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Lung-directed delivery of a ligand-mediated chimeric lysin has an enhanced ability to eradicate pulmonary and intracellular *Staphylococcus aureus*

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Abstract

Background Bacteriophage lysins have high antimicrobial activities with many advantages as alternatives to antibiotics, however, lysins generally do not exhibit intracellular bactericidal capabilities due to a lack of cell-penetrating properties and/or reduced activity under the intracellular environment. To address this problem, p-ClyC, an engineered chimeric lysin with a lung cell-targeting peptide, was used to kill *Staphylococcus aureus* (*S. aureus*) in vitro and in vivo.

Methods p-ClyC was constructed by fusing ClyC with a lung-directed peptide. Antimicrobial activities of the two lysins (ClyC, p-ClyC) against *S. aureus* were evaluated in vitro and in a murine lung infection model. The cell internalization of the lysins was explored using laser confocal imaging. The intracellular bactericidal efficacies of the lysins and gentamicin were evaluated using intracellular growth inhibition studies. The risk of generating antimicrobial resistance after the lysin or antibiotics treatment was investigated by deep sequencing, MIC and growth rate monitoring.

Results The bactericidal activity against pulmonary intracellular *S. aureus* of p-ClyC was obviously promoted. The treatment with p-ClyC made the surviving intracellular bacteria generate less tendence to resistance in terms of growth rates and minor alleles in genomes than the treatment with gentamicin. In murine lung infection model, the survival rate for the group of p-ClyC was significantly improved, and more pulmonary bacteria were killed by the p-ClyC than those by the ClyC.

Conclusions The lung-directed peptide-fused ClyC (p-ClyC) is a novel and effective lysin to be against intracellular *S. aureus* and a potential antimicrobial agent for therapeutics against the pulmonary infections by *S. aureus*.

Keywords Intracellular bacteria, Pulmonary infection, *Staphylococcus aureus*, Lysin, Minor alleles, Deep sequencing

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Background

Bacterial infections in the lungs can cause the severe lung injury and inflammatory reaction, leading to the acute respiratory distress syndrome (ARDS) and sepsis that pose a huge threat to patients [1]. The continuous emergence of multidrug resistant (MDR) bacteria further increases the difficulty in the clinical treatment of pulmonary infection and results in high mortality. For example, *Staphylococcus aureus* is a main etiology of community-acquired pneumonia (CAP) over the past 2 decades, with severe community-acquired pneumonia (SCAP) caused by methicillin-resistant *S. aureus* (MRSA) leading to critical illness and death [2]. Even worse, *S. aureus* can survive inside host cells and evade the immune system [3]. The persistence of intracellular *S. aureus* may lead to reinfection and treatment failure.

Bacteriophage lysins (or endolysins) exhibit rapid and efficient bactericidal activity, a low propensity for inducing drug resistance, and the ability to target multidrug-resistant bacteria, making them highly promising alternatives to traditional antibiotics [4, 5]. Although there are multiple studies reporting endolysins with high bactericidal activity, many of these reported endolysins do not exhibit intracellular bactericidal capabilities due to a lack of cell-penetrating properties and/or reduced activity under the intracellular environment [6]. Therefore, some scientists attempted to modify or construct the endolysins or antibacterial peptides to enhance intracellular bactericidal activity [6–8]. But all these mentioned endolysins or antibacterial peptides with enhanced cell-entry ability do not show the specific lung-targeting capability. Although Lin et al. used lactic-co-glycolic acid microspheres (LyIR@MS) to effectively encapsulate the bacteriolytic enzyme lysostaphin to enhance the lung-targeting ability [9], the pulmonary-targeting delivery of antibacterial agents still has inherent challenges. Developing antibacterial agents with the both advantages of lung-targeting capability and intracellular bactericidal activity could improve the therapy outcome during pulmonary infection.

At the same time, although a variety of resistance mechanisms have been documented, little is known if similar resistance would happen in the intracellular microbes during treatment. In our previous studies, we found the genetic polymorphism was a novel mechanism that could drive the bacteria adaptive evolution to obtain resistance capability in a short time after interaction with its lytic phage. Genetic polymorphism means that there are alternative bases with minor frequencies (minor allele frequency, MAF) at certain sites of the genomes besides major frequency base [10]. Studies have shown that bacteria are less likely to develop resistance to lysin than antibiotics [11, 12]. However, the genetic polymorphism of intracellular pathogens before and after lysin or antibiotic treatment has not been investigated. The study of genetic polymorphism features of intracellular pathogens would help to understand the responses of intracellular bacteria to lysin or antibiotic stress and assess the resistance risks.

In the current study, the *S. aureus* ATCC 29,213 was chosen as a model bacteria, and we attempted to engineer a protein p-ClyC, which fused a high bactericidal activity chimeric lysin ClyC [13] with a peptide reported targeting lung. This peptide has a good delivery capability targeting lung through α3β1 integrin receptor, on the surface of cells lining the lung airways and alveoli [14, 15]. The intracellular bactericidal activity of ClyC and p-ClyC were compared with gentamicin since gentamicin showed high intracellular bactericidal activity in A549 cell after relatively long treatment. Both ClyC and p-ClyC showed general good intracellular bactericidal activities in most cell lines, and p-ClyC displayed an enhanced intracellular bactericidal performance in A549 cells. The results from the deep sequencing analysis demonstrated that the intracellular bacteria were less evolved to counter the stress from both ClyC and p-ClyC than those from gentamicin. Further animal experiments indicated that p-ClyC and ClyC can obviously inhibit the growth of pulmonary bacteria in murine model and the survival rate of mice treated with p-ClyC was higher than that treated with ClyC at the same dose.

Materials and methods

Bacterial strains and culture conditions

S. aureus ATCC 29,213 were grown from a single colony in Luria-Bertani (LB) at 37 °C. *Escherichia coli* BL21 (DE3), used for gene cloning and protein expression, was grown in LB medium supplemented with 50 μg/mL of kanamycin. Bacterial loads in mice lung were counted in Baird-Parker agar plates containing 5% egg-yolk tellurite emulsion [16, 17].

Construction of expression plasmids

ClyC is a chimeric lysin with high activity of killing *Staphylococcus aureus in vivo* and in vitro constructed in our laboratory [13], and p-ClyC was constructed by fusing ClyC with the C-terminal ligand CAKSMGDIVC with lung-directed delivery capability. The plasmid constructs used in this work were generated using homologous recombination cloning techniques. DNA fragments of the ligand and ClyC were amplified with the Phusion high-fidelity DNA polymerase (New England BioLabs, Allschwil, Switzerland), using the primers listed in Supplementary Table 1. p-ClyC gene were obtained using ligand-F and ligand-R as primers and pET28a-ClyC as a template. Then the fragment amplified by p-ClyC-F and p-ClyC-F using p-ClyC as a template was cloned into the pET28a (+) plasmid (GenBank: MK847907.1) through

NcoI and XhoI sites using ClonExpress Ultra One Step Cloning Kit (Nanjing Vazyme Biotech Co., Ltd, Nanjing, China). Finally, the recombinant plasmid pET28a-p-ClyC was transformed into *E. coli* BL21 (DE3). The sequence of the p-ClyC gene was confirmed by Sanger sequencing.

