

ORIGINAL RESEARCH ARTICLE

Engineering the chimeric lysin Art-15 via NZ2114–PlySs2 fusion enhances strain-specific antibacterial activity

Meng-Lin Lee[†] , Zhitao Wu[†], Xingyu Zhou, Yishu Xiao, Ruilin Lai, Shuang Song, Bingjie Yan, and Jinmei Huang*

Department of Research and Development, Green Long Biotech Co., Ltd., Wuhan, Hubei, China

Abstract

The global rise of multidrug-resistant Gram-positive pathogens demands new antimicrobial strategies. Phage-derived endolysins such as PlySs2 show promising cell-wall targeting activity but may be limited by strain-dependent cell-envelope accessibility. We engineered Art-15, a chimeric lysin that fuses the lipid II-targeting peptide NZ2114 to PlySs2, to test whether enhanced targeting improves antibacterial activity. Art-15 was constructed using a (GGGS)₃ linker, expressed in *E. coli*, and purified by nickel affinity chromatography. Structural features were analyzed using AlphaFold2 and a transmembrane hidden Markov model. Antibacterial activity was evaluated through spot-on-lawn assays, minimum inhibitory concentration (MIC) determination, and five-hour OD₆₀₀ turbidity assays. Art-15 was successfully expressed, with a recoverable soluble fraction corresponding to a ~36 kDa recombinant protein. Structural prediction indicated a compact fold without transmembrane helices. In spot assays, Art-15 showed a narrower activity spectrum than PlySs2 (12/51 vs. 27/51 isolates). However, against strain STA2505001, Art-15 exhibited a lower MIC (64 µg/mL) than PlySs2 or NZ2114 alone (128 µg/mL). In OD₆₀₀ assays, Art-15 suppressed growth more effectively over five hours than either parental component. Art-15 appears to trade broader-spectrum activity for increased strain-specific potency. The findings suggest NZ2114 may contribute to altered cell-envelope targeting and local exposure of the PlySs2 catalytic domain to peptidoglycan, although host-range narrowing indicates target accessibility remains strain-dependent.

[†]These authors contributed equally to this work.

*Corresponding author:

Jinmei Huang
(jinmei.huang@greenlongbio.com)

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1. Introduction

The rapid global spread of multidrug-resistant Gram-positive pathogens, notably methicillin-resistant *Staphylococcus aureus* and various *Streptococcus* species, poses a formidable challenge to contemporary medicine and agriculture.^{1,2} As the developmental pipeline for conventional small-molecule antibiotics continues to stagnate, there is a critical imperative to establish alternative antimicrobial paradigms.³ Bacteriophage-derived endolysins have emerged as highly promising candidates in this domain.⁴⁻⁶ Recent advances in molecular engineering and translational studies have further expanded their potential as precision antimicrobial agents against multidrug-

resistant pathogens.⁷ Unlike traditional antibiotics that inhibit intracellular macromolecular synthesis, endolysins actively hydrolyze the highly conserved bonds within the bacterial peptidoglycan sacculus, inducing rapid osmotic lysis.⁸ Because they target structures essential for bacterial viability and operate independently of the pathogen's metabolic state, resistance to lysins is fundamentally low.⁹

Among characterized endolysins, PlySs2 (Figure S1), isolated from the streptococcal phage Ss2, has demonstrated notable efficacy.⁹ It features a typical bipartite architecture comprising an N-terminal catalytic cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain and a C-terminal SH3b cell-wall binding domain that confers target recognition.^{10,11} While PlySs2 exhibits a relatively broad lytic spectrum against several Gram-positive genera *in vitro*, the absolute therapeutic efficacy of native lysins is frequently constrained under complex physiological conditions.^{12,13} Structural barriers within bacterial envelopes, such as dense capsular polysaccharides, varying degrees of peptidoglycan cross-linking, and modified wall teichoic acids, can sterically hinder the accessibility of the catalytic domain to its substrate, thereby dampening overall bactericidal kinetics and *in vivo* potency.^{14–16}

To circumvent these inherent structural limitations, rational protein engineering strategies have been increasingly employed.^{17,18} Chimeric lysins, often termed artilysins, generated by fusing native endolysins with highly cationic or amphipathic antimicrobial peptides, provide a robust mechanism to enhance outer envelope permeabilization and target affinity.^{19–24} In this context, NZ2114 (Figure S2) represents an ideal fusion candidate. A well-characterized derivative of the fungal defensin plectasin, NZ2114 exhibits exceptional targeted potency against staphylococci and streptococci.^{25,26} Its mechanism of action is distinctly decoupled from mere generalized electrostatic disruption; instead, it specifically binds to lipid II, the highly conserved, membrane-anchored precursor of peptidoglycan synthesis.²⁷ We hypothesized that covalently linking NZ2114 to the N-terminus of PlySs2 via a flexible (GGGGS)₃ linker²⁸ would synergize these complementary mechanisms, tethering a potent peptidoglycan hydrolase directly to critical lipid II targets at the bacterial membrane.

