Master's Thesis of Science
in Agricultural Biotechnology

Isolation and characterization of bacteriophages and endolysins targeting *Clostridium perfringens*

클로스트리디움 퍼프린젠스를 감염하는 박테리오파지와
이로부터 분리된 엔도라이신의 특성 분석

February, 2019

The Graduate School

Seoul National University

Department of Agricultural Biotechnology

Daeun Shin

Isolation and characterization of bacteriophages and endolysins targeting *Clostridium perfringens*

Advisor: Sangryeol Ryu

Submitting a Master's Thesis of Science in Agricultural Biotechnology

February, 2019

The Graduate School
Seoul National University
Department of Agricultural Biotechnology

Daeun Shin

Confirming the master's thesis written by Daeun Shin
February, 2019

Chair	Pahn-Shick Chang (seal)
Vice Chair	Sangryeol Ryu (seal)
Examiner	Ki Won Lee (seal)

ABSTRACT

Clostridium perfringens is responsible for a variety of diseases in humans and animals. Since incidence of *C. perfringens* in the poultry industry has been increasing and the prevalence of antibiotic-resistant bacteria has also increased, it has been required to develop alternatives to typical antimicrobial treatments. Bacteriophages are bacterial viruses and endolysins are phage-encoded peptidoglycan hydrolases, and they both have received considerable attention as promising antibacterial agents. In this study, I newly isolated and characterized seven bacteriophages showing lytic activity against *C. perfringens*. Among these phages, CPD2 had the remarkable thermal stability and CPD7 had inhibition activity against *C. perfringens* FORC 25, that carries chromosomal *cpe* gene considered to be the virulence factor responsible for causing the several common gastrointestinal diseases. Thus, they were selected for further study. Bioinformatic analysis of CPD2 and CPD7 genome revealed a putative endolysins, LysCPD2 and LysCPD7, which had homology with *N*-acetylmuramoyl-L-alanine amidase. The antimicrobial spectrum was relatively broad since LysCPD2 and LysCPD7 could infect not only *C. perfringens* strains but also other Gram-positive bacteria such as *B. cereus* and *B. subtilis* strains. Also, as expected, LysCPD2 retained about 80% of the lytic activity up to 95°C, indicating remarkable thermal stability and LysCPD7

exhibited considerable antimicrobial activity against *C. perfringens* FORC 25 than LysCPD2. Hence, LysCPD2 was assumed to maintain its activity at the heat-shock condition (75°C for 20 min) for *C. perfringens* spore germination and it showed significant antibacterial ability against heat- activated germinating spores. Moreover, the bactericidal activity of LysCPD7 against *C. perfringens* FORC 25 was determined in food such as milk and beef broth. The data presented here suggest that these phages and endolysins can be used as an alternative biocontrol agent for *C. perfringens*.

Keywords: *Clostridium perfringens*, bacteriophage, endolysin

Student Number: 2017-26630

CONTENTS

List of Figure

List of Table

Ⅰ. INTRODUCTION

Clostridium perfringens is a Gram-positive, spore-forming, non-motile, and rod-shaped bacterium that grows well in anaerobic environments (Granum 1990). *C. perfringens* can cause human gas gangrene, foodborne diseases, and non-foodborne gastrointestinal diseases (Myers, Rasko et al. 2006). Usually, *C. perfringens* causes the diseases by producing various types of toxins (Songer 1996). About 5% of all *C. perfringens* strains produce *C. perfringens* enterotoxin (CPE), which is an important sporulation-associated virulence factor causing diarrhea and abdominal cramping symptoms (Kokai-Kun, Songer et al. 1994, Freedman, Shrestha et al. 2016). In addition, *C. perfringens* is also the most important cause of many animal diseases such as enterotoxaemia and necrotic enteritis in bird (Brynestad and Granum 2002). Economic losses by increased mortality and contamination of poultry products are significant concerns regarding *C. perfringens.* Furthermore, the incidence of *C. perfringens* in the poultry industry has increased in many countries that stopped using antibiotic growth promoters due to the risk of spreading of antibiotic resistance (Immerseel, Buck et al. 2004). The increasing emergence of antibiotic-resistant bacteria has become a serious worldwide problem and alternative methods for the control of bacterial diseases need to be investigated (Harbarth and Samore 2005, Norrby, Nord et al. 2005)

Recently, bacteriophages and their gene products such as endolysins have been reconsidered as an alternative to antibiotics. Bacteriophages (phages) are viruses that recognize and target bacterial cells in order to replicate (Bragg, van der Westhuizen et al. 2014). The main advantages of phages are the host specificity, which reduces the impact on commensal bacteria other than the host, and amplification at the site on infection (Deresinski 2009). Endolysins, also termed phage lysins, are phage-encoded peptidoglycan hydrolases employed by phages to degrade the peptidoglycan layer of the host bacterium from within, resulting in cell lysis and release of progeny virions (Schmelcher, Donovan et al. 2012). In Gram-positive bacteria, endolysins are expected to be effective bio-control agents resulting in immediate lysis of bacteria when the purified endolysin protein is added externally (Fischetti 2010). The important advantage of endolysins over classical antibiotics is that they have the high enzymatic activity such that bacterial cells lyse within minutes or even seconds and have ability to lyse antibiotic resistant cells or persister cells (Fischetti 2008, Gutierrez, Ruas-Madiedo et al. 2014). Also, they have high target specificity and low chance of developing bacterial resistance (Schuch, Nelson et al. 2002, Loessner 2005). Besides, many reports have already shown that bacteriophages and endolysins have high potentials as strong therapeutic agents against a number of

pathogens (Schuch, Nelson et al. 2002, Cheng, Nelson et al. 2005, Witzenrath, Schmeck et al. 2009, Gupta and Prasad 2011).

To date, there have been few reports on phages and endolysins that can infect *C. perfringens* due to the relative difficulties in isolating the phage from anaerobic *C. perfringens* (Ha, Son et al. 2018). In addition, although there are several phages and endolysins such as ΦCP34O, Φ24R, ΦCP39O, and ΦCP26F, examined for the lytic activity against *C. perfringens* strains, detailed information on these phages and their endolysins have not been reported (Oakley, Talundzic et al. 2011, Seal 2013). Thus, more bacteriophages and endolysins targeting *C. perfringens* should be isolated and characterized to provide additional candidates for *C. perfringens* biocontrol agents.

In this study, seven novel *C. perfringens* phages were isolated from environmental samples such as retail chicken and sewage. Through their characterization, the four phages were selected for genome sequencing. Among them, two endolysin genes were cloned and expressed in *Escherichia coli*, respectively, and the purified endolysins were biochemically characterized for its potential as an antimicrobial agent. I demonstrated that LysCPD7 shows strong lytic activity against *C. perfringens* in food and LysCPD2 also has lytic activity against heat-activated spores due to its thermal

stability. This is the first study to examine the possibility of using endolysin as a food antimicrobial agent for *C. perfringens* and an antibacterial agent for heat-activated spores.

Ⅱ. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions.

The bacterial strains used in this study are listed in Table 1. Thirty *Clostridium perfringens* strains were used for isolation of bacteriophages, and *C. perfringens* isolates 2589 and 2722 were utilized as hosts for propagation of bacteriophages. All *Clostridium* strains were cultured at 37°C in brain heart infusion (BHI) broth or on agar medium under anaerobic condition. *Bacillus* strains were grown in BHI broth at 37°C and *Staphylococcus* strains were grown in tryptic soy broth (TSB) or on agar at 37°C. All media used in this study were purchased from Difco. The strains named as isolate were all originated from Ulsan Institute of Health and Environment.

2.2. Isolation of bacteriophages.

Sewage samples were collected from Guri, Anyang and Jungnang Water Reclamation Center and retail chicken samples from markets in South Korea were used to screen bacteriophages using *Clostridium* spp. as host strains. Briefly, filtered 4-ml samples were mixed with 1-mL of 5x BHI broth and 200 μL of *Clostridium* strains overnight cultures. Also 5 mM of $MgCl_2$

and $CaCl_2$ were added and the mixture was incubated at 37°C for 24 h under anaerobic condition. After chloroform treatment and centrifugate (21,130 x g for 5 min) 1-mL of the culture, the supernatant was filtered using a 0.22-μm-pore-size filter (Millipore) to remove bacterial cells. Then, presence of bacteriophages was confirmed using a plaque-forming assay with molten 0.7% DW soft agar inoculated with *Clostridium* strains overnight cultures. After incubation at 37°C for 12 h, a single phage plaque was picked with a sterile pipette tip and eluted in 100 μL of SM buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl and 10 mM MgCl). These plaque isolation and elution steps were repeated at least three times to purify single phage.

2.3. High-titer stock preparation of bacteriophages.

Isolated bacteriophages were amplified by serial propagation and precipitated with 10% polyethylene glycol 6000 (Sigma) in the presence of 1 M NaCl at 4°C overnight. After centrifugation (15,000 x g for 15 min at 4°C), the precipitated bacteriophages were resuspended in SM buffer and purified by CsCl density gradient ultracentrifugation (78,500 x g for 2 h at 4°C). The concentrated bacteriophages were dialyzed using 2 L of standard dialysis buffer (10 mM NaCl, 10 mM $MgSO_4$ and 1 M Tris-HCl [pH 8.0]) for 1 h. The phage stock obtained was stored in glass vials at 4°C.

2.4. Transmission Electron Microscopy (TEM) Analysis.

Each purified bacteriophage samples (1 x 10^9 PFU/mL) in SM buffers were placed on carbon-coated copper grids and negatively stained with 2% aqueous uranyl acetate (pH 4.0) for 20 s. Bacteriophages were visualized by TEM (Leo 912AB transmission electron microscope; Carl Zeiss, Wezlar, Germany) at 80 kV. Images were scanned at the National Instrumentation Center for Environmental Management (Seoul, South Korea). Bacteriophages were classified into their relative family according to the guidelines of the International Committee on Taxonomy of Viruses based on the morphology of phages (Fauquet and Fargette 2005).

2.5. Bacterial-challenge test in liquid culture.

All tested bacteria were cultivated to the exponential phase in 50 mL BHI broth. The bacteriophages (50 μL) were added to 1-mL of cell resuspension (3 x 10^7 CFU/mL) with mineral oil (500 μL) to prevent oxygen transmission and incubated for another 18 h at 37°C. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) at various time points. As a negative control, one bacterial culture was inoculated with equal volume of SM buffer instead of bacteriophages.

