



Design and characterization of a novel lytic protein against *Clostridium difficile*

Meng Wang¹ · Zifeng Deng¹ · Yanmei Li¹ · Yi Ma^{1,2} · Jufang Wang^{1,2}

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Abstract

Clostridium difficile (*C. difficile*) is a Gram-positive, spore-forming, toxin-producing anaerobe that can cause nosocomial antibiotic-associated intestinal disease. Autolysin is a lytic enzyme that hydrolyzes peptidoglycans of the bacterial cell wall, with a catalytic domain and cell wall-binding domains, proven to be involved in bacterial cell wall remodeling and cell division. Although autolysins in *C. difficile* have been reported, the autolysins have failed to yield impressive results when used as exogenous lytic agents. In this study, we expressed and characterized the binding domains (Cwp19-BD and Acd-BD) and catalytic domains (Cwp19-CD, Acd-CD, and Cwl-CD) of *C. difficile* autolysins, and the domains with the best binding specificity and lytic activity were selected towards *C. difficile* to design a novel lytic protein Cwl-CWB2. Cwl-CWB2 showed good biosafety with significantly low hemolysis and without cytotoxicity. The results of fluorescence analysis and lytic assay demonstrated that Cwl-CWB2 has higher binding specificity and stronger lytic activity with a minimum inhibitory concentration at 13.39 ± 5.80 $\mu\text{g/mL}$ against living *C. difficile* cells, which is significantly stronger than commercial lysozyme (3333.33 ± 1443.37 $\mu\text{g/mL}$) and other reported *C. difficile* autolysins. Besides, Cwl-CWB2 exhibited good stability as about 75% of the lytic activity was still retained when incubated at 37 °C for 96 h, which is considered to be a potential antimicrobial agent to combat *C. difficile*.

Key points

- Several binding domains and catalytic domains, deriving from several *Clostridium difficile* autolysins, were expressed, purified, and functionally characterized.
- A novel *C. difficile* lytic protein Cwl-CWB2 was designed from *C. difficile* autolysins.
- The binding specificity and lytic activity of Cwl-CWB2 against *C. difficile* showed advantages compared with other reported *C. difficile* autolysins.
- Cwl-CWB2 exhibited significantly low hemolysis and cytotoxicity against normal-derived colon mucosa 460 cell.

Keywords *Clostridium difficile* · Lytic protein · Autolysin · Antibiotic · *Clostridium difficile* infection

Introduction

Clostridium difficile (*C. difficile*), a strictly anaerobic, spore-forming Gram-positive (G^+) bacterium, is the major cause of *C. difficile* infections (CDI) such as antibiotic-associated

diarrhea, pseudomembranous colitis, and *C. difficile*-associated diarrhea (Burke and Lamont 2014). In the past decades, the emergence of *C. difficile* high virulence epidemic strains had resulted in increased morbidity and mortality worldwide (Tamez-Torres et al. 2017). According to a statistics from 2011 to 2013 in the USA, CDI infected an average of 250,000 people a year, with a mortality rate of 5.6% (Lessa et al. 2015). The incidence of CDI among hospitalized patients in the USA has nearly doubled, to 8.2%, and the overall mortality rate is 7.2% since the twenty-first century and the three most common *C. difficile* subtypes are type 027, type 020, and type 026 (Reveles et al. 2014). Nowadays, in the face of the COVID-19 pandemic, antibiotic use during hospitalization increases the risk of CDI

✉ Jufang Wang
jufwang@scut.edu.cn

¹ School of Biology and Biological Engineering, South China University of Technology, Guangzhou 510006, China

² Guangdong Key Laboratory of Fermentation and Enzyme Engineering, South China University of Technology, Guangzhou 510006, China

infection in COVID-19 patients (Marinescu et al. 2021; Spigaglia 2022). Until now, the broad-spectrum antibiotics like vancomycin, metronidazole, or fidaxomicin are still the main treatment for CDI clinically (Chahine 2018; Debast et al. 2014; Okumura et al. 2020). However, these antibiotics always adversely affect the gut microbiota, costing billions of dollars to combat CDI (Collins and Auchtung 2017; Rao and Malani 2020). Hence, it is still of urgent need to explore novel strategies to combat *C. difficile* and CDI.

In recent decades, bacteriolytic enzymes are drawing increasing interest as potential alternatives to traditional small-molecule antimicrobials because of their high cellular specificity and strong lytic activity (Szweda et al. 2012). Moreover, they are active against drug-resistant strains with a low rate of bacteria resistance (Pastagia et al. 2013). Bacteria autolysin and endolysin are such well-known lytic enzymes, which can hydrolyze peptidoglycans of the bacterial cell wall, with a catalytic domain and cell wall-binding (CWB2) domains or Src-homology 3 (SH3b) domains that are expected to specifically recognize the bacterial cell walls (Bradshaw et al. 2018; Mayer 2001; Usenik et al. 2017; Vollmer et al. 2008). Many studies have shown that lytic enzymes are also involved in bacteria cell division, adhesion, peptidoglycan recycling, and virulence (Eldholm et al. 2009). However, limited studies have been given to the development of bacterial autolysins as a potential source of antibacterial agents even though wide studies were carried out on autolysin or endolysin.

