Lim *et al. Appl Biol Chem* (2020) 63:40
https://doi.org/10.1186/s13765-020-00523-z

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Characterization of a novel endolysin from bacteriophage infecting *Vibrio parahaemolyticus*, vB_VpaP_KF2

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Abstract

The antimicrobial resistance of food-borne pathogenic bacteria, including *Vibrio parahaemolyticus,* has been reported globally, warranting the need to identify promising alternative antibiotics such as endolysins that originate from bacteriophages. In our previous study, we characterized a bacteriophage infecting *V. parahaemolyticus*, vB_VpaP_KF2, at the molecular level. In this study, an open reading frame encoding putative endolysin was cloned from the complete genome data and expressed in the *Escherichia coli* expression system. The recombinant endolysin, vB_VpaP_KF2_Lys, exhibited a novel lytic property against Gram-negative bacteria regardless of pretreatment with an outer-membrane permeabilizer. It was also stable over a wide range of temperatures, pH, and NaCl concentrations, and its hydrolytic spectrum was broader than that of the parent bacteriophage. From the results, vB_VpaP_KF2_Lys could be used as a biocontrol agent against food-borne pathogens in the field of food safety.

Keywords: Bacteriophage, Endolysin, Lytic activity, *Vibrio parahaemolyticus*, Food safety

Introduction

Antimicrobial resistance arising from antibiotic abuse has been recognized as a critical global issue that poses a threat to public health and food safety, and the incidence of multidrug-resistant food-borne bacteria has been increasing [1]. Bacteriophages have been proposed as natural antimicrobial agents not synthetic antibiotics, which have lytic properties against target bacterial hosts by invading them and disrupting the metabolism. Some phage formulations, including ListShield™ and SalmoFresh™ for the control of *Listeria monocytogenes* and *Salmonella enterica*, respectively, have been given the Generally Recognized as Safe (GRAS) status for use in the food industry [2].

Endolysins, phage-encoded lytic enzymes produced during the late phase of gene expression in the lytic cycle, are responsible for the enzymatic cleavage of peptidoglycans [3]. Increasing attention is being directed to them as effective candidates for alternative antibiotics as they do not incur bacterial resistance and have a high host specificity without host natural microbial community interruption [4]. The use of external recombinant endolysins as antimicrobial materials against Gram-positive bacteria has been attempted in various applications, while its use against Gram-negative bacteria is limited, as the outer-membrane of the bacteria prevents endolysin from accessing the peptidoglycan [5].

Vibrio parahaemolyticus, a major Gram-negative food-borne pathogen that is widely distributed in marine and estuarine environments, leads to gastrointestinal infections on consumption of raw or undercooked seafood [6]. *V. parahaemolyticus* accounts for nearly 34,664 food poisoning incidents annually in the United State and 11 cases in South Korea in 2018 [7, 8]. Continuous reports of antimicrobial resistance of *V. parahaemolyticus* as well as other food-borne pathogens have warranted the need to find promising antimicrobial agents and broaden the pool of compounds that can substitute antibiotics [6].

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In our previous study, we isolated the bacteriophages infecting *V. parahaemolyticus* from the western and southern coastal areas of Korea, investigated their growth inhibitory effect against target host in manila clam, and analyzed the comparative genomic properties of six *V. parahaemolyticus* phages [9, 10].

In this present study, we characterized a novel endolysin from *V. parahaemolyticus* phage, vB_VpaP_KF2, and showed that its lytic activity against bacterial cells whose outer-membrane has not been treated with a permeabilizer, is effective. These results elucidate the fundamental properties of vB_VpaP_KF2 endolysin and its potential as an antibacterial agent that can be applied in the food chain.