2.6. DNA purification and whole genome sequencing of bacteriophages.

To extract genomic DNA from bacteriophages, host DNA of virion was removed by treatment with DNaseI and RNaseA (1 μg/mL each) at room temperature for 30 min. The virions were then lysed by reacting with proteinase K mixture (50 μg/mL proteinase K, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS) at 56°C for 1 h. After lysis, the DNA was purified by the phenol-chloroform extraction (Kirby 1956) and concentrated by ethanol precipitation (Zeugin and Hartley 1985). The purified genomic DNA of bacteriophages were sequenced using the GS-FLX Titanium sequencer (Roche Holding AG, Basel, Switzerland). Sequencing reads obtained were assembled using the GS De Novo Assembler version 2.9 (Roche Holding AG, Basel, Switzerland) with default parameters. The position of open reading frames (ORFs) was predicted by bioinformatics tools, including Glimmer 3.02 (Altschul, Gish et al. 1990) and Rapid Annotation using Subsystem Technology (RAST) software (Aziz, Bartels et al. 2008). The function of each ORF was predicted using NCBI BLASTP and InterProScan databases (Zeugin and Hartley 1985). Based on the information, each ORF's name was annotated manually. The gene encoding endolysin was identified, and its domain structure was investigated using InterProScan databases.

2.7. Endolysin production.

The endolysin gene from the CPD2 genome was PCR amplified with the following primers: GCGGGATCCATGAAAATAGGTATTAGAGACGG AC (forward) and GCGGTCGACTTAATTACATTCCTCCACAAAACAAT AC (reverse) (where underlined sequences represent restriction enzyme recognition sites). And the other endolysin gene from the CPD7 genome also was PCR amplified with the following primers: GCGGGATCCATGTATATA GAAAATATTTTAAAAACTAGAAA (forward) and GCGAAGCTTCTATAT TTTCTCGGTGAAACAAT AT (reverse). Each PCR product was cloned into pET28a (Novagen), which has an N-terminal hexa-histidine (His) tag sequence. Plasmid with a correct insert was transformed into competent *E. coli* BL21 (DE3) including pRARE plasmid to overcome the codon bias. To express the endolysins (LysCPD2 and LysCPD7), each clone was grown in LB broth containing 50 μg/mL kanamycin and 25 μg/mL carbenicillin to OD_{600} of 0.8 and induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Then, the induced cultures were shaken for 3 h at 30°C for LysCPD2 and 37°C for LysCPD7. The harvested cells were resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl) and disrupted by sonication (Branson Ultrasonics, Shanghai, China). After centrifugation at 21,330 x g for 40 min, the supernatant was collected, mixed with 1 mL of nickel-

nitrilotriacetic acid (Ni-NTA) super flow (Qiagen) and incubated at 4°C for 1 h with gentle shaking. The flow-through was discarded and the resin was serially washed with 10 mL of 10 mM imidazole and 5 mL of 20 mM imidazole. An elution buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl and 250 mM imidazole) was used to elute the proteins. The purified LysCPD2 and LysCPD7 were stored at 4°C until use after the buffer was changed to the storage buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl and 30% glycerol) using PD Miditrap G-25 (GE Healthcare, Amersham, Bucks, UK).

2.8. Lytic activity assay.

The lytic activity of the endolysin against bacterial cells was assayed by monitoring the decrease in OD_{600}. All tested bacteria were cultivated to the exponential phase with adjusted OD_{600} 0.8 and resuspended with the reaction buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl). The endolysin (50 μL) was added to the cell suspension (950 μL), followed by incubation at room temperature, unless indicated otherwise. The OD_{600} values were monitored over time. The lytic activity was calculated after 40 min as follows: [ΔOD_{600} tested (endolysin added)-ΔOD_{600} control (buffer only)]/initial OD_{600}. Antimicrobial spectrum was tested by plate lysis assay as previously described (Chang, Kim et al. 2017). Briefly, 20 μL of diluted endolysin in reaction buffer

was spotted onto a freshly prepared bacterial lawn on BHI agar plates. Spotted plates were air-dried in a laminar flow hood for 15 min and incubated overnight at 37°C. To evaluate the effect of pH on LysCPD2 and LysCPD7 enzymatic activity, the endolysin (250 nM) was added to *C. perfringens* ATCC 13124 cells suspended with a universal pH buffer (Walmagh, Boczkowska et al. 2013). The universal buffer consists of 50 mM KCl, 10 mM KH_2PO_4, 10 mM Na-citrate and 10 mM H_3BO_4, and was adjusted to different pH values—between 3 and 10—using 5 M NaOH or 5 M HCl. The influence of NaCl on lytic activity of LysCPD2 and LysCPD7 (250 nM) was tested with the addition of concentrations of 0, 100, 200, 300, 400, 500 and 1000 mM NaCl. Different temperatures (4–65°C) were applied to test the effect of temperature on LysCPD2 and LysCPD7 (250 nM) enzymatic activity. To evaluate the stability of the endolysins, the lysis assays were performed against *C. perfringens* ATCC 13124 at room temperature after the enzymes were incubated for 10 min and 30 min at different temperatures.

2.9. Antimicrobial activity of LysCPD7 in food samples.

The lytic activity of LysCPD7 against *C. perfringens* FORC 25 strain was tested in commercial sterilized milk and beef broth purchased from a local market (Seoul, Korea). Each sample (10 mL) of fresh milk and beef broth was

inoculated with 100 μL of *C. perfringens* FORC 25 cells (10^6 CFU/mL) at exponential growth phase. Subsequently, LysCPD7 was added at 0, 125, 250, and 500 nM, and each sample was incubated at 25°C. After then, viable bacterial cells (CFU/mL) were counted every 1 hr by plating each sample on a BHI agar plate and incubating at 37°C overnight under anaerobic condition.

2.10. Amidase assay.

N-acetylmuramoyl-L-alanine amidase activity was measured by quantification of endolysin-mediated release of acetaldehyde from peptidoglycan. *C. perfringens* peptidoglycan was prepared as follows. *C. perfringens* FORC 25 cells harvested from a stationary-phase culture were suspended in 1 mL of PBS and disrupted by sonication. After low-speed centrifugation (1400 x g, 10min) to remove unbroken cells, the resultant supernatant was centrifuged again at high speed (21,330 x g, 10 min, 4°C). The crude cell wall pellet was resuspended in 7 mL SM buffer per grams and boiled for 10 min in 4% SDS solution (wt/vol). After three washes with distilled water, the peptidoglycan fraction was resuspended in endolysin reaction buffer. Peptidoglycan solution and 1 μM of LysCPD2 and LysCPD7 solution were used as negative controls; reaction buffer alone was used as the reference. For the experimental group, 200 μL of peptidoglycan solution (5

mg/mL) containing 1 μM of LysCPD2 and LysCPD7 was prepared. All reactions were incubated at RT for 1 h, after which 1.0 M NaOH was added to each reaction to stop the reaction. After 30 min incubation at 38°C, 500 μL of 0.5 M H_2SO_4 and 5 mL of concentrated sulfuric acid were added sequentially to each reaction. The reaction tubes were placed in boiling water for 5 min. After cooling on ice for more than 20 min, 4% $CuSO_4 \cdot 5H_2O$ and 1.5% ρ-hydroxydiphenyl solutions were added, and the mixtures were incubated for 30 min at 30°C (Hadžija 1974, Hazenberg 1992). Then the activity of *N*-acetylmuramoyl-L-alanine amidase was colorimetrically assessed by measuring the OD_{560}.

2.11. Spore preparation and purification

C. perfringens FORC 25 was grown overnight at 37°C in 10 mL of fluid thioglycolate medium (FTG; Oxoid). Subsequently, 1 mL of cell culture was inoculated in 50 mL of modified Duncan and Strong medium (m-DS medium; HiMedia) with 0.05 mM caffeine and incubated overnight at 37°C (Jong and Beumer et al. 2002). After inducing sporulation, spores were harvested by centrifugation (10,000 x g, 10 min) and suspended in 3 mL of PBS. The suspended spores were centrifuged (10,000 x g, 30min) on 10 mL of 10% Histodenz (Sigma-Aldrich) and then the pellet was resuspended in 1 mL of PBS. The purified spores were stored at 4°C until use.

2.12. Nucleotide sequence accession number

The nucleotide sequences of bacteriophage CPD1, CPD2, CPD4, and CPD7 were deposited to GenBank under the accession number MH999280, MH999279, MK017819 and MK017820, respectively.

2.13. Statistical analysis

GraphPad Prism 5 was used for all statistical analyses. All experiments were done in triplicate or more. Data are means ± standard error of the mean (SEM) from independent experiments. One-way analysis of variance (ANOVA) and then Tukey's Multiple Comparison test were used for all experiments.

Ⅲ. RESULTS

3.1. Isolation and host range of bacteriophages.

Three phages, CPD1, CPD2, and CPD4, were isolated from chicken samples and four phages, CPD3, CPD6, CPD7, and CPD8, were isolated from sewage samples. Three phages, CPD3, CPD6, and CPD7, were amplified using *C. perfringens* isolate 2589 and *C. perfringens* isolate 2722 was used for the others. Since each phage has its own host range, an essential factor for developing biocontrol agents, I performed a plaque assay with a variety of bacterial species to check the host range. As shown in Table 1, most phages have narrow host range and in particular CPD4 and CPD7 can infect *C. perfringens* FORC 25 that carries *cpe* gene, the major virulence factor of *C. perfringens,* on its chromosome.

TABLE 1. Host range of bacteriophages.