G⁺ bacteria possess a thick cell wall that surrounds their cytoplasmic membranes and provides physical protection. The basic structure of the bacterial cell wall is a mesh polymer of peptidoglycans, in which N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) are alternately connected by β -1,4-glycosidic bonds to form the linear glycan backbones that are cross-linked by species-specific peptide side chains (Peltier et al. 2011; Popham 2013). Interestingly, *C. difficile* has a unique peptidoglycan structure compared with other G⁺ bacteria which has tetrapeptide side chains, L-Ala- γ -D-Glu-*meso*-2,6-diaminopimelic acid-D-Ala, and glycan backbones were connected by direct 3–4 cross-links (Kirk et al. 2017; Peltier et al. 2011), indicating autolysin in *C. difficile* would be highly specific compared with other G⁺ bacteria autolysins. In recent decades, increasing amounts of novel *C. difficile* autolysins were discovered. Recently, Cwp19 was characterized as a lytic transglycosylase, which can contribute to toxin release through bacteriolysis (Bradshaw et al. 2017; El Meouche and Peltier 2018; Wydau-Dematteis et al. 2018); Cwl0971, involved in cell wall lysis and cell viability, affects toxin release, sporulation, germination, and pathogenicity of *C. difficile* (Zhu et al. 2021); CwlA, which was the first substrate of PrkC in *C. difficile*, controls hydrolytic activity in the cell wall (Cuenot et al. 2019; Garcia-Garcia et al. 2021); Acd24020

exhibited *C. difficile*-specific lytic activity with an hour-glass-shaped substrate-binding groove across the molecule, which is responsible for recognizing the cross-linking structure unique to *C. difficile* peptidoglycan (Sekiya et al. 2021). However, all these autolysins are not directly used for the treatment of CDI because of their weak lytic activity or low cellular specificity. Hence, novel lysins with strong lytic activity and high cellular specificity are still of urgent need.

In this study, with the principle of high binding property and strong lytic activity against *C. difficile*, the binding activity of binding domains and the lytic activity of catalytic domains in several autolysins (Cwp19, Acd24020, and Cwl0971) were carried out. By the protein fusion strategy, a novel lytic protein Cwl-CWB2 with strong lytic and high specific binding activity against *C. difficile* was designed and expressed. Besides, the biosafety and stability of Cwl-CWB2 were also determined and it demonstrated that it has the potential for further application.

Materials and methods

Materials and organisms

Common chemical materials were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and Macklin (Shanghai, China). Enzymes and reagents for cloning were from Thermo Fisher Scientific (Waltham, MA, USA) and oligonucleotides (25 nmol scale, PAGE purification) were from Sangon Biotech. Unless noted, all strains used in this study were from American Type Culture Collection (ATCC, Manassas, VA), which are listed in Table S1. Luria–Bertani (LB) medium used in this study is one of the most common media for *E. coli* growth and protein expression. Antibiotics such as 25 μ g/mL chloramphenicol (Cm⁺), 50 μ g/mL kanamycin (Kan⁺), and 100 μ g/mL ampicillin (Amp⁺) were also used. The *C. difficile* strains were cultured in brain heart infusion (BHI) broth in an anaerobic chamber, with 250 mg/L D-cycloserine and 8 mg/L cefoxitin.

Recombinant plasmid construction

The genomic DNA of *C. difficile* was extracted using TIANamp Bacteria DNA Kit (Tiangen, Beijing, China). The Phanta Flash Master Mix (Vazyme Biotech, Nanjing, China) was used to PCR-amplify DNA fragments for cloning purposes. The *C. difficile* Cwp19, Acd24020, and Cwl0971 genes were separately cloned into vector pET28a by restriction-free cloning technology (RF cloning) method according to their sequences from NCBI (Gene ID: CDIF630erm_03030, CDIF630erm_02644, and CDI-F630erm_01282) (Unger et al. 2010). The overlap PCR to connect the Cwl-CD sequence and Cwp19-BD sequence

was carried out between a linker (glycine-glycine-serine-glycine, GGSG) and the Cwl-CWB2 sequence was cloned into pColdTF vector at the C terminal of the trigger factor (TF) following an enterokinase restriction site (aspartic acid—aspartic acid—aspartic acid—aspartic acid—lysine). The recombinant plasmids were transformed into *E. coli Trelief*®5 α Chemically Competent Cells (TsingKe, Beijing, China) for amplification with appropriate antibiotics. Then, the plasmids were extracted using the FastPure Plasmid Mini Kit (Vazyme Biotech, Nanjing, China) and analyzed by 1% agarose gel electrophoresis before DNA sequencing. Sequence similarity searches were performed with the BLAST program. All plasmids used in this study are listed in Table S1 and all primers are supplied in Table S2.

Protein expression and purification

Recombinant plasmids containing Cwp19-CD and Acd-BD were separately transformed into *E. coli* BL21 (DE3) Chemically Competent Cells (TsingKe, Beijing, China). For efficient protein expression, plasmids pET28a-Cwp19-BD, pET28a-sumo-Acd-CD, pET28a-sumo-Cwl-CD, and pColdTF-Cwl-CWB2 were transformed into *E. coli* Rosetta (DE3) Competent Cells (Tiangen, Beijing, China), respectively. When the OD₆₀₀ of these recombinant strains reached 0.4–0.6, isopropyl- β -D-thiogalactopyranoside (IPTG) was added and incubated at 16 °C for 24 h to induce the soluble expression. Cell cultures were separated by centrifugation at 8000 \times g for 20 min and the cell pellets were resuspended in binding buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.8), and cell disruption was performed by sonication (100 W, 3 s with a 5-s interval for 5 min) in an ice-water bath. The suspension was centrifuged (12,000 \times g at 4 °C for 30 min), and the supernatants were filtered through a 0.22- μ m filter and purified with a Ni-NTA 6FF (Pre-Packed Gravity Column) (Sangon, Shanghai, China). All proteins were purified using the standard nickel affinity chromatography procedure with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.8). Additionally, the Cwl-CWB2 was re-purified after digesting by Recombinant Bovine Enterokinase Light Chain with His-tag (Sangon, Shanghai, China) in digesting buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM CaCl₂, pH 7.8) at 4 °C for 36 h. Purified proteins were assessed by mixing samples with 4 \times Laemmli sample buffer (Bio-Rad, Hercules, CA) and running them on a 12% SDS-PAGE. The protein purities were analyzed by BandScan software and their concentrations were determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Hemolytic test and cytotoxicity assay

For hemolytic test, erythrocytes separated from mice blood were washed with pre-cold PBS for two times and resuspended in PBS with a final concentration at 8% (v/v). Then, the diluted erythrocytes were treated with different concentrations (5.02–321.43 μ g/mL) of Cwl-CWB2 and incubated at 37 °C for 1 h. After incubation, the supernatant was separated by centrifugation at 1200 \times g for 5 min and the absorbance at 540 nm was recorded. The hemolysis activity of Cwl-CWB2 was calculated by the percentage of hemoglobin contents in Cwl-CWB2 samples to hemoglobin content in 0.1% Triton X-100 sample. PBS and 0.1% Triton served as negative and positive control. This assay was performed in triplicate. Normal-Derived Colon Mucosa 460 (NCM 460) was used to assess the cytotoxicity of Cwl-CWB2. Briefly, NCM460 cells were adjusted to 0.5 \times 10⁵ cells/mL with RPMI1640 medium and dispensed into 96-well plates per 100 μ L. Then, increased concentrations (5.02–321.43 μ g/mL) of Cwl-CWB2 were added into 96-well plates separately and incubated with 5% CO₂ for 24 h at 37 °C. Cell viability was examined by cell counting kit-8 (CCK-8). PBS served as control and the assay was performed in triplicate.