Protein structure prediction and structural alignment

The protein structure predictions of both ClyC and p-ClyC were performed by the Alphafold2 pipeline [18]. Structure confidence was analysis by Chimera X software through predicted aligned error measure [19]. As mentioned in the study of Zhou et al., the root-mean-square-deviation (RMSD) based method is a golden rule to measure the structural similarity when the protein structures are highly identical [20]. Thus, our protein three-dimensional structure comparison between ClyC and p-ClyC was analyzed via the pymol software (version: 2.52, https://pymol.org/) with the default options, and the structural similarity between these two proteins was evaluated by the RMSD values in the best-superimposed atomic coordinates.

Protein expression and purification

Protein ClyC and p-ClyC were expressed in *E. coli* BL21 (DE3) cells and purified essentially as previously described [13]. In brief, cultures were grown to an optical density at 600 nm (OD_{600}) of 0.45 at 37 °C under agitation in LB medium containing 50 μg/mL of kanamycin. Protein expression was induced with 0.25 mM isopropyl β-D-thiogalactoside (IPTG) for 18 h at 16 °C under agitation. Cells were then harvested by centrifugation at 8000 × g for 5 min, resuspended in lysis buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}H_2O$, 1.4 mM KH_2PO_4, 20 mM imidazole, pH 7.4), lysed by a cell disrupter on ice, and finally centrifuged at 10,000 × g for 30 min to remove cell debris. His-tagged proteins were purified by affinity chromatography using nickel nitrilotriacetic acid columns. Target proteins were collected by washing and eluting with 40- and 250-mM imidazole, respectively. Collected proteins were dialyzed against PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}H_2O$, 1.4 mM KH_2PO_4, pH 7.4). The protein was then passed through a Detoxi-Gel™ Endotoxin Removing Gel (Thermo Scientific, Waltham, MA, USA) and quantified by a ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Nanjing, China). To test for protein identity and purity, protein was analyzed by SDS-PAGE. All purified proteins were sterile filtered and stored at 4 °C.

Extracellular lytic activity assay

The bacteriolytic activity of ClyC and p-ClyC against *S. aureus* ATCC 29,213 was determined as described previously with minor modifications [13]. Briefly, bacterial cells were grown to an OD_{600} of 1.0-1.2, centrifuged at 10,000 × g for 1 min, and resuspended in PBS to a final OD_{600} of 0.6. Then, 190 μL bacterial suspension was mixed with ClyC and p-ClyC (10 μL, a final concentration of 20 μg/mL) in 96-well plates. The turbidity was monitored at 1 min intervals using a Synergy H1 microplate reader (BioTek, USA) for 45 min at 37 °C. PBS treated wells were used as controls. All experiments were performed in triplicate.

Epithelial cell lines and culture conditions

We used the following cell lines in this study: (a) A549, human lung epithelial cell line; (b) Caco-2, human intestinal epithelial cell line; (c) Hela, human cervical epithelial cell line. A549 (ID: IVCAS9.096), Caco-2 (ID: IVCAS9.106) and Hela (ID: IVCAS9.090) were obtained from Preservation Center, Wuhan Institute of Virology, CAS. A549 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Thermo Scientific) supplemented with 10% Fetal Bovine Serum (FBS) at 37 °C under 5% CO_2. Caco-2 and Hela cells were grown in Dulbecco's modified Eagles medium (DMEM, Thermo Scientific) with the same conditions as above. The number of passages was not more than 15 times to ensure the genetic stability of the cells.

Cytotoxicity assay

A549 (1×10^4 cells/well), Caco-2 (1.8×10^4 cells/well), and Hela (1×10^4 cells/well) cells were seeded into 96-well plates and cultured for 24 h. Different concentrations of ClyC and p-ClyC (10, 20, 40, 80, and 120 μg/mL) were added to each well for 24 h. The viability of cells was determined using Cell Counting Kit-8 (CCK-8) assay. After culture, 10 μL of CCK-8 solution was added to all tested wells and control wells and incubated for 4 h at 37 °C under 5% CO_2. The absorbance was recorded at 450 nm on a microplate reader. All experiments were performed in triplicate.

Intracellular microscopy

Fluorescently labeling of proteins and microscopy refer to previous study [7]. The epithelial cells were incubated with 40 μg/mL AlexaFluor 488 succinimidyl ester dye (Invitrogen, Carlsbad, CA) labeled ClyC and p-ClyC in serum-free medium for 4 h. The cells were then fixed by 4% paraformaldehyde (PFA) for 15 min. After that, the cells were stained with ActinRed555 (Invitrogen, Carlsbad, CA) for 30 min and 1 μg/ml DAPI for 10 min. Images were obtained by an STELLARIS 8 confocal fluorescence microscope (Leica, German). The fluorescence was measured from three independent fields of each sample and the lysin intensity per cell was calculated using LAS X Core 3.7.4 software.

Intracellular bacteria killing assay

The intracellular bacteria killing activity of lysins and gentamicin were evaluated as described previously [7]. Briefly, 1×10^5 A549 or Hela cells, 1.8×10^5 Caco-2 cells were seeded into 24-well plates for 24 h before infection, respectively. On the day of infection, a log-phase culture of *S. aureus* strains ATCC 29,213 was washed twice with PBS and resuspended in serum-free medium. The MOI used for the infection was 10. After cocultured for 1.5 h, cells were washed three times with PBS to remove extracellular bacteria and treated with 100 μg/ml gentamicin in serum-free medium for 1 h to kill adherent bacteria. Cells were washed three times with serum-free medium and then different concentrations of ClyC, p-ClyC or gentamicin in fresh serum-free medium were added to the cells and incubated for 4 h at 37 °C and 5% CO2. Control without treatment was included in the assay. Subsequently, cells were washed three times with PBS, digested with 100 μL trypsin (trypsin-0.25% EDTA, Gibco) and lysed by adding 400 μL of 0.025% Triton X-100 solution for 5 min at 37 °C [7]. The cell lysate was serially diluted in PBS, plated on LB plates, and incubated at 37 °C overnight. The clearance rate of intracellular bacteria = $(N_{control} - N_{treatment}) / N_{control}$. Where $N_{control}$ is the number of viable bacteria in the control group and $N_{treatment}$ is the number of viable bacteria in the treatment group. All experiments were performed in triplicate.

Intracellular bacteria killing assay by serial passage

2 mL of A549 (1×10^6 cells/well) were seeded into 6-well plates and incubated for 24 h before infection. At the first infection, a log-phase culture of *S. aureus* ATCC 29,213 was washed once and resuspended in DMEM/F12 medium. The MOI used for the infection was 20. After co-cultured for 1.5 h, remove the extracellular bacteria and kill the adherent bacteria as described above. Then the cells were washed three times with the medium and then ClyC (20 μg/mL), p-ClyC (10 μg/mL) or gentamicin (4 μg/mL) were added at half lethal concentrations of intracellular *S. aureus* ATCC 29,213 and incubated at 37 °C and 5% CO_2. The group without treatment of lysin or gentamicin served as the control. After the incubation for 4 h, the intracellular bacteria were collected into 1.5 mL sterile centrifuge tube as described above. After centrifuged at 10,000 × g for 5 min and washed with PBS once, the bacteria were resuspended with 2 mL fresh DMEM/F12 medium, respectively. For the serial passage experiments, the intracellular bacteria that survived from the last generation of experiments were co-cultured with fresh cells in new round of experiments and treated in the same way as before (Fig. 1A). After four-passage experiments, the surviving bacteria were collected for further analysis.