Bacterial strain[a]	Plaques[b]						
	CPD1	**CPD2**	**CPD3**	**CPD4**	**CPD6**	**CPD7**	**CPD8**
***Clostridium perfringens* strains**							
C. perfringens H3	-	-	-	I	-	-	-
C. perfringens H9	-	-	-	I	-	-	-
C. perfringens FD1	-	-	-	-	-	-	-
C. perfringens ATCC 3624	I	I	I	I	I	I	I
C. perfringens ATCC 13124	I	I	+	+++	++	-	+
C. perfringens FORC 25	-	-	I	++	I	+	-
Isolate							
human stool isolate 2582	I	-	-	I	-	-	-
human stool isolate 2585	-	-	-	+	-	-	-
human stool isolate 2589	+++	+++	++	+++	++	+++	+++
human stool isolate 2722	-	I	+++	++	+++	+++	I
Other Gram-positive bacteria							
Listeria monocytogenes EGDe	-	-	-	-	-	-	-
Staphylococcus aureus RN4220	-	-	-	-	-	-	-
Staphylococcus aureus Newman	-	-	-	-	-	-	-
Bacillus cereus ATCC 10987	-	-	-	-	-	-	-
Bacillus cereus ATCC 13061	-	-	-	-	-	-	-
Bacillus subtilis ATCC 23857	-	-	-	-	-	-	-
Clostridium histolyticum ATCC 19401	-	-	-	-	-	-	-
Clostridium indolis ATCC 25771	-	-	-	-	-	-	-

[a] ATCC, American Type Culture Collection; FORC, Food-borne pathogen Omics Research Center

[b] +++, EOP range 1-0.01; ++, EOP range 0.01-0.0001; +, EOP range 0.0001-0.000001; I, bacterial growth inhibition zone; -, no plaque

3.2. Morphology of the phages and challenge assay.

Morphological observation of phages revealed that five phages, CPD2, CPD3, CPD6, CPD7 and CPD8, belong to the family *Podoviridae* due to the presence of short and non-contractile tails (Nelson, Schuch et al. 2003) and two phages, CPD1 and CPD4, belong to the family *Siphoviridae* due to the presence of long and non-contractile tail (Maniloff and Ackermann 1998) (Fig. 1).

The bacterial challenge assay was performed to evaluate the growth inhibition ability of phages. In order to compare the characteristic of the seven phages roughly, strains that can be commonly infected by most phages was selected. Four phages were shown the growth inhibition of *C. perfringens* isolate 2589 and three phages were shown the growth inhibition of *C. perfringens* isolate 2722. All phages inhibited host bacterial growth within 1 h to 7 h (Fig. 2), and generally, grown inhibition started faster slightly at MOI (multiplicity of infection) 1.0 than at MOI 0.1. Additionally, CPD4 and CPD7 showed the growth inhibition of *C. perfringens* FORC 25 only at MOI 1.0, and little inhibition at MOI 0.1. Although CPD7 lysed the host bacteria rapidly, the regrowth of host occurred immediately.

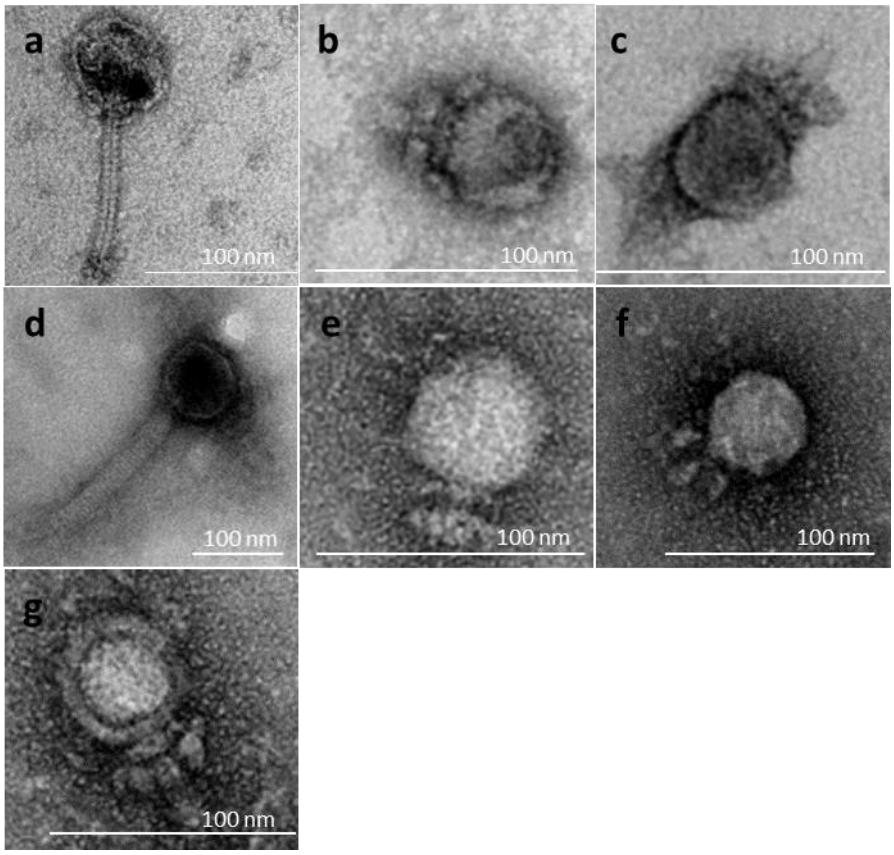
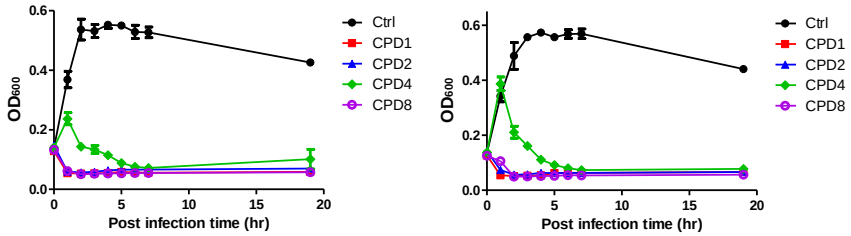
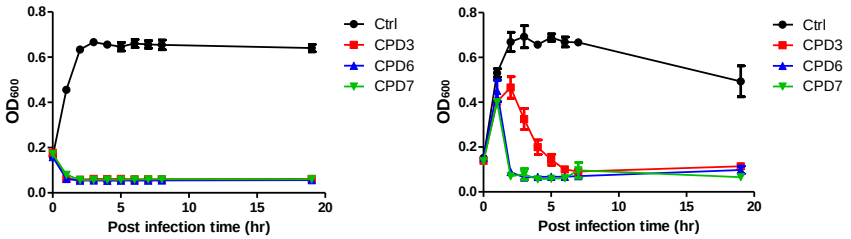


Figure 1. TEM analysis of phages. Transmission electron microscope of panels **a** to **g**, phage CPD1, CPD2, CPD3, CPD4, CPD6, CPD7 and CPD8, respectively. They had head with a diameter of 60, 43, 39, 84, 48, 43 and 49 nm, respectively.

- *C. perfringens* isolate 2589



- *C. perfringens* isolate 2722



- *C. perfringens* FORC 25

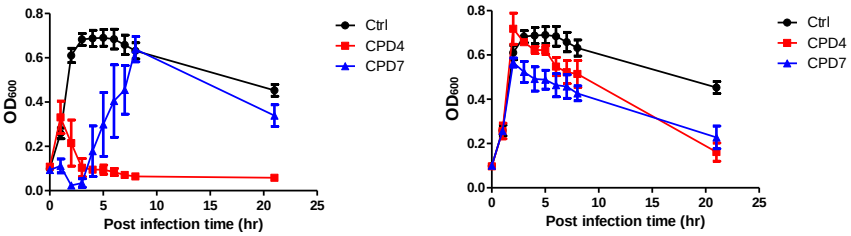


Figure 2. Bacterial challenge assay of *C. perfringens*-infecting phages. When optical density at 600 nm reached 0.3 (3 x 10^7 CFU/mL), phage lysates were added to the cell culture at a multiplicity of infection (MOI) of 1.0 (left) and 0.1 (right).

3.3. Thermal stability of the phages.

To evaluate the thermal stability of phages, each of phage suspensions (10^6 PFU/ml) were incubated at each temperature (4, 25, 37, 45, 50, 55, 60, 65, 70, 75 and 80°C) for 30 min. Then surviving phages were diluted and enumerated by double-layer plaque assay. The thermal stability tests suggest that all the phages were relatively heat stable at temperatures between 4°C to 60°C and CPD2 was significantly heat stable as it retained the phage titer up to 75°C (Fig. 3).

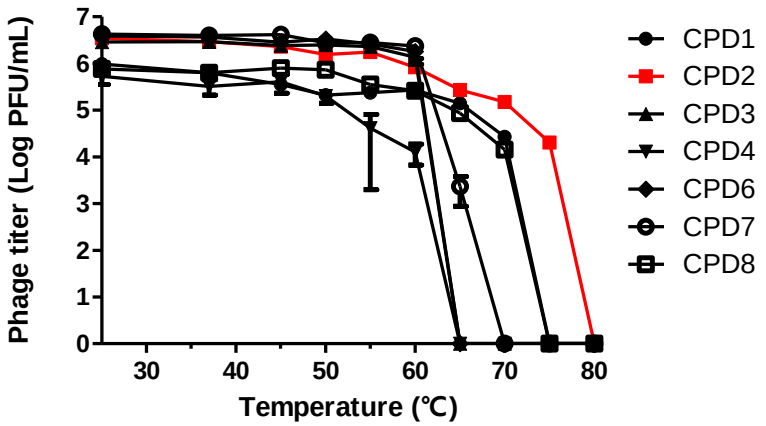


Figure 3. The thermal stability of phages. It showed changes in phages infectivity following the heating-holding-cooling trial in SM buffer at each temperature.

3.4. Genome analysis of the phages.

Based on the characteristics of phages four phages, CPD1, CPD2, CPD4 and CPD7, were selected and their whole genomes were sequenced. CPD1 and CPD2 showed remarkable thermal stability, and CPD4 and CPD7 infected *C. perfringens* FORC 25. The complete genome comprises 38,905-bp, 18,212-bp, 52,658-bp and 18,958-bp, with an overall G + C content of 32.06%, 27.11%, 33.3% and 29.52%, respectively. The sequence analysis showed that CPD1 and CPD4 had circular double-stranded DNA and CPD2 and CPD7 had linear double-stranded DNA. The functional ORFs were clustered into six functional groups of integration, regulation, DNA replication, packaging, structure, lysis and additional function. Only CPD1, especially, had integrase associated with lysogenization, suggesting CPD1 is a temperate phage and the others are virulent phages (Volozhantsev, Oakley et al. 2012). Importantly, the toxin production- and bacterial virulence-associated genes were not identified in these phages.

