A Thesis for the Degree of Doctor of Philosophy

Characterization of Endolysins from *Salmonella* Typhimurium-infecting Bacteriophages for Bio-control agents

살모넬라 타이피뮤리움을 숙주로 하는 박테리오파지로부터 분리된 엔도라이신의 생물학적 제재로서의 특성 연구

August, 2012

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Abstract

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Concerns over drug-resistant bacteria have stimulated interest in developing alternative methods to control bacterial infections. Endolysin, which is phage-encoded enzyme that breaks down bacterial peptidoglycan at terminal stage of phage reproduction cycle, is reported to be effective for the treatment of bacterial infectious diseases. From the DNA libraries of bacteriophage SPJ2 and iEPS5, three different lysis proteins were screened. All three proteins did not have any homology to known proteins, but they showed very strong lysis activity when they were expressed with holin. However, they could not be characterized further because the expression levels were too low or most of them were insoluble, finally they could not be purified. Then, two additional endolysins could be obtained from bioinformatic analyses of the full genome sequences of the newly isolated bacteriophages. The full genome sequence of bacteriophage SPN1S

contains genes encoding homologues of holin, endolysin, and Rz/Rz1-like accessory proteins, four phage lysis proteins. The ability of these proteins to lyse an *Escherichia coli* host cell when overexpressed was evaluated. In contrast to other endolysins, expression of holin and endolysin did not cause lysis, but expression of endolysin and the Rz/Rz1-like proteins, or coexpression of all three proteins was sufficient to cause lysis. The endolysin was tagged with oligo-histidine at the N-terminus and purified by affinity chromatography. The endolysin has lysozyme-like superfamily domain, and its activity was much stronger than that of lysozyme from chicken egg white. The chelating agent, ethylenediaminetetraacetic acid (EDTA) was used to increase outer membrane permeability and it greatly enhanced the lytic activity of SPN1S endolysin. The antimicrobial activity of endolysin was stable over broad pH and temperature ranges, and was active from pH 7.0 to 10.5 and from 25°C to 45°C. The SPN1S endolysin could kill most tested Gram-negative bacteria, but Gram-positive bacteria were resistant. SPN1S endolysin, like lysozyme, showed muramidase activity that cleaves the glycosidic bond of peptidoglycan. When extra sequences for helix structure was added to C-terminus of SPN1S endolysin, the recombinant protein showed antimicrobial activity against intact DH5α. Similar to SPN1S endolysin, bioinformatic

analysis of SPN9CC genome revealed a putative endolysin which had homology with lysozyme of P22-like phage ST104 and *E. coli* K-12 DLP12 prophage. SPN9CC endolysin was purified with C-terminal oligo-histidine tag. The antimicrobial activity of SPN9CC endolysin was stable at 37°C for two hours, and it was active over broad temperature ranges (from 24°C and 65°C) showing maximal activity at 50°C. At neutral and basic pH, SPN9CC endolysin showed lytic activity and its optimum pH range were from pH 7.5 to pH 8.5. SPN9CC endolysin showed antimicrobial activity against only Gram-negative strains like SPN1S endolysin by cutting glycosidic bond of peptidoglycan. Interestingly, SPN9CC endolysin can lyse intact *E. coli*, and moreover lysis rate was enhanced in the presence of EDTA as outer membrane permeabilizer. These results suggested that SPN1S endolysin and SPN9CC endolysin have potential as therapeutic agents against Gram-negative bacteria.

Key words: bacteriophage, endolysin, *Salmonella* Typhimurium, bio-control

Student Number: 2005-23634

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I. Introduction

Antibiotic resistance and bio-control agents

Over the last decade, a dramatic increase in the prevalence of antibiotic resistance has been noted in several medically significant bacterial species, especially *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, as well as *Staphylococcus aureus*, coagulase-negative staphylococci, enterococci, and *Streptococcus pneumoniae* (Hawkey, 2008). This unfavorable situation is further aggravated by a shortage of new classes of antibiotics with novel modes of action that are essential to contain the spread of antibiotic-resistant pathogens (Livermore, 2004). Therefore, there is an urgent need to develop novel antibacterial agents to eliminate multidrug-resistant bacteria (Breithaupt, 1999).

Furthermore, food borne diseases are among the most serious and costly public health concerns worldwide, being a major cause of morbidity. In spite of modern technologies, good manufacturing practices, quality control and hygiene and safety concepts such as risk assessment and HACCP, the reported numbers of food-borne illnesses and intoxications still increased over the past decade. The most common food-borne infections in the European Union (EU) are caused

by bacteria, namely *Campylobacter*, *Salmonella* and *Listeria*, and viruses.

Food market globalization, the introduction of novel foods, new manufacturing processes and the growing demand for minimally processed, fresh-cut and ready-to-eat products may require a longer and more complex food chain, increasing the risk of microbiological contamination. Thus, novel and complementary food preservation technologies that comply with these demands from “farm to fork” are continuously sought. Among alternative food preservation technologies, particular attention has been paid to biopreservation to extent the shelf-life and to enhance the hygienic quality, minimizing the impact on the nutritional and organoleptic properties of perishable food products. Biopreservation rationally exploits the antimicrobial potential of naturally occurring (micro-) organisms in food and/or their metabolites with a long history of safe use. Bacteriophages and bacteriophage-encoded enzymes fall in this concept.

Bacteriophage and endolysin

Bacteriophage and endolysin are regarded as effective antimicrobial agents (Loessner, 2005). In general, double-stranded DNA bacteriophages lyse their host cells using two proteins, holin and endolysin. Endolysin synthesized without a signal sequence accumulates in the cytosol in active form. Holin makes pores in the inner membrane which allows endolysin to reach its target, peptidoglycan. Endolysins typically cleave a glycosidic bond, peptide bond, or amide bond of peptidoglycan (Pritchard *et al.*, 2004; Baker *et al.*, 2006; Mikoulinskaia *et al.*, 2009). In contrast to canonical endolysins, there were some papers about other types of endolysins. Endolysins of phages P1 and 21 which have N-terminal signal-anchor-release (SAR) domains are secreted to the periplasm by the host *sec* system. They accumulate in an inactive membrane-tethered form and activated by membrane depolarization by the holin (Xu *et al.*, 2005; Sun *et al.*, 2009). The *Oenococcus oeni* phage fOg44 endolysin which has a typical signal sequence was changed to active form after cleavage of N-terminal signal peptide in a SecA-dependent manner (Sao-Jose *et al.*, 2000). Some phages do not encode an apparent endolysin (Krupovic *et al.*, 2007).

In case of Gram-negative bacteria, there are two more proteins,

Rz/Rz1 equivalent, those can contribute to the efficiency of lysis (Summer *et al.*, 2007; Berry *et al.*, 2008; Krupovic *et al.*, 2008). In the lambda phage lysis cassette, there are four lysis genes; *S* encoding holin and antiholin, *R* encoding endolysin, and *Rz/Rz*1. Rz is a type II membrane protein with a hydrophobic N-terminal transmembrane domain and a periplasmic C-terminus. Rz1 is a lipoprotein attached to the inner leaflet of the outer membrane, and the Rz1 coding region is entirely embedded within *Rz* in the +1 reading frame (Summer *et al.*, 2007).

Bacteriophages have a number of advantages that make them compelling alternatives to chemical antibiotics including their bactericidal rather than bacteriostatic activity, equal effectiveness against antibiotic-sensitive and antibiotic-resistant bacteria, and ability to disrupt bacterial biofilms. Disadvantages include phage resistant bacteria and the presence of bacterial toxins in phage lysates (Gupta and Prasad, 2011). To address these limitations, utilization of purified endolysin has been studied. The lytic activities of purified endolysins have shown potential as therapeutic agents against *Streptococcus pneumoniae, Bacillus anthracis, B. subtilis, Staphylococcus aureus, Lactobacillus fermentum, Listeria monocytogenes, Enterococcus faecalis* and *Clostridium perfringens* (Schuch *et al.*, 2002; Loeffler *et al.*, 2003; Turner *et al.*, 2004; Fukushima *et al.*, 2007; Kikkawa *et al.*,

2007; Sass and Bierbaum, 2007; Turner *et al.*, 2007; Nariya *et al.*, 2011; Uchiyama *et al.*, 2011). But most studies of purified endolysin have focused on Gram-positive bacteria, since the peptidoglycan is directly accessible to exogenous endolysin. In case of Gram-negative bacteria, activity of purified phage lysis proteins against *Pseudomonas aeruginosa* has been reported (Miroshnikov *et al.*, 2006; Briers *et al.*, 2007; Paradis-Bleau *et al.*, 2007), and just a few studies about endolysins of *Klebsiella* phage, *Acinetobacter baumannii* phage, and Enterobacteria phage T5 have been reported (Junn *et al.*, 2005; Miroshnikov *et al.*, 2006; Mikoulinskaia *et al.*, 2009; Lai *et al.*, 2011).

Several *Salmonella* Typhimurium-targeting bacteriophages were isolated and genomes of two phages were completely sequenced. SPN1S bacteriophage isolated from environmental water has a circular genome consisting of 38,684 bp with GC content of 50.16%, 52 ORFs. And it, belonging to the *Podoviridae* family, infects *S.* Typhimurium strains and a few *S.* Paratyphi strains (Shin *et al.*, 2012). SPN9CC bacteriophage was isolated from a chicken skin sample, and it also belongs to the *Podoviridae* family. It has a genome consisting of 40,128 bp with GC content of 47.33%, 63 putative ORFs, and 2 tRNAs (tRNA_Thr and tRNA_Asn) (Shin *et al*., unpublished data). SPN9CC showed specific inhibition against *S.* Typhimurium, *S.* Paratyphi, and *S.* Dublin. However, various Gram-positive and Gram-negative bacteria

including other *Salmonella* strains were not inhibited by this phage.

In this study, two endolysins from *S.* Typhimurium-infecting bacteriophages were purified and their lysis activities were analyzed. And three putative endolysins were screened from bacteriophage SPJ2 and iEPS5 DNA libraries.

II. Materials and Methods

1. Putative endolysins from phage libraries

Bacteriophage propagation and extraction of phage DNA

Bacteriophage SPJ2 and iEPS5 were propagated using *Salmonella* Typhimurium SL1344 as host at 37°C for 12 h with shaking. For purification of propagated phages, cell debris was removed by subsequent centrifugation at 6,000 g for 10 min and filtration using 0.22 μm pore size filters and phage particles were precipitated by treatment of polyethylene glycol (PEG) 6,000 (Sigma, St. Louis, MO, USA). Finally, CsCl density gradient ultracentrifugation (himac CP 100β, Hitachi, Japan) with different CsCl steps (step density = 1.3, 1.45, 1.5, and 1.7 g ml^{-1}) at 25,000 g for 2 h was conducted at 4°C.

Bacteriophage genomic DNA was extracted from the phage lysate as previously described (Sambrook J, 2001). Phage lysate was treated with RNase A and DNase I at 37°C for 1 h to remove bacterial DNA and RNA and then treated with lysis buffer (0.5% sodium dodecyl sulfate (SDS), 20 mmol l^{-1} EDTA and 50 μg ml^{-1} proteinase K) for 2 h at 56℃. Standard phenol-chloroform DNA purification was followed and ethanol precipitation was performed.

Phage library construction and screening of lysis genes

For negative or positive control, holin or holin and endolysin coexpression vectors were constructed. T4 phage holin gene and T7 phage endolysin gene were amplified by PCR using pBluelysis plasmid as template (Yun *et al.*, 2007) and hol F, R or end F, R primers. The PCR product for holin coding gene was ligated with SmaI-digested pBAD18. The recombinant plasmid was transformed into *E. coli* DH5α. PCR product of T7 endolysin coding gene was digested with EcoRI and cloned into EcoRI-digested pLT1 which is holin expression vector.

The purified phage DNA of iEPS5 and SPJ2 was partially digested with restriction enzyme EcoRI and ligated with EcoRI-digested arabinose inducible plasmid pBAD18 and pLT1, respectively. The recombinant plasmid was transformed into *E. coli* DH5α. Transformants were picked and inoculated into Luria Bertani medium supplemented with 50 μg ml^{-1} ampicillin in 96-well plates. When stationary phase was reached, the libraries were added with glycerol (final concentration 15%) and stored in a deep-freezer until screening.

For screening the clones showing lysis activity, the libraries were replica plated on a LB agar supplemented with ampicillin or ampicillin with 0.2% L-arabinose. After 8 h incubation, the clones which showed growth inhibition in only arabinose contained media were selected.

Lysis gene confirmation by sub-cloning

The inserted genes in the selected clones were sequenced, and the possible open reading frames (ORFs) were analyzed by ORF finder. To confirm the activity of each ORF product, several vectors were constructed. The PCR products of SPJ2 bacteriophage used primer combinations (SPJ2P2 sub F1, 2, 3 and R1, 2, 3 or SPJP1 sub F1, 2, 3, 4 and R1, 2, 3) were digested with EcoRI and ligated into EcoRI-digested pLT1. The PCR products of iEPS5 bacteriophage used primer set iEPS5 F1, 2, 3 and R1, 2, 3 were digested with EcoRI and ligated into EcoRI-digested pBAD18 or pLT1, respectively. The recombinant plasmids were transformed into DH5α, and the transformants were selected on LB agar supplemented with ampicillin. The plasmids and primers used in screening method are listed in Table 1 and Table 2.

Each sub-clones were cultured in LB medium supplemented with 50 μg ml^{-1} ampicillin until mid-exponential phase. L-arabinose was added to a final concentration of 0.2% to induce the expression of lysis proteins. Lysis activity was assessed by measuring optical density reduction.

Table 1. Oligonucleotide primers used in screening method

primer name	Sequence (5′→3′)
SPJ2P2 sub F1	GCTAGCGAAT TCATCGCCG
SPJ2P2 sub F2	CGGTGGCGAA TTCCGCATTT
SPJ2P2 sub F3	GACTGCCAGA ATTCATGGGA C
SPJ2P2 sub R1	CATTGGCTTG AATTCTCATA TCTC
SPJ2P2 sub R2	GTTGTCCGAA TTCAGCACGT A
SPJ2P2 sub R3	CGAGCTCGAA TTCAACGATG TT
SPJ2P1 sub F1	TGACTATGAA TTCGGTTGCA
SPJ2P1 sub F2	GGCTAGCGAA TTCTATGAAA
SPJ2P1 sub F3	CTATCTGGAA TTCTTCTGCA
SPJ2P1 sub F4	GCAAAACGAA TTCTGGCGAG
SPJ2P1 sub R1	GAGCTCGAAT TCATCAAGG
SPJ2P1 sub R2	GAGTAGAGAA TTCTAACCTC
SPJ2P1 sub R3	CAGTGTTGAA TTCGTTACAA
iEPS5 sub F1	GCGGCCACAA TCGGAATTCG CCTGTACA
iEPS5 sub F2	CAAAGCGTGA ATTCCCTATC T
iEPS5 sub F3	CTTTGTGTAC TGAGAATTCA CGGGTTAAT
iEPS5 sub R1	CTGTCAGCAC GAATTCCTCG CCGATG
iEPS5 sub R2	CCGTCTGAAT TCGGGCGA
iEPS5 sub R3	TTGTTATTGC GAATTCCGCG CGGCT
hol F	GCATGCATGG CAGCACCTAG AATAT
hol R	GCATGCTTAT TTAGCCCTTC CTAATA
end F	CACCGCTGAA TTCCGGGTCC
end R	CATCCGGAAT TCCTGTGGTC TC
SPJ2P2 F1	GAATTGAAGC ATATGGCGGA AGT
SPJ2P2 F2	ATTGAAGCCC ATGGCGGAAG T
SPJ2P2 R2	GGTCTGCTCG AGCTTATCG
SPJ2P2 R4	TCGACGCCAG ATCTGTATTC G
SPJ2P1 F	AGTTGGCAGA GTGCATATGT GC
SPJ2P1 R	GTCAGTGTTG GATCCGTTAC AATG
iEPS5 F1	CGGGGCCGCC ATATGACGGG G
iEPS5 F2	CGCTCCATGG CGGGGCTACT
iEPS5 R1	GAGCTCGGAT CCCTCGCCGA T
iEPS5 R3	ACAGAAAGTC GACGCAGGGT AAATCCTT
iEPS5 R4	CTAGGTGCTG CCATGCATGC GG

Table 2. Plasmids used in screening method

Name	Description	Source or reference
pBAD18	Amp^r, *araC*, P_{BAD}, pBR322 *ori*, expression vector	(Guzman *et al.*, 1995)
pET15b	Amp^r, T7 promoter, N-terminal His-Tag	Novagen, Wisconsin, USA
pET29b	Amp^r, T7 promoter, C-terminal His-Tag	Novagen, Wisconsin, USA
pGST parallel 1	Amp^r, T7 promoter, N-terminal GST-Tag	
pMBP parallel 1	Amp^r, T7 promoter, N-terminal MBP-Tag	
pLT1	pBAD18 expressing T4 phage holin	This study
pLT2	pBAD18 expressing T4 phage holin and T7 phage endolysin	This study
pLT10	pLT1 containing DNA fragment included SPJ2P1 (from phage library)	This study
pLT13	pBAD18 expressing SPJ2P2	This study
pLT14	pLT1 expressing SPJ2P2	This study
pLT15	pET15b containing SPJ2P2 (for N-terminal his-tagged SPJP2)	This study
pLT16	pET29b containing SPJ2P2 (for C-terminal his-tagged SPJP2)	This study
pLT17	pET29b containing SPJ2P2 (no tagging SPJP2)	This study
pLT18	pGST parallel 1 containing SPJ2-2 lysis gene (for N-terminal GST tagging SPJP2)	This study
pLT20	pLT1 containing DNA fragment included SPJ2P2 (from phage library)	This study
pLT21	pBAD18 expressing SPJ2P1	This study
pLT23	pLT1 expressing SPJ2P1	This study
pLT24	pET15b containing SPJ2P1 (for N-terminal his-tagged SPJP2)	This study
pLT33	pBAD18 expressing iEPS5 ORF2	This study
pLT34	pBAD18 expressing iEPS5 ORF3	This study
pLT35-1	pBAD18 expressing iEPS5 ORF1	This study
pLT36-1	pET15b containing iEPS5 ORF1 (for N-terminal his-tagged ORF1)	This study
pLT37-1	pET29b containing iEPS5 ORF1 (for C-terminal his-tagged ORF1)	This study
pLT38-1	pMBP parallel 1 expressing iEPS5 ORF1 (for N-terminal MBP-tagged ORF1)	This study
pLT39	pLT1 expressing MBP-tagged iEPS5 ORF1	This study

Lysis protein overexpression

For expression of an N-terminal or C-terminal oligo-histidine tagged endolysin, the SPJ2P2 gene was amplified by polymerase chain reaction using SPJ2P2 F1 and R2 primers and digested with NdeI and XhoI. And then it was cloned into pET15b or pET29b digested with the same restriction enzyme, respectively. For expression of SPJ2P2 protein without tag, PCR product used SPJ2P2 F1 and R4 primers containing its stop codon was digested with NdeI and BglII and ligated with the same enzyme treated pET29b. For N-terminal GST (glutathione *S*-transferase) tagged-SPJ2P2, PCR product used SPJ2P2 F2 and R2 primers was digested with NcoI and XhoI and cloned into pGST parallel 1 vector treated the same restriction enzyme (Sheffield *et al.*, 1999). In case of SPJ2P1, the PCR product was amplified by PCR using SPJ2P1 F and R primers. After enzyme digestion with NdeI and BamHI, lysis protein coding gene was ligated with pET15b digested with the same enzyme.

For expression of an N-terminal or C-terminal oligo-histidine tagged iEPS5 ORF1 protein, the gene was amplified by PCR using primers iEPS5 F1 and R1 or F1 and R3 and digested with NdeI and BamHI or NdeI and SalI. And then they were cloned into pET15b or pET29b digested with the same restriction enzyme, respectively. For an N-

terminal maltose binding protein (MBP) tagged lysis protein, the PCR product used iEPS5 F2 and R4 primers was digested with NcoI and SphI and ligated with pMBP parallel 1 treated with the same enzyme (Sheffield *et al.*, 1999). Finally, the recombinant plasmids were transformed into BL21(DE3), BL21 Star^{TM} (DE3) (Invitrogen, Carlsbad, CA, USA), C41(DE3), C43(DE3) (Miroux and Walker, 1996), or Rosetta(DE3) (Novagen, Wisconsin, USA).

Expression of the recombinant lysis proteins were induced with 50 μmol l^{-1}, 100 μmol l^{-1}, 300 μmol l^{-1}, or 1 mmol l^{-1} Isopropyl β-D-1-thiogalactopyranoside (IPTG) at optical density (600 nm) reached 0.4, 0.7, 1.0 or 1.5, followed by incubation for additional 2 h, 4 h or 6 h. To improve protein solubility, concentrations of IPTG were reduced to 20 μmol l^{-1} and culture temperatures were lowered to 25℃. In each condition, total protein profiles were analyzed by SDS-PAGE or Tricine SDS-PAGE (SPJ2P1).

