



# *Staphylococcus aureus* Bacteriophage 52 Endolysin Exhibits Anti-Biofilm and Broad Antibacterial Activity Against Gram-Positive Bacteria

Mujib Abdulkadir Abdurahman<sup>1,5</sup> · İnci Durukan<sup>1</sup> · Tuba Dinçer<sup>2</sup> · Serap Pektaş<sup>3</sup> · Ersin Karataş<sup>4</sup> · Ali Osman Kiliç<sup>1</sup>

Accepted: 31 July 2023 / Published online: 27 August 2023

© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2023

## Abstract

Bacteriophage endolysins have been shown to hold great promise as new antibacterial agents for animal and human health in food preservation. In the present study, endolysin from *Staphylococcus aureus* subsp. *aureus* ATCC 27692-B1 bacteriophage 52 (LysSA52) was cloned, expressed, and characterized for its antimicrobial properties. Following DNA extraction from bacteriophage 52, a 1446-bp DNA fragment containing the endolysin gene (*lysSA52*) was obtained by PCR amplification and cloned into pET SUMO expression vector. The positive clone was validated by sequencing and open-reading frame analysis. The LysSA52 sequence shared high homology with staphylococcal phage endolysins of the SA12, SA13, and DSW2 phages and others. The cloned *lysSA52* gene encoding 481 amino acids endolysin was expressed in *Escherichia coli* BL21 with a calculated molecular mass of 66 kDa (LysSA52). This recombinant endolysin LysSA52 exhibited lytic activity against 8 of 10 Gram-positive bacteria via agar spot-on lawn antimicrobial assay, including methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecium*, *Enterococcus faecalis*, and *Bacillus atrophaeus*. In addition, the 0.50 mg/mL, LysSA52 endolysins reduced about 60% of the biofilms of *S. aureus* and *S. epidermidis* established on a microtiter plate in 12 h treatment. The data from this study indicate that LysSA52 endolysin could be used as an antibacterial protein to prevent and treat infections caused by staphylococci and several other Gram-positive pathogenic bacteria irrespective of their antibiotic resistance.

**Keywords** Bacteriophage · Cloning · Endolysin · *Staphylococcus*

✉ Ali Osman Kiliç  
akilic@ktu.edu.tr

Mujib Abdulkadir Abdurahman  
mujibe07@gmail.com

İnci Durukan  
idurukan87@hotmail.com

Tuba Dinçer  
tdincer@ktu.edu.tr

Serap Pektaş  
serapektas@gmail.com

Ersin Karataş  
hdersin@gmail.com

<sup>1</sup> Department of Medical Microbiology, Faculty of Medicine, Karadeniz Technical University, Trabzon 61080, Turkey

<sup>2</sup> Department of Medical Biology, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey

<sup>3</sup> Department of Chemistry, Faculty of Arts and Sciences, Recep Tayyip Erdogan University, Rize, Turkey

<sup>4</sup> Department of Medical Services and Techniques, Patnos Vocational School, Ağrı İbrahim Çeçen University, Ağrı, Turkey

<sup>5</sup> Department of Microbial, Cellular, and Molecular Biology, Faculty of Natural Science, Addis Ababa University, Addis Ababa, Ethiopia

## 1 Introduction

Based on the current literature, it is well-known that more than thirty different *Staphylococcus* species could be responsible for a great number of infections. Many of these infections are caused by *Staphylococcus aureus*, and a large number of them colonize the human body [1]. *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), are generally transported via contaminated apparatus or through directly contaminated persons more often result in nosocomial infections. These infections usually lead to an additional prolonged hospital stay as well as substantial economic burden [2]. Therefore, alternative approaches are needed to prevent and/or treat these infections. One of such approaches include the use of lytic bacteriophages alone or in combination with conventional antimicrobial agents have been reported [3]. Although phage resistance is less common compared to the antibiotic resistance in bacteria, the narrow host range due to phage receptor variability on the host cell surface limits the use of phages for infections [4]. They can interact with the immune system, potentially leading to harmful immune responses [5–7]. So far, little evidence suggests this is a significant concern during phage treatment [8]. However, some phage therapy protocols may require highly purified phage preparations to avoid allergic reactions to bacterial components, like endotoxins found in crude phage lysates. Phages can also release bacterial components when they kill bacteria, similar to how cell-wall-disrupting antibiotics work [6]. In recent years, studies have been conducted regarding the therapeutic efficacy of endolysins for infections that are caused by Gram-positive bacteria including those caused by antibiotic-resistant ones [9–11]. Endolysins are enzymes that are encoded and released by phages at the final stage of their life cycle. At this stage, phages degrade host bacterial cell wall to release the newly produced phage particles [12]. Exogenous recombinant endolysin causes bacterial disintegration within a short period of time without the help of holin proteins, which is different from their usual life cycle [13, 14]. Considering the remarkable combined effects of endolysin and antibiotics, their synergistic use demonstrates the potential to enhance the endolysin's broad-spectrum activity while concurrently preventing the development of resistance in target cells [15–17]. Presently, several clinical trials are underway, and some phage endolysin-related preparations have already entered the market, indicating their potentials being better antibacterial agents [18, 19]. The benefits of using phage endolysins over other antimicrobial agents include their specificity, their ability to damage cells without harming the normal microflora, and the rarity of bacteria that develop resistance [20]. Therefore endolysins offer new hope for treating superbugs that regular antibiotics are unable prevent due to

their resistance to multiple drugs. These properties of phage endolysins make them alternative antibacterial proteins in medicine, veterinary care, agriculture, food production, and industrial biotechnology [11]. The use of phage endolysins for the treatment of infections and biofilms caused by MRSA [21, 22] as well as free-floating *Staphylococcus* cells and biofilms, regardless of the surface they were on [23].

In the present study, it was aimed to clone and express the endolysin from *S. aureus* subsp. *aureus* ATCC 27,692-B1 bacteriophage 52 and demonstrate its antibacterial potential against Gram-positive bacteria including *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. pyogenes*, *S. pneumoniae*, *E. faecalis*, *E. faecium*, and *B. atrophaeus*.

## 2 Materials and Methods

### 2.1 Bacterial Strains, Media, and Growth Conditions

*S. aureus*, *S. epidermidis*, and *S. haemolyticus* previously isolated from various clinical specimens were obtained from the Department of Medical Microbiology Culture Collection, Faculty of Medicine, Karadeniz Technical University, and the bacteriophage host *S. aureus* subsp. *aureus* ATCC 27692, and ATCC 27692-B1 bacteriophage 52 was obtained from the American Type Culture Collection (ATCC, USA). Bacterial strains used in this study were presented in Table 1. Chemically competent *Escherichia coli* One Shot™ Mach1™ cells used for the preparation of recombinant plasmids and the *E. coli* BL21 (DE3) strain used as a recombinant endolysin expression host were obtained from Thermo Fisher Scientific (USA) and Invitrogen (USA), respectively. All Gram-positive strains except lactobacilli were grown in tryptic soy agar (TSA) and tryptic soy broth (TSB). Lactobacilli were cultured in Demand Rogosa agar (MRSA) and MRSA broth. Gram-negative bacteria were cultured in Luria-Bertani (LB) media. Except for lactobacilli cultures, all microorganisms were grown at 37 °C with shaking aerobically unless otherwise indicated. Lactobacilli cultures were incubated statically in a candle jar at 37 °C.