Genome sequencing and analysis

DNA extraction and sequencing

The genome of surviving bacteria after four-passage experiments was extracted according to the instruction of QIAamp DNA Mini Kit (Qiagen, German). The libraries were constructed using TruePrep DNA Library Prep Kit V2 (Vazyme, Nanjing). Genome sequencing was done using Illumina NovaSeq 6000 sequencer.

Variation and polymorphism analyses of the bacteria genomes

The genetic variation and polymorphism analysis of the bacteria was performed according to the pipelines established in our previous publications [10, 21–23]. The reference genome and genes annotation information of *S. aureus* ATCC 29,213 were downloaded from NCBI with the accession ID CP094857.1. The bowtie2 program was performed to align the sequencing reads to the reference genome [24]. The sequencing depth was calculated based on the aligned reads. The jellyfish software (version 2.3.0) was utilized to evaluate the sequencing error rate at each site in the genome of *S. aureus* [25]. The VarScan software [26] was used to calculate minor allele frequency (MAF) based on the default options, and the genetic polymorphism sites with high confidence were identified. Finally, genes with polymorphism sites in the genomes were identified by an in-house script [22]. KEGG enrichment analysis was performed through the R package ClusterProfiler (https://cran.r-project.org/bin/windows/base/old/3.6.1/) with default options based on the genes with the polymorphism sites. All statistical analysis was performed by the R program (version 3.5.3) (https://cran.r-project.org/bin/windows/base/old/3.5.3/).

Gentamicin resistance evaluation of evolved intracellular *S. aureus*

In order to investigate whether the tolerance of the intracellular *S. aureus* after the four-passage experiments to gentamicin changed, the MICs (the minimum inhibition concentration) of gentamicin against ancestral and the fourth round evolved *S. aureus* were measured using a broth dilution method [27]. The MIC was determined as the lowest concentration of gentamicin inhibiting visible growth. In addition, we also monitored the growth rate of ancestral and evolved *S. aureus* under 1/2 MIC gentamicin and PBS, respectively, as described previously [28]. *S. aureus* prepared in LB to a concentration of 10^6 CFU/mL was incubated in a 96-well plate for 24 h in the presence of 1/2 MIC gentamicin (2 μg/mL). PBS with the same volume as gentamicin was added to *S. aureus* as the growth control. The optical density of the wells at 600 nm (OD600) was tracked using a microplate reader (Biotek, United States). All experiments were performed in triplicate.

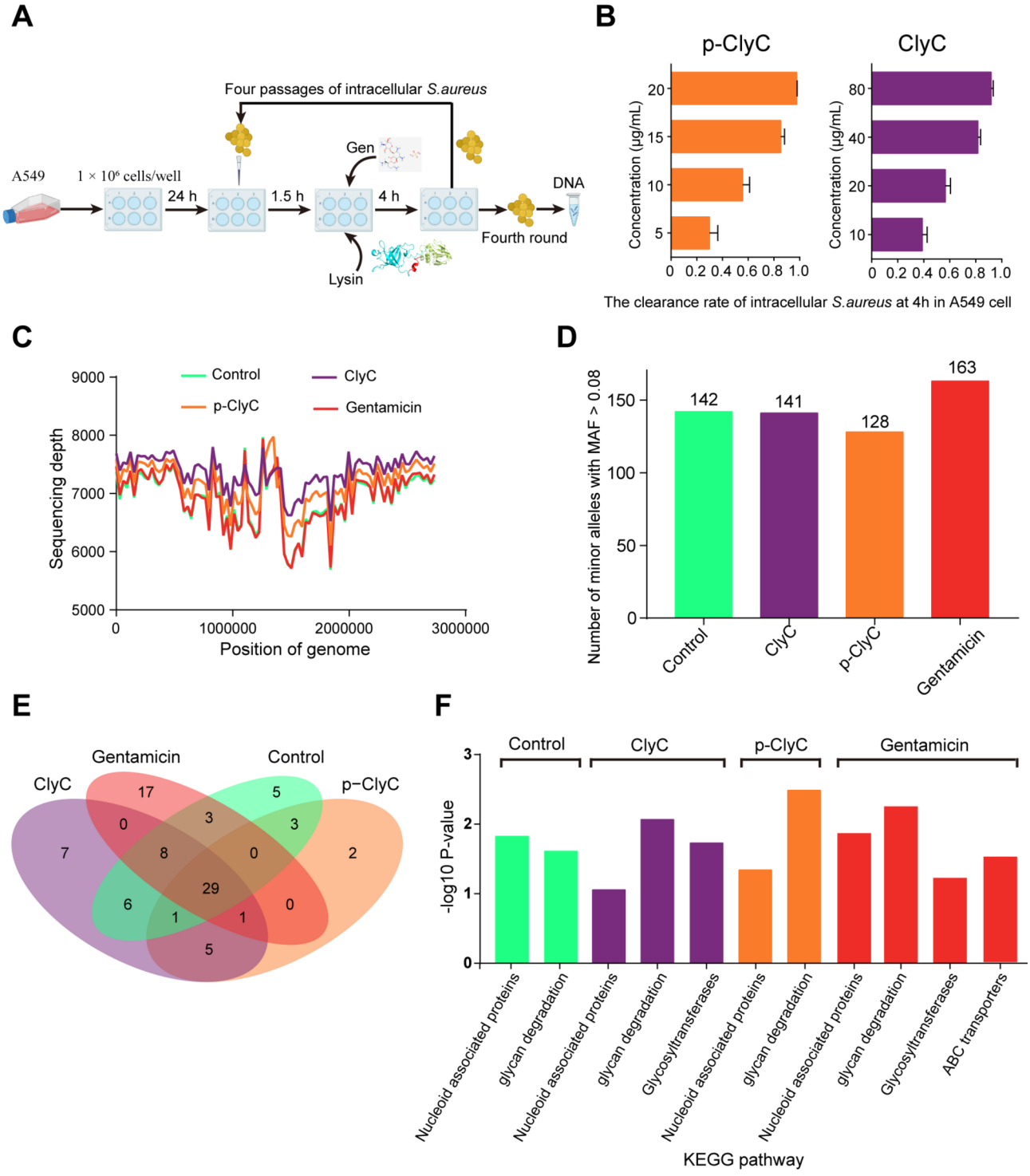

Fig. 1 Evaluation of the effect of ClyC and p-ClyC on minor alleles in the genome of *S. aureus* by deep genome sequencing. (**A**) Schematic illustration of the experiment carried out here. (**B**) The clearance rate of intracellular bacteria after treatment with different concentrations of p-ClyC or ClyC for 4 h. (**C**) Genome coverage and sequencing depth of intracellular bacteria *S. aureus* from the four groups. (**D**) Number of minor alleles with minor allele frequency (MAF) larger than 0.08 in the genomes of *S. aureus* after different treatments. (**E**) Overlap relationships among the genes with MAF larger than 0.08 in the *S. aureus* genomes from the four groups. (**F**) The most enriched KEGG pathways based on the genes with MAF larger than 0.08 in the *S. aureus* genomes from the four groups

Bactericidal efficacy in murine lung infection model

Murine lung infection model was established using BALB/C mice (18 to 22 g, 6 to 8 weeks old, female). All mice used were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were anesthetized before nasal drip injection. Anesthesia was conducted by the intraperitoneal injection of tribromoethanol (400 mg/kg).