Materials and methods

Purification of recombinant vB_VpaP_KF2 endolysin

The endolysin (vB_VpaP_KF2_Lys, abbreviated as KF2_Lys) gene was amplified using PCR and cloned into pET-28a at the *Nco*I and *Xho*I sites. This recombinant plasmid was transformed into *Escherichia coli* Rosetta 2 (DE3) pLysS. Expression of the endolysin was induced by adding 0.1 mM IPTG and incubating for 16 h at 18 °C. The KF2_Lys was then purified using Ni–NTA affinity chromatography under native conditions and stored at − 20 °C in storage buffer containing 50 mM Tris–HCl (pH 8.0), 200 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 50% glycerol [11, 12].

Antimicrobial activity of KF2_Lys

The antimicrobial activity of KF2_Lys was determined by measuring the decrease in optical density (OD) of the bacterial cell suspension (*V. parahaemolyticus* isolate KF1) after the addition of endolysin [13]. The bacterial cells in the log phase were harvested and resuspended in 50 mM Tris–HCl (pH 8.0). Next, they were treated with 1, 10, and 100 mM EDTA for 5 min at 37 °C and washed four times with the buffer. Then, 100 µl of endolysin (20 µg/ml) was added to 900 µl of EDTA pre-treated cell suspension and incubated for 30 min at room temperature, following which, its optical density at 600 nm was measured. For the negative control, 100 µl of resuspension buffer was used instead of the endolysin. The results of the experiment performed in triplicate are presented as a representative or mean ± SD.

The lytic activitis of different concentrations of KF2_Lys (1–30 µg/ml) were measured in the same way as the above experiment except for using the intact bacterial cell suspension instead of the EDTA pre-treated cell suspension as substrate.

Effects of temperature, pH, and ionic strength on the lytic activity of KF2_Lys

The lytic activities of the KF2_Lys (10 µg/ml) were compared at different temperatures (4–65 °C) and NaCl concentrations (0–200 mM) [13]. The lytic activity under different pH conditions was measured using the following pH buffers instead of the resuspension buffer: 0.1% trifluoroacetic acid (TFA) for pH 2.0; 50 mM sodium acetate for pH 4.3; 50 mM 2-(N-morpholino) ethanesulfonic acid (MES hydrate) for pH 6.0; 50 mM potassium phosphate for pH 7.0; 50 mM Tris–HCl for pH 8.0 and 8.5; 50 mM glycine for pH 9.0 and 9.5; and 50 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) for pH 10.0 and 10.5 [13]. The mean values of the experiment performed in triplicate are presented as mean ± SD of relative lytic activity (%), calculated as 100–(OD after 30 min × 100/OD at 0 time).

Antimicrobial spectrum of KF2_Lys

One hundred microliters of endolysin (10 µg/ml) was added to 900 µl of bacterial cell suspension and incubated for 30 min at room temperature before measuring OD_{600}. The bacterial strains used are shown in Table 1. The mean values of the experiment performed in triplicate are presented as mean ± SD of the relative lytic activity (%) [13].

Results and discussion

From the complete genome sequence of *V. parahaemolyticus* phage, vB_VpaP_KF2, it was found that the phage has two putative endolysin genes encoding a zinc peptidase and a glycosyl hydrolase, respectively, [10]. We focused on the putative peptidase (KF2_Lys) because it had a higher lytic activity than the glycosyl hydrolase (data not shown). Purified KF2_Lys revealed a band of 42.5 kDa by SDS-PAGE, which correlated with the predicted mass of the KF2_Lys protein (Fig. 1a). The yield of KF2_Lys was 10.22 mg from 1 L of culture.

