A Thesis for the Degree of Master of Science

Isolation and Characterization of Bacteriophage SAP4 and Its Endolysin LysSAP4 targeting *Staphylococcus aureus*

황색포도상구균을 감염시키는 박테리오파지 SAP4 및 이로부터 분리된 엔도라이신 LysSAP4의 특성 분석

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석사학위논문

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이 논문을 석사학위논문으로 제출함
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박승희

박승희의 석사학위논문을 인준함
2015 년 8 월

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ABSTRACT

Staphylococcus aureus is one of the most common foodborne pathogens that cause serious threats to human and animals. Prevalence of antibiotic-resistant *S. aureus*, such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), increases the necessity of alternative antibacterial agents those can replace traditional antibiotics. Bacteriophage and endolysin have been regarded as new bio-control agents against *S. aureus*. In this study, bacteriophage SAP4 targeting *S. aureus* was isolated and characterized. SAP4 is a prophage obtained from *S. aureus* clinical isolate. It belongs to *Siphoviridae* family with non-contractile tail and prolonged head, while most staphylococcal phages had icosahedral head. SAP4 had strong lytic activity against *S. aureus* and its activity was maintained over 29 h. It could infect 7 *S. aureus* strains including one MRSA and one additional staphylococcal strain (*S. haemolyticus*) among 18 staphylococcal strains tested. Genome analysis showed that phage SAP4 genome contains 43,120-bp double stranded DNA, encoding 61 open reading frames (ORFs) with no tRNA. Among 61 genes, a putative endolysin gene, which was highly homologous to *N*-acetyl-muramoyl-L-alanine amidase, was identified. LysSAP4, the endolysin of SAP4, contains two enzymatically

active domains (EADs) such as cysteine, histidine-dependent aminohydrolase/peptidase (CHAP) and *N*-acetyl-muramoyl-L-alanine amidase (MurNAc-LAA, amidase_3) and one cell wall binding domain (CBD) homologous to the SH3b domain. LysSAP4 was highly active between pH 8.0 and 37℃ in broad salt concentration conditions. Moreover, lytic activity was dependent on divalent metal ions, especially Zn^{2+}. The antimicrobial spectrum of LysSAP4 was broader than that of phage SAP4 that it could specifically kill all staphylococcal strains tested. Also the biofilm was reduced by about 70% with LysSAP4 treatment. CBD of LysSAP4 showed specific binding to staphylococcal strains. Taken together, LysSAP4 is a good candidate as a bio-control agent for *S. aureus*.

Keywords: *Staphylococcus aureus,* Bacteriophage, Endolysin

CONTENTS

Ⅰ. INTRODUCTION

Staphylococcus aureus is a major human and animal pathogen that causes both nosocomial and community-acquired infections. It is also cause of food poisoning. According to statistic, the Statistics Korea estimated in their fact sheet of December 2013 that there are over 60 illnesses annually caused by *S. aureus* (about 4% of total foodborne illnesses) in Korea, and based on Centers for Disease Control and Prevention (CDC) data, there are roughly 40 illnesses of *Staphylococcus spp.* (below 1% of total illnesses) annually in USA among 48 million illnesses caused by foodborne pathogens. Though the percentage of illnesses occupied only small portion, the control of *S. aureus* is very important because of prevalence of antibiotic-resistant *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA). Therefore, an alternative antibacterial agent is necessary to replace traditional antibiotics.

Bacteriophages (phages) are viruses specifically infecting bacteria and the most common and diverse entities in the biosphere (Bergh O 1989, Whitman WB 1998). Most of the phages belong to three families; the *Siphoviridae*, a long and non-contractile tail, the *Myoviridae*, a contractile tail with neck and sheath, and the *Podoviridae*, a short and non-contractile

tail (Ackermann HW 2009). Phages are generally divided into two basic classes, virulent and temperate (Lenski 1998). Virulent phages bring about rapid lysis and death of the host bacterial cell, whereas temperate phages spend part of their life cycle in a quiescent state called prophage (Hanlon 2007). Phages have been regarded as new bio-control agents for *S. aureus* because of high specificity to target their host determined by bacterial cell wall receptors, so each phage can only attack one species or in some cases a single strain of bacterium (Hanlon 2007, Sillankorva *et al*. 2012). Many papers to control MRSA were studied in vitro and in vivo (Borysowski J 2011, Chhibber *et al.* 2013). And phage application to dairy foods such as cheese (Bueno *et al*. 2012) and curd (Pilar García 2007), and for reducing biofilm (Drilling A 2014, Rahman M 2011) were conducted to control *S. aureus*.

Recently, endolysins (lysins) have been considered as an important therapeutic alternative to phages. Lysins are bacteriolytic enzymes encoded by phage that hydrolyze the peptidoglycan bacterial cell wall. It do not have signal sequence so can not work alone endogeneously, whereas holins make holes in the cytoplasmic membrane allowing the lysin to degrade the peptidoglycan at a specific scheduled time defined as the canonical holin-endolysin lysis system (Catalao *et al*. 2013, Young I 2000, Ziedaite *et al*.

2005). Because of the peptidoglycan- degradable property of lysins, there are able to rapidly lyse specifically targeting to Gram-positive bacteria when treated exogenously. Moreover, it shows fewer side effects compared with antibiotics and have a low probability of developing resistance (Fischetti 2010). Several studies about endolysin showed that a high bactericidal activity against various Gram-positive strains including VRSA (Pauline Yoong 2004, Proenca *et al*. 2012).

In general, domain modular architecture of lysins largely divided in two parts; an N-terminal enzymatically active domain and a C-terminal cell wall binding domain. The N-terminal catalytic domain have been classified into four different groups according to the cleavage sites; *N*-acetyl-muramidases (lysozymes), *N*-acetyl-β-D-glucosaminidases (glycosidases), which hydrolyze the β-1-4 glycosidic bond in the sugar moiety of the cell wall, *N*-acetylmuramoyl-L-alanine amidases, which cleave the amide bond connecting the sugar and peptide moieties of the bacterial cell wall L-alanoyl-D-glutamate endopeptidases and interpeptide bridge-specific endopeptidases, which attack the peptide moiety of the cell wall peptidoglycan (Catalao *et al*. 2013, Fischetti 2008). In some cases of staphylococcal lysins, two and even three different enzymatically active domains may be linked to a single cell wall binding domain (William Wiley

Navarre 1999). The C-terminal cell wall binding domain of the enzyme attach to its specific substrate in the bacterial cell wall via non-covalent binding of carbohydrate ligands (LoessnerMJ *et al*. 2002).

To data, most characterized *S. aureus Siphoviridae* phage had an isometric head (Xia and Wolz 2014). In addition, the endolysin of *S. aureus* bearing two of enzymatically active domains; CHAP and Amidase_3 linked to SH3b as a single cell wall binding domain was not well characterized yet, whereas the endolysin consisted of CHAP, Amidase_2 (PGRP) and SH3b was already characterized (Donovan *et al*. 2006, Garcia *et al*. 2010).

In this study, I isolated and characterized a novel phage SAP4 and its endolysin LysSAP4 targeting *S. aureus*. This phage had prolonged head, which are not easily found in *S. aureus* phage, and effective lytic activity persist more than a day. LysSAP4 endolysin composed of CHAP, Amidase_3 and SH3b domains showed high lytic activity and broadly lyse staphylococcal strains tested.