Experiment schedule of mice study showed in Fig. 2A-B. To compare the survival rates and body weight changes among the groups with different treatments, a minimum lethal dose of *S. aureus* ATCC 29,213 at 6×10^8 CFU in 50 μL of PBS was intranasally inoculated per mouse and then held for 30 s to guarantee that each mouse inhaled the bacteria into its lungs. After 1 h of infection, each mouse was injected 50 μL lysins (15–20 μg/mouse) or PBS by nasal drip injection. The health score of mice was performed every day, and the disease status and survival number of mice were recorded. If the mice were close to the endpoint, they were euthanized. To determine the bacterial loads in the lungs of the group with different treatments, the mice were intranasally infected with 4×10^7 CFU *S. aureus* ATCC 29,213 suspensions to maintain consistent and good growth state

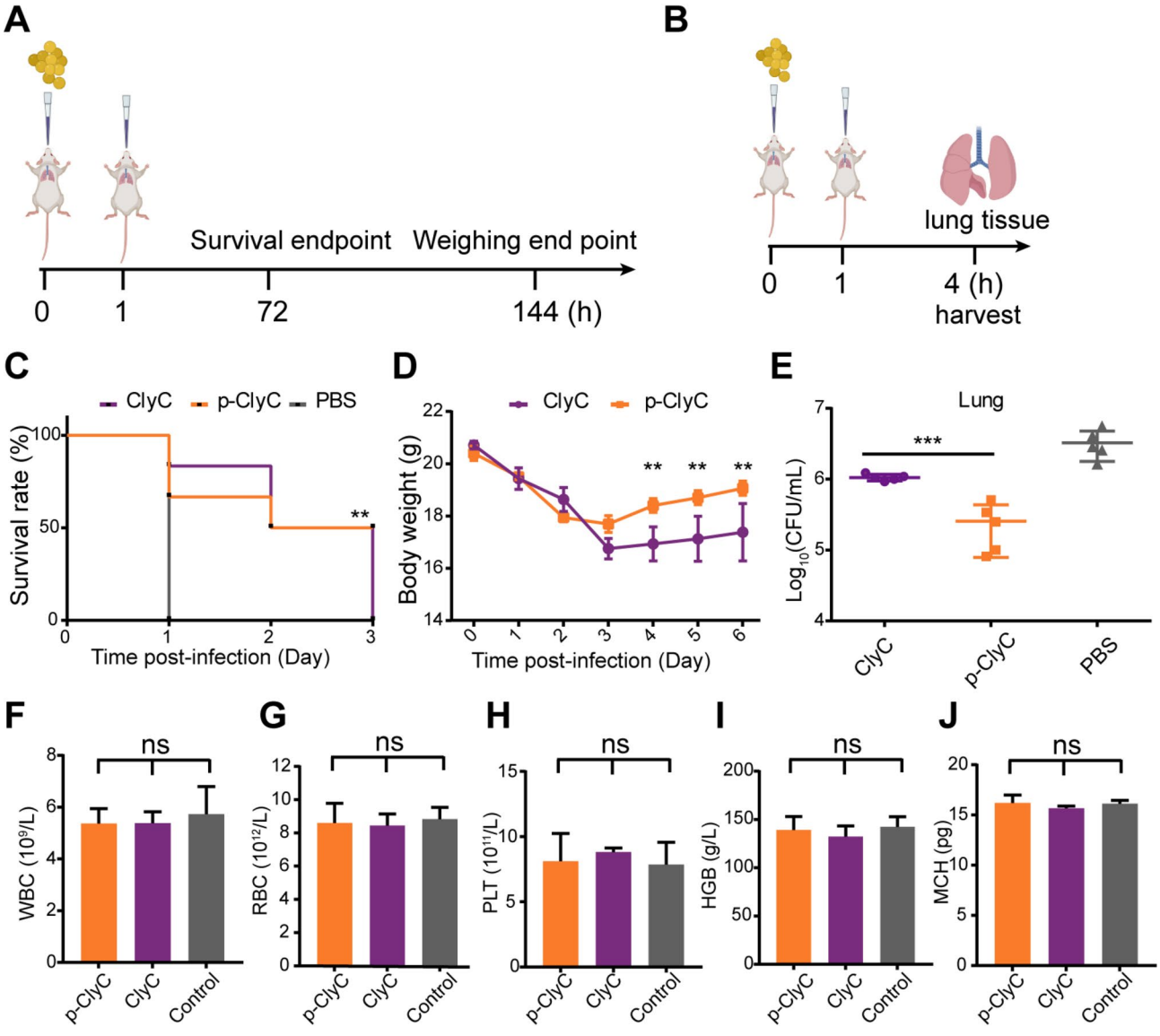

Fig. 2 Efficacy of ClyC and engineered lysin p-ClyC against *S. aureus* infection in a murine lung infection model. (**A**) Experimental scheme for the treatment of the *S. aureus* infection in a murine lung infection model. (**B**) Experimental schedule to determine the bacterial load after treatment with the lysins. (**C**) Survival rate of BABL/c mice ($n = 6$) infected with lethal dose of *S. aureus* under different treatment groups (the lysin concentration was 15 μg/mouse) and PBS). (**D**) The body weight change of survival mice ($n = 5$) treated with ClyC (20 μg/mouse) or p-ClyC (20 μg/mouse) after lethal dose infection. (**E**) The numbers of viable *S. aureus* after the different treatments were determined in the whole lung of mice ($n = 5$). F-J. Blood panel data of normal mice (Control) and mice post ClyC or p-ClyC injection ($n = 5$) after one day. All values are expressed as means ± SD

during the experiment. Then the mice were injected 50 μL lysins (20 μg/mouse) or PBS by nasal drip injection at 1 h post-infection. After 4 h, mice were sacrificed using the cervical dislocation method, and the lungs were collected, weighed, and homogenized in 1 mL of PBS along with sterile broken beads with a tissue grinder (Tissue cell-destroyer MD1000, NovaStar, Wuhan, China). The homogenates were then serially diluted in PBS, spread on Baird-Parker agar plates containing 5% egg-yolk tellurite emulsion, and incubated at 37 °C for 24 h, and then bacterial CFU was counted. Besides, the toxicity of lysins in mice was also investigated. After intranasally injected with lysins (20 μg/mouse) at 1 day, blood was collected from all the mice to detect white blood cell (WBC), red blood cell (RBC), platelets (PLT), hemoglobin (HGB), mean corpuscular hemoglobin (MCH) by an auto hematology analyzer (Mindray, Shenzhen, China). The untreated group was used as control.