In the present study, we hypothesized that fusing NZ2114 to PlySs2 via a flexible (GGGGS)₃ linker could enhance local targeting at the bacterial surface while maintaining the catalytic activity of the endolysin domain. We therefore constructed a novel engineered lysin, designated Art-15, and evaluated its structural characteristics and antibacterial performance. We further observed a notable functional shift in Art-15 compared with its parental components.

While the engineered enzyme exhibited a reduced lytic spectrum relative to PlySs2, it demonstrated significantly enhanced bactericidal potency against susceptible strains. This apparent trade-off between host range and killing efficiency suggests that peptide-mediated targeting may alter initial cell-envelope recognition events and enzyme–substrate interactions. Through structural modeling and dynamic functional assays, we aim to map this functional shift to a synergistic disruption mechanism, offering new mechanistic insights for the rational design of precision enzibiotics.^{29–32}

2. Materials and methods

2.1. Study design and bacterial strains

This study evaluated the antibacterial activity of engineered lysin Art-15 compared with NZ2114 and PlySs2 against *Staphylococcus* spp. A panel of clinical isolates of *Staphylococcus* spp. was included to ensure broad relevance and reproducibility of the results. Clinical strains were obtained from routine laboratory collections. Detailed information on all bacterial strains, oligonucleotide primers, and plasmids used in this study is provided in Supplementary Table 1.

All bacterial strains were routinely cultured in Luria–Bertani broth or brain heart infusion medium depending on their growth requirements. Cultures were incubated at 37 °C with shaking at 200 rpm to maintain adequate aeration and homogeneous growth, unless otherwise specified. For experimental assays, overnight cultures were subcultured into fresh medium and grown to the logarithmic phase to ensure consistent physiological states prior to treatment.

2.2. Construction, expression, and purification of the engineered lysin Art-15, NZ2114, and PlySs2

The engineered lysin Art-15 was designed by fusing the antimicrobial peptide NZ2114 to the N-terminus of the phage lysin PlySs2 via a flexible (GGGGS)₃ linker. This glycine–serine-rich linker was selected because its flexibility and low immunogenicity minimize steric interference between fused domains, allowing the NZ2114, CHAP, and SH3b modules to fold and function independently while providing sufficient spacer length to preserve their conformational mobility.²⁸ The fusion gene was constructed using overlap extension polymerase chain reaction. The PCR amplification was performed using PrimeSTAR® GXL Premix Fast, Dye Plus (Takara Bio Inc., Japan) under the following conditions: initial denaturation at 98 °C for 2 min; 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 5 s, and extension at 68 °C for 10 s; followed by a final extension at 68 °C for 2 min and

a hold at 4 °C. The resulting product, along with the genes encoding NZ2114 and PlySs2 individually, was cloned into the pET-28a expression vector (2× MultiF Seamless Assembly Mix; catalog no. RK21020, Technology Co., Ltd., China) and verified by DNA sequencing. The Sanger sequencing was outsourced to Tsingke Biotechnology Co., Ltd. (China). Clones were sequenced using the T7 promoter (T7P) and T7 terminator (T7t) primers. Because NZ2114 alone is poorly expressed in soluble form, an N-terminal His-SUMO tag was fused to NZ2114 for standalone expression and purification, whereas Art-15 and PlySs2 each carry only a shorter N-terminal His-tag; all tags were encoded by the pET-28a vector and were not part of the amplified coding sequences. Therefore, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis in Figure 1 represents the purified recombinant forms His-SUMO-NZ2114, His-PlySs2, and His-Art-15. The amino acid lengths listed in Table 1 correspond to the recombinant proteins, including affinity tags, whereas the amplified DNA fragments correspond only to the target coding regions. The complete amino acid sequences of recombinant proteins, including affinity tags and linker regions, are provided in Supplementary Table 2. Recombinant plasmids were transformed into *Escherichia coli* BL21(DE3).³³ Protein expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside at mid-logarithmic phase. Cells were harvested, lysed by sonication, and the soluble and insoluble fractions were separated by centrifugation. The soluble fraction of each protein was purified using nickel affinity chromatography. Nickel affinity chromatography was performed using nickel affinity resin (catalog no. HP201; Vazyme Biotech Co., Ltd., China) packed into an empty chromatography column (catalog no. FCL12; Beyotime Biotechnology, China). Purity and molecular weight of the pellet, soluble supernatant, flow-through, wash, elution, and stripping fractions were assessed by SDS–PAGE for each protein. Prior to use in the functional assays described in Sections 2.4–2.6, the His-SUMO tag was removed from purified NZ2114 by digestion with SUMO protease (Shanghai Yuanye Bio-Technology Co., Ltd, China), and the cleaved, untagged NZ2114 (~40 aa) was separated from the released His-SUMO tag and any uncleaved protein by a second nickel–nitrilotriacetic acid pass. The His-tags on Art-15 and PlySs2 were retained throughout all functional assays.

