has demonstrated synergism and would require lower concentrations of either the lysin or the antibiotic, which may address toxicity issues if they arise (13, 52).

In summary, we report here a novel chimeric lysin, ClyJ, consisting of a unique CHAP domain and a classical CBM, exhibits robust lytic activity against *S. pneumoniae* both *in vitro* and *in vivo*. Taking into account its high lytic activity, low resistance occurrence, and safety profile, ClyJ may represent a promising alternative to combat pneumococcal infections.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table S3. *S. pneumoniae* strains were cultured in THY (Todd-Hewitt broth containing 0.5% yeast extract) medium statically at 37°C with 5% CO₂. Other Gram-positive bacteria were grown in BHI (brain heart infusion; Becton, Dickinson and Company, Sparks, MD) broth at 37°C. *Escherichia coli* BL21(DE3), used for gene cloning and protein expression, was grown in LB (Luria broth) medium. Kanamycin (50 µg/ml) was added when necessary.

Sequence screening and cloning. The putative sequence coding a LytA-like murein hydrolase (Gene ID 24725347) was identified in the genome of the *Streptococcus* phage SP5L1 by SWISS-MODEL using LytA as the template. The candidate gene was chemically synthesized by GenScript (Nanjing, China) and cloned into a pET28b(+) vector using primers GP-F/GP-R (Table S4), and the corresponding gene product was termed gp20. The CD of gp20 (GPC, amino acids 1 to 158), and the CBD of gp20 (GPB, amino acids 148 to 316) fused with EGFP were amplified by primer-specific PCR and cloned into the NcoI and XhoI restriction sites of a pET28b(+) plasmid (Table S4). In addition, the chimeric lysin, ClyJ, was constructed by fusing GPB coding sequence to the C terminus of the CHAP domain of PlyCA (amino acids 314 to 465) using sequence-specific primers (Table S4). The pneumococcal Cpl-1 lysin coding sequence was chemically synthesized by GenScript (Nanjing, China) and cloned into a pET28b(+) vector using primers Cpl-1-F/Cpl-1-R (Table S4). All constructs were transformed into *E. coli* BL21(DE3) cells and verified by direct DNA sequencing analysis.

Protein expression and purification. For production of recombinant proteins, *E. coli* BL21(DE3) cells were incubated at 37°C to mid-log phase and then induced with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated at 16°C for 18 h to allow for protein expression. Cells were collected by centrifugation at 8,000 × g for 10 min and lysed by using a cell disrupter (JNBIO, JN-10C) on ice. His-tagged proteins were purified by nickel nitrilotriacetic acid columns according to the supplier's instructions. Briefly, columns were equilibrated with 20 mM imidazole before loading the protein samples. Target proteins were collected by washing and elution with 60 and 250 mM imidazole, respectively. Collected proteins were dialyzed against PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·H₂O, 1.4 mM KH₂PO₄ [pH 7.4]) and then passed through a Detoxi-Gel endotoxin removing gel (Thermo Scientific), and quantified by an EndoLISA (enzyme-linked immunosorbent assay [ELISA]-based endotoxin detection assay; Hyglos, GmbH) according to the manufacturer's instructions. After quantitation by the Bradford assay, the protein solution was filter sterilized and stored at 4°C until used.

Bactericidal assays. Lytic activity of ClyJ was measured via the turbidity reduction assay as described previously (53). Bacteria were cultured to an optical density at 600 nm (OD₆₀₀) of 0.8 to 1, washed once with PBS (pH 7.4), and resuspended in PBS to a final OD₆₀₀ of 0.5 to 0.8. Next, 190 µl of the bacterial suspension was mixed with 10 µl of purified proteins (at a final concentration of 25 µg/ml) in 96-well plates (Corning). The decrease in absorbance at OD₆₀₀ was monitored for 60 min at 37°C by using a Synergy H1 microplate reader (BioTek) at 1-min intervals or was measured every 15 s for 20 min on a

SpectraMax 190 spectrophotometer (Molecular Devices, San Jose, CA). Controls were run in parallel and used an equal volume of PBS buffer in place of the enzyme. The activity of each lysin was expressed as the difference in the percent decrease in OD₆₀₀ (% decrease in OD₆₀₀) from lysin-treated wells and the PBS-treated control wells.

