Using a Novel Lysin To Help Control *Clostridium difficile* Infections

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As a consequence of excessive antibiotic therapies in hospitalized patients, *Clostridium difficile*, a Gram-positive anaerobic spore-forming intestinal pathogen, is the leading cause of hospital-acquired diarrhea and colitis. Drug treatments for these diseases are often complicated by antibiotic-resistant strains and a high frequency of treatment failures and relapse; therefore, novel nonantibiotic approaches may prove to be more effective. In this study, we recombinantly expressed a prophage lysin identified from a *C. difficile* strain, CD630, which we named PlyCD. PlyCD was found to have lytic activity against specific *C. difficile* strains. However, the recombinantly expressed catalytic domain of this protein, PlyCD₁₋₁₇₄, displayed significantly greater lytic activity (>4-log kill) and a broader lytic spectrum against *C. difficile* strains while still retaining a high degree of specificity toward *C. difficile* versus commensal clostridia and other bacterial species. Our data also indicated that noneffective doses of vancomycin and PlyCD₁₋₁₇₄ when combined *in vitro* could be significantly more bactericidal against *C. difficile*. In an *ex vivo* treatment model of mouse colon infection, we found that PlyCD₁₋₁₇₄ functioned in the presence of intestinal contents, significantly decreasing colonizing *C. difficile* compared to controls. Together, these data suggest that PlyCD₁₋₁₇₄ has potential as a novel therapeutic for clinical application against *C. difficile* infection, either alone or in combination with other preexisting treatments to improve their efficacy.

Clostridium difficile, a Gram-positive anaerobic spore-forming bacterium, is the leading cause of hospital-acquired diarrhea and colitis in Europe and North America (1, 2). According to a new study released by the Centers for Disease Control and Prevention, *C. difficile* causes almost half a million infections in the United States per year and costs up to \$4.8 billion each year in excess health care costs for acute-care facilities alone (3). The pathogen's ability to exist in spore form allows it to persist in the hospital environment, with patients and health care workers acting as the reservoirs that spread the spores and contaminate hospital rooms and equipment. When the gastrointestinal tract microbiota of a patient becomes impaired or unbalanced, most often because of antibiotic treatment, *C. difficile* spores germinate to the vegetative form in the colon. *C. difficile* pathogenesis occurs as a result of the production of two major exotoxins, TcdA and TcdB (4), which cause inflammation, tissue damage, and disruption of the mucosal barrier of the gastrointestinal tract. This damage leads to various forms of disease, from mild to moderate diarrhea and colitis to much more severe diseases such as pseudomembranous colitis and toxic megacolon, collectively called *C. difficile*-associated disease (CDAD). In the past decade, the emergence of new highly virulent strains, such as North American pulsed-field type 1 (NAP1/027/III) isolates, have significantly increased the severity of *C. difficile* infections (CDI), causing lengthy hospitalizations and substantial morbidity and mortality. In 2013, *C. difficile* was among the top three antibiotic-resistant bacteria that were classified as an urgent threat by CDC, drawing attention to the need for better methods of treatment and prevention of CDI (4–8).

The current standard treatment for CDAD is metronidazole or vancomycin antibiotics, which can be effective to some extent but are often accompanied with treatment failures or episodes of post-treatment relapse (1, 9–11). Data suggest that patients can have up to a 25% recurrence of CDI after antimicrobial treatment of a first episode and up to 60% recurrence after the subsequent treatments (12–14). However, highly antibiotic-resistant strains of *C. difficile* have emerged, further complicating treatment (15, 16). Evolving

therapeutics include new antibiotics such as fidaxomicin, which have improved therapeutic effect and lead to less relapses (2), fecal microbiota transplantation (FMT), probiotic therapy, and monoclonal antibodies toward the toxins. While FMT seems to be the most effective treatment against relapsing CDI, the specific route of administration of the FMT has risks, particularly when dealing with a damaged colon, and the procedure can cause patient discomfort and is often complicated by a lack of standardization in procedures, facilities, stool processing, and donor screening (17, 18). Further, the act of transferring the fecal contents from one person to the next often dissuades some from this procedure. Results of probiotic therapy and monoclonal antibody treatment are either inconclusive or still in clinical trials, respectively (19, 20). Therefore, there is a need for new treatments which better target *C. difficile* without collateral damage to the protective commensal species.

In this regard, bacteriophage therapy has potential, and it has been investigated in *in vitro* models (21–23). To date, four temperate bacteriophages have been identified that are active against *C. difficile*, namely, ΦC2, ΦCD119, ΦCD27, and ΦCD6356 (24–28). While bacteriophage therapy has potential, there are many limitations that may constrain their clinical application. Bacteriophage treatment often selects for resistant mutants, and the vi-

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ruses usually have a relatively narrow host range (strain specific), which has been observed with *C. difficile* bacteriophages (29, 30). This forces the use of a cocktail of different phages for treatment, increasing the complexity of drug development and treatment regulations.

Alternatively, using single components of bacteriophages, such as bacteriophage lytic enzymes, or endolysins, to treat infections may reduce some of these constraints. Endolysins or lysins are highly evolved molecules produced by bacteriophages to digest the bacterial cell wall for bacteriophage progeny release (31). For the past decade, phage endolysins have been widely investigated as novel antimicrobial agents to treat bacterial infections by a number of Gram-positive species. This phenomenon of “lysis from without,” utilizing bacteriophage lysins to attack pathogens, has been quite well established (32–39). Lysins normally consist of two domains: an N-terminal catalytic domain, which provides lytic activity against the host species, and a C-terminal binding domain, which binds to particular cell wall structures. The specificity of a lysin is usually dictated by the binding domain, which recognizes a cell wall feature specific to the bacteria that it targets (40).

