Degradation of methicillin-resistant *Staphylococcus aureus* biofilms using a chimeric lysin

Hang Yang, Yun Zhang, Yanling Huang, Junping Yu and Hongping Wei*

Center for Emerging Infectious Diseases and State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, PR China

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for a large number of chronic infections due to its ability to form robust biofilms. Herein, the authors evaluated the anti-biofilm activity of a *Staphylococcus* specific chimeric lysin ClyH on MRSA biofilms. ClyH is known to be active against planktonic MRSA cells *in vitro* and *in vivo*. The minimum concentrations for biofilm eradication (MCBE) of ClyH were 6.2–50 mg l\(^{-1}\), much lower than those of antibiotics. Scanning electron microscope (SEM) analysis revealed that ClyH eliminated MRSA biofilms through cell lysis activity in a time-dependent manner. Viable plate counts and kinetic analysis demonstrated that biofilms of different ages displayed varying susceptibility to ClyH. Together with previously demonstrated *in vivo* efficacy of ClyH against MRSA, the degradation efficacy against biofilms of different ages indicates that ClyH could be used to remove MRSA biofilms *in vivo*.

**Keywords**: *Staphylococcus aureus*; methicillin-resistant *Staphylococcus aureus* (MRSA); chimeric lysin; biofilm; anti-biofilm; susceptibility

**Introduction**

Along with the emergence and dissemination of multidrug-resistant *Staphylococcus aureus* (Lowy 2003; de Lencastre et al. 2007; Arias & Murray 2009), hospital-, community- and livestock-acquired infections caused by methicillin-resistant *S. aureus* (MRSA) are becoming increasingly difficult to manage (Klevens et al. 2007; Gould et al. 2012). The difficulty comes not only from multidrug resistance but also the ability of MRSA to form robust biofilms, which could increase resistance to antimicrobial agents and contribute to several potential infection pathways, such as infections mediated by medical devices for internal or external use (Kallen et al. 2010; Peters et al. 2012). Therefore, there is an urgent need to develop anti-biofilm agents against MRSA biofilms, and this may be key for the successful treatment of MRSA-associated chronic infections (Davies 2003).

Although many antibiotics, alone or in combination, are reported to have the ability to inhibit *S. aureus* biofilm formation (Mataraci & Dosler 2012; Parra-Ruiz et al. 2012; Singh et al. 2012; Estes et al. 2013), their ability to eliminate established biofilms is limited. Bacteriophages and their derivatives, alone or in combination with antibiotics, can eliminate established *S. aureus* biofilms (Rahman et al. 2011; Kelly et al. 2012). However, the use of phages still suffers from drawbacks when used *in vivo*, including bacterial resistance, inactivation by the immune system, and the presence of endotoxin from impure phage preparations (Donlan 2009). Due to their unique advantages over antibiotics and phages (O’Flaherty et al. 2009), lysins have been found to be promising in the elimination of bacterial biofilms (Daniel et al. 2010; Domenech et al. 2011; Meng et al. 2011; Shen et al. 2013). Several research groups have attempted to remove *S. aureus* biofilms using lysins (Wu et al. 2003; Sass & Bierbaum 2006; Son et al. 2010). However, these studies mainly focused on the inhibitory effects of lysins to biofilm formation. Little is known about the activity of lysins for the elimination of mature biofilms.

The anti-biofilm activity of ClyH, a staphylococci specific chimeric lysin, was evaluated by monitoring its kinetics for disrupting MRSA biofilms *in vitro*. ClyH was constructed in the authors’ laboratory by fusing the catalytic domain of Ply187 with the cell wall binding domain of phiNM3 lysin, which is known to be highly active against planktonic MRSA cells *in vitro* and *in vivo* (Yang et al. 2014).

**Materials and methods**

**Bacterial strains**

The bacterial strains (Table S1; Supplementary material is available via a multimedia link on the online article webpage) used in this work were routinely grown at 37°C. All the *S. aureus* strains were grown in trypticase soy broth (TSB) medium. MRSA strain N315 was used as a standard for evaluating the degradation efficacy of ClyH against MRSA biofilms. Clinical *S. aureus* isolates
were collected from hospitals in Wuhan, China. The medium for biofilm production was TSB supplemented with 1% glucose (TSBG). *Escherichia coli* BL21(DE3) was used to express ClyH as described previously (Yang et al. 2014).