Ⅱ. MATERIALS AND METHODS

2.1. Bacterial strains, media and growth conditions

Staphylococcus aureus RN4220 strain was used as a host for phage SAP4 and the substrate for the LysSAP4 endolysin. Bacterial strains used in this study are 21 of staphylococcal strains, 4 of Gram-positive bacteria including *Enterococcus faecalis*, *Bacillus cereus, Bacillus subtilis* and *Listeria monocytogenes* and 5 of Gram-negative bacteria including *Salmonella enterica serovar Typhimurium, Escherichia coli, Cronobacter sakazakii* and *Pseudomonas aeruginosa* (Table 1). All staphylococcal strains were grown in Tryptic Soy Broth (TSB) (Difco, Deroit, MI, USA) at 37℃ with shaking for 12 h. The other non-staphylococcal strains were grown in Luria-Bertani (LB) broth medium (Difco) under same growth conditions of staphylococcal strains. *E.coli* DH5α and *E.coli* BL21(DE3)star were used as the host for construction and expression of recombinant LysSAP4.

2.2. Phage SAP4 isolation and propagation

To isolate prophage within a chromosome of *S. aureus* clinical isolate 0136 from Gyeongsang National University Hospital, 1 mM

mitomycinC (Sigma) was treated in *S. aureus* cell culture under early-exponential phase, and further incubated for 3 h at 37℃ with shaking condition. After centrifugation of the cultures (10,000 g, 10 min, 4℃) and filtration of the supernatants using 0.45 μm pore size filters (Millipore, Billerica, MA). To confirm the presence of phages in the supernatant, 1 mL of filtrates were mixed with molten 0.4 % TSB soft agar containing 1% *S. aureus* RN4220 as a host and then poured on TSA plate. The plate was incubated for overnight at 37℃ and monitored for a plaque formation. The plaques were picked sterile tip and eluted in 0.5 μL of SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$) with the filtration. 10-fold serial dilutions of these filtrates were dotted on molten 0.4 % TSB soft agar containing 1% *S. aureus* RN4220 and incubated for 12 h at 37℃. Each single plaque was picked using sterile tip and eluted in 0.5 μL of SM buffer, followed by filtration. This purification step was repeated at least three times.

The cell culture of *S. aureus* RN4220 under early-exponential phase was infected with phage at a multiplicity of infection (M.O.I) of 1.0 and incubated for 3 h at 37℃ with shaking. Centrifugation at 10,000 g for 15 min and filtration using 0.45 μm pore size filters and phage particles were precipitated by treatment of polyethylene glycol (PEG) 6,000 (Sigma).

Finally, CsCl density gradient ultracentrifugation (himac CP 100β, Hitachi, Japan) with different CsCl densities (1.3, 1.45, 1.5 and 1.7 g/mL) at 25,000 g, 4℃ for 2 h. Viral particles were recovered and dialyzed using dialysis buffer (50 mM Tris-Cl, pH 7.5, 10 mM $MgSO_4$) and stored at 4℃.

2.3. Transmission electron microscopy (TEM)

Diluted phage sample in SM buffer (approximately 10^9 CFU/mL) was put on carbon-coated copper grids and negatively strained with 2% aqueous uranyl acetate (pH 4.0) for 30 sec. The strained phages were observed by transmission electron microscope (LIBRA 120, Carl Zeiss, Germany) at 80kV at the National Academy of Agricultural Science (South Korea). The phage was identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses (Fauquet and Fargette 2005, Shin et al. 2011)

2.4. Bacterial challenge assay

To confirm the lytic activity of phage SAP4, 1% overnight culture of *S. aureus* RN4220 was added in 50 mL TSB broth and incubated at 37℃ with shaking until an early-exponential phase (OD_{600} value is about 0.3). At

that time, phage was added in it at M.O.I of 1.0 with 10 mM $CaCl_2$. And then, OD_{600} was monitored every hour up to emerging *S. aureus* resistance against phage, and the culture without phage was used as a control sample.

2.5. Bacteriophage host range

A 100 μL of overnight bacterial seed culture was added to 5 mL of the molten 0.4% TSB soft agar containing 10 mM $CaCl_2$. The mixture was overlaid on the 1.5% TSA agar plate. 10 μL of serial dilutions of phage SAP4 stock (from 10^2 to 10^{10}) was dotted on the overlaid bacterial lawns. After drying for 20 min at room temperature, these plates were incubated at 37℃ for overnight. The formation of a single plaque or the inhibition zone was observed to determine the sensitivity of each bacterium.

2.6. Receptor analysis

To identify receptor of phage SAP4, phage SAP4 which titer is about 10^9 PFU/mL was serially diluted and spotted onto the each molten 0.4% TSB soft agar lawn of *S. aureus* RN4220, *S. aureus* RN4220 *ΔtagO*, which is deficient in wall teichoic acid (WTA) and *ΔtagO* pBR474::*tagO* which is WTA complemented strain followed by incubation at 37℃ for overnight. The formation of a single plaque was observed to determine the infection

ability of each strain.

2.7. Bacteriophage genomic DNA purification

Phage genomic DNA was isolated from the phage lysate (10^9 PFU/mL) (SA Wilcox 1996). To remove bacterial DNA and RNA, phage sample was treated with 1 μg/mL of DNase and RNaseA for 30 min at 37℃, respectively. Lysis buffer (0.5% of Sodium dodecyl sulfate, 20 mM of EDTA, pH 8.0 and 50 μg/mL of proteinase K) was treated to the samples for 15 min at 65℃. A standard phenol-chloroform DNA purification and ethanol precipitation were carried out.

2.8. Full-genome sequencing of bacteriophage and bioinformatics analysis

To sequencing of extracted whole genomic DNA of phage SAP4, a pyrosequencing was conducted using the Genome Sequencer FLX titanium sequencer (Roche, Mannheim, Germany) and assembled with GS *de novo* assembler software (v. 2.60) at Macrogen Inc., South Korea. Prediction of all open reading frames (ORFs) was carried out using GeneMarkS (J Besemer 2001), Glimmer 3.02 (Delcher et al. 2007), and FgenesB (Softberry, Inc., USA) softwares. The predicted ORFs were annotated according to the results

of BLASTP (SF Altschul 1990), InterProScan (EM Zdobnov 2001), and NCBI Conserved Domain Database (Marchler-Bauer et al. 2007). tRNAs were predicted by tRNAscan-SE (TM Lowe 1997). RBSfinder which predicts ribosomal binding sites (RBS) was used to confirm ORFs prediction (J. Craig Venter Institute, USA). Comparison of amino acid sequences was conducted using ClustalX (JD Thompson 1997) and GeneDoc program (KB Nicholas 1997).