The study of *C. difficile* phage lysin is still in its early stages. The only characterized *C. difficile* phage endolysin, CD27L, was identified in the genome of a temperate phage, ΦCD27, from *C. difficile* strain NCTC 12727 (27). Recombinantly expressed CD27L is active against diverse strains of *C. difficile* *in vitro*, and its lytic activity is more specific to *C. difficile* than to other commensal species that inhabit the gastrointestinal tract. However, even though many mouse models of *C. difficile* infection have been developed to mimic the clinical symptoms of CDI in humans, there have been no *in vivo* or *ex vivo* studies that implement *C. difficile* phage lysin as an alternative therapy for treating CDI (41, 42).

In this study, we describe a putative amidase lysin, PlyCD, identified from the prophage genome of multidrug-resistant *C. difficile* strain CD630. The sequences of both PlyCD and its catalytic domain, PlyCD_{1–174}, are considerably different from those for the previously described lysin specific for *C. difficile* (27). PlyCD and PlyCD_{1–174} were recombinantly expressed and purified from *Escherichia coli*, and their biochemical activities were characterized *in vitro*. We found that PlyCD_{1–174} was significantly more effective than PlyCD in killing *C. difficile*, and we exploited this effectiveness in a series of *in vitro* and *ex vivo* experiments to ascertain the effectiveness of this lysin as a new treatment for *C. difficile* infections or as an agent to remove residual *C. difficile* prior to FMT.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. difficile* strains ATCC 43255 (ribotype 087, a high-level toxin-producing strain isolated from an abdominal wound), ATCC 9689 (ribotype 001), ATCC 43598 (ribotype 017, isolated from infant stool) were obtained from ATCC. Recent clinical isolates of *C. difficile*, strains 112C and 139B and hypervirulent MLST2 type strains 217B and 615H, were kindly provided by Eric Pamer (Memorial Sloan-Kettering Cancer Center, NY). *C. difficile* strain UK1 (ribotype 027) was kindly provided by Xingmin Sun (The University of South Florida). *Clostridium novyi* (VPI 2383) and *Clostridium perfringens* (VPI 2641) were obtained from ATCC. *Clostridium septicum* (ATCC 12464), *Clostridium sporogenes* (ATCC 3584), *Clostridium bifermentans* (ATCC 638), and *Clostridium sordellii* (ATCC 9714) were purchased from Microbiologics. *Streptococcus pyogenes* D471, group G streptococcus 14-DA, *Enterococcus*

faecalis V583, *Enterococcus faecium* EFSK2, *Pseudomonas aeruginosa* RS1, *Streptococcus suis* 6112, *Bacillus subtilis* SL4, *Bacillus anthracis* 1659 and ΔSterne, *Bacillus cereus* ATCC 14579, *Bacillus thuringiensis*, *Lactobacillus rhamnosus* ATCC 21052, *Listeria monocytogenes* HER 1083, and *Staphylococcus aureus* RN4220 belong to the Rockefeller University collection. The methicillin-susceptible *S. aureus* (MSSA) Newman strain was obtained from Olaf Schneewind (University of Chicago, IL). The vancomycin-intermediate *S. aureus* (VISA) strain IV was obtained from Alexander Tomasz (The Rockefeller University, NY). *Staphylococcus epidermidis* HER 1292 was obtained from Barry Kreiswirth (Public Health Research Institute, NJ). All strains were stored at –80°C and cultivated at 37°C. *Staphylococcus*, *Streptococcus*, *Listeria*, *Enterococcus*, *Pseudomonas*, and *Bacillus* strains were cultivated in Difco brain heart infusion (BHI) broth (Spectrum). *Lactobacillus* strains were cultivated in de Mas, Rogosa, and Sharpe (MRS) broth (Sigma), *Escherichia coli* was grown in Luria-Bertani (LB) broth (BD Biosciences). *Clostridium* strains were cultured in BHIS medium (BHI supplemented with yeast extract [0.5%, wt/vol] and L-cysteine [10%, wt/vol]) and incubated in a Whitley A35 anaerobic chamber (Microbiology International, MD) supplied with an anaerobic gas mixture (10% CO₂, 85% N₂, 5% H₂) (T.W. Smith).

Subcloning of the *C. difficile* PlyCD gene and its subdomains. The nucleotide sequence of PlyCD gene was acquired from the NCBI database (NCBI reference sequence [YP_001088405.1](#)), synthesized, and inserted into a pUC57 vector after codon optimization for *E. coli* expression (GenScript, NJ). After product verification by sequencing, the PlyCD gene insert was amplified with primer set 5′-GGAGATATATCCATGAAAGT AGTAATAATACCAGGGCACACTTAAATTG and 3′-CTAGAGGATC CCCGGTTATAATTATCTATTTCTTGTAATGCTAATTTAAACAG TTTC and subcloned into a pBAD24 expression vector using the CloneEZ PCR cloning kit (GenScript, NJ), to yield the expression vector pQW1. The catalytic domain of PlyCD, namely, PlyCD_{1–174}, was generated by inserting a stop codon at the end of the amino acid sequence of the catalytic domain, Cys₁₇₄, using a site-directed mutagenesis kit (Agilent Technologies) with primer set 5′-CTAAACAAATCTATATCATTAATCTCAA GGGGGAGGGG and 3′-CCCCTCCCCCTTGAGAATTATGATATAGA TTTGTTTAG). The binding domain of PlyCD, namely, PlyCD_{BD}, was generated by replacing the N-terminal sequence (M₁ to Q₁₇₇) with a His tag followed by two E-tag (GAPVYPDPLEPR) sequences in tandem. All constructs were transformed into NEB 5-α F′I⁺ competent *E. coli* (New England BioLabs). Positive clones were identified by colony PCR and sent for DNA sequencing (Genewiz, NJ).