Cell binding assay and western blot analysis

The binding specificity of proteins was tested with some modifications of the method described and previously reported (Sekiya et al. 2021). Briefly, *C. difficile* strains cultured in BHI medium at 37 °C for 16 h were washed twice with PBS and suspended in the same buffer to adjust OD₆₀₀ to 50. Then, 15 μ g of purified proteins was incubated with 20 μ L of the above suspended cells for 15 min on ice. The samples were centrifuged at 8000 \times g and 4 °C for 2 min. Afterward, the supernatant samples were analyzed by 12% Tris-Gly-SDS-PAGE at 80 V for 2.5 h. For western blot, proteins were blotted onto a nitrocellulose membrane (0.2 μ m, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) using the Bio-Rad TransBlot Turbo system at 80 V for 1 h. After incubating with the blocking buffer (5% (w/v) skimmed milk powder in TBST) for 1 h, the membrane was incubated with HRP-Conjugated 6*His-Tag Monoclonal Antibody (Proteintech, Wuhan, China) at dilution of 1:5000 in TBST at 4 °C overnight. The next day, the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize the bands on the membrane.

Minimum inhibitory concentration (MIC) assay

C. difficile and the other strains were cultured in BHI medium at 37 °C overnight. Cells were harvested by centrifugation, washed once with BHI, and then suspended in BHI

to a final OD₆₂₅ at 0.08 to 0.13, which contains 10⁸ CFU/mL cells in most of the bacteria (Wiegand et al. 2008). The assay was performed in 96-well plates, each well consisting of 50 µL of bacteria suspension with a final concentration of 10⁶ CFU/mL, and 50 µL of gradient diluted proteins. Then, the plates were incubated at 37 °C overnight. The contents of the plates were pipette-mixed to re-suspend any material which may have deposited at the bottom of the wells and the optical density at 600 nm was measured using Kaiao K6600 (Beijing, Kaiao) microplate reader and the MIC value is the lowest concentration at which no bacterial growth is observed. Means and standard deviations were calculated by three independent experiments, each with triplicate samples.

Lytic activity assay

The lytic activity was tested with some modifications according to the method previously described (Gerova et al. 2011). *C. difficile* strains cultured in BHI medium at 37 °C for 16 h were washed twice with PBS and adjusted OD₆₀₀ at 1.25. The lytic activity was started by the addition of 100 µL protein or assay buffer into 100 µL of the pre-incubated cell suspensions. Their optical density at 600 nm was measured at 37 °C and 3-min intervals for 150 min (K6600, Kaiao, Beijing, China). The lytic activity was calculated by the following formula: lysis efficiency = $1 - [\Delta OD_{600} \text{ test (samples added)} - OD_{600} \text{ (PBS buffer only)}] / [\text{initial } OD_{600} - OD_{600} \text{ (PBS buffer only)}] \times 100\%$. The protein concentration for the lytic activity measurement was optimized by preliminary measurements using various concentrations. All experiments were conducted with three biological replicates, and means and standard deviations were calculated.

Statistical analysis

All statistical analyses were performed with IBM SPSS Statistics 22 software and all graphs were performed with GraphPad Prism 8.2.1 software. Data were expressed in the format of mean ± S.D.

Results

Expression and purification of binding domains of autolysin

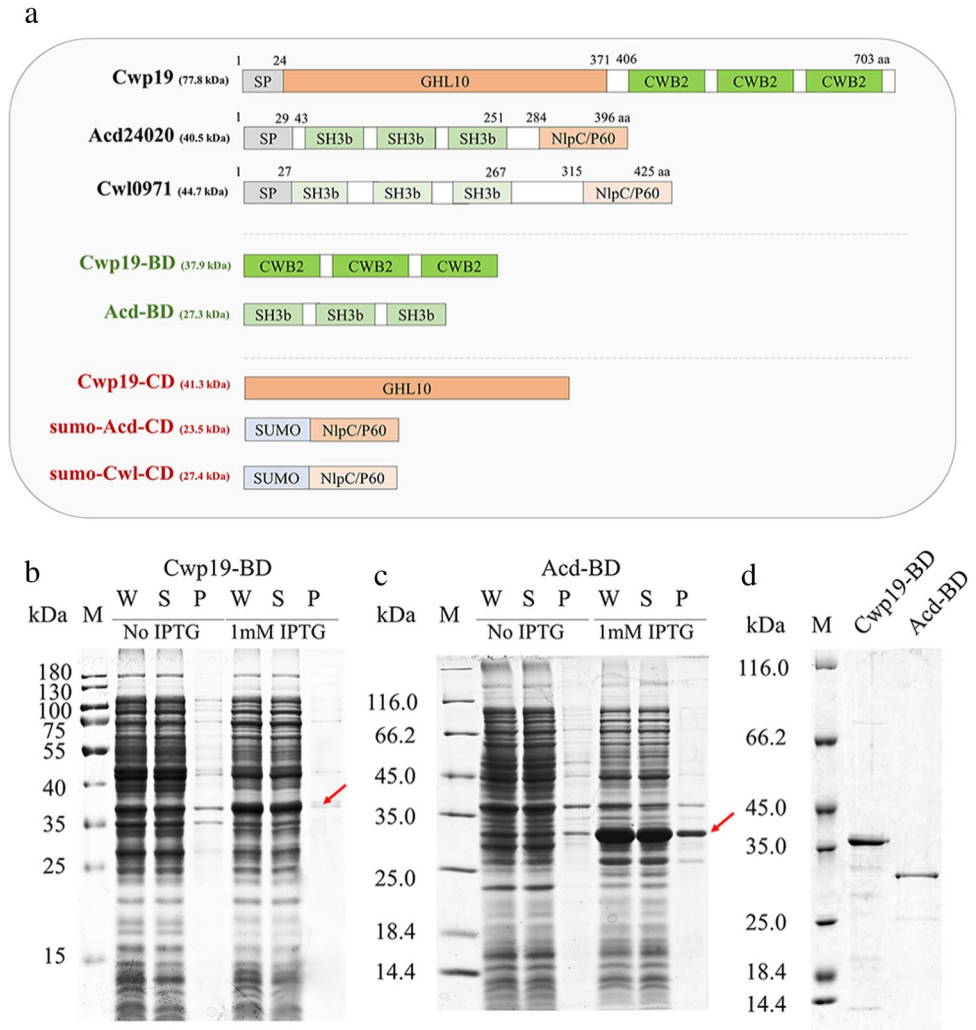
The genes ID Cwp19 and CD24020 in the *C. difficile* 630 genomes were identified as autolysins in previous studies (Sekiya et al. 2021; Wydau-Dematteis et al. 2018). Cwp19 of *C. difficile* 630 has a signal peptide (SP) sequence at the N terminus, followed by an endopeptidase catalytic domain of the GH10 family, and three CWB2 domains at the C terminus (Fig. 1a). Acd24020 has a signal peptide sequence

