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Characterization of endolysin from a *Salmonella* Typhimurium-infecting bacteriophage SPN1S

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Abstract

The full genome sequence of bacteriophage SPN1S, which infects *Salmonella,* contains genes that encode homologues of holin, endolysin and Rz/Rz1-like accessory proteins, which are 4 phage lysis proteins. The ability of these proteins to lyse *Escherichia coli* cells when over-expressed was evaluated. In contrast to other endolysins, the expression of endolysin and Rz/Rz1-like proteins was sufficient to cause lysis. The endolysin was tagged with oligohistidine at the N-terminus and purified by affinity chromatography. The endolysin has a lysozyme-like superfamily domain, and its activity was much stronger than that of lysozyme from chicken egg white. We used the chelating agent, ethylenediaminetetraacetic acid (EDTA), 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 of the tested Gram-negative strains, but the Gram-positive strains were resistant. SPN1S endolysin, like lysozyme, cleaves the glycosidic bond of peptidoglycan. These results suggested that SPN1S endolysin has potential as a therapeutic agent against Gram-negative bacteria.

Keywords: Endolysin; Peptidoglycan hydrolase; Bacteriophage; *Salmonella* Typhimurium

1. Introduction

As antibiotic-resistant bacteria become more prevalent, alternative therapeutic methods are required. Bacteriophages and endolysins are regarded as effective antimicrobial agents (Loessner, 2005). In general, double-stranded DNA bacteriophages lyse their host cells with 2 proteins, holin and endolysin. Endolysin synthesized without a signal sequence accumulates in the cytosol in the active form. Holin makes pores in the inner membrane, which allows the endolysin to reach its target, peptidoglycan. Endolysins typically cleave glycosidic bonds, peptide bonds, or amide bonds of the peptidoglycan, causing host cell lysis (Baker et al., 2006; Callewaert et al., 2011; Mikoulinskaia et al., 2009; Pritchard et al., 2004). Contrary to canonical endolysins, studies on other types of endolysins exist. The 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 are activated by membrane depolarization through holin (Sun et al., 2009; Xu et al., 2005). The *Oenococcus oeni* phage fOg44 endolysin, which has a typical signal sequence, was converted into an active form after cleavage of the N-terminal signal peptide in a SecA-dependent manner (Sao-Jose et al., 2000). Some phages do not encode an apparent endolysin (Krupovič et al., 2007).

In the case of Gram-negative bacteria, 2 additional proteins, Rz/Rz1 equivalents, contribute to the efficiency of the lysis (Berry et al., 2008; Krupovič et al., 2008; Markov et al., 2004). In the lambda phage lysis cassette, there are 4 lysis genes: *S*-encoding holin and anti-holin, *R*-encoding endolysin and Rz and Rz1. Rz is a type II membrane protein with a hydrophobic

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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 the 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 the 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, the use of purified endolysins has been studied. The lytic activities of purified endolysins have shown potential as therapeutic agents against *Streptococcus pneumoniae, Bacillus anthracis, Bacillus subtilis, Staphylococcus aureus, Lactobacillus fermentum, Listeria monocytogenes, Enterococcus faecalis,* and *Clostridium perfringens* (Fukushima et al., 2007; Kikkawa et al., 2007; Loeffler et al., 2003; Nariya et al., 2011; Sass and Bierbaum, 2007; Schuch et al., 2002; Turner et al., 2004, 2007; Uchiyama et al., 2011). However, most studies on purified endolysins have focused on Gram-positive bacteria, since the peptidoglycan is directly accessible to exogenous endolysin. In the case of Gram-negative bacteria, the activity of purified phage lysis proteins against *Pseudomonas aeruginosa* has been reported (Briers et al., 2007; Miroshnikov et al., 2006; Paradis-Bleau et al., 2007), while only a few studies on endolysins from the *Klebsiella* phage, *Acinetobacter baumannii* phage and Enterobacteria phage T5 have been reported (Junn et al., 2005; Lai et al., 2011; Mikoulinskaia et al., 2009; Miroshnikov et al., 2006).

Previously, *Salmonella* Typhimurium-targeting bacteriophage SPN1S was isolated from environmental water and its genome was completely sequenced (Shin et al., 2012). It has a circular genome consisting of 38,684 bp with a GC content of 50.16% and 52 ORFs (open reading frames). In addition, SPN1S, belonging to the *Podoviridae* family, infects *S.* Typhimurium strains and a few *S.* Paratyphi strains.