**Biofilm growth conditions**

*S. aureus* biofilms were cultured as described by Mataraci and Dosler (2012), with some modifications. Briefly, strains were subcultured onto TSB agar plates and a single colony was inoculated into TSB medium for 24 h at 37°C. Then the overnight culture was diluted 1:40 with fresh TSBG, and 10 μl of the diluted culture were pipetted into each well of a 96-well polystyrene microtiter plate (about 1 × 10^7 colony forming units (CFU) per well), which had been pre-filled with 190 μl of fresh TSBG medium. Finally, plates were incubated for 24–72 h at 37°C to induce biofilm formation. For experiments assessing biofilms on glass coverslip surfaces, the experimental setup was exactly the same as described above, except that biofilms were grown in the wells of 6-well polystyrene plates on sterilized 18 mm × 18 mm glass coverslips as described previously (Absalon et al. 2011).

**MCBE determination**

The minimum concentration for biofilm eradication (MCBE) of each antibiotic or ClyH was determined as described previously (Ceri et al. 1999, 2001; Shen et al. 2013), using 96-well plates and not the MBEC device. Briefly, N315 cultures were allowed to develop biofilms in 96-well plates (PE, USA) at 37°C for 24 h. Next, the supernatant containing planktonic bacteria was removed by aspiration and the attached biofilms were subsequently washed twice with sterile phosphate buffered saline (PBS), at pH 7.4. Biofilms were then challenged with TSBG medium or TSBG medium containing serial dilutions of antibiotics or ClyH. After incubation at 37°C for 2 h, each well was again aspirated, washed twice with PBS and incubated overnight in fresh. The lowest concentration of antibiotic or ClyH which resulted in a clear supernatant was defined as the MCBE.

**Crystal violet assay**

Biofilm biomass was quantified by crystal violet assay. After washing twice with PBS, biofilms formed in 96-well plates were treated with either serial concentrations of ClyH or PBS for different treatment times. After treatment, biofilms were washed twice with PBS and allowed to dry overnight at 4°C. Then, wells were stained with 200 μl of 0.1% crystal violet for 5 min. After rinsing off excess stain with tap water, plates were dried at 37°C. Finally, the stain was eluted in 200 μl of 95% ethanol, at room temperature for 30 min and absorbance was measured at OD_{595}nm (Cires et al. 2009). To evaluate the degradation efficacy of ClyH against clinical *S. aureus* isolates, 24 h biofilms were treated with ClyH (10 μg ml⁻¹) for 1 h before crystal violet analysis.

**Viable plate count analysis**

MRSA strain N315 was allowed to develop biofilms for 24, 48 and 72 h as described above. These biofilms were washed twice with PBS and then treated with ClyH (10 μg ml⁻¹) or PBS for 15, 30, 60 and 105 min, respectively. After the treatment, biofilms were washed twice with PBS and analyzed using either the crystal violet assay, or CFU analysis.

For viable plate count analysis, the biofilms were aseptically transferred to tubes containing 1 ml of PBS and gently sonicated in a Scientz 5200 water bath sonicator (Ningbo, Zhejiang, China) at 40 kHz for 5 min to disaggregate biofilm-embedded cells. Serial dilutions were made in sterile PBS and plated onto TSB agar plates for CFU enumeration.

**Scanning electron microscopy (SEM)**

N315 biofilms grown on glass coverslips for 24 h were washed twice with PBS and then treated with ClyH (100 μg ml⁻¹) or PBS for 15, 30 and 60 min. After treatment, the biofilms were washed twice with PBS, sampled as described previously (Peters et al. 2013), and analyzed using a scanning electron microscope (JSM-6390, JEOL, Japan).

**Dynamic analysis of biofilm eradication**

N315 biofilms were grown in 96-well plates for 24, 48 and 72 h. After washing twice with PBS, biofilms were treated with serial concentrations of ClyH (0.46–30 μg ml⁻¹), or different concentrations of antibiotics (including

| Strain | Amp | Kan | Gen | Oxa | Van | ClyH |
|--------|-----|-----|-----|-----|-----|------|
| N315   | >6400 | >6400 | >6400 | 3200 | 200 | 50   |
| D402   | >6400 | >6400 | >6400 | 1600 | 1600 | 12.5 |
| B304   | >6400 | >6400 | >6400 | >6400 | 800 | 25   |
| JM591  | >6400 | >6400 | >6400 | 1600 | 800 | 6.2  |
| JM603  | >6400 | >6400 | >6400 | 3200 | 200 | 12.5 |
Figure 1. The degradation efficacy of ClyH against N315 biofilms. Mature biofilms aged 24 to 72 h were treated with 10 μg ml⁻¹ of ClyH or PBS for different times, respectively. After washing twice with PBS, the biomass of each well was determined by crystal violet assay, and the CFU of each well was counted by plating onto TSB agar plates. Data were analyzed by one-way ANOVA test. Error bars represent SD; *p < 0.05; **p < 0.01.
2 mg ml\(^{-1}\) ampicillin, 1 mg ml\(^{-1}\) kanamycin, 1 mg ml\(^{-1}\) gentamicin, 1 mg ml\(^{-1}\) vancomycin, and 1 mg ml\(^{-1}\) oxacillin), or PBS buffer. Absorbance at OD\(_{600}\) was monitored using a microplate reader (Synergy H1, BioTek, Winooski, VT, USA) at 37°C for 5 h.