at the N terminus, three SH3b domains, and an endopeptidase catalytic domain of the NlpC/P60 family at the C terminus (Fig. 1a). As can be seen in Fig. 1b and c, proteins Cwp19-BD and Acd-BD were successfully expressed and the majority of proteins were in the soluble form. The band positions in SDS-PAGE match the theoretical molecular weight of Cwp19-BD and Acd-BD calculated from the amino acid sequence (37.9 kDa for Cwp19-BD, 27.3 kDa for Acd-BD), respectively. As Cwp19-BD and Acd-BD coding sequences were each appended with an N terminal 6* His-tag, the Cwp19-BD and Acd-CD can be purified by nickel affinity chromatography. As shown in Fig. 1d, high purities of Cwp19-BD (89.1%) and Acd-BD (92.8%) were eventually achieved.

Characterization of binding specificities of Cwp19-BD and Acd-BD

To examine the binding specificity of Cwp19-BD and Acd-BD to bacterial cells, a binding assay combined with western blot was performed. Five intestinal bacteria, namely *C. difficile* 630Δ*ermB*, *Lactococcus lactis* ATCC 19,435, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 53,103, and *Bifidobacterium breve* ATCC15700, at logarithmic growth phase (growth for 12 h) were selected. In this binding assay, protein with high binding activity to a specific bacteria would bind with the bacteria cells and caused a decrease in the amounts of protein in the centrifuged supernatant after incubation. On the contrary, there was no difference in the protein content in the supernatant after incubation if this protein had low or no binding activity towards a certain bacterium. As shown in Fig. 2a, Cwp19-BD showed high binding specificity to *C. difficile* 630Δ*ermB* compared with the other four probiotics as there was an obvious decrease of Cwp19-BD content in the supernatant after incubation with *C. difficile*, while no significant differences were observed after incubation with the other four probiotics. Additionally, Acd-BD exhibited weak binding activity towards all the tested bacteria (Fig. 2b). To further investigate whether this binding property of Cwp19-BD is related to *C. difficile* growth state, *C. difficile* cells at early stationary phase (24 h), late stationary phase (48 h), and decline phase (72 h) were also analyzed. As expected, Cwp19-BD could be bound to *C. difficile* 630Δ*ermB* cultured for 24 h, 48 h, and 72 h, indicating that Cwp19-BD has a good binding activity with *C. difficile* cells regardless of bacteria growth state (Fig. 2c). In addition, for a more intuitive understanding of the binding specificity of Cwp19-BD, a GFP-Cwp19-BD fusion protein was constructed. Furthermore, 13 selected bacteria were applied for testing the binding specificity of Cwp19-BD. Based on the intrinsic weak green fluorescence of *C. difficile*, the Δfluorescence intensity after removing the background from *C. difficile*

Fig. 1 Schematic diagrams of *C. difficile* autolysin structures and expression and purification of Cwp19-BD and Acd-BD. Schematic diagrams of the Cwp19, Acd24020, and Cwl0971 structures (a). Cwp19 of *C. difficile* 630Δ*ermB* has a signal peptide (SP) sequence at the N terminus, an endopeptidase catalytic domain of the GHL10 family, and three CWB2 domains at the C terminus. Both Acd24020 and Cwl0971 have a signal peptide (SP) sequence at the N terminus, three SH3b domains, and an endopeptidase catalytic domain of the NlpC/P60 family at the C terminus. Expression of Cwp19-BD (b) and Acd-BD (c) was analyzed by 12% SDS-PAGE, where positive bands are marked in a red box. The purified Cwp19-BD and Acd-BD were verified by SDS-PAGE (d); Cwp19-BD (352 amino acids, 37.9 kDa) and Acd-BD (259 amino acids, 27.3 kDa) were shown



was recorded. As depicted in Fig. 3, GFP-Cwp19-BD protein showed good binding specificity with *C. difficile* as the high level of Δfluorescence intensity (nearly 8000) was detected. However, the Δfluorescence intensity values of other tested bacteria were all below 1000, indicating that Cwp19-BD indeed had high binding specificity towards *C. difficile*.