To examine the biochemical properties of ClyJ, *S. pneumoniae* NS26 was resuspended in PBS with various concentrations of NaCl (0, 50, 100, 150, 300, 500, and 750 mM) or EDTA (0, 50, 100, 200, 300, 400, and 500 μ M) or was resuspended in phosphate buffer with different pHs (3.5, 5, 6, 6.5, 7, 8, 8.5, 9, 9.5, 10, 11, and 11.5). ClyJ (25 μ g/ml) was then added to bacterial suspensions, and the change in turbidity (OD₆₀₀) under each condition was monitored by using a microplate reader at 37°C for 60 min. The stability of ClyJ at different temperatures was evaluated by testing the residual activity of each lysin (at a final concentration of 25 μ g/ml) against *S. pneumoniae* NS26 after incubation at various temperatures (25, 37, 42, and 45°C) for 60 min in the presence or absence of 50 mM choline. The lytic activity of ClyJ (0, 4, 8, 16, 32, 64, and 128 μ g/ml) against *S. pneumoniae* NS26 under different temperatures (37, 42, and 45°C) was further assessed by a microplate reader (BioTek) for 60 min. The effect of each factor (NaCl, EDTA, pH, and temperature) on the lytic activity of ClyJ was normalized by comparison to the maximum activity of ClyJ among different levels of that factor and was expressed as the relative activity.

The effect of choline on the lytic activity of ClyJ was assessed by adding different concentrations of choline (0, 5, 10, 20, 40, 80, and 160 mM) to the reaction mixture of ClyJ (at a final concentration 25 μ g/ml) and *S. pneumoniae* NS26. Identical experiments were performed with Cpl-1 as a control. The relative activities of ClyJ and Cpl-1 under different choline conditions were normalized by comparison to the lytic activity of each lysin in the absence of choline.

The decrease of *S. pneumoniae* in viable cell numbers after treatment with different lysins was determined by plating series of dilutions on THY agar. Specifically, a series of 10-fold dilutions was prepared in PBS, and a 10- μ l aliquot of each dilution was spotted on THY agar to visualize viable cells after an overnight incubation at 37°C. In the time- and dose-dependent bactericidal assay for ClyJ, *S. pneumoniae* NS26 was treated with 25 μ g/ml ClyJ for 0, 1, 5, 15, 30, or 60 min, or treated with 0, 2.5, 5, 10, 25, 50, or 100 μ g/ml ClyJ for 60 min, respectively, before dilution and plating on THY agar. In comparing the log killing capacity of ClyJ to other lysins, *S. pneumoniae* NS26 or *S. pneumoniae* NS63 were treated with an equal molar concentrations (0, 0.69, 1.38, and 2.76 μ M) of either ClyR, ClyJ, Cpl-1, or an equal combination of ClyJ and Cpl-1 for 30 min at 37°C, and residual CFU were counted on THY agar. All experiments included at least three biological replicates.

Fluorescence microscopy. The binding specificity of GPB to various bacterial strains was examined by fluorescence microscopy after incubation with an EGFP-fused GPB protein, EGFP-GPB (EGPB). Briefly, various bacterial strains, including *S. pneumoniae* NS26, *S. pneumoniae* NS63, *S. agalactiae*, *S. dysgalactiae*, *S. pyogenes*, and *S. mutans*, were grown overnight, washed and suspended in PBS to an OD₆₀₀ of 0.5 to 0.8. Next, 100 μ l of each bacterial suspension was incubated with an equal concentration (20 μ M) of EGPB or EGFP protein for 60 min at 37°C. After three washes with PBS, the samples were imaged by a Nikon fluorescence microscope (TS2-FL) at $\times$ 100 magnification and with a 0.4-s exposure time.

Transmission electron microscopy. The change in morphology of *S. pneumoniae* after exposure to ClyJ was observed by using a transmission electron microscope. *S. pneumoniae* NS26 was washed twice with PBS, resuspended in PBS to an OD₆₀₀ of 0.8 to 1.0, and treated with 100 μ g/ml ClyJ for 0, 30, or 60 s. The bacterial suspensions were then cross-linked with 2.5% glutaraldehyde and analyzed by a transmission electron microscope (Tecnai G² 20 Twin; Fei).

