Eradication of drug-resistant *Acinetobacter baumannii* by cell-penetrating peptide fused endolysin§

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Antimicrobial agents targeting peptidoglycan have shown successful results in eliminating bacteria with high selective toxicity. Bacteriophage encoded endolysin as an alternative antibiotics is a peptidoglycan degrading enzyme with a low rate of resistance. Here, the engineered endolysin was developed to defeat multiple drug-resistant (MDR) *Acinetobacter baumannii*. First, putative endolysin PA90 was predicted by genome analysis of isolated *Pseudomonas* phage PBPA. The His-tagged PA90 was purified from BL21(DE3) pLysS and tested for the enzymatic activity using Gram-negative pathogens known for having a high antibiotic resistance rate including *A. baumannii*. Since the measured activity of PA90 was low, probably due to the outer membrane, cell-penetrating peptide (CPP) DS4.3 was introduced at the N-terminus of PA90 to aid access to its substrate. This engineered endolysin, DS-PA90, completely killed *A. baumannii* at 0.25 μM, at which concentration PA90 could only eliminate less than one log in CFU/mL. Additionally, DS-PA90 has tolerance to NaCl, where the ~50% of activity could be maintained in the presence of 150 mM NaCl, and stable activity was also observed with changes in pH or temperature. Even MDR *A. baumannii* strains were highly susceptible to DS-PA90 treatment: five out of nine strains were entirely killed and four strains were reduced by 3–4 log in CFU/mL. Consequently, DS-PA90 could protect waxworm from *A. baumannii*-induced death by ~70% for ATCC 17978 or ~44% for MDR strain 1656-2 infection. Collectively, our data suggest that CPP-fused endolysin can be an effective antibacterial agent against Gram-negative pathogens regardless of antibiotics resistance mechanisms.

**Keywords**: bacteriophage, endolysin, cell-penetrating peptide, *Acinetobacter baumannii*, multiple drug-resistant

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**Introduction**

Bacterial infections can be efficiently cured by treatment with antibiotics, but a shortage of available antibiotics is closely related with infection-induced death, which is a result of the emergence of multiple drug-resistant (MDR) bacteria. As a representative MDR bacteria, *A. baumannii* is resistant to most clinical antibiotics by using various mechanisms such as efflux pumps, β-lactamas, aminoglycoside-modifying enzymes, permeability defects, and the alteration of target sites (Lin and Lan, 2014; Lee et al., 2017). In particular, the high genetic plasticity of *A. baumannii* results in a reservoir of resistance genes by horizontal transfer, particularly using mobile genetic elements including integrons (Lin and Lan, 2014; Pagano et al., 2016). Therefore, it is urgent to develop alternative antimicrobials for the treatment of *A. baumannii* infection, with possibly low frequency of resistance.

Attention has been paid to bacteriophage as an alternative agent(s) for the control of bacterial infection. As viruses of bacteria, phages can infect specific hosts depending on receptor binding proteins and kill the host bacteria when reproduction is completed. Even biofilms and extracellular matrix can be disrupted by phages (Azeredo and Sutherland, 2008; Walsh et al., 2021). Additionally, the genetic engineering is relatively less troublesome due to simple genetic composition (Meile et al., 2022). Indeed, there is increasing evidence that shows the potential of phages or engineered phages as a treatment option without or together with antibiotics for bacterial infections including MDR bacteria (Bhargava et al., 2021; Walsh et al., 2021; Blasco et al., 2022; Sisakhtpour et al., 2022; Xuan et al., 2022). Despite the benefits of phages as antimicrobials, drawbacks such as restricted host range and the emergence of phage resistance are also evident (Meile et al., 2022).

Phage endolysins are peptidoglycan hydrolases that mediate the release of phage progeny (Fischetti, 2010). The addition of purified recombinant endolysin causes immediate lysis and death of Gram-positive bacteria *in vitro* (Fenton et al., 2010). In addition, administration of endolysin was shown to be effective in reducing a colonized Gram-positive pathogen in an animal model (Loeffler et al., 2001; Nelson et al., 2001; Cheng et al., 2005). Currently, endolysins targeting Gram-positive pathogens are in the development stage for clinical treatment in humans where natural endolysins were mined and improved by protein engineering with increased antimicrobial activity, followed by additional protein and biochemical engineering. Also, formulations have been designed for improving half-life, bioavailability, and antimicrobial activity (De Maesschalck et al., 2020). However, the activity of endolysin is less effective against Gram-negative bacteria due to the outer membrane.**
Pseudomonas aeruginosa