Statistical analysis

The lysin intensity was assessed with a student's t test. CFU counts in lung tissue and blood tests were analyzed using a one-way ANOVA with Tukey's correction for multiple comparisons. The clearance rates of intracellular *S. aureus*, differences in bacterial growth between ancestral and evolved *S. aureus* and the weight data of mice were analyzed by a two-way analysis of variance (ANOVA). Survival curves were generated using the Kaplan – Meier method, and significance was assessed by employing the log-rank (Mantel – Cox) test. Significant differences are indicated by asterisks (ns = no significant difference; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). All the experimental results are represented as means ± S.D and $n = 3$, if not mentioned specifically. All statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Inc., San Diego, CA).

Results

Engineering the C-Terminus of ClyC

As shown in Fig. 3A, a new chimeric lysin, p-ClyC, was constructed, where the lung intracellular-targeting peptide was linked to the C-terminal of the ClyC and near to its cell-wall binding domain (CBD). The three-dimensional structures of ClyC and p-ClyC were predicted by Alphafold2 pipeline with high confidence (Fig. 3B). SDS-PAGE showed that ClyC (31 kDa) and p-ClyC (33 kDa) could be expressed as soluble proteins in *E. coli* and the purified proteins displayed high purity (> 90%) (Fig. 3C). Further, the structural comparison between ClyC and p-ClyC was analyzed. The root-mean-square-deviation (RMSD) value 0.864 calculated from the structural alignment revealed the lung intracellular-targeting peptide has almost no effect on the structure of the lysin (Fig. 3D).

Enhanced internalization ability and intracellular bactericidal activity of p-ClyC to lung epithelia A549 cells

To determine whether the bactericidal activity of engineered ClyC (p-ClyC) was affected by the short peptide, the bactericidal activity of lysins in PBS was continuously monitored. As shown in Fig. 4A, the bactericidal rate of

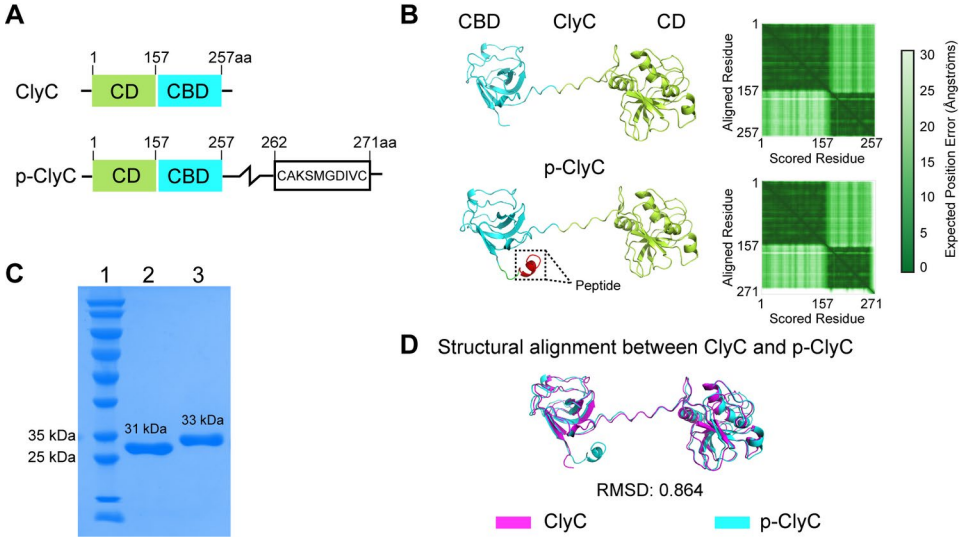

Fig. 3 Construction of p-ClyC. (**A**) Design of a C-terminal engineered lysin p-ClyC. (**B**) Three-dimensional structure of ClyC and p-ClyC predicted by Alphafold2 pipeline with high confidence based on the predicted aligned error plot. Both lysins include CHAP catalytic domain (CD) (limon) and Non-SH3b cell-wall binding domain (CBD) (cyan), while p-ClyC has an extra lung intracellular-targeting peptide CAKSMGDIVC (red). (**C**) SDS-PAGE analysis of ClyC and p-ClyC. Lane 1: Standard protein marker. Lane 2: Purified ClyC (31 kDa). Lane 3: Purified p-ClyC (33 kDa). (**D**) Structure pairwise alignment between ClyC and p-ClyC, the amino acid residues of ClyC are marked with magenta, and the amino acid residues of p-ClyC are marked with cyan. The root-mean-square-deviation (RMSD) is performed to evaluate the structural similarity. The CD and CBD regions of ClyC are referred from our previous study [13]