Recombinant protein expression and purification. After nucleotide sequence verification, the aforementioned clones were propagated in LB broth containing 100 μg/ml ampicillin to mid-log phase. The culture was then induced with 0.2% arabinose at 30°C overnight. Cells were then pelleted, resuspended in 20 mM phosphate buffer (pH 7.0) containing EDTA-free complete Mini protease inhibitor cocktail (Roche), and lysed with an EmulsiFlex C-5 homogenizer (Avestin, Ottawa, Canada). Lysate debris was removed via ultracentrifugation at 4°C (17,000 rpm, 45 min), and the supernatant was sterile filtered through a 0.2-μm filter. The target protein was then purified from the supernatant with a HiTrap cation-exchange column (GE Healthcare, Uppsala, Sweden). Lysin was eluted using a stepwise gradient of 0.0 to 1.0 M NaCl in 20 mM phosphate buffer (PB) (pH 6.0). Extra salt was removed by filtration with a 10-kDa-cutoff Ultra Centricon (Amicon). The binding domain of PlyCD, PlyCD_{BD}, was fused to a His tag and dual E tags at the N-terminal end and inserted into a pBAD24 vector, named pQW2. PlyCD_{BD} was expressed from pQW2 in *E. coli* and purified from the whole-cell lysate using a nickel column as previously described (43). Fractions were analyzed by SDS-PAGE to determine the purity of the lysin in each fraction. Those with high concentrations of purified lysin were collected, concentrated, and buffer exchanged against 20 mM PB (pH 7.0) via Ultra Centricon filtration (10 kDa; EMD Millipore, MA).

Lytic activity assays. The lytic activities of *C. difficile* phage lysin against *C. difficile* strains and other bacteria were assessed based on pre-

viously described methods (44). Basically, *C. difficile* strains were grown to mid-log phase under anaerobic conditions, and cells were harvested by centrifugation ($3,000 \times g$, 5 min). Pellets were washed twice and resuspended in PB to generate a final optical density at 600 nm (OD_{600}) of approximately 0.9. The lytic activity of lysin was calculated based on reduction in OD_{600} as measured in 96-well plates using a SpectraMax Plus reader (Molecular Devices, Sunnyvale, CA). For each sample, 25 μ l of lysin (12.5 μ M final concentration) or an equal volume of 20 mM PB was added to 180 μ l of cell resuspension. The drop in OD_{600} at 37°C was measured once per minute for 60 min.

To study the effect of pH on the activity of lysin, the same experimental conditions and optical drop assays described above were utilized with *C. difficile* strain ATCC 43255 and buffers of different pH, i.e., 20 mM PB (pH 6.0, 7.0, and 8.0) or 20 mM sodium acetate buffer (pH 4.0 and 5.0). To study the effect of salt and cation concentrations on the activity of lysin, PB (pH 7.0) that contained various concentrations of NaCl, KCl, $MgSO_4$, or $CaCl_2$ (each at 5 mM, 20 mM, 50 mM, 100 mM, or 200 mM) was used. All experiments were performed in triplicate, and results are shown as mean $\pm$ standard deviation (SD).

Rhodamine labeling of lysin constructs. *N*-Hydroxysuccinimide (NHS)-rhodamine (Thermo Scientific) was chemically linked to PlyCD and PlyCD₁₋₁₇₄ following the manufacturer's instructions. The lysin construct at 1 mg was mixed with rhodamine (10 mg/ml in dimethyl sulfoxide [DMSO]) at a calculated lysin/rhodamine molar ratio of 1:10. The mixture was incubated on ice for 2 h. Excess dye was then removed by passage through a desalting column (GE Healthcare, Sweden), and fractions containing the labeled lysin were collected, pooled, and stored at 4°C until use.

Fluorescence microscopy. Fluorescence microscopy procedures were adapted from a previously described method (43). Briefly, cells from an overnight culture of *C. difficile* strain ATCC 43255 were fixed with 2.6% paraformaldehyde in PB on ice for 45 min. After washing with 20 mM PB (pH 7.0), bacteria were fixed onto poly-L-lysine-coated coverslips. Attached cells were then washed with PB and blocked for 15 min with goat serum (Sigma, MO) supplemented with 1% gelatin from cold-water fish skin (Sigma, MO). To visualize the binding of PlyCD and PlyCD₁₋₁₇₄ to *C. difficile*, rhodamine-conjugated full-length PlyCD or rhodamine-conjugated PlyCD₁₋₁₇₄ was added to the slides, left for 10 min, and then washed with PB. To visualize the binding of PlyCD_{BD}, cells were pretreated either with or without unlabeled PlyCD₁₋₁₇₄ for 10 min and then washed 3 times with PB. Recombinantly expressed PlyCD_{BD} containing dual E tags was then added to the slides, left for 10 min, and washed again with PB. Following this, cells were sequentially incubated with rabbit anti-E-tag antibody (Abcam) for 1 h at a 1:500 dilution and anti-rabbit-fluorescein isothiocyanate (FITC) (Sigma, MO) at a 1:1,000 dilution, with 3 PB washes in between. All slides were sealed with coverslips and mounting medium before viewing under the microscope (Eclipse E400; Nikon, Japan).

Collaborative effect of PlyCD₁₋₁₇₄ and vancomycin. To highlight the role of *C. difficile* lysin PlyCD₁₋₁₇₄ in combination with vancomycin, a preparation of lysin with low lytic activity was used in this experiment (see Fig. S5 in the supplemental material). An overnight culture of *C. difficile* ATCC 43255 was diluted to 1×10^7 CFU/ml in BHIS medium and divided into two equal volumes, one of which was pretreated with 10 μ g/ml vancomycin (Sigma, MO) for 20 min. Both sets were then centrifuged at $4,000 \times g$ for 5 min and resuspended in 50 mM PB (pH 7.0). The resuspended cells were treated with 25 μ g, 12.5 μ g, 6.25 μ g, or 3.125 μ g of PlyCD₁₋₁₇₄ in 250 μ l PB for 30 min under anaerobic conditions. Cells from each treatment were then plated on a BHIS agar plate and incubated overnight for CFU enumeration.