Lysis protein purification

Bacterial cells were suspended with buffer containing 20 mmol l^{-1} Tris-HCl (pH 8.0) and 300 mmol l^{-1} NaCl and disrupted by sonication (Bioruptor, Diagenode, Denville, NJ, USA). N-terminal his-tagged lysis protein was purified using nickel-nitriloacetic acid agarose resin (Qiagen, Hilden, Germany) with imidazole gradient according to manufacturer`s instructions. MBP-tagged lysis protein was purified using amylose resin (New England Biolabs, Beverly, MA, USA) with maltose gradient according to manufacturer`s instructions. ProTEV Protease (Promega, Madison, WI, USA) reactions are carried out to remove MBT-tag from the lysis protein. After cleaving the fusion protein, ProTEV Protease can be removed from the reaction by incubating with nickel-nitriloacetic acid agarose resin (Qiagen, Hilden, Germany).

2. SPN1S endolysin

Bioinformatic analysis

Prediction of open reading frames (ORFs) in the lysis cluster of SPN1S genome was performed using Glimmer 3.02 (Delcher *et al.*, 2007), GeneMark.hmm (Lukashin and Borodovsky, 1998) and FgeneV software (http://www.softberry.com). Ribosomal binding sites (RBS) were predicted using RBSfinder (ftp://ftp.tigr.org/pub/software/RBSfinder) for confirmation of predicted ORFs. Annotation of predicted ORFs was conducted using the results of BLASTP (Altschul *et al.*, 1990), NCBI Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2007) and InterProScan analyses (Zdobnov and Apweiler, 2001).

DNA manipulation

Bacteriophage SPN1S DNA was prepared as previously described (Sambrook J, 2001). The holin, endolysin, and Rz/Rz1-like proteins coding genes were amplified by polymerase chain reaction and cloned into pBAD18 expression vector. For expression of an N-terminal oligo-histidine tagged endolysin, the endolysin gene was amplified by polymerase chain reaction and cloned into pET15b. The plasmids and primers used for SPN1S endolysin are listed in Table 3.

Table 3. Plasmids, and oligonucleotide primers for SPN1S endolysin

Plasmid or primer	description or sequence	Source or reference
Plasmids		
pBAD18	Amp^r, *araC*, P_{BAD}, pBR322 *ori*, expression vector	(Guzman *et al.*, 1995)
pET15b	Amp^r, T7 promoter, N-terminal His-Tag	Novagen
pBAD-hol	pBAD18 encoding SPN1S holin	This study
pBAD-lys	pBAD18 encoding SPN1S endolysin	This study
pBAD-Rz	pBAD18 encoding SPN1S Rz/Rz1-like proteins	This study
pBAD-hollys	pBAD18 encoding SPN1S holin and endolysin	This study
pBAD-holRz	pBAD18 encoding SPN1S holin and Rz/Rz1-like proteins	This study
pBAD-lysRz	pBAD18 encoding SPN1S endolysin and Rz/Rz1-like proteins	This study
pBAD-hollysRz	pBAD18 encoding SPN1S holin, endolysin, and Rz/Rz1-like proteins	This study
pET15-lys	pET15b encoding N-terminal his-tagged SPN1S endolysin	This study
Primers		
hol F	5`-GTACGTATTGGCATGCGGCTCTATC-3`	This study
hol R	5`-ATTGATACCGCATGCGCGCCG-3`	This study
lys F	5`-CCGTCCTGAATTCGCGCGGT-3`	This study
lys R	5`-AAGCTTTGACGAATTCCCAGATCATAAC-3`	This study
Rz F	5`-CAGAACGGTGTCGACGACCG-3`	This study
Rz R	5`-TTTATATACCGTCGACGGAAATCGGT-3`	This study
hislys F	5`-GAGGTCTCGAGATGGACATTA-3`	This study
hislys R	5`-GTAACTGTCTCGAGTAAGCTTTG-3`	This study
T4 hol F	5`-GCATGCATGGCAGCACCTAGAATAT-3`	This study
T4 hol R	5`-GCATGCTTATTTAGCCCTTCCTAATA-3`	This study
T7 lys F	5`-CACCGCTGAATTCCGGGTCC-3`	This study
T7 lys R	5`-CATCCGGAATTCCTGTGGTCTC-3`	This study

Lysis gene confirmation

E. coli DH5α harboring recombinant plasmid expressing each of the three lysis genes or combinations of them was cultured in Luria Bertani medium supplemented with 50 μg ml^{-1} ampicillin until mid-exponential phase. L-arabinose was added to a final concentration of 0.2% to induce the expression of lysis proteins. Lysis activity was assessed by measuring optical density.

SPN1S endolysin purification

The BL21 Star^{TM} (DE3) (Invitrogen, Carlsbad, CA, USA) was transformed with pET15-lys plasmid. Expression of the recombinant endolysin was induced with 1 mmol IPTG at optical density (600 nm) reached 0.6, followed by incubation for additional 3 h. Bacterial cells were suspended with buffer containing 20 mmol l^{-1} Tris-HCl (pH 8.0) and 300 mmol l^{-1} NaCl and disrupted by sonication (Bioruptor, Diagenode, Denville, NJ, USA). N-terminal his-tagged endolysin was purified using nickel-nitriloacetic acid agarose resin (Qiagen, Hilden, Germany) with imidazole gradient according to manufacturer`s instructions. The purified protein was stored at -80°C in 20 mmol l^{-1} Tris-HCl (pH 8.0), 300 mmol l^{-1} NaCl, and 30% glycerol.

Antimicrobial activity assay

An exponentially growing *E. coli* DH5α culture was washed and incubated in buffer containing 20 mmol l^{-1} Tris-HCl (pH 8.0) and 100 mmol l^{-1} EDTA for 5 min. After centrifugation to remove the EDTA, the cell pellet was resuspended with 20 mmol l^{-1} Tris-HCl (pH 8.0) (Ayres *et al.*, 1998; Junn *et al.*, 2005). To analyze the antimicrobial activity of endolysin, 10 ng, 50 ng, 100 ng, or 500 ng of SPN1S endolysin was added to 1 ml DH5α suspension and reduction of optical density at 600 nm were measured. The same volume of 20 mmol l^{-1} Tris-HCl (pH 8.0) was used as a negative control. The antimicrobial activity of 2 μg, 3 μg, 4 μg, or 5 μg of lysozyme from chicken egg white (Sigma, St. Louis, MO, USA) was measured similarly. In an alternative assay, an exponentially growing DH5α culture was washed with 20 mmol l^{-1} Tris-HCl (pH 8.0) and diluted 100-fold in the same buffer, without pretreating with EDTA. Endolysin (3 μg, 5 μg or 10 μg) was mixed with 1, 5, or 10 mmol l^{-1} EDTA, and added to 100 μl cell suspension. For negative control, an equivalent volume of buffer was used instead of endolysin or EDTA. After 20 min, 1 h, or 2 h reaction times, the numbers of residual viable cells were measured by determining colony forming units.

Effects of pH and temperature on SPN1S endolysin enzymatic activity

To test the thermostability of endolysin, aliquots of enzyme were incubated at different temperatures (30, 37, 45, 50, 55, 65, or 75°C) for 10 min or 30 min. The pH stability of endolysin was estimated after 12 days at 4°C in the following buffers: 0.1% trifluoroacetic acid (pH 2.0), 50 mmol l^{-1} sodium acetate (pH 4.3), 50 mmol l^{-1} 2-(N-morpholino)ethanesulfonic acid (MES, pH 6.0), 50 mmol l^{-1} Bis-Tris (pH 7.0), 50 mmol l^{-1} Tris-HCl (pH 7.5, pH 8.0 and pH 8.8), 50 mmol l^{-1} glycine (pH 9.5), or 50 mmol l^{-1} N-cyclohexyl-3-aminopropanesulfonic acid (pH 10.5). After each treatment, residual lysis activity was assayed. To determine the optimum conditions for antimicrobial activity, the enzyme reaction was performed in different pH buffers and at different temperatures. As controls, cell suspensions that had not been treated with endolysin were included in the assays.

Antimicrobial spectrum of SPN1S endolysin

Bacteria strains that used for antimicrobial spectrum determination were described in Table 6 with the result. Gram-positive strains were grown to mid-exponential phase then washed with 20 mmol l^{-1} Tris-HCl (pH 8.0) and 1 ml of each cell suspension was used in the endolysin assay. Gram-negative strains were grown to mid-exponential phase and treated with 100 mmol l^{-1} EDTA for 5 min. After centrifugation, the cell pellet was resuspended in 20 mmol l^{-1} Tris-HCl (pH 8.0). The cell suspensions were treated with 50 ng endolysin for 10 min and the optical density was measured.

Target site identification

A crude peptidoglycan preparation of *E. coli* DH5α was prepared as previously described (Fein and Rogers, 1976). *E. coli* cell cultures were disrupted by sonication and unbroken cells were removed by low-speed centrifugation (1400 g, 10 min). The supernatant was centrifuged (27,000 g, 5 min) and the pelleted crude cell wall was resuspended in 4% SDS solution and boiled for 10 min. After cooling, the crude peptidoglycan was washed several times. In 100 μl peptidoglycan solution suspended in 20 mmol l^{-1} Tris-HCl (pH 8.0), 100 ng, 500 ng, or 1 μg of SPN1S endolysin was added and the change in optical density at 600 nm was measured using a Sunrise microplate absorbance reader (Tecan, Switzerland). After hydrolysis, unbroken peptidoglycan was removed by centrifugation, and the supernatant was assayed using a modified Park and Johnson`s method (Park and Johnson, 1949) to investigate whether endolysin cleaves the polysaccharide backbone of peptidoglycan. Briefly, aliquots (0.2 ml) were mixed with 0.05% (w/v) aqueous potassium ferricyanide (0.2 ml) and 0.53% (w/v) sodium carbonate/0.065% (w/v) potassium cyanide in water (0.2 ml). The mixtures were boiled for 15 min. Upon cooling, 1.0 ml of a ferric ion reagent (final 0.15% (w/v) ferric ammonium sulfate/0.1% (w/v) SDS in 0.025 M sulfuric acid) was added. A stable Prussian blue color was

measured at 690 nm. To confirm the peptidase activity in the peptidoglycan hydrolysate, final 4% $NaHCO_3$ and 0.1% TNBS (trinitrobenzene sulfonic acid) solutions were used. In the dark for 2 h, 1N HCl was added for acidification and then OD_{340} was measured (Mikoulinskaia *et al.*, 2009). For determination of amidase activity, 1.0 M NaOH was mixed with the hydrolysate up to 0.5 ml. After 30 min incubation, 0.5 ml of 0.5 M H_2SO_4 and 5 ml of concentrated H_2SO_4 were added. The well-stoppered tube was placed in boiling water bath for 5 min. After cooling, 0.05 ml $CuSO_4$ and 0.1 ml PHD solutions (1.5% *p*-hydroxyphenyl in 96% ethanol) were added. After incubation for 30 min, the OD_{560} was measured (Hadzija, 1974; Hazenberg and de Visser, 1992). Peptidoglycan suspension that had not been treated with endolysin and the endolysin solutions were used as negative controls.

The glucosaminidase activity was determined by cleaving 4-Nitrophenyl N-acetyl-β-D-glucosaminide (Sigma). SPN1S endolysin was added to 0.2 ml of substrate solution (0.3 mg ml^{-1}) in 0.05 mol l^{-1} Tris-HCl buffer, pH 8.0, and incubated at 37℃. The activity was determined by the increase in the absorbance at 400 nm. The muramidase activity was determined by the cleavage of 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside (Sigma). SPN1S endolysin was added to 0.2 ml of a substrate solution (0.2 mg ml^{-1}) in

0.05 mol l^{-1} Tris-HCl buffer, pH 8.0, and incubated at 37℃. The activity was determined by the increase in the absorbance at 400 nm (Tsfasman *et al.*, 2007).

SPN1S endolysin improvement for introduction into the bacterial cell

At the front of SPN1S endolysin stop codon the DNA sequence related to helix structure (CTTACTAAAA TCGCTAAAAA G) was added by PCR using primer which has those sequences (Morita *et al.*, 2001). The PCR product was cloned with pET15b, and the ligated plasmid transformed into BL21 Star^{TM} (DE3). After selection, the recombinant endolysin which has N-terminal his-tagged and C-terminal helix structure was purified by Ni-NTA affinity chromatography as mentioned above.

3. SPN9CC endolysin

Bioinformatic analysis

Annotation of predicted ORFs was conducted using the results of BLASTP (Altschul *et al.*, 1990), NCBI Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2007) and InterProScan analyses (Zdobnov and Apweiler, 2001).

SPN9CC endolysin purification

Bacteriophage SPN9CC DNA was prepared as previously described (Sambrook J, 2001). For expression of an C-terminal oligo-histidine tagged endolysin, the endolysin gene was amplified by polymerase chain reaction using end F, R primers and cloned into pET29b. The plasmids and primers used for SPN9CC endolysin are listed in Table 4.

The *E. coli* BL21 (DE3) was transformed with pET29-lys plasmid. Expression of the recombinant endolysin was induced with 1 mmol l^{-1} IPTG at optical density (600 nm) reached 0.6, followed by incubation for additional 4 h. Bacterial cells were suspended with buffer containing 50 mmol l^{-1} sodium phosphate buffer (pH 7.0) and 300 mmol l^{-1} NaCl and disrupted by sonication (Bioruptor, Diagenode, Denville, NJ, USA). C-terminal his-tagged endolysin was purified using nickel-nitriloacetic acid agarose resin (Qiagen, Hilden, Germany) with imidazole gradient according to manufacturer`s instructions. The purified protein was stored at -80°C in 50 mmol l^{-1} sodium phosphate buffer (pH 7.0), 300 mmol l^{-1} NaCl, and 30% glycerol.

Table 4. Plasmids, and oligonucleotide primers for SPN9CC endolysin

Plasmid or primer	description or sequence	Source or reference
Plasmids		
pET29b	Kan^r, T7 promoter, C-terminal His-Tag	Novagen, Wisconsin, USA
pET29b-lys	pET29b encoding C-terminal his-tagged SPN9CC endolysin	This study
Primers		
end F	5`-AGCGTGAGGACAGATCATATGGCAATGT-3`	This study
end R	5`-AATCGCGGTTACTCGAGTCCTTGCTGCC-3`	This study

Antimicrobial activity assay

An exponentially growing *E. coli* DH5α culture was washed and incubated in buffer containing 20 mmol l^{-1} Tris-HCl (pH 8.0) and 100 mmol l^{-1} EDTA for 5 min. After centrifugation to remove the EDTA, the cell pellet was resuspended with 50 mmol l^{-1} Tris-HCl buffer (pH 8.0) (Ayres *et al.*, 1998; Junn *et al.*, 2005). To analyze the antimicrobial activity of endolysin, 0.1 μg, 0.5 μg, 1 μg, or 5 μg of SPN9CC endolysin was added to 1 ml DH5α suspension and reduction of optical density at 600 nm were measured. The same volume of 50 mmol l^{-1} Tris-HCl buffer (pH 8.0) was used as a negative control.

In an alternative assay, an exponentially growing *E. coli* MG1655 culture was washed with phosphate buffered saline (PBS) and diluted 100-fold in the same buffer, without pretreating with EDTA. Endolysin (10 μg or 30 μg) was mixed with EDTA and added to 100 μl cell suspension (final concentrations of EDTA were 1 mmol l^{-1} or 5 mmol l^{-1}). For negative control, an equivalent volume of buffer was used instead of endolysin or EDTA. After 1 h, or 2 h reaction times, the numbers of residual viable cells were measured by determining colony forming units.

Effects of pH and temperature on SPN9CC endolysin enzymatic activity

To test the thermostability of SPN9CC endolysin, aliquots of enzyme (0.5 μg) were incubated at different temperatures (4, 24, 37, 45, 55, or 65°C) for 3 min, 10 min or 30 min. To measure the thermostability for long time, SPN9CC endolysin aliquots were incubated at 4, 24, 37, or 50°C for 1 h or 2 h. After each treatment, residual lysis activity was assayed.

To determine the optimum conditions for antimicrobial activity, the enzyme reaction was performed in the JBScreen pH-2D broad range pH-buffer (Jena Bioscience, Germany) at room temperature. The relative lysis activity was calculated by combining the results obtained with six different kinds of buffers covering from pH 4 to 10 (SSG buffer, Succinic Acid : Sodium dihydrogen Phosphate : Glycine = 2 : 7 : 7; CHC buffer, Citric acid : HEPES : CHES = 2 : 3 : 4; MIB buffer, Malonic Acid : Imidazole : Boric Acid = 2 : 3 : 3) or with three different kinds of buffers covering from pH 4 to 9 (SAB buffer, Sodium Acetate : ADA : Bicine = 1 : 1 : 1; MMT buffer, L-Malic Acid : MES : Tris = 1 : 2 : 2; SBG buffer, Sodium Tartrate dehydrate : Bis-Tris : Glycylglycine = 3 : 2 : 2). To determine the optimum temperature of SPN9CC endolysin, antimicrobial activity assay was performed at

different temperatures (24, 30, 37, 45, 50, 55, 60, or 65°C) for 3 min using EDTA treated DH5α as a substrate. By measuring OD_{600} of substrate lysate, relative lysis activity was calculated in proportion to the highest activity. As controls, cell suspensions that had not been treated with endolysin were included in the assays.

Antimicrobial spectrum of SPN9CC endolysin

Total eight Gram-positive strains were grown to mid-exponential phase then washed with 50 mmol l^{-1} Tris-HCl (pH 8.0) and 1 ml of each cell suspension was used in the endolysin assay. Total twenty-three Gram-negative strains containing *Salmonella* (10 strains) and *E. coli* (6 strains) were grown to mid-exponential phase and treated with 100 mmol l^{-1} EDTA for 5 min. After centrifugation, the cell pellet was resuspended in 50 mmol l^{-1} Tris-HCl (pH 8.0). The cell suspensions were treated with 0.5 μg endolysin for 5 min and the optical density was measured. The relative lysis activity was calculated from the differences of optical densities at 600 nm between samples treated with buffer and endolysin.

Target site identification

A crude peptidoglycan preparation of *E. coli* DH5α was prepared as previously described (Fein and Rogers, 1976). *E. coli* cell cultures were disrupted by sonication and unbroken cells were removed by low-speed centrifugation (1400 g, 10 min). The supernatant was centrifuged (27,000 g, 5 min) and the pelleted crude cell wall was resuspended in 4% SDS solution and boiled for 10 min. After cooling, the crude peptidoglycan was washed several times. In 160 μl peptidoglycan solution suspended in 50 mmol l^{-1} Tris-HCl (pH 8.0), 5 μg of SPN9CC endolysin was added and the change in optical density at 600 nm was measured using a Sunrise microplate absorbance reader (Tecan, Switzerland). Peptidoglycan suspension that had not been treated with endolysin and the endolysin solutions were used as negative controls.

After hydrolysis, unbroken peptidoglycan was removed by centrifugation, and the supernatant was assayed using a modified Park and Johnson`s method (Park and Johnson, 1949) to investigate whether endolysin cleaves the polysaccharide backbone of peptidoglycan. Briefly, aliquots (40 μl) were mixed with 0.05% (w/v) aqueous potassium ferricyanide (40 μl) and 0.53% (w/v) sodium carbonate/0.065% (w/v) potassium cyanide in water (40 μl). The mixtures were boiled for 15 min. Upon cooling, 200 μl of a ferric ion

reagent (final 0.15% (w/v) ferric ammonium sulfate/0.1% (w/v) SDS in 0.025 M sulfuric acid) was added. A stable Prussian blue color was measured at 690 nm.

For determination of amidase activity, 1.0 mol l^{-1} NaOH was mixed with the hydrolysate. After 30 min incubation, 0.5 ml of 0.5 mol l^{-1} H_2SO_4 and 5 ml of concentrated H_2SO_4 were added. The well-stoppered tube was placed in boiling water bath for 5 min. After cooling, 0.05 ml $CuSO_4$ and 0.1 ml PHD solutions (1.5% *p*-hydroxyphenyl in 96% ethanol) were added. After incubation for 30 min, the OD_{560} was measured (Hadzija, 1974; Hazenberg and de Visser, 1992).

To confirm the peptidase activity in the peptidoglycan hydrolysate, peptidoglycan was acetylated. Peptidoglycan suspension in half-saturated sodium acetate is cooled in an ice bath and mixed by gentle shaking. An equal weight of acetic anhydride (10 mmole l^{-1}) is added in five equal portions over 1 h. The acetylated peptidoglycan was washed with distilled water for three times (27,000g, 5 min). To determine the peptidase activity final 4% $NaHCO_3$ and 0.1% TNBS (trinitrobenzene sulfonic acid) solutions were used. In the dark for 1 h at 40°C, 1N HCl was added for acidification and then OD_{340} was measured (Mikoulinskaia *et al.*, 2009).

In all activity assays, peptidoglycan suspension that had not been

treated with endolysin and the same amount of endolysin solutions were used as negative controls.