MIC determination. The MICs of Cpl-1, ClyJ, and penicillin against *S. pneumoniae* NS26 and NS63 were determined by the broth dilution method as described by the Clinical and Laboratory Standards Institute (54), with some modifications. Briefly, *S. pneumoniae* was inoculated at a final concentration of 1×10^5 cells/well in a 96-well microtiter plate and allowed to incubate in cation-adjusted Mueller-Hinton broth (CAMHB) for 24 h at 37°C in the presence of various concentrations of ClyJ (0, 1, 2, 4, 8, 16, 32, and 64 μ g/ml), Cpl-1 (0, 1, 2, 4, 8, 16, 32, and 64 μ g/ml), or penicillin G (0, 0.25, 0.5, 1, 2, 4, 8, and 16 μ g/ml). The resulting cell viability within each well was determined by adding 0.04% resazurin, followed by incubation for 4 h at 37°C for colorimetric detection as described previously (55). The MIC was defined as the lowest concentration of antimicrobial that completely abolished the bacterial viability (corresponding to lowest concentration that causes the color from blue to red).

In vitro resistance development assay. The resistance development for *S. pneumoniae* NS26 and NS63 to ClyJ and penicillin G were performed according to established methods (56, 57), with minor modifications. Briefly, bacterial cells at a concentration of 5×10^8 CFU/ml were cultured overnight in THY broth in the presence of 1/32 $\times$ initial MIC of ClyJ at 37°C with 5% CO₂. The cells were pelleted and subdivided into two aliquots. One aliquot was diluted 10-fold into fresh THY medium and allowed to grow to log phase with 1/16 $\times$ initial MIC of ClyJ (2-fold higher than the former day); the other portion was applied to determine the actual MIC of ClyJ. The above procedure was repeated over an 8-day period, and the concentration of ClyJ in the liquid culture was serially doubled from 1/32 $\times$ to 4 $\times$ the initial MIC. The resistance development of these two *S. pneumoniae* strains to penicillin G was also conducted according to the same method.

Toxicity of ClyJ. The cytotoxicity of ClyJ was evaluated against two different cell lines. Briefly, HLMEC cells (Institute of Immunology and Experimental Therapy Cell Culture Collection) and FaDu cells (ATCC HTB-43) were seeded on 96-well plated at 10^4 cells per well in 100 μ l of medium (recommended by the manufacturer) and allowed to adhere to the surface for 3 h. Next, the medium was removed, and new medium containing various concentrations of ClyJ (0, 62.5, 125, 250, and 500 μ g/ml) was added and allowed to coculture for 48 h at 37°C in 5% CO₂. Subsequently, a sulforhodamine B (SRB) assay was conducted as described previously (58). Briefly, 50 μ l/well of cold 50% (wt/vol) trichloroacetic acid was

added to each well, followed by incubation for 1 h at 4°C. Wells were washed with PBS, and 50 μ l/well of a 0.05% (wt/vol) SRB solution was added and allowed to incubate for 1 h at room temperature. Wells were washed with 1% (vol/vol) acetic acid and then incubated with 10 mM Tris base solution (pH 10.5, 100 μ l/well) for 30 min at room temperature. Finally, the absorbance was measured at 510 nm in a Synergy H4 microplate reader (BioTek). The relative viability of cells after each treatment was normalized and compared to PBS-treated control wells.

The effects of ClyJ on the gene expression profile of FaDu cells was further evaluated using the SurePrint G3 Human Gene Expression v3 8x60K microarrays (Agilent Technologies). Briefly, FaDu cells were cocultured with 50 μ g/ml ClyJ for 5 h before detection, using 50 μ g/ml albumin-treated cells as a control. For detailed information, see the supplemental material.

