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ORIGINAL ARTICLE

Biofilm formation and eradication of *Staphylococcus aureus*: a study of culture conditions and endolysin ZAM-CS effect

Yasaman Ahmadbeigi[1], Neda Soleimani[1], Farzaneh Azizmohseni[2], Zahra Amini-Bayat[2*]

[1]Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
[2]Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran

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ABSTRACT

Background and Objectives: *Staphylococcus aureus* significantly contributes to healthcare-associated infections, with biofilm formation causing chronic, antibiotic-resistant cases. Because biofilms show high resistance to conventional antibiotics, endolysins have emerged as a promising alternative for treating antibiotic-resistant, biofilm-associated infections. This study evaluated the effects of four culture media and different incubation times on biofilm formation in methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) *S. aureus* strains and assessed the anti-biofilm efficacy of a novel chimeric endolysin called ZAM-CS (catalytic domain of SAL-1 endolysin and binding domain of lysostaphin).
Materials and Methods: Biofilms were grown for 24, 48, and 72 hours in Mueller-Hinton broth (MHB), Luria broth (LB), terrific broth (TB), and tryptic soy broth (TSB). The crystal violet assay was used to assess the biomass of the biofilm. The optimal biofilm conditions were then used to test ZAM-CS's activity at different concentrations.
Results: MSSA formed the strongest biofilms in TB. MRSA formed stable, high-biomass biofilms in TSB, TB, and LB, while MHB was the least supportive medium for both strains. ZAM-CS significantly reduced biofilm biomass in both MSSA and MRSA (up to 77%).
Conclusion: ZAM-CS's rapid and potent anti-biofilm activity at low concentrations highlights its potential as a promising treatment against antibiotic-resistant *S. aureus* biofilm infections.

Keywords: Biofilms; *Staphylococcus aureus*; Culture media; Anti-bacterial agents; Lysostaphin

INTRODUCTION

The continued rise of multidrug-resistant (MDR) organisms across medical environments has significantly undermined the effectiveness of clinical antibiotics (1). This global challenge has heightened concerns about bacterial pathogens that resist multiple drugs and possess sophisticated survival mechanisms, making infections increasingly difficult to treat (2). Among these, *Staphylococcus aureus* has emerged as a particularly critical threat due to its widespread prevalence, clinical impact, and prominent role as a leading contributor to healthcare-associated infections across the world (3, 4). This adaptable bacterium combines an array of virulence factors with antibiotic resistance, enabling it to cause a diverse range of infections, from mild skin conditions to severe, potentially fatal bloodstream

*Corresponding author: Zahra Amini-Bayat, Ph.D, Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. Tel: +98-2157416815 Fax: +98-2156276636 Email: Amini-bayat@irost.org