III. Results

1. Putative endolysins from phage libraries

Establishment of lysis protein screening system

Two plasmids for confirmation of lysis protein screening system were constructed. pLT1 is T4 bacteriophage holin expression vector, and the gene expression was tightly controlled by arabinose inducible promoter (Guzman *et al.*, 1995). To confirm the holin function of pLT1, endolysin coding gene of T7 bacteriophage was cloned into pLT1, and the recombinant plasmid was named pLT2. In pLT2 plasmid, the expressions of both proteins are controlled by the same promoter. *E. coli* DH5α harboring each vector was added with 0.2% L-arabinose at the optical density (600 nm) reached 0.6. When the holin and endolysin were expressed together, *E. coli* cell growth was very strongly inhibited. But when the holin was expressed alone, it did not show any effect on cell growth compared to non-induced control culture (Figure 1). Thus, it could confirm that pLT1 is useful vector for phage lysis protein screening.

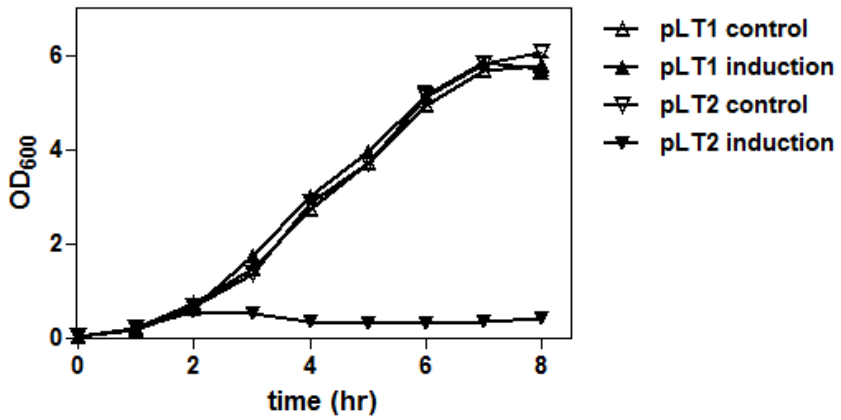


Figure 1. Confirmation of holin expression vector

E. coli DH5α harboring holin expression vector pLT1 or holin and endolysin coexpression vector pLT2 were added with L-arabinose (final 0.2%) to induce protein expression at the 2 h after inoculation.

Phage DNA library construction and lysis gene screening

DNA library of SPJ2 and iEPS5 bacteriophage were constructed. SPJ2 DNA was cloned with holin expression vector pLT1. In contrast, iEPS5 DNA was cloned with pBAD18 to screen whole lysis system. They were transformed into *Salmonella* Typhimurium SL1344. From the transformants, phage DNA libraries were constructed and several lysis proteins were screened by replica plating into two kinds of agar medium, one contained only ampicillin the other contained ampicillin with L-arabinose (final 0.2%). After 8 h incubation at 37℃, it could be found out several clones which showed cell growth inhibition. By sequencing the inserted genes, total three DNA fragments, two fragments from SPJ2 (959 bp and 276 bp) and one fragment from iEPS5 (1,873 bp), were could be obtained.

Lysis proteins from SPJ2 bacteriophage; sub-cloning

DNA fragments which showed lysis activity were sequenced and compared to the sequences in the GenBank by Blast. In the first fragment of SPJ2, there were four complete and one incomplete open reading frames (Figure 2a). Since all open reading frames did not have any similarity to known proteins, several plasmids containing each ORF and holin gene by using primer set shown in figure 2b were constructed. To test the lytic activity of these clones, 0.2% L-arabinose was added to the cell culture at 2 h after inoculation. OD_{600} values were measured as time goes by to compare lysis activity, finally it was confirmed that 333 bp ORF in +1 frame was related to host cell lysis (Figure 3a). The lysis activities of the clone which have whole DNA fragments (pLT10/DH5α) or 333 bp ORF were similar, so other ORFs might be not involved in lytic activity. The lysis protein was named SPJ2P2. In general, among the bacteriophage lysis proteins, endolysin need to holin for its activity. So, lysis activities of SPJ2P2 were compared in the present or absent conditions of holin. As shown in Figure 3b, SPJ2P2 showed lysis activity only in the holin present condition.

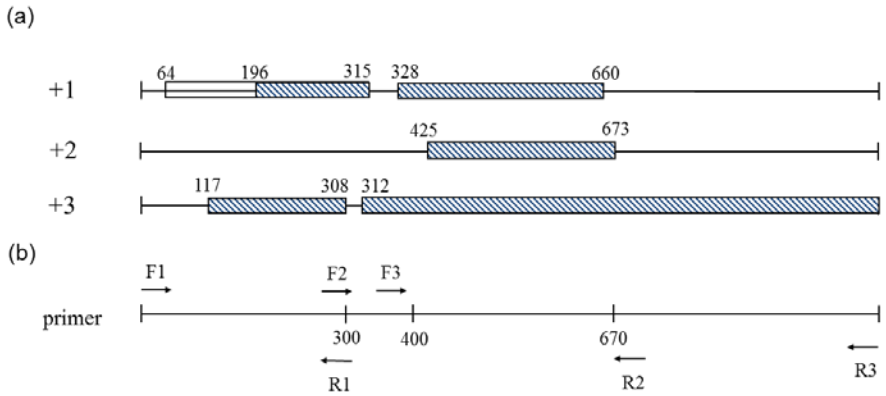


Figure 2. Schematic diagram of the insert DNA in pLT10

(a) Possible open reading frames were shown. The bar with diagonal line has ATG start codon and empty bar has CTG or TTG as start codon.

(b) Several primers for sub-cloning were shown.

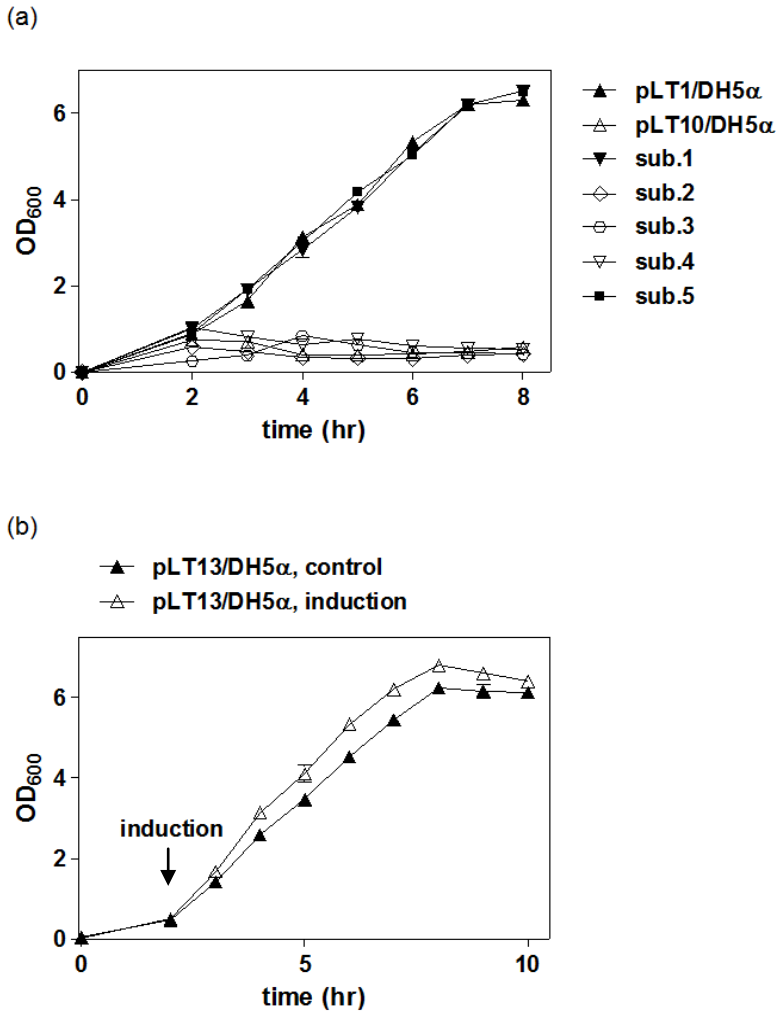


Figure 3. Growth curve of pLT10 sub-clones

(a) The growth of pLT10 sub-clones were compared with DH5α harboring pLT1 as negative control or pLT10 as positive control. L-arabinose (final 0.2%) was added to cell culture at 2 h after inoculation. (b) The effect of SPJ2P2 expression without holin on the host cell growth was analyzed.

The second clone from the SPJ2 phage library has three complete ORFs (Figure 4a). Similar to the first one, all the three ORFs did not have any homology with known proteins. So, sub-cloning using primer set shown in Figure 4b was carried out. As a result, sub-clone 3 showed lytic activity similar to DH5α harboring pLT20 (Figure 5a). The insert DNA of sub-clone 3 has two start sites. One is CTG which is 114 bp from the restriction enzyme site the other is ATG which is located in 162 bp from the restriction site. Two sub-clones which contain both of the start sites or only the second start site showed similar activity, so it was concluded that the lysis protein was started to translate from the second start site, ATG. The lysis protein was named SPJ2P1. SPJ2P1 showed lysis activity only when it acted with holin, like SPJ2P2 (Figure 5b).

Two lysis protein coding sequences from SPJ2 phage were shown in Figure 6.

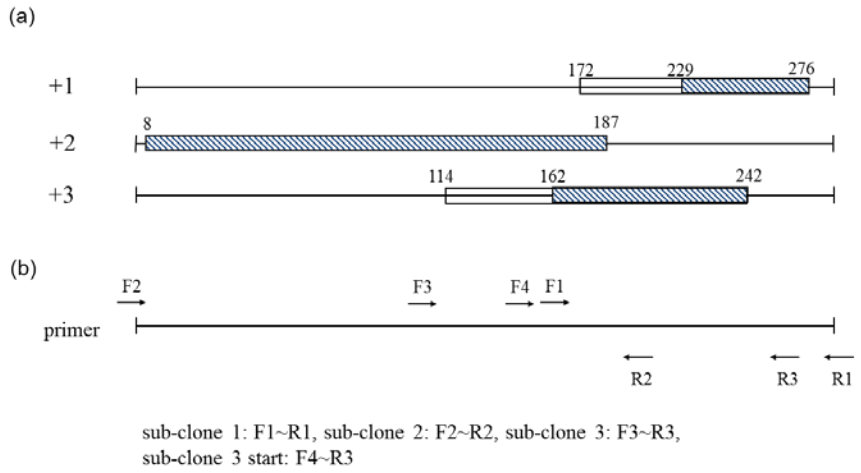


Figure 4. Schematic diagram of the insert DNA in pLT20

(a) Possible open reading frames were shown. The bar with diagonal line has ATG as start codon and empty bar has CTG or TTG as start codon.

(b) Several primers for sub-cloning were shown.

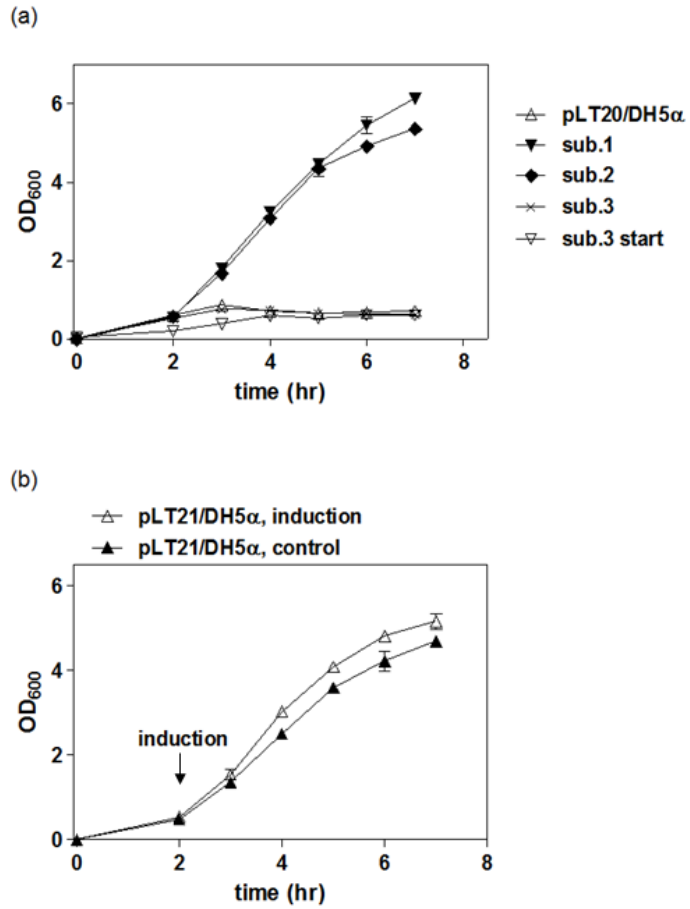


Figure 5. Growth curve of pLT20 sub-clones

(a) The lysis activity of pLT20 sub-clones were compared to DH5α harboring pLT20. In all culture, L-arabinose (final 0.2%) was added to induce protein expression at 2 h after inoculation.

(b) The effect of SPJ2P1 expression without holin on the host cell growth was analyzed.

(a)

```
ATGGCGGAAG TCATTTATCC GTCGTGCAGC ATAGCGACCA GTTTTATTGT  50
CGCGACTGCC AGAAGACATG GGACGCGAAT GACATCGACC CGCCGCCATG  100
TACCGCGCCG GAGTCAGACG AGCCGAAGAA AACGATCTGG AAACGCTGGC  150
AAGGCGGCCC GGCCTGTCTC CAGAACCTTC CGAAAGACGA GCCGAAAGCA  200
ACTGTTACGA CCACGCCCAC CGGACGTCGC GTTTCGGCCA GCCCGGCTTT  250
CGCCTATCCG TACGGCACCC CGGCGTCCGG AGAATGTCAA GCGGTACCGC  300
CAGTGCCGCC GGAGTGGATC GCCGAATACA TGA                    333
```

(b)

```
ATGTGCTCGG TTGCAACCCG AGTTAGAGAG GTTAGAATCC TCTACTCGCC  50
TCCAAACTGG AATCCTTATG AAGCATTGTA A  81
```

Figure 6. Sequences of the lysis protein coding genes of SPJ2 bacteriophage

(a) SPJ2P2 (b) SPJ2P1

Lysis protein from SPJ2 bacteriophage; overexpression

To characterize lysis proteins of SPJ2, protein expression vectors were constructed. In case of SPJ2P1, protein coding gene was cloned with pET15b to produce N-terminal his-tagged SPJ2P1. The recombinant plasmid pLT24 was transformed into BL21 Star^{TM} (DE3), a high-performance host designed for improved protein yield in T7 promoter-based expression systems (Aghera and Udgaonkar, 2011). The culture was added with IPTG (final concentration 1 mmol l^{-1}) to induce protein expression at OD_{600} reached 0.4, 0.7 or 1.3. After 2, 4, or 6 h further incubation, total protein profiles were analyzed by Tricine SDS-PAGE. The expected protein size is about 5.3 kDa, but there was not overexpressed protein (data not shown).

In case of SPJ2P2, several plasmids were used for protein expression. Firstly, for N-terminal his-tagged SPJ2P2 the gene was cloned into pET15b. The recombinant plasmid pLT15 was transformed into Rosetta(DE3) which harboring pRARE plasmid to overcome the codon bias of *E. coli* for enhanced protein expression. By trial and error changing induction time, IPTG concentration for protein expression, incubation temperature, or incubation time, protein expression conditions could be set up although the amount of protein did not much compared to common overexpressed proteins (Figure 7).

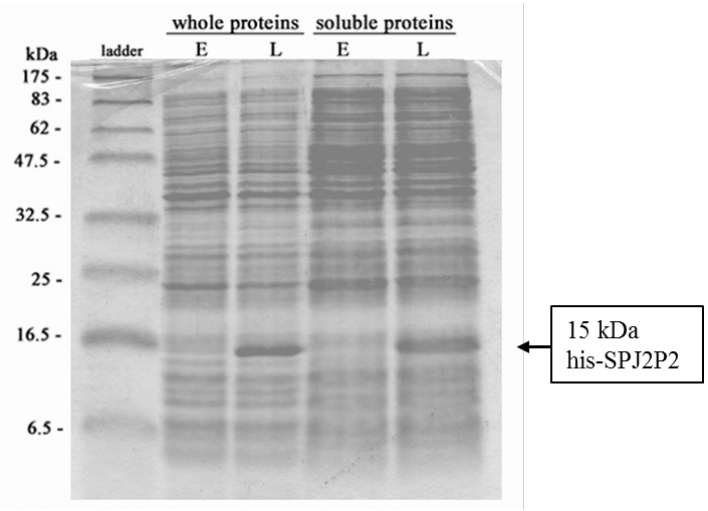


Figure 7. Protein profiles of *E. coli* harboring pLT15

The cultures of Rosetta(DE3) harboring pET15b or pLT15 were added with 50 μmol l^{-1} IPTG at OD_{600} reached 1.5. After further incubation for 2 h at 30℃, total and soluble protein profiles were analyzed by SDS-PAGE. E, pET15b/Rosetta(DE3) as negative control;

L, pLT15/Rosetta(DE3).

To purify the recombinant protein, Ni-NTA (Nitrilotriacetic acid) affinity chromatography was performed, but there was no eluted protein by imidazole gradient. It might be that most of the proteins did not bind to Ni-NTA resin because the protein band in the size of his-tagged SPJ2P2 could be detected in flow through. So, other affinity resins like Ni-TED (tris-carboxymethyl ethylene diamine), Ni-IDA (iminodiacetic acid), TALON, or Ni-Sepharose were used. But the results were not changed (data not shown).

Two more vectors, pLT16 or pLT18 for C-terminal his-tagged or N-terminal GST-tagged SPJ2P2 were constructed. They were also transformed into Rosetta(DE3) for protein expression, but it did not reach to purification step since the expression level was too low (C-terminal his-tagged SPJ2P2) or overexpressed protein was insoluble (GST-tagged SPJ2P2) (data not shown).

SPJ2P2 has very high isoelectric point value, about 12.4. Thus, purification of the protein was tried to by ion exchange chromatography. From the pLT17 plasmid in Rosetta(DE3), intact SPJ2P2 was expressed. Since the protein was suspended with pH 8.0 buffer, cation exchanger such as SP or MONO-S was used for ion exchange chromatography. With FPLC system, purification of SPJ2P2 was tried with NaCl gradient, but the protein concentration of eluted fractions was too low. The fractions were concentrated, but there were too many protein bands

(data not shown).

Lysis protein from iEPS5 bacteriophage; sub-cloning

Recently, the genome of bacteriophage iEPS5 was fully sequenced (Ryu *et al*., unpublished data). The insert DNA fragment of clones which showed lysis activity from iEPS5 phage DNA library was compared to iEPS5 full genome. As a result, in 1,873 bp DNA fragment there are three ORFs, ORF1 (255 bp), ORF2 (714 bp), and ORF3 (339 bp) (Figure 8). ORF1 and ORF2 are in different frames overlapping 4 bp. ORF2 and ORF3 are in the same frame at a 3 bp distance. To find out lysis related genes, three ORFs were expressed alone or with holin. As a result, ORF1 showed activity only when it acted with holin, on the other hand, lytic activity ORF3 protein was independent on holin function (Figure 9).

To investigate whether the action of lysis protein is ‘inhibition’ or ‘lysis’, viable cell counts of *E. coli* DH5α and *S*. Typhimurium SL1344 harboring lysis protein expression vector were measured. As shown in Figure 10, the numbers of viable cells were reduced after protein expression. Thus, it could be concluded that the activity of iEPS5 lysis protein was not just ‘growth inhibition’ but ‘cell lysis’.