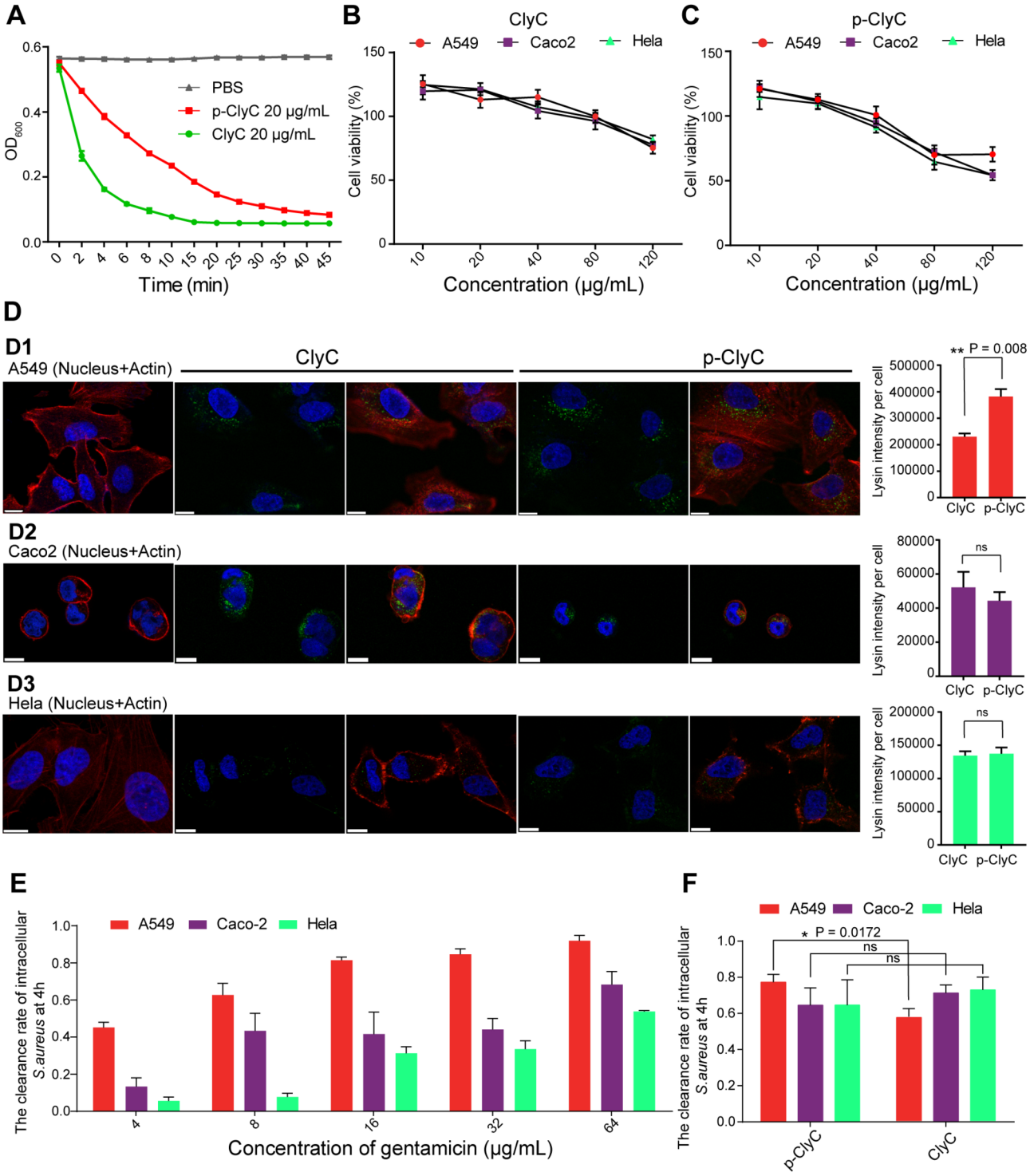

Fig. 4 Characterization of internalization ability and intracellular bactericidal activity of ClyC and p-ClyC. (**A**) Characterization of the bactericidal activities of ClyC and p-ClyC in PBS at 20 μg/mL. (**B**) Cytotoxicity of ClyC to different cell lines at different concentrations. (**C**) Cytotoxicity of p-ClyC to different cell lines at different concentrations. (**D**) Internalization of ClyC and p-ClyC by (D1) A549 cells, (D2) Caco-2 cells and (D3) Hela cells. Both ClyC and p-ClyC were labeled with AlexaFluor 488 and co-cultured with the corresponding cells for 4 h. After staining with DAPI, cells were imaged by laser confocal microscopy. The scale in D1 to D3 is 10 μm. The lysin intensity per cell was calculated and analyzed. (**E**) The clearance rate of intracellular bacteria treated with different concentrations of gentamicin for 4 h. (**F**) The clearance rate of intracellular bacteria treated with 20 μg/mL ClyC or p-ClyC for 4 h. All values are expressed as means ± SD and $n = 3$

p-ClyC was slower compared to ClyC, however, after 45 min, both lysins reduced bacterial turbidity to near the minimum detection limit. We further evaluate the cytotoxicity of ClyC and p-ClyC against to different cell lines. As shown in Fig. 4B, C and p-ClyC revealed similar cytotoxicity to ClyC, and both of the lysins showed no cytotoxicity below 40 μg/mL. Because the lung cell-targeting peptide used in this study was reported to be specifically targeted and internalized into lung cells through α3β1 integrin receptor, three epithelial cell lines (A549, Caco-2 and Hela) with different α3β1 integrin expression levels, where the lung epithelia A549 has the most abundance, were selected to explore the ability of antibacterial agents into the cell and intracellular bactericidal effects. The confocal imaging showed both ClyC and p-ClyC were internalized in A549, Caco-2 and Hela cells (Fig. 4D). In comparison, p-ClyC showed a more obvious cell entry capability to A549 cell than that of ClyC (Fig. 4D1), while in the other two cell lines, this enhanced cell entry capability was not significant (Fig. 4D2 and 2D3). Subsequently, we performed additional experiments to test and compare the intracellular bactericidal activities among gentamicin, ClyC and p-ClyC. Comparing with the group only treated with PBS, the clearance of intracellular bacteria by gentamicin, ClyC and p-ClyC was obtained. As shown in Fig. 4E, gentamicin only had a superior intracellular bactericidal activity in A549 cell at 4 h treatment. The intracellular bactericidal ability of gentamicin decreased obviously in Caco-2 and Hela cells even for 4 h treatment. In addition, gentamicin had no intracellular bactericidal ability in a short time of 1 h (data not shown). However, the lysins had a relatively stable intracellular bactericidal activity in these three cell lines. In particular, p-ClyC also showed an enhanced intracellular bactericidal activity in A549 cell compared with ClyC, but there is no significant difference in bactericidal activity in the other two cell lines (Fig. 4F). Above all, both ClyC and p-ClyC had good abilities to internalize epithelial cells and kill intracellular bacteria, and p-ClyC showed enhanced bactericidal activity of intracellular bacteria in A549 than ClyC and gentamicin showed different intracellular bactericidal ability in various cell lines.

Exploration of the genetic polymorphisms of *S. aureus* under the treatment of lysin or gentamicin

As mentioned above, *S. aureus* can evolve and adapt various conditions, and our previous study [10] also highlighted that it can also rapidly get the phage resistance under the positive driving by its genetic polymorphisms in the genome. Whether antibiotics or lysins may affect the genetic polymorphisms in the genome of bacteria still remains a gap. Hence, a deep genome sequencing approach was used to investigate the mutual effect between the anti-bacteria agents ClyC, p-ClyC or gentamicin and the genetic polymorphisms of A549 intracellular bacteria. Firstly, we determined the clearance rate of A549 intracellular bacteria with different concentrations of p-ClyC or ClyC. As shown in Fig. 1B, in the case of the same clearance rate, the concentration of p-ClyC is obviously lower than that of ClyC. 20 μg/mL ClyC, 10 μg/mL p-ClyC and 4 μg/mL gentamicin were respectively selected to keep almost the same clearance rate of intracellular bacteria to ensure that the numbers of viable bacteria in the different treatment groups were as similar as possible. Then, deep sequencing for the four groups treated with PBS, ClyC, p-ClyC and gentamicin were performed. As shown in Fig. 1C, the 100% genome coverage (the reference length is 2.9 M) and an average of 7000 depth were obtained for each group. The genomics data and variation analysis indicated no mutation (including point mutation, deletion or truncation) identified in each sample. Besides, the average sequencing error rate estimated by Jellyfish software was about 0.004. We selected the minor allele frequency (MAF) 0.08, which is 20 times larger than the error rate, to determine the minor alleles with high confidence. Then, with the threshold of MAF value, we found the number of minor alleles of the intracellular *S. aureus* treated with gentamicin was obviously larger than that of control group. While, the numbers of minor alleles of the intracellular *S. aureus* treated with ClyC and p-ClyC were close to that of control group. Among them, bacteria treated with p-ClyC had the lowest number of minor alleles (Fig. 1C). More detailed information is provided in Supplementary Table 2. From the functional gene level, all of the *S. aureus* strains from the four groups shared most minor alleles on 29 functional genes (Fig. 1E), and the KEGG enrichment results based on these functional genes were similar. For example, all of them significantly enriched the glycan degradation pathway (Fig. 1F). While, the bacteria treated with gentamicin has some specific minor alleles belonging to 17 functional genes (Fig. 1E), and some of them belong to the ABC transporters pathway (Fig. 1F), which could contribute to multidrug resistance [29]. The functional genes with minor alleles of lysin group were not involved in any virulence or drug resistance pathway.