2.3. Physicochemical and bioinformatic analysis

The molecular weight, isoelectric point, instability index, and hydrophobicity of Art-15, NZ2114, and PlySs2 were predicted using the ExPASy ProtParam online tool. The structural model of Art-15 was generated with AlphaFold2, and the top-ranked model (rank 0) with the highest average

predicted Local Distance Difference Test score was selected for subsequent structural analysis and visualization using PyMOL (version 3.1.6.1). Transmembrane helices were predicted using the transmembrane hidden Markov model (TMHMM) server v2.0. Functional domains were identified based on conserved sequence motifs and domain databases, including Pfam (version 37.0) and InterPro (version 108.0).

2.4. Lytic spectrum analysis

The lytic activity of Art-15, NZ2114, and PlySs2 was evaluated using a double-layer agar method. Briefly, an overnight culture of *Staphylococcus* was mixed with molten soft agar (0.6%–0.7% agar) and overlaid onto solidified base agar plates to form a uniform bacterial lawn. After the top agar solidified, 10 µg of each antibacterial agent (Art-15, NZ2114, and PlySs2) in 50 µL, corresponding to 200 µg/mL was carefully spotted onto the surface of the bacterial lawn. The plates were then incubated at 37 °C for 16–20 h. Lytic activity was assessed based on the appearance of clear or turbid lysis zones. Clear zones indicated strong bacteriolytic activity, and turbid zones indicated partial or weaker activity.

2.5. Minimum inhibitory concentration determination

The minimum inhibitory concentrations (MICs) of Art-15, NZ2114, and PlySs2 against *Staphylococcus* strains were determined by broth microdilution in 96-well plates. Briefly, two-fold serial dilutions of each antibacterial agent were prepared in Mueller–Hinton broth across the wells using a multichannel pipette to ensure consistency and efficiency. Bacterial cultures were grown to the logarithmic phase and adjusted to a standardized inoculum (approximately 5×10^5 CFU/mL), then added to each well to achieve a uniform final volume. The plates were incubated at 37 °C for 16–20 h under appropriate conditions. After incubation, bacterial growth was evaluated visually based on turbidity. The MIC was defined as the lowest concentration of the tested agent that completely inhibited visible bacterial growth in the well.

2.6. Turbidity reduction assay

Turbidity reduction kinetics of Art-15, NZ2114, and PlySs2 were evaluated using *Staphylococcus* cultures in a shake-flask system. Briefly, bacterial cells were grown to the logarithmic phase and diluted into fresh Luria–Bertani broth to obtain a standardized initial inoculum. A total volume of 200 mL of culture was prepared in 500 mL Erlenmeyer flasks to ensure sufficient oxygen transfer and proper mixing during incubation. Art-15, NZ2114, and PlySs2 were added at final concentrations corresponding