(a)

```
ATGACGGGGC TACTCGCCCG GATCAAGACG GGCGTCCTTG CGGCGCTCGT  50
TTTCGTTGTA GCCCTCTTCG GCGTCTGGCG GGCAGGACGC ACGAAGGGCA  100
AACAGGATCA GATTAATAAC CAGAACTACG ACACGTTGCG GGAACAGGCA  150
AACGCGGATA AAAATGTGGC CGAGGTGCAT AATGAAATCA ATAAACTCCC  200
TGATGGTGGC GCTAACGATC TGCTTCGCCG TAAGTGGATG CGTCGAAAGG  250
ATTAA  255
```

(b)

```
ATGGCTAAAC AGAAGCCGCG CGGGATTCGC AATAACAACC CGGGCAACAT  50
CGAGTGGGGG TCGCCGTGGC AAGGTCTGAT CCCGCGTAAC GAGGCGACGG  100
ACAGCCGCTT CGCGCAGTTC AAAGACCCGG CATCCGGCAT CCGCGCCATT  150
GCTGTCACCC TGACCACCTA CTACGACAAG CGCAAGGCCA ACGACGGCAG  200
CAAAATCGAC TCGGTTCGCG AAGTGATCGA GCGGTGGGCA CCCGCCGTTG  250
AGAACAACGT ATCTGCATAC GCTAAACAGG TGGCCGCCGT TCTGGCCGTT  300
GACCCGAATA GCGAAACGCT GAATCTCCAC GACTACGACA CCATGCGCGG  350
TCTGGTGGAA GGCATCATCC GCCACGAAAA CGGGAACCCG GAAGCGTTCG  400
GCCTGACGCC GTACAGCAAC GCGAATATGT GGTACTCGGA CGAAGTGATC  450
GAGGAAGGTC TGCGCCGGGC GGGCATCGTT AAGGCGGCCA AGCCTGTTAA  500
CCGTACCACC GTGGCCGCGA CGAGCGTGGC CGGACTCGGT GCGGCTCAAC  550
TGGTGGACAT GGTTCAGCCC GTCAAGGCTG CGATGGACAG CGCCCACGGC  600
GATATTTCTT CCGGTGATTG GGTACGCATC GCATTCGGCG CGGCCACAAT  650
CGCGATTGGC CTGTACATGG GATGGGTCGC GTACCGGAAG CATCGGGCCG  700
GGGCCGCTGC ATGA  714
```

(c)

```
ATGAGCGAAA TGGAACGGGA ACGGCCGGGG GATACCTCCG GCGGCATCAA  50
GTTAGACTTG TCTGTCAATC TCCCCACGAT TCTGACGATG GCTTCGATGC  100
TGGTGGCGGC GGTACTGTAC GTCAATAACC GCTTCGCCGA CCTATCAAAT  150
CAGAACACGC AGACGGACAC CCGGCTTACC AACGTAGAGA AACGTCAGGA  200
CTCCACTGAA GCGGCGTTCA CGGTTCTCCG GGCCGAAACC GTCTCGCAAA  250
ACTCCGCCCT TCGTTCAGAC CTTCGCGCCG ACATGCGCGA CCTGAAACAA  300
AGCGTGGATA CCCTATCTTC CCAAATGATG AGGAAATAA  339
```

Figure 8. Sequences of the lysis genes of iEPS5 bacteriophage

(a) ORF1 (b) ORF2 (c) ORF3

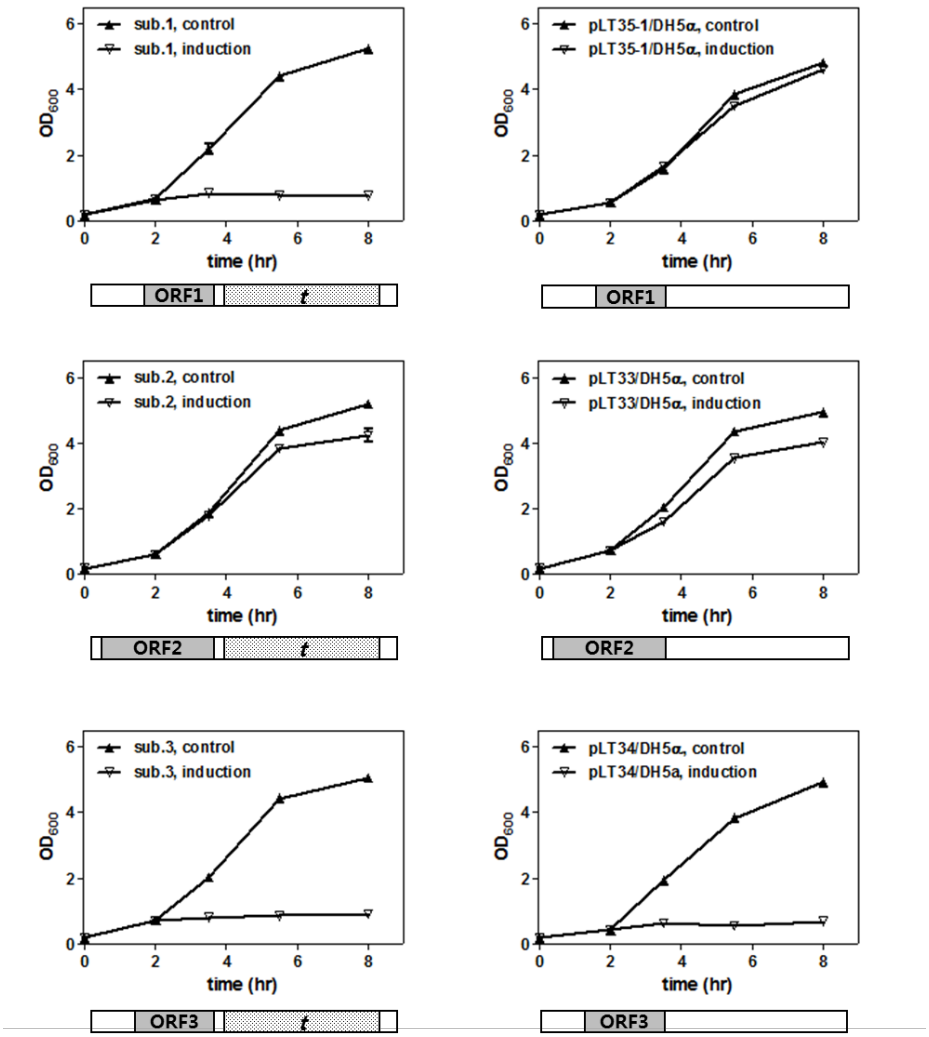
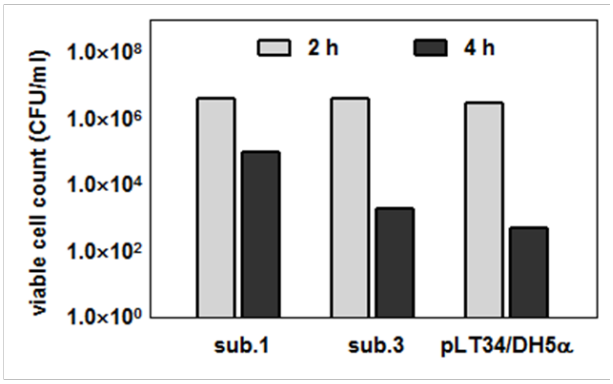


Figure 9. Growth curves of iEPS5 ORFs sub-clones

Three ORFs of iEPS5 were expressed alone or with holin by addition with 0.2% L-arabinose at 2 h after inoculation.

t, holin coding gene of bacteriophage T4.

(a)



(b)

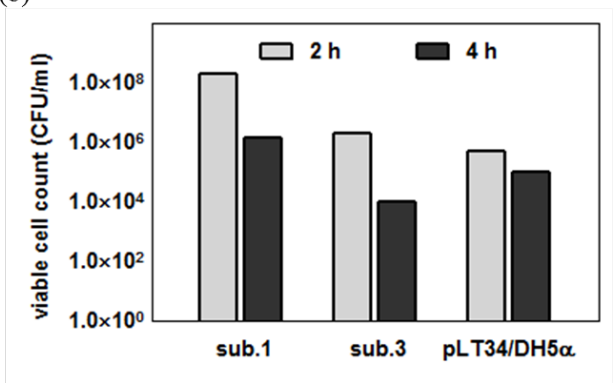


Figure 10. Lysis activities confirmation of iEPS5 proteins

(a) *E. coli* DH5α harboring ORF1 and holin coexpression vector (sub.1), ORF3 and holin coexpression vector (sub.3), or ORF3 expression vector (pLT34) were induced to protein expression. The viable cell numbers before or after induction were measured and compared to each other.

(b) *S.* Typhimurium SL1344 was used as host instead of DH5α.

Lysis protein from iEPS5 bacteriophage; overexpression

To purify iEPS5 ORF1, several vectors were constructed. Firstly, for oligo-histidine tagged protein at N-terminal or C-terminal, ORF1 was cloned into pET15b or pET29b. The recombinant plasmids (pLT36-1 and pLT37-1) were transformed into C41(DE3) or C43(DE3) harboring pRARE plasmid. The strain C41(DE3) was derived from BL21(DE3) as described in before (Miroux and Walker, 1996). This strain has at least one uncharacterized mutation, which prevents the cell death associated with the expression of toxic recombinant proteins. The strain C43(DE3) was further derived from C41(DE3) transformed with the F-ATPase subunit gene and cured. Thus, these strains are effective in overexpressing toxic proteins. As a results, the expressed levels of his-tagged ORF1 protein were not low, but they were insoluble (data not shown).

To improve the protein solubility, a vector for expression of N-terminal MBP-tagged ORF1 protein was constructed. For proteins which have solubility problems, the MBP fusion system might be suitable due to the inherent solubility of the MBP-tag itself (Sheffield *et al.*, 1999). Actually, MBP-tagged ORF1 protein was expressed very well in soluble form, finally, purification of recombinant lysis protein was succeed (Figure 11).

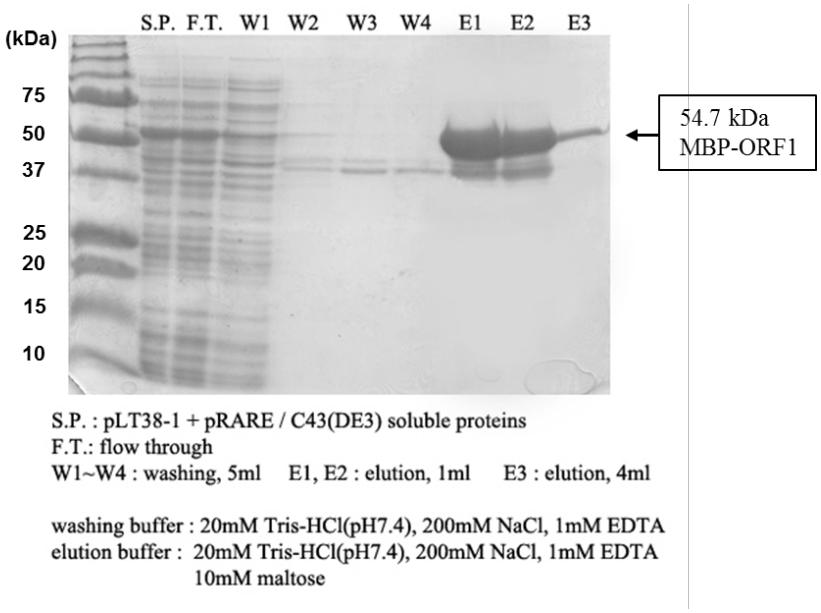


Figure 11. Purification of MBP-tagged iEPS5 ORF1 protein

MBP-tagged iEPS5 ORF1 protein was bound to amylose resin and eluted with 10 mM maltose.

iEPS5 ORF1 encodes very small protein (9.5 kDa). On the contrary, MBP-tag is about 45 kDa. To confirm an impact of big difference of their size between two proteins, MBP-tagged ORF1 protein was expressed with holin. Figure 12 showed lysis activity of MBP-tagged ORF1 protein produced in the DH5α. Optical density of cell culture and viable cell numbers were decreased after protein expression. And the activity of MBP-tagged ORF1 protein was similar to that of intact ORF1 protein.

Lysis activity of MBP-tagged ORF1 protein in purified form was checked using EDTA-pretreated DH5α as substrate. Unfortunately, it was not functional (Figure 13). Lysozyme used as positive control reduced the optical density of substrate cell dramatically, but MBP-tagged ORF1 protein did not show any difference compared to buffer control.

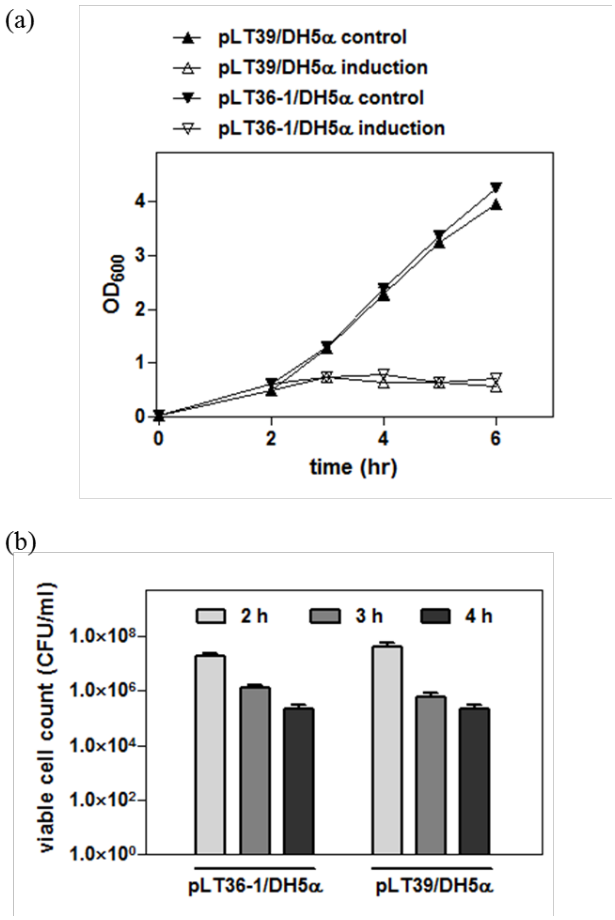


Figure 12. **Lytic activity of MBP-tagged ORF1 protein from inside**

(a) *E. coli* DH5α harboring ORF1 protein and holin expression vector (pLT36-1) or MBP-tagged ORF1 protein and holin expression vector (pLT39) were incubated with or without L-arabinose. OD_{600} values were measured as time goes by.

(b) Viable cell numbers of DH5α harboring pLT36-1 or pLT39 were measured at just before or 1 h and 2 h after induction time.

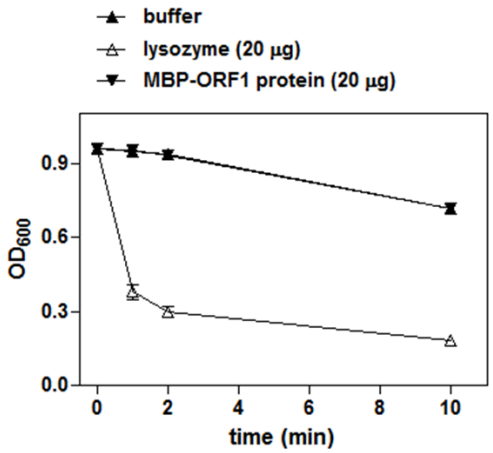


Figure 13. Lytic activity of MBP-tagged ORF1 protein from outside

In the EDTA pretreated DH5α suspension, 20 μg MBP-ORF1 protein was added. As time goes by, OD_{600} were measured. Buffer and lysozyme (20 μg) were also used as negative and positive controls.

2. SPN1S endolysin

Lysis genes of SPN1S bacteriophage

The genome of bacteriophage SPN1S was fully sequenced (GenBank ID: JN391180). In the host lysis cluster of SPN1S bacteriophage genome, there are four open reading frames (ORFs) encoding putative holin, putative endolysin and putative Rz/Rz1-like proteins. Annotation and functional analysis of ORFs in this cluster revealed that two ORFs (SPN1S_0027, 309-bp and SPN1S_0028, 630-bp) are homologous to holin and endolysin of phage epsilon 15 (amino acid identity, 99% and 94%), a serotype-converting, group E1 *Salmonella enterica*-specific bacteriophage (Kropinski *et al.*, 2007). The other two ORFs (SPN1S_0029, 483-bp and SPN1S_0029_1, 276-bp) have homology with the Rz/Rz1-like proteins of phage phiV10 (amino acid identity, 80% and 85%), an *E. coli* O157:H7-specific temperate phage (Perry *et al.*, 2009) (Table 5). InterProScan analysis in this gene cluster showed that only putative endolysin has functional domains. The putative endolysin of SPN1S has homology to Lysozyme-like domain (SSF53955) and the C-terminal region of that has family 19-glycoside hydrolase catalytic domain (PF00182). On the other hand, a putative holin and Rz/Rz1-like proteins have transmembrane region(s) and a

Table 5. ORF analysis of lysis cluster genes in the SPN1S genome

ORF	Predicted function	Length[a]	Results of InteProScan (Domain)	Location[b]	Matches	BLASTP best matches	% Identity[c]
SPN1S_0027	Holin	102	Bacteriophage D3, ORF90 (PF05449)	10-95	1.2E-30	holin from *Enterobacteria* phage epsilon15	99%
			Signal-peptide	1-20	-	holin protein from *Escherichia coli* O127:H6 str. E2348/69	98%
			Transmembrane_regions	2-24, 39-61	-	putative holin from *Escherichia* phage phiV10	93%
SPN1S_0028	Endolysin	209	Glyco_hydro_19 (PF00182)	130-172	1.5E-9	endolysin from *Enterobacteria* phage epsilon15	94%
			Lysozyme-like (SSF53955)	7-207	1.4E-44	endolysin from *Escherichia coli* O127:H6 str. E2348/69	90%
						chitinase class I family protein from *Escherichia coli* EPECa14	82%
SPN1S_0029	Rz protein	160	DUF2514 (PF10721)	10-159	4.1E-34	endopeptidase from *Escherichia coli* O127:H6 str. E2348/69	84%
			Prokar_lipoprotein (PS51257)	1-26	5.0	putative Rz-like protein from *Enterobacteria* phage epsilon15	84%
			Signal-peptide	1-28	-	putative lysis accessory protein from *Escherichia* phage phiV10	80%
			Transmembrane_regions	13-31	-		
SPN1S_0029_1	Rz1 protein	91	Prokar_lipoprotein (PS51257)	1-26	7.0	Rz1 from *Enterobacteria* phage epsilon15	92%
			Signal-peptide	1-29	-	putative Rz1-like protein from *Escherichia* phage phiV10	85%
			Transmembrane_regions	13-35	-	lipoprotein precursor from *Escherichia coli* O127:H6 str. E2348/69	89%

[a] Number of amino acids; [b] Amino acids sequence location; [c] Amino acids sequence identity.

signal-peptide region. And a putative holin and Rz/Rz1-like proteins have unknown function domains (PF05449 and PF10721) and Rz/Rz1-like proteins have PROKAR_LIPOPROTEIN entry (PS51257) using PROSITE. ClustalW alignment and phylogenetic analysis of putative endolysin were performed with similar sequence from various prophages or bacteriophages (Figure 14, 15).

The four genes are in tandem in SPN1S with sequence overlaps of 11 bp between the holin and the endolysin and 4 bp between the endolysin and the Rz-like proteins. And the Rz1-like protein is overlapped with Rz-like protein in other frame. To confirm the functions of these genes, they were cloned into expression vector pBAD18 separately or together, and expressed in *E. coli* DH5α. As shown in Figure 16, none of the 4 genes affected growth of *E. coli* when expressed alone. When all four were expressed, there was strong lysis activity. Interestingly, expression of endolysin and the Rz/Rz1-like proteins also yielded strong lysis activity. SPN1S endolysin showed lysis activity when it was expressed with T4 holin, but the expression of T7 endolysin with SPN1S holin or Rz/Rz1-like proteins did not show any growth defect (Figure 17). Thus, it was possible that SPN1S Rz/Rz1-like proteins affects bacterial cell lysis only when it acted with SPN1S endolysin and SPN1S phage has unusual lysis system.

Figure 14. CLUSTALW alignment of SPN1S endolysin with similar sequences from various prophages or bacteriophages.

Identical (*), highly similar (:), and similar () amino acids are indicated. Numbers refer to the amino acids positions.

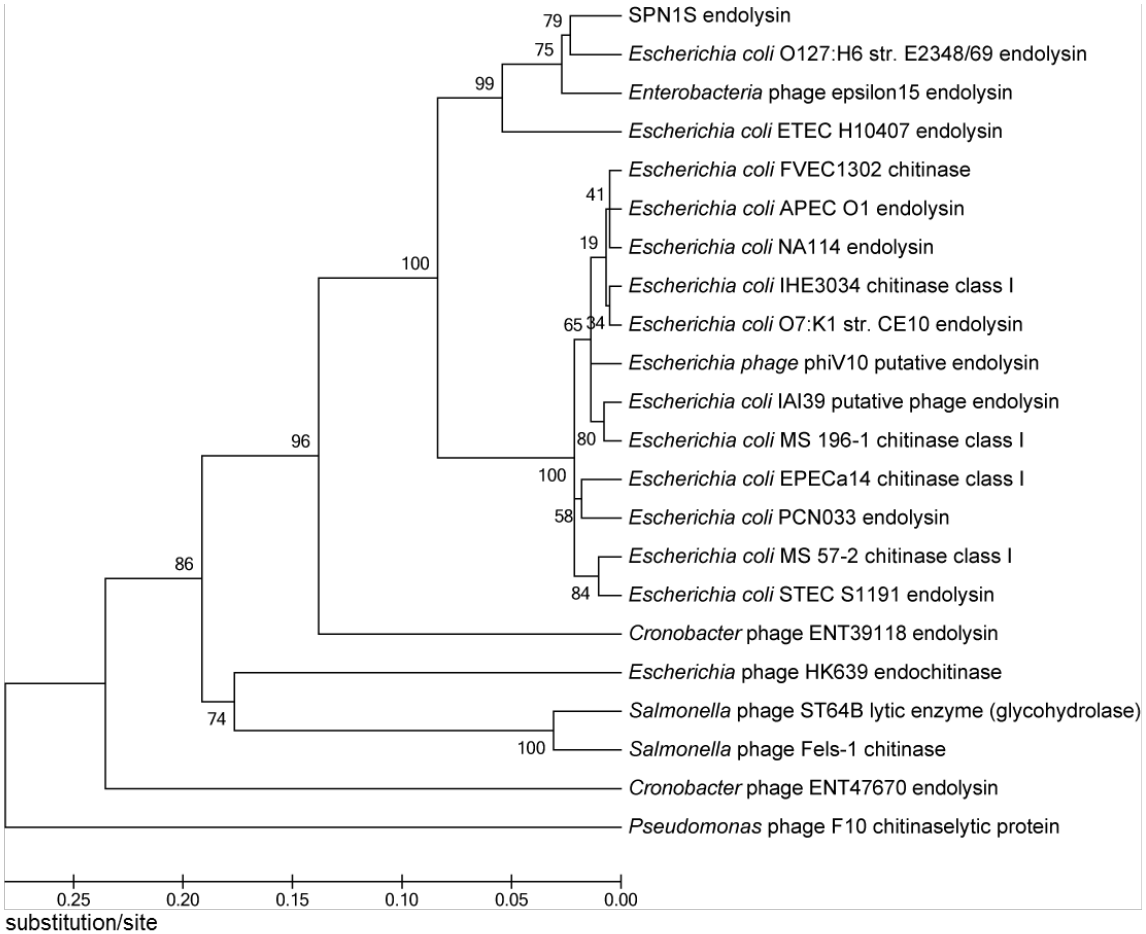


Figure 15. Phylogenetic analysis of SPN1S endolysin with similar sequences from various prophages or bacteriophages. Endolysins were compared by ClustalW multiple alignments and the phylogenetic tree was generated by MEGA5 program using the neighbor-joining method with *P* distance values.