**Statistics**

All experiments were performed in triplicate and repeated at least three times. The crystal violet assay data and CFU data were analyzed using a one-way analysis of variance (ANOVA). The dynamic analysis of treated biofilms was compared with the controls using one-way ANOVA also. Differences at \( p < 0.05 \) and \( p < 0.01 \) were considered significant and very significant, respectively.

**Results**

**High anti-biofilm activity of ClyH**

A previous study showed high lytic activity of ClyH against planktonic MRSA cells *in vitro* and *in vivo* (Yang et al. 2014). However, its ability to degrade or reduce MRSA biofilms was unclear. Therefore, the anti-biofilm activity of ClyH against MRSA strain N315 was initially tested in comparison with that of antibiotics (including ampicillin, kanamycin, gentamicin, vancomycin, and oxacillin). The MCBE values of ampicillin, kanamycin, gentamicin, and oxacillin were found to be > 3,200 mg l\(^{-1}\) against MRSA strain N315 biofilms (Table 1). Vancomycin showed an MCBE of 200 mg l\(^{-1}\). In contrast, the MCBE of ClyH was only 50 mg l\(^{-1}\), indicating greater anti-biofilm activity than the antibiotics tested. The MCBE assay was then expanded to several clinical MRSA isolates (Table 1). MCBE values were > 3,200 mg l\(^{-1}\) for ampicillin, kanamycin, gentamicin, and oxacillin; 200–1,600 mg l\(^{-1}\) for vancomycin; and 6.2–50 mg l\(^{-1}\) for ClyH for the strains tested. These results showed that ClyH has significantly greater anti-biofilm activity than conventional antibiotics.

**Degradation efficacy of ClyH against MRSA biofilms of different ages**

Next, the degradation activity of a low concentration of ClyH (10 µg ml\(^{-1}\)) was evaluated against established N315 biofilms. As shown in Figure 1, degradation efficacy increased with increased treatment time, as shown in the results of the crystal violet staining. This is also consistent with the results of viable plate count analysis. After treatment for 30 min, there was a reduction in biomass in the 24 h and 48 h biofilms. However, for 72 h biofilms, a reduction in biomass was observed only after treatment for 60 min. This indicates that the 72 h biofilms may be difficult to eliminate, due to the presence of more extracellular matrix composed of proteins, polysaccharides, extracellular DNA (eDNA), and presumably host factors. The crystal violet assay indicated that there was no obvious difference in the biofilm degradation efficacy for the 24 h biofilms between the groups treated for 60 min and 105 min. However, there was a difference in the viable plate counts from treated wells between these two treatment groups as revealed by CFU analysis. Viable plate counts for 48 h biofilms did not show any differ-

![Figure 2](https://example.com/figure2.png)

Figure 2. Comparison of the anti-biofilm activities of antibiotics and ClyH against N315 biofilms. N315 biofilms aged 24 h (A), 48 h (B) and 72 h (C) were washed twice with PBS and then treated with antibiotics (Amp: 2 mg ml\(^{-1}\) ampicillin; Kan: 1 mg ml\(^{-1}\) kanamycin; Gen: 1 mg ml\(^{-1}\) gentamicin; Van: 1 mg ml\(^{-1}\) vancomycin; Oxa: 1 mg ml\(^{-1}\) oxacillin) or ClyH (0.46–30 µg ml\(^{-1}\)) for 2 h. The changes in OD\(_{600}\) were analyzed by one-way ANOVA test. Error bars represent SD; *\( p < 0.05 \); #\( p < 0.01 \).
ences for the different treatment times. The CFU analysis also indicated that the viable cells in the 72 h biofilms decreased with increased treatment time. This phenomenon was different from the biofilms aged 24 and 48 h, indicating that ClyH is less effective at lysing cells in older biofilms.

Furthermore, the degradation kinetics of different concentrations of ClyH (0.46–30 μg ml⁻¹) were evaluated on biofilms of different ages (Figure S1). The results showed that a ClyH concentration as low as 0.46 μg ml⁻¹ still had degradation efficacy on all the biofilms, indicating that ClyH could be used as a potential anti-biofilm agent against MRSA. The changes in biomass as measured by absorbance within 2 h of the biofilms treated with antibiotics and serial concentrations of ClyH were analyzed in comparison with PBS treated controls (Figure 2). Decreases (p < 0.01) were observed between ClyH treated wells and the control wells for all the biofilms.