Bacterial strains and growth conditions

Materials and Methods

to endolysin can enhance the function against Gram-negative

Therefore, our study suggests that the introduction of CPP

waxworm from

of endolysins, was improved. Finally, DS-PA90 could protect

to high concentrations of NaCl, which limited the application

analysis of endolysin PA90

of cultured P. aeruginosa at log phase (OD_{600} = 0.5) were

added to an LB soft agar (final 0.7%) and then the mixture

was overlayed on an LB agar plate. After overnight incuba-

tion at 37°C, a single plaque was selected for the purification

of phages by centrifugation using 10% (v/v) polyethylene glycol

8000 (PEG 8000) (Kim et al., 2015). The purified phage

was named PBPA90. The genomic DNA of PBPA90 was

isolated using a phage DNA isolation kit (Norgen #46800)

and whole genome sequences were identified by Illumina Miseq

(LAS) using the genomic library prepared by the TrueSeq®

Nano DNA sample preparation kit with 150 bp paired-end DNA

reads. SAVAGE was used for genome assembly and annotation

(Baaijens et al., 2017), followed by remapping to sequence

reads and manual curation. Open reading frames (ORFs) were

analyzed using BLAST and the putative endolysin was

identified by comparison to the COG, SwissProt, and Pfam
database: endolysin PA90 (GenBank accession number: MW815133). The domain architecture of PA90 was

predicted by the InterPro tool (Quevillon et al., 2005) and the

secondary/tertiary structure was predicted by Phyre server

(Kelley and Sternberg, 2009).

Construction of the PA90 or DS-PA90 expression plasmid

The putative endolysin PA90 was amplified by PCR with the

primers forward 5'-aaAGATCCCATGCGATCCTTTATCCTCAACGTGCGCT-3' and reverse 5'-aCTCGAGTCCGCGGATGTTTCGAAACCTTATCCTTC-3'. The 793 bp PCR product was

cleaved up with Qagen clean up kit (#28204) and digested with

BamH1 (NEB#R316S) and Xho1 (NEB#R0146L). The digested

DNA fragment was ligated with pET21-a (+), generating pAS-

025. The cell-penetrating peptide DS4.3 (RIMRILRILKLAR)

was introduced by PCR using pAS025 as a template with

forward primer 5'-GCATTCTGAACAATCGGCGTGATGCTACTGACTCATCTCAACGTGCGCT-3' and reverse primer 5'-

GCAGAATACGCTAAATGCGGCCGCGCCCTGGAAA GTAAA-3'. The PCR product was treated with Dpn1 (NEB

#R0176L) for the removal of the template and a DNA band

with the expected size was extracted from the agarose gel.
The cleaned DNA fragment was circularized by ligation in the

presence of polynucleotide kinase (PNK; NEB #M026L),
yielding pAS033. The plasmids were confirmed by sequenc-
ing analysis. Each plasmid, pAS025 or pAS033, was intro-
duced into BL21 (DE3) pLysS or SoluBL21™, respectively,

for the purification of endolysins.

Purification of PA90 or DS-PA90

BL21 strain carrying pAS025 or pAS033 was grown in 1.5 L

of LB broth containing ampicillin (100 mg/ml) at 37°C until

the optical density at 600 nm reached 0.6. Protein expression

was induced by the addition of 1 mM isopropyl-β-D-thio-
galactopyranoside (IPTG) and bacteria were further cultured

at 37°C for 4 h. The cells were harvested by centrifugation

at 5,000 × g for 10 min at 4°C. The bacterial pellet was resus-
pended in 100 ml of lysis buffer [20 mM Tris-HCl; pH 7.5,

0.5 M NaCl, 10 mM imidazole] and disrupted by sonica-
nation on ice. The unbroken cells were separated by centrifu-
gation at 14,000 × g for 30 min at 4°C and the supernatant

fraction was filtered through a 0.4 μm pore size filter (GVS

#FJ25ASCMA004FL01). The collected fraction containing

Materials and Methods

Bacterial strains and growth conditions

Pseudomonas aeruginosa ATCC 13388, Escherichia coli ATCC
8739, Acinetobacter baumannii ATCC 17978, Klebsiella pneu-
moniae KTCC 2208, Enterobacter aerogenes CCARM 16006,

Enterobacter cloacae CCARM 0252 were purchased from the

American Type Culture Collection (ATCC), the Korean

Collection for Type Cultures (KCTC), and the Culture Collec-
tion of Antimicrobial Resistance Microbes (CCARM). The

clonal isolates of A. baumannii were obtained from the

Kyungpook National University Hospital National Culture

Collection for Pathogens (KNUH-NCCP). All bacterial strains

were grown in Luria Bertani (LB; MBCell MB-L4488) broth

at 37°C with vigorous aeration at 200 rpm.