infections and pneumonia (3). One of the most significant contributors to the persistence and severity of *S. aureus* infections is its ability to form biofilms (3, 5). Biofilms are structured microbial communities in which bacterial cells adhere to surfaces and become embedded within a self-produced extracellular polymeric substance (EPS) made up of DNA, proteins, and carbohydrates (6). This matrix creates a safeguarded environment that protects bacteria from antibiotics and immune responses, while also supporting nutrient exchange and enabling coordinated microbial activity (6, 7). As a result, biofilm-related infections are typically persistent, recurrent, and difficult to treat, posing substantial health risks and high fatality rates (8). In the context of *S. aureus,* biofilm formation exacerbates the clinical challenge, as it not only increases bacterial survival but also promotes infection spread within the host (5). The biofilm structure allows bacterial cells to disseminate from one site to another, contributing to both localized and systemic infections (6). In addition, antibiotics have been shown to induce changes in planktonic bacteria that increase their propensity to form biofilms (9). Furthermore, the proximity of cells within a biofilm promotes the exchange of genetic material, including genes responsible for antibiotic resistance, compounding the problem (10). The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) and biofilm-associated infections present a major therapeutic challenge. The rise in antibiotic resistance, coupled with the difficulty in producing and developing new antibiotics, has shifted research focus toward discovering new compounds to replace traditional antibiotics for treating *S. aureus* infections (11). Among these alternatives, endolysins have been recommended as an effective treatment option (12). Unlike antibiotics, which are often hindered by biofilm permeability issues and resistance development, endolysins target the structural elements of the bacterial cell wall. This unique mechanism of action allows them to rapidly degrade the peptidoglycan layer, causing cell lysis and death even in dormant or slow-growing biofilm cells (13). Other advantages of endolysins include their high potency, rapid antimicrobial action, ability to target antibiotic-resistant strains, and the absence of bacterial resistance to endolysins (12). Several studies have shown that endolysins have the potential to disrupt biofilms and reduce the bacterial load (14-16). Despite their reported efficacy, issues such as effectiveness time (time required to remove biofilms) and high concentration required for performance, necessitate the design and evaluation of new endolysins. The modular structure of *S. aureus* endolysins allows for the design of more dynamic and effective endolysins against antibiotic-resistant strains through the integration of catalytic domains using protein engineering (17). In our earlier research, we designed and produced a new chimeric endolysin called ZAM-CS (GenBank accession number PP739730), comprising the CHAP domain from the SAL-1 endolysin, known for its powerful lytic activity, and the SH3b domain from lysostaphin, which ensures a stable and effective binding to the peptidoglycan of *S. aureus,* this combination demonstrated remarkable stability and strong antimicrobial effectiveness, even at low enzyme concentrations. In current study, we evaluated the effect of this endolysin on the reduction and removal of biofilm formed by MSSA and MRSA strains. In vitro studies and evaluation of the efficacy of the recombinant antimicrobial compound require the preparation of biofilms with stable and appropriate biomass (18). Strong and stable biomass indicates a well-established biofilm, which is typically more resistant to antimicrobial treatments and environmental stresses. Various protocols have been evaluated for the laboratory assessment of biofilms formed by *S. aureus* (19-21), but these protocols yield different results depending on the bacterial strain. In this study, to form a stronger biofilm and to evaluate the antibiofilm activity of the new chimeric endolysin, we assessed the effect of four different culture media and incubation time on biofilm formation by *S. aureus* (MSSA) and (MRSA) strains.

MATERIALS AND METHODS

Bacterial strains and culture media. The strains investigated in this research were sourced from the Persian Type Culture Collection (PTCC), which is overseen by the Iranian Research Organization for Science and Technology (IROST). In the current study, two reference strains were tested. ATCC 29213 and ATCC 33591 were used as MSSA and MRSA strains respectively. The biofilm formation of both strains was evaluated in four culture media: Mueller Hinton broth (MHB), tryptic soy broth (TSB), terrific broth (TB), and Luria broth (LB).

Biofilm formation and quantification of biofilm biomass. MSSA and MRSA strains were grown on TSA plates at 37°C overnight. A single colony from each culture was picked and transferred to a selective medium containing 1% glucose, then incubated at 37°C for 16 hours. Glucose was added to MHB, TSB, LB, and TB at a concentration of 1%. A total of 198 µL of sterile, prepared broth was dispensed into each well of a 96-well plate. Then, 2 µL of preculture was added to each well and incubated at 37°C for 24, 48, and 72 hours. After incubation, the plate was rinsed with PBS buffer to remove planktonic cells. The biofilms were stabilized at 60°C for 40 minutes, then the wells were stained with 0.1% crystal violet for 15 minutes. After washing, the absorbance of the samples was measured at 595 nm after adding 33% acetic acid to the wells, using a microplate spectrophotometer (Agilent BioTek Epoch, USA) (21).

Antibiofilm activity of chimeric endolysin ZAM-CS. The chimeric endolysin ZAM-CS (GenBank accession number PP739730) was designed, expressed, and purified as described in our prior work. To examine how ZAM-CS impacts MSSA and MRSA biofilm reduction, the optimal conditions for biofilm formation of ATCC 29213 and ATCC 33591 were established and biofilms were fixed according to the previous section. After fixation, the test wells were exposed to different concentrations (1-4 µM) of ZAM-CS for 2.5 hours, and the control group contained only Tris buffer. After the incubation period, the wells were rinsed once more and then subjected to crystal violet staining as described above. After the addition of 33% acetic acid, the absorbances were taken at 595 nm using an Epoch microplate reader.