The results for the minor allele analyses indicated that the bacteria strain *S. aureus* (*S. a*) ATCC 29,213 evolved from the four rounds at the stress of gentamicin provided some minor mutations in the genes related to the ABC transporters pathway. To further investigate whether these minor alleles could enhance the resistance ability of the evolved strain, MIC of gentamicin against ancestral strain and the evolved strains under four rounds intracellular challenges from gentamicin, ClyC and p-ClyC was determined. The results revealed there was no significant MIC difference among the four strains (Fig. 5A), which agrees with the results of that there is no mutation

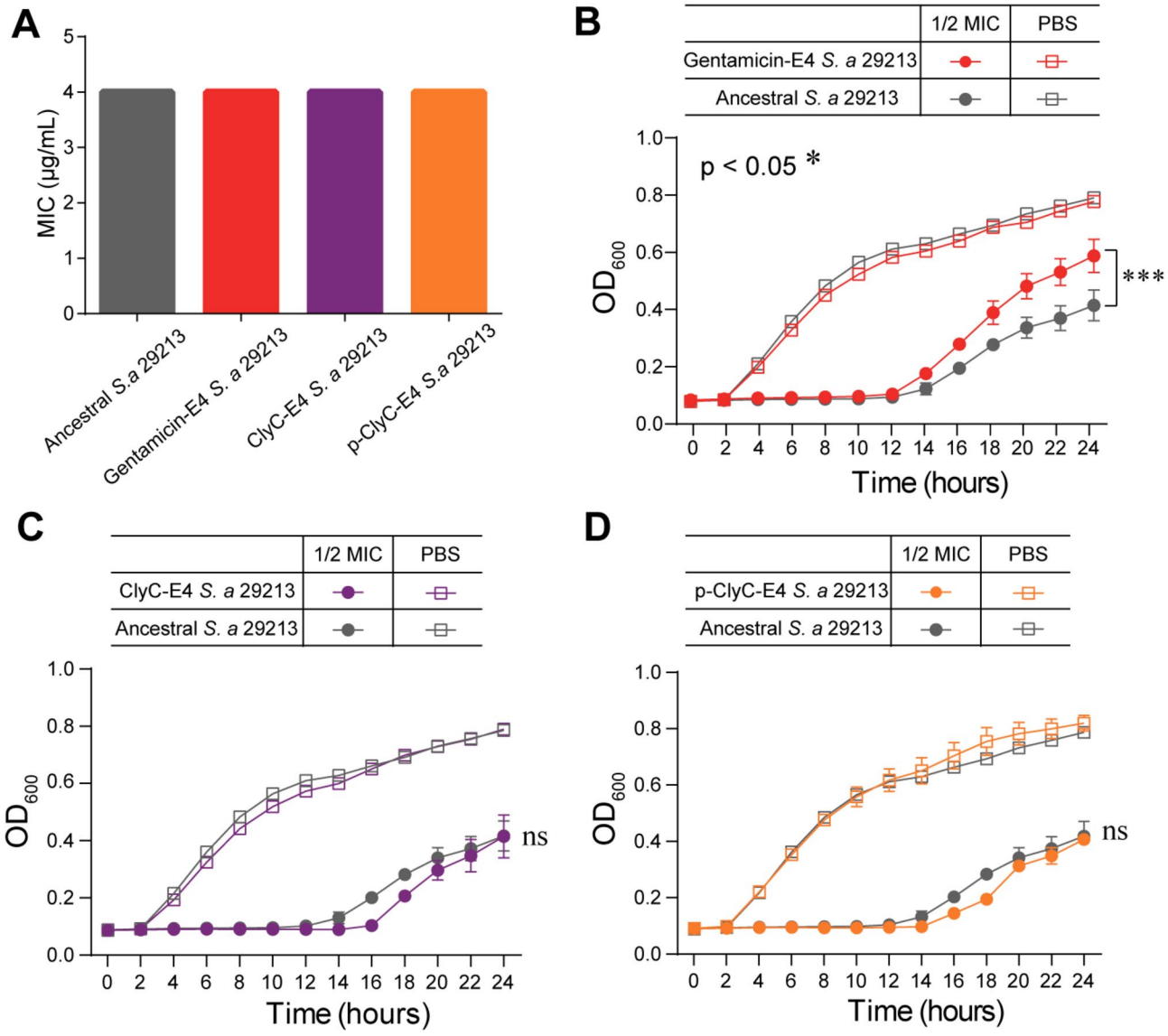

Fig. 5 Resistance evaluation of *S. aureus* (*S. a*) 29,213 strains to gentamicin, ClyC and p-ClyC driven by minor alleles. (**A**) MIC tests of the ancestral *S. a* 29,213 strain and the adaptive evolved strains of *S. a* 29,213 under the stresses from gentamicin (Gentamicin-E4 *S. a* 29213), ClyC (ClyC-E4 *S. a* 29213) and p-ClyC (p-ClyC-E4 *S. a* 29213), respectively. Growth rate comparisons of ancestral *S. a* 29,213 strain with the three evolved strains, respectively, in the condition of 1/2 MIC gentamicin (Gentamicin-E4 *S. a* 29213) (**B**), (ClyC-E4 *S. a* 29213) (**C**) and (p-ClyC-E4 *S. a* 29213) (**D**). All values are expressed as means ± SD and $n = 3$

between each of them. However, when we monitored the growth curves of the four strains under 1/2 MIC gentamicin condition, we found that compared with the ancestral *S. aureus*, the strain evolved from four rounds gentamicin stress had a significantly faster growth rate (Fig. 5B), while the evolved strains under four rounds challenges from the two lysins showed no significant difference to the ancestral *S. aureus* (Fig. 5C-D).

Engineered lysin p-ClyC has an enhanced ability to kill *S. aureus* in a murine lung infection model

Using a murine lung infection model, we assessed the ability of ClyC and p-ClyC to treat *S. aureus* infection. The infected mice were treated with PBS, ClyC and p-ClyC and monitored their survival rate. Figure 2A displayed the whole experimental process for the treatment of the infected mice with the two lysins. All mice in the PBS group died on the first day after infection, while all mice in the ClyC group died on the third day after infection. However, after 72 h post infection and treated with 15 μg lysin per mouse, the survival rate of mice in p-ClyC was 50% (Fig. 2C), indicating that p-ClyC could

improve the survival rate of infected mice. When treated with 20 μg lysin per infected mouse, the mice treated the two lysins had the same survival rates of 83.3% at 144 h post infection, so we monitored weight changes in the surviving mice during this time. Mice treated with ClyC and p-ClyC both showed decreases in body weights on the one to three days post infection and ClyC treated mice had a significantly lower body weight than p-ClyC treated mice on day 3 and subsequent days after infection (Fig. 2D). In addition, we evaluated the ability of ClyC and p-ClyC to eliminate bacteria in lung of mice. Figure 2B illustrated the bacterial loads determination after infection by the bacteria and the treatment of the two lysins. As exhibited in Fig. 2E, compared with ClyC, p-ClyC significantly reduced the load of bacteria in the lung. To investigate whether the treatment of lysins on mice has toxic or side effects, the routine blood test of each group was conducted. The results in Fig. 2F-J showed that WBC, RBC, PLT, HGB and MCH demonstrated no significant difference among the groups, indicating that the dose of lysin used in this study had no apparent side effects on mice. Detailed blood test results can be found in Supplementary Table 3. However, p-ClyC as a potential novel agent for the treatment of lung infections requires specific and ongoing safety evaluations of large samples and multiple doses in the future.