In this study, an endolysin from the bacteriophage SPN1S was purified and its lysis activity was analyzed.

2. Materials and methods

2.1. Bioinformatic analysis

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

2.2. DNA manipulation

Bacteriophage SPN1S DNA was prepared as previously described (Sambrook and Russell, 2001). The holin, endolysin, and Rz/Rz1-like protein coding genes were amplified by the polymerase chain reaction and cloned into the pBAD18 expression vector. For expression of an N-terminal oligohistidine-tagged endolysin, the endolysin gene was amplified by the polymerase chain reaction and cloned into pET15b. The plasmids and primers used in this study are listed in Table 1.

2.3. Lysis gene confirmation

Escherichia coli DH5α harboring a recombinant plasmid expressing each of the 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 expression of lysis proteins. Lysis activity was assessed by measuring the optical density.

2.4. Endolysin purification

The BL21 Star™ (DE3) (Invitrogen, Carlsbad, CA, USA) strain was transformed with the pET15-lys plasmid. Expression of the recombinant endolysin was induced with 1 mmol l^{-1} isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density (600 nm) reached 0.6, followed by incubation for an 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 with nickel-nitriloacetic acid agarose resin (Qiagen, Hilden, Germany) with an imidazole gradient according to the 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.

2.5. Antimicrobial activity assay

An exponentially growing *E. coli* DH5α culture (10^8 CFU ml^{-1}) was washed and incubated in a 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 the endolysin, 10 ng, 50 ng, 100 ng, or 500 ng of endolysin was added to a 1 ml DH5α suspension (10^8 CFU ml^{-1}) and the reduction in optical density at 600 nm was measured. The same volume of 20 mmol l^{-1} Tris–HCl (pH 8.0) was used as the 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 in a similar manner. 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

Table 1
Plasmids, and oligonucleotide primers used in this study.

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, Wisconsin, USA
pBAD-hol	pBAD18 encoding holin	This study
pBAD-lys	pBAD18 encoding endolysin	This study
pBAD-Rz	pBAD18 encoding Rz/Rz1-like proteins	This study
pBAD-hollys	pBAD18 encoding holin and endolysin	This study
pBAD-holRz	pBAD18 encoding holin and Rz/Rz1-like proteins	This study
pBAD-lysRz	pBAD18 encoding endolysin and Rz/Rz1-like proteins	This study
pBAD-hollysRz	pBAD18 encoding holin, endolysin, and Rz/Rz1-like proteins	This study
pET15-lys	pET15b encoding N-terminal his-tagged 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

10 μg) was mixed with EDTA and added to a 100 μl cell suspension (final concentrations of EDTA were 1, 5, or 10 mmol l^{-1} EDTA). For the negative control, an equivalent volume of buffer was used instead of endolysin or EDTA. After a 20 min, 1 h or 2 h reaction time, the numbers of residual viable cells were measured by determining the colony-forming units.

2.6. Effects of pH and temperature on enzyme activity

To test the thermostability of endolysin, aliquots of the 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 the 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)ethane sulfonic 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-aminopropane sulfonic acid (pH 10.5). After each treatment, the residual lysis activity was assayed. To determine the optimal conditions for antimicrobial activity, the enzyme reaction was carried out in different pH buffers and at different temperatures. As controls, cell suspensions that were not treated with endolysin were included in the assays.

2.7. Antimicrobial spectrum

The bacterial strains used for antimicrobial spectrum determination are described in Table 2 along with the results. 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 optical density was measured.

2.8. 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 a 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 with a Sunrise microplate absorbance reader (Tecan, Switzerland). After hydrolysis, unbroken peptidoglycan was removed by centrifugation and the supernatant was assayed with 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.

Table 2
Antimicrobial activity of SPN1S endolysin against various Gram-negative or Gram-positive strains.

Strain		Relative lysis activity[a]
Escherichia coli	BL21	++++
	DH5α	+++
	DH10B	+++
	GI698	+++
	JM109	+++
	MC4100	+++++
	MG1655	+++
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.

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 1 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). The peptidoglycan suspension that had not been treated with endolysin and the endolysin solutions were used as negative controls.