2.9. Cloning, overexpression and purification of LysSAP4 and EGFP-LysSAP4_CBD

The endolysin gene (*lysSAP4*) was amplified from the genome of the phage SAP4 by polymerase chain reaction using the primer pairs *lysSAP4*_F (5'-GGTAGGTGAGGATCCATGTTGATA-3') and *lysSAP4*_R (5'-GTAAGATTCCCTCGAGTTAATGTAGC-3'). The 5' ends of the genes are appended with a BamHI restriction site and the 3' ends with a XhoI restriction site. These restriction sites were then used to clone into a pET28a vector (Novagen, Madison, WI), which coded for a N-terminal hexahistidine (His)-tag. The correctly cloned plasmid was transformed into competent *E. coli* BL21(DE3)star. Expression of the recombinant LysSAP4 was inducted with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at OD_{600} of 1.0,

followed by incubation for additional 5 h at 30℃. Bacterial cells were suspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride) and disrupted for 15 min with 5 sec pulses and 15 sec rest by sonication (Branson Ultrasonics, Danbury, CT). After centrifugation at 9,000 g for 30 min, the supernatant was passed through a Ni-NTA Superflow column (Qiagen Gnbh, Germany), and purification of the recombinant LysSAP4 was performed according to the manufacturer's instruction. The purified protein was stored at - 80℃ until use after the buffer was changed to the storage buffer (50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 30% glycerol) using PD Midirtap G-25 (GE Healthcare, Amersham, Bucks, UK).

2.10. SyTox kinetic assay

250 μL of an overnight seed culture of *S. aureus* RN4220 grown in TSB was added into 25 mL fresh TSB, and grown at 37℃ with shaking for 2 h 30 min, the OD_{600} of the cells was about 1.0. Cells were harvested by centrifugation, washed once with PBS buffer (Green 2012), and then suspended in reaction buffer (50 mM sodium phosphate, pH 8.0) to a OD_{600} of 2.0. The assay was performed in black 96-well plates (BD Falcon™), and

the wells consisted of 300 μL reaction buffer containing cells at a final OD_{600} of 1.0, SyTox Green at 5 μM (Invitrogen), and enzyme at the specified concentrations. Mean fluorescence intensity readings were taken every 1 min on a SpectraMax i3 multimode mcroplate reader (Molecular Devices, Sunnyvale, CA) using an excitation of 504 nm and emission of 524 nm. Statistical analyses and data presentation were produced in GraphPad Prism v.5 software (La Jolla, CA).

In all variations of the assay, the total volume of 300 μL and final SyTox Green concentration of 5 μM were maintained. To check the effect of pH, temperature and sodium chloride (NaCl) concentration on the lytic activity of LysSAP4, the wells were supplemented with appropriate buffer in test conditions. In salt dependency assay, salt stocks at pH 8.0, 25℃ were diluted appropriately into the assay. In pH dependency assay, reaction buffer was replaced by 0.1% trifluoroacetic acid at pH 2.0, 50 mM sodium acetate at pH 4.0, 50 mM 2-ethanesulfonic acid at pH 6.0, 50 mM bis-tris at pH 7.0, 50 mM Tris-HCl at pH 8.0 and 50 mM *N*-cyclohexyl-3-aminopropanesulfonic acid at pH 10.0. In temperature dependency assay, LysSAP4 was added into reaction buffer (50 mM sodium phosphate, pH 8.0). The residual enzyme activity was determined by using *S. aureus* RN4220 as substrate for 30 min in all assay and compensated control effect when needed.

To check antimicrobial spectrum of LysSAP4, previously described SyTox kinetic assay was conducted using various bacterial strains tested in Table 1.

2.11. Biofilm reduction assay

1% overnight seed culture of *S. aureus* Newman grown in TSB added 0.25% D-(+)-glucose was added into 200 μL fresh media in 96-well plate (SPL), and grown at 37℃ with shaking for 24 h. The supernatant was removed and incubated at 60℃ for 1 h to fix the biofilm. According to the test conditions, PBS buffer treated only as a control and 30 μg of LysSAP4 was treated at experimental group followed by incubation at 37℃ with shaking for 1 h. After removing supernatant, remaining biofilm was stained by 0.1% crystal violet at room temperature with no shaking for 5 min. Carefully PBS washing three times and eluted biofilm using 33% acetic acid and finally measured OD_{570} values to quantify the amount of biofilm.

2.12. GFP fluorescence assay

Cell wall binding activity of EGFP-LysSAP4_CBD was observed by fluorescence microscopy. 1% of seed culture of bacteria tested was added

into fresh TSB and incubated at 37℃ for 2 h (OD_{600} of 0.6 ~ 0.8). 1 mL of incubated cell culture was harvested with centrifugation (16,000 g, 1 min), then washed and re-suspended with PBS buffer to a concentration of about $1x10^7$ CFU/mL. After mixing 200 µL of the cell solution with 100 µL of 0.8 µM EGFP-LysSAP4_CBD diluted in PBS buffer, the mixture was incubated at room temperature for 5 min. After incubation, the cells were separated from the supernatants by centrifugation (16,000 g, 1 min), washed three times with PBS and re-suspended in PBS before fluorescence microscopy. The fluorescence images were obtained using a Carl Zeiss DE/Axio Imager A1 (ZEISS, Germany) with a 100x magnification oil-immersion objection lens. The exposure time used was set at 0.8 s when capturing all the fluorescence images. Individual images were obtained using suitable filter settings, and colour channels.

III. RESULTS

3.1. Isolation and characterization of bacteriophage SAP4

Staphylococcal phage SAP4 was prophage that was obtained by mitomycin C induction from *Staphylococcus aureus* clinical isolate 0136. The isolated phage SAP4 formed clear and small size (1 ~ 2 mm) plaques against *S. aureus* RN4220.

Transmission electron microscopy (TEM) analysis of phage SAP4 revealed an elongated head with a non-contractile tail (Fig. 1). Its head was approximately 131.5 nm in height and 55.7 nm in width. The tail length was about 303.5 nm. Based on these morphological characteristics, phage SAP4 was assigned to *Siphoviridae* family, serogroup A which features a prolate head and non-contractile long tail (Ackermann H. W. 2001).

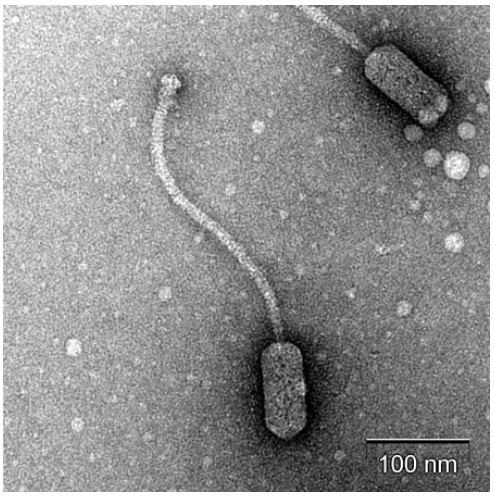


Figure 1. Electron micrograph of phage SAP4. Purified phage particles were negatively stained with 2% uranyl acetate. Transmission electron microscopy of phage SAP4 revealing that it belongs to *Siphoviridae* family with elongated head. The scale bar appeared at the bottom right corner of the image.

3.2. Challenge assay

The effect of phage SAP4 infection on *S. aureus* RN4220 was observed through the inoculation of a bacterial culture in early-exponential phage (OD_{600} of 0.3) at M.O.I of 1.0. As a result, phage SAP4 was highly effectively inhibited the growth of *S. aureus* cells and the antibacterial activity was maintained over 29 h (Fig. 2).