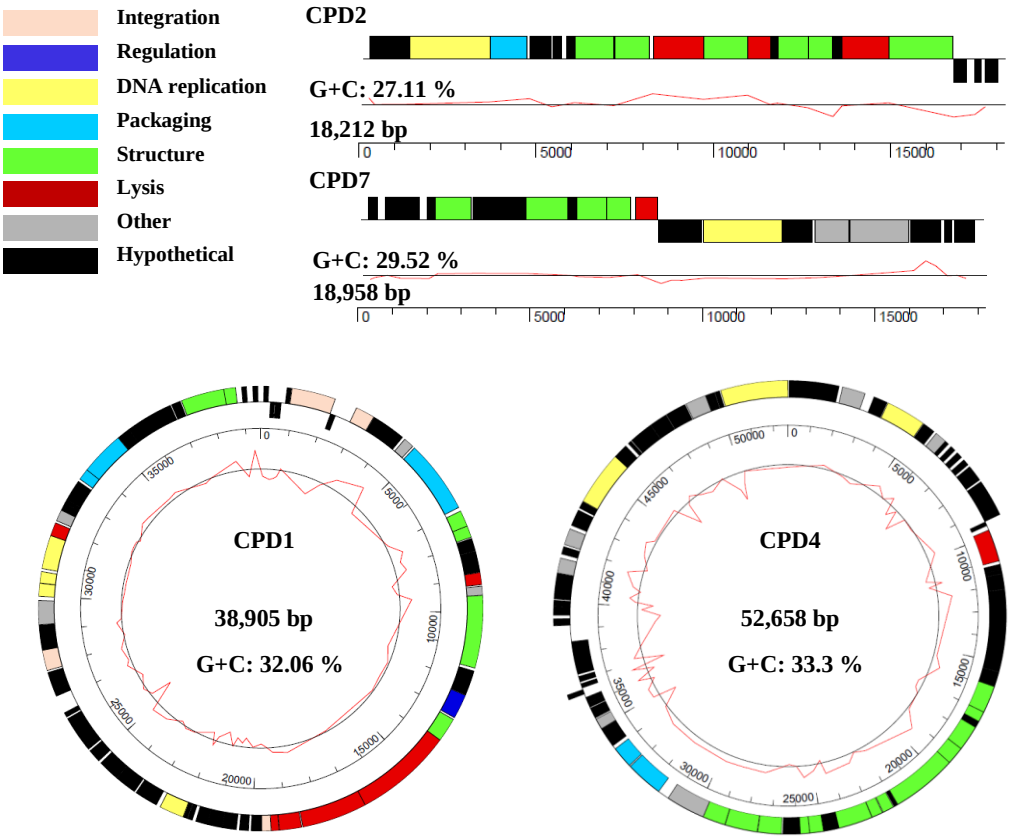


Figure 4. Genome map of phages. The color of each gene refers to the functional categories. Total sixty-seven, twenty-two, seventy-seven and twenty-five ORFs were identified in the CPD1, CPD2, CPD4 and CPD7 genome.

3.5. Identification and expression of endolysins.

The putative endolysin genes identified from the CPD2 genome and CPD7 genome were designated as LysCPD2 and LysCPD7, respectively. Amino acid sequence alignment revealed that LysCPD2 and LysCPD7 were homologous to endolysins of *C. perfringens* phage phi24R, CPS1 and phiCP34O at the amino acid sequence level (Fig. 5a). Pfam and Conserved Domain Database analysis revealed that LysCPD2 and LysCPD7 are putative *N*-acetylmuramoyl-L-alanine amidase that contains a type 3 amidase domain at its N-terminus. This is the enzymatically active domain (EAD) that cleaves the amide bond between *N*-acetylmuramic acid and L-alanine in the bacterial cell wall (Szweda, Schielmann et al. 2012). The LysCPD2 and LysCPD7 were cloned and expressed in *E. coli* with an N-terminal His_6-tag. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed single bands of the purified endolysins, LysCPD2 (27.41 kDa) and LysCPD7 (29.15 kDa), which were consistent with the calculated molecular mass (Fig. 5c). Only 25 nM of purified LysCPD2 could lyse type strains of *C. perfringens* cells substantially in 20 min and exceptionally C. perfringens FORC 25 was lysed entirely in 20 min with 250 nM of the LysCPD2 (Fig. 6a). Likewise, only 25 nM to 50 nM of purified LysCPD7 could lyse all tested type strains of *C. perfringens* cells considerably in 20 min (Fig. 6b).

To confirm the predicted amidase activity of LysCPD2 and LysCPD7, I performed amidase assay that colorimetrically measured the amount of free muramic acid generated after endolysin treatment of peptidoglycan. As shown in Fig. 7, the amount of free muramic acid released from *C. perfringens* FORC 25 peptidoglycan was significantly increased by treatment of LysCPD2 and LysCPD7 compared to the non-treated or substrate-free control groups. These results suggest that LysCPD2 and LysCPD7 have conserved type 3 amidase domain and have amidase activity.

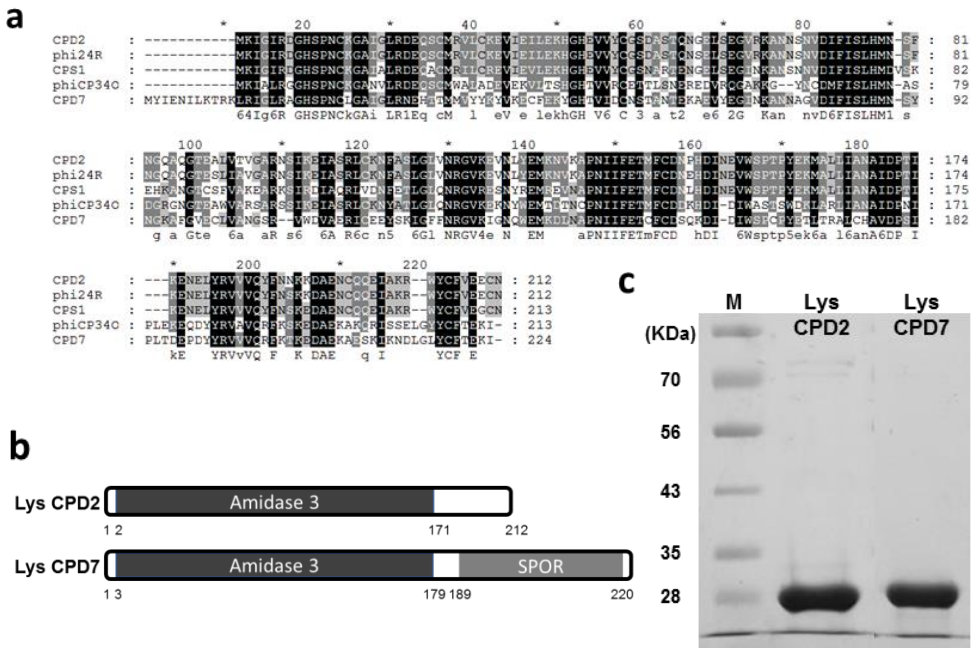
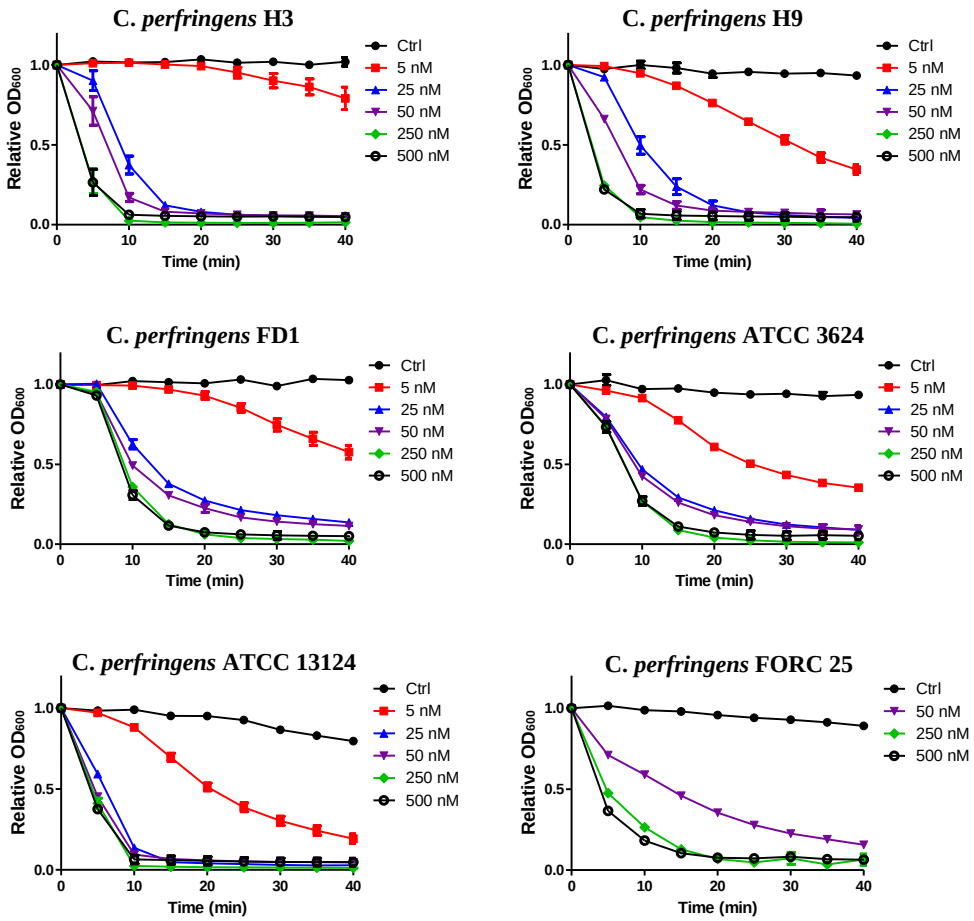


Figure 5. Sequence analysis and purification of LysCPD2 and LysCPD7. (a) Multiple-sequence alignment of amino acid sequence of various Clostridial phage endolysins. The alignment was performed using the ClustalX2 computer program. Dark shading represents sequence identity and lighter shading represents similarity. (b) Schematic representation of LysCPD2 and LysCPD7. The conserved type 3 amidase domain and SPOR domain are shown. (c) SDS-PAGE analysis of purified LysCPD2 and LysCPD7. M, standard molecular weight marker; LysCPD2, purified LysCPD2 fraction; LysCPD7, purified LysCPD7 fraction.