### 2.2 Phage DNA Isolation, Primer Design, and PCR Amplification of the Endolysin Gene

Phage purification was performed according to Carroll-Portillo et al., 2021 with some modification [24]. DNA isolation from phage was performed by using the phenol-chloroform isoamyl extraction method. Briefly, 450 µL purified phage stock (ca.  $1 \times 10^{10}$  PFU mL<sup>-1</sup>) of bacteriophage 52 was treated with lysis buffer (500 mM Tris-HCl; 250 mM EDTA; 2.5% SDS, 20 mg/mL RNase A; and 10 mg/mL DNase I) for about 30 min at 37°C. The lysed phage particles were

**Table 1** Lytic efficacy of the recombinant LysSA52 against different clinical isolates of *Staphylococcus* and various Gram-positive and Gram-negative bacteria

No	Isolates/Strains	Origin	Lysis	No	Isolates/Strains	Origin	Lysis
1	<i>S. aureus</i> TRSA1	Pus	+	47	<i>S. aureus</i> TRSA47	Wound	+
2	<i>S. aureus</i> TRSA2	Wound	+	48	<i>S. aureus</i> ATCC25923	Clinical isolates	+
3	<i>S. aureus</i> TRSA3	Blood	+	49	<i>S. aureus</i> ATCC 6538	Human lesion	+
4	<i>S. aureus</i> TRSA4	Tracheal aspirate	+	50	<i>S. aureus</i> MRSA1	Tracheal aspirate	+
5	<i>S. aureus</i> TRSA5	Tracheal aspirate	-	51	<i>S. aureus</i> MRSA2	Tracheal aspirate	+
6	<i>S. aureus</i> TRSA6	Tracheal aspirate	+	52	<i>S. aureus</i> MRSA3	Sputum	-
7	<i>S. aureus</i> TRSA7	Wound	+	53	<i>S. aureus</i> MRSA4	Wound	+
8	<i>S. aureus</i> TRSA8	Tracheal aspirate	+	54	<i>S. aureus</i> MRSA5	Wound	+
9	<i>S. aureus</i> TRSA9	Ear	+	55	<i>S. aureus</i> MRSA6	Tracheal aspirate	+
10	<i>S. aureus</i> TRSA10	Wound	+	56	<i>S. aureus</i> MRSA7	Wound	+
11	<i>S. aureus</i> TRSA11	Ear	+	57	<i>S. aureus</i> MRSA8	Tracheal aspirate	+
12	<i>S. aureus</i> TRSA12	Wound	+	58	<i>S. aureus</i> MRSA9	Wound	+
13	<i>S. aureus</i> TRSA13	Tracheal aspirate	+	59	<i>S. aureus</i> MRSA10	Tracheal aspirate	+
14	<i>S. aureus</i> TRSA14	Wound	+	60	<i>S. aureus</i> MRSA 89	Beef meatball	+
15	<i>S. aureus</i> TRSA15	Tracheal aspirate	+	61	<i>S. aureus</i> MRSA 90	Beef meatball	+
16	<i>S. aureus</i> TRSA16	Wound	+	62	<i>S. aureus</i> MRSA 126	Milk	+
17	<i>S. aureus</i> TRSA17	Sputum	+	63	<i>S. aureus</i> MRSA 127	Milk	+
18	<i>S. aureus</i> TRSA18	Urine	-	64	<i>S. aureus</i> MRSA 137	hand wash	+
19	<i>S. aureus</i> TRSA19	Tracheal aspirate	+	65	<i>S. aureus</i> MRSA 138	hand wash	+
20	<i>S. aureus</i> TRSA20	Wound	+	66	<i>S. aureus</i> MRSA 148	Cheese	+
21	<i>S. aureus</i> TRSA21	Pus	-	67	<i>S. aureus</i> MRSA 151	Cheese	+
22	<i>S. aureus</i> TRSA22	Wound	+	68	<i>S. epidermidis</i> TRSE1	Blood	+
23	<i>S. aureus</i> TRSA23	Wound	+	69	<i>S. epidermidis</i> TRSE2	Blood	-
24	<i>S. aureus</i> TRSA24	Tracheal aspirate	+	70	<i>S. epidermidis</i> TRSE6	Urine	+
25	<i>S. aureus</i> TRSA25	Tracheal aspirate	-	71	<i>S. epidermidis</i> TRSE7	Urine	+
26	<i>S. aureus</i> TRSA26	Wound	+	72	<i>S. epidermidis</i> TRSE9	Wound	+
27	<i>S. aureus</i> TRSA27	Nose swab	+	73	<i>S. epidermidis</i> TRSE13	Blood	+
28	<i>S. aureus</i> TRSA28	Wound	+	74	<i>S. epidermidis</i> TRSE14	Wound	+
29	<i>S. aureus</i> TRSA29	Wound	+	75	<i>S. epidermidis</i> TRSE15	Wound	+
30	<i>S. aureus</i> TRSA30	Pus	+	76	<i>S. epidermidis</i> TRSE16	Blood	+
31	<i>S. aureus</i> TRSA31	Tracheal aspirate	+	77	<i>S. epidermidis</i> TRSE22	Urine	-
32	<i>S. aureus</i> TRSA32	Wound	+	78	<i>S. epidermidis</i> TRSE23	Wound	+
33	<i>S. aureus</i> TRSA33	Tracheal aspirate	+	79	<i>S. epidermidis</i> TRSE24	Urine	+
34	<i>S. aureus</i> TRSA34	Wound	+	80	<i>S. epidermidis</i> ATCC 12228	ATCC	+
35	<i>S. aureus</i> TRSA35	Wound	+	81	<i>S. haemolyticus</i> TRSH1	Wound	+
36	<i>S. aureus</i> TRSA36	Blood	+	82	<i>S. haemolyticus</i> TSHE2	Blood	-
37	<i>S. aureus</i> TRSA37	Tissue	+	83	<i>S. haemolyticus</i> TRSH3	Urine	+
38	<i>S. aureus</i> TRSA38	Blood	+	84	<i>S. pyogenes</i> ATCC 19615	Pharynx of child	+
39	<i>S. aureus</i> TRSA39	Wound	+	85	<i>S. pneumoniae</i> ATCC 49619	Sputum	+
40	<i>S. aureus</i> TRSA40	Wound	+	86	<i>E. faecalis</i> ATCC 29212	Urine	+
41	<i>S. aureus</i> TRSA41	Tracheal aspirate	+	87	<i>E. faecium</i> ATCC 6057	Dairy products	+
42	<i>S. aureus</i> TRSA42	Wound	+	88	<i>B. atrophaeus</i> ATCC 9372	ATCC	+
43	<i>S. aureus</i> TRSA43	Pus	+	89	<i>L. plantarum</i> ATCC 14917	Pickled cabbage	-
44	<i>S. aureus</i> TRSA44	Blood	+	90	<i>L. monocytogenes</i> ATCC 43251	ATCC	-
45	<i>S. aureus</i> TRSA45	Wound	+	91	<i>E. coli</i> ATCC 35218	Canine, Tennessee	-
46	<i>S. aureus</i> TRSA46	Wound	+	92	<i>S. typhimurium</i> ATCC 14028	Tissue of chicken	-

-; no lysis, +; lysis

then centrifuged at 9000 g (Eppendorf Centrifuge 580 R, Germany) for 10 min, and the supernatant was transferred to a new microcentrifuge tube followed by the addition of phenol in a one-to-one ratio before mixing and centrifugation at 9000 g for 10 min. The DNA extraction from supernatant was performed by treatment with a 1:1 volume of chloroform: isoamyl alcohol (24:1) for 10 min at room temperature. After precipitation with two volumes of 96% ethanol, DNA was washed with 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). The purity and concentration of DNA were measured using a NanoDrop 2000/2000c (Thermo Scientific, USA) spectrophotometer. The forward (Lys52F: 5'- ATG CAA GCA AAA CTA ACT AAA AAA G -3') and reverse (Lys52R: 5'- TTA ACT GAT TTC TCC CCA TAA GTC-3') primers were designed based on the coding sequence of five staphylococcal phage endolysin sequences (accession numbers; NC\_021801.1, NC\_010147, NC\_011614.1, NC\_024391.1, and NC\_021863.1) available from the National Center for Biotechnology Information (NCBI) website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)), and were employed to amplify the phage 52 endolysin gene using PCR. The PCR reaction was carried out in 25  $\mu$ L reaction volume containing 1x PCR buffer (Promega 5x), 2.5  $\mu$ M of each nucleotide, 10  $\mu$ M of each primer, 25 mM MgCl<sub>2</sub>, 0.5 U Taq DNA polymerase (Promega, USA), and 5–10 ng DNA template. A PCR condition was adjusted to an initial denaturation (5 min at 94 °C), 30 cycles of denaturation (1 min at 94 °C), primer annealing (1 min at 52 °C), and extension (1 min at 72 °C), plus a final extension (7 min at 72 °C). The amplified PCR products were analyzed by electrophoresis in 1% agarose gel and visualized under a UV transilluminator.