Bacteriophage PBPA90 genome analysis and bioinformatic

analysis of endolysin PA90

Bacteriophage was isolated from Opo Wastewater Facility

in Gwangju, Gyeonggi-do, Korea by the soft agar overlay

method. In brief, three milliliters of wastewater and 100 ml

(OM), which limits the accessibility of endolysin to peptido-
glycan.

Cell-penetrating peptide (CPP) is a short peptide with the

ability of crossing the cell membrane and is widely applied

for the intracellular delivery of molecules such as peptides,

proteins, nucleic acids, and nanoparticles (Derkhshankhah

and Jafari, 2018; Sadeghian et al., 2022). For medical appli-
cations, CPP has been used as a delivery tool for cancer ther-
apy including DS4.3 (Jeong et al., 2014), inflammation ther-

apy (Lee et al., 2012), and vaccine development (Sadeghan

et al., 2022), and is expanding to other areas. More import-
anty, CPP has been used against bacteria by binding and

disturbing the membrane, which is more negatively charged

than mammalian cells (Oikawa et al., 2018; Drexelius et al.,

2021). Also, conjugation of CPP to antibacterial agents has

been used for the delivery of agents into the cytoplasm of

infected cells where the agents kill intracellular pathogens

efficiently (Chen et al., 2021; Frimodt-Møller et al., 2022; Rüter, 2022).

Here, we mined a novel endolysin from a newly identified

Pseudomonas phage in an endeavor to develop an alternative

control agent for MDR A. baumannii infection. From ge-

nome analysis, one gene was predicted to be endolysin and

named as PA90, which was then cloned for overexpression.

The purified recombinant protein was shown to have moderate

antibacterial activity against A. baumannii. Subsequently,

CPP DS4.3 was introduced at the N-terminus of PA90 to

increase the efficiency of endolysin. This engineered endo-

lysin could completely kill not only laboratory strains but also

drug-resistant strains of A. baumannii in vitro and tolerance to

high concentrations of NaCl, which limited the application of

endolysins, was improved. Finally, DS-PA90 could protect

waxworm from A. baumannii infection including MDR strains.

Therefore, our study suggests that the introduction of CPP
to endolysin can enhance the function against Gram-negative

pathogens such as MDR A. baumannii.
N-terminal 6× histidine tagged endolysin was applied to 5 ml of HisTrap HP column (Cytiva #17524802) installed in an ÄKTA go fast protein liquid chromatography (FPLC) system (Cytiva) controlled by UNICORN 5.1 software. The bound endolysins were eluted using a buffer [20 mM Tris-HCl; pH 7.5, 0.5M NaCl] with gradient of imidazole concentration from 10 mM to 0.5 M and the eluted fractions containing endolysins were then applied into a 5 ml of HisTrap SP column (Cytiva #17-1152-01). The proteins were bound to the resin in 20 mM Tris-HCl; pH 7.5 and eluted with another buffer [20 mM Tris-HCl; pH 7.5, 1 M NaCl]. Fractions were collected and dialyzed overnight in 20 mM Tris-HCl; pH 7.5 and 150 mM NaCl at 4°C. The protein concentration was measured with a Bradford assay kit (Bio-Rad 5000006).

Measurement of antibacterial activity of PA90 or DS-PA90 by CFU reduction assay

The lytic spectrum of PA90 was determined using Pseudomonas aeruginosa, Escherichia coli, Acinetobacter baumannii, Klebsiella pneumoniae, Enterobacter aerogenes, and Enterobacter cloacae as host strains. Bacterial cells were grown in exponential phase (OD600 = 0.8). The cell pellet was washed and resuspended in 20 mM Tris-HCl; pH 7.5 then mixed with 0, 0.2, 2 μM of the purified PA90 in 96-well. After 2 h incubation at 37°C, cells were serially diluted in 1 × PBS and plated on LB agar to determine the surviving number of bacteria. For antibiotic-resistant A. baumannii strains including clinical isolates, the bacteria were treated with 0 and 0.25 μM of PA90 or DS-PA90, respectively, for the comparison of enzyme activity.