Statistical analysis. The data analysis and visual representations in this study were conducted using GraphPad Prism software, version 9.1.0 (GraphPad Software, Boston, MA, USA). To evaluate statistical significance, one-way and two-way ANOVA were conducted, followed by post hoc multiple comparisons with Tukey's test ($p < 0.05$).

RESULTS

Biofilm formation and quantification of biofilm biomass. The study on the impact of culture media on biofilm development in ATCC 29213 indicated that TB was more conducive to biofilm formation and produced more biomass than the other media. The results for TSB and LB were nearly identical. MHB was found to be the weakest medium for biofilm formation. The optimum time for the highest biofilm biomass formation in ATCC 29213 was 24 hours, while the lowest biomass of all cultures was observed at 48 hours. After 72 hours of incubation, the biomass in TB increased compared to the 48 hours, while the results for LB and TSB remained similar to those at 48 hours. Therefore, the most effective biofilm was formed in the TB medium after 24 hours by ATCC 29213 (Fig. 1a).

For ATCC 33591, the biofilm formed in MHB was weaker than in the other culture media, while the amount of biomass formed in the other three cultures was almost the same. Additionally, the biofilm biomass at 48 and 72 hours was greater than the biofilm formed at 24 hours. Based on the obtained results, no substantial difference was detected in the biofilm formation between LB, TB, and TSB at 48 and 72 hours. All three cultures formed a stable biofilm with strong biomass during these two incubation times (Fig. 1b).

Antibiofilm activity of chimeric endolysin ZAM-CS. Based on the results of the effect of culture medium on stable biofilm formation, the 24-hour biofilm of ATCC 29213 in TB medium and the 48- and 72-hour biofilms of ATCC 33591 in TSB medium were selected as suitable models to evaluate the antibiofilm effect of different concentrations of ZAM-CS. The results demonstrated that ZAM-CS effectively reduced biofilm biomass in both strains. Moreover, the antibiofilm effect of ZAM-CS was found to be concentration-dependent. As shown in Fig. 2a and c, 4 µM (124 µg/mL) of ZAM-CS reduced the biofilm by 73% and 77% for MSSA and MRSA, respectively, in just 2.5 hours.

DISCUSSION

The global rise of MDR bacteria has created a pressing challenge for modern medicine, diminishing the effectiveness of antibiotics and complicating the treatment of bacterial infections (2). Among these resistant pathogens, *S. aureus* stands out as a particularly concerning threat due to its high prevalence in healthcare settings and its capacity to cause diverse types of infections (3, 4). A key factor underlying the

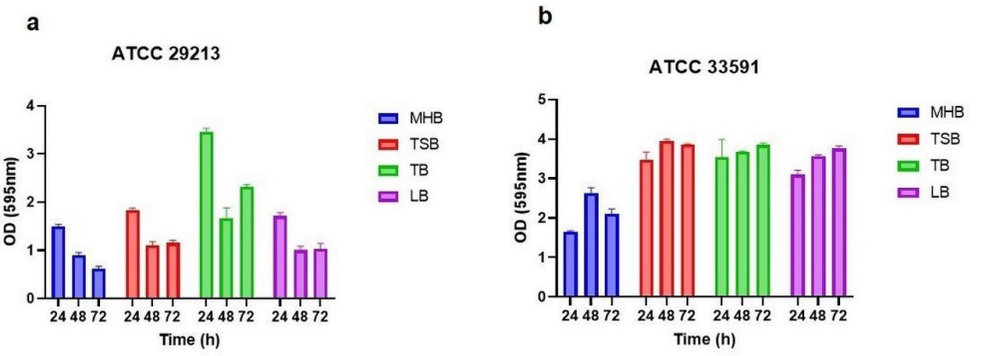

Fig. 1. Biofilm formation in different media and incubation times. (a) ATCC 29213. (b) ATCC 33591.