a

- **LysCPD2**


C. perfringens H3
C. perfringens H9
C. perfringens FD1
C. perfringens ATCC 3624
C. perfringens ATCC 13124
C. perfringens FORC 25
Relative OD600
Time (min)
Ctrl
5 nM
25 nM
50 nM
250 nM
500 nM

b

- **LysCPD7**

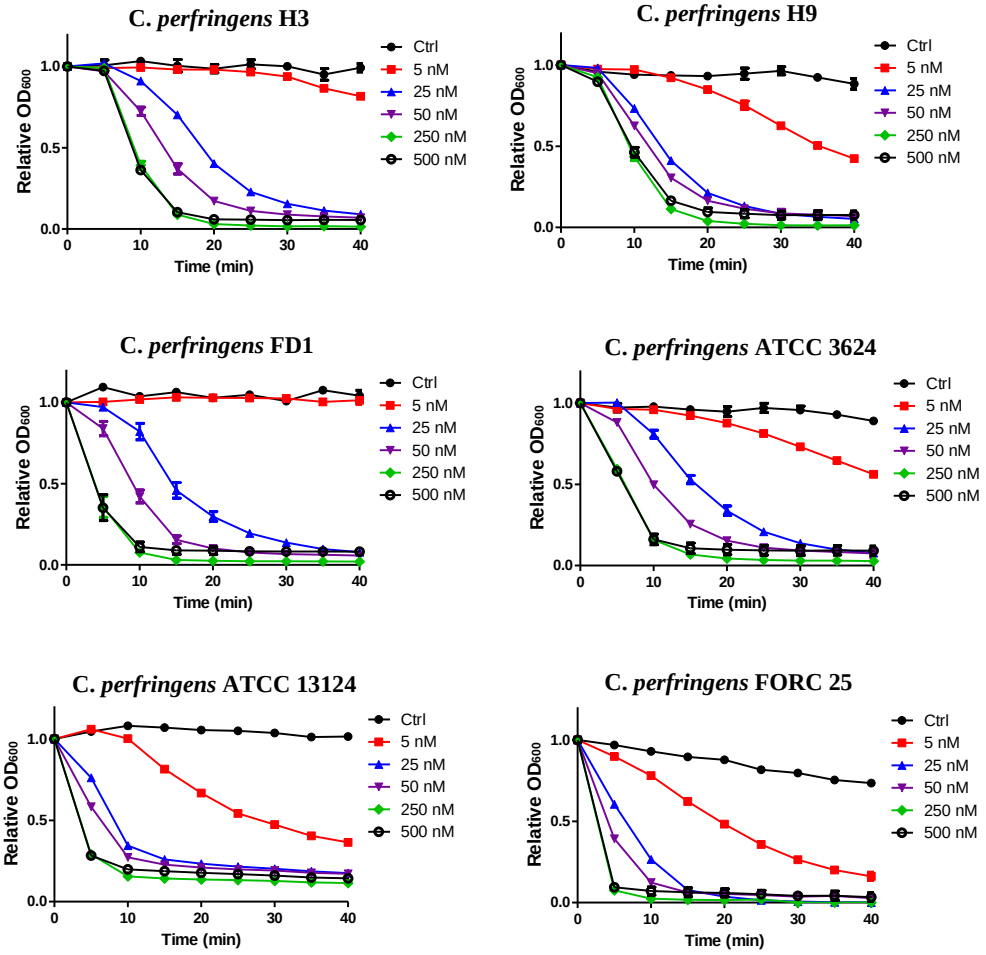


Figure 6. Lytic activity of (a) LysCDP2 and (b) LysCPD7. Various concentration of LysCPD2 and LysCPD7 were added to the suspension of *C. perfringens* cells. Optical density was measured periodically after endolysins treatment.

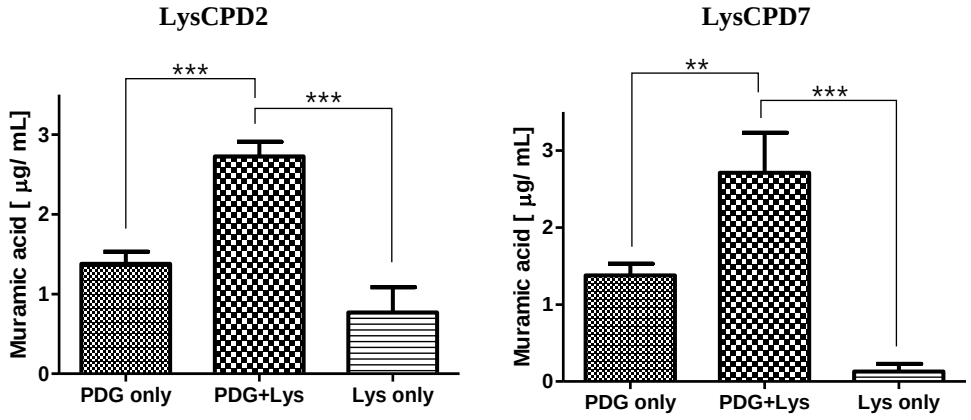


Figure 7. *N*-acetylmuramoyl-L-alanine amidase activity of LysCPD2 and LysCPD7. PDG only, peptidoglycan only; Lys only, treatment with 1 μM endolysin without peptidoglycan; PDG + Lys, treatment with 1 μM endolysin and peptidoglycan. Each column represents the mean and standard deviation of triplicate assays, and the asterisks indicate significant differences (***, $P < 0.0001$; **, $P < 0.001$).

3.6. Antimicrobial spectrum of the endolysins.

Antimicrobial activity against *Clostridium* and other Gram-positive bacterial strains was examined. While the phages CPD2 and CPD7 had the narrow host range, LysCPD2 and LysCPD7 lysed most of the *C. perfringens* strains tested, indicating a broader antimicrobial spectrum, and even exhibited lytic activity against *Bacillus* strains (Table 2). However, these endolysins did not show lytic activity against other Gram-positive bacteria, such as *Listeria monocytogenes, Staphylococcus aureus* and other *Clostridium* species, suggesting the genus- or species-specific moieties of the cell wall are required for the enzymatic activity of LysCPD2 and LysCPD7.

TABLE 2. The antimicrobial spectrum of LysCPD2 and LysCPD7.

Bacterial host [a]	LysCPD2	LysCPD7
***Clostridium perfringens* strain**		
C. perfringens H3	+	+
C. perfringens H9	+	+
C. perfringens FD1	+	+
C. perfringens ATCC 3624	+	+
C. perfringens ATCC 13124	+	+
C. perfringens FORC 25	+	+
Isolate		
human stool isolate 2582	-	+
human stool isolate 2585	+	+
human stool isolate 2589	+	+
human stool isolate 2722	+	+
Other Gram-positive bacteria		
***Listeria monocytogenes* EGDe**	-	-
***Staphylococcus aureus* RN4220**	-	-
***Staphylococcus aureus* Newman**	-	-
***Bacillus cereus* ATCC 10987**	-	+
***Bacillus cereus* ATCC 13061**	+	+
***Bacillus subtilis* ATCC 23857**	+	+
***Clostridium histolyticum* ATCC 19401**	-	-
***Clostridium indolis* ATCC 25771**	-	-

[a] ATCC, American Type Culture Collection; FORC, Food-borne pathogen Omics Research Center; +, positive activity; -, negative activity

3.7. Effects of NaCl, pH, and temperature on the endolysins.

To determine optimum conditions for the endolysin activity, the biochemical properties of LysCPD2 and LysCPD7 were evaluated. Analysis of lytic activity at various concentrations of NaCl, ranging from 0 to 1000 mM, showed that LysCPD2 had the highest lytic activity at 100 mM NaCl and retained more than 50% of its lytic activity even at 500 mM NaCl (Fig. 8a), while LysCPD7 also exhibited the highest lytic activity at 100 mM NaCl and maintained more than 50 % of its lytic activity up to 300 mM NaCl (Fig. 8b). The effect of pH on the lytic activity of LysCPD2 and LysCPD7 was determined at pH 3 to pH 10. As a result, both LysCPD2 and LysCPD7 showed the highest lytic activity at pH 9, and the activity was decreased sharply at pH 6 or lower. In addition, the maximal lytic activity was observed at 37°C and there was no drastic decrease in lytic activity at higher temperatures, showing more than 40% residual lytic activity in the temperature range from 25°C to 65°C.