3. Results

3.1. Lysis genes of the SPN1S bacteriophage

The genome of bacteriophage SPN1S was fully sequenced (GenBank ID: JN391180). In the host lysis cluster of the SPN1S bacteriophage genome, there are 4 ORFs encoding the putative holin, putative endolysin, and putative Rz/Rz1-like proteins. Annotation and functional analysis of the ORFs in this cluster revealed that 2 ORFs (SPN1S_0027, 309-bp and SPN1S_0028, 630-bp) were homologous to the holin and endolysin of phage epsilon15 (amino acid identity, 99% and 94%), a serotype-converting, group E1 *Salmonella enterica*-specific bacteriophage (Kropinski et al., 2007). The other 2 ORFs (SPN1S_0029, 483-bp and SPN1S_0029_1, 276-bp) had homology with 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 S1). InterProScan analysis in this gene cluster showed that only the putative endolysin had any functional domains. The putative endolysin of SPN1S had homology to the lysozyme-like domain (SSF53955) and its C-terminal region had a family 19 glycoside hydrolase catalytic domain (PF00182). On the other hand, the putative holin and Rz/Rz1-like proteins had transmembrane region(s) and a signal peptide region. In addition, the putative holin and Rz-like protein had unknown function domains (PF05449 and PF10721) and the Rz/Rz1-like proteins had a PROKAR_LIPOPROTEIN entry (PS51257) determined by PROSITE. ClustalW alignment and phylogenetic analysis of the putative endolysin were done with similar sequences from various prophages or bacteriophages (Figs. S1 and S2).

The 4 genes were 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 protein. Moreover, the Rz1-like protein overlaps the Rz-like protein in the other frame. To confirm the functions of these genes, they were cloned into expression vector pBAD18 separately or together and expressed in the *E. coli* DH5α strain. As shown in Fig. 1, none of the 4 genes affected growth of the *E. coli* strain when expressed alone. When all 4 were expressed together, there was strong lysis activity. Interestingly, the expression of endolysin and the Rz/Rz1-like proteins also yielded strong lysis activity. The SPN1S endolysin exhibited lysis activity

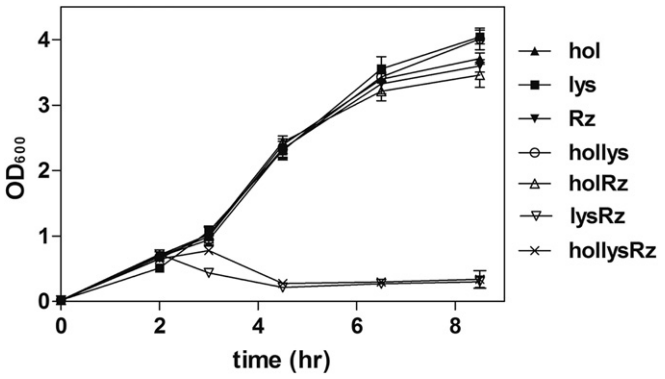

Fig. 1. Expression of holin, endolysin, and Rz/Rz1-like proteins of bacteriophage SPN1S. Holin, endolysin and Rz/Rz1-like accessory proteins were expressed in *E. coli*. Protein expression was induced 2 h after inoculation with 0.2% L-arabinose. Lysis of the induced cells was measured by the decrease in absorbance at 600 nm. Hhol; holin, lys; endolysin, Rz; Rz/Rz1-like proteins.

when it was expressed with T4 holin, but expression of T7 endolysin with SPN1S holin or Rz/Rz1-like proteins did not show any growth defect (Fig. 2). Thus, it is possible that SPN1S Rz/Rz1-like proteins affect bacterial cell lysis only when acting together with SPN1S endolysin, indicating that the SPN1S phage has an unusual lysis system.

3.2. Enzymatic lysis activity of purified endolysin

The endolysin coding region was cloned into pET15b and transformed into BL21 Star™ (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 (Fig. 3A). 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, we 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 the 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 lysis of most cells within 1 min (Fig. 3B). This activity was very strong and the activity was approximately 30 times higher than the activity of chicken egg white lysozyme (Fig. 3C).

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 compared to the control with only 5 mmol l^{-1} EDTA added, and a more than 4-log reduction in CFUs when 100 ng μl^{-1} endolysin and 10 mmol l^{-1} EDTA were added (Fig. 4A). The lysis activity varied not only with the concentration of endolysin and EDTA, but also with the reaction time. Fig. 4B 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 the cell numbers in the untreated suspensions at 100%. Within 20 min, 50 ng μl^{-1} endolysin with 10 mmol l^{-1} EDTA reduced the bacterial cell numbers by 60% and at 2 h by 99.8% compared to the control with only 10 mmol l^{-1} EDTA added. In any condition, when the enzyme reaction time was longer, many more viable cells were degraded. The concentrations of EDTA used in this study and the oligohistidine tag did not affect the endolysin itself (data not shown).