Expression and characterization of lytic domains

The genes ID Cwp19, CD24020, and Cwl0971 in the *C. difficile* 630Δ*ermB* genome were identified as a putative autolysin by sequence similarity searching. Cwl0971 has a signal peptide sequence at the N terminus, three SH3b domains, and an endopeptidase catalytic domain of the NlpC/P60 family at the C terminus (Fig. 1a). For comparative analysis of the lytic activity of these autolysins, the catalytic domains of Cwp19, CD24020, and Cwl0971 were named Cwp19-CD, Acd-CD, and Cwl-CD, respectively. Besides, a SUMO tag was fused at the N terminal of Acd-CD and Cwl-CD to obtain the soluble protein expression. As

shown in Fig. 4a, b, and c, all the proteins were successfully expressed as soluble fractions. Interestingly, target proteins were also expressed in *E. coli* Rosetta cells without IPTG induction; it implied that the leaky expressions existed in these recombinant bacteria. As reported by previous studies, this leaky expression was caused by the low level of cellular β-galactosidase which competitively combined with repressor protein to open the lac operon (Gatti-Lafranconi et al. 2013; Shariati et al. 2021). After purification by one-step Ni-NTA affinity chromatography, high purities of Cwp19-CD (80.2%), sumo-Acd-CD (93.8%), and sumo-Cwl-CD (70.6%) were obtained (Fig. 4d). MIC measurements against various bacteria were performed to examine the species-specific lytic activities of these proteins. As shown in Table 1, sumo-Cwl-CD showed the strongest lytic activity ($16.49 \pm 7.14 \mu\text{g/mL}$) against *C. difficile* 630Δ*ermB* among these lytic proteins (Cwp19-CD was $34.91 \pm 12.09 \mu\text{g/mL}$, sumo-Acd-CD was $22.27 \pm 7.71 \mu\text{g/mL}$, and lysosome was $4166.67 \pm 1804.22 \mu\text{g/mL}$, respectively). On the species-specific assessment of these lytic proteins, Cwp19-CD and

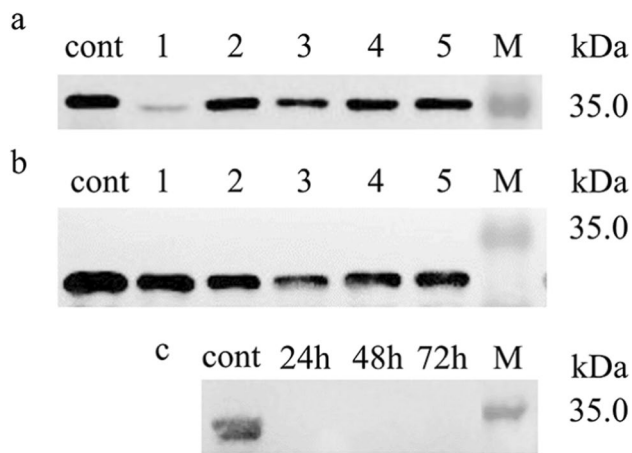


Fig. 2 Species-specific binding and binding activity of proteins were detected by western blot. Species-specific assays were detected by western blot for Cwp19-BD (a) and Acd-BD (b) with different strains. cont: negative control; 1: *C. difficile* 630ΔermB; 2: *Lactococcus lactis* subsp ATCC 19,435; 3: *Lactobacillus acidophilus* ATCC4356; 4: *Lactobacillus rhamnosus* ATCC53103; 5: *Bifidobacterium breve* ATCC15700. Cultured time of the best binding activity was also detected by western blot for Cwp19-BD (c) with *C. difficile* 630ΔermB; cont: negative control

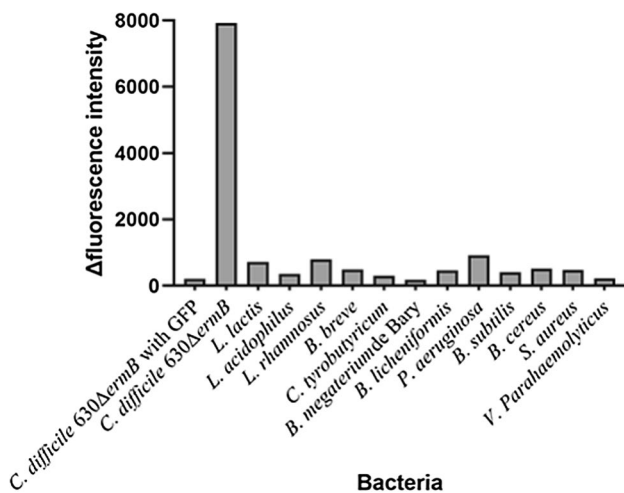


Fig. 3 The binding specificity of GFP-Cwp19-BD was detected by fluorescence analysis. Thirteen kinds of Gram-positive and Gram-negative bacteria were applied in this assay. *C. difficile* 630 with GFP protein served as control

sumo-Cwl-CD exhibited lytic activity towards four bacteria cells, which were more specific than lysosome (with lytic activity against almost all tested bacteria except *V. parahaemolyticus* and *P. aeruginosa*) and sumo-Acd-CD (with lytic activity towards 8 bacteria). Based on these results, sumo-Cwl-CD exhibited the lowest MIC to *C. difficile* and high MIC to other bacteria, indicating that the lytic activity of sumo-Cwl-CD is more specific to *C. difficile*.

Design, expression, and purification of novel lytic protein

Based on the above optimized results, Cwp19 binding domain (Cwp19-BD) and Cwl0971 lytic domain (Cwl-CD) were eventually selected to construct the novel lytic protein, named as Cwl-CWB2. In the design of Cwl-CWB2, Cwl-CD was placed at the N terminal and Cwp19-BD was set at the C terminal, and a GGSG linker was inserted between them (Fig. 5a). To obtain a highly soluble expression of Cwl-CWB2, a trigger factor (TF) in pColdTF vector was fused to the N terminal of Cwl-CWB2. Besides, an enterokinase restriction site was inserted between TF and Cwl-CWB2 to acquire the tag-less Cwl-CWB2 protein; the recombinant plasmid is illustrated in Fig. 5b. SDS-PAGE analysis result (Fig. 5c) suggested that the TF-Cwl-CWB2 protein was successfully expressed in soluble form in *E. coli* Rosetta cell. TF-Cwl-CWB2 was initially purified by Ni-NTA affinity chromatography and then re-purified after digesting by the Recombinant Bovine Enterokinase Light Chain to remove the TF (Fig. 5d). Eventually, high purity (88.4%) of Cwl-CWB2 was obtained and the concentration of purified Cwl-CWB2 was estimated to be 321.43 μg/mL (Fig. 5e).