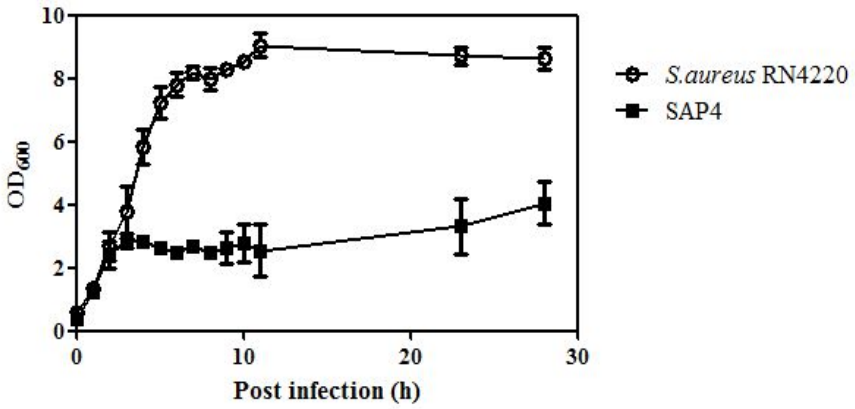


Figure 2. Growth curves of *S. aureus* in the presence or absence of phage SAP4. When optical density at 600 nm reached 0.3 (10^8 CFU/mL), phage lysates were added to the cell culture at M.O.I of 1.0. After that incubated at 37℃ with shaking and OD_{600} was measured at each time. ○, bacterium-only control; ■, phage SAP4 added to the bacteria.

3.3. Host range of bacteriophage SAP4

To check up antimicrobial spectrum of phage SAP4, various bacterial strains of staphylococcal and other species were used at the spotting assay. Phage SAP4 could form single plaque in 7 *S. aureus* strains including MRSA and one *S. haemolyticus* among 18 staphylococcal strains tested. It formed the inhibition zone in 5 *S. aureus* strains. However, other Gram-positive bacteria without *Staphylococcus* and Gram-negative bacteria were not infected (Table 1).

Table 1. The host range of phage SAP4

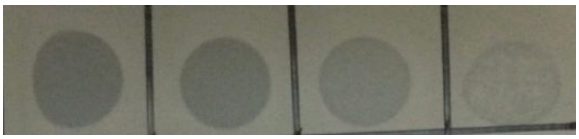
Bacterial host	SAP4[a]	Reference of source[b]
Staphylococcal strains		
S. aureus RN4220	+	(Park K. H. *et al*. 2010)
S. aureus Newman	+	(Baba *et al*. 2008)
S. aureus ATCC 13301	+	ATCC
S. aureus ATCC 23235	+	ATCC
S. aureus ATCC 33586	+	ATCC
S. aureus ATCC 33593	+	ATCC
S. aureus KCTC 1916	**I**	KCTC
S. aureus ATCC 6538	**I**	ATCC
S. aureus ATCC 29213	**I**	ATCC
S. aureus ATCC 12600	**I**	ATCC
MRSA CCARM 3793	+	CCARM
MRSA CCARM 3090	**I**	CCARM
MRSA CCARM 3089	**-**	CCARM
S. haemolyticus ATCC 29970	+	ATCC
S. epidermidis ATCC 35983	**-**	ATCC
S. hominis ATCC 37844	-	ATCC
S. warneri ATCC 10209	**-**	ATCC
S. sapitis ATCC 35661	**-**	ATCC
Other Gram positive bacteria		
Enterococcus faecalis ATCC 29212	-	ATCC
Bacillus cereus ATCC 14579	-	ATCC
Bacillus subtilis ATCC 23857	-	ATCC
Listeria monocytogenes ATCC 19114	-	ATCC
Gram negative bacteria (EDTA-treated)		
Salmonella enterica serovar Typhimurium SL1344	-	NCTC
Escherichia coli MG1655 ATCC 47076	-	ATCC
Escherichia coli O157:H7 ATCC 35150	-	ATCC
Cronobacter sakazakii ATCC 29544	-	ATCC
Pseudomonas aeruginosa ATCC 27853	-	ATCC

[a], +, single plaque; I, inhibition zone; -, no plaque.

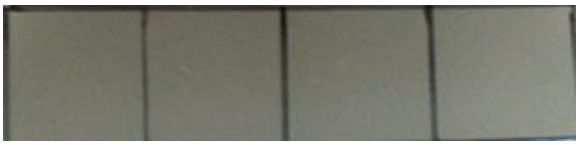
[b], ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CCARM, Culture Collection of Antimicrobial Resistant Microbes.

3.4. Receptor determination

Phage SAP4 formed plaques on the bacterial lawn of wild-type RN4220 but didn't form plaques on the *ΔtagO* mutant, indicating that the infections dependent on wall teichoic acid (WTA). WTA complemented strain recovered the susceptibility to *S. aureus* RN4220 (Fig. 3).



S. aureus RN4220



S. aureus RN4220 *ΔtagO* mutant

S. aureus RN4220 *tagO* complemented

(*ΔtagO* pBR474::*tagO*)

Figure 3. Phage receptor determinization. Phage SAP4 could infect *S. aureus* RN4220 but not infect *tagO* mutant strain. When complemented *tagO*, phage SAP4 recovered infection ability against *S. aureus*.

3.5. Whole genome analysis of bacteriophage SAP4

The complete genome of phage SAP4 showed a double stranded circular DNA, 43,120 bp in size with a GC content of 34 %, 61 open reading frames (ORFs) and tRNA did not exist (Fig. 4). Phage genome consisted with ORFs arranged in a modular structure and assigned to different functional groups such as phage structure, packaging, replication, regulation, cell lysis, integration and additional function.

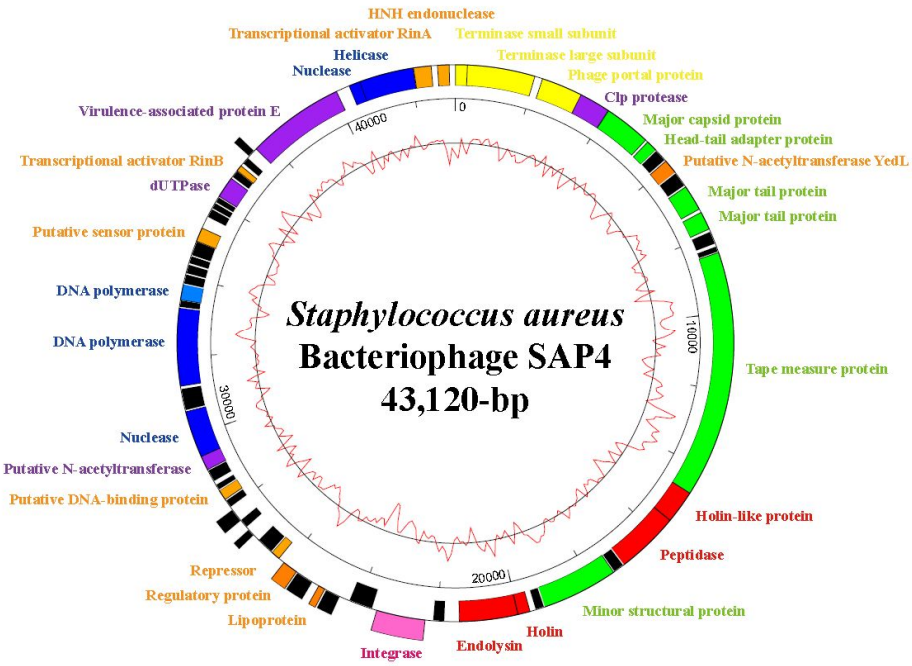


Figure 4. Genome map of phage SAP4. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories such as phage structure (green), packaging (yellow), replication (blue), regulation (orange), cell lysis (red), integration (pink) and additional function (purple). The inner circle line indicate the GC content. Scale unit is base pair.