Determination of optimal salt concentration, pH, and temperature for the activity of DS-PA90

Freshly grown A. baumannii ATCC 17978 cells were prepared as mentioned above and treated with 2 μM of PA90 or 0.125 μM of DS-PA90. For the optimal pH test, each endolysin was incubated in 20 mM Tris-HCl solution adjusted to pH 5.0, 6.5, 7.5, 8.5 and 10.0, respectively, at room temperature for 1 h and then used for a CFU reduction assay. Sodium chloride was added to 20 mM Tris-HCl; pH 7.5 buffer as final 0, 25, 50, 100, and 150 mM for a salt concentration test and the mixture of bacterial cells and each endolysin was incubated at 37°C for 2 h. The thermal stability of endolysins was determined by the pre-incubation of endolysins at 15, 25, 37, 45, and 55°C for 1 h. The bacteria resuspended in 20 mM Tris-HCl; pH 7.5 was then treated with preincubated PA90 or DS-PA90 at 37°C. After 2 h, the diluted samples were plated on LB agar and incubated at 37°C overnight. The next day, the bacterial numbers were counted and the percent of relative reduction was calculated as the percent of bacteria killed under the given condition referring to the assay conditions in 20 mM Tris-HCl; pH 7.5 at 37°C.

Determination of minimum inhibitory concentrations (MICs) of A. baumannii clinical isolates

The MIC of antibiotics were determined by broth microdilution method in 96-well plates as described previously (Hong et al., 2022). Each clinical isolate of A. baumannii was grown in LB overnight and inoculated into fresh CAA medium (5 g/L casamino acids, 5.2 mM KH₂PO₄, and 1 mM MgSO₄). The culture was then grown at 37°C for 3.5 h. The cells were diluted to 1 × 10⁶ CFU/well in CAA medium and incubated with antibiotics (colistin, cefotaxime, ciprofloxacin, meropenem, gentamicin, and tetracycline) at 37°C for 20 h. The MIC values were determined as the lowest concentration for inhibiting bacterial growth completely.

Galleria mellonella infection model for DS-PA90 treatment

Healthy larvae of greater wax moth, Galleria mellonella, were obtained from Sworn. Larvae were selected to be in the final instar stage and weigh from 80 to 140 mg. Before bacterial infection, larvae were fastened and kept in the dark at 30°C for 24 h. Overnight culture of A. baumannii ATCC 17978 or clinical isolate 1656-2 (Park et al., 2011) strain was inoculated into fresh LB and cultured for 2 h for infection into the larvae. The bacterial cultures were then harvested by centrifugation at 4,500 rpm for 3 min and resuspended in 1 × PBS. Immediately before injection, 10 μM of DS-PA90 was mixed with 2 × 10⁶ CFU of each bacterial suspension and the mixture was injected into the last-left-proleg using a 10R-GT 10 ml syringe (Trajan Scientific and Medical, 002200) (n = 10). Larvae were then incubated at 30°C for 72 h and their survival were monitored by checking the response to touch and melanization of the whole body. As a control, the same amount of 1 × PBS was mixed with bacterial suspension and injected into larvae (mock; n = 10). The experiment was repeated at least three times.

Statistical analysis

Data analysis was carried out using GraphPad Prism software version 9.3.0. A two-tailed Student’s t-test was used for the analysis of the differences between the two groups and a log-rank (Mantel-cox) test was used for survival experiments. All data are presented as mean ± SD, and differences were considered significant at P < 0.05.

Results

Identification of new Pseudomonas phage PBPA and its endolysin PA90

A library of bacteriophages from a sewage water treatment facility was screened and a phage from the library formed a single plaque on a soft agar plate containing Pseudomonas aeruginosa ATCC 13388, which was named PBPA. A circular genome of PBPA was 304,052 bp in length, which has 98.53% identity compared to Pseudomonas phage PA1C with 99% coverage by BLAST analysis with whole genome sequencing results. There were 241 predicted ORF by NCBI ORF finder and analyzed by BLAST (Supplementary data Fig. S1) and one of them was a putative endolysin, PA90. BlastP analysis showed that the amino acid sequence of PA90 is 99.23% identical to the putative endolysin of Pseudomonas phage PA1C (GenBank accession number: QBX32324.1) and 57.2% identical to the putative endolysin of Pseudomonas phage KTN4 (GenBank accession number: ANM44938.1) (Fig. 1A). Func-
tional domain analysis and structural alignment revealed a peptidoglycan-binding domain (aa 10-64) and a lysozyme-like domain (aa 98-180) (Fig. 1B). The predicted tertiary structure was 55.8% similar to the *Pseudomonas* bacteriophage PhikZ lytic transglycosylase (PDB ID: 3BKH) (Fig. 1C). Therefore, a new *Pseudomonas* phage, PBPA, was isolated and a putative endolysin, PA90, was identified.