Hemolytic activity and cytotoxicity of Cwl-CWB2

The high hemolysis and cytotoxicity of antimicrobial agents are obstacles for clinical application. It is important for further application of this fusion protein. As shown in Fig. 6a, Cwl-CWB2 had negligible hemolytic activity at concentrations ranging from 5.02 to 321.43 μg/mL when compared with 0.1% Triton X-100 treatment (positive control, 100% hemolysis). The results of Fig. 6b showed that the cell viability of NCM460 cells was almost unaffected when incubated with increasing concentrations (5.02 ~ 321.43 μg/mL) of Cwl-CWB2 when compared with the PBS treatment group. These results demonstrated that the designed novel lytic protein Cwl-CWB2 had good biosafety and has great potential for clinical application.

Lytic activity and stability analysis of Cwl-CWB2

MIC method was applied for investigating the binding specificity and lytic activity of Cwl-CWB2. As shown in Table 2, compared with commercial lysozyme which has lytic activity against almost all of the tested bacteria except *V. parahaemolyticus* and *P. aeruginosa*, Cwl-CWB2 showed high specificity towards *C. difficile* with a MIC value of 13.39 ± 5.80 μg/mL. In addition, we observed that Cwl-CWB2 also exhibited lytic activity against *Bifidobacterium breve*; however, the high MIC (267.87 ± 92.79 μg/mL) value indicated the weak lytic activity against *Bifidobacterium breve* when compared with *C. difficile*. To better

Fig. 4 Expression and purification of Cwp19-CD, sumo-Acd-CD, and sumo-Cwl-CD. Expression of Cwp19-CD (a), sumo-Acd-CD (b), and sumo-Cwl-CD (c) was analyzed by 12% SDS-PAGE, where positive bands are marked in a red box. SDS-PAGE analysis of purified proteins (d); Cwp19-CD (395 amino acids, 44.6 kDa), sumo-Acd-CD (215 amino acids, 23.9 kDa), and sumo-Cwl-CD (251 amino acid, 27.7 kDa) were shown

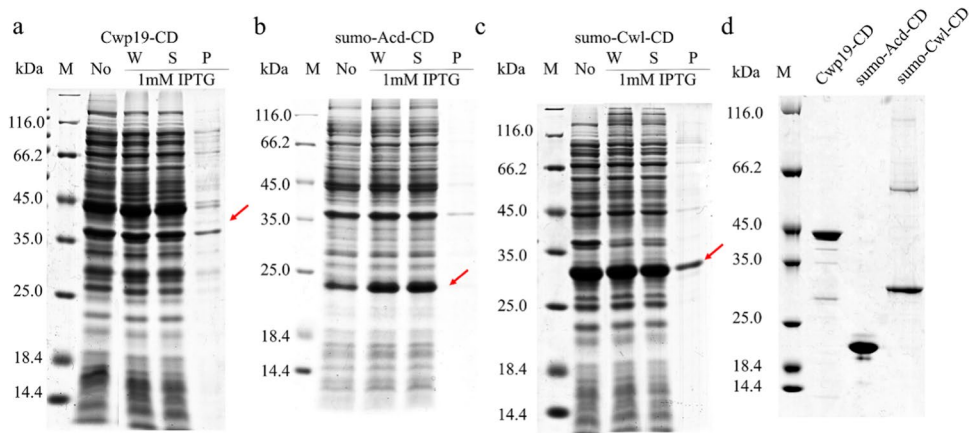


Table 1 Lytic activities of lysozyme, Cwp19-CD, sumo-Acd-CD, and sumo-Cwl-CD

Bacteria	Lysozyme (µg/mL)	sumo-Acd-CD (µg/mL)	Cwp19-CD (µg/mL)	sumo-Cwl-CD (µg/mL)
<i>Clostridium difficile</i> 630ΔermB	4166.67 ± 1804.22	22.27 ± 7.71	34.91 ± 12.09	16.49 ± 7.14
<i>Lactococcus lactis</i>	5208.33 ± 1804.22	712.50 ± 246.82	\	\
<i>Lactobacillus acidophilus</i>	130.21 ± 56.38	\	55.86 ± 24.19	82.49 ± 28.57
<i>Lactobacillus rhamnosus</i>	10,461.67 ± 3680.44	712.50 ± 246.82	\	\
<i>Bifidobacterium breve</i>	9375.00 ± 5412.66	570.00 ± 246.82	558.63 ± 193.52	\
<i>Bacillus megaterium</i> de Bary	3385.42 ± 2743.66	71.25 ± 30.85	\	\
<i>Vibrio parahaemolyticus</i>	\	\	\	\
<i>Pseudomonas aeruginosa</i>	\	\	\	65.99 ± 28.57
<i>Staphylococcus aureus</i>	1041.67 ± 451.05	\	\	\
<i>Bacillus subtilis</i>	113.93 ± 74.59	44.53 ± 15.43	195.52 ± 128.00	20.62 ± 7.14
<i>Bacillus licheniformis</i>	7291.67 ± 4773.52	17.81 ± 7.71	\	\