The antimicrobial activity of KF2_Lys endolysin was verified via the decrease in optical density of the target bacterial suspension. The optical density of the *V. parahaemolyticus* suspension decreased from 1.01 to 0.313 within 30 min after adding KF2_Lys (20 µg/ml) (Fig. 1b), and the lytic activity was dependent on the concentration of KF2_Lys (1–20 µg/ml) (Fig. 1c). The activity of KF2_Lys was slightly higher than that of other reported endolysins obtained from *V. parahaemolyticus* bacteriophage (400 µg/ml or 1 mg/ml) [11, 14]. Generally, to verify the lytic activity of endolysin against Gram-negative bacteria, outer-membrane permeabilizers such as EDTA, are often used to destabilize the outer-membrane. For example, for endolysin qdvp001 and LysVPp1 to lyse their target bacteria *V. parahaemolyticus*, EDTA pretreatment

Table 1 Antimicrobial spectrum of KF2_Lys endolysin

Bacterial strains			Relative lytic activity[a]
Gram positive	*Bacillus cereus*	ATCC 14,579	+
	Listeria monocytogenes	KCCM 40,307	–
	Staphylococcus aureus	ATCC 25,923	–
Gram negative	*Escherichia coli* K12	ER2738	+
	Escherichia coli O157:H7	NCCP 1109-047	–
	Lactobacillus acidophilus	KCTC 3145	–
	Lactobacillus delbrueckii subsp. *bulgaricus*	ATCC 7995	–
	Lactobacillus delbrueckii subsp. *bulgaricus*	ATCC 11,842	–
	Salmonella Enteritidis	ATCC 13,076	–
	Salmonella Enteritidis	KCCM 12,021	+
	Salmonella Typhimurium	KCCM 11,862	–
	Vibrio alginolyticus	Isolate	++++
	Vibrio cholera	Isolate	+++
	Vibrio fluvialis	Isolate	+
	Vibrio metschnikovii	Isolate	+++
	Vibrio mimicus	Isolate	++++
	Vibrio parahaemolyticus	ATCC 27,969	++
	Vibrio parahaemolyticus	ATCC 17,802	+
	Vibrio parahaemolyticus	Isolate KF1	+++
	Vibrio parahaemolyticus	Isolate KF2	+++
	Vibrio parahaemolyticus	Isolate KF3	++++
	Vibrio parahaemolyticus	Isolate KF4	++++
	Vibrio parahaemolyticus	Isolate KF5	++++
	Vibrio parahaemolyticus	Isolate KF6	+++
	Vibrio parahaemolyticus	Isolate KF7	+++
	Vibrio parahaemolyticus	Isolate KF8	+++
	Vibrio vulnificus	ATCC 27,562	++
	Vibrio vulnificus	Isolate	+

[a] Relative lytic activity (%) = 100 − (OD_{600} after 30 min * 100/OD_{600} at 0 time)

0–10% −; 10–20% +; 20–30% ++; 30–40% +++; > 40% ++++

is essential [11, 14]. Thus, the necessity of pretreatment with outer-membrane permeabilizers can act as a hurdle in the application of endolysin as a biocontrol agent against Gram-negative bacteria. Hence, studies are being carried out to overcome this limitation [15, 16]. KF2_Lys showed lytic activity against *V. parahaemolyticus* without EDTA pretreatment, and it showed the highest activity compared to the OD change of groups treated with different concentrations of EDTA (Fig. 1b). A few studies have shown the lytic activity of endolysin against Gram-negative bacteria without an outer-membrane permeabilizer such as SPN9CC endolysin against *E. coli* and *Bacillus amyloliquefaciens* phage endolysin against *P. aeruginosa* [17, 18]. These endolysins have hydrophobic transmembrane regions that may allow them to pass through the outer-membrane. However, unlike them, KF2_Lys does not possess a predicted transmembrane domain (data not shown). Further study is needed to analyze the lytic mechanism of KF2_Lys.

The antimicrobial activity of KF2_Lys endolysin was similar at temperatures ranging from 4 to 50 °C but decreased at temperatures above 55 °C (Fig. 2a). KF2_Lys was relatively stable under a wide pH range (2.0–10.5) and had the highest lytic activity at pH 8.5–10.5 (Fig. 2b). NaCl concentrations did not significantly affect lytic activity until 200 mM although activity slightly decreased as concentration of NaCl increased (Fig. 2c). These results show that KF2_Lys is stable at a broad range of temperatures, pH, and NaCl concentrations, which is furthers its potential as an antimicrobial agent.