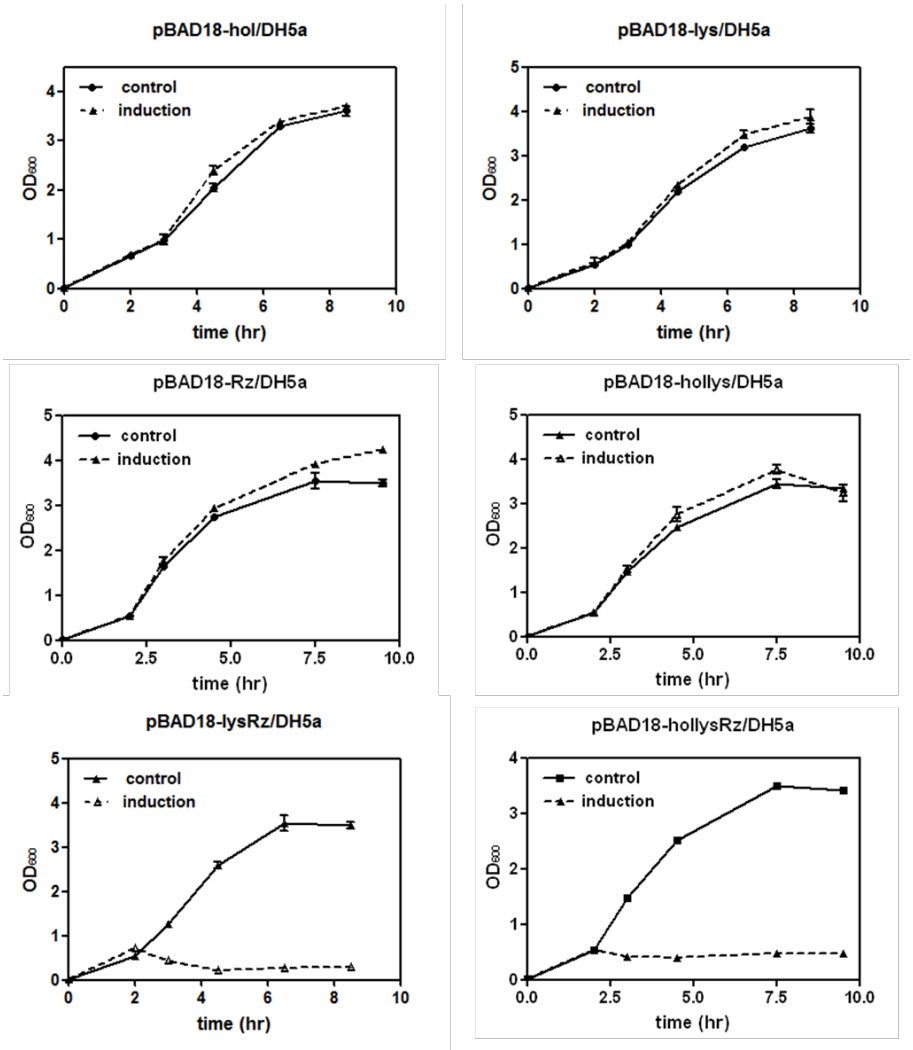


Figure 16. Expression of the holin, endolysin and Rz/Rz1-like proteins of bacteriophage SPN1S

Holin, endolysin, and Rz/Rz1-like accessory proteins were expressed in *E. coli*. Protein expression was induced 2 h after inoculation with 0.2% L-arabinose. Lysis of induced cells was measured by the decrease in absorbance at 600 nm. hol; holin, lys; endolysin, Rz; Rz/Rz1-like proteins.

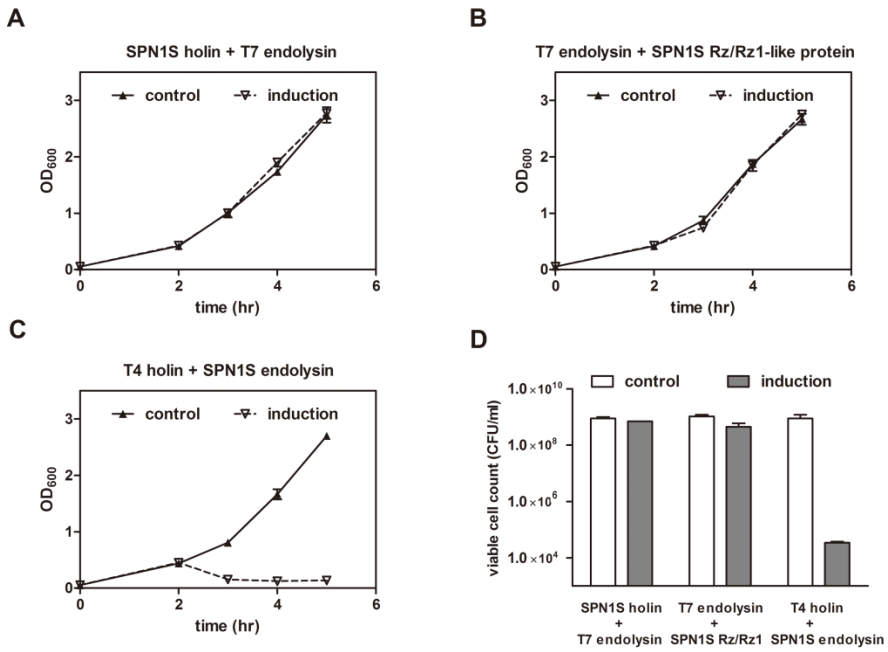


Figure 17. Lysis activities of SPN1S lysis proteins with known lysis proteins.

SPN1S holin and T7 endolysin (A), T7 endolysin and SPN1S Rz/Rz1-like proteins (B), T4 holin and SPN1S endolysin were expressed with 0.2% L-arabinose at 2 h after inoculation. The viable cell numbers at 5 h incubation of each culture were measured (D).

Enzymatic lysis activity of purified endolysin

The endolysin coding region was cloned into pET15b and transformed into BL21 Star^{TM} (DE3), a high-performance host designed for improved protein yield in T7 promoter-based expression systems (Aghera and Udgaonkar, 2011). Total protein profiles were analyzed by SDS-PAGE (Figure 18). An overexpressed protein band of the expected size (approximately 26 kDa) was detected.

The addition of purified SPN1S endolysin to *E. coli* is not expected to affect cell viability because endolysin is hydrophilic and cannot cross the Gram-negative outer membrane to access the peptidoglycan (Hermoso *et al.*, 2007). Thus, I used EDTA to destabilize the outer membrane and allow endolysin to pass through it and reach the peptidoglycan.

At first, the lysis activity of endolysin was assayed by measuring the effect of endolysin addition on the optical density of cell suspensions pretreated with 100 mmol l^{-1} EDTA. Addition of 10 ng ml^{-1} endolysin did not have a strong effect compared to buffer alone, however, gradually higher activities were detected beginning with 50 ng ml^{-1}. Addition of 500 ng ml^{-1} endolysin led to the lysis of most cells within 1 min (Figure 19a). This activity was very strong, and is approximately 30 times higher activity of chicken egg white lysozyme action (Figure

19b).

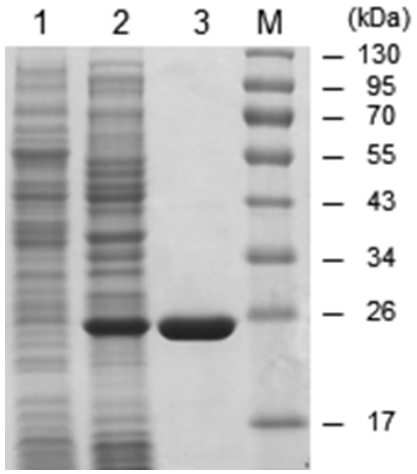


Figure 18. SDS-PAGE analysis of total soluble proteins or purified SPN1S endolysin

Total lysate of IPTG-induced *E. coli* cells harboring backbone plasmid (lane 1) and recombinant plasmid (lane 2) were separated on a 12% acrylamide gel. The overexpressed endolysin was purified (lane 3). M, molecular weight markers

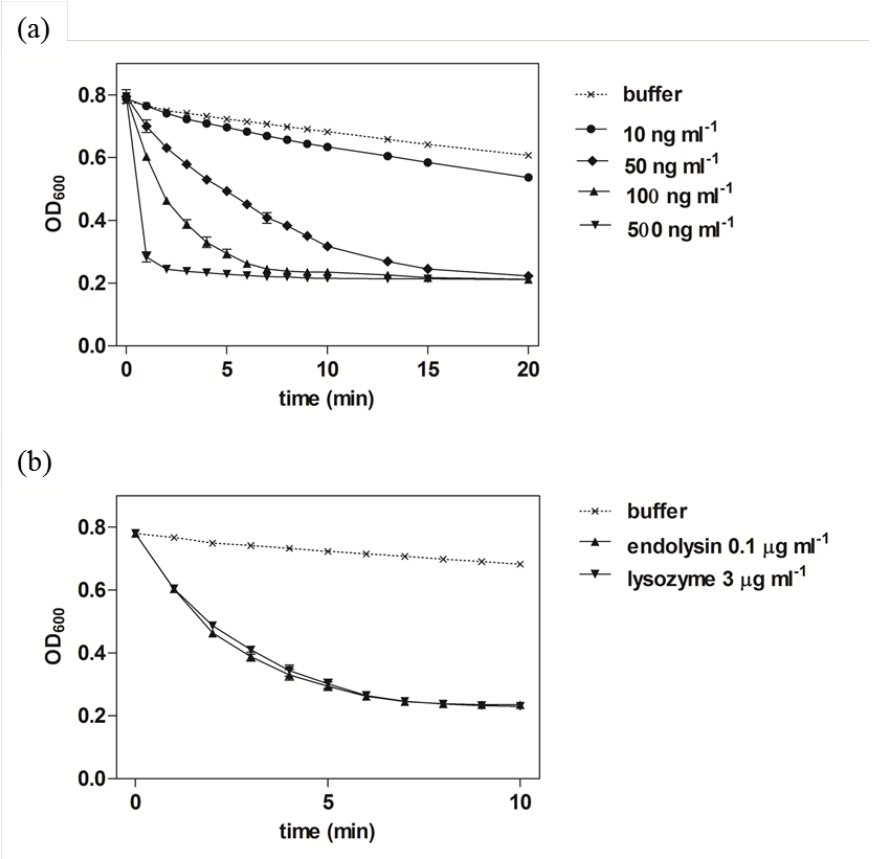


Figure 19. Lysis activities of purified SPN1S endolysin against EDTA pretreated substrate cell

(a) The activities of various concentrations of purified SPN1S endolysin were detected using *E. coli* cell suspensions, with EDTA. (b) Enzymatic activity of 0.1 μg ml^{-1} SPN1S endolysin was similar to that of 3 μg ml^{-1} lysozyme from chicken egg white.

SPN1S endolysin was then added with EDTA to intact *E. coli* cells, instead of pretreating the cells with EDTA (Briers *et al.*, 2011). Without EDTA, endolysin did not affect the number of colony forming units (CFUs) in the cell suspension. However, when added with EDTA, endolysin caused a dramatic reduction in bacterial viability. A combination of 30 ng μl^{-1} endolysin and 5 mmol l^{-1} EDTA resulted in an approximate 2 log reduction in CFUs after a 2 h reaction time comparison with the control with only 5 mmol l^{-1} EDTA added, and a more than a 4 log reduction in CFUs when 100 ng μl^{-1} endolysin and 10 mmol l^{-1} EDTA were added (Figure 20a). The lysis activity varied with not only with concentration of endolysin and EDTA but also with reaction time. Figure 20b shows the relative viable cell numbers after treatment with 50 ng μl^{-1} endolysin and different concentrations of EDTA for 20 min, 1 h, or 2 h. Relative cell counts were calculated after setting cell numbers in untreated suspensions at 100%. Within 20 min, 50 ng μl^{-1} endolysin with 10 mmol l^{-1} EDTA reduced bacterial cell numbers by 60%, and at 2 h by 99.8% comparison with the control with only 10 mmol l^{-1} EDTA added. In any conditions, when the enzyme reaction time was longer, much more viable cells were degraded. The concentrations of EDTA used in this study and oligohistidine tag did not affect endolysin itself.

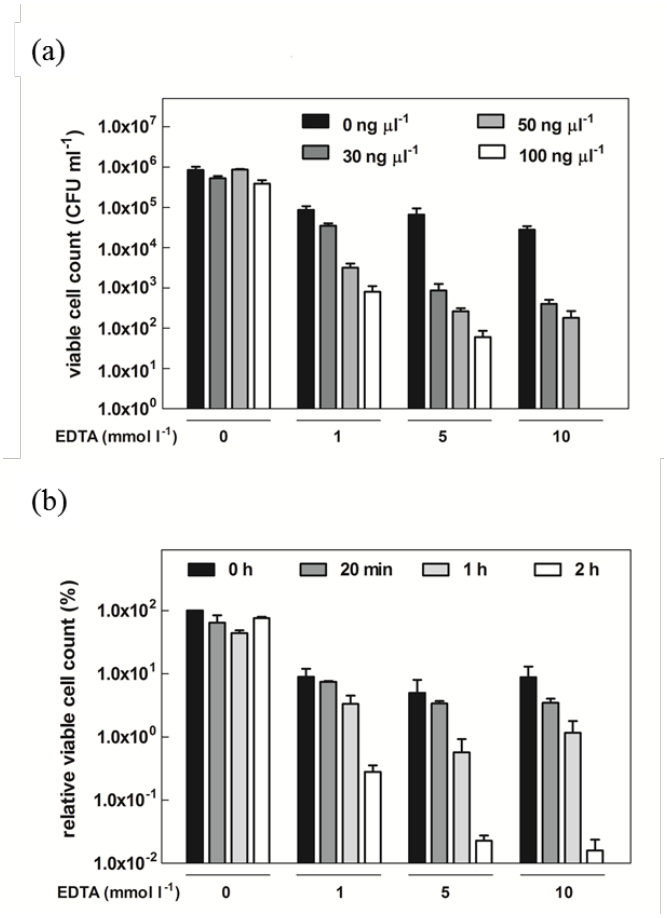


Figure 20. Lytic activity of SPN1S endolysin over a range of EDTA concentrations.

(a) The viable cell numbers (CFUs) of *E. coli* upon addition of 30 ng μl^{-1}, 50 ng μl^{-1}, or 100 ng μl^{-1} endolysin were examined for three EDTA concentrations (1 mmol l^{-1}, 5 mmol l^{-1}, 10 mmol l^{-1}) after a 2 h reaction time.

(b) Relative viable cell numbers upon addition of 50 ng μl^{-1} endolysin and EDTA for different time points were calculated relative to cell numbers after treatment with buffer.

Conditions for enzyme function

SPN1S endolysin was stable from pH 4.3 to 10.5 for 12 days, but was unstable in pH 2.0 buffer with only 13% of its lytic activity remaining. Although thermal treatment at temperatures up to 75°C for 10 min did not inhibit enzyme function, 30 min at elevated temperatures had a negative effect on activity (Figure 21a,b). Endolysin was active to some extent over a broad range of pH and temperature (pH 7.0 to 10.5 and temperature 25°C to 45°C), but showed maximal activity in pH 9.5 and 37°C (Figure 21c, d).

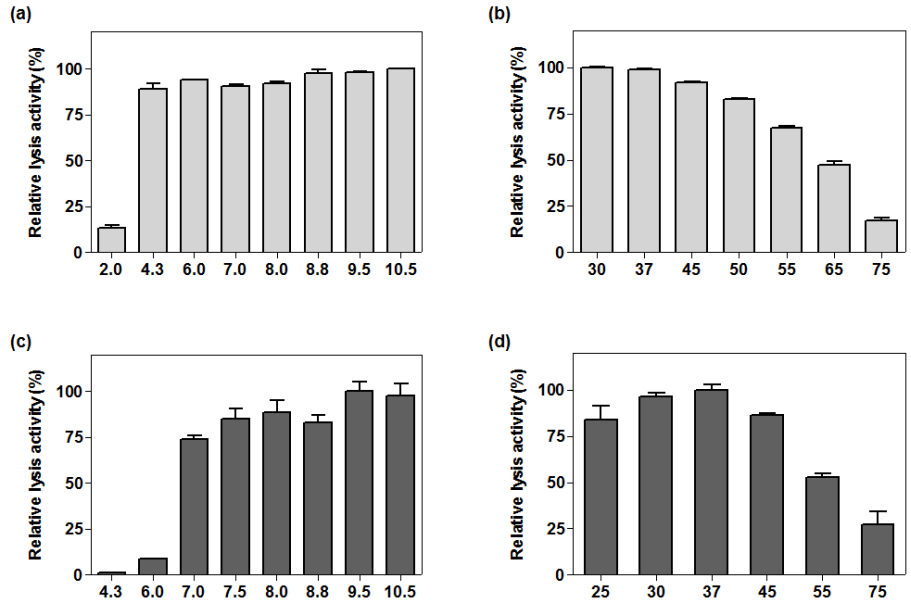


Figure 21. The effect of temperature and pH on enzymatic activity

(a) The enzyme stability at different pH and temperature were measured. The endolysin aliquots were stored in various pH buffer and the residual activities were measured in pH 8.0.

(b) To confirm thermostability, endolysin aliquots were incubated in each temperatures. After 30 min treatment the antimicrobial activities were detected.

The antimicrobial activity of deep-freezed endolysin were assayed in different pH buffer (c) and different temperature (d).

Antimicrobial spectrum

Nine Gram-positive and 21 Gram-negative strains were examined for their susceptibility to SPN1S endolysin (Table 6). SPN1S endolysin was able to lyse all of the tested Gram-negative strains at 50 ng ml^{-1} within 10 min with the highest activity against *E. coli*. However, no activity was detected against the Gram-positive strains. Even at 1000 ng ml^{-1} endolysin for 1 h, there was no effect on the Gram-positive strains tested. Thus, I conclude that SPN1S endolysin had a wide spectrum of antimicrobial activity against Gram-negative bacteria.

Table 6. Antimicrobial activity of SPN1S endolysin

strain		relative lysis activity[a]
Escherichia coli	BL21	++++
	DH5α	+++
	DH10B	+++
	GI698	+++
	JM109	+++
	MC4100	+++++
	MG1655	+++
Escherichia coli O157:H7	ATCC 35150	++
	ATCC 43888	+
	ATCC 43890	++
	ATCC 43894	++
	ATCC 43895	++
Salmonella Typhimurium	LT2	+++
	SL1344	+++
	UK1	+++
	ATCC 14028	++
	KCTC[b] 1925	++
Salmonella Typhi	Ty2	++
Salmonella Paratyphi A		++
Salmonella Paratyphi B		++
Salmonella Paratyphi C		+
Shigella flexneri	2a 2457T	+
Pseudomonas aeruginosa	ATCC 27853	+
Pseudomonas putida	KCTC 1643	++
Cronobacter sakazakii	ATCC 29544	++
Vibrio vulnificus	ATCC 29307	++
Bacillus cereus	ATCC 21772	-
	ATCC 21768	-
	ATCC 27348	-
	ATCC 10876	-
Bacillus subtilis	ATCC 23857	-
Listeria monocytogenes	ATCC 19114	-
Enterococcus faecalis	ATCC 29212	-
Staphylococcus aureus	ATCC 29213	-
Staphylococcus epidermidis	ATCC 35983	-

[a] relative lysis activity;

ΔOD_{600} of endolysin treated sample/ΔOD_{600} of buffer treated sample

-, 1; +, 1~2; ++, 2~3; +++, 3~4; ++++, 4~5; +++++, 5~6

[b] KCTC ; Korean Collection for Type Culture

Target site identification

Bioinformatic analysis shows that SPN1S endolysin has lysozyme-like superfamily domain (E-value, 10^{-44}). Lysozyme damages bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan (Nakimbugwe *et al.*, 2006). To confirm the predicted glycoside bond cleaving activity of SPN1S endolysin, I analyzed the quantity of reducing sugar after peptidoglycan hydrolysis. *E. coli* peptidoglycan was treated with 1 μg ml^{-1}, 5 μg ml^{-1}, or 10 μg ml^{-1} endolysin, and dose-dependent degradation was observed (Figure 22a). After lysis, the amounts of reducing sugar in each sample were measured. As with peptidoglycan degradation, the amount of reducing sugar in enzyme digests was proportional to endolysin concentration (Figure 22b). Buffer and enzyme itself at any concentration used in this experiment did not affect the results. To examine whether endolysin might have other activities, a peptidase assay and an amidase assay were also carried out using enzyme digests but found no difference between endolysin lysate and buffer (data not shown). Thus, it was concluded that SPN1S endolysin cleaves the glycosidic bond of peptidoglycan.