In vivo murine model. The Rockefeller University's Institutional Animal Care and Use Committee approved all *in vivo* protocols. All experiments were conducted at The Rockefeller University's animal housing facility, an AAALAC-accredited research facility, with all efforts to minimize suffering. All mice used in the experiments were housed in groups of

5 per cage. Autoclaved drinking water, bedding, and cages were changed every day. Chow food was radiated and kept in individual packs. Six-week-old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Preparation of *C. difficile* spores. An overnight culture of *C. difficile* strain VPI 10463 (ATCC 43255, a high-level toxin-producing strain) was inoculated into Difco cooked meat broth (BD Diagnostic Systems, MD) and incubated at 37°C in an anaerobic chamber. After 5 days, the meat broth culture was filtered through a sterile cell strainer (40 μ M; Fisher) to remove large meat particles. *C. difficile* spores in the flowthrough were then pelleted by centrifugation (4,000 rpm, 5 min). The pellet was resuspended, washed 3 times in PB, and heated at 80°C for 30 min. Spores were then pelleted again and resuspended in PB at a final concentration of 1×10^7 spores/ml.

***C. difficile* murine infection model.** The protocol used to establish the *in vivo* murine CDI model was modified from a method previously described by Chen et al. (42). An antibiotic mixture of kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml), and vancomycin (0.045 mg/ml) was prepared in drinking water (all antibiotics were purchased from Sigma-Aldrich, St. Louis, MO). Mice were allowed to drink the antibiotic cocktail *ad libitum* for 5 days and then regular autoclaved water for 2 days. Next, mice received a single dose of clindamycin (20 mg/kg) intraperitoneally 1 day before *C. difficile* challenge. At day zero, mice were inoculated with 200 μ l of 10^7 CFU/ml *C. difficile* spores via gavage. Noninfected control mice were given PB via gavage instead of spores. The actual spore inoculation titer was verified by serial dilution and plating to BHIS plates containing 10% (wt/vol) taurocholate acid (Sigma-Aldrich, St. Louis, MO) for each experiment. At 24 h and 48 h after spore gavage, 400 μ g of PlyCD₁₋₁₇₄ or PB (vehicle alone) was delivered intrarectally to mice under ketamine-xylazine anesthesia. Intrarectal injection was performed by inserting 20-gauge polyethylene gavage tubing (Braintree Scientific, MA) about 3.5 cm proximal to the anus. A total volume of approximately 250 μ l was injected via a syringe attached to the gavage tubing. Mice were then held in a head-down vertical position for 1 min after the administration to ensure that the entire volume remained in the colon. Noninfected control mice were also treated intrarectally with 400 μ g of PlyCD₁₋₁₇₄ to test the safety of the lysin. All mice were followed for 7 days, with daily monitoring for weight loss, diarrhea, morbidity, and mortality. Mouse survival data were analyzed by Kaplan-Meier curves using the Prism computer program (GraphPad Software, La Jolla, CA).

Ex vivo treatment of *C. difficile* colon infection. Mice were purchased and treated with antibiotic cocktails in the same fashion and fed with 200 μ l of 10^7 CFU/ml *C. difficile* ATCC 43255 spores as described for the *in vivo* model, except that mice were euthanized 48 h after spore gavage. The colon from each mouse was then excised and further processed in an anaerobic chamber, where it was cut into small (approximately 3-mm) tissue segments. Tissue pieces from each infected mouse colon that were free of solid stool pellets were randomly divided into two equal sets, which were mixed with either 250 μ l of 500 μ g PlyCD₁₋₁₇₄ diluted in previously reduced (by incubating in an anaerobic chamber) PB buffer or reduced PB vehicle in separate Stomacher 80 Biomaster bags, whose contents became isolated from oxygen once liquid was added. Lysin-treated and nontreated tissues from the same mouse were processed simultaneously in a Stomacher Biomaster (Seward, United Kingdom) for 90 s to ensure sufficient mixing between buffer and colon tissue. The bags were then placed back inside an anaerobic chamber for incubation. After 1 h, an aliquot from each bag was sampled, diluted, and plated onto BHIS agar plates containing *C. difficile* selective supplement SR0096 (Thermo Scientific, United Kingdom). Plates were incubated in the anaerobic chamber for 24 h to determine CFU. Differences in bacterial survival between the control and treated groups were analyzed statistically by Student's *t* test using Prism.

FIG 1 Amino acid sequence of PlyCD. (A) Full sequence of PlyCD with the catalytic domain highlighted in red; (B) amino acid sequence alignment of PlyCD with a previously described *C. difficile* lysin, CD27L (27); (C) amino acid sequence alignment of the PlyCD catalytic domain, PlyCD₁₋₁₇₄, with the catalytic domain of CD27L (double dots symbolize identity, single dots symbolize similarity, and dashes represent gaps in the alignment).

RESULTS

Sequence alignment. Through online database searches, we identified the sequence of a putative phage lysin (NCBI reference sequence YP_001088405.1) (Fig. 1A) from a prophage in *C. difficile* strain 630 and termed it PlyCD (Phage lysin from *C. difficile*). The sequences of PlyCD and its catalytic domain PlyCD₁₋₁₇₄ were compared with those for the sequence of the previously described *C. difficile* lysin CD27L (Fig. 1B and C). There was 33% identity in amino acid sequence between PlyCD and CD27L and 34.6% identity between the catalytic domain of PlyCD (PlyCD₁₋₁₇₄) and CD27L, indicating that PlyCD is a unique *C. difficile* lysin.

