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RESEARCH ARTICLE

LysJEP8: A promising novel endolysin for combating multidrug-resistant Gram-negative bacteria

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Abstract

Antimicrobial resistance (AMR) is an escalating global health crisis, driven by the overuse and misuse of antibiotics. Multidrug-resistant Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, are particularly concerning due to their high morbidity and mortality rates. In this context, endolysins, derived from bacteriophages, offer a promising alternative to traditional antibiotics. This study introduces LysJEP8, a novel endolysin derived from *Escherichia* phage JEP8, which exhibits remarkable antimicrobial activity against key Gram-negative members of the ESKAPE group. Comparative assessments highlight LysJEP8's superior performance in reducing bacterial survival rates compared to previously described endolysins, with the most significant impact observed against *P. aeruginosa*, and notable effects on *A. baumannii* and *K. pneumoniae*. The study found that LysJEP8, as predicted by in silico analysis, worked best at lower pH values but lost its effectiveness at salt concentrations close to physiological levels. Importantly, LysJEP8 exhibited remarkable efficacy in the disruption of *P. aeruginosa* biofilms. This research underscores the potential of LysJEP8 as a valuable candidate for the development of innovative antibacterial agents, particularly against Gram-negative pathogens, and highlights opportunities for further engineering and optimization to address AMR effectively.

INTRODUCTION

Antimicrobial resistance (AMR) has become a serious concern due to the excessive and inappropriate utilization of antibiotics in recent decades (Tang et al., 2023). Pathogens of the ESKAPE group such as *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and various species within the Enterobacteriaceae family, pose the greatest threat among the so-called superbugs (Rice, 2008; Shrivastava et al., 2018). Multidrug-resistant (MDR) Gram-negative bacteria, such as *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*, are of particular concern, often leading to significantly increased rates of morbidity and mortality (Troeger et al., 2018). This

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escalating problem threatens to push us closer to a post-antibiotic era, where common infections could turn lethal (Şen Karaman et al., 2020). Consequently, there is an urgent need to develop innovative antimicrobial strategies to address this pressing global health crisis.

In this context, endolysins emerge as one of the most promising protein-based alternatives to conventional antibiotics. Endolysins, derived from viruses infecting bacteria, known as bacteriophages, assume a pivotal role in the ultimate stage of the bacteriophage infection cycle. Their primary function is to facilitate the lysis of bacterial cells, resulting in the release of newly assembled viral progeny. This intricate process involves the disruption of the bacterial cell wall through the degradation of the peptidoglycan barrier. The disruptive impact that leads to bacterial lysis has captured the attention of researchers who are actively investigating the external application of recombinantly expressed endolysins, a concept known as "lysis from without" (Schmelcher et al., 2012).

Although certain endolysins can be remarkably specific (Dams & Briers, 2019), others may possess a lower degree of selectivity, resembling the broad-spectrum trait of antibiotics (Jiang et al., 2021; Kim et al., 2020). This variability in specificity comes from their capacity to recognize and cleave either common or less common linkages found in peptidoglycan, which varies depending on the bacterial species (Vollmer et al., 2008). Furthermore, their capacity to identify and bind to specific patterns in the cell wall structure is an additional factor contributing to specificity, particularly notable in the case of modular endolysins featuring a cell wall binding domain (CBD) (Loessner, 2005). On the other hand, the processes involved in developing resistance against endolysins occur at much lower rates compared to those observed with antibiotics (São-José, 2018), making it less likely to emerge de novo. Indeed, mechanisms conferring resistance to endolysins have been demonstrated to pre-exist. Consequently, the true peril arises from the horizontal transfer of specific determinants from intrinsically resistant species (Grishin et al., 2020). However, there seems to be evidence that modifications enabling bacteria to acquire resistance to endolysins may have a detrimental impact on bacterial fitness, potentially compromising their overall virulence (Kusuma et al., 2007).

The accessibility of peptidoglycan in Gram-positive bacteria has played a pivotal role in highlighting the importance of Gram-positive endolysins. This has led to a multitude of innovative discoveries and advancements in protein engineering, all with a primary focus on combating Gram-positive bacterial infections, with a special emphasis on *S. aureus* (Becker et al., 2016; Kaur et al., 2020; Lee et al., 2021; Li et al., 2021; Manoharadas et al., 2021; Son et al., 2021). Indeed, most endolysin-based formulations that progress to advanced preclinical and clinical development stages are those featuring recombinant endolysins designed against *S. aureus* (Fowler et al., 2020; Jun et al., 2017; Totté et al., 2017). In contrast, the presence of an outer membrane in Gram-negative bacteria presents a significant barrier to the effective action of Gram-negative endolysins, frequently leading to constraints or complete inhibition of their function. However, recent studies have demonstrated the existence of naturally occurring endolysins capable of overcoming the limitations imposed by the outer membrane and exhibiting notable activity against relevant Gram-negative species (Chu et al., 2022; Larpin et al., 2018; Plotka, Kapusta, et al., 2019). Furthermore, innovative engineering techniques, such as "Artilysation," empower specific endolysins with the ability to penetrate the outer membrane by designing fusion chimeras with outer membrane permeabilizing peptides (Carratalá et al., 2023). All these discoveries have been moving forward research into the identification of novel Gram-negative endolysins with an inherent capacity to exhibit antibacterial activity even in the presence of the outer membrane barrier, which simultaneously represents potential candidates for further modification.

In the present work, the authors characterize a new endolysin termed LysJEP8, which demonstrates significant activity against key Gram-negative members of the ESKAPE group. This investigation explores various aspects, including optimal dose concentrations, pH, and salt tolerance, along with its ability to promote biofilm disruption and eliminate bacteria residing within them. The results highlight the potential of endolysin LysJEP8 as an excellent candidate for further investigation in the development of innovative antibacterial agents against both Gram-negative and Gram-positive bacteria.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

The bacterial strains used in our antimicrobial assays included carbapenem-resistant *K. pneumoniae* (KPC) (kindly provided by Dr. Lourdes Migura-Garcia, IRTA), *A. baumannii* (ATCC-15308), methicillin-resistant *S. aureus* (MRSA, ATCC-33592), and *P. aeruginosa* (ATCC-10145). Two strains of *Escherichia coli* were employed in this study: *E. coli* DH5α for cloning purposes and *E. coli* BL21(DE3) for the production of recombinant proteins. These strains were cultivated in Luria–Bertani (LB) medium at 37°C. Liquid cultures were agitated at 250 rpm using an orbital shaker, while solid agar plates were incubated under the same temperature conditions without agitation. Ampicillin was added to the media at 100 μg/mL when necessary. All strains were stored at −80°C for preservation.

Production and purification of recombinant proteins

The LysJEP8 (QOC55652.1), LysSi3 (YP_009150069.1), and Ts2631 (AIM47292.1) genes were synthesized and incorporated into the pET22b (ampR) expression vector by GeneArt (GeneArt®, Life Technologies, Regensburg, Germany). The nucleotide sequences were codon-optimized specifically for the *E. coli* expression system to enhance soluble expression efficiency. *E. coli* BL21(DE3) cells carrying the pET22b vector with the respective C-terminal 6xHis-Tag endolysin genes were used as the host for recombinant protein expression. Transformed cells were cultivated in LB medium at 37°C, supplemented with 100 μg/mL of ampicillin, until they reached an OD_{600} of 0.6–0.7. Subsequently, protein production was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and carried out for 3 h at 37°C. Cell pellets were collected through centrifugation at 6000 *g* for 20 min at 4°C and stored at −80°C for subsequent purification procedures.