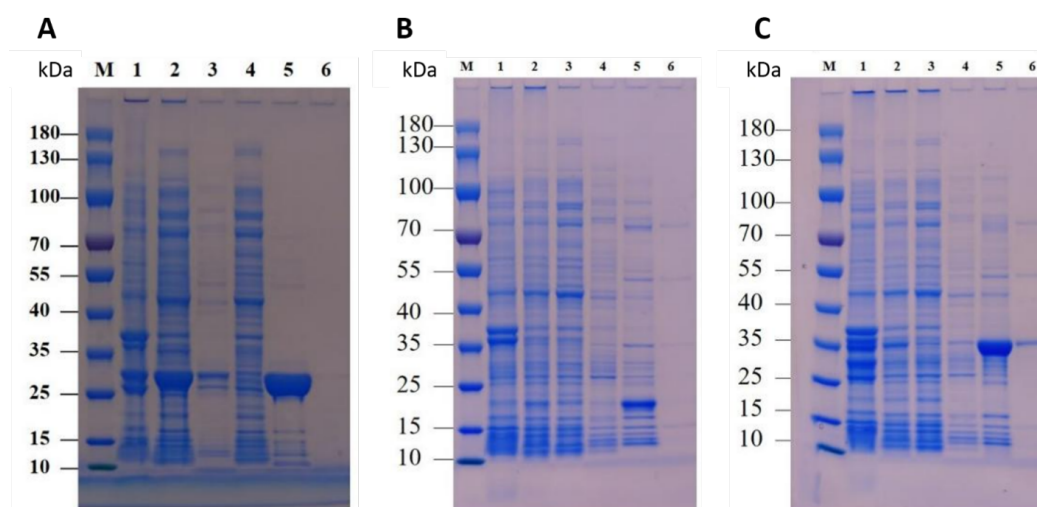


Figure 1. Expression and purification of recombinant His-Art-15 (36.69 kDa), His-SUMO-NZ2114 (19.94 kDa), and His-PlySs2 (29.60 kDa). (A) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of His-PlySs2 expression and purification. M: protein marker; Lane 1: pellet fraction; Lane 2: soluble supernatant fraction; Lane 3: column flow-through fraction; Lane 4: wash fraction (20 mM Tris–hydrochloric acid [HCl], 300 mM sodium chloride [NaCl], 20 mM imidazole); Lane 5: elution fraction (20 mM Tris–HCl, 300 mM NaCl, 250 mM imidazole); Lane 6: column stripping fraction (20 mM Tris–HCl, 300 mM NaCl, 500 mM imidazole). (B) SDS–PAGE analysis of His-SUMO-NZ2114 expression and purification. M: protein marker; Lane 1: pellet fraction; Lane 2: soluble supernatant fraction; Lane 3: column flow-through fraction; Lane 4: wash fraction; Lane 5: elution fraction; Lane 6: column stripping fraction. (C) SDS–PAGE analysis of His-Art-15 expression and purification. M: protein marker; Lane 1: pellet fraction; Lane 2: soluble supernatant fraction; Lane 3: column flow-through fraction; Lane 4: wash fraction; Lane 5: elution fraction; Lane 6: column stripping fraction.

to $2\times$ MIC values for the tested strain, and the flasks were incubated at 37 °C with shaking at 200 rpm. At predetermined time points, aliquots were withdrawn, and bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (752N UV–visible spectrophotometer; Shanghai Yoke Instrument Co., Ltd., China). Growth curves were generated to assess the growth-suppressive effects of each agent over time, with reductions or delays in OD_{600} compared to the untreated control indicating inhibitory activity.

2.7. Statistical analysis

Experiments were executed in biological triplicates. Variance was evaluated using standard parametric tests, with data expressed as mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad Prism version 9.5 (GraphPad Software, United States). A p -value < 0.05 was considered statistically significant.

3. Results

3.1. Construction and architectural profiling of Art-15

Overlap extension polymerase chain reaction successfully generated the Art-15 fusion construct, producing a single

903 bp DNA fragment consistent with the theoretical size of the fused gene. Agarose gel electrophoresis showed a clear and specific band at the expected position, with no detectable nonspecific amplification, confirming the accuracy and efficiency of the fusion process (Figure 2).

Following isopropyl β -D-1-thiogalactopyranoside induction in *Escherichia coli* BL21 (DE3), Art-15 was expressed, with a substantial fraction retained in inclusion bodies; 59.57% of the target band intensity was in the soluble supernatant versus 40.43% in the pellet fraction by densitometric analysis (Figure 1C). Sufficient soluble protein was recovered for downstream nickel–nitrilotriacetic acid purification. SDS–PAGE analysis showed a dominant protein band at approximately 36 kDa in the soluble fraction, consistent with the expected molecular mass of His-Art-15 (Figure 1C). Compared with His-PlySs2 (~30 kDa), the increased molecular size of His-Art-15 confirmed successful fusion of the NZ2114 module to the N-terminus of PlySs2 (Figure 1A and 1C).