Expression and purification of PlyCD and PlyCD₁₋₁₇₄ from *E. coli*. The DNA sequences of PlyCD and PlyCD₁₋₁₇₄ were separately cloned into a pBAD24 expression vector, and after arabinose induction, the whole-cell lysates of PlyCD or PlyCD₁₋₁₇₄ were individually purified by cation-exchange chromatography. Eluted fractions that contained the purified target proteins (PlyCD [28 kDa] and PlyCD₁₋₁₇₄ [20 kDa]) were pooled and run on SDS-PAGE to reveal that both final purified products were >90% pure (Fig. 2A and B). The average yield for both lysins was about 5 mg protein per liter of *E. coli* culture. These purified molecules were used in all subsequent experiments.

Molecular characterization of PlyCD. (i) **Effects of pH and salt.** The activity of a lysin is often affected by salt concentration,

cations, and pH. To investigate the lytic activity of PlyCD under various conditions, the reduction in the OD₆₀₀ of *C. difficile* suspensions was monitored over 60 min. PlyCD displayed the strongest activity at pH 7.0 and pH 8.0 (Fig. 3A), showed moderate

FIG 2 Purification of PlyCD and PlyCD₁₋₁₇₄. (A) Gel analysis of the final purified PlyCD. Lanes: 1, Precision dual-color protein standard (Bio-Rad); 2, purified PlyCD. (B) Gel analysis of the final purified PlyCD₁₋₁₇₄. Lanes: 1, Precision dual-color protein standard (Bio-Rad); 2, purified PlyCD₁₋₁₇₄.

FIG 3 Molecular characterization of PlyCD activity. (A) Effect of pH on the lytic activity of PlyCD by analysis of OD₆₀₀ decrease over time. Closed symbols, PlyCD treatment; open symbols, buffer controls. (B) Effect of different concentrations of NaCl on the lytic activity of PlyCD. (C) Substrate specificity of PlyCD among clostridia. The ratios between the OD₆₀₀ values of PlyCD-treated against buffer-treated bacteria were determined at 30 min (open bars) and 60 min (closed bars) postreaction; all results are expressed as means $\pm$ standard deviations (SD) from duplicate assays. The standard deviation was plotted for all experiments; however, the error bars may sometimes be too small to be seen.

activity at pH 6.0, and lost lytic activity at pH 5.0 and pH 4.0. To test salt sensitivity, the lytic activity of PlyCD was measured with different NaCl concentrations. While PlyCD displayed equivalent lytic activity at the end of 60 min for all concentrations tested (0 to 100 mM NaCl), greater lytic activity was observed during the initial 20 min of the reaction with increasing concentrations of NaCl (Fig. 3B). At 200 mM NaCl, the lytic activity of PlyCD was inhibited. At 400 mM NaCl, *C. difficile* start to display autolysis, making analysis at or above this concentration unreliable (data not shown).

(ii) Host specificity. Though phage lysins with broader activity do exist (34), lysins generally exhibit high specificity (45), displaying elevated activity against a few closely related species. To study the specificity of PlyCD, we tested its lytic action against multiple *C. difficile* strains and *Clostridium* spp. All species were cultured anaerobically until mid-exponential phase, washed, and resuspended in 20 mM PB, pH 7.0. PlyCD was then added to the bacterial suspensions at a final concentration of 12.5 μ M (50 μ g), and the OD₆₀₀ values of each culture were recorded over 60 min. The ratio of OD₆₀₀ at 30 and 60 min versus time zero was calculated. Of all the *Clostridium* spp. tested, PlyCD demonstrated the most effective lytic activity against *C. difficile* strain ATCC 43255 and had moderate activity against two clinical strains 139B and 112C. Interestingly, the full-length lysin did not have activity against the other *Clostridium* spp. tested or against *C. difficile* strains ATCC 9689 and ATCC 43598, suggesting that the full-length PlyCD has a very specific and narrow range of activity against *C. difficile* strains (Fig. 3C).

Molecular characterization of the catalytic domain of PlyCD.

Similar to the intact PlyCD, PlyCD₁₋₁₇₄ also displayed very effective lytic efficiency at neutral to basic pH, (i.e., pH 6.0, pH 7.0, and pH 8.0) (Fig. 4A) and no activity below pH 5.0. The salt sensitivity of PlyCD₁₋₁₇₄, was also similar to that of PlyCD, where PlyCD₁₋₁₇₄ had maximum lytic activity in the absence of NaCl (Fig. 4B). A similar pattern was also observed in the presence of KCl (Fig. 4C). The lytic activity of PlyCD₁₋₁₇₄ was more sensitive to the presence of Ca²⁺ and Mg²⁺ than to that of Na⁺ and K⁺ cations. A trace amount of MgSO₄ significantly inhibited the lytic activity of PlyCD₁₋₁₇₄ (Fig. 4D). A similar event was observed with CaCl₂ (see Fig. S4 in the supplemental material). For subsequent experiments, PlyCD₁₋₁₇₄ was used in 20 mM PB at pH 7.0.

The lytic activity of PlyCD₁₋₁₇₄ was directly compared to that of PlyCD by measuring the change in OD₆₀₀ of a *C. difficile* (ATCC 43255) suspension over 60 min (Fig. 5). While the OD₆₀₀ value of the buffer control did not change in 60 min, the OD₆₀₀ of samples treated with 12.5 μ M (50 μ g) PlyCD displayed an $\sim$ 30% decrease after 30 min compared to the initial OD value, and baseline was not achieved until $\sim$ 60 min. In contrast, the OD₆₀₀ of PlyCD₁₋₁₇₄ dropped significantly and quickly when 12.5 μ M (50 μ g) PlyCD was added to the bacterial cells (Fig. 5A), and within 20 min, the OD₆₀₀ value dropped to baseline. This comparison showed that PlyCD₁₋₁₇₄ has greater lytic activity toward *C. difficile* than full-length PlyCD; therefore, PlyCD₁₋₁₇₄ was chosen for subsequent experiments.