**Endolysin activity of PA90 and engineered PA90 (DS-PA90) against *A. baumannii***

To verify endolysin activity, the recombinant PA90 with 6× His tag at the C-terminus was purified from BL21 (DE3) pLysS strain using FPLC with a HisTrap HP column (Fig. 2A). The antibacterial activity of the purified PA90 was tested against *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, *E. aerogenes*, *E. cloacae*, and *E. coli* (Fig. 2B). Most bacterial strains were not affected by 0.2 μM PA90, except *E. cloacae*, and *E. coli*, the numbers of which decreased 10- and 7.9-fold, respectively. The number of bacteria further decreased by treatment with 2 μM PA90 for 2 h for all tested strains, where the reduction fold ranged from 2 to 150. Among the tested bacteria, *P. aeruginosa*, the original host for PBPA, and *A. baumannii* were most effectively killed by treatment with PA90, and their numbers were reduced 150- and 60-fold, respectively. However, PA90 could not eliminate either bacteria completely. Therefore, engineering of PA90 was pursued to increase lytic activity, especially against our target bacteria, *A. baumannii*.

To this end, the cell-penetrating peptide DS4.3 (RIMRIL-RILKLAR; Jeong et al., 2014) was introduced at the N-terminus of PA90 to aid the penetration of endolysin into the outer membrane of Gram-negative bacteria (Fig. 2C). The engineered PA90, named DS-PA90, was purified from BL21 (DE3) SoluBL21™ using His tag at the C-terminus by affinity purification (Fig. 2D). Of note, DS4.3 fused PA90 slightly formed aggregates when it was induced by 1 mM IPTG at 37°C for 5 h (Supplementary data Fig. S2). Next, the antibacterial activity of DS-PA90 was determined using *A. baumannii* 17978 grown at exponential phase in comparison with PA90 (Fig. 2E). The number of *A. baumannii* slightly de-
Antibacterial activity of DS-PA90 increased from $1 \times 10^6$ to $3.4 \times 10^5$ by 0.25 μM PA90, whereas more bacteria were killed by 0.0312 μM DS-PA90 and half of the bacterial population was removed by 0.125 μM DS-PA90. Finally, bacterial cells were completely eliminated by 0.25 μM DS-PA90. Of note, antibacterial activity of DS-PA90 was also improved against P. aeruginosa, K. pneumoniae, A. baumannii, E. aerogenes, E. cloacae, and E. coli. Each strain was used in a CFU reduction assay with 0.125 μM DS-PA90 using freshly grown A. baumannii ATCC 17978 cells in buffer containing different concentrations of NaCl (A). DS-PA90 was pre-incubated in buffer with different pH (B) or at different temperatures (C) for 1 h and then used for the assay. The percent of relative reduction was calculated as the percent of bacteria killed in a given condition, referring to the assay condition in 20 mM Tris-HCl; pH 7.5 at 37°C. Significance is indicated as *$P < 0.04$; **$P = 0.0042$. Experiments were repeated at least three times and data are presented as mean ± SD.

Effect of salinity, pH and temperature on lytic activity of DS-PA90

Next, the endolysin activity of DS-PA90 was tested under environmental stresses including salinity, pH and temperature. The maximal activity of DS-PA90 was observed when the assay was performed in the reaction buffer either without NaCl or in the presence of 25 mM NaCl, but gradually decreased as the concentration of NaCl was increased. The