The degradation efficacy of ClyH against MRSA biofilms was also assessed by scanning electron microscopy (SEM). After 24 h static incubation, N315 cells formed thick, dense layers of biofilms on the coverslips (Figure 3A). As shown in Figure 3B, after treatment with 100 μg ml⁻¹ of ClyH for 15 min, the thickness of the treated biofilms decreased and cell deformation was visible (arrows). Increased treatment time led to an increased reduction in established biofilms, and to S. aureus cells undergoing a process from coarsening to rupture, and the formation of dead ghost cells (Figure 3C and D).

**Degradation efficacy of ClyH against clinical S. aureus biofilms**

Finally, the anti-biofilm activity of ClyH was evaluated against several S. aureus clinical isolates using the crystal violet assay. Treatment with ClyH (10 μg ml⁻¹) resulted in a reduction in the biofilm biomass against both methicillin sensitive S. aureus (MSSA) and MRSA isolates (Figure S2). As shown in Figure 4, > 60% of the biofilm biomass was eliminated for the strains tested using PBS treated plates as controls, indicating the degradation efficacy of ClyH against clinical S. aureus biofilms.

**Discussion**

Previous studies have demonstrated that S. aureus cells within biofilms are up to 1,000 times more resistant to antimicrobial agents than their planktonic counterparts (Hall-Stoodley et al. 2004). Although high concentrations of antibiotics were able to kill MRSA cells within biofilms and inhibit the regrowth of MRSA biofilms, such high doses of antibiotics are impractical for clinical care. In this study, it was found that ClyH showed anti-biofilm activity against MRSA biofilms, even at a low concentration of 0.46 μg ml⁻¹. ClyH was able to lyse the MRSA cells in biofilms of different ages and disperse the biofilms as shown by the OD₆₀₀ drop in absorbance (Figure 2). In contrast, all the biofilms treated with high concentrations of antibiotics could not be dispersed as shown by the minimum decrease in OD₆₀₀ absorbance. These differences may be due to the fact that the antibiotics tested are bacteriostatic and do not directly lyse bacteria. Therefore, it seems that ClyH could eradicate biofilms more rapidly than antibiotics.

CFU analysis also indicated that the established MRSA biofilms of different ages displayed varying susceptibility to ClyH (Figure 1), indicating a different therapeutic efficacy may be obtained at different therapeutic time points. However, further study is needed to understand whether such variations in susceptibility are due to differences between the structure of the extracellular matrix, or the susceptibility of the cells within the biofilms at different ages.

It has been reported that lysin only disrupts biofilms formed by lysin-sensitive bacteria, but not lysin-resistant bacteria (Wu et al. 2003; Sass and Bierbaum 2006), indicating that the anti-biofilm activity of a lysin has a direct connection with its antibacterial spectrum. Previous data have shown that ClyH has some improved efficacy over other lysins, including an improved lytic activity, broad spectrum, and a low probability of developing resistance (Yang et al. 2014). Therefore, ClyH should have greater anti-biofilm activity than other lysins for removing MRSA biofilms. Although the present results for ClyH were very encouraging, since ClyH displayed biofilm degradation activity against both MSSA and MRSA clinical isolates in vitro (Figure 4), the in vivo anti-biofilm activity of ClyH still needs to be evaluated.

Like phages, the mechanism of lysin to disrupt biofilms was previously hypothesized to be mediated by direct cell lysis (Fenton et al. 2013; Shen et al. 2013). As evidence, researchers observed ATP efflux from lysed biofilm cells (Okuda et al. 2013). In the present work, cell disruption was also observed using SEM of ClyH treated biofilms (Figure 3). It was hypothesized that the enzyme probably utilized its lytic activity against cells to work through biofilms, layer by layer, ultimately resulting in the breakdown of the whole biofilm, which is consistent with the observation of Shen et al. (2013) on the degradation of Streptococcus pyogenes biofilms by endolysin PlyC.

In conclusion, the present data show that MRSA cells within biofilms are much more susceptible to ClyH
than to antibiotics. Together with the \textit{in vivo} efficacy of ClyH against MRSA, as was demonstrated previously, the degradation efficacy of ClyH against MRSA biofilms of different ages indicates that ClyH has the potential to be used as an antimicrobial agent for the removal of MRSA biofilms \textit{in vivo}.

Figure 3. SEM analysis of the degradation efficacy of ClyH against N315 biofilms. Mature N315 biofilms grown on coverslips for 24 h were washed twice with PBS and then treated with 100 μg ml$^{-1}$ ClyH for different times at 37°C. After washing twice more with PBS, the coverslips were analyzed by SEM. The arrows indicate cells with obvious asperity and deformation.
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