\: No significant MIC value could be observed

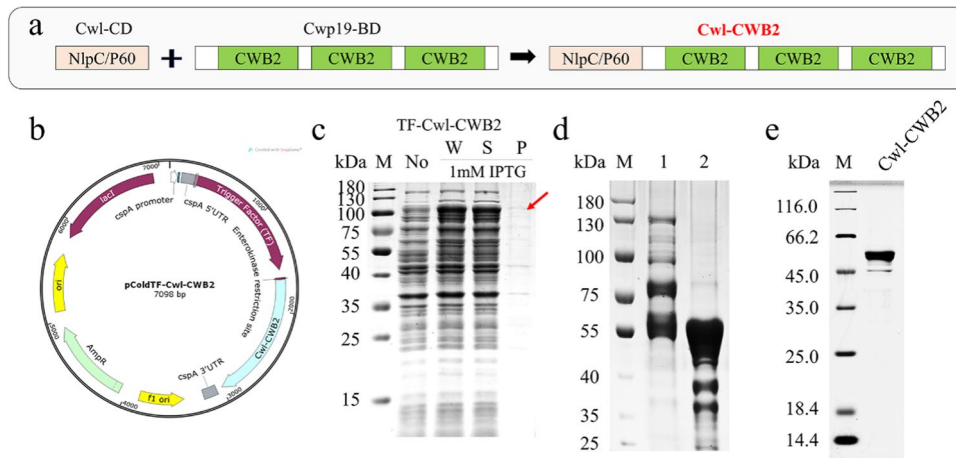


Fig. 5 Schematic diagrams of the fusion protein Cwl-CWB2 (a). Cwl-CWB2 contains an endopeptidase catalytic domain of the NlpC/P60 family from Cwl0971 at the N terminus, and three CWB2 domains from Cwp19 at the C terminus. Construction of pColdTF-Cwl-CWB2 plasmid (b) and expression of Cwl-CWB2 (c) were analyzed by 12% SDS-PAGE, where positive bands are marked in a red

box. Digestion and purification of TF-Cwl-CWB2 were analyzed by 10% SDS-PAGE (d). Band 1: purification of TF-Cwl-CWB2; band 2: protein mixture after digested by Recombinant Bovine Enterokinase; SDS-PAGE analysis of the purified Cwl-CWB2 protein (437 amino acid, 46.8 kDa) is shown (e)

understand the lytic activity of Cwl-CWB2, living *C. difficile* cells were also applied. As depicted in Fig. 7a, Cwl-CWB2 showed high lytic activity (88.3%) against *C. difficile* 630 Δ ermB compared with Triton X-100 (a positive control) in the concentration of 321.43 μ g/mL. As predicted, the lytic activity of Cwl-CWB2 showed as a dose-dependent

manner in the tested concentrations. Besides, to examine the temperature stability for Cwl-CWB2, various temperatures (-80 $^{\circ}$ C, -20 $^{\circ}$ C, 4 $^{\circ}$ C, 25 $^{\circ}$ C, and 37 $^{\circ}$ C) and incubation time (24 h and 96 h) were carried out on Cwl-CWB2. As can be seen in Fig. 7b, Cwl-CWB2 placed in -80 $^{\circ}$ C showed 89.7% activity compared with the Triton X-100 treatment,

Fig. 6 Hemolytic activity and cytotoxicity of Cwl-CWB2. **a** Hemolytic activity in mouse red blood cells. 0.1% Triton X-100 was used as the positive control (PC). **b** Cytotoxicity in NCM460 cells. PBS was used as negative control

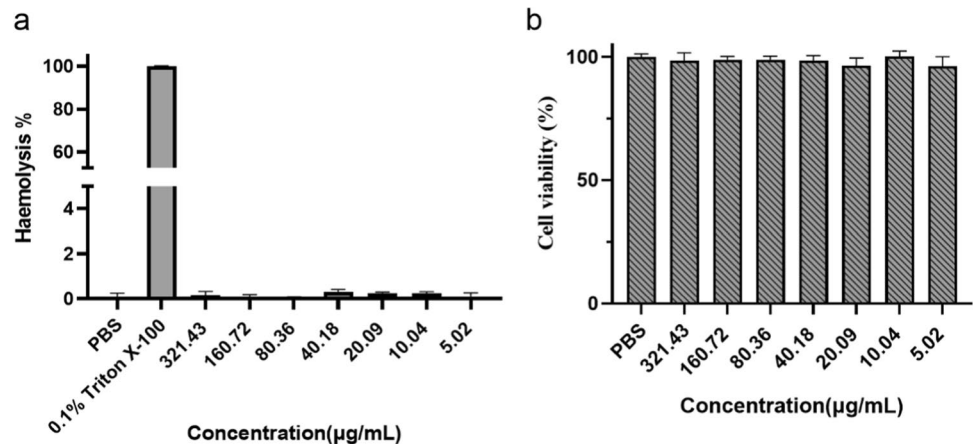
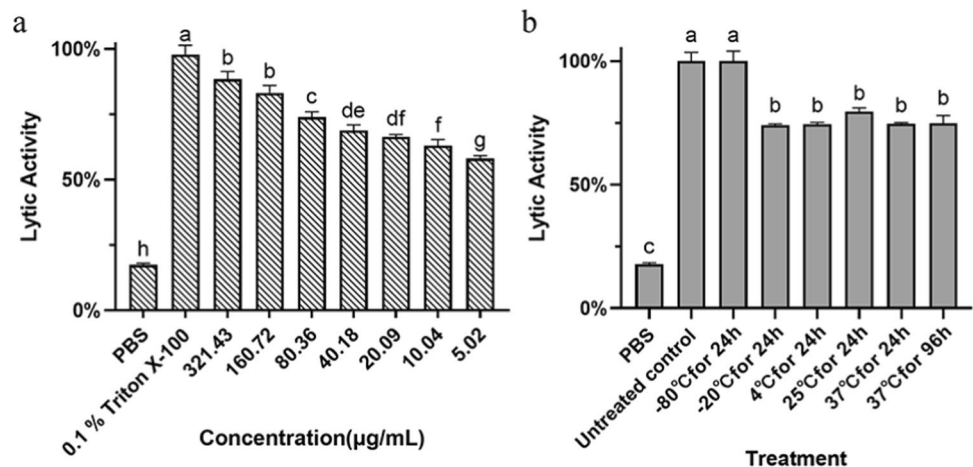


Table 2 Species specificity and lytic activities of lysozyme and Cwl-CWB2

Bacteria	Lysozyme (μ g/mL)	Cwl-CWB2 (μ g/mL)
<i>Clostridium difficile</i> 630 Δ ermB	3333.33 \pm 1443.37	13.39 \pm 5.80
<i>Lactococcus lactis</i>	6666.67 \pm 2886.75	\
<i>Lactobacillus acidophilus</i>	104.17 \pm 45.11	\
<i>Lactobacillus rhamnosus</i>	16,666.67 \pm 5773.50	\
<i>Bifidobacterium breve</i>	16,666.67 \pm 5773.50	267.87 \pm 92.79
<i>Bacillus megaterium</i> de Bary	4166.67 \pm 1443.38	\
<i>Vibrio parahaemolyticus</i>	\	\
<i>Pseudomonas aeruginosa</i>	\	\
<i>Staphylococcus aureus</i>	3333.33 \pm 1443.37	\
<i>Bacillus subtilis</i>	22.79 \pm 14.91	\
<i>Bacillus licheniformis</i>	5833.33 \pm 3818.81	\