To further evaluate the lytic activity of the catalytic domain, various concentrations of PlyCD₁₋₁₇₄, were mixed with *C. difficile*

FIG 4 Molecular characterization of PlyCD₁₋₁₇₄ activity. (A) Effect of pH on the lytic activity of PlyCD₁₋₁₇₄. Closed symbols, PlyCD₁₋₁₇₄ treatment; open symbols, buffer control. (B) Effect of different NaCl concentrations on the lytic activity of PlyCD₁₋₁₇₄. Closed symbols, PlyCD₁₋₁₇₄ treatment; open symbols, buffer control. (C) Effect of different KCl concentrations on the lytic activity of PlyCD₁₋₁₇₄. Closed symbols, PlyCD₁₋₁₇₄ treatment; open symbols, buffer control. (D) Effect of different MgSO₄ concentrations on the lytic activity of PlyCD₁₋₁₇₄. Closed symbols, PlyCD₁₋₁₇₄ treatment; open symbols, buffer control. All experiments were performed using *C. difficile* ATCC 43255, and results expressed as means $\pm$ standard deviations (SD) from duplicate assays.

ATCC 43255, and the OD₆₀₀ was monitored over 60 min. The results revealed that the lytic activity of PlyCD₁₋₁₇₄ functions in a dose-dependent manner (Fig. 5B). To determine the direct bactericidal activity of PlyCD₁₋₁₇₄ on *C. difficile* cells, 80 μ g of PlyCD₁₋₁₇₄ was mixed with 10^8 /ml *C. difficile* ATCC 43255 in PB. The cell suspension was incubated anaerobically for 30 and 60 min, and at each time point the surviving bacteria were plated for enumeration. After 30 and 60 min of lysis exposure, 3- and 4-log reductions, respectively, in *C. difficile* survival were observed (Fig. 5C).

Host specificity of PlyCD₁₋₁₇₄. PlyCD₁₋₁₇₄ was tested against a variety of *C. difficile* strains, other clostridia, and non-*Clostridium* species. All species were cultured until mid-exponential phase, washed, and resuspended in 20 mM PB, pH 7.0. PlyCD₁₋₁₇₄ was added to the buffer at a final concentration of 12.5 μ M (50 μ g). Unlike the full-length molecule, PlyCD₁₋₁₇₄ displayed strong activity in an OD₆₀₀ reduction assay against all of the *C. difficile* reference laboratory strains tested, i.e., ATCC 43255, ATCC 9689, and ATCC 43598, as well as two clinical strains, 139B and 112C, and two clinical hypervirulent strains, 217B and 615H, but not against other *Clostridium* spp. tested, with the exception of *C. sordellii* (Fig. 6A). This indicates that PlyCD₁₋₁₇₄ has better lytic activity against a wider *C. difficile* strain spectrum than PlyCD, yet it is not active against other potential intestinal *Clostridium* spp., such as *C. novyi*, *C. perfringens*, *C. bifermentans*, *C. sporogenes*, and *C. septicum*. The lytic activity of PlyCD₁₋₁₇₄ against hypervirulent MLST2 strains 217B, 615H, and UK1 (R027) was further verified by the CFU reduction assay (Fig. 6B). In 60 min, the CFU of all the hypervirulent strains tested decreased over 4 logs in the presence of 120 μ g of PlyCD₁₋₁₇₄. This reduction was dose dependent and

comparable to what was found with the other laboratory reference strains. To determine if PlyCD₁₋₁₇₄ is effective against other, non-*Clostridium* species, strains of *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Bacillus*, *Lactobacillus*, and *Listeria* were also tested. Except for *B. subtilis*, PlyCD₁₋₁₇₄ did not display lytic activity, as determined by OD₆₀₀ reduction, against any of these other species (Fig. 6C). These data suggest that compared to PlyCD, PlyCD₁₋₁₇₄ is more active against many *C. difficile* strains while still retaining a majority of its species specificity.

Collaborative effect of lysis and vancomycin. Vancomycin, an antibiotic that inhibits cell wall synthesis, is often used to treat severe forms of *C. difficile* infection as the first-line drug. The minimal recommended trough concentration in adults is 5 to 10 μ g/ml (46). To explore whether there could be a synergistic therapeutic effect of PlyCD₁₋₁₇₄ and a low dose of vancomycin, 1×10^7 CFU/ml of *C. difficile* (ATCC 43255) was grown in the presence of a minimal physiological trough concentration of vancomycin (10 μ g/ml) or medium alone for 20 min. The cells were then centrifuged, resuspended in 50 mM PB (pH 7.0), and treated with a series of low doses of PlyCD₁₋₁₇₄ or buffer control for 30 min before being plated to enumerate CFU. We found that by themselves, neither the minimal trough dose of vancomycin nor the low doses of lysis reduced the titer of *C. difficile*; however, cells pretreated with vancomycin and then 25 μ g of lysis were significantly reduced, by two logs, and this response appeared to be lysis dose dependent (Fig. 7).

Reduction of *C. difficile* bacteria in mouse colon by ex vivo treatment. In a mouse model of *C. difficile* intestinal disease, we attempted to treat the infected colon with PlyCD₁₋₁₇₄ delivered

FIG 5 Effect of truncation on lytic activity. (A) Comparison of the lytic activities of PlyCD₁₋₁₇₄ (closed circles) and PlyCD (triangles) against *C. difficile*. Open circles, buffer control. Lysis assay mixtures comprised cells incubated with 50 μg purified protein or PB. (B) The lytic activity of PlyCD₁₋₁₇₄ functions in a dose-dependent manner against *C. difficile*. (C) Lytic activity of PlyCD₁₋₁₇₄ determined by the decrease in *C. difficile* titer (80 μg of PlyCD₁₋₁₇₄ per sample was used in the assay). All experiments were performed using *C. difficile* ATCC 43255, and results are expressed as means ± standard deviations (SD) from duplicate assays. The standard deviation was plotted for all experiments; however, the error bars may sometimes be too small to be seen.