3.6. Identification of a phage endolysin, LysSAP4

According to BlastP analysis (Altschul *et al*. 1997), a putative endolysin gene was predicted at ORF 19 of 1455-bp lengths which had high homology with *N*-acetylmuramoyl-L-alanine amidase of *Staphylococcus aureus* A9765 (EFB98990.1) and it was designated as *lysSAP4*. Using the web site of InterProScan proGram (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) and the Conserved Domain Database (Marchler-Bauer *et al*. 2007), LysSAP4 consist two enzymatically active domains (EADs) and one cell wall binding domain (CBD). The predicted EADs of LysSAP4 contain CHAP (cysteine, histidine-dependent aminohydrolase/peptidase) domain (PF05257) and *N*-acetyl-muramoyl-L-alanine amidase (MurNAc-LAA, Amidase_3) (PF01520) and the CBD had high homology with SH3b domain (PF08460) (Fig. 5). The domain composition of N-terminal EAD and C-terminal CBD had most endolysins isolated from phages infecting Gram-positive bacteria (Fischetti 2008). However, LysSAP4 composed of Amidase_3 domain was not similar to the well-characterized staphylococcal phage endolysin which had Amidase_2 domain such as phiSauS-IPLA88 (YP_002332536.1), phiMR11 (YP_001604156.1), GRCS (YP_009004305.1) and K (YP_009041293.1). A subset of the MurNAc-LAA family binds the three Zn^{2+} coordinating residues (His187, Glu198, His254) to exhibit Zn^{2+} dependent amidase

activity like T7 lysozyme (Xiaodong Cheng 1994).

To characterize biochemical properties of LysSAP4, *lys*SAP4 was cloned and expressed in *E. coli* with N-terminal His-tag followed by purification using affinity chromatography. SDS-PAGE showed a single band with the purified LysSAP4, about 57 kDa in size and pI was 9.35. (Fig. 6a). The purified recombinant LysSAP4 showed lytic activity against *S. aureus* cells (Fig. 6b).

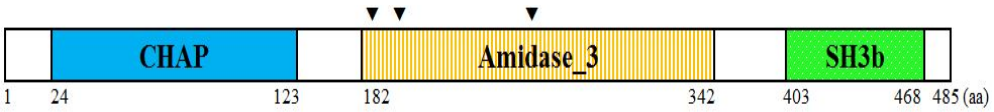


Figure 5. Schematic diagram of LysSAP4. LysSAP4 is composed of two EADs; CHAP and Amidase_3 domains and one CBD;.SH3b. Three reversed triangles indicate Zn^{2+} binding residues (His187, Glu198, His254) in amidase_3 domain.

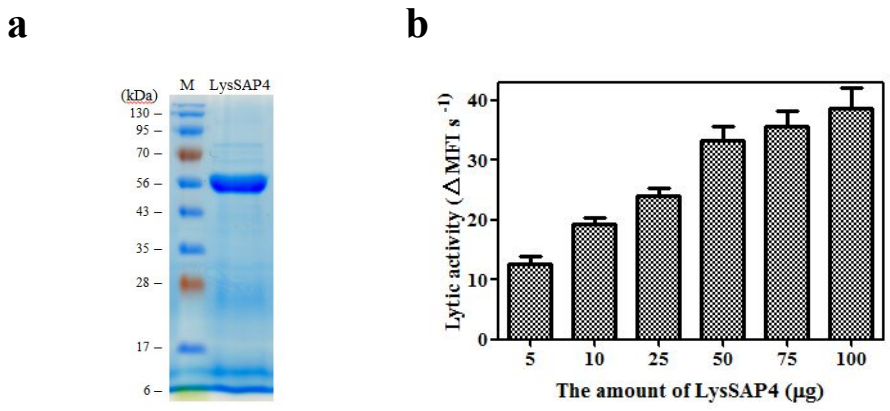


Figure 6. Purification and lytic activity of LysSAP4. (a) SDS-PAGE of the purified LysSAP4. Lane M; molecular weight marker. (b) Lytic activity of the various concentrations of LysSAP4 against *S. aureus* RN4220 in exponential growth phase as determined by a SyTox kinetic assay. ΔMFI s^{-1} means the change of mean fluorescence intensity per second.

3.7. Effect of pH, temperature and ionic strength of LysSAP4

Exogenous lytic activity of LysSAP4 was examined under different conditions. The lytic activity was highest at pH 8.0 (Fig. 7a), 37℃ (Fig. 7b) and a wide range of salt concentrations from 0 mM to 400 mM (Fig. 7c).

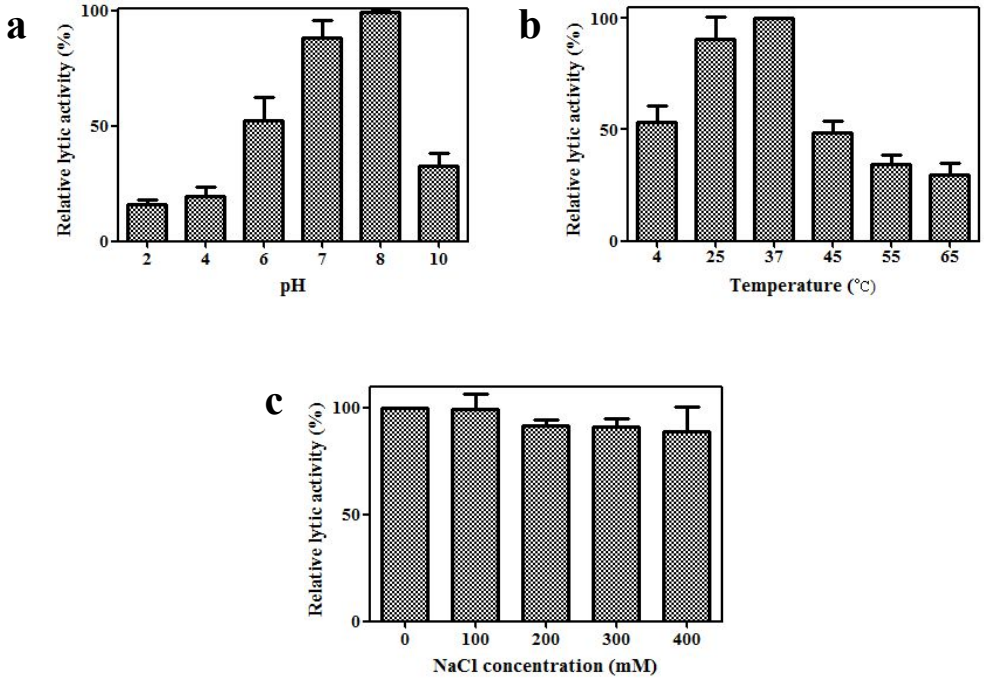


Figure 7. The effect of pH, temperature and NaCl concentration on the lytic activity of LysSAP4. Aliquouts of the enough endolysin (30 μg) were added in *S. aureus* cell cultures followed by measured lytic activity by using SyTox kinetic assay for 30 min. (a) pH, (b) temperature and (c) NaCl concentration. Each column represents the mean of triplicate assays within standard deviation.