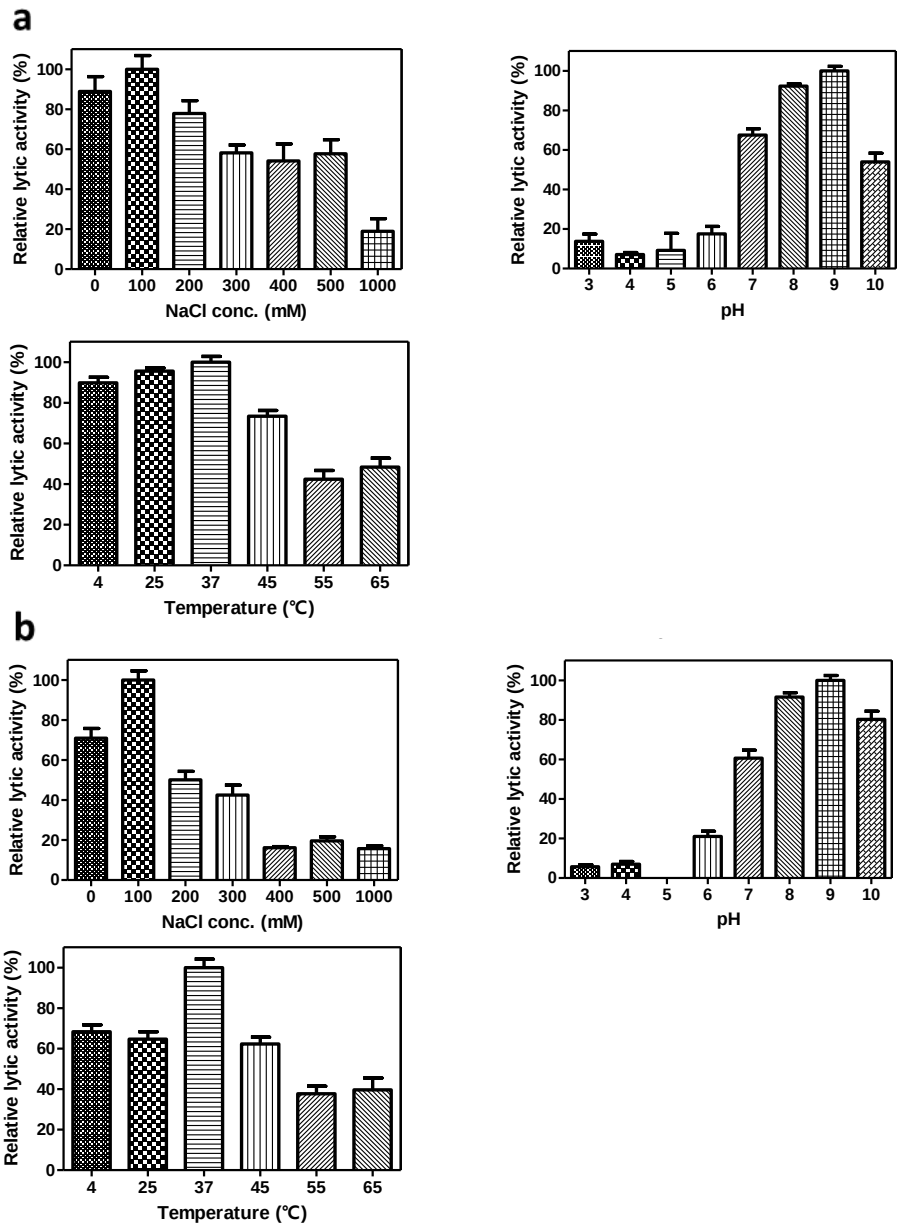


Figure 8. The effect of NaCl concentration, pH, and temperature on the lytic activity of (a) LysCPD2 and (b) LysCPD7 against *C. perfringens* ATCC 13124 cells. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation.

3.8. Determination of thermal stability.

In general, phage lysins are not known to be thermostable, and most endolysins were inactivated or retained only a minor fraction of its activity at temperatures in excess of 50-60°C due to inappropriate refolding after heat treatment (Lavigne, Briers et al. 2004). However, endolysin LysCPD2 was expected to have high heat resistance because the phage CPD2 the phage CPD2 exhibited considerable thermal stability. To estimate the thermostability of LysCPD2 and LysCPD7, the endolysins were incubated for 10 min at various temperatures, ranging from 4°C to 95°C. The results shown in Fig. 9 indicate that LysCPD2 is highly thermostable. After incubation at 95°C for 10 min, LysCPD2 retained approximately 80% of its original activity and remained (Fig. 9a) more than 20% residual activity after 30 min at 95°C (Fig. 9b). On the other hand, LysCPD7 maintained less than 20% of the maximal lytic activity after 10 min at 95°C (Fig. 9c).

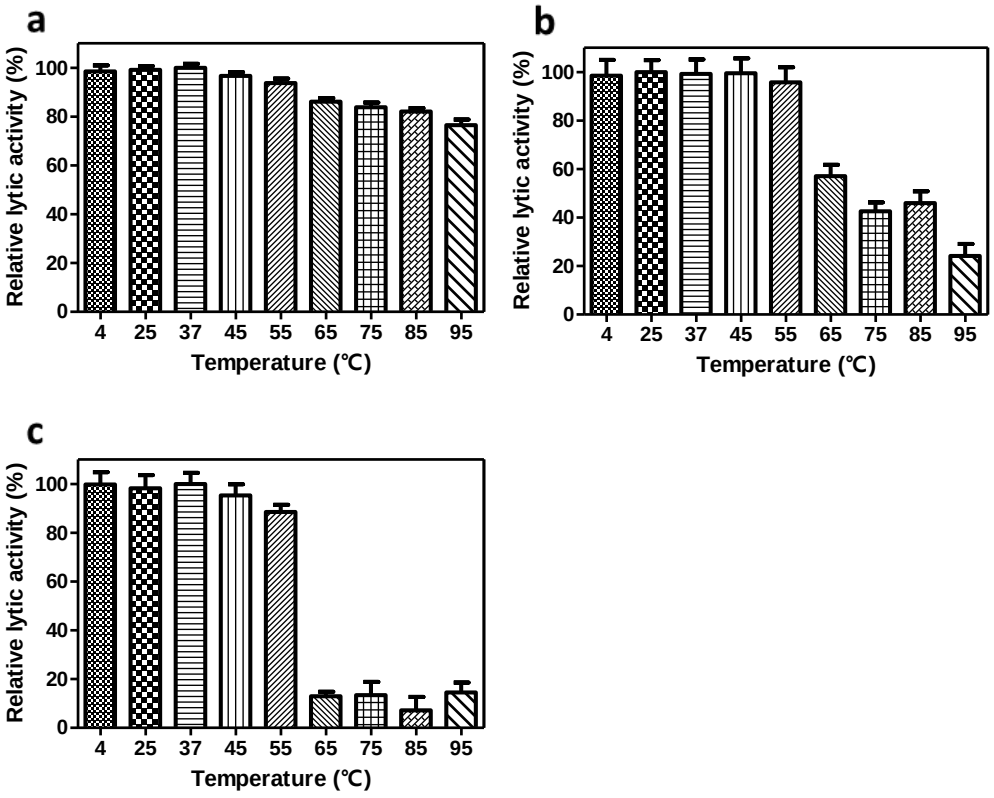


Figure 9. The thermal stability of LysCPD2 and LysCPD7. LysCPD2 was incubated in reaction buffer at various temperature for (a) 10 min and (b) 30 min. (c) LysCPD7 was incubated at various temperature for 10 min. Each experiment was repeated in triplicate and the error bars indicate standard deviation.

3.9. Antibacterial ability of LysCPD7 against *C. perfringens* in milk and beef broth.

I assessed the suitability of LysCPD7 as a biocontrol agent for *C. perfringens* in food considering that it was superior in antibacterial activity against *C. perfringens* FORC25 to LysCPD2. Antibacterial activity against *C. perfringens* FORC 25 strain carrying the major virulence gene, *cpe,* on its chromosome in sterilized milk and beef broth was examined at various endolysin concentrations and times. There are many reports associated with occurrence of *Clostridium* species in dairy products including milk and they showed the contamination of *Clostridium* species could be hazardous for the human health (El-Bassiony 1980). In addition, there are many reported outbreaks of *C. perfringens* from beef dishes like beef soup, beef broth and ground beef. Moreover, these foodstuffs are appropriate models for determining LysCPD7 activity due to their neutral/moderately acidic condition (milk and beef broth; pH 6-7). *C. perfringens* FORC 25 cells was inoculated into milk and beef broth and the number of cells was significantly reduced by LysCPD7 treatment, whereas the number of viable cells were not reduced in the negative control group.

In milk, considerable inhibitory effects ($P < 0.05$) were shown within 2 h by all tested concentrations of LysCPD7. Furthermore, viable cells were

reduced to undetectable levels at 2 h by treatment with 500 nM of LysCPD7 and at 3 h by treatment with 125, 250 and 500 nM of LysCPD7 (Fig. 10a). Similarly, when *C. perfringens* cells were inoculated into beef broth and treated with LysCPD7, the cells were significantly inhibited ($P < 0.05$) within 1 h. Also, treatment with 500 nM of LysCPD7 for 2 h reduced the number of viable cells to an undetectable level of 4-log reduction (Fig. 10b). These data suggest that LysCPD7 is a promising biocontrol agent as a food antimicrobial to control *C. perfringens* in dairy of beef products.

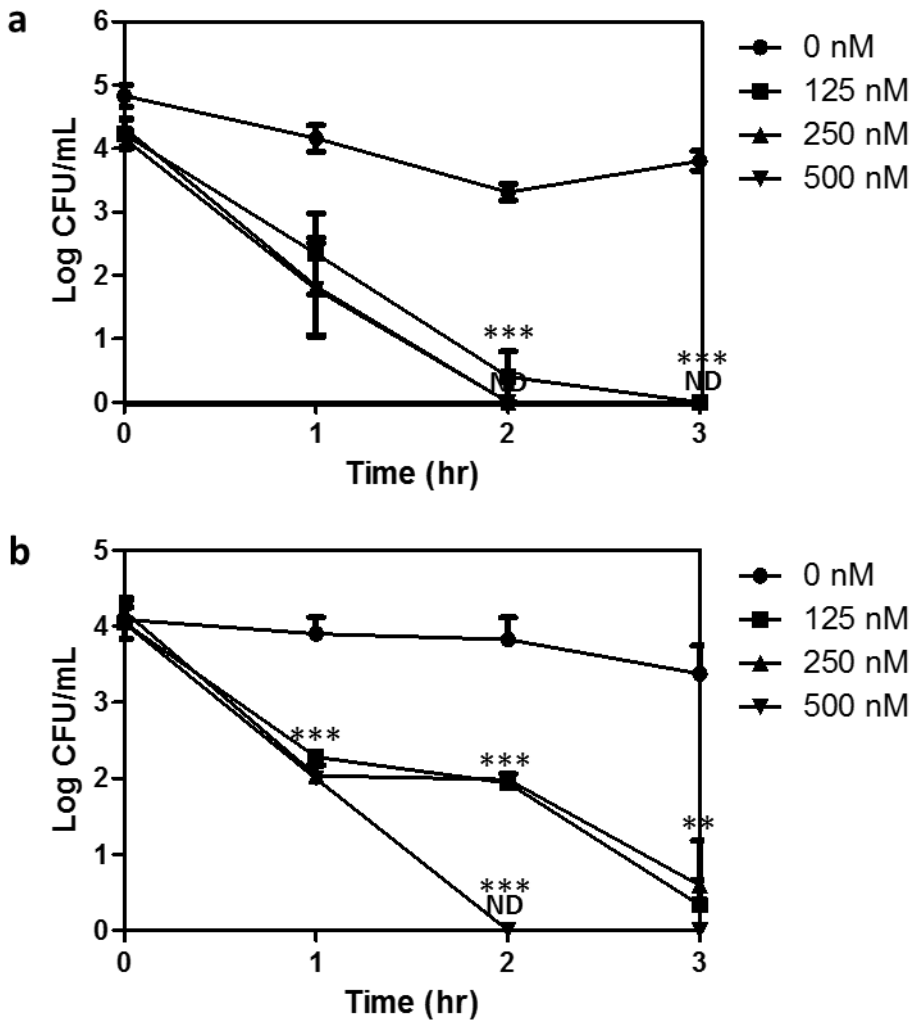


Figure 10. LysCPD7-mediated elimination of *C. perfringens* FORC 25 in (a) sterilized whole milk and (b) beef broth. The numbers of *C. perfringens* FORC 25 cells without LysCPD7 treatment (negative control) and with LysCPD7 125, 250 and 500 nM treatment were counted. Values are the means of more than three independent experiments with standard deviation. The asterisks indicate significant differences (***, $P < 0.0001$; **, $P < 0.001$). ND, not detected.