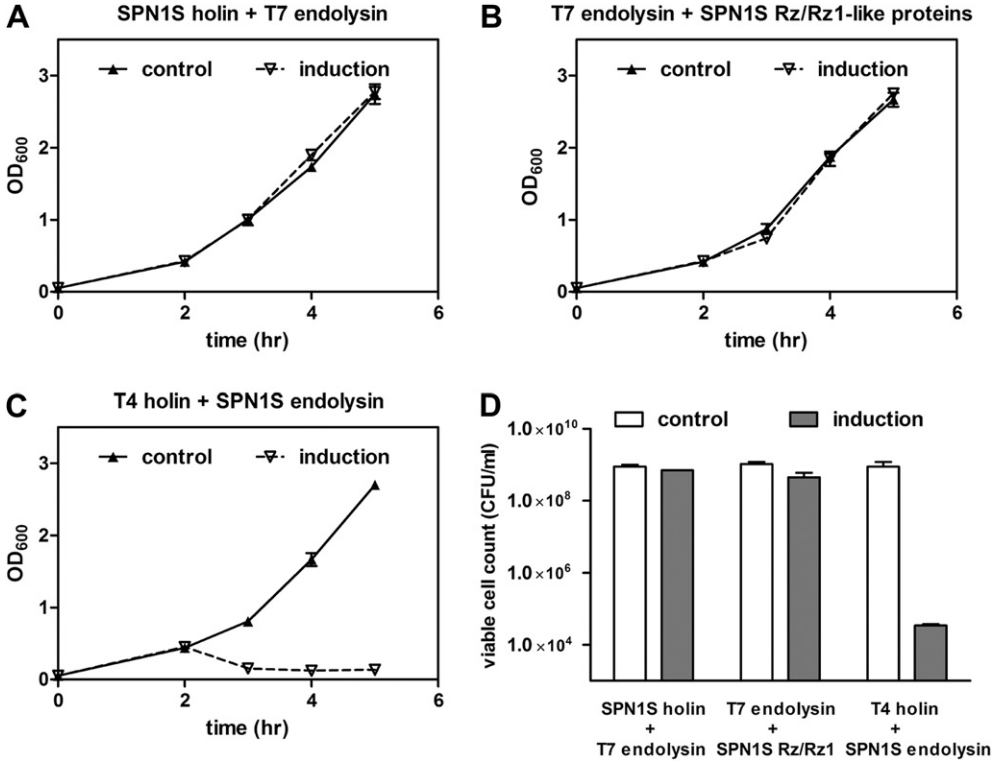

Fig. 2. Lysis activities of SPN1S lysis proteins with known lysis proteins. SPN1S holin and T7 endolysin (A), T7 endolysin and SPN1S Rz/Rz1-like proteins (B), T4 holin and SPN1S endolysin were expressed with 0.2% L-arabinose at 2 h after inoculation. Viable cell numbers after 5 h of incubation for each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