Bacterial cell lysis was achieved via high-pressure homogenization using the continuous flow CF1 cell disruptor (Constant Systems). Subsequently, the soluble and insoluble fractions were separated by centrifugation (45 min, 15,000 *g* at 4°C). The soluble fractions were then filtered through a 0.2 μm pore diameter filter and subjected to purification using Immobilized Metal Affinity Chromatography (IMAC) on an ÄKTA Start system (GE Healthcare) equipped with 1 mL HiTrap chelating HP column (GE Healthcare). Elution of proteins was carried out using a linear gradient of elution buffer (20 mM Tris–HCl, pH 8.00, 500 mM NaCl, 500 mM Imidazole). The eluted fractions were subsequently analysed using SDS electrophoresis (TGX™ FastCast™, Bio-Rad) and Western Blot. The selected protein fractions were dialyzed against 20 mM Tris–HCl (pH 7.4). The yield of purified soluble protein was determined using the NanoDrop™ One Microvolume UV–Vis Spectrophotometer (Thermo Scientific) and the Qubit™ Protein Assay Kit (Thermo Scientific). Additionally, the integrity and purity of the protein were assessed through SDS electrophoresis (TGX™ FastCast™, Bio-Rad). The purified proteins were aliquoted and stored at −80°C for future use.

In vitro bactericidal activity assays

The effects of different endolysins (LysJEP8, LysSI3, and Ts2631) were assessed against *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, and *S. aureus*. Bacterial cultures were prepared by inoculating 15 mL of LB broth with previously inoculated LB agar solid medium. These cultures were then incubated at 37°C with agitation at 250 rpm for 1 h. Subsequently, a fresh subculture was initiated with an initial OD_{600} of 0.05 and allowed to grow until it reached the early logarithmic phase (OD_{600} 0.3–0.4). Following this, cultures were centrifuged at 2500 *g* for 10 min at 16°C, subjected to two washes with 20 mM Tris–HCl (pH 7.4), and adjusted to a final concentration of 10^6 CFU/mL. Next, 250 μL of the bacterial suspension was mixed with an equal volume of the corresponding endolysin at the appropriate concentration in 1.5 mL Eppendorf tubes. A negative control, containing only the buffer (20 mM Tris–HCl, pH 7.4) without endolysins, was prepared. The mixtures were incubated at 37°C for 2 h with gentle agitation. Bacterial colony-forming units (CFUs) in the samples were determined by plating 10 μL of serial 10-fold dilutions in normal saline (0.9% w/v of NaCl) onto LB agar plates. Raw CFU counts were then converted into base 10 logarithm values. Each concentration sample was evaluated in quintuplicate.

The influence of pH and salt concentrations on the specific activity of LysJEP8 was assessed against *P. aeruginosa* (ATCC-10145). Employing the same protocol described earlier, bacteria were incubated with LysJEP8 in a 20 mM Tris–HCl buffer with pH values ranging from 4.0 to 10.0 or supplemented with various NaCl concentrations ranging from 0 to 500 mM.

Determination of the minimum inhibitory concentration of LysJEP8

The assay utilized a broth microdilution method. In 96-well plates, after a two-fold dilution process, each well contained a specific amount of LysJEP8, with concentrations ranging from 7.8 to 250 μg/mL. These protein samples were dissolved in Mueller Hinton Broth Cation-adjusted medium (MHB-2, Sigma-Aldrich, Saint Louis, MO, USA). Subsequently, 50 μL of MHB-2 containing 10^6 colony forming units per mL (CFU/mL) was inoculated into each well. Following inoculation, the plates were incubated at 37°C for 18 h without agitation. Bacterial growth was quantified by measuring OD_{620}. Control wells without any protein exhibited the highest growth, and each concentration was evaluated in triplicates. To determine the minimum inhibitory concentration (MIC), we identified the lowest concentration that resulted in no bacterial growth through visual inspection, supported by OD_{620} measurement.

Biofilm assay

A biofilm assay was conducted in a 96-well plate to assess the lytic activity of LysJEP8. Overnight cultures of *P. aeruginosa* (ATCC-10145) were adjusted to a concentration of 10^6 CFU/mL in LB medium supplemented with 0.5% w/v glucose. Subsequently, 100 μL of this suspension was added to each well, and the

plate was incubated at 37°C. After 24 h incubation, the supernatant was removed with a pipette and the wells were washed three times with sterile distilled water to eliminate non-adhered cells. Formed biofilms were subsequently exposed to 150 μL containing 7.8–250 μg/mL of LysJEP8 in 20 mM Tris–HCl (pH 7.4). For control wells, 20 mM Tris–HCl (pH 7.4) was added instead of LysJEP8. After a 24-h incubation, the wells were washed twice with distilled water, fixed at 60°C for 1 h, and subsequently stained for 15 min with 200 μL of a 0.1% CV (Crystal Violet) solution. The stained biofilms were rinsed with distilled water, allowed to air dry at 37°C for 30 min, and then extracted with 200 mL of 30% acetic acid. The amount of biofilm was quantified by measuring the OD_{550} of CV using a LUMIstar Omega Microplate Reader (BMG LABTECH). Each concentration was evaluated in triplicate.

Scanning electron microscope (SEM) analysis

The ultrastructural effects of LysJEP8 were assessed in *P. aeruginosa* (ATCC-10145). For this evaluation, 500 μL of the bacterial suspension (10^6 CFU/mL) was mixed with an equal volume of LysJEP8 at a concentration of 62.5 μg/mL and incubated (37°C) for various durations: 15 min, 30 min, and 1 h. After the specified incubation times, the samples were filtered through 0.22 μm filters and these filters were fixed in 2.5% (v/v) glutaraldehyde in 100 mM phosphate buffer (PB) for 2 h at 4°C. Subsequently, the filters were postfixed in a 1% (w/v) osmium tetroxide solution (TAAB) in PB for 2 h at 4°C, followed by dehydration using a graded series of ethanol (50, 70, 90, 96 and 100% v/v) at RT and finally dried with CO_2 in a Bal-Tec CPD030 critical-point dryer. The samples were mounted on metal stubs and observed without coating using a FESEM Merlin (Zeiss) operating at 0.8 kV.