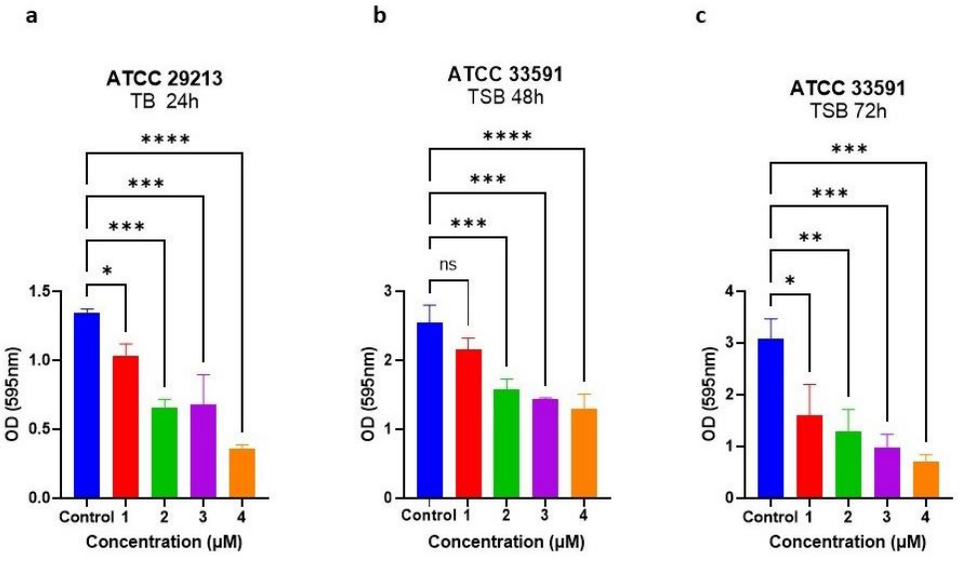

Fig. 2. Anti-biofilm efficacy of ZAM-CS. (a) On 24-hour biofilm of ATCC 29213 in TB medium. (b) On 48-hour biofilm of ATCC 33591 in TSB medium. (c) On 72-hour biofilm of ATCC 33591 in TSB medium. Significance was calculated using one-way ANOVA followed by Tukey's multiple comparison test. NS=not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P ≤ 0.0001.

chronic and severe progression of *S. aureus* infections is its remarkable ability to form biofilms (5). Biofilms play a key role in supporting bacterial persistence, facilitating the transmission of infections, and promoting the transfer of resistance genes, which significantly hinders efforts to eliminate the infection (10). Understanding the factors that influence *S. aureus* biofilm formation is therefore critical for developing more effective antimicrobial strategies (9). We investigated how different growth media and incubation times impact biofilm development, as nutrient availability is a key environmental factor shaping biofilm formation and stability. To explore this, we selected MHB, TSB, LB, and TB because they represent a range of nutrient compositions and are widely used in both clinical and laboratory settings. MHB, the standard medium for antimicrobial susceptibility testing, offers a balanced nutrient profile with defined cation levels (19). TSB is a nutrient-rich medium often used in biofilm studies due to its ability to support robust bacterial growth (19, 22). LB, a general-purpose medium with comparatively lower nutrient content (22), and TB, a highly enriched medium designed to maximize biomass, provide valuable contrasts in nutrient availability. By comparing biofilm formation in these environments, we were able to assess how differences in nutrient conditions affect the biofilm development of various *S. aureus* strains. As shown in Fig. 1, MSSA formed the strongest biofilms in TB. MRSA formed stable, high-biomass bio-

films in TSB, TB, and LB, while MHB was the least supportive medium for both strains. It can be concluded that the composition of the culture medium affects biofilm growth and the formation of substantial biomass (20). In LB and TB, tryptone supplies bacteria with nutrients, nitrogen, and growth factors, with concentrations of 1% in LB and 1.2% in TB. Yeast extract, present at 0.5% in LB and 2.4% in TB, provides essential amino acids and vitamins. TB is more nutrient-rich than LB due to the inclusion of glycerol as a carbon source. In TB, potassium phosphate (1.25%) helps maintain potassium levels, while NaCl in LB (1%) and TSB (0.5%) supports osmotic balance. In TSB, peptides and free amino acids are supplied by the pancreatic digest of casein (1.7%) and the papaic digest of soybean meal (0.3%). Glucose (0.25%) acts as a readily accessible energy source for microorganisms. In MHB, beef infusion (0.2%) and acid casein peptone (1.75%) offer essential nitrogen, vitamins, minerals, and amino acids necessary for microbial growth. Additionally, starch (0.15%) absorbs any toxic byproducts generated during the process. As confirmed by previous studies, glucose concentration affects biofilm formation (19, 22, 23). Specifically, it was found that adding 1% glucose to the TSB resulted in stronger biofilms and improved the repeatability of the test under laboratory conditions (23). Therefore, 1% glucose was added to all cultures in the current research. Lopes, L.Q.S., et al (19). evaluated the efficacy of different concentrations of glucose on 24-hour biofilm formation of the methicillin-susceptible *S. aureus* ATCC 25923 in MHB, and TSB. The findings indicated that MHB containing 1% glucose formed a weaker biofilm than TSB supplemented with 1% glucose. In another study, the effect of LB, TSB, and BM (basic medium: consisting of 1% peptone, 0.5% yeast extract, 0.1% glucose, 0.5% NaCl, and 0.1% dipotassium phosphate) on the biofilm formation of the *S. aureus* P7 was assessed. The findings revealed that all three cultures were suitable for biofilm formation (22).