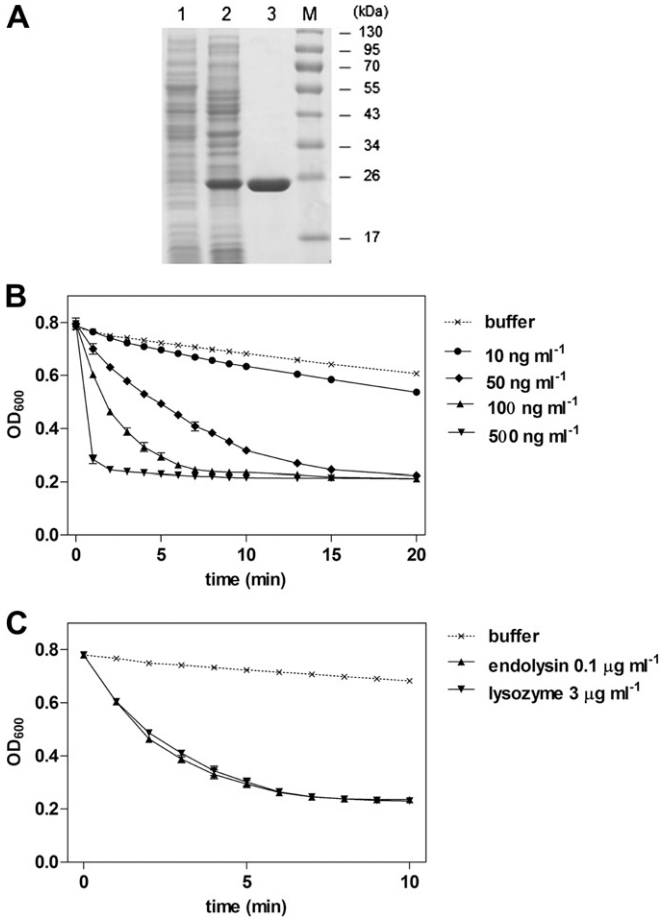

Fig. 3. Lysis activity of purified SPN1S endolysin. Total lysate of IPTG-induced *E. coli* cells harboring backbone plasmid (lane 1) and recombinant plasmid (lane 2) were separated on a 12% acrylamide gel (A). The overexpressed oligohistidine-tagged endolysin was purified (lane 3). M, molecular weight markers. The activity of various concentrations of purified SPN1S endolysin was detected using *E. coli* cell suspensions, with EDTA (B). The enzymatic activity of 0.1 μg ml^{-1} SPN1S endolysin was similar to that of 3 μg ml^{-1} lysozyme from chicken egg white (C).

3.3. 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 its activity. Endolysin was active to some extent over broad pH range and temperature (pH 7.0 to 10.5 and temperature 25 °C–45 °C) ranges but exhibited maximum activity in pH 9.5 and at 37 °C. This data are shown in Fig. S3.

3.4. Antimicrobial spectrum

Nine Gram-positive and 21 Gram-negative strains were examined for their susceptibility to SPN1S endolysin (Table 2). 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, we conclude that SPN1S endolysin has a wide spectrum of antimicrobial activity against Gram-negative bacteria.

3.5. Target site identification

Bioinformatic analysis showed that SPN1S endolysin has a lysozyme-like superfamily domain (E-value, 10^{-44}). Lysozyme damaged bacterial cell walls by catalyzing the hydrolysis of 1,4-beta-linkages between N-acetyl muramic acid and N-acetyl-D-glucosamine residues in the peptidoglycan (Nakimbugwe et al., 2006). To confirm the predicted glycosidic bond cleaving activity of SPN1S endolysin, we 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 (Fig. 5A). After lysis, the amounts of reducing sugar in each sample were measured. As with peptidoglycan degradation, the amount of reducing sugar in the enzyme digests was proportional to the endolysin concentration (Fig. 5B). The buffer and enzyme itself at any concentration used in this experiment did not affect the results. To examine whether endolysin might have other activities, we performed a peptidase assay and an amidase assay with the same enzyme digests, but found no difference between the endolysin lysate and buffer (data not shown). Thus, we concluded that SPN1S endolysin cleaved the glycosidic bond of peptidoglycan.

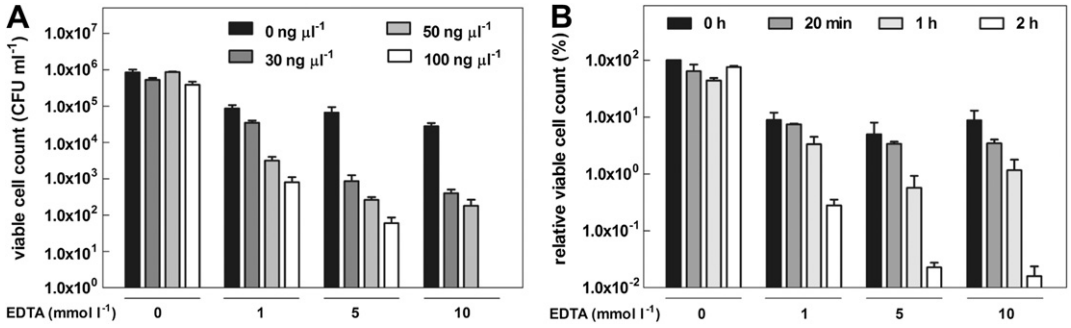

Fig. 4. Lytic activity of SPN1S endolysin over a range of EDTA concentrations. The viable cell numbers (CFUs) of *E. coli* upon addition of 30 ng μl^{-1}, 50 ng μl^{-1}, or 100 ng μl^{-1} endolysin were examined for 3 EDTA concentrations (1 mmol l^{-1}, 5 mmol l^{-1}, 10 mmol l^{-1}) after a 2 h reaction time (A). Relative viable cell numbers upon addition of 50 ng μl^{-1} endolysin and EDTA for different time points were calculated relative to the cell numbers after treatment with buffer (B).