RESULTS AND DISCUSSION

Comparing antimicrobial activities: LysJEP8, LysSi3, and Ts2631 endolysins

From the complete genome sequence of *Escherichia* phage JEP8 (KY379853.1), the possible DNA sequence encoding endolysin LysJEP8 was identified (Kim et al., 2021). Since this endolysin has never been characterized, we explored its functional properties by comparing LysJEP8 with two other endolysins of similar size and architectural structure, named LysSi3 and Ts2631, which have previously demonstrated efficacy against Gram-negative bacteria (Plotka, Kapusta, et al., 2019; Vasina et al., 2021). All of them were composed of an enzymatically active domain (EAD) but differed in their catalytic activities due to the endolysins functional diversity. More specifically, these candidates are predicted to belong to specific domain families, typically associated with endopeptidase (LysJEP8, Peptidase M15C—IPR039561 InterPro), muramidase (LysSi3, Glycoside hydrolase, family 24—IPR002196 InterPro), and amidase (Ts2631, Peptidoglycan recognition protein—IPR015510 InterPro) functionalities (Figure 1A). All these proteins were successfully produced in high yields in their soluble forms (LysJEP8: 47.2 mg/L, LysSi3: 68.6 mg/L, and Ts2631: 25.5 mg/L) and were effectively purified, demonstrating a remarkable level of purity (Figure 1B).

Compared to LysSi3 and Ts2631, LysJEP8 outperforms them by exhibiting a significantly increased

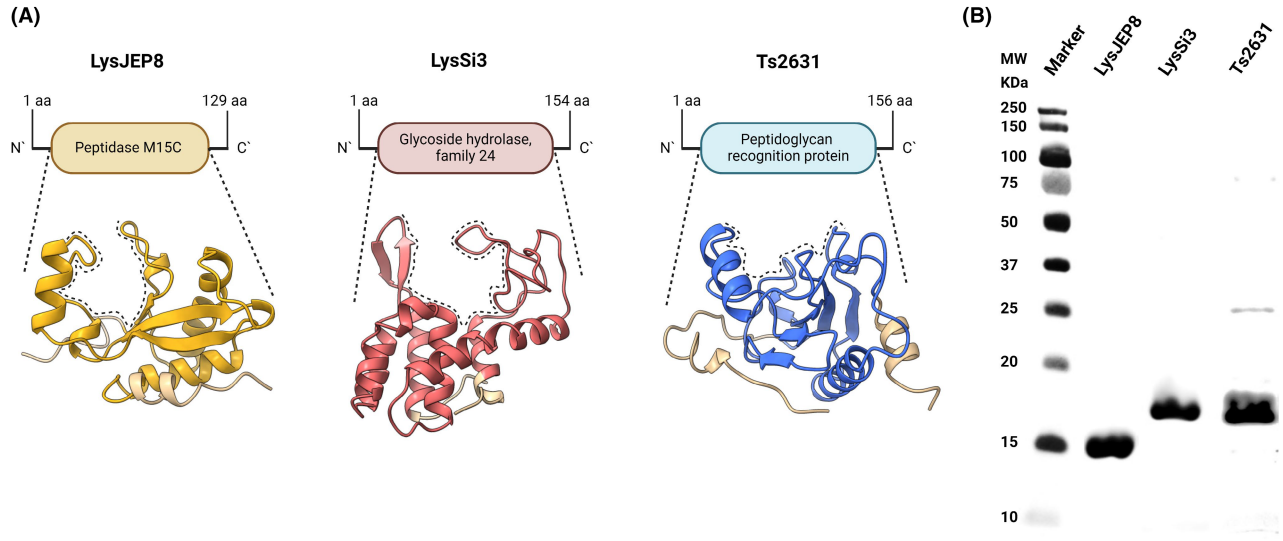


FIGURE 1 Characterization and purification of endolysins LysJEP8, LysSi3, and Ts2631. (A) Schematic illustration of the 3D structure of phage endolysins. (B) SDS-PAGE gel image displaying the purified proteins.

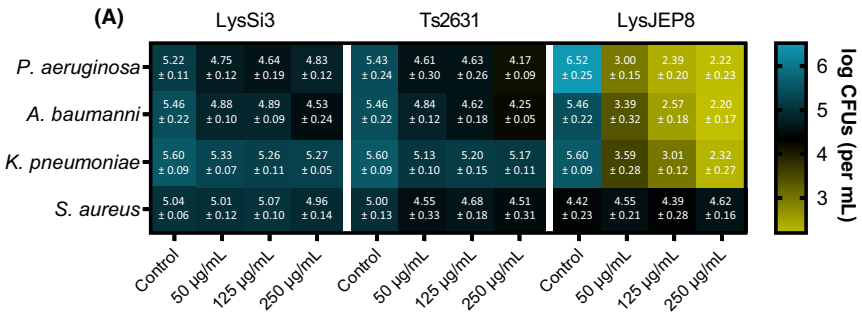
bactericidal effect, as reflected in a lower survival rate among bacterial cells (Figure 2A). LysJEP8 showed superior antimicrobial activity against three Gram-negative members of the ESKAPE group, with the highest activity observed against *P. aeruginosa*, followed by *A. baumannii*, and finally *K. pneumoniae* (Figure 2). This pattern is also evident when considering the bacteriostatic effect, as represented by the minimum inhibitory concentration (MIC), where LysJEP8 demonstrates the lowest value against *P. aeruginosa* (Figure 2B).

In relation to *S. aureus*, the tested endolysins demonstrate comparable levels of efficacy. Although Ts2631 appears to be the most effective, none of them seems to have a noteworthy impact on this bacterium (Figure 2A). In the cases of LysSi3 and Ts2631, their reduced efficacy may be attributed to various factors resulting from the differences in peptidoglycan in Gram-positive and Gram-negative bacteria. Among these factors, we can include variations in peptidoglycan modifications, unique three-dimensional arrangements, or thickness. Specifically, the peptidoglycan barrier in Gram-positive bacteria is thicker than that in Gram-negative bacteria, consequently, higher concentrations of endolysin might be required to achieve the same effect. On the other hand, endolysins that target Gram-positive bacteria are predominantly modular endolysins consisting of an EAD and a CBD (Kashani et al., 2017; Rahman et al., 2021). This modular domain can cooperate in the lysis process by recognizing specific patterns or components in the peptidoglycan barrier, increasing the local concentration of endolysins at the bacterial cell wall, and aid in the catalytic process as a supporting module to the EAD. Given this context, globular endolysins, such as the ones under study, may not be suitable for effectively targeting *S. aureus*.

In the case of LysJEP8, along with the factors mentioned earlier, analysis of the protein sequence using the NCBI Conserved Domain Database (CDD) indicates that it belongs to the peptidase M15 superfamily, specifically the L-Ala-D-Glu peptidase family. This suggests that LysJEP8 functions as an L-alanyl-D-glutamate endopeptidase, cleaving between these two amino acids in the peptidoglycan stem peptide (Figure 3). It is important to highlight that Gram-positive bacteria exhibit a variation in their peptidoglycan structure, wherein the D-isoglutamate (D-iGlu) present in the stem peptides is replaced by D-isoglutamine (D-iGln) (Figure 3) (Vollmer et al., 2008). This modification is likely a critical factor contributing to the activity of LysJEP8, thereby influencing its effectiveness not only against *S. aureus* but also against all Gram-positive bacteria.