The antimicrobial spectrum of KF2_Lys against several Gram-positive and Gram-negative bacteria

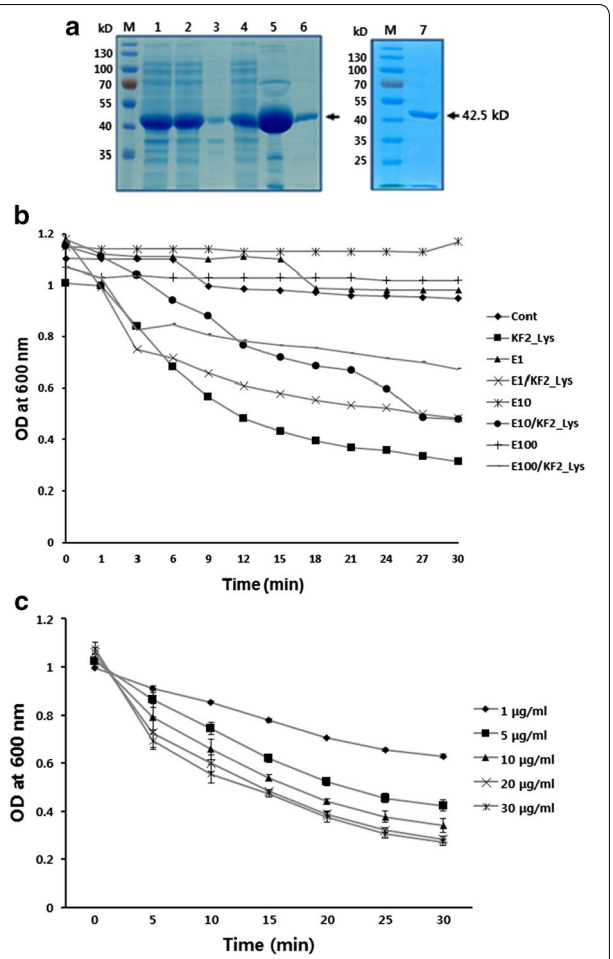

Fig. 1 Purification of KF2_Lys endolysin and its lytic activity. **a** 10% SDS-PAGE gel picture of KF2_Lys endolysin after purification. Lane 1: total lysate; lane 2: supernatant; lane 3: pellet; lane 4: flow-through; lane 5: eluate #1; lane 6: eluate #2; lane 7: buffer-exchanged eluate. **b** Effect of EDTA pretreatment of *V. parahaemolyticus* on lytic activity of KF2_Lys endolysin (20 μg/ml). E1, E10, E100: 1, 10, and 100 mM EDTA. The results of the experiment performed in triplicate are presented as a representative. **c** The lytic activities of various concentrations of KF2_Lys endolysin. Results of the experiment performed in triplicate are presented as mean ± SD