Fig. 3. Lytic activity of DS-PA90 upon changes in NaCl concentration, pH and temperature. A CFU reduction assay was performed with 0.125 μM DS-PA90 using freshly grown A. baumannii ATCC 17978 cells in buffer containing different concentrations of NaCl (A). DS-PA90 was pre-incubated in buffer with different pH (B) or at different temperatures (C) for 1 h and then used for the assay. The percent of relative reduction was calculated as the percent of bacteria killed in a given condition, referring to the assay condition in 20 mM Tris-HCl; pH 7.5 at 37°C. Significance is indicated as *$P < 0.049$; **$P < 0.007$. Experiments were repeated at least three times and data are presented as mean ± SD.
activity of DS-PA90 decreased approximately 30% by the addition of 100 mM NaCl, and 58% of activity was maintained in the buffer with 150 mM NaCl compared to the buffer without NaCl for CFU reduction assay (Fig. 3A). Thus, the engineered PA90 was shown to be stable under osmotic stress. Of note, the remaining activity of PA90 was only 10% in the buffer containing 150 mM NaCl for CFU reduction assay (Supplementary data Fig. S4). The stability of DS-PA90 was also tested with changes in pH or temperature. The antibacterial activity of DS-PA90 decreased less than 8% after pre-incubation of endolysin in the reaction buffer with different pH ranging from 5 to 10. Also, the thermal stability of DS-PA90 was measured by CFU reduction assay after pre-treatment of endolysin at different temperatures. The lytic activity of DS-PA90 was maintained well after pre-treatment of protein at 15, 25, 37, and 45°C, but the activity decreased up to 60% after pre-treatment at 55°C. Collectively, the stable lytic activity of DS4.3 fused PA90 was observed upon the challenges of pH and temperature stress.

**Antibacterial activity of DS-PA90 in waxworm, Galleria mellonella**

Since the potential of DS-PA90 as an alternative antibiotics was tested using a standard laboratory strain of *A. baumannii* (ATCC 17978), the antibacterial activity was further investigated against clinical isolates of *A. baumannii*. First, a total of nine clinical isolates were screened for susceptibility to antibiotics by the measurement of minimum inhibitory concentrations (MICs) (Table 1). All tested strains were either intermediate or resistant to cefotaxime, meropenem, gentamicin, except strain 365 for gentamicin. Several strains were susceptible to ciprofloxacin and tetracycline. Collectively, each strain was shown to be resistant to at least two antibiotics; thus, all clinical isolates were regarded as multiple drug-resistant strains. Next, the antibacterial activity of DS-PA90 was tested against such antibiotic-resistant clinical isolates (Fig. 4A). Strains 365, 1656-2, 216, and 643 were completely killed, and ~4 log number of four strains (3097, 4316, 2496, and 205) were eliminated by DS-PA90 treatment. Therefore, the lytic activity of DS4.3 fused PA90 was also effective in multiple drug-resistant strains of *A. baumannii* in vitro. Finally, the efficacy of DS-PA90 was determined using waxworm, *G. mellonella*, as an *in vivo* model system with *A. baumannii* ATCC 17978 or clinical isolate 1656-2. Each strain was diluted in PBS containing 2 × 10⁶ CFU and DS-PA90 was added to the cell suspension immediately before injection, which did not lead to bacterial killing (data not shown). Larval death was observed in the group infected with either strain and treatment with DS-PA90 increased the survival of infected larvae up to ~70% with ATCC 17978 or ~44% with clinical isolate 1656-2 strain (Fig. 4B and C). Therefore, it was clearly shown that DS-PA90 can kill mult erad-resistant strains of *A. baumannii* and effectively control bacterial infection in waxworm.

**Discussion**

Endolysin is a promising agent to replace or support antibiotics with high specificity against bacteria and low probability of resistance (Dams and Briers, 2019). However, the enzymatic activity of endolysin is restricted to its substrate, peptidoglycan, resulting in the limited application of endo-

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**Table 1. Minimum inhibitory concentrations (μg/ml) of the clinical isolates of A. baumannii**

| Strains  | Colistin | Cefotaxime | Ciprofloxacin | Meropenem | Gentamicin | Tetracycline |
|---------|----------|------------|---------------|-----------|------------|--------------|
| 1656-2  | 16       | 64 ≥       | 64 ≥          | 64 ≥      | 64 ≥       | 64 ≥         |
| 3097    | 64       | 64 ≥       | 63 ≥          | 64 ≥      | 64 ≥       | 64 ≥         |
| 365     | 8        | 32         | 1             | 32        | 4          | 4            |
| 643     | 64 ≥     | 64 ≥       | 64 ≥          | 64 ≥      | 64 ≥       | 64 ≥         |
| 4316    | 64 ≥     | 32         | 8             | 64 ≥      | 32         |
| 2496    | 64 ≥     | 32         | 1             | 32        | 8          | 4            |
| 205     | 8        | 64         | 1             | 4         | 8          | 4            |
| 216     | 8        | 32         | 4             | 64        | 8          | 1            |
| 3680    | 64       | 32         | 4             | 64        | 16         | 2            |