Differential effects of LysJEP8 on various Gram-negative bacteria

LysJEP8 demonstrates varying degrees of activity against the different Gram-negative bacteria tested, with *P. aeruginosa* being the most significantly affected by its action. At its highest concentration (50 μg/mL), LysJEP8 exhibited notable efficacy by reducing *P. aeruginosa* bacterial loads from 5.00×10^5 to 2.14×10^2 CFU/mL, achieving a remarkable over 1000-fold reduction (Figure 4A). Similarly, in the case of *A. baumannii*, bacterial loads decreased from 5.20×10^5



(B)

Bacteria	MIC (μg/mL)	MIC (μM)
P. aeruginosa	62.5	4
A. baumanni	125	8
K. pneumoniae	250	16
S. aureus	ND	ND

FIGURE 2 Antimicrobial activity of endolysins on various Gram-negative or Gram-positive bacteria. (A) Identification of the lytic activity of endolysins LysSi3, Ts2631, and LysJEP8 against *Pseudomonas aeruginosa*, *Acinetobacter baumanni*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* by CFU reduction assay. Exponentially grown bacterial cells were adjusted as 1×10^6 CFU in 20 mM Tris–HCl pH 7.5 and treated with 50, 125, and 250 μg/mL of purified endolysin at 37°C for 2 h. The surviving bacterial cells were counted by plating on an LB plate. (B) Minimum inhibitory concentrations (μg/mL) and (μM) of LysJEP8 against *P. aeruginosa*, *A. baumanni*, *K. pneumoniae* and *S. aureus*.

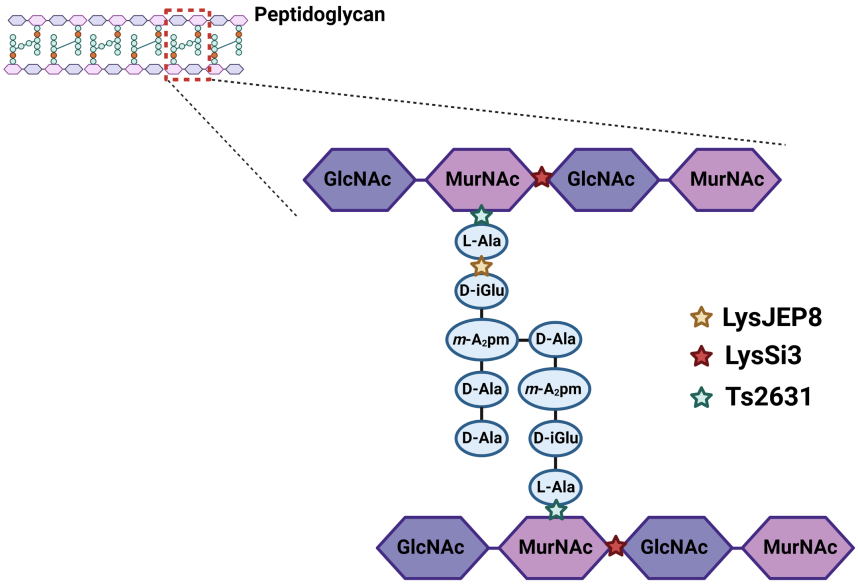


FIGURE 3 Schematic representation of the peptidoglycan structure in *Escherichia coli*, including the different enzymatic activities of endolysins. LysJEP8, an L-alanyl-D-glutamate endopeptidase, catalyses the cleavage of the peptide bond between the amino acid residues L-alanine and D-glutamic acid in the stem peptide region. LysSi3, an *N*-acetylmuramidase, catalyses the hydrolysis of the β-1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine. Ts2631, an *N*-acetylmuramoyl-L-alanine amidase, catalyses the cleavage of the amide bond that links the *N*-acetylmuramic acid of the glycan strand to the L-alanine of the peptide chain.

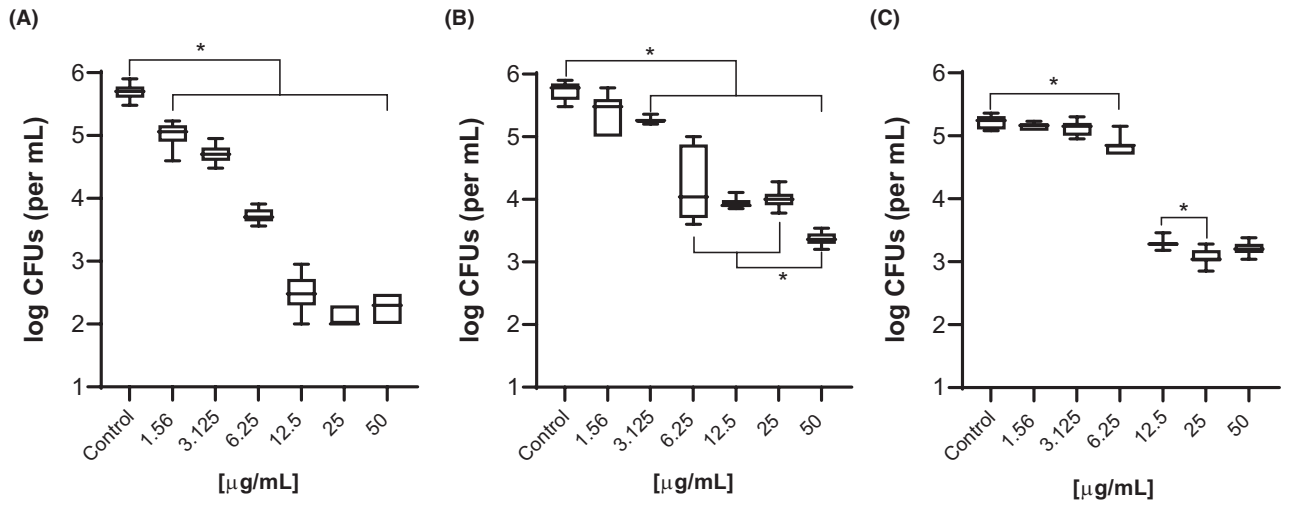


FIGURE 4 Evaluation of the lytic activity of endolysin LysJEP8 against *Pseudomonas aeruginosa* (A, C) and *Acinetobacter baumanni* (B) by CFU reduction assay. Exponentially grown bacterial cells were adjusted as 1×10^6 CFU in 20 mM Tris–HCl pH 7.5 (for A and B) or Sorbitol 10% pH 7.5 (C) and then treated with different concentrations ranging from 50 to 1.56 μg/mL of purified endolysin at 37°C for 2 h. The data ($n = 10$) were summarized in terms of central tendency and dispersion. Asterisks denote significant differences (p-value ≤ 0.01).

to 2.44×10^3 CFU/mL, reflecting a 100-fold reduction at the same concentration (Figure 4B).

When utilizing a different buffer solution in the analysis of *P. aeruginosa*, specifically an isotonic solution containing 10% of the non-ionic solute Sorbitol, the activity was reduced by approximately 24%–31% across the three highest concentrations, yet it still maintained a high degree of effectiveness (Figure 4C). This suggests an influence of the medium on the activity of the endolysin, a factor that could be further optimized in future studies.