3.10. Antibacterial ability of endolysins against germinating spores.

Based on the thermal stability of endolysins, I next examined the antibacterial ability of LysCPD2 and LysCPD7 against heat-activated germinating spores of *C. perfringens* FORC 25. In the dormant state, spore peptidoglycan, or cortex is protected from lysozymes and amidases by a proteinaceous coat (Henriques and Moran Jr 2000). However, coat porosity increases after inducing germination (Santo and Doi 1974). Furthermore, LysCPD2 showed remarkable thermostability as it had more than 80% of residual activity at 75°C for 10 min. I therefore reasoned that spores might be rendered susceptible to LysCPD2 under the general spore germination condition, treatment at 75°C for 20 min. To evaluate this, 1 mL aliquots including about 10^6 spores were treated with LysCPD2 and LysCPD7, respectively, at 75°C for 20 min and incubated at 37°C for 12 h. As the result, the LysCPD2-treated spores showed about 6-log CFU/mL lower viability and the LysCPD7-treated spores exhibited approximately 2-log CFU/mL lower viability compared to only heat-treated spores, respectively (Fig. 11).

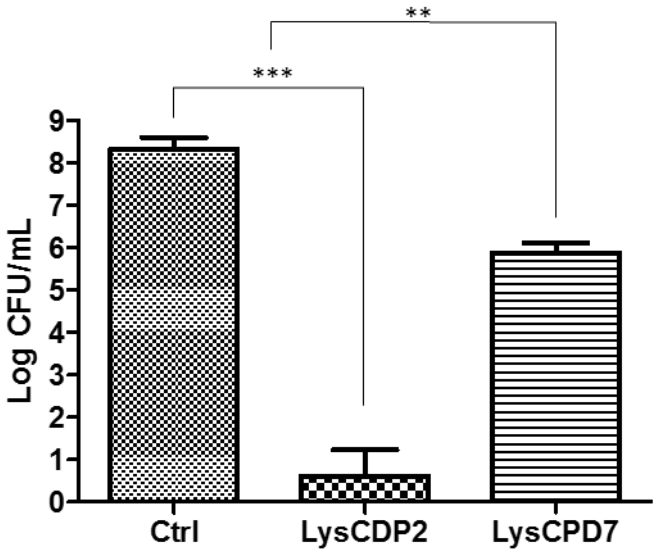


Figure 11. The effect of endolysins on spore viability. Spores (10^6 CFU/mL) suspensions containing endolysin (500 nM) were heat-treated at 75°C for 20 min. Then, they were incubated at 37°C for 12 h to be completely germinated and the number of germinated cells were counted. Each column represents the mean and standard deviation of triplicate assays, and the asterisks indicate significant differences (***, $P < 0.0001$; **, $P < 0.001$).

Ⅳ. DISCUSSION

In general, *C. perfringens* infecting phages and their endolysins have not been studied yet, since *C. perfringens* is an anaerobe and difficult to study. In this study, seven bacteriophages were isolated showing lytic activity against *C. perfringens* strains. Among these phages, phage CPD1 and CPD2 showed remarkable thermal stability, and phage CPD4 and CPD7 had the most effective inhibition activity. In particular, CPD4 and CPD7 can infect *C. perfringens* FORC 25, a virulent strain. These four phages were selected and their whole genomes were sequenced. Sequence analysis showed that CPD1 was not a virulent phage but a temperate phage. In addition, BLASTP analysis showed that endolysin of CPD4 showed 65% sequence identity and that of CPD7 showed 56% sequence identity with previously reported *Clostridium* phages endolysin. Unfortunately, the endolysin of CPD4 was insoluble even though it was attempted to express and purify under several experimental conditions. Therefore, endolysins of CPD2 and CPD7, designated as LysCPD2 and LysCPD7, were selected for further study.

The newly discovered endolysins LysCPD2 and LysCPD7 had homology with *N*-acetylmuramoyl-L-alanine amidase of other *C. perfringens* phages endolysins and their amidase activity was confirmed by the amidase assay. Compared with the narrow host specificity of the phages, LysCPD2 and

LysCPD7 showed a much broader lytic spectrum and even had lytic activity against *Bacillus* strains, although not as much as *C. perfringens*. It is note that *C. perfringens* and *Bacillus* species have a similar peptidoglycan composition of MurNAc- $_{L}$-Ala- $_{D}$-Glu-Dmp- $_{D}$-Ala, and the type 3 amidase, EAD of LysCPD2 and LysCPD7, cleaves the peptide bond between the MurNAc and $_{L}$-Ala (Schleifer and Kandler 1972) (Fig. 12).

The biochemical characteristics of LysCPD2 and LysCPD7 showed that the optimal pH was pH 9.0, but over 60% activity was retained at pH 7.0 and less than 20% activity was retained below pH 6.0, indicating that they are alkalophilic. The maximal lytic activity occurred in the presence of 100 mM NaCl. Furthermore, LysCPD7 showed approximately more than 4-log reduction of *C. perfringens* cells per milliliter in milk and beef broth. These data suggest that LysCPD7 is a promising biocontrol agent as a food antimicrobial to control *C. perfringens* in milk or beef broth products. However, LysCPD7 showed only less than 2-log reduction in food below pH 6, such as pork and gravy sauce (data not shown), suggesting that pH of food would be an important factor in determining the food to which LysCPD7 will be applied as a biocontrol agent.

Endolysins are generally not heat stable, and most lose their lytic activity above 50-60°C (Lavigne, Briers et al. 2004). However, few endolysins

such as *Salmonella* phage endolysin Lys68 and *Stenotrophomonas maltophilia* phage endolysin P28 have been reported to be thermostable. Lys68 maintained 54.7% of its residual activity after 30 min incubation at 80°C (Oliveira, Thiagarajan et al. 2014), and endolysin P28 retained 55% of its activity after treatment at 70°C for 30 min (Dong, Zhu et al. 2015). LysCPD2 maintained about 80% of its residual activity after heating at 95°C for 10 min and 30% of its activity after incubation at 95°C for 30 min. In addition, LysCPD2 showed much more lytic activity than LysCPD7 against heat-activated *C. perfringens* FORC 25 spores even after LysCPD2 and LysCPD7 were heated with spores at 75°C for 20 min, the general spore germination condition. These data suggest that LysCPD2 will provide powerful tools for many applications in molecular biology, biotechnology and medicine (Loessner 2005).

In conclusion, seven phages targeting *C. perfringens* were isolated and characterized. They commonly had the narrow host specificity and it may limit their efficiency as a biocontrol agent. Meanwhile, their endolysins LysCPD2 and LysCPD7 showed the broader lytic spectrum and LysCPD2 had remarkable thermal stability. Moreover, it was shown that LysCPD2 can control *C. perfringens* when heated with their spores and LysCPD7 can inhibit

C. perfringens in milk and beef broth. Taken together, these phages and endolysins have potential to be effective antibacterial agents to *C. perfringens*.

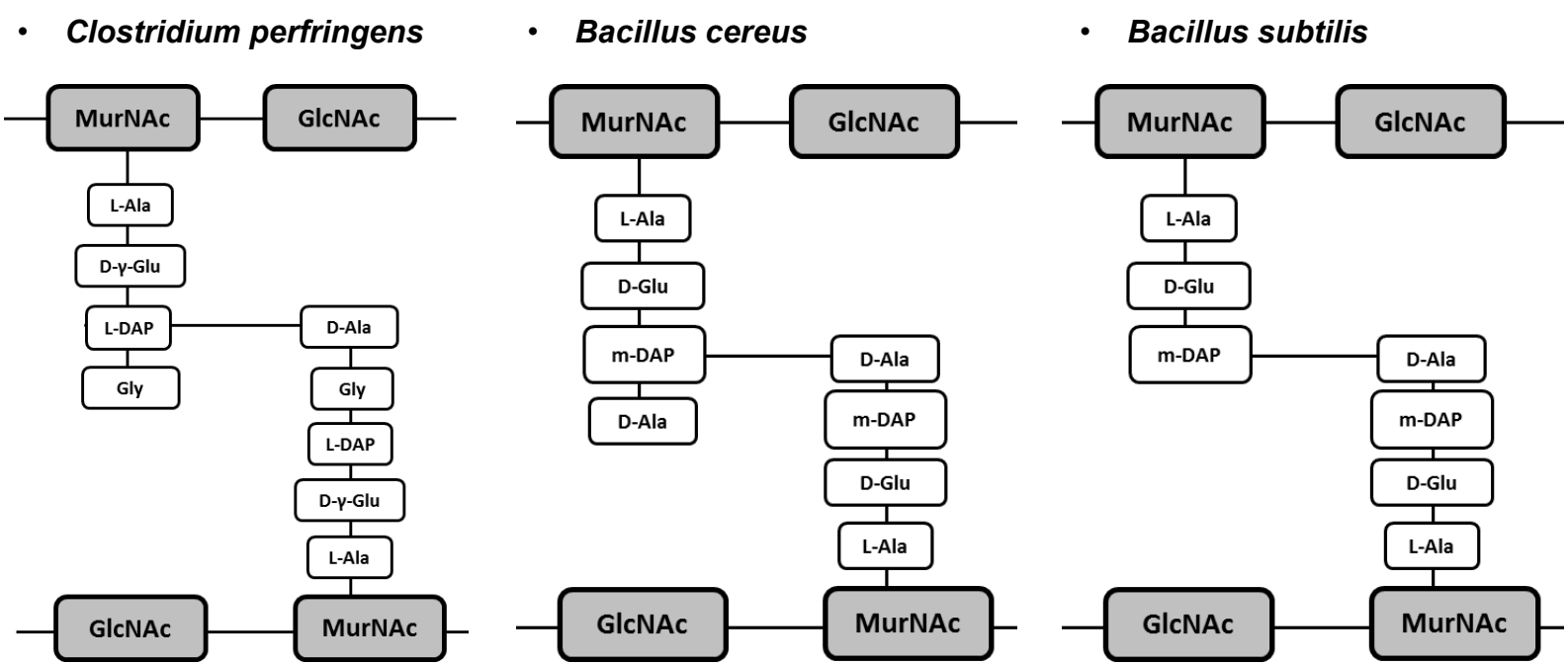


Figure 12. Fragment of the primary structure of a peptidoglycan of *C. perfringens*, *B. cereus and B. subtilis*.