### 2.3 Cloning and Sequencing of the Endolysin Gene

The amplified PCR product was purified using the High Pure PCR Product Purification kit (Sigma-Aldrich, USA) and cloned into the pET-SUMO expression vector (Invitrogen, USA) by following the recommendations of the manufacturer. The cloned plasmid was used to transform *E. coli* One Shot Mach1-T1R competent cells and then transformed cells were plated on LB agar plates containing kanamycin (50  $\mu$ g/mL), and incubated at 37 °C for overnight. Several colonies were picked and inoculated into 5 mL of LB containing kanamycin (50  $\mu$ g/mL) for plasmid DNA isolation using a plasmid Miniprep kit (Qiagen, Germany), according to the manufacturer's protocol. The clones were confirmed by sequencing using an Applied Biosystem ABI 310 DNA Analyzer (Applied Biosystem®, USA) with vector-specific forward and reverse primers. One clone with the correct sequence designated as pET-SUMO-LysSA52 was selected for expression.

### 2.4 Over-expression and Purification of the Endolysin

The pET-SUMO-LysSA52 plasmid with correct orientation was transformed into competent *E. coli* BL21 (DE3) cells for endolysin protein expression using a standard calcium chloride transformation procedure. A single colony from the transformed cells was grown in 10 mL LB medium with appropriate antibiotic (50  $\mu$ g/mL of kanamycin) and incubated overnight at 37 °C with shaking. Then, 100 mL of LB media containing kanamycin (50  $\mu$ g/mL) and 1% glucose was inoculated with 1 mL of the overnight culture and grown at 37 °C with shaking until it reached an optical density at 600 nm (OD<sub>600 nm</sub>) of 0.5. Then, culture was induced for 18 h with 1 mM IPTG at 20 °C with shaking. The induced cells were collected by centrifugation at 5000 xg at 4 °C for 30 min, weighed, and resuspended as a 10 mL/g wet pellet in lysis buffer (400 mM NaCl; 50 mM NaH<sub>2</sub>PO<sub>4</sub> buffer pH 7.8; 0.5% Triton X-100; 10% glycerol; and 10 mM imidazole) and performed three freeze-thaw cycles at -80 °C and 37 °C. The cells were sonicated (Vibra Cell™ Sonics, USA) at 50% amplitude via 18  $\times$  10 s bursts with 10 s rest time. Lastly, the homogenized suspension was centrifuged at 9000 xg for 30 min and the clear lysate was filtered through Millex® Syringe Filters (Merck KGaA, Germany) with a 0.2  $\mu$ m pore size, and polyhistidine-containing recombinant fusion protein (SUMO-LysSA52) was purified using a ProBond™ Purification System kit (Novex, USA), in accordance with the manufacturer's recommendations. All extracted fractions including fusion protein were collected and analyzed using 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing the fusion protein were combined and dialyzed (SnakeSkin™ Dialysis Tubing, Thermo Scientific, USA) at 4 °C against 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl. The protein concentration was measured using Bradford Assay and stored in a -20 °C freezer until use.

### 2.5 Lytic Activity and Lytic Spectrum of Endolysin

The expressed endolysin (LysSA52) activity was determined via agar spot-on lawn antimicrobial assay. In brief, 10  $\mu$ L of purified endolysin (0.5-1 mg/mL) was spotted onto agar plates pre-seeded with mid-log phase bacterial culture and grown at 37 °C for 24 h. Lytic activity was determined as the presence of bacterial lysis with clear spots on the agar cultures was considered to indicate positive endolysin activity.

The host spectrum of LysSA52 endolysin was tested against 90 strains of 10 Gram-positive species including 49 *S. aureus* strains, 18 of which were MRSA, 13 *S. epidermidis*, 3 *S. haemolyticus*, and *S. pneumonia* ATCC 49,619, *S.*

*pyogenes* ATCC 19,615, *E. faecium* ATCC 6057, *E. faecalis* ATCC 29,212, *B. atrophaeus* ATCC 9372, *Listeria monocytogenes* ATCC 43,251, *Lactobacillus plantarum* ATCC 14,917, and two Gram-negative bacterial strains including *E. coli* ATCC 35,218, and *Salmonella typhimurium* ATCC 14,028 (Table 1).

## 2.6 Effects of pH and Heat on the Activity of LysSA52 Endolysin

The effects of pH and heat sensitivity (thermostability) on LysSA52 activity were analyzed by using the *S. aureus* TRSA 2 strain cultured to the logarithmic growth phase. To evaluate the effect of pH on LysSA52, bacterial host cells were suspended in three different pH buffers (sodium acetate (pH 4.0–6.0), HEPES (pH 6.8–8.2), and Tris (pH 8.0–9.0)), mixed with an equal volume of endolysin solution (1 mg/mL) in a 96-well plate with a 200  $\mu$ L total volume, and incubated plates at 37 °C for 12 h. The buffer concentration was 20 mM for all experiments. Surviving cells were determined by colony count and the cell killing rate of the enzymes was expressed as a percentage.

The heat sensitivity of endolysin was reported previously [25]. Briefly, LysSA52 was incubated at various temperatures between 30 and 90 °C at 10 °C intervals for 30 min followed by a 20 min cooling step on ice, and was spotted on an agar culture of the *S. aureus* TRSA 2 strain. Endolysin solution without heat treatment was used as a positive control. The inhibition zone diameters of heat-treated endolysins were measured and compared to that of positive control which was kept on ice.

## 2.7 Storage Stability

Storage stability of the LysSA52 endolysin was examined by exposing it to various temperatures (room temperature, 4 °C, and –20 °C) for nine weeks. The enzyme samples were stored in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.1 mM EDTA, and 50% (v/v) glycerol. Endolysin samples were assessed for their antimicrobial activity using the agar spot-on lawn antimicrobial assay following nine weeks of storage.

## 2.8 Anti-biofilm Properties of Endolysin

The biofilm removal performance of the LysSA52 endolysin was performed by using a microtiter plate assay as described previously with slight modifications [26]. Briefly, *S. aureus* and *S. epidermidis* strains were diluted with 0.25% wv D-(+)-glucose to attain an initial inoculum level of 10<sup>6</sup> CFU/mL. From each strain, 200  $\mu$ L of prepared inoculums was added into 96 wells of a flat-bottomed microtiter plate

and 200  $\mu$ L TSB was added in separate wells as a negative control. Biofilms were established by incubating the plate at 37 °C without agitation for 48 h. Then, the content of the wells was discarded, and biofilms were washed two times with sterile phosphate-buffered saline (PBS). Following this, biofilms were treated with LysSA52 endolysin for 12 h at 37 °C. The content of the wells was discarded and washed two times with PBS. Wells were stained for 30 min by adding 200  $\mu$ L of 1% (v/v) crystal violet solution. The unbound stain was removed, and stained biofilms were washed with PBS, and then solubilized by the addition of 200  $\mu$ L of ethanol-acetone (80:20) solution to each well. The remaining biofilms after endolysin treatment were quantified using a plate reader at OD<sub>595 nm</sub> and the percentage reduction in biofilms was compared with untreated control. The mean of four wells was used and the experiments were repeated three times.