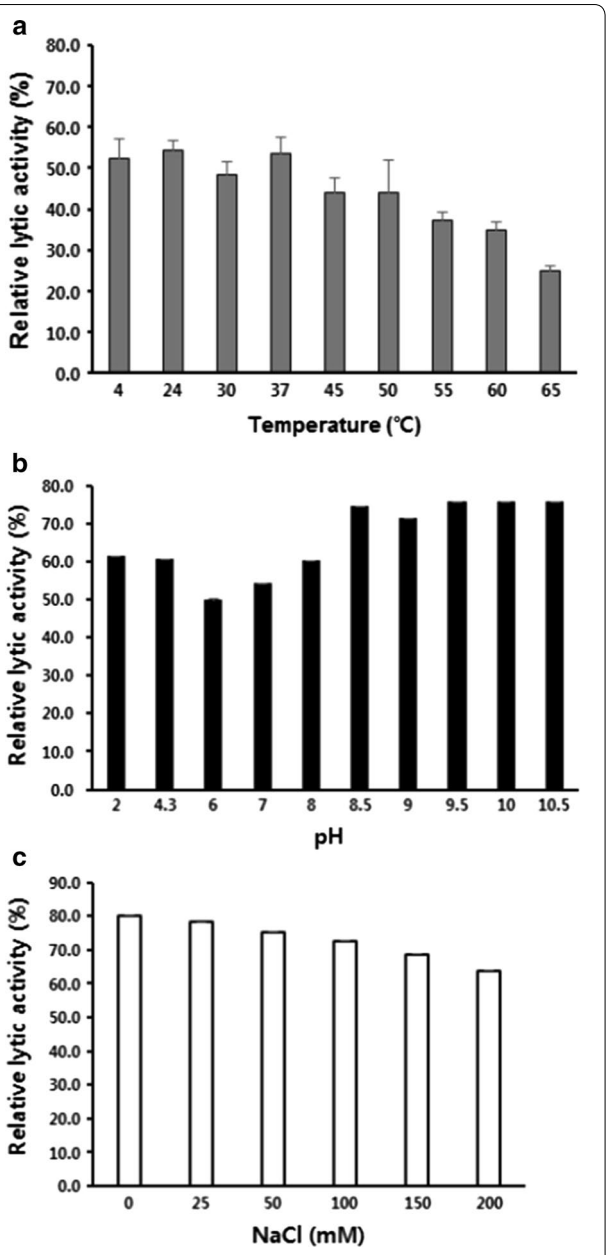

Fig. 2 Effects of different temperatures (**a**), pH (**b**), and NaCl concentrations (**c**) on the lytic activities of KF2_Lys endolysin. Results of the experiment performed in triplicate are presented as mean ± SD

was examined (Table 1). The lytic activity of KF2_Lys tended to be concentrated in *Vibrio* species. All *Vibrio* strains used in the experiment were susceptible to KF2_Lys although its activity against each strain differed. KF2_Lys also showed lytic activities against three strains classified in *E. coli* K12, *S.* Enteritidis, and *B. cereus*, respectively but those activities were very weak. All other Gram-negative and Gram-positive bacterial strains were resistant to KF2_Lys. In our previous studies, the bacteriophage vB_VpaP_KF2, had a narrow host range as it infected only 5 of the 18 tested *Vibrio* strains [9]. However, KF2_Lys endolysin from that phage showed a much broader range as it lysed 18 of the 18 tested strains. This implies that the applicability of endolysin has increased.

In conclusion, the recombinant endolysin KF2_Lys was overexpressed and purified to identify its lytic characteristics. As the endolysin has a broader lytic spectrum than its original vB_VpaP_KF2 phage and independently exhibits high lytic activities without the requirement of an outer-membrane permeabilizer, it

is considered a promising antimicrobial candidate for food chain applications.

Acknowledgements
This study was supported by the Main Research Program of the Korea Food Research Institute funded by the Ministry of Science and ICT (E0193120-02).

Authors' contributions
NL, HSC and HJC developed ideas and designed experiments. JAL and HJC carried out experiments and wrote the manuscript. NL, HSC and HJC revised the manuscript. All authors read and approved the final manuscript.

Funding
Ministry of Science and ICT (Korea Food Research Institute, Grant No. E0193120-02).

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate
Not applicable because we did not work with animals or humans.

Consent for publication
Not applicable.

Competing interests
All authors declare that there is no conflict of interests.