To investigate target site of SPN1S more specifically, two chemical

substrates were used. One is 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside as muramidase substrate the other is 4-Nitrophenyl N-acetyl-β-D-glucosaminide as glucosaminidase substrate. In the substrate solutions, 50 μg of SPN1S endolysin and lysozyme were added. Since lysozyme is known as muramidase so it was used as a positive control. When EDTA pretreated DH5α was used as substrate, SPN1S endolysin showed about 30 times higher activity than that of lysozyme. So, the results of 30 times higher amounts of lysozyme (1.5 mg) was also compared. As a result, SPN1S endolysin and lysozyme showed muramidase activity as expected because SPN1S endolysin has lysozyme like superfamily domain, but toward glucosaminidase substrate there were no difference between buffer added sample and enzyme added sample (Figure 23).

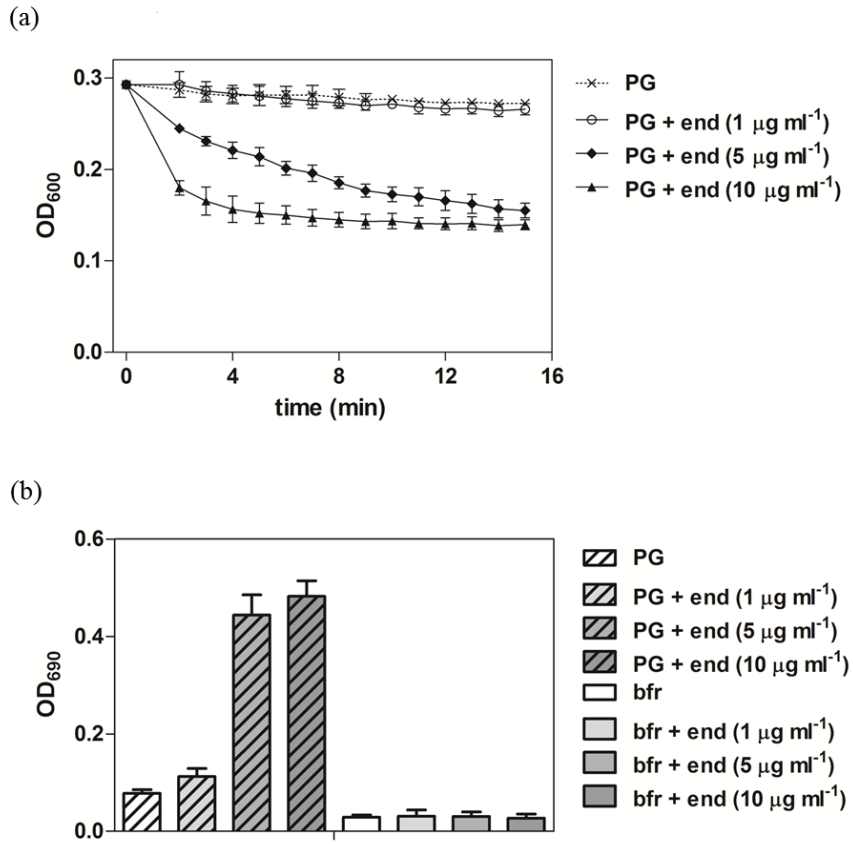
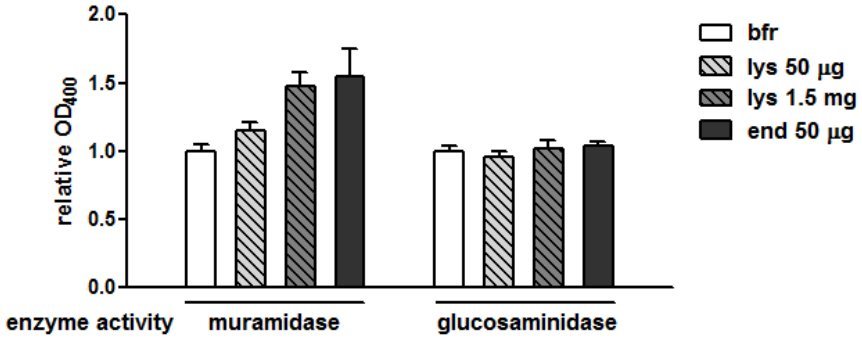


Figure 22. Degradation of *E. coli* peptidoglycan by the SPN1S endolysin

(a) Peptidoglycan degradation was detected as a decrease in optical density.

(b) Reducing sugar was detected after endolysin treatment of *E. coli* peptidoglycan

(a)



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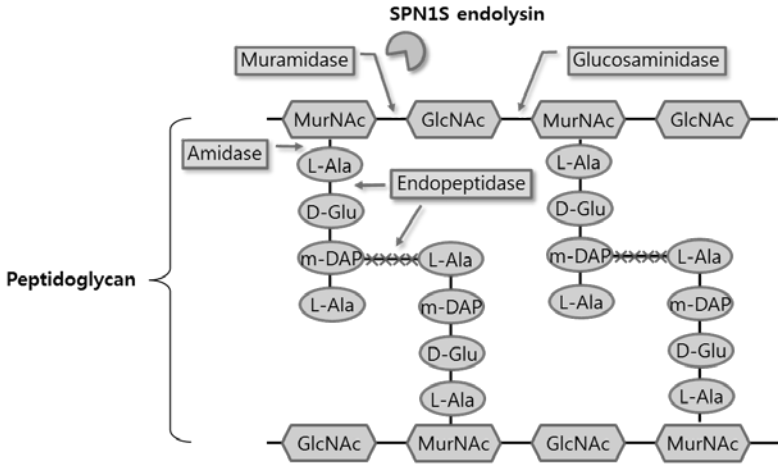


Figure 23. Muramidase activity of SPN1S endolysin

(a) SPN1S endolysin and lysozyme were mixed with muramidase substrate and glucosaminidase substrate, respectively. After reaction at 37℃, OD_{400} value were detected.

(b) Schematic diagram of target bond of SPN1S endolysin

Endolysin improvement for introduction into the bacterial cell

In general endolysins can not introduce into the Gram-negative bacterial cell from the outside because outer membrane acts as physical barrier. However, two endolysins, are capable of affecting the Gram-negative bacterial cells were reported. *Bacillus amyloliquefaciens* phage Morita2001 endolysin has a C-terminal region (99 bp) for enhancement permeabilization of the outer membrane. *Acinetobacter* phage phiAB2 endolysin (lysAB2) has the region of the putative amphipathic helices (aa 113–145) in the C terminus (Lai *et al.*, 2011). From the phage Morita2001 DNA sequence, 21 bp sequences (CTTACTAAAA TCGCTAAAAA G) were added to at the front of stop codon of SPN1S endolysin. The recombinant SPN1S endolysin which has N-terminus his-tag and C-terminus helix structure was purified. Firstly, EDTA pretreated DH5α was used as substrate to confirm that enzymatic function was stand up to added C-terminal sequence. As a result, SPN1S endolysin containing C-terminus helix structure showed still lysis activity although it was about 5 times lower than that of intact endolysin (Figure 24). To confirm the C-terminus sequence improved the endolysin function against intact Gram-negative bacterial cells, DH5α cell culture was mixed with intact endolysin or recombinant endolysin, respectively. In each condition, the effect of outer membrane

permeabilizer was checked by addition of EDTA. As shown in Figure 25, in EDTA absent condition intact SPN1S hardly affected cell viability. Fortunately, C-terminal extra sequences made SPN1S endolysin inhibit cell growth. In EDTA present condition, SPN1S endolysin showed lysis activity similar to previous results. In this case, SPN1S endolysin containing C-terminus helix structure showed dramatic lytic activity. Just for 20 min reaction, the viable cell numbers were decreased about 3.5 log by addition of 50 ng μl^{-1} endolysin. When the amount of endolysin was increased to 100 ng μl^{-1} or reaction time was longer up to 1 h, there was no viable cell. SPN1S containing C-terminal extra sequence had lower enzymatic activity compared to intact endolysin, but when it added to substrate with EDTA at the same time its activity was higher than that of endolysin. It might be that contrary to EDTA pretreated DH5α, the effect of EDTA which added to the substrate at the same time with endolysin was a little bit small, therefore outer membrane permeability was lower than that of EDTA pretreated DH5α. As a result, SPN1S endolysin might show higher lysis activity.

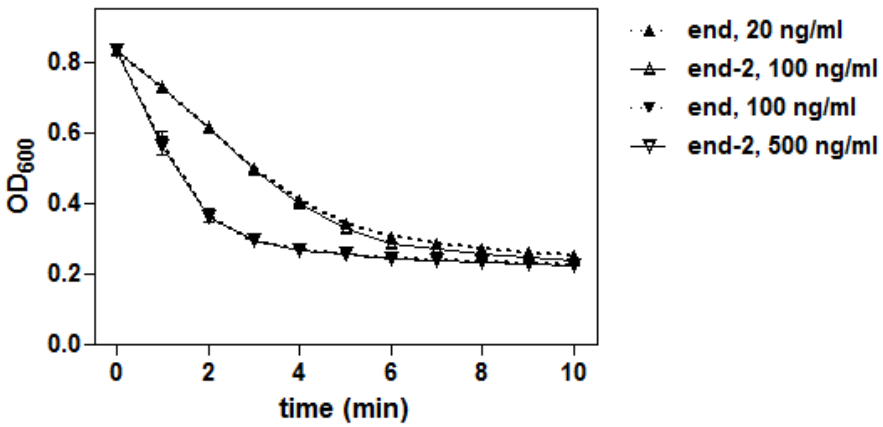


Figure 24. Lysis activity confirmation of SPN1S endolysin containing helix structure

SPN1S endolysin containing C-terminal extra sequence for helix sturcutre was added to EDTA pretreated DH5α as substrate. The activity was compared to that of 5 times lower amounts of intact endolysin. end, intact SPN1S endolysin; end-2, SPN1S endolysin containing C-terminal extra sequence

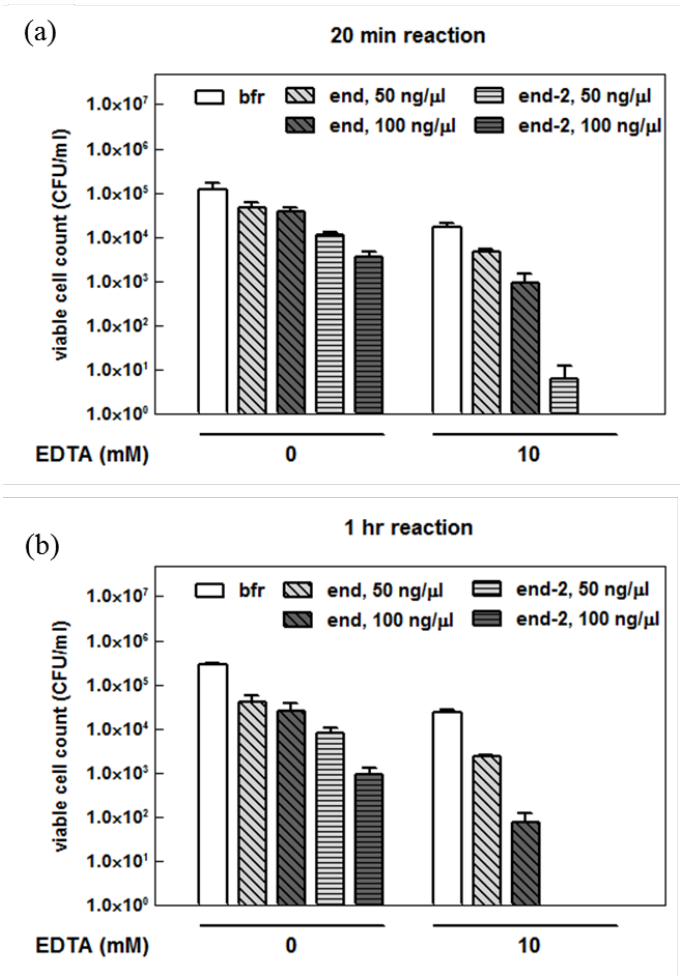


Figure 25. Lytic activity of improved SPN1S endolysin

SPN1S endolysin with or without C-terminal extra sequences for helix structure were treated to intact DH5α. The reactions were continued for 20 min (a) or 1 h (b) in absent or present conditions of EDTA. After reactions, viable cell number were measued by spreading them. end, intact SPN1S endolysin; end-2, SPN1S endolysin containing C-terminal extra sequence

3. SPN9CC endolysin

Expression and purification of SPN9CC endolysin

Bioinformatic analysis of the sequence of SPN9CC (GenBank ID: JF900176) and the 63 ORFs identified lysis gene cluster of SPN9CC phage which is very similar to those of P22-like phage ST104 and *E. coli* K-12 DLP12 prophage. In the host lysis cluster of SPN9CC bacteriophage, SPN9CC_0043 identified as a putative endolysin.

The endolysin coding region was cloned into pET29b and transformed into BL21 (DE3). Final 1 mmol l^{-1} IPTG was added for induction of recombinant endolysin overexpression at OD_{600} reached 0.6. After 4 h further incubation total soluble protein profiles were analyzed by SDS-PAGE (Figure 26). An overexpressed protein band of the expected size (approximately 17 kDa) was detected.

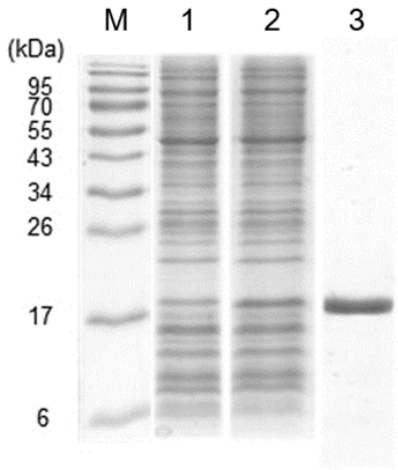


Figure 26. SDS-PAGE analysis of total soluble proteins or purified SPN9CC endolysin

Total lysate of IPTG-induced *E. coli* cells harboring backbone plasmid (lane 1) and recombinant plasmid (lane 2) were separated on a 15% SDS-PAGE. The purified endolysin showed single band (lane 3).

M, molecular weight markers

Lysis activity of SPN9CC endolysin

The lysis activity of SPN9CC endolysin was assayed by measuring the effect of endolysin addition on the optical density of substrate suspensions pretreated with 100 mmol l^{-1} EDTA. Addition of 0.1 μg ml^{-1} endolysin showed a little lysis effect compared to buffer alone, and, the enzyme activity increased gradually as the endolysin concentration was increased up to 5 μg ml^{-1} (Figure 27a). However, the same amount of SPN9CC endolysin showed lower lysis effect against intact *E. coli* cell suspension without EDTA pretreatment (Figure 27b). Confirmation of lysis activity against intact *E. coli* could be done when the amount of endolysin and incubation time were increased. For 1 h reaction, SPN9CC endolysin (300 ng μl^{-1}) reduced the viable cell numbers about a 2 logs in CFUs (Figure 28a). In longer incubation time to 2 h, EDTA addition enhanced lysis activity a lot against *E. coli*. A combination of 300 ng μl^{-1} endolysin and 1 mmol l^{-1} EDTA resulted in an approximate 4 logs reduction in CFUs compared with the control with only 1 mmol l^{-1} EDTA added (Figure 28b). The concentration of EDTA (1 mmol l^{-1} or 5 mmol l^{-1}) did not affect lysis activity.

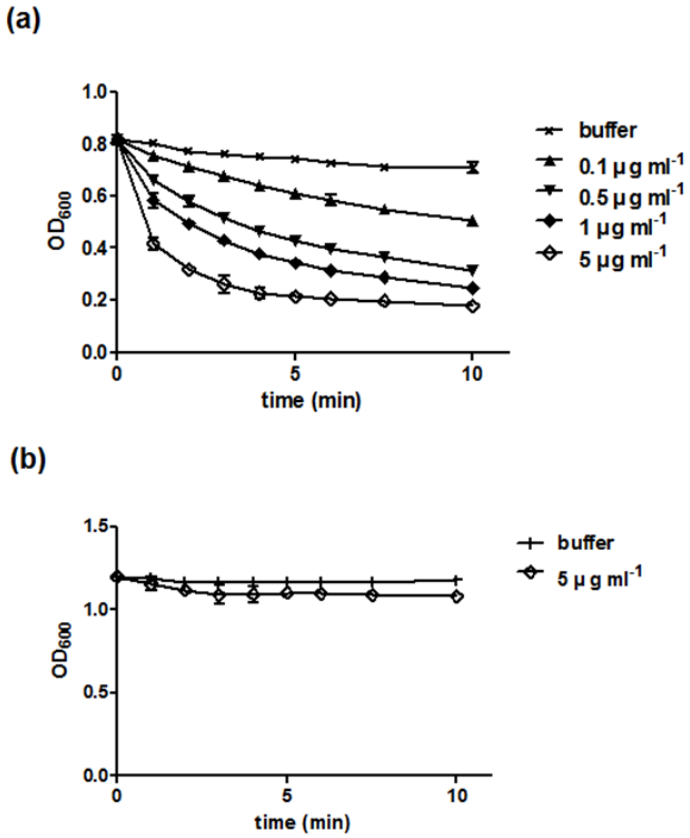


Figure 27. Lysis activities of purified SPN9CC endolysin (optical density)

(a) The activities of various concentrations of purified SPN9CC endolysin were detected using *E. coli* cell suspensions pretreated with EDTA.

(b) Enzymatic activity of 5 μg ml^{-1} SPN9CC endolysin against intact *E. coli* cell suspensions was measured.

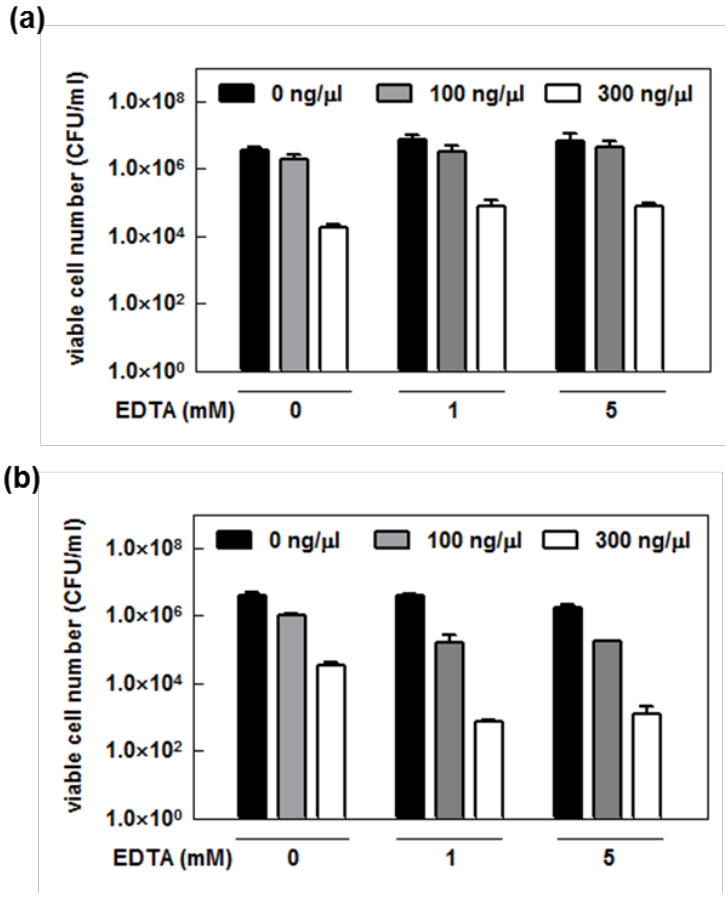


Figure 28. Lysis activities of purified SPN9CC endolysin (viable cell number)

The viable cell numbers (CFUs) of *E. coli* upon addition of 100 ng μl^{-1}, or 300 ng μl^{-1} endolysin were examined for two EDTA concentrations (1 mmol l^{-1}, 5 mmol l^{-1}) after 1 h (a) or 2 h (b) reaction times.

pH and temperature conditions for SPN9CC endolysin

SPN9CC endolysin was active to some extent over a broad range of temperature (24°C to 65°C). It showed similar lysis activities in neutral and basic pH (pH 7.0 to 9.5), but showed maximal activity from pH 7.5 to 8.5 and 50°C (Figure 29).

Thermal treatment at temperatures up to 45°C for 30 min or 37°C for 2 h did not cause significant inhibition of enzyme function, however, elevated temperature (55°C or 65°C) or longer incubation time (1 h or 2 h) had a negative effect on activity (Figure 30).