rectally by enema (see Fig. S6 in the supplemental material). However, due to the unpredictable presence of solid fecal stool pellets in the mouse colons, despite severe diarrheal disease, lysin could not be reliably distributed in an effective manner throughout the infected colon, resulting in inconsistent results. Thus, a *C. difficile* mouse *in vivo* infection model could not adequately serve as an appropriate platform to determine the therapeutic effect of lysin enema (see Fig. S6 in the supplemental material). Therefore, to continue to pursue whether *C. difficile* infection could be controlled by PlyCD₁₋₁₇₄ in a colonic environment, we modified the *in vivo* infection model to develop an *ex vivo* mouse treatment model. In this model, we asked whether the PlyCD₁₋₁₇₄ lysin was able to function and kill *C. difficile* vegetative cells in the mouse colonic environment and whether there were any inhibitory substances that would block its activity. Two days after *C. difficile* infection, mouse large intestines between the cecum and anus were excised and divided into two groups to be treated with either PlyCD₁₋₁₇₄ or PB alone (Fig. 8A). In a 500-μl total reaction volume, 600 μg of PlyCD₁₋₁₇₄ decreased the titer of intestinal *C. difficile* from an average of 6.5×10^6 CFU/ml to 5.2×10^4 CFU/ml and 4.5×10^4 CFU/ml (>2 logs) after 30 and 60 min of incubation, respectively (Fig. 8B). Since the intestinal volume between the cecum and anus of a 6- to 8-week-old mouse is about 250 μl (unpublished results), we repeated the experiment by adding 250 μl PB to the intestinal pieces with or without 600 μg PlyCD₁₋₁₇₄ to be more physiologically relevant. Intestines that were treated with 250 μl of PlyCD₁₋₁₇₄ also displayed an approximate two-log decrease in *C. difficile* CFU after 60 min of incubation (Fig. 8C).

These data suggest that PlyCD₁₋₁₇₄ is active against *C. difficile* vegetative cells infecting the large intestine in the presence of its contents.

DISCUSSION

C. difficile infection (CDI) is the major identifiable cause of antibiotic-associated diarrhea and colitis in hospitals. The potential use of bacteriophage endolysins as treatment against bacterial infections has long been recognized since their initial discovery (47), and they have been used against a number of different human pathogens in a variety of animal models (38, 47, 48). In this study, we characterized a novel *C. difficile* endolysin (PlyCD) and its catalytic domain PlyCD₁₋₁₇₄.

PlyCD₁₋₁₇₄ displayed stronger lytic activity than the full-length PlyCD containing both the catalytic and binding domains. The role of the binding domain is to bind tightly to its cell wall receptor and provide specificity, thus positioning the catalytic domain adjacent to its substrate for cleavage. While some lysins require the binding region for activity (49), other catalytic domains have been reported to work equally well or better on their own (50). This feature was also found in the only other characterized *C. difficile* lysin, CD27L (27). However, the reason for the increased activity of the CD27L catalytic subunit was not explored in the previous report (27). Since lysins are utilized by the phage to act from the inside a bacterial cell wall to release its viral progeny, structures on the outer surface of the *C. difficile* cell could hinder peptidoglycan accessibility of the entire lysin when it is added from the outside. The presence of secondary structures on the cell surface, such as

FIG 6 Substrate specificity of PlyCD₁₋₁₇₄. (A) The ratios between the OD₆₀₀ values of PlyCD₁₋₁₇₄-treated against buffer-treated *Clostridium* strains were determined at 30 min (open bars) and 60 min (closed bars) postreaction. (B) Comparison of CFU changes between hypervirulent clinical strains and the laboratory strain with different doses of PlyCD₁₋₁₇₄ over the course of 60 min. (C) The ratios between PlyCD₁₋₁₇₄-treated and buffer-treated non-*Clostridium* strains were determined at 30 min (open bars) and 60 min (closed bars) postreaction. Results are the means $\pm$ standard deviations (SD) from duplicate assays.

S-layer proteins, of which *C. difficile* has several, could act as a filter preventing the larger PlyCD from entering the wall (51). Furthermore, other, nonsteric cell wall factors could reduce the ability of PlyCD to bind to the cell. Our microscopic analysis of fluorescently tagged PlyCD showed that PlyCD bound intact *C. difficile*

FIG 7 Collaborative effect of PlyCD₁₋₁₇₄ and vancomycin. *C. difficile* cells (1×10^7) were pretreated with or without 10 µg/ml vancomycin for 20 min and then were centrifuged and subjected to increasing amounts of PlyCD₁₋₁₇₄ in 50 mM PB (to help approximate the local intestinal ionic environment) for 30 min (3.125 µg, 6.25 µg, 12.5 µg, and 25 µg). CFU of remaining bacteria from each treatment group were counted after overnight incubation. Results are the means $\pm$ standard deviations (SD) from duplicate assays.

cells poorly, with fluorescence found only on cells that were degraded (see the supplemental material), suggesting that the binding receptor in the wall was not surface accessible. Previous studies have observed a correlation between the positive charge on a lysin's catalytic domain and its bactericidal activity in the absence of the binding domain, where an inactive catalytic domain, without a binding domain, was made active by engineering it to have more of a positive net charge (52). Likewise, the pIs of the catalytic domain and binding domain of PlyCD are 8.78 and 5.76, respectively, making the pI of the full-length protein 8.31. Thus, in a reaction buffer of pH 7.0, the catalytic domain would assume a greater net positive charge than the full-length protein. This difference in the net charge between PlyCD and its catalytic domain may help explain the difference in their lytic activity and host range.