\: No significant MIC value could be observed

Fig. 7 Lytic activity of protein Cwl-CWB2. **a** Lytic activity with different concentrations on *C. difficile*. **b** Lytic activity of Cwl-CWB2 with different temperature treatment on *C. difficile*. The significance was calculated by the Kruskal–Wallis one-way ANOVA with Tukey's test for multiple comparisons, $P \leq 0.05$



and approximately 75% of the lytic activity remained even at 37 °C for 96 h. It showed that this fusion protein has good stability in conventional methods for protein storage.

Discussion

Several aspects of CDI pathology suggest that autolysin may be a particularly promising alternative to antibiotics. Firstly, the high degree of host specificity and the ability to disrupt cell wall endow autolysin with great potential as antibacterial agent to combat *C. difficile* (Haddad Kashani et al. 2018). Secondly, maintaining a healthy host microbiome is of particular concern in the successful treatment of CDI, as most CDI is caused by an imbalance of the gut microbiome mediated by antibiotics treatment (Theriot and Young 2015). Autolysin generally exhibits a high degree of specificity for target bacteria (Wang et al. 2015), suggesting that *C. difficile* autolysins might help reduce collateral damage to healthy microorganisms and enhance protection against recurrent infection. Therefore, *C. difficile* autolysin implies great potential in fighting against *C. difficile* and the treatment of CDI.

However, on the currently discovered *C. difficile* autolysins there exist several undesirable drawbacks associated with binding specificities and lytic activities. For example, the autolysin CD27L exhibited low binding specificity towards *C. difficile* since it could also bind with *Bacillus amyloliquefaciens*, *Bacillus cereus*, and *Listeria ivanovii* (Mayer et al. 2011); the enzymes Sle1, LytN, and Atl of *S. aureus* are all specifically localized to the transmembrane septum of dividing cells in subcellular localization, thus severely limiting the exposure time of cell wall to autolysis and inhibiting the lysis potential of autolysin (Frankel and Schneewind 2012; Gotz et al. 2014; Lee et al. 2020; Nega et al. 2020). To address this limitation, we designed combinatory protein with autolysin CWB2 targeting regions and the autolysin peptidoglycan hydrolase regions to form novel lytic protein, the former has been shown to bind cell membrane proteins of *C. difficile* (Biazzo et al. 2013; Ferreira et al. 2017). An earlier validation study by using the LytM autolysin in *S. aureus* fusion with the peptidoglycan binding domain of lysostaphin demonstrated this approach is feasible (Osipovitch et al. 2015), and here it was extended to develop *C. difficile* autolysin candidates.

Therefore, we initially analyzed and identified novel *C. difficile* autolysins bearing cell wall-binding domain and peptidoglycan hydrolytic domain, and then, the three characterized autolysin candidates Cwp19, Acd24020, and Cwl0971 were obtained. By characterization of the binding activity of the three autolysins, Cwp19-BD exhibited high binding specificity to *C. difficile* cells while Acd-BD showed weak binding activity among all tested bacteria. Besides,

Cwp19-BD candidate has binding activity towards live *C. difficile* cells compared with Acd-BD, where it was recently reported that Acd-BD could bind with the heat-inactivated cells (Sekiya et al. 2021). We speculate that this might be due to Cwp19-CD could bind externally to the cell wall peptidoglycan while Acd-CD could only bind internally to the cell wall peptidoglycan when the cell wall permeability was altered. As the great potential of Cwp19-BD on *C. difficile* clinically, we selected Cwp19-BD as the binding domain of the designed novel lytic protein. In the determination of lytic activities of Cwp19-CD, Acd-CD, and Cwl-CD, all proteins exhibited strong lytic activities (34.91 ± 12.09 µg/mL, 22.27 ± 7.71 µg/mL, and 16.49 ± 7.14 µg/mL, respectively) against *C. difficile* compared with commercial lysozyme (4166.67 ± 1804.22 µg/mL). In addition, Cwp19-CD and Cwl-CD showed higher binding specificity than Acd-CD, and Cwl-CD was eventually selected as the lytic domain for novel lytic protein design as it has stronger lytic activity than the other two proteins.

The designed novel lytic protein Cwl-CWB2 has stronger lytic activity and higher binding specificity towards *C. difficile* with a lytic MIC of 13.39 ± 5.80 µg/mL. As the high hemolysis and cytotoxicity of antibacterial agents are critical factors that considerably hinder their application, we initially investigated the hemolytic activity and cytotoxicity of Cwl-CWB2. Fortunately, the negligible hemolytic activity and no cytotoxicity of Cwl-CWB2 implied the great potential of Cwl-CWB2 as an antibacterial agent for the clinical application to combat *C. difficile*. Besides, Cwl-CWB2 exhibited good stability under different incubation temperatures. Thus, we have shown here that fusion of highly functional cell wall-binding domains with high lytic activity autolysin catalytic domains is an effective strategy for enhancing their antibacterial activity. In addition, we expect that this strategy will apply to other bacterial pathogens as almost all bacterial genomes encode multiple autolysins, and it was believed that this strategy will yield a large number of candidates for CDI treatment, and more specific effective antibacterial agents will be investigated in the future.

To sum all, our data suggested that Cwl-CWB2 has a strong lytic activity and high species specificity against *C. difficile*. The low concentration of MIC to *C. difficile* indicates that Cwl-CWB2 could be a novel potential candidate antimicrobial agent for CDI treatment.

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Author contribution JW conceived and designed the research. MW and ZD conducted the experiments. YL contributed to the analysis

of data. YM prepared the materials. MW wrote the manuscript. JW modified the manuscript. All the authors read and approved the submitted version.

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Data availability The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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