Moreover, in the case of *P. aeruginosa*, scanning electron microscopy (SEM) images demonstrate that these effects initiate as early as 15 min following in vitro treatment with LysJEP8 (Figure 5). Regardless of the incubation time (15, 30, and 60 min), the treated bacterial cells exhibit evident indications of cell damage, featuring notable ultrastructural alterations such as bleb-like protrusions and cytoplasmic content leakage (Figure 5). Additionally, as the incubation time prolongs, the presence of cell debris around the bacterial cells becomes

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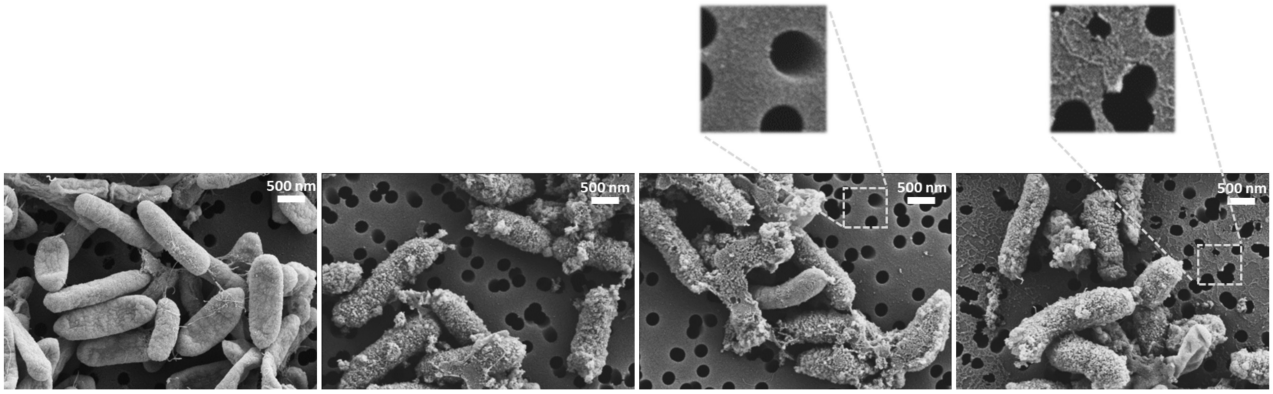

FIGURE 5 Scanning electron microscopy micrographs of *Pseudomonas aeruginosa* after incubation with LysJEP8 for different durations. For all tests, 1×10^6 CFU of bacterial cells was treated with or without 62.5 μg/mL LysJEP8 dissolved in Tris–HCl pH 7.5 at 37°C. From left to right: Control, 15 min, 30 min, and 1 h. Dotted squares serve as visual indicators, highlighting the presence of cellular debris within the sample.

more prominent, serving as a clear indicator of a more extensive degree of bacterial cell disintegration.

Two out of the three Gram-negative bacteria evaluated in this study, *K. pneumoniae* and *A. baumannii*, were both known to produce capsular structures. Previous studies have revealed that these structures can grant resistance to antimicrobial peptides like polymyxin and lactoferrin (Campos et al., 2004). Additionally, other studies have highlighted the essential role of these structures in providing resistance against disinfectants and lysozyme (Tipton et al., 2018). However, the impact of endolysins on bacterial capsules remains uncertain, with conflicting findings in the literature. While some argue that the expression of thick polysaccharide capsules by streptococci or *Bacillus anthracis* species does not hinder endolysin lytic activity (Liu et al., 2023), others speculate on the possibility that thick capsules produced by *K. pneumoniae* may impede the access of endolysins to peptidoglycan (Hong et al., 2022). In fact, a very recent study has demonstrated that the presence of capsular polysaccharides may indeed modulate endolysin activity (Alreja et al., 2024). Due to the insufficient evidence available, it is not possible to exclusively attribute the differential activity observed among the various Gram-negative bacteria solely to the presence or absence of these protective structures. Other contributing factors must also be considered in understanding the variations in bacterial responses. Factors such as the specific composition of the bacterial cell wall, genetic variations, and potential adaptations to environmental pressures could all play a role in influencing the effectiveness of endolysins across different bacterial species.

Impact of pH, ionic strength, and divalent cations on LysJEP8 functionality

LysJEP8's bactericidal activity against *P. aeruginosa* was assessed at different pH levels using a concentration of 6.25 μg/mL, which was chosen based on previous experiments (Figure 4A) to ensure that any observed effects were primarily attributed to pH differences rather than the lysin concentration itself. The results revealed that LysJEP8 displayed significant bactericidal activity at pH 4.0, 5.0, and 6.0, effectively reducing the number of bacterial cells below the limit of detection (Figure 6A). However, beyond pH 7.0, there was a progressive decrease in its bactericidal activity, leading to an almost complete loss of activity at pH 10.0.

To gain further insights into the pH-dependent variations, in silico analysis (using ProtParam from Expasy) predicted an isoelectric point of 9.55 for LysJEP8. This prediction underscores the potential loss of activity at pH levels around 9.0 and 10.0, supporting the observed increase in activity at lower pH values, corresponding to the positively charged state of the protein. These in silico evidence complement and align with the experimentally observed pH-dependent trends.

The protein's positively charged nature likely plays a crucial role in its antimicrobial activity. Recently, the presence of naturally occurring N-terminal cationic peptides on endolysins has been reported (Plotka, Sancho-Vaello, et al., 2019; Wang et al., 2020). These regions would serve two important functions: electrostatic interactions with negatively charged compounds in the bacterial surface and enhancing membrane permeability to facilitate improved penetration. Remarkably, the 25-residue N-terminal tail of LysJEP8 (MFRLSQRSKDRLVGVHPDLVKVVHR) comprises six positively charged residues (underlined), mirroring the observations made in the aforementioned reports.

Next, we examined the halotolerance of LysJEP8 and its antibacterial activity under different NaCl concentrations. For this experiment, *P. aeruginosa* cells were exposed to 6.25 μg/mL of LysJEP8 at varying NaCl concentrations, ranging from 0 to 500 mM. In the absence of NaCl, LysJEP8 displayed its maximum killing potential. However, at a NaCl concentration