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Received: 7 April 2020 Accepted: 13 July 2020
Published online: 21 July 2020

References

1. CDC (2013) Antibiotic resistance threats in the United States, 2013. Current 114. https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-2508.pdf
2. O'Sullivan L, Bolton D, McAuliffe O, Coffey A (2019) Bacteriophages in food applications: from foe to friend. Annu Rev Food Sci Technol 10:151–172
3. Schmelcher M, Donovan DM, Loessner MJ (2012) Bacteriophage endolysins as novel antimicrobials. Future Microbiol 7:1147–1171
4. Borysowski J, Weber-Dabrowska B, Gorski A (2006) Bacteriophage endolysins as a novel class of antibacterial agents. Exp Biol Med 231:366–377
5. Oliveira H, Melo LD, Santos SB, Nóbrega FL, Ferreira EC, Cerca N, Azeredo J, Kluskens LD (2013) Molecular aspects and comparative genomics of bacteriophage endolysins. J Virol 87:4558–4570
6. Elmahdi S, DaSilva LV, Parveen S (2016) Antibiotic resistance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in various countries: a review. Food Microbiol 57:128–134
7. Lee LH, Ab Mutalib NS, Law JW, Wong SH, Letchumanan V (2018) Discovery on antibiotic resistance patterns of *Vibrio parahaemolyticus* in Selangor Reveals Carbapenemase producing *Vibrio parahaemolyticus* in Marine and freshwater fish. Front Microbiol 9:2513
8. Korea Food and Drug Administration (2019) Annual report of food and drug statistics. Vol. 21. www.mfds.go.kr/brd/m_371/list.do
9. Chang HJ, Hong J, Lee N, Chun HS, Kim HY, Choi SW, Ok G (2016) Growth inhibitory effect of bacteriophages isolated from western and southern coastal areas of Korea against *Vibrio parahaemolyticus* in Manila clams. Appl Biol Chem 59:359–365
10. Yu J, Lim JA, Kwak SJ, Park JH, Chang HJ (2018) Comparative genomic analysis of novel bacteriophages infecting *Vibrio parahaemolyticus* isolated from western and southern coastal areas of Korea. Arch Virol 163:1337–1343
11. Li M, Jin Y, Lin H, Wang J, Jiang X (2018) Complete Genome of a Novel Lytic *Vibrio parahaemolyticus* Phage VPp1 and Characterization of Its Endolysin for Antibacterial Activities. J Food Prot 81:1117–1125
12. Yu JH, Lim JA, Chang HJ, Park JH (2019) Characteristics and lytic activity of phage-derived peptidoglycan hydrolase, LysSAP8, as a potent alternative biocontrol agent for *Staphylococcus aureus*. J Microbiol Biotechnol 29:1916–1924
13. Son B, Yun J, Lim JA, Shin H, Heu S, Ryu S (2012) Characterization of LysB4, an endolysin from the *Bacillus cereus*-infecting bacteriophage B4. BMC Microbiol 12:33–41
14. Wang W, Li M, Lin H, Wang J, Mao X (2016) The *Vibrio parahaemolyticus*-infecting bacteriophage qdvp001: genome sequence and endolysin with a modular structure. Arch Virol 161:2645–2652
15. Gerstmans H, Rodriguez-Rubio L, Lavigne R, Briers Y (2016) From endolysins to Artilysin(R)s: novel enzyme-based approaches to kill drug-resistant bacteria. Biochem Soc Trans 44:123–128
16. Heselpoth RD, Euler CW, Schuch R, Fischetti VA (2019) Lysocins: bioengineered antimicrobials that deliver lysins across the outer membrane of gram-negative bacteria. Antimicrob Agents Chemother 63:e00342
17. Lim JA, Shin H, Heu S, Ryu S (2014) Exogenous lytic activity of SPN9CC endolysin against gram-negative bacteria. J Microbiol Biotechnol 24:803–811
18. Orito Y, Morita M, Hori K, Unno H, Tanji Y (2004) *Bacillus amyloliquefaciens* phage endolysin can enhance permeability of *Pseudomonas aeruginosa* outer membrane and induce cell lysis. Appl Microbiol Biotechnol 65:105–109

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