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**Fig. 4. Antibacterial activity of DS-PA90 against MDR A. baumannii in vitro and in Galleria mellonella.** (A) The lytic activity of DS-PA90 was determined using clinical isolates of *A. baumannii* strains by CFU reduction assay. Significance is indicated as *P < 0.05; **P < 0.008. Data are presented as mean ± SD (n = 3). The larvae of *Galleria mellonella* were infected by 2 × 10⁶ CFU *A. baumannii* ATCC 17978 (B) or clinical isolate 1656-2 (C) strain. For endolysin treatment, 10 μM of DS-PA90 was mixed with bacterial suspension immediately before injection. As a control, the same amount of 1× PBS was used (mock). The survival of larvae was monitored while the larvae were kept at 30°C for 72 h (n = 10 per group). The experiment was repeated three times. Significance is shown as ****P < 0.0001; **P = 0.0047.
lys in for Gram-negative pathogens containing an outer membrane. Therefore, engineering is required to develop endolysin to eliminate Gram-negative bacteria including A. baumannii.

Here, we identified a new Pseudomonas bacteriophage, PBPA-90, from the environment and mined a putative endolysin, PA90, by bioinformatic analysis. Since the bactericidal activity of the purified PA90 was shown to be weak, the endolysin was engineered where a cell-penetrating peptide (CPP) DS4.3 was introduced at the N-terminus of PA90. The resulting endolysin, DS-PA90, could completely eliminate A. baumannii cells in vitro. Although the role of DS4.3 is known for the eukaryotic cell membrane, this peptide can help the penetration of the outer membrane of bacteria, which has positive charge and was suggested for other CPPs against bacteria (Okawa et al., 2018; John et al., 2019). Also, its increased stability might be related to the improved activity of DS4.3 fused endolysin. In particular, the activity of PA90 was hindered by a high concentration of NaCl, but fusion of DS4.3 buffered the impact of the increased concentration of NaCl on the enzymatic activity of PA90. However, the role of DS4.3 should be addressed in future studies to elucidate the feasibility of this CPP for the development of engineered endolysins against Gram-negative pathogens. It was shown that certain CPPs can efficiently disrupt bacterial membranes due to their chemical characteristics such as net positive charge, which aid the binding of peptides to negatively charged bacterial membranes (Deraikhshannkah and Jafari, 2018; Okawa et al., 2018). By fusing CPP to endolysin, this enzyme can be a more powerful antibacterial agent.

Most importantly, the antibacterial activity of DS-PA90 was extremely effective against clinical isolates of A. baumannii with multiple drug resistance such as β-lactam antibiotics, aminoglycoside and tetracycline. Furthermore, the endolysin activity of DS-PA90 could rescue waxworm from A. baumannii infection-induced death. But the in vivo activity of DS-PA90 should be tested in an animal model to prove the potential of this engineered endolysin as alternative agent for A. baumannii infection in a future study. Also, the synergistic effect of DS-PA90 can be determined in combination with a variety of antibiotics, which is also pursued for other endolysins (De Maeschalck et al., 2020).

There are many excellent features of endolysin, which include a broad spectrum of hosts, high antibacterial activity, and low rates of resistance (Lai et al., 2020). But when it comes to practical application, endolysins are not as effective as in the in vitro condition. It was observed that the majority of endolysins were deposited in the kidneys like other peptide- or protein-based drugs (Seijsing et al., 2018; Sobieraj et al., 2020). A short half-life in serum was reported, and low accessibility to intracellular pathogens was also noticed (Seijsing et al., 2018; Schmelcher and Loessner, 2021; Sobieraj et al., 2020). Protein engineering can help overcome such limitations, including the fusion of albumin binding domain (ABD) or domain shuffling of multiple endolysins or addition of CPP (Sobieraj et al., 2020).

In our study, we demonstrated that the addition of CPP to endolysin enhanced antibacterial activity against MDR A. baumannii and stability of protein in vitro. Therefore, our findings pave the way for new designed drugs to treat infection by drug-resistant pathogens.

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Conflict of Interest

The authors have no conflict of interest to report.

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