For NZ2114 production, the recombinant protein was expressed as His-SUMO-NZ2114 to improve soluble expression and purification efficiency. The SDS–PAGE profile shown in Figure 1B therefore represents the His-SUMO-tagged NZ2114 construct rather than the untagged NZ2114 peptide used in subsequent functional assays.

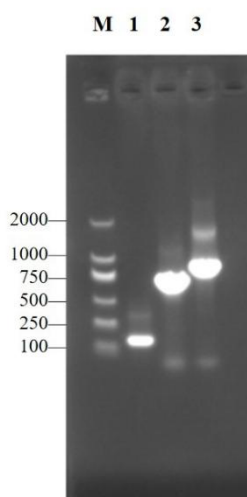


Figure 2. Construction of the Art-15 fusion gene. Agarose gel electrophoresis analysis of overlap extension polymerase chain reaction products showing successful amplification of the Art-15 fusion construct (903 bp). M: DNA marker; Lane 1: amplified NZ2114 fragment; Lane 2: amplified PlySs2 fragment; Lane 3: amplified Art-15 fusion fragment.

Physicochemical analysis (Table 1) showed that Art-15 exhibits a theoretical isoelectric point ($pI = 9.14$), which is comparable to that of PlySs2 ($pI = 9.34$) but substantially higher than that of NZ2114 ($pI = 8.62$).

In terms of hydrophobicity, the grand average of hydropathicity value of Art-15 (-0.366) is slightly lower than that of PlySs2 (-0.315), but markedly higher than that of NZ2114 (-0.672). This shift indicates that Art-15 possesses intermediate amphipathic properties, suggesting a balance between hydrophilic and hydrophobic regions that may facilitate membrane interaction while maintaining solubility.

Functional domain mapping (Figure 3A) showed that Art-15 preserves the canonical CHAP catalytic domain and the SH3 cell-wall binding domain originating from PlySs2, while incorporating the NZ2114 peptide at the N-terminus as an additional module. Structural modeling (Figure 3B) suggests that Art-15 adopts a well-folded, compact three-dimensional structure, with the catalytic and cell-wall binding domains maintaining their canonical architectures. The overall model indicates that the N-terminal fusion does not disrupt the global folding of the protein, supporting the preservation of enzymatic functionality.

Transmembrane topology prediction using the TMHMM server indicates that Art-15 lacks any predicted transmembrane helices (Figure 3C). The posterior

probability plot shows consistently high probabilities for an extracellular location and very low probabilities for transmembrane or intracellular regions, suggesting that Art-15 is unlikely to span membranes. Instead, it may transiently associate with bacterial membranes, promoting membrane destabilization and allowing its catalytic domain to access the peptidoglycan substrate.

3.2. Restricted lytic spectrum but amplified specific potency

To map the host range of the engineered chimera, spot-on-lawn diffusion assays were conducted across a panel of 51 clinical isolates. The resulting lysis heatmap (Figure 4A) revealed that Art-15 exhibited a narrower lytic spectrum than the broad-acting wild-type PlySs2, with Art-15 lysing only 12/51 strains compared to 27/51 for PlySs2. Several strains previously susceptible to native PlySs2 were insensitive to Art-15, indicating a shift in targeted recognition.

Despite this restricted host range, Art-15 showed a 2-fold lower MIC than both parental components against one of the three MIC-tested strains (Table 2). This enhancement was not consistent across all strains tested: against JP2409004, PlySs2 alone was 8-fold more potent than Art-15, and against JP231001, Art-15 and PlySs2 showed equal potency (Table 2). Representative clearing zones (Figure 4B) on these susceptible lawns were also notably larger and more distinct.

Table 1. Physicochemical characteristics of Art-15, NZ2114, and PlySs2 predicted using bioinformatic analysis tools

Parameters	Art-15	NZ2114	PlySs2
Molecular weight/kDa	36.69	4.4	29.6
Instability index	26.49	25.49	23.81
Grand average of hydropathicity	-0.366	-0.672	-0.315
Number of amino acids	334	40	279
Total number of positively charged residues	29	3	23
Total number of negatively charged residues	20	6	17
Theoretical isoelectric point	9.14	8.62	9.34

Note: Values shown are theoretical parameters of the proteins as expressed and purified, including their affinity tags: His-PlySs2 (279 aa, 29.6 kDa), His-Art-15 (334 aa, 36.69 kDa), and NZ2114 (40 aa, 4.4 kDa).