## 3 Results

### 3.1 PCR Amplification, Cloning, and Sequence Analysis of LysSA52 Genes

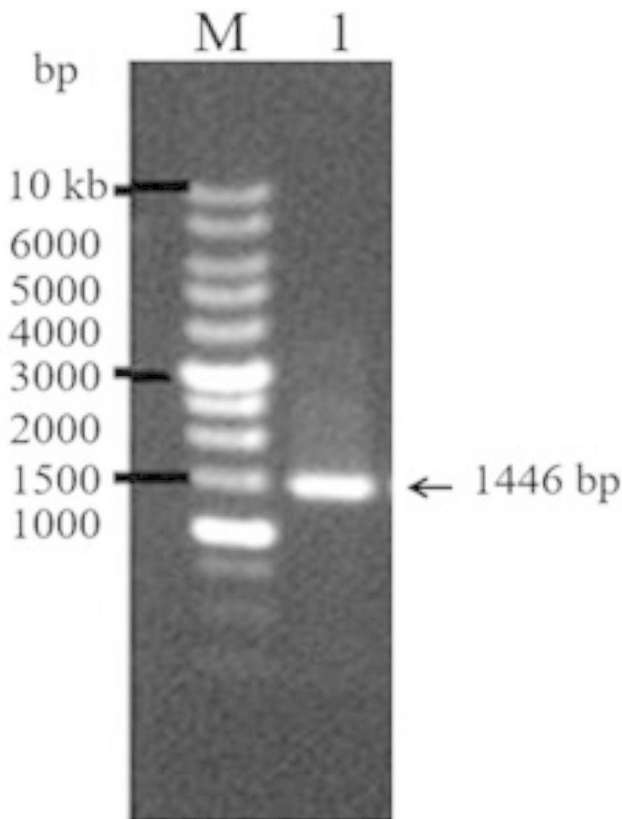
The DNA from *S. aureus* subsp. *aureus* bacteriophage 52 (ATCC®27692-B1™) obtained from ATCC was used to amplify the endolysin gene of 1446 bp (Fig. 1). The amplified product was cleaned and cloned directly into the pET-SUMO vector (Invitrogen, USA) and the pET-SUMO-LysSA52 recombinant clone was sequenced to confirmed for the presence of the 1446 bp insert with correct orientation encoding a single open reading frame beginning with a start codon (ATG) and ending with a stop codon (TAA). The nucleotide sequence data were submitted to the GenBank database (NCB, USA) and assigned accession number: (MH104949). Neighbor-joining phylogenetic tree analysis showed that the LysSA52 endolysin gene was closely related to the staphylococcal phages DW2, SA12, and SA13 (Fig. 2).

The amino acid sequence (481 amino acids) analysis using Simple Modular Architecture Research Tool (SMART) available at <http://smart.embl-heidelberg.de/> revealed that LysSA52 has three distinct domains; CHAP (cysteine, histidine-dependent) domain, amidase (Ami-2) domain, and SH3b domain (Fig. 3).

### 3.2 Expression of LysSA52 Endolysin

The culture of *E. coli* BL21 (DE3) carrying the pET-SUMO-LysSA52 plasmid was induced with 1 mM IPTG. The LysSA52 endolysin with a molecular mass of approximately 53 kDa was successfully co-expressed with 13 kDa





**Fig. 1** PCR amplification of LysSA52 gene from *S. aureus* subsp. *aureus* bacteriophage 52 DNA. M; molecular weight marker, 1; endolysin gene product

SUMO protein and polyhistidine [6-His]-tag at the N terminus resulting in a 66 kDa fusion protein (Fig. 4A). The expressed his-tagged protein was purified from crude cell lysate by nickel chelating affinity chromatography. The eluted polyhistidine-containing recombinant SUMO-fused endolysin LysSA52 was demonstrated in SDS-PAGE analysis (Fig. 4B). The LysSA52-SUMO fusion protein was used for the lytic activity assays without further purification since the SUMO protease cleavage was ineffective despite the manufacturer's recommendations.

### 3.3 Lytic Activity and Host Range of LysSA52 Endolysin

The LysSA52 endolysin lytic activity and the lytic spectrum were demonstrated by the agar spot-on lawn antimicrobial assay and indicated by a clear lysis zone on the endolysin spotted area compared to the control, containing total cell proteins of uninduced *E. coli* BL 21 cells carrying the pET-SUMO-LysSA52 plasmid (Fig. 5). With respect to the lytic spectrum, the recombinant endolysin LysSA52 showed lytic activity against 74 (89%) out of 83 staphylococcal strains, including *S. aureus*, MRSA, *S. epidermidis*, and *S.*

*haemolyticus*, irrespective of their origin (Table 1). In addition, LysSA52 lysed *E. faecium* ATCC 6057, *E. faecalis* ATCC 29,212, *S. pneumonia* ATCC 49,619, *S. pyogenes* ATCC 19,615, and *B. atrophaeus* ATCC 9372 but did not show any lytic activity against *L. plantarum* ATCC, 14917, *L. monocytogenes* ATCC 43251, and *S. Typhimurium* ATCC 14,028 (Table 1).

### 3.4 Enzymatic Properties of LysSA52

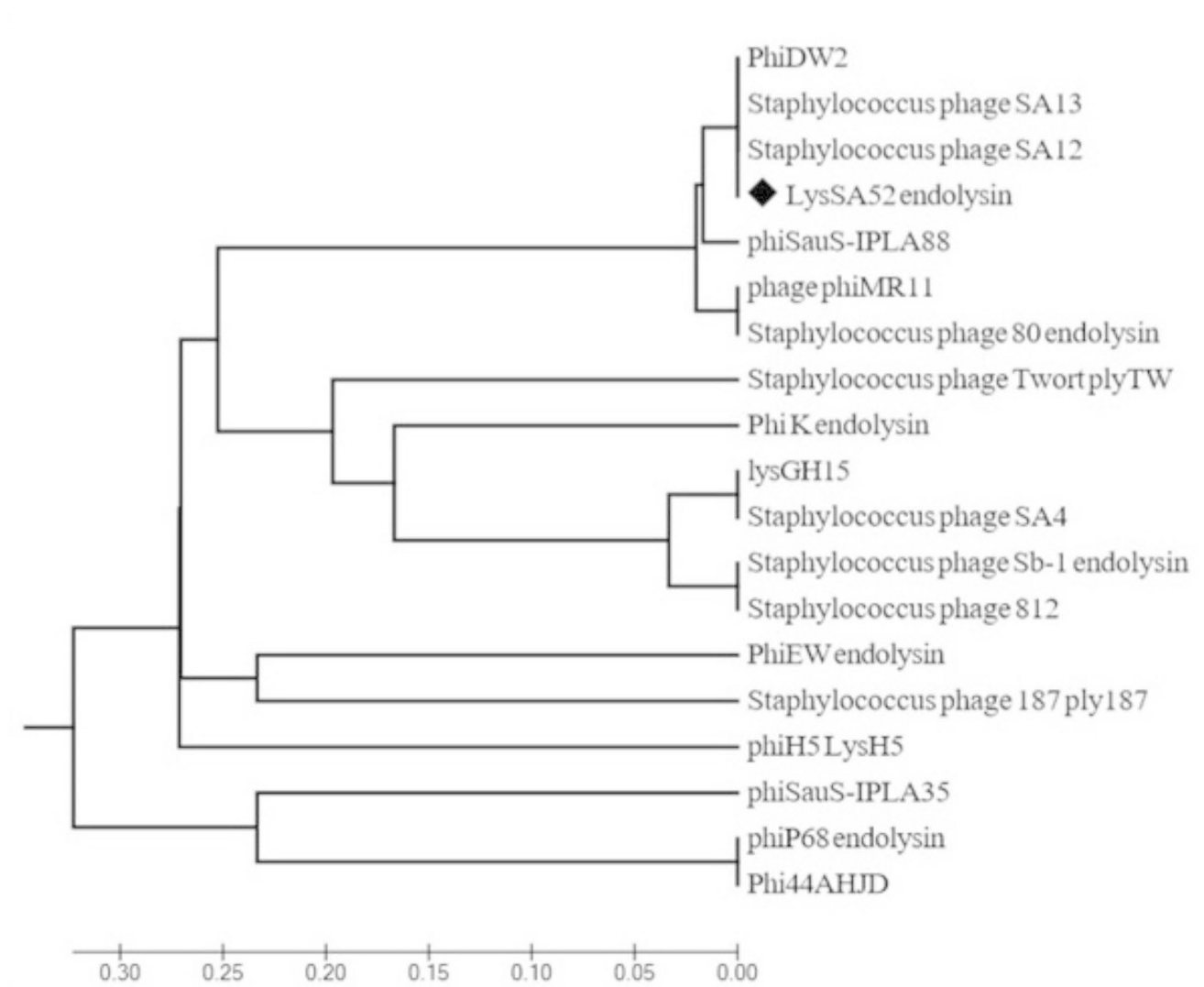
Using the agar spot-on lawn antimicrobial assay and colony count, the activity of LysSA52 was evaluated over a range of pH and temperature, (Fig. 6A-B). The LysSA52 endolysin works best in the alkaline pH range with maximum or optimum activity at pH 8.0, and its activity decreased significantly below pH 7.0 (Fig. 6A). The LysSA52 endolysin retained more than 85% of its antibacterial activity at 30–50 °C for 30 min (Fig. 6B). However, its activity sharply decreased at or above 60 °C. The storage stability of LysSA52 endolysin was examined by exposing the enzyme at room temperature, 4 °C, and –20 °C and assessing its activity after nine weeks. The result showed that LysSA52 retained its activity for 9 weeks of storage at 4 °C (Fig. 7).