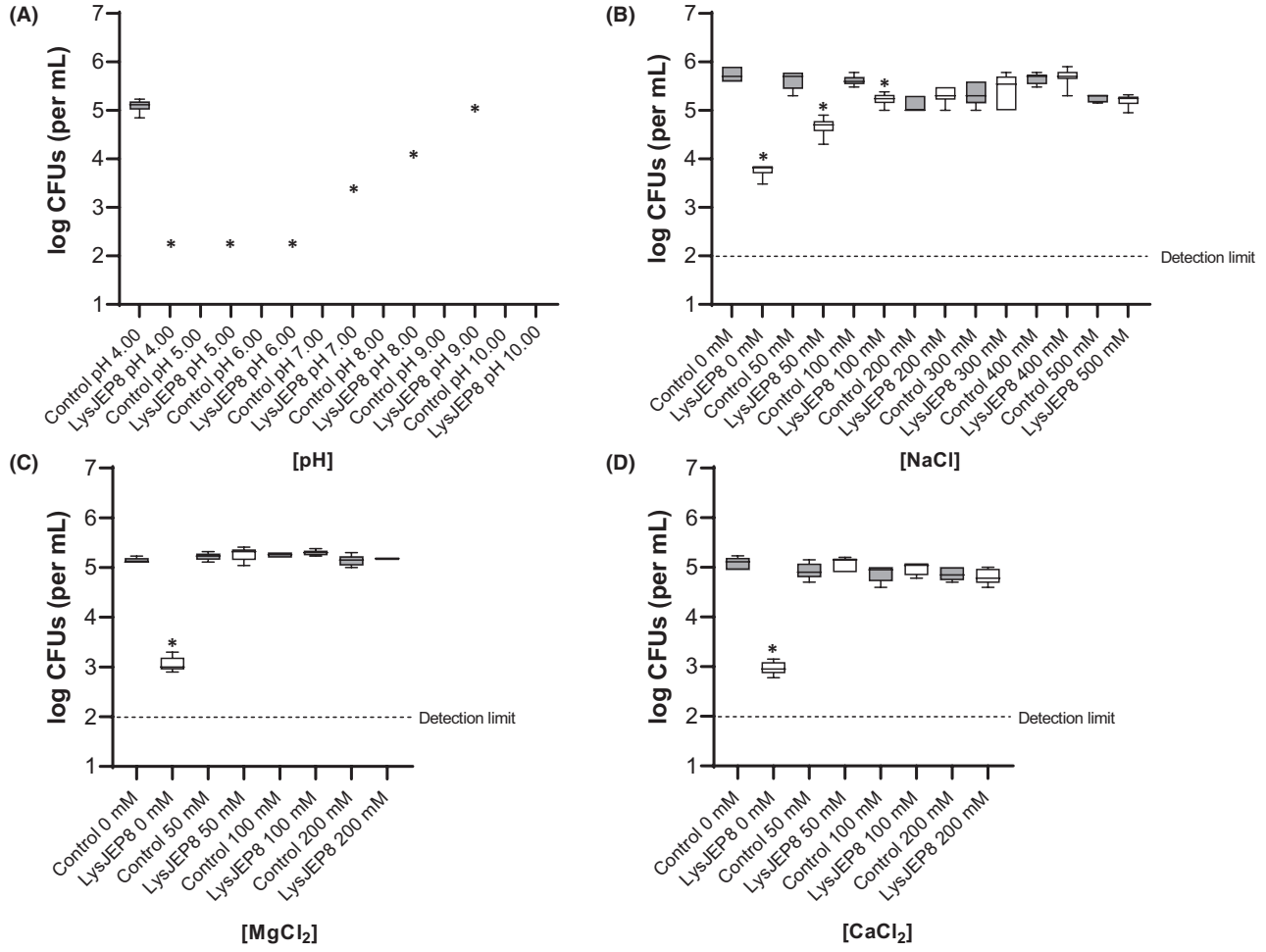

FIGURE 6 Effect of pH, NaCl, $MgCl_2$, and $CaCl_2$ on the ability of LysJEP8 to lyse *Pseudomonas aeruginosa*. A CFU reduction assay was performed by the incubation of LysJEP8 with target cells over a range of pH and NaCl, $MgCl_2$, and $CaCl_2$, values. Exponentially grown bacterial cells were adjusted as 1×10^6 CFU in the corresponding buffer and then treated with 6.25 μg/mL (A) or 12.5 μg/mL (B–D) of purified endolysin at 37°C for 2 h. The surviving bacterial cells were counted by plating on an LB plate. The shaded areas in the figure represent the control conditions. Dotted line denotes the detection limit. The data ($n = 10$) were summarized in terms of central tendency and dispersion. Asterisks denote significant differences compared to the corresponding control (p-value ≤ 0.01). Results without asterisks did not exhibit significant differences.

of 50 mM, the lytic efficacy of LysJEP8 notably decreased. Furthermore, at 100 mM, it became nearly inactive, marking the upper limit where substantial activity remains observable, with concentrations surpassing 100 mM showing no distinguishable variation (Figure 6B).

The limited halotolerance of LysjEP8 may arise from the excessive presence of salt ions, creating an electrostatic screening effect. Normally, the negatively charged bacterial cell wall interacts with positively charged segments of the endolysin. However, surplus ions weaken these interactions and form a protective barrier around the peptidoglycan. As a result, the ability of the endolysin to efficiently bind to the cell wall is reduced, ultimately compromising its potential to induce cell lysis (Shen et al., 2016). Nonetheless, despite this limitation, advanced engineering techniques like artilysation have the potential to enable modified endolysins to function effectively at higher salt concentrations (Lim et al., 2022; Rodríguez-Rubio et al., 2016). For that reason, LysJEP8 stands out as a promising candidate for further investigation and enhancement through these engineering methods.

Finally, we explored the influence of divalent cations on LysJEP8's activity. Unexpectedly, while some endolysins show enhanced activity with divalent cations (Melo-López et al., 2021; Son et al., 2012), the addition of Mg^{2+} and Ca^{2+} adversely affected the bactericidal function of LysJEP8. This resulted in a complete blockade of its functionality, even at the lowest tested concentration (50 mM) (Figure 6C,D) (Ding et al., 2020). This contrasts with our observations with NaCl, where the activity was diminished but not fully inhibited at the same concentration. It is well-known that Mg^{2+} and Ca^{2+} ions, in particular, play a crucial role in maintaining the stability of the outer membrane by binding between

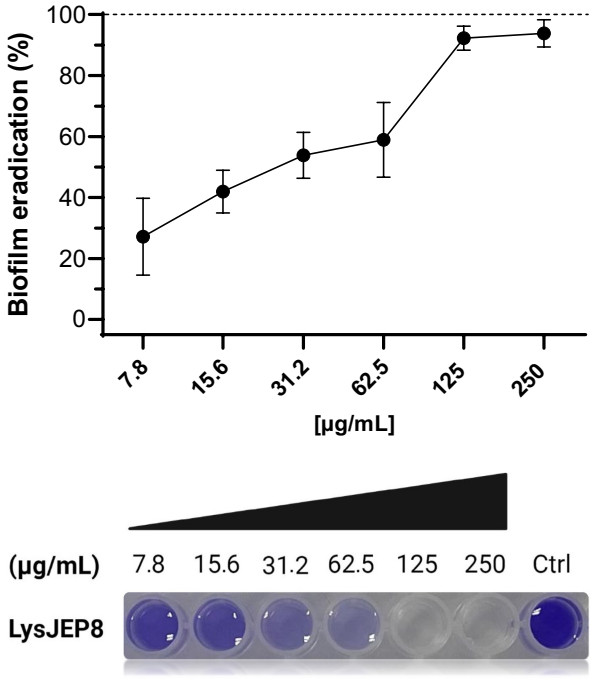

FIGURE 7 Identification of the disruption ability of endolysin LysJEP8 toward bacterial biofilm. Biofilms formed by *Pseudomonas aeruginosa* were treated with various concentrations of LysJEP8 and visualized by staining biofilms with crystal violet. No staining indicates successful removal of the biofilm. Control indicates the sample treated with buffer without endolysins. Data were presented as mean ± standard deviation ($n = 3$).

adjacent LPS molecules (Clifton et al., 2015). An excess of these divalent cations may further enhance this stabilization, consequently reducing the permeability of the outer membrane and diminishing the action of the endolysin.