Hemolysis assay. *In vitro* assessment of ClyJ cytotoxicity was also studied using human RBCs as described previously (59). Briefly, fresh human blood was obtained from healthy volunteers ($n = 3$) to tubes containing EDTA to prevent coagulation and centrifuged at $3,000 \times g$ for 10 min at 4°C. Plasma and leukocytes were discarded, and erythrocytes were washed three times ($3,000 \times g$ for 10 min at 4°C) with PBS (pH 7.2) supplemented with 10 mM glucose. Erythrocyte suspensions (1.6×10^8 cells/ml) were incubated with different concentrations of ClyJ (62.5, 125, 250, and 500 μ g/ml) for 60 min at 37°C with gentle shaking. Endotoxin free water served as the positive control (PC), while PBS with 10 mM glucose served as the negative control (NC). After incubation, the erythrocytes were centrifuged at $3,000 \times g$ for 10 min, and the degree of hemolysis was estimated by measuring the absorbance at 540 nm of hemoglobin in the supernatant. The percentage of hemolysis (% hemolysis) was calculated as follows: % hemolysis = $100 \times (\text{OD}_{\text{sample}} - \text{OD}_{\text{NC}}) / (\text{OD}_{\text{PC}} - \text{OD}_{\text{NC}})$.

Mouse infection models. All mouse infection experiments were carried out in an ABSL-2 lab, and all experimental methods were carried out in accordance with the regulations and guidelines set forth by the Animal Experiments Committee of Wuhan Institute of Virology, Chinese Academy of Sciences. All experimental protocols were approved by the Animal Experiments Committee of Wuhan Institute of Virology, Chinese Academy of Sciences (WIVA17201602). For hematological parameter detection, animal experiments were performed in the Animal Breeding Centre of the Institute of Immunology and Experimental Therapy according to EU directive 2010/63/EU for animal experiments and were approved by the 1st Local Committee for Experiments with the Use of Laboratory Animals, Wrocław, Poland. Animals were randomized and cared in individually ventilated cages, following a set of animal welfare and ethical criteria during the experiment, and euthanized at the end of observation.

In the mouse systemic infection model, the virulence of *S. pneumoniae* NS26 was determined initially. Female BALB/c mice (6 to 8 weeks old) were randomly separated into four groups ($n = 5$), three of which were injected intraperitoneally with different concentrations of *S. pneumoniae* NS26 (6.7×10^6 to 26.8×10^6 CFU/mouse). The fourth group was challenged with PBS only. To test the bacterial burden in blood and organs, female BALB/c mice (6 to 8 weeks old) were injected intraperitoneally with a bacterial dose that caused 100% mortality within 24 h. Animals were euthanized at 1 or 3 h after infection ($n = 5$). Blood was collected and diluted for the enumeration of CFU on THY agar. Kidney, liver, and lungs were aseptically removed from each mouse and homogenized in 1 ml PBS with 0.1% Triton X-100 by a Qiagen TissueLyse II homogenizer at 1/20 frequency for 90 s, and serial dilutions of each organ were plated on THY agar for enumeration of CFU. To test the protective efficacy of ClyJ, female BALB/c mice (6 to 8 weeks old) were injected intraperitoneally with a bacterial dose that caused 100% mortality within 24 h and divided randomly into eight groups. Three groups ($n = 10$) intraperitoneally received a single dose of 0.3 mg/mouse of ClyJ, 0.4 mg/mouse of ClyJ, or 0.25 mg/mouse of penicillin G at 1 h postinfection, respectively. Another three groups ($n = 10$) intraperitoneally received a single dose of 0.4 mg/mouse of ClyJ, 0.8 mg/mouse of ClyJ, or 0.25 mg/mouse of penicillin G at 3 h postinfection, respectively. The final two groups ($n = 10$) were intraperitoneally injected with PBS buffer at 1 or 3 h postinfection and served as controls. To evaluate the potential toxicity of ClyJ, another group of mice ($n = 5$, without *S. pneumoniae* infection) were intraperitoneally injected with a high dose of ClyJ (1 mg/mouse). The survival rates for all groups were recoded for 10 days.

For detection of hematological parameters, 16-week-old female BALB/c mice ($n = 5$ each) were inoculated intraperitoneally with a single ClyJ dose of 0.4 mg/mouse (the residual LPS content was 5 EU/mouse) and an equal volume of PBS containing 5 EU/mouse of LPS (negative control) or 3,000 EU/mouse of LPS (inflammatory control). Six hours later, the mice were sacrificed, blood was collected into EDTA-containing tubes, and blood parameters were tested by a computerized Mythic 18 hem analyzer (C2 Diagnostics, France). Serum was separated from the blood by double centrifugation ($2,250 \times g$ for 5 min and $10,000 \times g$ for 10 min) and subjected to interleukin-1 β (IL-1 β) and IL-6 detection using commercial ELISA kits (PeproTech).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02043-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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