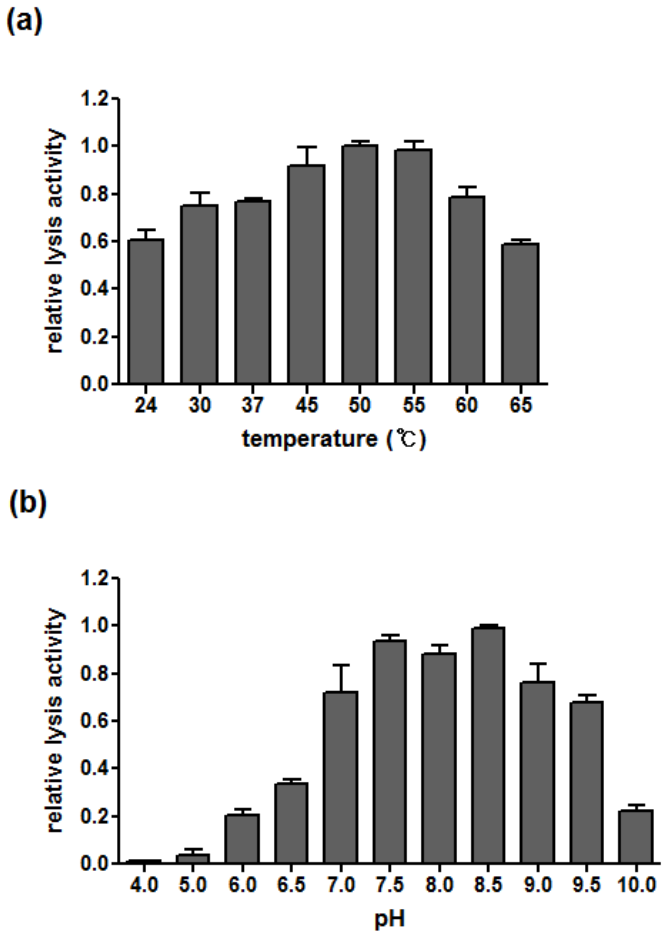


Figure 29. The optimum conditions for enzymatic activity of SPN9CC endolysin

The antimicrobial assay was carried out at different temperature for 3 min in 50 mM Tris-Cl buffer, pH 8.0 (a), and at different pH buffer for 10 min at room temperature (b). Lysis activity was detected by measuring optical density and relative lysis activity was calculated in proportion to the highest one.

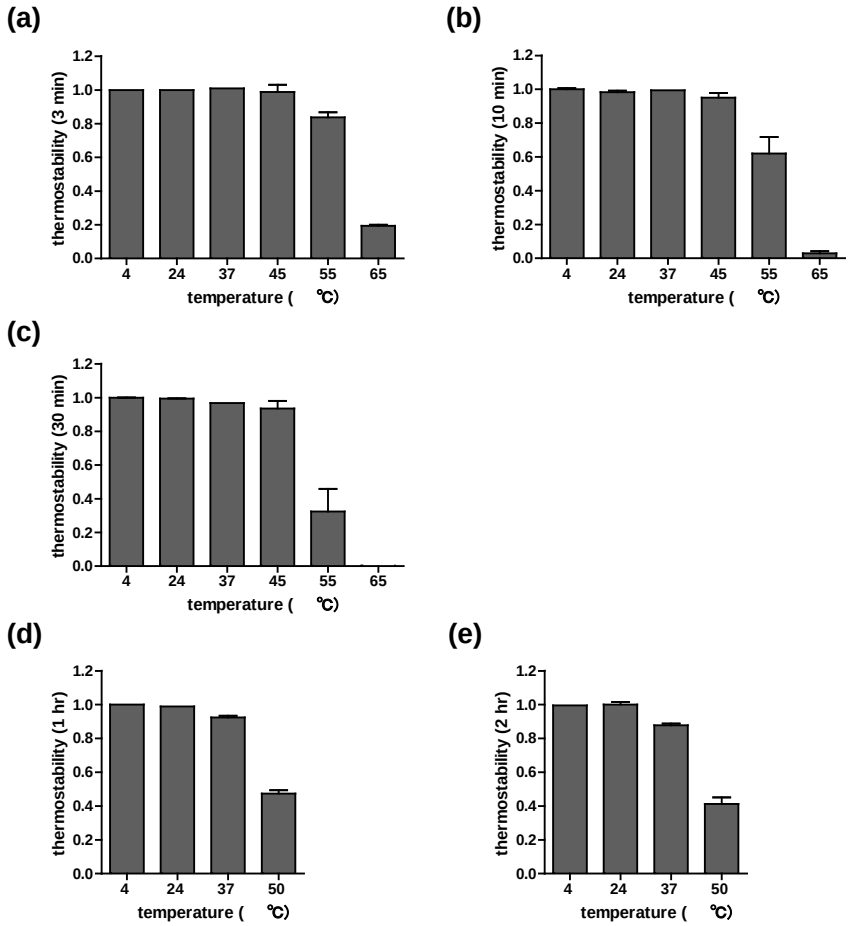


Figure 30. The thermostability of SPN9CC endolysin

The SPN9CC endolysin aliquots were incubated in each temperatures. After 3 min (a), 10 min (b), 30 min (c), 1 h (d), or 2 h (e) treatment the residual antimicrobial activities were detected at room temperature.

Antimicrobial spectrum of SPN9CC endolysin

Eight Gram-positive and 23 Gram-negative strains were examined for their susceptibility to SPN9CC endolysin (Figure 31). SPN9CC endolysin was able to lyse all of the tested Gram-negative strains at 0.5 μg ml^{-1} within 5 min with the highest activity against *E. coli* JM109, MG1655, and *Pseudomonas aeruginosa* ATCC 27853. However, no activity was detected against the Gram-positive strains. Thus, it was concluded that SPN9CC endolysin had a wide spectrum of antimicrobial activity against Gram-negative bacteria.

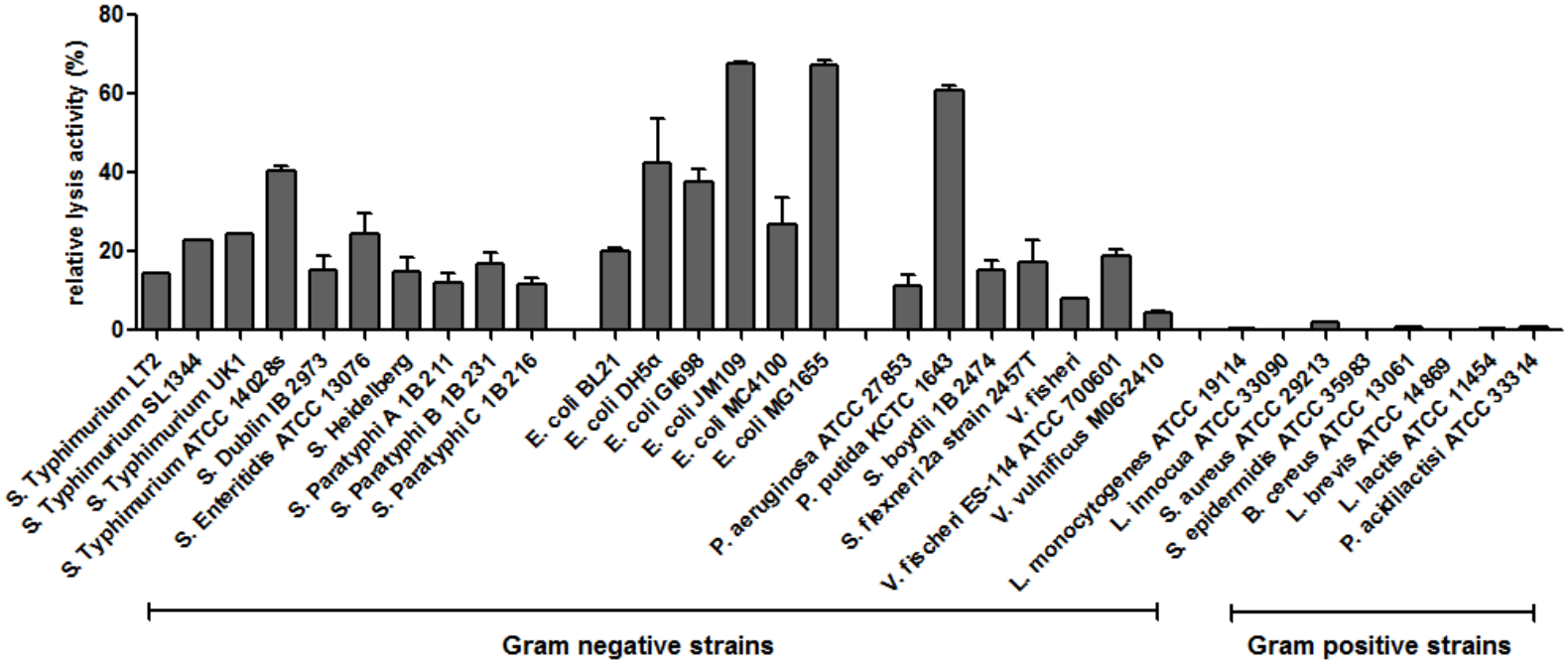


Figure 31. The antimicrobial spectrum of SPN9CC endolysin

Target site identification

Bioinformatic analysis shows that SPN9CC endolysin has a lytic transglycosylase activity. Lytic transglycosylases are widely distributed among Gram-negative bacteria and cleave the β-1,4 glycosidic bond between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). In contrast to structurally and functionally related lysozymes, the reaction catalyzed by lytic transglycosylase is not a hydrolysis but rather a transglycosylase reaction which results in formation of an internal 1,6 anhydro bond in the N-acetylmuramic acid residue (Holtje *et al.*, 1975)

To confirm the predicted glycoside bond cleaving activity of SPN9CC endolysin, the quantity of reducing sugar in peptidoglycan hydrolysate was analyzed. *E. coli* peptidoglycan was treated with SPN9CC endolysin, and degradation of peptidoglycan was observed (Figure 32a). After lysis, the amounts of reducing sugar in each sample were measured. As with peptidoglycan degradation, the amount of reducing sugar was significantly increased by enzyme digestion. It showed glycosidase activity of SPN9CC endolysin (Figure 32b). Buffer and enzyme itself used in this experiment did not affect the results.

To examine whether SPN9CC endolysin might have other target sites, amidase activity was analyzed using the same enzyme digests but

found no difference between endolysin lysate and peptidoglycan suspension (Figure 32c). To analyze peptidase activity, peptidoglycan was acetylated with acetic anhydride to remove preexistent amine residue. It was hard to observe the hydrolysis the acetylated peptidoglycan with SPN9CC endolysin by measuring optical density of peptidoglycan suspension because the lysis activity was decreased against acetylated peptidoglycan, but the hydrolysis could be confirmed by detecting reducing sugar. Figure 32d showed that SPN9CC endolysin did not have peptidase activity. Thus, it was concluded that SPN9CC endolysin cleaves only the glycosidic bond of peptidoglycan.

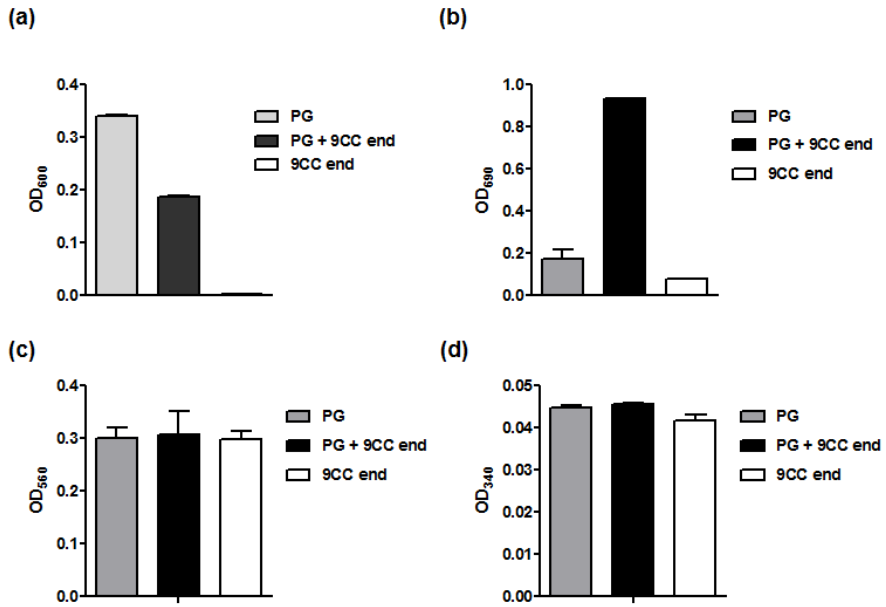
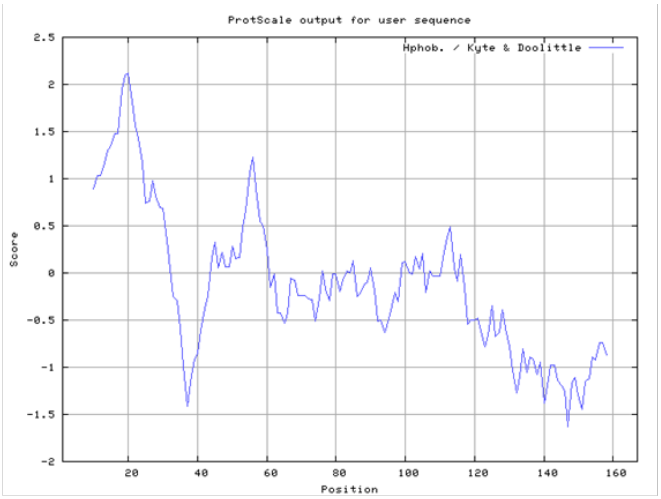


Figure 32. Target site of SPN9CC endolysin

(a) Peptidoglycan of *E. coli* DH5α was extracted and added with SPN9CC endolysin. After 1 h reaction at room temperature, optical density (600 nm) was measured. Using peptidoglycan hydrolysate digested with SPN9CC endolysin, glycosidase activity (b), amidase activity (c), and peptidase activity (d) were analyzed.

(a)



(b)

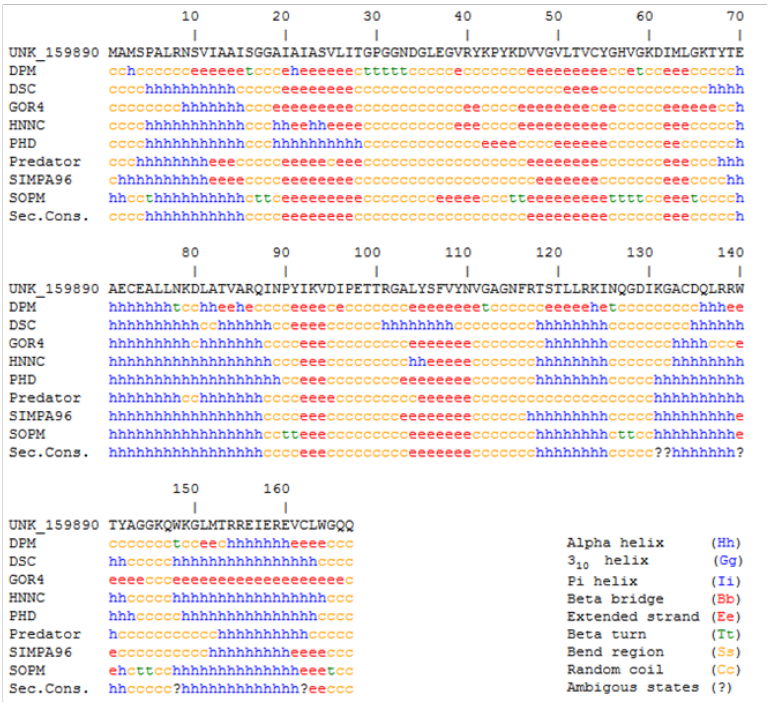


Figure 33. Prediction of hydrophicity and secondary structure

(a) Hydrophobic (+)/hydrophilic (-) profile of the SPN9CC endolysin

(b) Prediction of secondary structure

IV. Discussion

In this study, the lysis activities of two endolysin homologues from SPN1S and SPN9CC, newly isolated bacteriophages infecting *S.* Typhimurium were confirmed. The endolysins have a lysozyme-like superfamily domain, but showed much stronger lysis activities than lysozyme from chicken egg white. Several endolysins with lytic activity against Gram-negative bacteria have been studied. The viable cell number of *P. aeruginosa* PAO1 was reduced approximately 4 logs by addition of 5 μg ml^{-1} lysis protein EL188 and 10 mmol l^{-1} EDTA (Briers *et al.*, 2011). The endolysin of *Klebsiella* phage K11 reduced optical density of sensitized cells approximately 10% in 10 μg ml^{-1} concentration (Junn *et al.*, 2005). The antibacterial activity of *A. baumannii* phage phiAB2 endolysin was approximately 30% of that of chicken egg white lysozyme (Lai *et al.*, 2011). SPN1S and SPN9CC endolysins showed strong lytic activity relative to these endolysins. Fifty nanograms of SPN1S endolysin and 0.5 μg of SPN9CC endolysin were sufficient to reduce the optical density of a 1 ml EDTA pretreated cell suspension approximately 50% within 10 min. Indeed, SPN1S endolysin appears to be superior to lysozyme, with approximately 30 times higher activity.

The peptidoglycan of all Gram-negative bacterial is (almost)

identical. And *Bacillus* contains A1γ types of peptidoglycan like Gram-negative bacteria (Schleifer and Kandler, 1972). The different lysis patterns between Gram-negative bacteria and *Bacillus* by SPN1S endolysin treatment might be caused by peptidoglycan deacetylation. *Bacillus* possesses deacetylase(s), and a high proportion of the acetylglucosamine residues in the peptidoglycan layer are de-*N*-acetylated by the action of peptidoglycan deacetylase (Araki *et al.*, 1972; Hayashi *et al.*, 1973). This modification has the effect of making the bacterial cell wall resistant to digestion by lysozyme, and these lysozyme-resistant cell walls were converted into a lysozyme-sensitive form by N-acetylation. Likewise, *Bacillus cereus* peptidoglycan with N-unsubstituted glucosamine residues, which is resistant to the bacteriophage T4 lysozyme, was converted into a sensitive form by N-acetylation (Kleppe *et al.*, 1981). Similar to these, N-acetylation of *B. cereus* peptidoglycan with acetic anhydride made it sensitive to lytic action of SPN1S endolysin.

In general, endolysin cannot act as exolysin against intact Gram-negative bacteria (Hermoso *et al.*, 2007), since the outer membrane acts as a physical barrier preventing access of endolysin to the peptidoglycan. The peptidoglycan of Gram-positive strains, in contrast, is easily accessible from the extracellular environment (Fischetti, 2010;

Lai *et al.*, 2011). Success in making Gram-negative bacteria sensitive to the antimicrobial effects of endolysin suggests that phage endolysins might be used as therapeutics for not only Gram-positive bacteria, but for Gram-negative bacteria as well. A few methods that increase outer membrane permeability in Gram-negative bacteria have been reported, including use of chelating agents like EDTA, high hydrostatic pressure or the attachment of hydrophobic peptides to endolysin (Morita *et al.*, 2001; Nakimbugwe *et al.*, 2006; Briers *et al.*, 2011). Some endolysins, especially those from phages of Gram-negative bacteria, are capable of affecting bacterial cells by means of a mechanism completely independent of their enzymatic activity (During *et al.*, 1999; Orito *et al.*, 2004). In these cases, it was found that helix-forming amphipathic peptides containing basic amino acid residues seem to interact with negatively charged membrane elements, such as lipopolysaccharide in Gram-negative bacteria (During *et al.*, 1999). Morita *et al*. elucidated the exogenous antibacterial action of the *B. amyloliquefaciens* phage endolysin against *P. aeruginosa* PAO1 (Morita *et al.*, 2001). Similarly, LysAB2 produced from *Acinetobacter baumannii* bacteriophage exhibited a bacteriolytic activity against a number of Gram-negative and Gram-positive bacteria when applied exogenously (Lai *et al.*, 2011). In both endolysins, C-terminal hydrophobic regions possess the putative amphipathic helix domains possibly play the roles to enhance

the permeability of bacterial outer membrane. They speculated that C-terminal region of endolysin interacts with or penetrates the cell envelope, subsequently, the N-terminus of endolysin, which may harbor the catalytic domain, approaches the peptidoglycan layer, causing the lysis of the bacterial cell. In this study, SPN9CC endolysin showed exogenous antibacterial activity without outer membrane permeabilizer. N-terminus of SPN9CC endolysin is composed of highly hydrophobic helix structure (Figure 33), and it could be possible that this region is responsible for outer membrane penetration. In case of SPN1S endolysin, it could not cause substrate lysis when it was treated exogenously. However, addition of EDTA along with endolysin or attachment of small peptide to C-terminus for helix structure allowed strong antimicrobial activity.