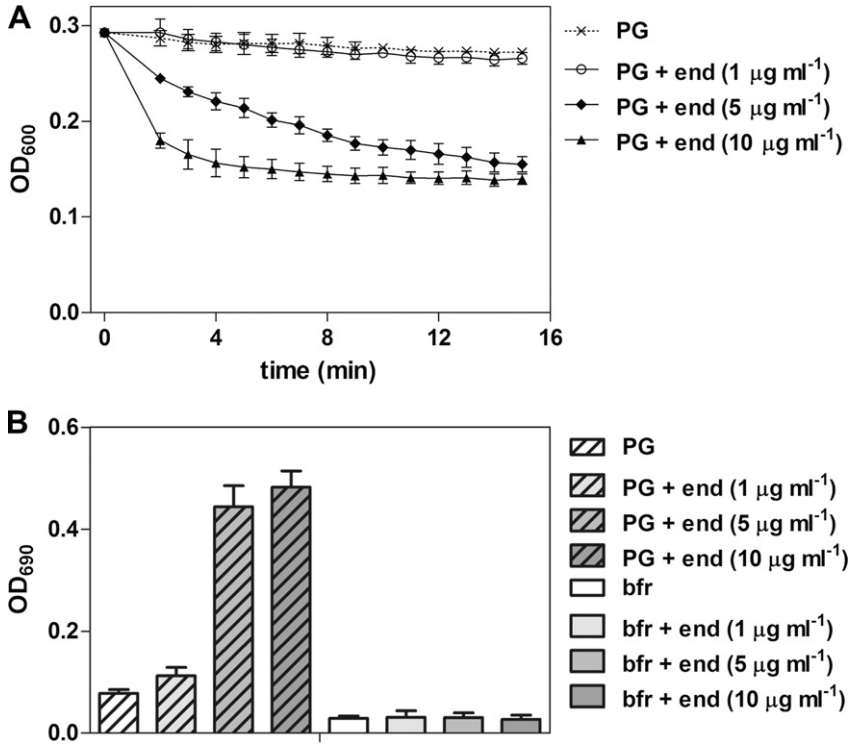

Fig. 5. Degradation of *E. coli* peptidoglycan by SPN1S endolysin. Peptidoglycan degradation was detected as a decrease in optical density (A). Reducing sugar was detected after endolysin treatment of *E. coli* peptidoglycan (B).

4. Discussion

In this study, we confirmed the lysis activity of an endolysin homologue from SPN1S, a newly isolated bacteriophage infecting *S.* Typhimurium. Endolysin has a lysozyme-like superfamily domain, but showed much stronger lysis activity than lysozyme from chicken egg white. Several endolysins with lytic activity against Gram-negative bacteria were 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 the optical density of sensitized cells approximately 10% at a 10 μg ml^{-1} concentration (Junn et al., 2005). The antibacterial activity of *A. baumannii* phage phiAB2 endolysin was approximately 30% of the antibacterial activity of chicken egg white lysozyme (Lai et al., 2011). SPN1S endolysin had greater activity than these endolysins. Fifty nanograms of endolysin were sufficient to reduce the optical density of a 1 ml EDTA pretreated cell suspension by approximately 50%. Indeed, SPN1S endolysin appears to be superior to lysozyme, with approximately 30 times higher activity.

The peptidoglycan of all Gram-negative bacteria is (almost) identical. In addition, *Bacillus* contains A1γ types of peptidoglycan like Gram-negative bacteria (Schleifer and Kandler, 1972). The different lysis pattern of 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 had 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, the *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 the lytic action of SPN1S endolysin (data not shown).

In general, endolysin cannot act as an 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). 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 (Briers et al., 2011; Morita et al., 2001; Nakimbugwe et al., 2006). 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. With SPN1S endolysin, we observed that the addition of EDTA along with endolysin allowed for 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 (Berry et al., 2008; Krupovič

et al., 2008; Summer et al., 2007). 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. (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 the 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 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 SPN1S, the expression of endolysin with Rz/Rz1-like proteins was sufficient for lysis. To our knowledge, this is the first example of phage lysis in which the Rz/Rz1-like proteins are critical. Further study will focus on determining the functions of the holin and Rz/Rz1-like proteins of SPN1S bacteriophage in lysis.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.resmic.2012.01.002.

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