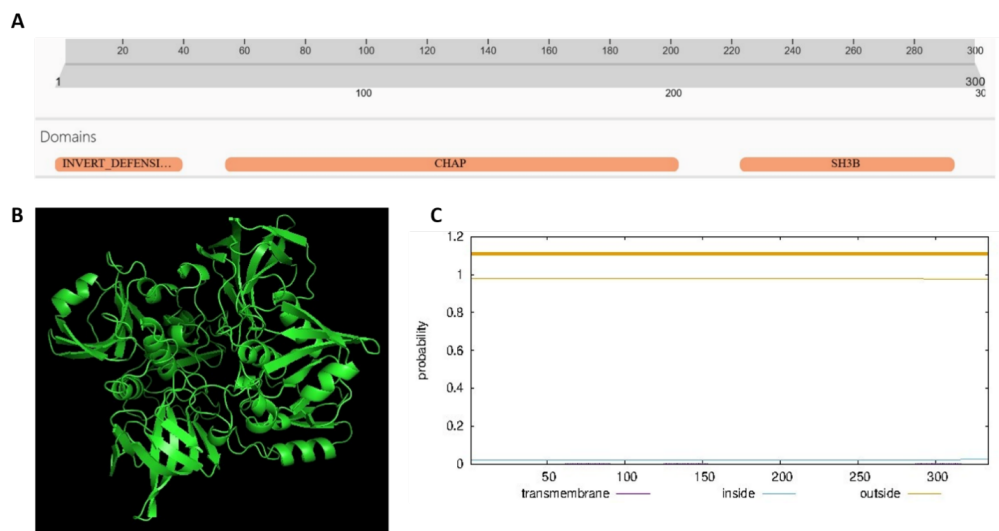


Figure 3. Structural and bioinformatic characterization of Art-15. (A) Schematic representation of the domain architecture of Art-15 showing the NZ2114 peptide, flexible linker, cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) catalytic domain, and SH3b cell-wall binding domain. (B) AlphaFold2-predicted three-dimensional structure of Art-15 visualized using PyMOL. (C) The transmembrane hidden Markov model prediction profile indicating the absence of transmembrane helices in Art-15.

Table 2. Minimum inhibitory concentrations of Art-15, PlySs2, and NZ2114 against different *Staphylococcus aureus* strains

Protein	STA2505001	JP2409004	JP231001	Art-15 vs. PlySs2	Art-15 vs. NZ2114
Art-15	64 µg/mL	64 µg/mL	128 µg/mL	2-fold better	2-fold better
PlySs2	128 µg/mL	8 µg/mL	128 µg/mL	8-fold worse	2-fold better
NZ2114	128 µg/mL	128 µg/mL	256 µg/mL	Equal	2-fold better

3.3. Enhanced growth suppression of Art-15 revealed by turbidity reduction kinetics

To evaluate the dynamic antibacterial activity, turbidity reduction assays were performed by monitoring OD₆₀₀ over a five-h period (Figure 5). The growth curve of the untreated *S. aureus* STA2505001 control increased steadily from an initial OD₆₀₀ of ~0.1 to ~0.6 at five h. In comparison, the Art-15-treated culture exhibited rapid growth suppression, with OD₆₀₀ decreasing slightly initially and remaining at 0.2 throughout the assay, demonstrating near-complete inhibition of bacterial growth. By contrast, PlySs2 and NZ2114 caused only moderate suppression: OD₆₀₀ increased gradually to ~0.29 and ~0.23, respectively, over the same period. These kinetic profiles indicate that although Art-15 has a narrower host range, its antibacterial efficiency against susceptible strains is substantially higher, achieving strong inhibition far faster than either parental component.

4. Discussion

The engineered lysin Art-15, generated by fusing the cationic antimicrobial peptide NZ2114 to the N-terminus of PlySs2 via a flexible (GGGGS)₃ linker, represents a rational design to enhance antibacterial potency without disrupting the canonical CHAP catalytic or SH3 cell-wall binding domains (Figure 3A–3C). The use of flexible glycine–serine linkers is a well-established strategy to ensure proper domain folding and preserve the independent functionality of fused modules.²⁸ Structural modeling and TMHMM analysis confirmed a well-folded architecture with no predicted transmembrane helices, suggesting that Art-15 transiently interacts with bacterial membranes to facilitate enzymatic access to peptidoglycan rather than spanning the membrane itself (Figure 3C).