Ⅴ. REFERENCES

Altschul, S. F., et al. (1990). "Basic local alignment search tool." Journal of molecular biology **215**(3): 403-410.

Aziz, R. K., et al. (2008). "The RAST Server: rapid annotations using subsystems technology." BMC genomics **9**(1): 75.

Bragg, R., et al. (2014). "Bacteriophages as potential treatment option for antibiotic resistant bacteria." Infectious Diseases and Nanomedicine I, Springer**:** 97-110.

Brynestad, S. and Granum, P. E. (2002). "Clostridium perfringens and foodborne infections." International journal of food microbiology **74**(3): 195-202.

Chang, Y., et al. (2017). "Characterization of a novel endolysin LysSA11 and its utility as a potent biocontrol agent against Staphylococcus aureus on food and utensils." Food microbiology **68**: 112-120.

Cheng, Q., et al. (2005). "Removal of group B streptococci colonizing the vagina and oropharynx of mice with a bacteriophage lytic enzyme." Antimicrobial agents and chemotherapy **49**(1): 111-117.

Deresinski, S. (2009). "Bacteriophage therapy: exploiting smaller fleas." Clinical infectious diseases **48**(8): 1096-1101.

Dong, H., et al. (2015). "Antibacterial Activity of Stenotrophomonas

maltophilia Endolysin P28 against both Gram-positive and Gram-negative Bacteria." Frontiers in microbiology **6**: 1299.

El-Bassiony, T. A. (1980). "Occurrence of Clostridium perfringens in milk and dairy products." Journal of Food Protection **43**(7): 536-537.

Fauquet, C. M. and Fargette, D. (2005). "International Committee on Taxonomy of Viruses and the 3,142 unassigned species." Virology journal **2**(1): 64.

Fischetti, V. A. (2008). "Bacteriophage lysins as effective antibacterials." Current opinion in microbiology **11**(5): 393-400.

Fischetti, V. A. (2010). "Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens." International Journal of Medical Microbiology **300**(6): 357-362.

Freedman, J. C., et al. (2016). "Clostridium perfringens enterotoxin: action, genetics, and translational applications." Toxins **8**(3): 73.

Granum, P. E. (1990). "Clostridium perfringens toxins involved in food poisoning." International journal of food microbiology **10**(2): 101-111.

Gupta, R. and Prasad, Y. (2011). "P-27/HP endolysin as antibacterial agent for antibiotic resistant Staphylococcus aureus of human infections." Current microbiology **63**(1): 39.

Gutierrez, D., et al. (2014). "Effective removal of staphylococcal biofilms by

the endolysin LysH5." PLoS One **9**(9): e107307.

Ha, E., et al. (2018). "Clostridium perfringens Virulent Bacteriophage CPS2 and Its Thermostable Endolysin LysCPS2." Viruses **10**(5): 251.

Hadžija, O. (1974). "A simple method for the quantitative determination of muramic acid." **60**(2): 512-517.

Harbarth, S. and Samore, M. H. (2005). "Antimicrobial resistance determinants and future control." Analytical biochemistry **11**(6): 794.

Hazenberg, M. (1992). "Assay for N-acetylmuramyl-L-alanine amidase in serum by determination of muramic acid released from the peptidoglycan of Brevibacterium divaricatum." European journal of clinical chemistry and clinical biochemistry: journal of the Forum of European Clinical Chemistry Societies.

Henriques, A. O. and Moran Jr, C. P. (2000). "Structure and assembly of the bacterial endospore coat." Methods **20**(1): 95-110.

Immerseel, F. V., et al. (2004). "Clostridium perfringens in poultry: an emerging threat for animal and public health." Avian pathology **33**(6): 537-549.

Kirby, K. S. (1956). "A new method for the isolation of ribonucleic acids from mammalian tissues." Biochemical Journal **64**(3): 405.

Kokai-Kun, J. F., et al. (1994). "Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic

isolates of Clostridium perfringens." Journal of clinical microbiology **32**(10): 2533-2539.

Lavigne, R., et al. (2004). "Identification and characterization of a highly thermostable bacteriophage lysozyme." **61**(21): 2753-2759.

Loessner, M. J. (2005). "Bacteriophage endolysins—current state of research and applications." Cellular and Molecular Life Sciences CMLS **8**(4): 480-487.

Maniloff, J. and Ackermann, H. W. (1998). "Taxonomy of bacterial viruses: establishment of tailed virus genera and the other Caudovirales." Archives of virology **143**(10): 2051-2063.

Myers, G. S., et al. (2006). "Skewed genomic variability in strains of the toxigenic bacterial pathogen, Clostridium perfringens." Genome Research **16**(8): 1031-1040.

Nelson, D., et al. (2003). "Genomic sequence of C1, the first streptococcal phage." Journal of bacteriology **185**(11): 3325-3332.

Norrby, S. R., et al. (2005). "Lack of development of new antimicrobial drugs: a potential serious threat to public health." The Lancet infectious diseases **5**(2): 115-119.

Oakley, B. B., et al. (2011). "Comparative genomics of four closely related Clostridium perfringens bacteriophages reveals variable evolution among core genes with therapeutic potential." BMC genomics **12**(1):

282.

Oliveira, H., et al. (2014). "A thermostable Salmonella phage endolysin, Lys68, with broad bactericidal properties against gram-negative pathogens in presence of weak acids." PLoS One **9**(10): e108376.

Santo, L. Y. and Doi, R. H. (1974). "Ultrastructural analysis during germination and outgrowth of Bacillus subtilis spores." Journal of Bacteriology **120**(1): 475-481.

Schleifer, K. H., & Kandler, O. (1972). "Peptidoglycan types of bacterial cell walls and their taxonomic implications." *Bacteriological reviews* **36**(4), 407.

Schmelcher, M., et al. (2012). "Bacteriophage endolysins as novel antimicrobials." Future microbiology **7**(10): 1147-1171.

Schuch, R., et al. (2002). "A bacteriolytic agent that detects and kills Bacillus anthracis." Nature **418**(6900): 884.

Seal, B. S. (2013). "Characterization of bacteriophages virulent for Clostridium perfringens and identification of phage lytic enzymes as alternatives to antibiotics for potential control of the bacterium1." Poultry science **92**(2): 526-533.

Songer, J. G. (1996). "Clostridial enteric diseases of domestic animals." Clinical microbiology **9**(2): 216.

Szweda, P., et al. (2012). "Peptidoglycan hydrolases-potential weapons against Staphylococcus aureus." Applied microbiology and biotechnology **96**(5): 1157-1174.

Volozhantsev, N. V., et al. (2012). "Molecular characterization of podoviral bacteriophages virulent for Clostridium perfringens and their comparison with members of the Picovirinae." PLoS One **7**(5): e38283.

Walmagh, M., et al. (2013). "Characterization of five novel endolysins from Gram-negative infecting bacteriophages." Applied microbiology and biotechnology **97**(10): 4369-4375.

Witzenrath, M., et al. (2009). "Systemic use of the endolysin Cpl-1 rescues mice with fatal pneumococcal pneumonia." Critical care medicine **37**(2): 642-649.

Zeugin, J. A. and Hartley, J. L. (1985). "Ethanol precipitation of DNA." Focus **7**(4): 1-2.

국문 초록

클로스트리디움 퍼프린젠스(*Clostridium perfringens*)는 식중독 유발균으로서 인간과 동물에 감염하여 다양한 질병을 유발할 수 있다. 특히, 항생제 내성의 확산을 막기위해 성장촉진용 항생제의 사용을 금지한 여러 유럽 국가에서 가금류의 클로스트리디움 퍼프린젠스로 인한 발병 증가로 인하여 기존의 항생제를 대체할 수 있는 새로운 항세균제제의 개발이 필요하다. 박테리오파지 및 파지에서 분리된 세포벽 분해 효소인 엔도라이신은 병원균에 대해 특이적으로 작용하고 내성균 생성 확률이 적어 새로운 항생물질로서 각광받고 있다. 본 연구에서는 클로스트리디움 퍼프린젠스를 감염할 수 있는 7 개의 파지를 분리하고 그 특성을 분석하였다. 그 중에서 내열성이 뛰어난 CPD2 와 주요 병원성 유전자에 속하는 *cpe* 유전자를 가진 균주를 제어할 수 있는 CPD7 을 선정해 엔도라이신 실험을 진행하였다. CPD2 와 CPD7 의 유전자를 분석한 결과 엔도라이신으로 추정되는 유전자를 밝혀냈고, 이들은 기존의 *N*-acetylmuramoyl-L-alanine amidase 와 상동성을 보였다. 각각의 엔도라이신 LysCPD2 와 LysCPD7 은 모두 클로스트리디움 퍼프린젠스 뿐만 아니라 바실러스 세레우스(*Bacillus cereus*)와 바실러스 서브틸리스 (*Bacillus subtilis*)에도 제어효과를 보였다. 또한 예상한 바와 같이 LysCPD2 는 95°C 에서 80%의 활성을 유지하여 뛰어난 내열성을

나타냈다. LysCPD2 의 내열성을 바탕으로 포자를 발아 조건인 75°C 에서 20 분간 LysCPD2 와 함께 배양하였고, 열에 의해 활성화된 포자에 대해 항균 능력을 확인하였다. 덧붙여 LysCPD7 은 LysCPD2 보다 클로스트리디움 퍼프린젠스 FORC 25 균주에 대해 높은 용해능을 보였고, 우유와 고기육수에서도 항균 활성을 확인하였다. 이러한 결과로 미루어 실험에 사용된 파지와 엔도라이신이 클로스트리디움 퍼프린젠스를 제어할 수 있는 새로운 미생물 제어 방안으로서 가능성을 가진 물질임을 알 수 있었다.

주요어: 클로스트리디움 퍼프린젠스, 박테리오파지, 엔도라이신

학번: 2017-26630