Unlike antibiotics, endolysins, such as PlyCD, display high specificity for their substrates (53). Compared to the low activity against other commensal gut flora tested, such as *C. septicum*, *C. novyi*, *E. faecalis*, *E. faecium*, and *Lactobacillus rhamnosus*, the catalytic domain of PlyCD had almost exclusively strong lytic activity against *C. difficile*, which included multiple ribotypes and toxin types of laboratory reference strains and highly virulent recent clinical isolates of *C. difficile*. The only exceptions were the lytic activities against *B. subtilis* and *C. sordellii*, suggesting a cell wall

FIG 8 PlyCD₁₋₁₇₄ decreases *C. difficile* colonization of mice colons in an *ex vivo* treatment model. (A) Schematics of the experimental design. (B and C) Decrease in *C. difficile* titer after 30 and 60 min of PlyCD₁₋₁₇₄ treatment compared to controls ($n = 11$ [B] or 7 [C]). After antibiotic treatment, C57BL/6 mice were fed 10^7 spores by gavage at day 0. At day 2 postinfection, mice were euthanized and colons removed and cut into 3-mm tissue pieces, which were equally divided into 2 sealed plastic pouches containing 500 μ l (B) or 250 μ l (C) of reduced PB or PlyCD₁₋₁₇₄ (1 mg/ml). Tissues were then homogenized for 90 s, incubated anaerobically for 1 h, and plated to BHIS agar to enumerate CFU. Data from two independent experiments were combined and analyzed for statistical significance with the Student *t* test. Means $\pm$ SDs are shown.

structure very similar to that of *C. difficile* (54). This high specificity has also been seen in other bacteriophage lysins, making them suitable candidates for clinical treatment of bacterial infections (31, 32, 48). Our *in vitro* specificity results suggest that intestinal delivery of PlyCD₁₋₁₇₄ should have a minimal effect on the commensal bacterial population, thus reducing potential complications seen with antibiotics.

Synergism between lysins and antibiotics has been shown previously in the growth inhibition of facultative anaerobic bacteria (33, 55). However, no synergism has been shown in the growth inhibition of strict anaerobic bacteria, possibly due to the challenge of maintaining the growth of anaerobic bacteria in minimal media. To evaluate whether there is a synergistic effect with PlyCD₁₋₁₇₄ and an antibiotic, we developed an *in vitro* assay to determine the bactericidal effect of PlyCD₁₋₁₇₄ and vancomycin. Under conditions of minimal concentration and exposure time, neither vancomycin nor PlyCD₁₋₁₇₄ alone significantly killed *C. difficile*. However, when the bacteria were pretreated for a short period with a minimal dose of vancomycin and then exposed to a minimal dose of PlyCD₁₋₁₇₄, there was a significant killing of *C. difficile* (>2-logs). These data suggest that a collaborative effect between vancomycin and lysin does exist and that *C. difficile* organisms exposed to vancomycin (the standard of care for CDI) could be sensitized to sublytic doses of lysin.

For the past 5 years, several mouse models of CDI have been established successfully, including a gnotobiotic model (56), an antibiotic cocktail model (42), a single-antibiotic model (57), and even a CDI relapse model (41). However, our mouse *C. difficile* infection model did not seem to be ideal for evaluating rectally applied therapeutics. In this study, we administered an enema of PlyCD₁₋₁₇₄ into the colons of *C. difficile*-infected mice in an attempt to reduce CDAD mortality. Our initial data showed that

delivery of 400 μ g lysin via enema resulted in no gross abnormal effects, such as diarrhea or mouse weight loss in uninfected controls (see Fig. S6B in the supplemental material). Further analysis of *C. difficile*-infected mice showed that compared to buffer-treated controls, the lysin-treated mice showed an increase in survival and a delay in the rate of morbidity and mortality. Unfortunately, the results were possibly confounded by the nature of the mouse model. Mice were often variable in the rates of *C. difficile* disease symptoms, and though some had wet perianal regions from diarrhea, the presence of solid stools in their colons was often randomly found upon necropsy. These stool pellets prevented the distribution of the lysin into the entire colon and made it difficult to consistently deliver an accurate volume of the enema into the rectum, thus hindering the protective effect of lysin and affecting the proper evaluation of the efficacy of PlyCD₁₋₁₇₄ in the colons of these mice. An unblocked colon is critical to any rectal delivery of drug or FMT and can be more easily achieved, observed, and confirmed in human procedures. Unfortunately, we found it difficult to fully accomplish this in mice, possibly due to differences in diet, physiology, morphology, and/or size of the colons. We attempted to eliminate hard stools in the colons by feeding mice a liquid diet and/or laxatives before infection, but we were unsuccessful. Therefore, we established an *ex vivo* treatment model, where colons were removed from infected mice and only colons from mice who had enough disease to have loose stools were treated with lysin or control buffer. In this model, we were able to show that the PlyCD₁₋₁₇₄ lysin functioned effectively in the presence of the infected large intestinal environment, significantly killing the colonized *C. difficile* organisms by >2 logs compared to the vehicle buffer control.

Because the use of antibiotics is often unsuccessful in curing CDI, FMT has emerged as a second-line therapy for recurrent

CDI. While almost 90% successful, transplantation failures in which *C. difficile* is not fully cleared from the individuals do occur (58). The success rates of FMT may be increased by reducing residual *C. difficile* remaining in the colon prior to administering the transplant; thus, lysin treatment of the colon prior to delivery of the transplant may prove effective. The idea of removing residual *C. difficile* prior to transplantation is supported by a randomized controlled trial of treatment of recurrent CDI (59), where colon lavage before FMT was significantly more effective (81%) at resolving CDI than FMT without bowel lavage (23%) or after vancomycin treatment (31%). Additionally, since PlyCD₁₋₁₇₄ may have little effect on normal commensal bacteria, including *Clostridia* species, the lysin could also be combined with the donor fecal microbiota and delivered with the FMT. Thus, PlyCD₁₋₁₇₄ treatment could represent another weapon in combating CDI, utilizing lysin either alone, in combination with antibiotics, or alongside FMT therapies to increase their efficacy and prevent CDI recurrence.

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