Functional assays revealed a narrowed lytic spectrum: Art-15 lysed 12 of 51 clinical isolates, compared to 27 for

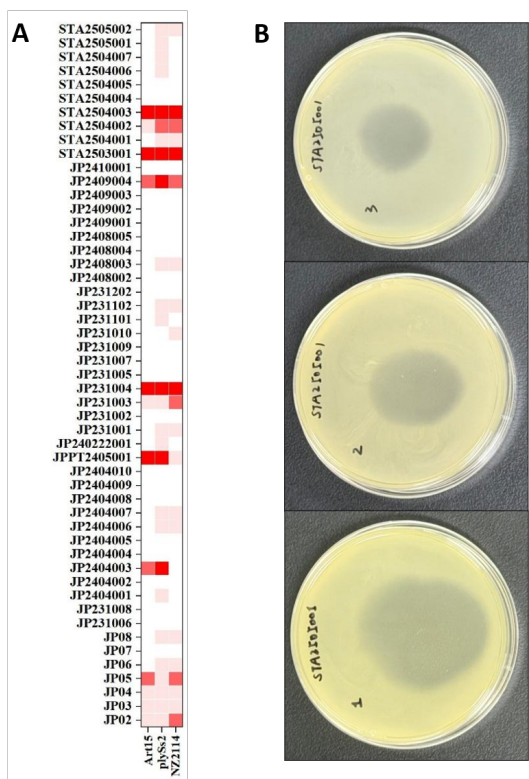


Figure 4. Lytic spectrum and antibacterial activity of Art-15, NZ2114, and PlySs2. (A) Heatmap summarizing lytic activity of Art-15, PlySs2, and NZ2114 against 51 clinical *Staphylococcus* isolates using spot-on-lawn assays. (B) Representative lysis zones produced by Art-15, PlySs2, and NZ2114 on susceptible *Staphylococcus aureus* lawns. Plate 1: Art-15; Plate 2: PlySs2; Plate 3: NZ2114. For each assay, 10 µg of recombinant protein in a total volume of 50 µL was spotted onto the bacterial lawn.

wild-type PlySs2 (Figure 4A). This narrowing likely results from cell-wall recognition mechanisms altered by the NZ2114 fusion. Native PlySs2 relies on its SH3b domain for broad recognition of conserved peptidoglycan motifs.¹⁰ In contrast, NZ2114, a derivative of the fungal defensin plectasin, specifically targets lipid II, an essential cell wall precursor located at the cytoplasmic membrane.^{26,31} We postulate that the high-affinity lipid II-targeting of the NZ2114 moiety energetically dominates the initial binding phase. Consequently, in strains where lipid II is sterically shielded by dense capsular polysaccharides or highly modified wall teichoic acids, Art-15 fails to anchor effectively, thereby restricting off-target binding and narrowing the overall host range.

Importantly, this restriction did not uniformly compromise potency against susceptible strains: against one of the three strains tested, it was accompanied by a two-fold potency gain, although this was not observed consistently across the strain panel (Table 2). MIC measurements showed that Art-15 achieved a two-fold lower MIC against the susceptible strain STA2505001 (64 µg/mL) compared with PlySs2 or NZ2114 alone (128 µg/mL) (Table 2). Representative clearing zones qualitatively supported this highly localized, enhanced lytic efficiency (Figure 4B).

Turbidity reduction assays further underscored Art-15's superior efficacy: the OD₆₀₀ of STA2505001 cultures remained near baseline (~0.2) over five h, whereas PlySs2- and NZ2114-treated cultures increased to ~0.29 and ~0.23, respectively (Figure 5). This rapid and sustained