### 3.5 Biofilm Removal Assay

In order to assay the biofilm removal capacity of LysSA52 endolysin using crystal violet assay, 48 h mature biofilms of *S. aureus* TRSA 8 and *S. epidermidis* TRSA 31 established in 96 well polystyrene microtiter plates were treated with LysSA52 endolysin at three different concentrations (0.20 mg/mL, 0.30 mg/mL, and 0.50 mg/mL) for 12 h. The remaining biofilm mass was stained with crystal violet and the remaining biofilm was estimated by spectrophotometric measurement of the solubilized biofilm. The data demonstrated that the LysSA52 treatment reduced biofilms of both *S. aureus* and *S. epidermidis* relative to the control group at all concentrations tested as shown in Fig. 8. The reduction was markedly sensitive to 0.50 mg/mL, which removed about 60% of established *S. aureus* and *S. epidermidis* biofilms in 12 h.

## 4 Discussion

*S. aureus* is one of the main human pathogens that cause local, systemic, and nosocomial infections amongst coagulase-positive staphylococci [27]. In previous studies, it has been reported that treatment of these infections has challenges because of the high rate of MRSA infection [28]. Lytic phages are used individually or in combination with conventional antimicrobial agents to treat bacterial



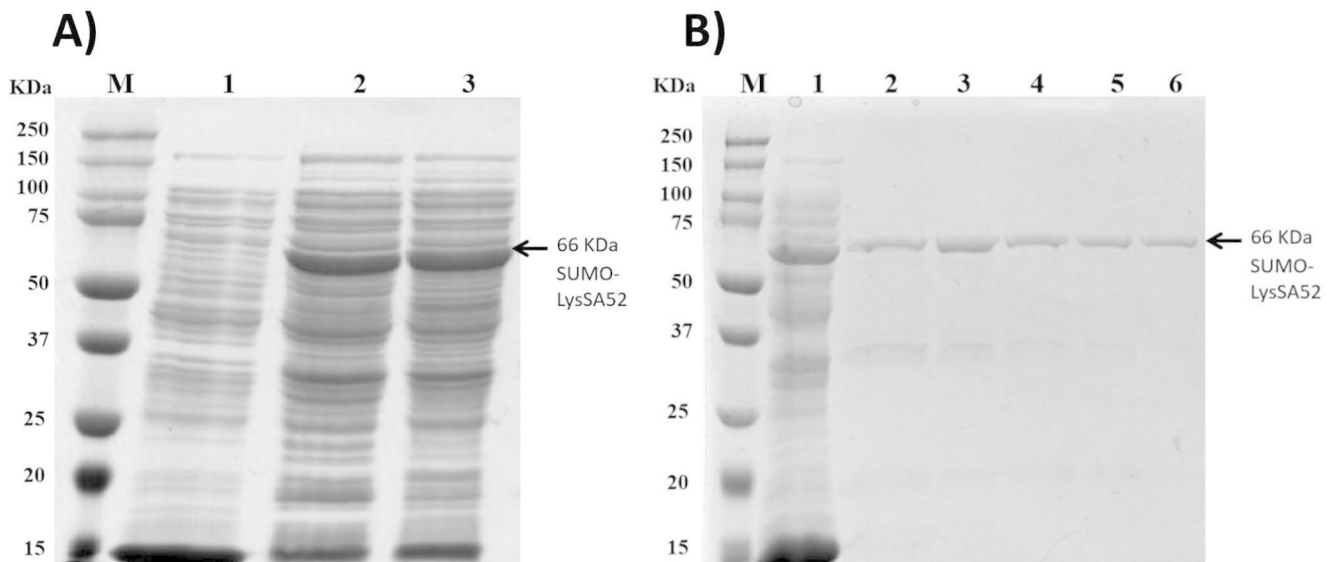
**Fig. 2** Phylogenetic position of LysSA52 endolysin gene derived from *S. aureus* subsp. *aureus* bacteriophage 52



**Fig. 3** Domain organization of the endolysins LysSA52. Two catalytic domains CHAP and Ami-2; catalytic domains, SH3b; cell wall binding domain Numbers indicate amino acid positions

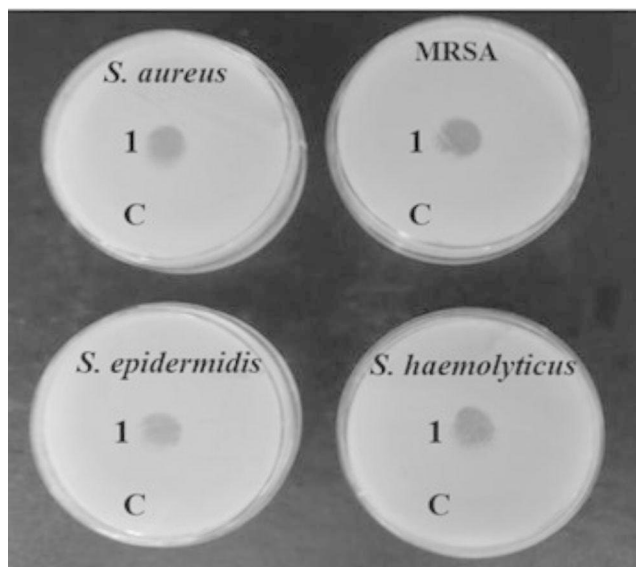
infections [3]. However, the narrow host range, which is largely due to the variability in phage receptor molecules on the host cell surface, limits the application of bacteriophage therapy [4]. Recently, the use of phage endolysin has attracted considerable interest in combating pathogenic bacteria as an alternative to conventional antibiotics [9]. Phage

endolysins can kill target bacteria upon exposure and do not harm the normal microflora, and it is very rare for bacteria to develop resistance to them [20]. The demand for recombinant antimicrobial proteins, including phage endolysins as enzybiotics, which can be produced with high quality in large amounts under lower production costs, has increased