There exist a few reports that phage accessory proteins like lambda phage Rz and Rz1 are involved in host cell lysis with respect to the outer membrane (Markov *et al.*, 2004; Berry *et al.*, 2008; Krupovic *et al.*, 2008). Lambda mutants lacking Rz/Rz1 have a conditional lysis-deficient phenotype. Under standard laboratory incubation conditions, holin and endolysin are sufficient for the lysis and release of the progeny virions. In the presence of high concentrations of divalent cations, however, bacterial cells infected with Rz/Rz1-deficient lambda phage do not lyse, but instead form mechanically fragile spheres

(Young *et al.*, 1979; Zhang and Young, 1999). In the case of the P22 bacteriophage, the Rz/Rz1 homologues are encoded by gene 15. P22 phage mutants lacking gene 15 display a 3- to 6-log decrease in plating efficiency in the presence of divalent cations (Casjens *et al.*, 1989). Krupovič *et al* (Krupovic *et al.*, 2008) reported that the Rz/Rz1-like accessory lysis genes of bacteriophage PRD1 were needed for full lysis. The lysis rate of PRD1 Rz/Rz1-deficient phage was only slightly less than that of wild type phage in the absence of divalent cations, but in the presence of 10 mmol l^{-1} $MgCl_2$, the lysis rate was reduced dramatically. The authors suggested that the Rz/Rz1 complex transmits mechanical stress associated with the lesions caused by holin (in the inner membrane) to the outer membrane, ultimately leading to lysis. An alternative model was suggested for lysis mediated by phage lambda in which Rz/Rz1 are required for the fusion of the inner and outer membranes, a step proposed to be necessary for the release of progeny virions (Berry *et al.*, 2008).

Summer *et al* (Summer *et al.*, 2007) reported that Rz/Rz1 equivalents are ubiquitous among phages infecting Gram-negative bacteria. In a comprehensive bioinformatics study, they found 120 Rz/Rz1 equivalents in 137 phage genomes. In most cases in which the function of Rz/Rz1 has been studied, they are not essential for lysis. In the SPN1S, the expression of endolysin with Rz/Rz1-like proteins was

sufficient for lysis. To my knowledge, this is the first example of phage lysis in which the Rz/Rz1-like proteins are critical.

V. References

Aghera, N. and Udgaonkar, J. B. (2011). Heterologous expression, purification and characterization of heterodimeric monellin. *Protein Expr Purif* **76** (2): 248-253.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215** (3): 403-410.

Araki, Y., Nakatani, T., Nakayama, K. and Ito, E. (1972). Occurrence of N-nonsubstituted glucosamine residues in peptidoglycan of lysozyme-resistant cell walls from Bacillus cereus. *J Biol Chem* **247** (19): 6312-6322.

Ayres, H. M., Furr, J. R. and Russell, A. D. (1998). Effect of divalent cations on permeabilizer-induced lysozyme lysis of *Pseudomonas aeruginosa*. *Lett Appl Microbiol* **27** (6): 372-374.

Baker, J. R., Liu, C., Dong, S. and Pritchard, D. G. (2006). Endopeptidase and glycosidase activities of the bacteriophage B30 lysin. *Appl Environ Microbiol* **72** (10): 6825-6828.

Berry, J., Summer, E. J., Struck, D. K. and Young, R. (2008). The final step in the phage infection cycle: the Rz and Rz1 lysis proteins link the inner and outer membranes. *Mol Microbiol* **70** (2): 341-351.

Breithaupt, H. (1999). The new antibiotics. *Nat Biotechnol* **17** (12): 1165-1169.

Briers, Y., Volckaert, G., Cornelissen, A., Lagaert, S., Michiels, C. W., Hertveldt, K. and Lavigne, R. (2007). Muralytic activity and modular structure of the endolysins of *Pseudomonas aeruginosa* bacteriophages phiKZ and EL. *Mol Microbiol* **65** (5): 1334-1344.

Briers, Y., Walmagh, M. and Lavigne, R. (2011). Use of bacteriophage endolysin EL188 and outer membrane permeabilizers against *Pseudomonas aeruginosa*. *J Appl Microbiol* **110** (3): 778-785.

Casjens, S., Eppler, K., Parr, R. and Poteete, A. R. (1989). Nucleotide sequence of the bacteriophage P22 gene 19 to 3 region: identification of a new gene required for lysis. *Virology* **171** (2): 588-598.

Delcher, A. L., Bratke, K. A., Powers, E. C. and Salzberg, S. L. (2007). Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics* **23** (6): 673-679.

During, K., Porsch, P., Mahn, A., Brinkmann, O. and Gieffers, W. (1999). The non-enzymatic microbicidal activity of lysozymes. *FEBS Lett* **449** (2-3): 93-100.

Fein, J. E. and Rogers, H. J. (1976). Autolytic enzyme-deficient

mutants of *Bacillus subtilis* 168. *J Bacteriol* **127** (3): 1427-1442.

Fischetti, V. A. (2010). Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens. *Int J Med Microbiol* **300** (6): 357-362.

Fukushima, T., Yao, Y., Kitajima, T., Yamamoto, H. and Sekiguchi, J. (2007). Characterization of new L,D-endopeptidase gene product CwlK (previous YcdD) that hydrolyzes peptidoglycan in *Bacillus subtilis*. *Mol Genet Genomics* **278** (4): 371-383.

Gupta, R. and Prasad, Y. (2011). P-27/HP endolysin as antibacterial agent for antibiotic resistant *Staphylococcus aureus* of human infections. *Curr Microbiol* **63** (1): 39-45.

Guzman, L. M., Belin, D., Carson, M. J. and Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* **177** (14): 4121-4130.

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

Hawkey, P. M. (2008). The growing burden of antimicrobial resistance. *J Antimicrob Chemother* **62 Suppl 1** i1-9.

Hayashi, H., Araki, Y. and Ito, E. (1973). Occurrence of glucosamine residues with free amino groups in cell wall peptidoglycan from bacilli as a factor responsible for resistance to lysozyme. *J*

Bacteriol **113** (2): 592-598.

Hazenberg, M. P. and de Visser, H. (1992). Assay for N-acetylmuramyl-L-alanine amidase in serum by determination of muramic acid released from the peptidoglycan of Brevibacterium divaricatum. *Eur J Clin Chem Clin Biochem* **30** (3): 141-144.

Hermoso, J. A., Garcia, J. L. and Garcia, P. (2007). Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Curr Opin Microbiol* **10** (5): 461-472.

Holtje, J. V., Mirelman, D., Sharon, N. and Schwarz, U. (1975). Novel type of murein transglycosylase in Escherichia coli. *J Bacteriol* **124** (3): 1067-1076.

Junn, H. J., Youn, J., Suh, K. H. and Lee, S. S. (2005). Cloning and expression of *Klebsiella* phage K11 lysozyme gene. *Protein Expr Purif* **42** (1): 78-84.

Kikkawa, H., Fujinami, Y., Suzuki, S. and Yasuda, J. (2007). Identification of the amino acid residues critical for specific binding of the bacteriolytic enzyme of gamma-phage, PlyG, to *Bacillus anthracis*. *Biochem Biophys Res Commun* **363** (3): 531-535.

Kleppe, G., Vasstrand, E. and Jensen, H. B. (1981). The specificity requirements of bacteriophage T4 lysozyme. Involvement of N-

acetamido groups. *Eur J Biochem* **119** (3): 589-593.

Kropinski, A. M., Kovalyova, I. V., Billington, S. J., Patrick, A. N., Butts, B. D., Guichard, J. A., Pitcher, T. J., Guthrie, C. C., Sydlaske, A. D., Barnhill, L. M., Havens, K. A., Day, K. R., Falk, D. R. and McConnell, M. R. (2007). The genome of epsilon15, a serotype-converting, Group E1 Salmonella enterica-specific bacteriophage. *Virology* **369** (2): 234-244.

Krupovic, M., Cvirkaite-Krupovic, V. and Bamford, D. H. (2008). Identification and functional analysis of the *Rz*/*Rz1*-like accessory lysis genes in the membrane-containing bacteriophage PRD1. *Mol Microbiol* **68** (2): 492-503.

Krupovic, M., Daugelavicius, R. and Bamford, D. H. (2007). A novel lysis system in PM2, a lipid-containing marine double-stranded DNA bacteriophage. *Mol Microbiol* **64** (6): 1635-1648.

Lai, M. J., Lin, N. T., Hu, A., Soo, P. C., Chen, L. K., Chen, L. H. and Chang, K. C. (2011). Antibacterial activity of *Acinetobacter baumannii* phage AB2 endolysin (LysAB2) against both gram-positive and gram-negative bacteria. *Appl Microbiol Biotechnol* **90** (2): 529-539.

Livermore, D. M. (2004). The need for new antibiotics. *Clin Microbiol Infect* **10 Suppl 4** 1-9.

Loeffler, J. M., Djurkovic, S. and Fischetti, V. A. (2003). Phage lytic

enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia. *Infect Immun* **71** (11): 6199-6204.

Loessner, M. J. (2005). Bacteriophage endolysins - current state of research and applications. *Curr Opin Microbiol* **8** (4): 480-487.

Lukashin, A. V. and Borodovsky, M. (1998). GeneMark.hmm: new solutions for gene finding. *Nucleic Acids Res* **26** (4): 1107-1115.

Marchler-Bauer, A., Anderson, J. B., Derbyshire, M. K., DeWeese-Scott, C., Gonzales, N. R., Gwadz, M., Hao, L., He, S., Hurwitz, D. I., Jackson, J. D., Ke, Z., Krylov, D., Lanczycki, C. J., Liebert, C. A., Liu, C., Lu, F., Lu, S., Marchler, G. H., Mullokandov, M., Song, J. S., Thanki, N., Yamashita, R. A., Yin, J. J., Zhang, D. and Bryant, S. H. (2007). CDD: a conserved domain database for interactive domain family analysis. *Nucleic Acids Res* **35** (Database issue): D237-240.

Markov, D., Christie, G. E., Sauer, B., Calendar, R., Park, T., Young, R. and Severinov, K. (2004). P2 growth restriction on an *rpoC* mutant is suppressed by alleles of the *Rz1* homolog *lysC*. *J Bacteriol* **186** (14): 4628-4637.

Mikoulinskaia, G. V., Odinokova, I. V., Zimin, A. A., Lysanskaya, V. Y., Feofanov, S. A. and Stepnaya, O. A. (2009). Identification and characterization of the metal ion-dependent L-alanoyl-D-glutamate peptidase encoded by bacteriophage T5. *FEBS J* **276**

(24): 7329-7342.

Miroshnikov, K. A., Faizullina, N. M., Sykilinda, N. N. and Mesyanzhinov, V. V. (2006). Properties of the endolytic transglycosylase encoded by gene 144 of *Pseudomonas aeruginosa* bacteriophage phiKZ. *Biochemistry (Mosc)* **71** (3): 300-305.

Miroux, B. and Walker, J. E. (1996). Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* **260** (3): 289-298.

Morita, M., Tanji, Y., Orito, Y., Mizoguchi, K., Soejima, A. and Unno, H. (2001). Functional analysis of antibacterial activity of *Bacillus amyloliquefaciens* phage endolysin against Gram-negative bacteria. *FEBS Lett* **500** (1-2): 56-59.

Nakimbugwe, D., Masschalck, B., Atanassova, M., Zewdie-Bosuner, A. and Michiels, C. W. (2006). Comparison of bactericidal activity of six lysozymes at atmospheric pressure and under high hydrostatic pressure. *Int J Food Microbiol* **108** (3): 355-363.

Nariya, H., Miyata, S., Tamai, E., Sekiya, H., Maki, J. and Okabe, A. (2011). Identification and characterization of a putative endolysin encoded by episomal phage phiSM101 of *Clostridium*

perfringens. *Appl Microbiol Biotechnol* **90** (6): 1973-1979.

Orito, Y., Morita, M., Hori, K., Unno, H. and Tanji, Y. (2004). *Bacillus amyloliquefaciens* phage endolysin can enhance permeability of *Pseudomonas aeruginosa* outer membrane and induce cell lysis. *Appl Microbiol Biotechnol* **65** (1): 105-109.

Paradis-Bleau, C., Cloutier, I., Lemieux, L., Sanschagrin, F., Laroche, J., Auger, M., Garnier, A. and Levesque, R. C. (2007). Peptidoglycan lytic activity of the *Pseudomonas aeruginosa* phage phiKZ gp144 lytic transglycosylase. *FEMS Microbiol Lett* **266** (2): 201-209.

Park, J. T. and Johnson, M. J. (1949). A submicrodetermination of glucose. *J Biol Chem* **181** (1): 149-151.

Perry, L. L., SanMiguel, P., Minocha, U., Terekhov, A. I., Shroyer, M. L., Farris, L. A., Bright, N., Reuhs, B. L. and Applegate, B. M. (2009). Sequence analysis of *Escherichia coli* O157:H7 bacteriophage PhiV10 and identification of a phage-encoded immunity protein that modifies the O157 antigen. *FEMS Microbiol Lett* **292** (2): 182-186.

Pritchard, D. G., Dong, S., Baker, J. R. and Engler, J. A. (2004). The bifunctional peptidoglycan lysin of *Streptococcus agalactiae* bacteriophage B30. *Microbiology* **150** (Pt 7): 2079-2087.

Sambrook J, R. D. (2001). Molecular Cloning: A laboratory manual.

New York, Cold Spring Harbor Laboratory Press.

Sao-Jose, C., Parreira, R., Vieira, G. and Santos, M. A. (2000). The N-terminal region of the Oenococcus oeni bacteriophage fOg44 lysin behaves as a bona fide signal peptide in Escherichia coli and as a cis-inhibitory element, preventing lytic activity on oenococcal cells. *J Bacteriol* **182** (20): 5823-5831.

Sass, P. and Bierbaum, G. (2007). Lytic activity of recombinant bacteriophage phi11 and phi12 endolysins on whole cells and biofilms of *Staphylococcus aureus*. *Appl Environ Microbiol* **73** (1): 347-352.

Schleifer, K. H. and Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36** (4): 407-477.

Schuch, R., Nelson, D. and Fischetti, V. A. (2002). A bacteriolytic agent that detects and kills *Bacillus anthracis*. *Nature* **418** 884-889.

Sheffield, P., Garrard, S. and Derewenda, Z. (1999). Overcoming expression and purification problems of RhoGDI using a family of "parallel" expression vectors. *Protein Expr Purif* **15** (1): 34-39.

Shin, H., Lee, J. H., Lim, J. A., Kim, H. and Ryu, S. (2012). Complete genome sequence of Salmonella enterica serovar

typhimurium bacteriophage SPN1S. *J Virol* **86** (2): 1284-1285.

Summer, E. J., Berry, J., Tran, T. A., Niu, L., Struck, D. K. and Young, R. (2007). *Rz*/*Rz1* lysis gene equivalents in phages of Gram-negative hosts. *J Mol Biol* **373** (5): 1098-1112.

Sun, Q., Kuty, G. F., Arockiasamy, A., Xu, M., Young, R. and Sacchettini, J. C. (2009). Regulation of a muralytic enzyme by dynamic membrane topology. *Nat Struct Mol Biol* **16** (11): 1192-1194.

Tsfasman, I. M., Sitkin, B. V., Lysanskaya, V. Y., Stepnaya, O. A. and Kulaev, I. S. (2007). Substrate specificity and some physicochemical properties of autolytic enzymes of the bacterium *Lysobacter* sp. XL 1. *Biochemistry (Mosc)* **72** (7): 760-765.

Turner, M. S., Hafner, L. M., Walsh, T. and Giffard, P. M. (2004). Identification, characterisation and specificity of a cell wall lytic enzyme from *Lactobacillus fermentum* BR11. *FEMS Microbiol Lett* **238** (1): 9-15.

Turner, M. S., Waldherr, F., Loessner, M. J. and Giffard, P. M. (2007). Antimicrobial activity of lysostaphin and a *Listeria monocytogenes* bacteriophage endolysin produced and secreted by lactic acid bacteria. *Syst Appl Microbiol* **30** (1): 58-67.

Uchiyama, J., Takemura, I., Hayashi, I., Matsuzaki, S., Satoh, M.,

Ujihara, T., Murakami, M., Imajoh, M., Sugai, M. and Daibata, M. (2011). Characterization of lytic enzyme open reading frame 9 (ORF9) derived from *Enterococcus faecalis* bacteriophage phiEF24C. *Appl Environ Microbiol* **77** (2): 580-585.

Xu, M., Arulandu, A., Struck, D. K., Swanson, S., Sacchettini, J. C. and Young, R. (2005). Disulfide isomerization after membrane release of its SAR domain activates P1 lysozyme. *Science* **307** (5706): 113-117.

Young, R., Way, J., Way, S., Yin, J. and Syvanen, M. (1979). Transposition mutagenesis of bacteriophage lambda: a new gene affecting cell lysis. *J Mol Biol* **132** (3): 307-322.

Yun, J., Park, J., Park, N., Kang, S. and Ryu, S. (2007). Development of a novel vector system for programmed cell lysis in *Escherichia coli*. *J Microbiol Biotechnol* **17** (7): 1162-1168.

Zdobnov, E. M. and Apweiler, R. (2001). InterProScan--an integration platform for the signature-recognition methods in InterPro. *Bioinformatics* **17** (9): 847-848.

Zhang, N. and Young, R. (1999). Complementation and characterization of the nested Rz and Rz1 reading frames in the genome of bacteriophage lambda. *Mol Gen Genet* **262** (4-5):

659-667.

초록

항생제 내성균의 출현과 관련하여 박테리아 감염을 저해할 수 있는 항생제 대체재의 개발이 각광받고 있다. 엔도라이신은 박테리오파지의 용균 생활사의 마지막 단계에서 숙주의 세포벽을 분해하기 위해 박테리오파지에 의해 만들어지는 효소이다. 박테리오파지 SPJ2 및 iEPS5의 DNA 라이브러리로부터 세 가지 용해 단백질이 발견되었다. 세 개의 단백질 모두 알려진 단백질과는 상동성이 없으며, 홀린과 함께 작용할 때에 강한 용해 활성을 보였다. 하지만 단백질 발현 정도가 너무 낮거나 대부분이 불용성 상태로 있었으므로 순수분리가 어려워 더 이상의 특성 분석은 되지 않았다. 박테리오파지 SPN1S의 유전자 서열로부터 박테리오파지의 용해 단백질인 홀린, 엔도라이신 및 보조 단백질 Rz/Rz1와 유사한 유전자를 발견하였다. 이 단백질들의 숙주 세균 용해능을 측정한 결과 일반적인 용해 단백질의 특성과 달리 홀린과 엔도라이신의 발현만으론 숙주가 용해되지 않고 세 개의 단백질 모두가 발현되거나 엔도라이신과 보조 단백질 Rz/Rz1가 함께 발현될 때에만 숙주의 성장이 저해되었다. 엔도라이신의 N- 말단에 히스티딘 태그가 붙도록 재조합 단백질을 발현시키고 친화 크

로마토그래피를 통해 순수 분리 하였다. SPN1S 엔도라이신은 라이소자임 유사 계열의 superfamily 도메인을 가지고 있으며, 라이소자임 보다 훨씬 큰 용균 활성을 보였다. 외부 막 투과성을 증가시키기 위해 EDTA(에틸렌 다이아민 테트라 아세트산)와 같은 킬레이트 물질이 사용됐을 때 SPN1S 엔도라이신의 용균 작용은 크게 증가하였다. 항균 활성은 넓은 산도 및 온도 범위에서 안정하게 유지되었고, 산도 7과 10.5 사이의 범위와 온도 25℃에서 45℃ 사이의 범위에서 가장 큰 항균 활성을 보였다. SPN1S 엔도라이신은 측정된 균주 중 대부분의 그람 음성 세균에 대해 항균 활성을 보였지만, 그람 양성 세균에는 효과가 없었다. SPN1S 엔도라이신의 작용 지점은 세포벽의 당 결합 부위이며 라이소자임과 같은 뮤라미다아제의 활성을 보였다. 단백질의 C- 말단에 나선 구조를 가지도록 재조합된 SPN1S 엔도라이신은 외부 막 투과율을 증가시키기 위한 물질 (EDTA) 없이도 항균 활성을 보였다. SPN9CC 박테리오파지의 유전체 분석 결과, P22 계열 박테리오파지 ST104와 대장균 K-12의 프로파지 DLP12의 라이소자임과 상동성을 보이는 엔도라이신을 찾을 수 있었다. SPN9CC 엔도라이신의 C- 말단에 올리고 히스티딘 택이 붙은 재조합 단백질을 분리 정제하였으며, EDTA로 외부 막 투과성을 높인 대장균에 처리하였을 때

강한 용균 활성을 확인할 수 있었다. 37도에서 2시간 동안 처리하여도 SPN9CC 엔도라이신의 활성은 안정하게 유지되고, 넓은 범위의 온도 조건에서 용균 활성을 보였으며 특히 50도에서 가장 높은 활성을 보였다. 중성과 약염기 조건에서 용균 활성을 보이며 최적 산도는 7.5에서 8.5 사이였다. SPN1S 엔도라이신과 유사하게 SPN9CC 엔도라이신은 세포벽의 당 결합 부위를 잘라 세포벽을 분해시키며, 그람 음성 세균에 대해서만 용균 활성이 나타났다. SPN9CC 엔도라이신은 막 투과성을 변화시키지 않은 대장균 대해서도 용균 활성을 보였으며, EDTA와 함께 처리할 때 증가된 용균 활성을 나타냈다. 이상의 결과들은 SPN1S 엔도라이신과 SPN9CC 엔도라이신의 그람 음성 세균의 제어를 위한 항생제 대체 물질로의 발전 가능성을 보여준다.