Discussion

p-ClyC, chimerizing a lung-targeting peptide with ClyC (p-ClyC), showed an improved intracellular bactericidal activity for the lung A549 cells compared with ClyC, but not for the other two cell lines (Caco-2 and Hela) (Fig. 4F). The peptide used in this study was reported to be specifically targeted and internalized into lung cells through α3β1 integrin receptor, of which the integrin subunit α3 played a very important role [14]. The lung A549 cells have much higher expression of integrin, especially integrin subunit α3, than Caco-2 and Hela, which may contribute to the improved intracellular bactericidal activity of p-ClyC. Integrin subunit α3 and β1 relative expressions in the different tissues of human body were referred to https://www.ncbi.nlm.nih.gov/gene/3675 (integrin subunit α3) and https://www.ncbi.nlm.nih.gov/gene/3688 (integrin subunit β1), respectively. If the enhanced intracellular bactericidal capacity is expected for most cell lines, more rational engineering is still needed, for example, designing the peptide that can recognize the receptor shared by most cells.

In our previous studies, we found and verified that the genetic polymorphism was a novel mechanism that drove the rapid adaptive evolution of bacteria to get the resistance capability under the phage stresses [10, 22]. In the current study, according to the analyses of the genome deep sequencing data, we found that the genetic polymorphism also existed in intracellular bacteria under the stress of gentamicin or lysins. It was shown that the population of bacteria, whether in vitro or in vivo, when encountering selection pressure, would produce some minor variations as a response. But the numbers of minor alleles that caused by gentamicin and lysin were different (Fig. 1D). Importantly, using the same bioinformatics pipeline to predict the relative biology pathways that might correlate with the minor alleles, we found the minor alleles caused by gentamicin were located in the ABC transporters related genes which were reported to play the roles in drug resistance (Fig. 1F) [29]. At the same time, we also noticed that the intracellular bacteria in all treatment groups did not develop any mutations after 4 rounds of treatment, which may be related to the short time of each round of treatment. Further experiments found the evolved strain treated with gentamicin showed higher growth rate under the condition of 1/2 MIC gentamicin (Fig. 5B). Combined with both sequencing results and the experimental results, we concluded that even though in a short period of time, the bacterial population was regulated by gentamicin, resulting in a small proportion of resistant progeny. The risk of the emergence of resistant intracellular strains in the treatment under antibiotics could not be ignored. In contrast, the progeny strains treated by lysins (both ClyC and p-ClyC) showed almost the same growth rate as its ancestor under the 1/2 MIC gentamicin, indicating a low risk of resistance in lysin treatment of intracellular bacteria (Fig. 5C and D). Although deep sequencing revealed that there were also minor alleles occurring in the populations treated with ClyC or p-ClyC, it was similar to that of the control group (Fig. 1D and E).A potential reason for the small impact of the lysins (ClyC and p-ClyC) on the genome of bacterial progenies might be that lysins targeted the essential and conserved elements of the bacterial cell wall to rapidly kill the bacteria, rather than entering the bacterial cell [30]. Gentamicin, as an aminoglycoside antibiotic, bound to the A site of the ribosome 30 S subunit to inhibit the synthesis of bacterial proteins and the use of gentamicin had predictably led to the emergence of multiple resistance mechanisms in pathogens [31]. Therefore, in the intracellular environment, bacteria were prone to mutation under the dual influences of the internal environment and gentamicin [32]. In addition, the rate of intracellular accumulation of gentamicin was relatively slow [33], so the time of action affected the accumulation of gentamicin. A previous study showed that gentamicin was taken up by macrophages after 8 h treatment, playing an intracellular bactericidal role [34]. In this study, we also found that gentamicin had no intracellular bactericidal effect after 1 h treatment (data not shown), but the intracellular bactericidal effect was significantly improved under the 4 h

treatment. As shown in Fig. 4E, gentamicin showed superior intracellular bactericidal ability only in A549 cell line. Therefore, the use of gentamicin to kill intracellular bacteria still had drawbacks due to cell lines preference [35] and high drug resistance risk, both in vivo and in vitro. Therefore, the lysins to eliminate the intracellular bacteria had better efficacy and more safety than gentamicin.

In the mouse model, the infected mice treated with p-ClyC had a higher survival rate than those treated with ClyC at 15 μg/mouse, which was due to the higher lung targeting ability of p-ClyC. In the future, it will be essential to conduct systematic studies on the mode of administration, dosing, safety, pharmacodynamics, and pharmacokinetics of p-ClyC in comparison with standard antibiotics to comprehensively evaluate its clinical potential for the treatment of pulmonary infections. The bactericidal rate and therapeutic effect of p-ClyC could be improved by further genetic engineering modification, which needed further researches. In addition, the lung targeting peptide could be used to modify other lysins to improve their activities to treat the lung infections caused by intracellular bacteria, especially multi-drug-resistant bacteria.

Abbreviations

ARDS	Acute respiratory distress syndrome
MDR	Multidrug resistant
CAP	Community-acquired pneumonia
SCAP	Severe community-acquired pneumonia
MRSA	Methicillin-resistant S. aureus
RMSD	Root-mean-square-deviation
IPTG	isopropyl β-D-thiogalactoside
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
FBS	Fetal Bovine Serum
DMEM	Dulbecco's modified Eagles medium
DAPI	4, 6-diamidino-2-phenylindole
MOI	Multiplicity of infection
MAF	Minor allele frequency
KEGG	Kyoto Encyclopedia of Genes and Genomes
MIC	Minimum inhibition concentration
CFU	Colony-forming units
WBC	White blood cell
RBC	Red blood cell
PLT	Platelets
HGB	Hemoglobin
MCH	Mean corpuscular hemoglobin

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03978-6.

Supplementary Table 1: Primers used in this study for the construction of expression plasmids

Supplementary Table 2: Detailed information of minor alleles of the intracellular ***S. aureus*** treated with different groups (ClyC, p-ClyC, gentamicin and control group)

Supplementary Table 3: Detailed results of the blood test of mice with different treatments (p-ClyC, ClyC and control group)

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Author contributions

Xiaoxu Zhang designed the study, performed all experiments and drafted the manuscript. Dongyan Xiong performed the data analysis, gave the advice on functional genomics analyses, and drafted the manuscript. Xiaohong Li gave the lysin ClyC. Heng Xue and Min Chen helped the animal experiments. Junping Yu supported the high-throughput sequencing and revised the manuscript. Hongping Wei guided the study, revised and approved the manuscript.

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Data availability

The raw sequencing data of all bacteria strains reported in this study have been deposited in the Genome Warehouse in the National Genomics Data Center https://ngdc.cncb.ac.cn/bioproject/browse/PRJCA020832, Beijing Institute of Genomics (China National Center for Bioinformation), Chinese Academy of Sciences. The BioProject accession number is PRJCA020832.

Declarations

Ethics approval and consent to participate

All mouse experiments were carried out in an ABSL-2 lab. The experimental protocols were carried out following the regulations and guidelines set forth by the Animal Experiments Committee of Wuhan Institute of Virology, Chinese Academy of Sciences, and approved by the committee (No: WIVA17202303). Animals were randomized and cared in individually ventilated cages following a set of animal welfare and ethical criteria during the experiment. After the experiment, cervical dislocation was performed for euthanasia, and the experimental animals were not given pain during the whole process. This research did not contain any studies with human participants.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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