**Fig. 4** SDS-PAGE analysis of expressed His-SUMO-LysSA52 endolysin. **A**; total cell proteins of *E. coli* BL 21 carrying pET-SUMO-LysSA52 plasmid, M; protein molecular weight marker, 1; uninduced cell proteins, 2 and 3; IPTG induced LysSA52 for 18 and 20 h, respec-

tively. **B**; SDS-PAGE analysis of His-SUMO-LysSA52 fusion protein before and after purifications. M; protein molecular weight marker, 1; total cell proteins before purification, 2 to 6; eluted fractions



**Fig. 5** Lytic activity of the LysSA52 endolysin against *S. aureus*, MRSA, *S. epidermidis*, and *S. haemolyticus* by the agar spot-on lawn antimicrobial assay. 1; LysSA52; C: control

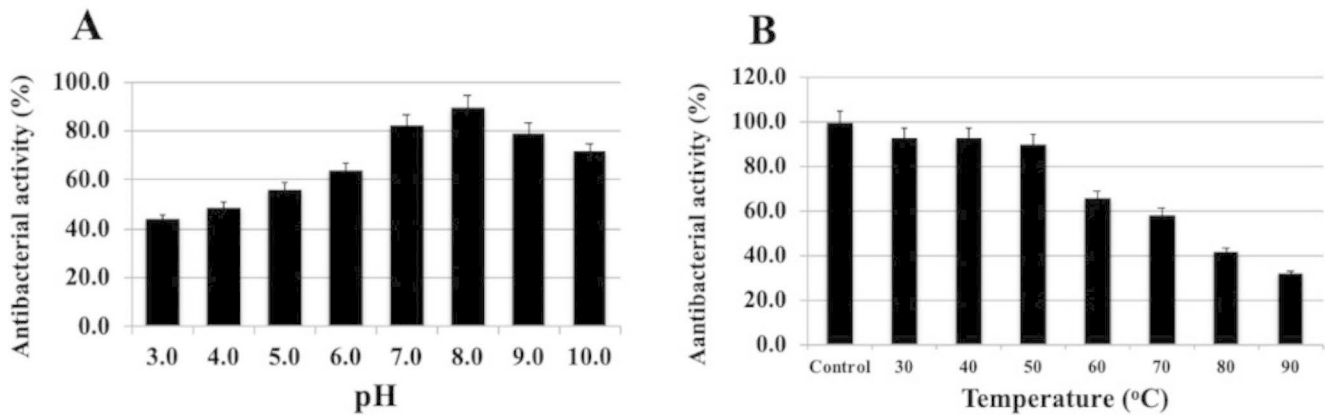
due to their utilization in various sectors, such as healthcare, agriculture, and the food industry [29].

In this study, we aimed to clone and express the endolysin gene from *S. aureus* subsp. *aureus* ATCC 27,692-B1 bacteriophage 52 and assess its antibacterial activity against staphylococci and other various Gram-positive pathogens. The lytic spectrum of LysSA52 was evaluated via the agar spot-on lawn antimicrobial assay method. LysSA52 displayed strong lytic activity against a wide range of

*Staphylococcus* isolates, including methicillin-sensitive *S. aureus* (MSSA), MRSA, *S. epidermidis*, and *S. haemolyticus* of clinical origin. LysSA52 also showed cross-species lytic efficacy for several Gram-positive bacteria, including *E. faecium*, *E. faecalis*, *S. pneumoniae*, *S. pyogenes*, and *B. atrophaeus*. These results demonstrated the therapeutic potential of this recombinant endolysin. On the other hand, it was ineffective against *L. monocytogenes* ATCC 43,251, *L. plantarum* ATCC 14,917, and it did not exhibit lytic activity against Gram-negative bacteria such as *E. coli* and *S. Typhimurium*.

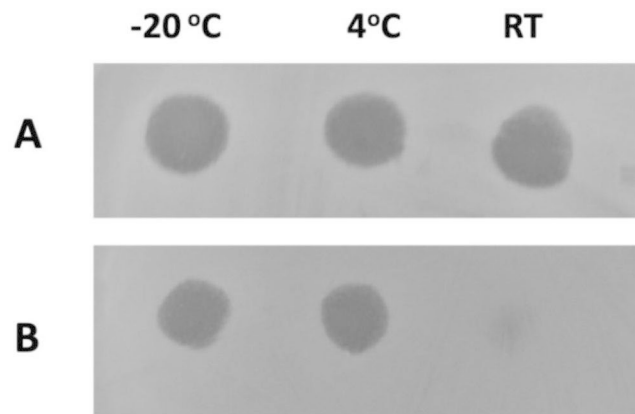
Numerous studies have been conducted regarding bacteriophage endolysins and their structures and applications as antibacterials in controlling and treating different bacterial infections [4, 25, 29–31]. Although many phage endolysins studied so far have demonstrated lytic activity against only their respective phage host strains, a number of endolysins from phages of Gram-negative and -positive bacteria exhibit cross-species lytic activity. The phage endolysin LysAB2 from *Acinetobacter baumannii* phage AB2 exhibits bacteriolytic activity against *S. aureus* in addition to (*A. baumannii* [32]. LysBPS13, an endolysin derived from *Bacillus cereus* bacteriophage was found to lyse (*B. cereus* and *Bacillus thuringiensis* and it was also active against EDTA-treated *E. coli*, *Shigella*, *Salmonella*, and *Coronobacter sakazakii* [33]. The *Streptococcus suis* phage endolysin, PlySs2, showed a broad lytic spectrum against a number of Gram-positive bacteria including vancomycin-intermediate *S. aureus*, MRSA, *Staphylococcus simulans*, *S. epidermidis*, *Listeria*, and enterococci as well as a number of streptococci [34].





**Fig. 6** Biochemical properties of LysSA52. A; Influence of pH on the bacterial activity of LysSA52. The activity was determined by viable cell count and expressed in percentage. B; Temperature stability of LysSA52 endolysin. The activity was determined by measuring inhibi-

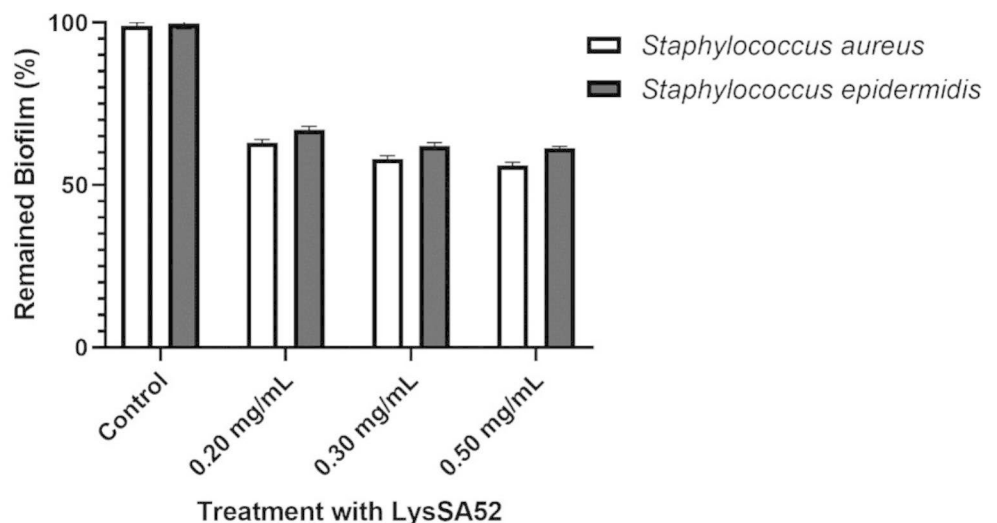
tion zones and expressing the results in percentage relative to control (non-heat treatment endolysin kept at room temperature). Each column represents the mean of triplicate assays, and error bars indicate the standard deviation