3.8. Effects of divalent metal ions

The lytic activity of LysSAP4 which treated 5.0 mM EDTA was significantly decreased because of removing a metal ion effect, which suggests this enzyme required metal ion for full catalytic function. When 1.0 mM Zn^{2+} was added to the EDTA-treated endolysin, the lytic activity of the enzyme was restored (Fig. 8). In case of other divalent metal ions, such as Ca^{2+}, Mn^{2+} and Mg^{2+} did not restore the lytic activity of the EDTA-treated endolysin. Taken together, LysSAP4 requires divalent metal ion, particularly Zn^{2+} for its full enzymatic activity.

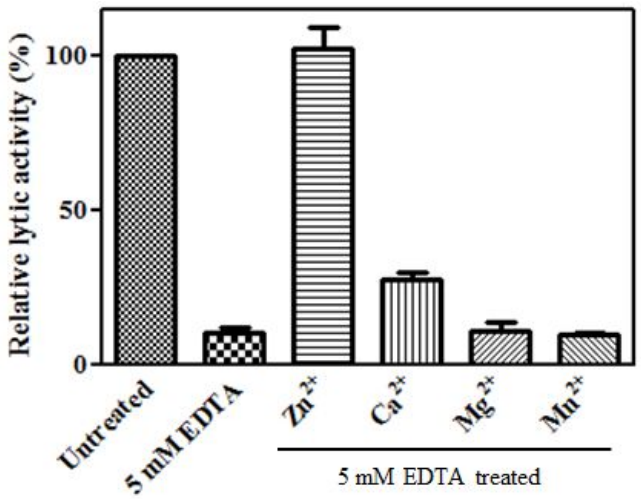


Figure 8. The effect of metal ions on lytic activity of LysSAP4 after treatment with EDTA. Untreated means LysSAP4 was not any treatment. After 5.0 mM EDTA treatment to LysSAP4, each divalent metal ions (1.0 mM) was added and measured OD_{600}. The lytic activity was measured by comparing with it of the no EDTA-treated control. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation.

3.9. Antimicrobial activity spectrum of LysSAP4

LysSAP4 showed lytic activity against all staphylococcal strains tested (Table 2) and it was more broader antimicrobial activity spectrum compared with phage SAP4. In particular, LysSAP4 could infect all 3 of MRSA strains tested and additional staphylococcal strains such as *S. epidermidis*, *S. warneri* and *S. sapitis*. The endolysin did not show lytic activity against non-staphylococcal Gram-positive bacteria and Gram-negative bacteria.

Table 2. Antimicrobial spectrum of endolysin LysSAP4

Bacterial host	LysSAP4[a]	Reference of source[b]
Staphylococcal strains		
S. aureus RN4220	+	(Park K. H. *et al*. 2010)
S. aureus Newman	+	(Baba *et al*. 2008)
S. aureus ATCC 13301	+	ATCC
S. aureus ATCC 23235	+	ATCC
S. aureus ATCC 33586	+	ATCC
S. aureus ATCC 33593	+	ATCC
S. aureus KCTC 1916	+	KCTC
S. aureus ATCC 6538	+	ATCC
S. aureus ATCC 29213	+	ATCC
S. aureus ATCC 12600	+	ATCC
MRSA CCARM 3793	+	CCARM
MRSA CCARM 3090	+	CCARM
MRSA CCARM 3089	+	CCARM
S. haemolyticus ATCC 29970	+	ATCC
S. epidermidis ATCC 35983	+	ATCC
S. hominis ATCC 37844	+	ATCC
S. warneri ATCC 10209	+	ATCC
S. sapitis ATCC 35661	+	ATCC
Other Gram positive bacteria		
Enterococcus faecalis ATCC 29212	-	ATCC
Bacillus cereus ATCC 14579	-	ATCC
Bacillus subtilis ATCC 23857	-	ATCC
Listeria monocytogenes ATCC 19114	-	ATCC
Gram negative bacteria (EDTA-treated)		
Salmonella enterica serovar Typhimurium SL1344	-	NCTC
Escherichia coli MG1655 ATCC 47076	-	ATCC
Escherichia coli O157:H7 ATCC 35150	-	ATCC
Cronobacter sakazakii ATCC 29544	-	ATCC
Pseudomonas aeruginosa ATCC 27853	-	ATCC

[a], +, lysis activity; -, no lytic activity.

[b], ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CCARM, Culture Collection of Antimicrobial Resistant Microbes.

3.10. Biofilm reduction assay

In order to identify biofilm reduction ability of LysSAP4, PBS buffer only as a control and LysSAP4 were treated in each walls. As a result, when treated LysSAP4, biofilm was reduced approximately 70 % compared with control (Fig. 9).

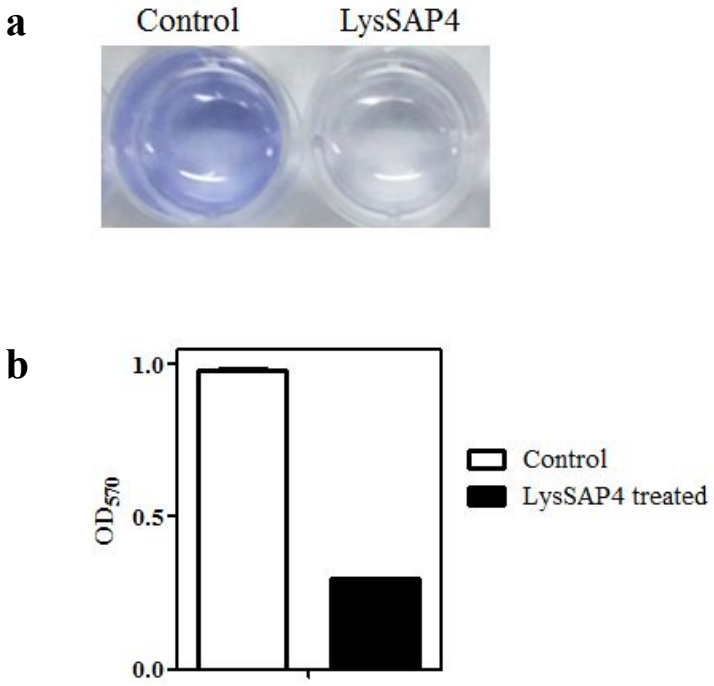


Figure 9. Biofilm reduction assay. Biofilm was stained by 0.1% crystal violet. OD_{570} was measured after each treatment. (a) The plate images of biofilm reduction assay, (b) Quantification of remaining biofilm measured by OD_{570} values. Biofilm was removed about 70% by LysSAP4 treatment. Each column represents the mean of triplicate assays within standard deviation.

3.11. EGFP-LysSAP4_CBD recombinant expression, purification and binding spectrum of the fusion protein.

To check binding ability of LysSAP4_CBD, *lysSAP4_CBD* was cloned with EGFP::pET28a and expressed in *E. coli* with N-terminal His-tag followed by purification using affinity chromatography. SDS-PAGE showed a single band with the purified EGFP-LysSAP4_CBD, about 42 kDa in size and pI was predicted as 8.19 (Fig. 10a). The purified EGFP-LysSAP4_CBD fusion protein bound to cells from 0.4 μM and measured fluorescence of EGFP-LysSAP4_CBD binding to the cell surface (Fig. 10b). When check binding spectrum, EGFP-LysSAP4_CBD had broad binding spectrum against all staphylococcal strains including MRSA (Table 3).