Endolysins are now considered a potential substitute for antibiotics because of their unique ability to disrupt or eradicate biofilms (12, 13). Given the superior efficacy of chimeric endolysins compared to natural endolysins, there has been increasing research interest in designing engineered endolysins with greater efficiency and specificity (17). ZAM-CS is a novel engineered endolysin that has demonstrated strong antibacterial activity. Results showed that this endolysin was more effective against *S. aureus* biofilms than phage-derived or other chimeric endolysins and was able to significantly reduce biofilm mass in a short time and at lower concentrations. For instance, the ability of LysGH15 to remove biofilm was dose-dependent, with a more pronounced lysis effect at 150 µg/mL compared to 50 µg/mL (24). LysSA52 reduced the biofilm by approximately 60% after 12 hours of incubation at a concentration of 0.5 mg/mL (25). The endolysin LysP108, which originates from phage P108, reduced the biofilm biomass by 66% when applied at a concentration of 250 µg/mL for 5 hours of incubation (14). Additionally, H. Ning and colleagues (15) demonstrated that a concentration of 10 µM of the endolysin Lys84, derived from phage qdsa002, successfully led to an 88% reduction in biofilm. The effectiveness of 200 µg/mL of two endolysins—CHAPk (from LysK) and the engineered endolysin CHAPk-SH3blys, which combines the CHAP domain from LysK and the lysostaphin's SH3b domain —against 24-hour biofilms of *S. aureus* DPC5246 strain was evaluated (26). The results showed that 4 hours of treatment with 200 µg/mL of CHAPk resulted in a 32.5% reduction in biofilm mass. In contrast, treatment of a 24-hour DPC5246 biofilm with the engineered endolysin CHAPk-SH3blys for 4 hours resulted in a 72% reduction in biofilm mass at the same concentration (26). Behera, et al. (27). also tested the performance of CHAPk-SH3bk, a recombinant endolysin combining the CHAP and cell wall-binding (CBD) domains of LysK, and CHAPk (without the CBD domain) in reducing biofilms of several *S. aureus* strains. The results showed that a concentration of 50 µg/ml of CHAPk was able to reduce 47% of the biofilm of strain SA1 and 40% of the biofilm of strain SA3 after 18 hours of treatment. CHAPk had no significant effect on the other strains. After 18 hours of treatment, biofilms of strains SA1, SA2, SA3, and strain ATCC®BAA-44™ were reduced by 40%, 20%, 30%, and 33%, respectively, upon exposure to 50 µg/ml of recombinant endolysin CHAPk-SH3bk (27). Therefore, it can be concluded that ZAM-CS is an effective option for treating *S. aureus* biofilm-associated infections.

CONCLUSION

In current study, we evaluated the effects of four common laboratory culture media and different in-

cubation times on the biofilm development of two strains of *S. aureus*. The results showed that the sensitive strain formed the most robust biofilm in TB after 24 hours of incubation. For the resistant strain, a stable and strong biofilm was observed in TSB, LB, and TB after 48 and 72 hours of incubation. We also evaluated the effect of chimeric endolysin on reducing biofilm formation. The findings indicated that the high lytic activity of the ZAM-CS and its efficacy at low concentrations, in comparison to other endolysins, position it as a promising candidate for combating *S. aureus* biofilms.

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