**Fig. 7** Storage stability of LysSA52 endolysin. A; LysSA52 test at the beginning of the experiment on *S. aureus* TRSA 8, B; LysSA52 test result after nine weeks on the same strain. In each case 5  $\mu$ L of LysSA52 was dropped on agar plate containing bacterial lawn (endolysin stored at room temperature started to losing activity only after nine weeks)

An endolysin originated from a *Stenotrophomonas maltophilia* bacteriophage, P28, demonstrated high lytic activity against three Gram-negative bacteria; *Xanthomonas citri* *Malvacearum* sp. *Shigella flexneri*, and *Klebsiella pneumonia* ssp. *pneumoniae*, in addition to *B. cereus*, *Bacillus subtilis*, *L. monocytogenes*, and *S. aureus* [35]. Similarly, the TSPphg lysin from *Thermus* sp. TC4 phage-displayed lytic activity against both Gram-positive (*Bacillus subtilis* and *S. aureus*) and Gram-negative bacteria (*K. pneumonia*, *E. coli* O157, and *Salmonella paratyphi* B) [36]. In the present study, we cloned, expressed, and assessed the lytic activity of the *S. aureus* phage 52 endolysin, LysSA52. We demonstrated the ability of LysSA52 to inactivate not only approximately 90% of clinical strains of staphylococci, such as MRSA, *S. epidermidis*, and *S. haemolyticus*, but also a number of Gram-positive bacteria, including *E. faecium*, *E. faecalis*, *S. pneumonia*, *S. pyogenes*, and *B. atrophaeus*. However, LysSA52 was ineffective against commensal probiotic bacteria, such as *L. plantarum*, *L. monocytogenes*, *E.*

**Fig. 8** Biofilm removal capacity of LysSA52 endolysins at different concentrations as determined by 96 well microtiter plate assay. Error bars represent the SD of three independent experiments



*coli*, and *S. Typhimurium*. The LysSA52 endolysin proved to be quite stable; retaining activity for at least 9 weeks of storage at 4°C. Stability was also found to be a property of several other characterized bacteriophage endolysins. The lysK endolysin derived from *S. aureus* phage K remained active for at least 60 days of storage at 4 °C [37]. The Cpl-1 lysin of *S. pneumoniae* phage Cp -1 was stable for more than 6 months at 4 °C [38].

The potential of LysSA52 as a therapeutic agent for biofilm-associated *S. aureus* and *S. epidermidis* was evaluated in vitro setup and showed about a 60% reduction of the already established biofilms. We considered this; it is one of the valuable characteristics of LysSA52 because those bacteria involved in biofilms are far more difficult to eradicate than their free-living counterparts [39].

In conclusion, the LysSA52 endolysin is a promising candidate for application, either individually or in combination with other antibacterial agents or each other with different cleavage specificities, as a disinfectant/therapeutic enzybiotic to control nosocomial infections caused by multiple drug-resistant bacteria. Further investigation is required to increase the lytic activity of this endolysin by genetic modifications of its relevant domains.

**Acknowledgements** This study was supported by the Scientific Research Project Unit at Karadeniz Technical University with Grant No.TDK-2015-5340, Türkiye.

**Author Contributions** MAA performed the cloning experiments, ID performed the phage experiments, TD made bioinformatic analysis, SP performed the expression experiments, EK wrote the manuscript and performed expression and purification of the protein, AOK wrote the manuscript. All authors reviewed the manuscript.

## Declarations

**Competing Interests** The authors declare no competing interests.

**Conflict of Interest** The authors declare that there are no conflicts of interest.

## References

- Kloos WE, Bannerman TL (1994) Update on clinical significance of coagulase-negative staphylococci. *Clin Microbiol Rev* 7:117–140. <https://doi.org/10.1128/CMR.7.1.117>
- Song X, Perencevich E, Campos J, Short BL, Singh N (2010) Clinical and economic impact of methicillin-resistant *Staphylococcus aureus* colonization or infection on neonates in intensive care units. *Infect Control Hosp Epidemiol* 31:177–182. <https://doi.org/10.1086/649797>
- Sulakvelidze A, Alavidze Z, Morris JG Jr (2001) Bacteriophage therapy. *Antimicrob Agents Chemother* 45:649–659. <https://doi.org/10.1128/AAC.45.3.649-659.2001>
- Drulis-Kawa Z, Majkowska-Skrobek G, Maciejewska B, Delattre AS, Lavigne R (2012) Learning from bacteriophages - advantages and limitations of phage and phage-encoded protein applications. *Curr Protein Pept Sci* 13:699–722. <https://doi.org/10.2174/138920312804871193>
- Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, Abedon ST (2010) Phage therapy in clinical practice: treatment of human infections. *Curr Pharm Biotechnol* 11:69–86. <https://doi.org/10.2174/138920110790725401>
- Skurnik M, Pajunen M, Kiljunen S (2007) Biotechnological challenges of phage therapy. *Biotechnol Lett* 29:995–1003. <https://doi.org/10.1007/s10529-007-9346-1>
- Abedon ST, Thomas-Abedon C (2010) Phage therapy pharmacology. *Curr Pharm Biotechnol* 11:28–47. <https://doi.org/10.2174/138920110790725410>
- Skurnik M, Strauch E (2006) Phage therapy: facts and fiction. *Int J Med Microbiol* 296:5–14. <https://doi.org/10.1016/j.ijmm.2005.09.002>
- Nelson DC, Schmelcher M, Rodriguez-Rubio L, Klumpp J, Pritchard DG, Dong S, Donovan DM (2012) Endolysins as antimicrobials. *Adv Virus Res* 83:299–365. <https://doi.org/10.1016/B978-0-12-394438-2.00007-4>
- O’Flaherty S, Coffey A, Meaney W, Fitzgerald GF, Ross RP (2005) The recombinant phage lysin LysK has a broad spectrum of lytic activity against clinically relevant staphylococci, including methicillin-resistant *Staphylococcus aureus*. *J Bacteriol* 187:7161–7164. <https://doi.org/10.1128/JB.187.20.7161-7164.2005>
- Schmelcher M, Donovan DM, Loessner MJ (2012) Bacteriophage endolysins as novel antimicrobials. *Future Microbiol* 7:1147–1171. <https://doi.org/10.2217/fmb.12.97>
- Loessner MJ (2005) Bacteriophage endolysins—current state of research and applications. *Curr Opin Microbiol* 8:480–487. <https://doi.org/10.1016/j.mib.2005.06.002>
- Fenton M, Ross P, McAuliffe O, O’Mahony J, Coffey A (2010) Recombinant bacteriophage lysins as antibacterials. *Bioeng Bugs* 1:9–16. <https://doi.org/10.4161/bbug.1.1.9818>
- Rashel M, Uchiyama J, Ujihara T, Uehara Y, Kuramoto S, Sugihara S, Yagyu K, Muraoka A, Sugai M, Hiramatsu K, Honke K, Matsuzaki S (2007) Efficient elimination of multidrug-resistant *Staphylococcus aureus* by cloned lysin derived from bacteriophage phi MR11. *J Infect Dis* 196:1237–1247. <https://doi.org/10.1086/521305>
- Djurkovic S, Loeffler JM, Fischetti VA (2005) Synergistic killing of *Streptococcus pneumoniae* with the bacteriophage lytic enzyme Cpl-1 and penicillin or gentamicin depends on the level of penicillin resistance. *Antimicrob Agents Chemother* 49:1225–1228. <https://doi.org/10.1128/AAC.49.3.1225-1228.2005>
- Schuch R, Lee HM, Schneider BC, Sauve KL, Law C, Khan BK, Rotolo JA, Horiuchi Y, Couto DE, Raz A, Fischetti VA, Huang DB, Nowinski RC, Wittekind M (2014) Combination therapy with lysin CF-301 and antibiotic is superior to antibiotic alone for treating methicillin-resistant *Staphylococcus aureus*-induced murine bacteremia. *J Infect Dis* 209:1469–1478. <https://doi.org/10.1093/infdis/jit637>
- Schmelcher M, Powell AM, Camp MJ, Pohl CS, Donovan DM (2015) Synergistic streptococcal phage lambdaSA2 and B30 endolysins kill streptococci in cow milk and in a mouse model of mastitis. *Appl Microbiol Biotechnol* 99:8475–8486. <https://doi.org/10.1007/s00253-015-6579-0>
- Jun SY, Jang IJ, Yoon S, Jang K, Yu K-S, Cho JY, Seong M-W, Jung GM, Yoon SJ, Kang SH (2017) Pharmacokinetics and tolerance of the phage endolysin-based candidate drug SAL200 after a single intravenous administration among healthy volunteers. *Antimicrob Agents Chemother* 61:02629–02616. <https://doi.org/10.1128/AAC.02629-16>
- Totte JEE, van Doorn MB, Pasmans S (2017) Successful treatment of chronic *Staphylococcus aureus*-related dermatoses with