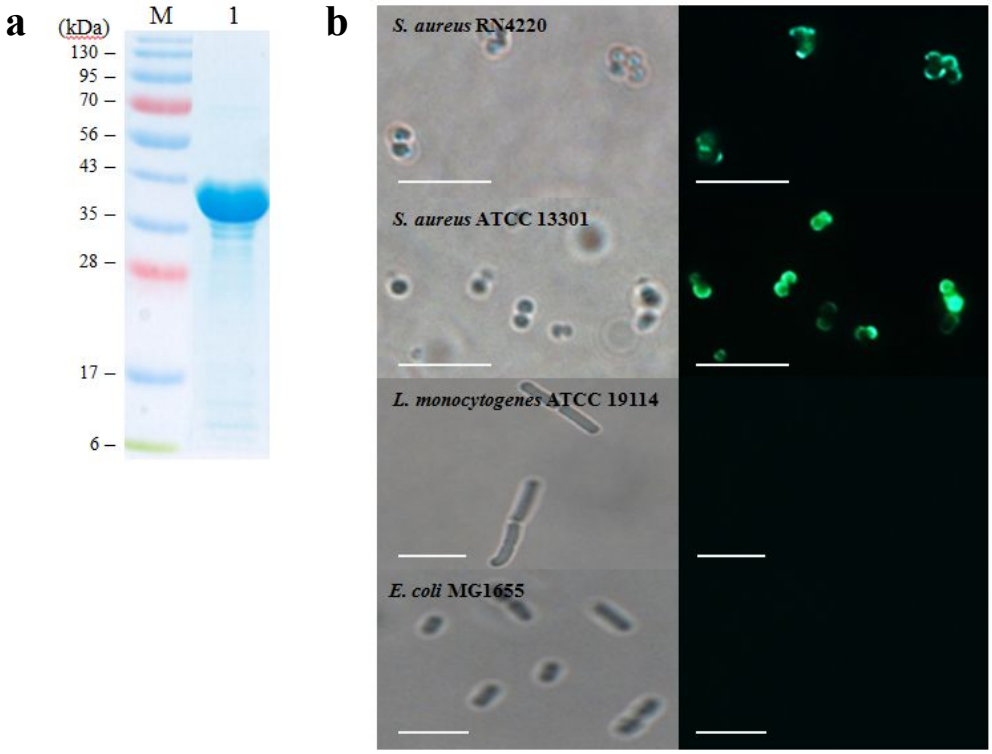


Figure 10. Purification and fluorescence images of bacterial cells strained with EGFP-LysSAP4_CBD fusion protein. (a) SDS-PAGE of the purified EGFP-LysSAP4_CBD. M, molecular weight marker; lane 1, the purified EGFP-LysSAP4_CBD fraction. (b) Fluorescence images of bacterial cells stained with EGFP-LysSAP4_CBD fusion protein. Bar size : 5 μm, all panels are viewed through a 100x magnifications oil-immersion objective lens. The exposure times is set at 0.8 s.

Table 3. Binding spectrum of EGFP-LysSAP4_CBD

Bacterial host	EGFP-LysSAP4_CBD[a]	Reference of source[b]
Staphylococcal strains		
S. aureus RN4220	+	(Park K. H. *et al*. 2010)
S. aureus Newman	+	(Baba *et al*. 2008)
S. aureus ATCC 13301	+	ATCC
S. aureus ATCC 23235	+	ATCC
S. aureus ATCC 33586	+	ATCC
S. aureus ATCC 33593	+	ATCC
S. aureus KCTC 1916	+	KCTC
S. aureus ATCC 6538	+	ATCC
S. aureus ATCC 29213	+	ATCC
S. aureus ATCC 12600	+	ATCC
MRSA CCARM 3793	+	CCARM
MRSA CCARM 3090	+	CCARM
MRSA CCARM 3089	+	CCARM
S. haemolyticus ATCC 29970	+	ATCC
S. epidermidis ATCC 35983	+	ATCC
S. hominis ATCC 37844	+	ATCC
S. warneri ATCC 10209	+	ATCC
S. sapitis ATCC 35661	+	ATCC
Other Gram positive bacteria		
Enterococcus faecalis ATCC 29212	-	ATCC
Bacillus cereus ATCC 14579	-	ATCC
Bacillus subtilis ATCC 23857	-	ATCC
Listeria monocytogenes ATCC 19114	-	ATCC
Gram negative bacteria		
Salmonella enterica serovar Typhimurium SL1344	-	NCTC
Escherichia coli MG1655 ATCC 47076	-	ATCC
Escherichia coli O157:H7 ATCC 35150	-	ATCC
Cronobacter sakazakii ATCC 29544	-	ATCC
Pseudomonas aeruginosa ATCC 27853	-	ATCC

[a], +, EGFP-LysSAP4_CBD was binding to the bacterial strains; -, no binding.
[b], ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CCARM, Culture Collection of Antimicrobial Resistant Microbes.

Ⅳ. DISCUSSION

In this study, *S. aureus* targeting prophage SAP4 was obtained from *S. aureus* clinical isolate 0136. Although it had effective inhibition ability, it could not directly use as a bio-control agent because of the possibility of the horizontal transfer of virulence or resistance genes by phages. In the lysogenic cycle, viral DNA is often integrated into the host cell DNA and the prophage DNA will be replicated when the host cell genome replicates, so daughter cells will inherit the viral DNA. For this reason temperate phages are not appropriate for phage therapy (Hanlon 2007). To find new target for bio-control agent, whole genome sequence was analyzed and the endolysin LysSAP4 was identified and characterized. Newly discovered endolysin LysSAP4 had homologous with *N*-acetyl-muramoyl-L-alanine amidase of *Staphylococcus aureus* A9765 and composed of three domains; CHAP, Amidase_3 (MurNAc-LAA) and SH3b. CHAP domain of LysSAP4 was identical with that of *Staphylococcus aureus subsp. aureus* IS-M (EID39378.1). Amidase_3 domain had highly homology with phage amidase of *Staphylococcus aureus* UCIM6115 (EVI42509.1). SH3b domain was homologous with that of *Staphylococcus aureus subsp. aureus* CO-23

(EID87328.1). As all three domains were homologous with genome of *S. aureus* strains, it can be inferred that phage SAP4 genes were widely horizontal transferred to *S. aureus* and presented in bacterial genome as a prophage.

Biochemical characterization of LysSAP4 endolysin showed that optimum pH was pH 8.0 but over 50% activity was remained at pH 6.0 and pH 7.0 and optimum temperature was 37℃ but also over 50% activity was remained at room temperature of 25℃ and refrigerator temperature of 4℃. There was a problematic issue of staphylococcal contamination in milk (Garcia *et al.* 2010) and the condition of pH and temperature of most distributed milk were about pH 7 and 4℃, respectively. Based on previously described data, these are suggested the possibility that LysSAP4 will be used as bio-control agent in milk industry to control staphylococcal strains. LysSAP4 could be also used as a therapeutic agent by effectively reducing *S. aureus* biofilm that is highly problem with clinical issue, such as catheter biofilm.