LysJEP8-mediated disruption of *P. aeruginosa* biofilm

Biofilm formation in *P. aeruginosa* serves as an alternative resistance mechanism, leading to increased tolerance to traditional antibiotics (Pang et al., 2019). Hence, the identification of an antimicrobial solution capable of overcoming this obstacle is of utmost importance.

To explore the efficacy of LysjEP8 in eliminating established biofilm, varying concentrations were incubated with a 24-h mature biofilm of *P. aeruginosa*. After a 2-h incubation period, the results demonstrated that LysjEP8 significantly reduced the developed biofilm across all tested concentrations in a dose-dependent manner (Figure 7). Notably, complete eradication was achieved at the highest concentrations of 125 (92.2% ± 4.0) and 250 µg/mL (93.8% ± 4.5), representing two and four times the MIC observed for *P. aeruginosa*, respectively (Figure 2B).

These results demonstrated the ability of LysjEP8 to infiltrate and lyse bacterial cells of *P. aeruginosa* that are embedded within the biofilm matrix. This process has been hypothesized to contribute to the destabilization of the biofilm's structural integrity (Sharma et al., 2018), ultimately resulting in the elimination of a well-established 24-h biofilm.

CONCLUSIONS

This study provides a comprehensive investigation into the novel endolysin, LysJEP8, and its potential as an innovative antimicrobial agent against Gram-negative bacteria, particularly within the ESKAPE group. The study compares LysJEP8 with other endolysins (Ts2631 and LysSi3) and highlights its bactericidal activity against *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, while also discussing its effectiveness against methicillin-resistant *S. aureus* (MRSA). The research reveals that LysJEP8's activity is influenced by pH, with higher effectiveness observed at lower pH values, and is adversely affected by elevated salt concentrations. Furthermore, the study demonstrates that LysJEP8 possesses significant biofilm-disrupting capabilities, suggesting its potential for combating bacterial infections associated with biofilm formation. Overall, LysJEP8 emerges as a promising candidate for further research and development as an alternative antimicrobial agent, particularly against Gram-negative bacteria that are often resistant to conventional antibiotics. Further studies and engineering approaches may enhance its efficacy and broaden its applicability in the fight against antimicrobial resistance, addressing this critical public health concern.

AUTHOR CONTRIBUTIONS

Jose Vicente Carratalá: Conceptualization; methodology; writing – original draft. **Neus Ferrer-Miralles:** Funding acquisition; supervision; writing – original draft. **Elena Garcia-Fruitós:** Funding acquisition; supervision; writing – original draft. **Anna Arís:** Funding acquisition; supervision; writing – original draft.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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REFERENCES

Alreja, A.B., Appel, A.E., Zhu, J.C., Riley, S.P., Gonzalez-Juarbe, N. & Nelson, D.C. (2024) SP-CHAP, an endolysin with enhanced activity against biofilm pneumococci and nasopharyngeal colonization. *mBio*, 15, e0006924.

Becker, S.C., Roach, D.R., Chauhan, V.S., Shen, Y., Foster-Frey, J., Powell, A.M. et al. (2016) Triple-acting lytic enzyme treatment of drug-resistant and intracellular *Staphylococcus aureus*. *Scientific Reports*, 6, 1–10.

Campos, M.A., Vargas, M.A., Regueiro, V., Llompart, C.M., Albertí, S. & Bengoechea, J.A. (2004) Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infection and Immunity*, 72, 7107–7114.

Carratalá, J.V., Arís, A., Garcia-Fruitós, E. & Ferrer-Miralles, N. (2023) Design strategies for positively charged endolysins: insights into artilysin development. *Biotechnology Advances*, 69, 108250.

Chu, J.J.K., Poh, W.H., Hasnuddin, N.T.B., Hew, E.Y., Dam, L.C., El Sahili, A. et al. (2022) Novel phage lysin Abp013 against *Acinetobacter baumannii*. *Antibiotics*, 11, 169.

Clifton, L.A., Skoda, M.W.A., Le Brun, A.P., Ciesielski, F., Kuzmenko, I., Holt, S.A. et al. (2015) Effect of divalent cation removal on the structure of Gram-negative bacterial outer membrane models. *Langmuir*, 31, 404–412.

Dams, D. & Briers, Y. (2019) Enzybiotics: enzyme-based antibacterials as therapeutics. In: Labrou, N. (Ed.) *Therapeutic enzymes: function and clinical implications*. Singapore: Springer Singapore, pp. 233–253.

Ding, Y., Zhang, Y., Huang, C., Wang, J. & Wang, X. (2020) An endolysin LysSE24 by bacteriophage LPSE1 confers specific bactericidal activity against multidrug-resistant *Salmonella* strains. *Microorganisms*, 8, 737.

Fowler, V.G.J., Das, A.F., Lipka-Diamond, J., Schuch, R., Pomerantz, R., Jáuregui-Peredo, L. et al. (2020) Exebacase for patients with *Staphylococcus aureus* bloodstream infection and endocarditis. *The Journal of Clinical Investigation*, 130, 3750–3760.

Grishin, A.V., Karyagina, A.S., Vasina, D.V., Vasina, I.V., Gushchin, V.A. & Lunin, V.G. (2020) Resistance to peptidoglycan-degrading enzymes. *Critical Reviews in Microbiology*, 46, 703–726.

Hong, H.-W., Kim, Y.D., Jang, J., Kim, M.S., Song, M. & Myung, H. (2022) Combination effect of engineered endolysin EC340 with antibiotics. *Frontiers in Microbiology*, 13, 821936.

Jiang, Y., Xu, D., Wang, L., Qu, M., Li, F., Tan, Z. et al. (2021) Characterization of a broad-spectrum endolysin LysSP1 encoded by a *Salmonella* bacteriophage. *Applied Microbiology and Biotechnology*, 105, 5461–5470.

Jun, S.Y., Jang, I.J., Yoon, S., Jang, K., Yu, K.-S., Cho, J.Y. et al. (2017) Pharmacokinetics and tolerance of the phage endolysin-based candidate drug SAL200 after a single intravenous administration among healthy volunteers. *Antimicrobial Agents and Chemotherapy*, 61, e02629-16.

Kashani, H.H., Schmelcher, M., Sabzalipoor, H., Hosseini, E.S. & Moniri, R. (2017) Recombinant endolysins as potential therapeutics against antibiotic-resistant *Staphylococcus aureus*: current status of research and novel delivery strategies. *Clinical Microbiology Reviews*, 31, e00071-17.

Kaur, J., Singh, P., Sharma, D., Harjai, K. & Chhibber, S. (2020) A potent enzybiotic against methicillin-resistant *Staphylococcus aureus*. *Virus Genes*, 56, 480–497.

Kim, J., Park, H., Ryu, S. & Jeon, B. (2021) Inhibition of antimicrobial-resistant *Escherichia coli* using a broad host range phage cocktail targeting various bacterial phylogenetic groups. *Frontiers in Microbiology*, 12, 699630.