- the Topical Endolysin Staphfect SA.100: a report of 3 cases. *Case Rep Dermatol* 9:19–25. <https://doi.org/10.1159/000473872>
20. Pastagia M, Euler C, Chahales P, Fuentes-Duculan J, Krueger JG, Fischetti VA (2011) A novel chimeric lysin shows superiority to mupirocin for skin decolonization of methicillin-resistant and -sensitive *Staphylococcus aureus* strains. *Antimicrob Agents Chemother* 55:738–744. <https://doi.org/10.1128/AAC.00890-10>
  21. Lu Y, Wang Y, Wang J, Zhao Y, Zhong Q, Li G, Fu Z, Lu S (2021) Phage endolysin LysP108 showed Promising Antibacterial potential against Methicillin-resistant *Staphylococcus aureus*. *Front Cell Infect Microbiol* 11:668430. <https://doi.org/10.3389/fcimb.2021.668430>
  22. Kuiper JWP, Hogervorst JMA, Herpers BL, Bakker AD, Klein-Nulend J, Nolte PA, Krom BP (2021) The novel endolysin XZ.700 effectively treats MRSA biofilms in two biofilm models without showing toxicity on human bone cells in vitro. *Biofouling* 37:184–193. <https://doi.org/10.1080/08927014.2021.1887151>
  23. Rahman MU, Wang W, Sun Q, Shah JA, Li C, Sun Y, Li Y, Zhang B, Chen W, Wang S (2021) Endolysin, a Promising solution against Antimicrobial Resistance. *Antibiot (Basel)* 10:1277. <https://doi.org/10.3390/antibiotics10111277>
  24. Carroll-Portillo A, Coffman CN, Varga MG, Alcock J, Singh SB, Lin HC (2021) Standard bacteriophage purification procedures cause loss in numbers and activity. *Viruses* 13:328. <https://doi.org/10.3390/v13020328>
  25. Gondil VS, Harjai K, Chhibber S (2020) Endolysins as emerging alternative therapeutic agents to counter drug-resistant infections. *Int J Antimicrob Agents* 55:105844. <https://doi.org/10.1016/j.ijantimicag.2019.11.001>
  26. Gutierrez D, Ruas-Madiedo P, Martinez B, Rodriguez A, Garcia P (2014) Effective removal of staphylococcal biofilms by the endolysin LysH5. *PLoS ONE* 9:e107307. <https://doi.org/10.1371/journal.pone.0107307>
  27. Gutierrez D, Fernandez L, Rodriguez A, Garcia P (2018) Kill *Staphylococcus aureus*? mBio 9. <https://doi.org/10.1128/mBio.01923-17>. Are Phage Lytic Proteins the Secret Weapon To10.1128/mbio. 01923 – 01917
  28. Denis O, Nonhoff C, Dowzicky MJ (2014) Antimicrobial susceptibility among Gram-positive and Gram-negative isolates collected in Europe between 2004 and 2010. *J Glob Antimicrob Resist* 2:155–161. <https://doi.org/10.1016/j.jgar.2014.05.001>
  29. Polaska M, Sokolowska B (2019) Bacteriophages—a new hope or a huge problem in the food industry. *AIMS Microbiol* 5:324–346. <https://doi.org/10.3934/microbiol.2019.4.324>
  30. Dams D, Briers Y (2019) Enzybiotics: enzyme-based antibacterials as therapeutics. *Adv Exp Med Biol* 1148:233–253. [https://doi.org/10.1007/978-981-13-7709-9\\_11](https://doi.org/10.1007/978-981-13-7709-9_11)
  31. Mirski T, Lidia M, Nakonieczna A, Gryko R (2019) Bacteriophages, phage endolysins and antimicrobial peptides - the possibilities for their common use to combat infections and in the design of new drugs. *Ann Agric Environ Med* 26:203–209. <https://doi.org/10.26444/aaem/105390>
  32. Lai MJ, Lin NT, Hu A, Soo PC, Chen LK, Chen LH, Chang KC (2011) Antibacterial activity of *Acinetobacter baumannii* phage varphiAB2 endolysin (LysAB2) against both gram-positive and gram-negative bacteria. *Appl Microbiol Biotechnol* 90:529–539. <https://doi.org/10.1007/s00253-011-3104-y>
  33. Park J, Yun J, Lim JA, Kang DH, Ryu S (2012) Characterization of an endolysin, LysBPS13, from a *Bacillus cereus* bacteriophage. *FEMS Microbiol Lett* 332:76–83. <https://doi.org/10.1111/j.1574-6968.2012.02578.x>
  34. Gilmer DB, Schmitz JE, Euler CW, Fischetti VA (2013) Novel bacteriophage lysin with broad lytic activity protects against mixed infection by *Streptococcus pyogenes* and methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 57:2743–2750. <https://doi.org/10.1128/AAC.02526-12>
  35. Dong H, Zhu C, Chen J, Ye X, Huang YP (2015) Antibacterial activity of *Stenotrophomonas maltophilia* Endolysin P28 against both Gram-positive and Gram-negative Bacteria. *Front Microbiol* 6:1299. <https://doi.org/10.3389/fmicb.2015.01299>
  36. Wang F, Ji X, Li Q, Zhang G, Peng J, Hai J, Zhang Y, Ci B, Li H, Xiong Y, Deng X, Lin L (2020) TSPphg Lysin from the Extremophilic *Thermus* Bacteriophage TSP4 as a potential Antimicrobial Agent against both gram-negative and gram-positive pathogenic Bacteria. *Viruses* 12:192. <https://doi.org/10.3390/v12020192>
  37. Becker SC, Foster-Frey J, Donovan DM (2008) The phage K lytic enzyme LysK and lysostaphin act synergistically to kill MRSA. *FEMS Microbiol Lett* 287:185–191. <https://doi.org/10.1111/j.1574-6968.2008.01308.x>
  38. Loeffler JM, Djurkovic S, Fischetti VA (2003) Phage lytic enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia. *Infect Immun* 71:6199–6204. <https://doi.org/10.1128/IAI.71.11.6199-6204.2003>
  39. Chen M, Yu Q, Sun H (2013) Novel strategies for the prevention and treatment of biofilm related infections. *Int J Mol Sci* 14:18488–18501. <https://doi.org/10.3390/ijms140918488>

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.