This enzyme required a divalent metal ion, such as Zn^{2+}, for full enzymatic activity. Based on domain search data, zinc binding site was in Amidase_3 domain of LysSAP4 (Fig. 5), the result was consistent with the

findings, that the lytic activity of LysSAP4 was restored when Zn^{2+} was treated (Fig. 8). A similar requirement for divalent cations was seen for Ply500 in *L. monocytogens* bactetiophage A500 (Loessner MJ *et al*. 1995) and LysB4 in *B. cereus* phage B4 (Son *et al*. 2012), but Zn^{2+} coordinating residues of these endolysins are His80, Asp87 and His133 different with that of LysSAP4 (His187, Glu198, His254). Instead Zn^{2+}, T5 endolysin requires Ca^{2+} for optimal enzymatic activity (Mikoulinskaia *et al*. 2009).

In some cases of lysins (LoessnerMJ *et al*. 2002, Sass and Bierbaum 2007), C-terminal domain is necessary for lytic activity but this is not always the case. A number of enzymes have shown increased lytic activity upon removal of the binding domain such as LysK (Horgan *et al*. 2009). To check these properties of LysSAP4, I tested the activity of each enzymatically active domains; CHAP only, Amidase_3 only and CHAP and Amidase_3 by engineering domains. However, unfortunately the expressed recombinants were insoluble so I couldn't determine that this constructs had more strong lytic activity than the original endolysin or not.

Most phages belong to the order *Caudovirales* or tailed phages are divided into three families: *Myoviridae* with a long, double-sheathed, contractile tails, *Podoviridae* with a very short tail and *Siphoviridae* with a long, non-contractile tail. According to the previous work (Ackermann H. W.

2001), siphoviruses can be assigned to the major serogroups A, B and F based on tail length, head size and head shape, and phage SAP4 could be classed as serogroup A, which features a prolate head and non-contractile long tail. This morphology is unique compared with the most staphylococcal siphovirus with icosahedral head.

Many Gram-positive cell envelopes are modified with a unique anionic glycopolymer, the peptidoglycan-anchored wall teichoic acid (WTA), which is one of the most abundant molecules at the bacterial surface. Most *S. aureus* strains express polyribitol phosphate WTA substituted with *N*-acetylglucosamine (GlcNAc) and D-alanine (Xia *et al*. 2011). In general, WTA of *S. aureus*, but not lipoteichoic acid (LTA), serves as a receptor for staphylococcal phage. Because the *tagO* gene is required for the first step of WTA biosynthesis (Weidenmaier *et al*. 2004), phage might not infect the cell which lacks WTA. In case of phage SAP4, it couldn't infect *S. aureus* RN4220 *ΔtagO* mutant strain. Moreover, SAP4 effectively infected the mutants deleted other genes related WTA biosysthesis such as *tagB*, so another receptor of staphylococcal phage would be hard to find because of critical role of *tagO* gene.

LysSAP4 had more broader antibacterial activity spectrum than the narrow host range of the phage SAP4. That's because of the unique cell wall

of staphylococcal stains compared with that of other Gram-positive bacteria (Weidenmaier and Peschel 2008). Staphylococcal endolysin target to the glycosidic bonds in the glycan strand of unique staphylococcal cell wall, not the species, therefore LysSAP4 might be specifically active in all staphylococcal stains tested.

In conclusion, phage SAP4 belongs to the *Siphoviridae* family with prolonged head was characterized. Moreover, endolysin composed of CHAP, Amidase_3 and SH3 was firstly characterized with LysSAP4. LysSAP4 could lyse all staphylococcal strains tested and also showed stability in wide range of salt concentration. Optimal pH and temperature of this enzyme was pH 8.0 and 37 ℃, respectively. It showed high biofilm reduction ability and its CBD specifically bind to all staphylococcal strains. Taken together, LysSAP4 has potential to be an effective antibacterial agent to control *S. aureus*.

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국문 초록

황색포도상구균 (*Staphylococcus aureus*)은 주요 식중독 유발균으로서 인간과 동물에 감염하여 심각한 질병을 유발할 수 있다. 메티실린 저항성 황색포도상구균 및 반코마이신 저항성 황색포도상구균과 같은 항생제 내성 균주의 출현으로 인하여 기존의 항생제를 대체할 수 있는 새로운 항세균제제의 개발이 필요하다. 박테리오파지 및 파지에서 분리된 세포벽 분해 효소인 엔도라이신은 새로운 항생제 대체제로서 각광받고 있다. 본 연구에서는 황색포도상구균에 특이적으로 감염할 수 있는 파지 SAP4를 분리하고 그 특성을 분석하였다. 파지는 병원성 황색포도상구균 내에 존재하는 프로파지를 유도하여 분리하였으며, 형태학적으로 분류하면 시포비리데 과에 속한다. 대부분의 황색포도상구균 시포비리데 파지는 정이십면체의 머리를 지니는 반면 새롭게 분리한 파지 SAP4는 보다 신장된 머리를 가지므로 형태학적으로 특이하다. 파지 SAP4는 29 시간 이상 지속적으로 황색포도상구균의 증식을 억제할 수 있으며, 검사한 18 종류의 포도상구균 중에 MRSA를 포함한 7 종류의 황색포도상구균과 1

종류의 다른 포도상구균인 스타파일로코쿠스 헤몰라이티쿠스에 사멸능을 보인다. 유전체 분석 결과, SAP4 유전체는 43 kb 크기의 이중나선 DNA로 구성되며 61 개의 ORF, 34% GC함량을 가지며, tRNA는 존재하지 않았다. 황색포도상구균의 제어함에 있어 가장 효과적일 것으로 추정되는 엔도라이신 유전자 *lysSAP4*를 선정하였고, 이는 포도상구균 파지 YMC/09/04/R1988의 *N*-acetylmuramoyl-L-alanine amidase와 가장 높은 상동성을 보였다. LysSAP4는 효소적 활성을 나타내는 2개의 도메인인 CHAP, Amidase_3과 세포 부착기능을 수행하는 도메인인 SH3b로 구성되며, pH 8.0과 37℃에서 최적의 효소 활성을 나타낸다. 또한 항균 활성은 특히 아연 이가 양이온에 의존적이며 항균 범위는 검사한 18 종의 포도상구균을 모두 사멸시킬 수 있었다. 이들의 활성은 포도상구균 내에서는 넓은 숙주 범위를 가질 뿐만 아니라 포도상구균만을 특이적으로 사멸시킬 수 있으며, 바이오 필름 저해능력 또한 우수하다. 세포 부착에 관여하는 SH3b 도메인을 녹색형광단백질과 융합하여 재조합한 후 포도상구균에 대한 부착 능력을 확인해 본 결과, 엔도라이신 LysSAP4의 항균 범위와

동일하게 검사한 모든 포도상구균에 SH3b 도메인이 부착하였다. 종합하면, 직접 항균제제로 쓰일 수 없는 프로파지 SAP4의 높은 용균 활성을 활용하고자 엔도라이신 LysSAP4를 연구하였으며, LysSAP4가 황색포도상구균을 제어할 수 있는 우수한 미생물 제어 후보 물질임을 확인하였다.

주요어 : 황색포도상구균, 박테리오파지, 엔도라이신

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