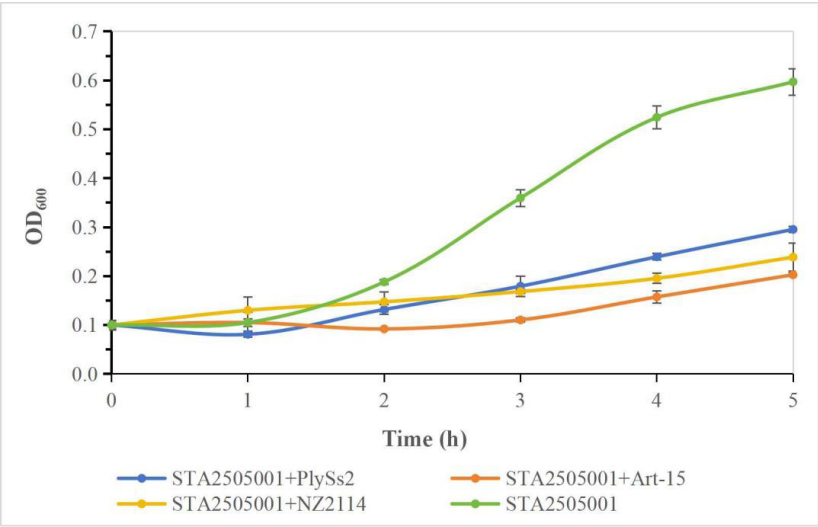


Figure 5. Growth Suppression of Art-15, PlySs2, and NZ2114 against *Staphylococcus aureus* STA2505001. Growth curves monitored by OD₆₀₀ measurements over five h following treatment with Art-15, PlySs2, or NZ2114. Untreated cultures served as controls. Data are presented as mean ± standard deviation from three independent experiments.

inhibition is consistent with a putative dual-targeting mechanism reported for other artilysins.³⁴ A non-covalent physical mixture of NZ2114 and PlySs2 was not tested in this study, so we cannot formally distinguish a synergistic interaction from an additive effect of the two fused modules; this is addressed as a limitation. We hypothesize that the NZ2114 moiety may act as a membrane-proximal anchor that increases the local concentration of the PlySs2 CHAP domain near its peptidoglycan substrate³⁵; this could, in principle, accelerate substrate access and reduce enzyme detachment. Similar cooperative effects between membrane-active peptides and endolysins have been reported, where membrane perturbation enhanced susceptibility to cell wall degradation.³⁶ Direct evidence for membrane depolarization, altered peptidoglycan-cleavage kinetics, and enzyme retention at the membrane was not obtained in the present study and would be needed to test this model directly. In other words, although the host range was narrowed due to more stringent lipid II-dependent targeting requirements³⁷, the fusion concentrated its dual-destructive activity on susceptible strains, producing stronger growth suppression.

These findings highlight a generalizable and highly valuable principle in lysin engineering: selective targeting via peptide fusion can trade broad-spectrum promiscuity for amplified, specific bactericidal potency. In the era of precision medicine, developing narrow-spectrum antimicrobials is particularly desirable to eradicate specific pathogens while preserving the patient's commensal microbiome.^{4,38,39} Future work should explore additional peptide modules, linker length optimizations⁴⁰, and systematic comparison with non-covalent physical mixtures to distinguish synergistic effects from additive interactions.⁴¹ Expanding MIC analyses across a broader panel of susceptible and resistant strains, together with colony-forming unit-based time-kill assays, will be essential to quantitatively validate bactericidal activity and define the therapeutic potential of such engineered lysins. In addition, *in vivo* efficacy studies should be conducted to further evaluate the translational potential of these engineered lysins against multidrug-resistant Gram-positive pathogens.

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Conflict of interest

Meng-Lin Lee, Zhitao Wu, Xingyu Zhou, Yishu Xiao, Ruilin Lai, Shuang Song, Bingjie Yan, and Jinmei Huang are employees of Green Long Biotech Co., Ltd. The company has filed a patent application related to the technology described in this manuscript. These interests have been disclosed and did not influence the design, execution, or interpretation of the study.

Author contributions

Conceptualization: Meng-Lin Lee

Formal analysis: Yishu Xiao

Investigation: Ruilin Lai, Shuang Song

Methodology: Zhitao Wu

Project administration: Meng-Lin Lee

Supervision: Jinmei Huang

Validation: Zhitao Wu, Xingyu Zhou, Ruilin Lai

Visualization: Yishu Xiao, Shuang Song

Writing–original draft: Meng-Lin Lee

Writing–review & editing: Bingjie Yan

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The underlying data are available through the published article “Engineering the Chimeric Lysin Art-15 via NZ2114-PlySs2 Fusion Enhances Strain-Specific Antibacterial Activity” (doi:10.5281/ZENODO.20077111). The extended data are available through the published article “Extended data for Engineering the Chimeric Lysin Art-15 via NZ2114-PlySs2 Fusion Enhances Strain-Specific Antibacterial Activity” (doi:10.5281/ZENODO.22037507). Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC BY 4.0).

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