Kim, S., Jin, J.-S., Choi, Y.-J. & Kim, J. (2020) LysSAP26, a new recombinant phage endolysin with a broad spectrum antibacterial activity. *Viruses*, 12, 1340.

Kusuma, C., Jadanova, A., Chanturiya, T. & Kokai-Kun, J.F. (2007) Lysostaphin-resistant variants of *Staphylococcus aureus* demonstrate reduced fitness in vitro and in vivo. *Antimicrobial Agents and Chemotherapy*, 51, 475–482.

Larpin, Y., Oechslin, F., Moreillon, P., Resch, G., Entenza, J.M. & Mancini, S. (2018) In vitro characterization of PlyE146, a novel phage lysin that targets Gram-negative bacteria. *PLoS One*, 13, e0192507.

Lee, C., Kim, J., Son, B. & Ryu, S. (2021) Development of advanced chimeric endolysin to control multidrug-resistant *Staphylococcus aureus* through domain shuffling. *ACS Infectious Diseases*, 7, 2081–2092.

Li, X., Wang, S., Nyaruaba, R., Liu, H., Yang, H. & Wei, H. (2021) A highly active chimeric lysin with a calcium-enhanced bactericidal activity against *Staphylococcus aureus* in vitro and in vivo. *Antibiotics*, 10, 461.

Lim, J., Jang, J., Myung, H. & Song, M. (2022) Eradication of drug-resistant *Acinetobacter baumannii* by cell-penetrating peptide fused endolysin. *Journal of Microbiology*, 60, 859–866.

Liu, H., Hu, Z., Li, M., Yang, Y., Lu, S. & Rao, X. (2023) Therapeutic potential of bacteriophage endolysins for infections caused by Gram-positive bacteria. *Journal of Biomedical Science*, 30, 29.

Loessner, M.J. (2005) Bacteriophage endolysins – current state of research and applications. *Current Opinion in Microbiology*, 8, 480–487.

Manoharadas, S., Altaf, M., Alrefaei, A.F., Ahmad, N., Hussain, S.A. & Al-Rayes, B.F. (2021) An engineered multimodular enzybiotic against methicillin-resistant *Staphylococcus aureus*. *Life*, 11, 1–19.

Melo-López, F.N., Zermeño-Cervantes, L.A., Barraza, A., Loera-Muro, A. & Cardona-Félix, C.S. (2021) Biochemical characterization of LysVpKK5 endolysin from a marine vibriophage. *Protein Expression and Purification*, 188, 105971.

Pang, Z., Raudonis, R., Glick, B.R., Lin, T.-J. & Cheng, Z. (2019) Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnology Advances*, 37, 177–192.

Plotka, M., Kapusta, M., Dorawa, S., Kaczorowska, A.K. & Kaczorowski, T. (2019) Ts2631 endolysin from the extremophilic *Thermus scotoductus* bacteriophage vB_Tsc2631 as an antimicrobial agent against Gram-negative multidrug-resistant bacteria. *Viruses*, 11, 657.

Plotka, M., Sancho-Vaello, E., Dorawa, S., Kaczorowska, A.K., Kozlowski, L.P., Kaczorowski, T. et al. (2019) Structure and function of the Ts2631 endolysin of *Thermus scotoductus* phage vB_Tsc2631 with unique N-terminal extension used for peptidoglycan binding. *Scientific Reports*, 9, 1261.

Rahman, M.U., Wang, W., Sun, Q., Shah, J.A., Li, C., Sun, Y. et al. (2021) Endolysin, a promising solution against antimicrobial resistance. *Antibiotics*, 10, 1277.

Rice, L.B. (2008) Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *The Journal of Infectious Diseases*, 197, 1079–1081.

Rodríguez-Rubio, L., Chang, W.L., Gutiérrez, D., Lavigne, R., Martínez, B., Rodríguez, A. et al. (2016) "Artilysation" of endolysin λSa2lys strongly improves its enzymatic and antibacterial activity against streptococci. *Scientific Reports*, 6, 1–11.

São-José, C. (2018) Engineering of phage-derived lytic enzymes: improving their potential as antimicrobials. *Antibiotics*, 7, 29.

Schmelcher, M., Donovan, D.M. & Loessner, M.J. (2012) Bacteriophage endolysins as novel antimicrobials. *Future Microbiology*, 7, 1147–1171.

Şen Karaman, D., Ercan, U.K., Bakay, E., Topaloğlu, N. & Rosenholm, J.M. (2020) Evolving technologies and strategies for combating antibacterial resistance in the advent of the postantibiotic era. *Advanced Functional Materials*, 30, 1908783.

Sharma, U., Vipra, A. & Channabasappa, S. (2018) Phage-derived lysins as potential agents for eradicating biofilms and persisters. *Drug Discovery Today*, 23, 848–856.

Shen, Y., Barros, M., Vennemann, T., Travis Gallagher, D., Yin, Y., Linden, S.B. et al. (2016) A bacteriophage endolysin that eliminates intracellular streptococci. *eLife*, 5, e13152.

Shrivastava, S.R., Shrivastava, P.S. & Ramasamy, J. (2018) World Health Organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *Journal of Medical Society*, 32, 76.

Son, B., Kong, M., Lee, Y. & Ryu, S. (2021) Development of a novel chimeric endolysin, Lys109 with enhanced lytic activity against *Staphylococcus aureus*. *Frontiers in Microbiology*, 11, 1–12.

Son, B., Yun, J., Lim, J.-A., Shin, H., Heu, S. & Ryu, S. (2012) Characterization of LysB4, an endolysin from the *Bacillus cereus*-infecting bacteriophage B4. *BMC Microbiology*, 12, 33.

Tang, K.W.K., Millar, B.C. & Moore, J.E. (2023) Antimicrobial resistance (AMR). *British Journal of Biomedical Science*, 80, 11387.

Tipton, K.A., Chin, C.Y., Farokhyfar, M., Weiss, D.S. & Rathera, P.N. (2018) Role of capsule in resistance to disinfectants, host antimicrobials, and desiccation in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, 62, e01188-18.

Totté, J.E.E., van Doorn, M.B. & Pasmans, S.G.M.A. (2017) Successful treatment of chronic *Staphylococcus aureus*-related dermatoses with the topical endolysin Staphefekt SA.100: a report of 3 cases. *Case Reports in Dermatology*, 9, 19–25.

Troeger, C., Blacker, B., Khalil, I.A., Rao, P.C., Cao, J., Zimsen, S.R.M. et al. (2018) Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory infections in 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Infectious Diseases*, 18, 1191–1210.

Vasina, D.V., Antonova, N.P., Grigoriev, I.V., Yakimakha, V.S., Lendel, A.M., Nikiforova, M.A. et al. (2021) Discovering the potentials of four phage endolysins to combat Gram-negative infections. *Frontiers in Microbiology*, 12, 748718.

Vollmer, W., Blanot, D. & De Pedro, M.A. (2008) Peptidoglycan structure and architecture. *FEMS Microbiology Reviews*, 32, 149–167.

Wang, F., Ji, X., Li, Q., Zhang, G., Peng, J., Hai, J. et al. (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.

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