HEALTH, ENVIRONMENT, DEVELOPMENT

EFFECT OF STAPHYLOCOCCAL BACTERIOPHAGE ON BIOFILMS OF STAPHYLOCOCCUS AUREUS STRAINS SENSITIVE AND RESISTANT TO CEFOTAXIME AND AZITHROMYCIN DEPENDING ON THE CHEMICAL COMPOSITION OF THEIR MATRIX

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Summary

Removal of mature biofilms is the main task of therapy, because biofilms are the basement of the pathogenic process. An influence on staphylococcal biofilms can be done by destruction of extracellular matrix. Phages produce a depolymerase that can destroy the exopolysaccharide matrix of biofilms. This treatment is more effective than standard antibiotic therapy.

It was showed that the effect of the drug "Bacteriophage staphylococcal liquid" on biofilms of sensitive to cefotaxime and azithromycin strains, characterized by decreasing of absorbance level of eluted dye on 19.1%. A more significant effect of the bacteriophage was found for biofilms of strains resistant to cefotaxime and azithromycin (33.9% decrease in optical density).

Study the composition of biofilms matrix based on the specific destruction of its components. It was found that the main component in the biofilm matrix of the studied strains is exopolysaccharides, and their amount is 12.02% higher than in the antibiotic-resistant strain. After treatment with sodium periodate the decreasing of optical density of eluted dye at 47.00±2.87% for antibiotic sensitive strain and 59.02±2.10% – for resistant strain.

The results demonstrate the effectiveness of the phages drugs use to control the growth of biofilms formed by *Staphylococcus aureus* strains, the matrix of that consisting of a large number of exopolysaccharide.

Key words: staphylococci, biofilm, matrix, exopolysaccharides, polysaccharidedepolimerase.

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1. Introduction

In recent years, a lot of information has been accumulated about bacterial biofilms. Thus, it has been shown that biofilms are highly ordered communities of bacteria that form on biological or artificial surfaces *(Muhammad et al., 2020)* as a result of adhesion, growth and reproduction of microorganisms and the formation of an extracellular polysaccharide matrix, which is produced by the microbes themselves for protection *(Goltermann et al., 2024).* The bacteria themselves make up only 5–35% of the mass of the biofilm, the rest of it is an interbacterial

matrix, which can account for more than 90% of the total organic carbon of biofilms *(Pinto et al., 2020)*. Microorganisms form biofilms both in nature and in the host organism, which creates major problems in medical practice and in various industries. This is especially true for opportunistic bacteria, *S.aureus* in particular.

It is known that from 67% to 78% of clinical isolates of *S. aureus* can form biofilms. The main structural components of the *S. aureus* biofilm matrix are exopolysaccharides, which can also include proteins, glycolipids and bacterial DNA *(Chang et al., 2022)*.

The main element of the polysaccharide matrix of staphylococci is the polysaccharide intercellular adhesin PIA (Polysaccharide Intercellular Adhesin), which is involved in both cell-substrate adhesion and the subsequent formation of cell clusters. PIA initiates hemagglutination and prevents phagocytosis by activating bacterial aggregation *(Nguyen et al., 2020)*. The polysaccharide matrix plays an important role in the formation of antibiotic resistance of staphylococci in biofilms. The surface membrane and components of the biofilm matrix prevent access, bind and inactivate antimicrobial drugs *(Xia et al., 2025)*. Therefore, the issue of finding additional or alternative means to antibiotics in the fight against bacterial biofilms is currently relevant. In this sense, the use of phage therapeutic drugs may be promising, which will exert their effect by destroying the polysaccharide matrix with phage-encoded polysaccharide depolymerases *(Joo et al., 2023)*.

The aim of the work was to study the correlation between the chemical composition of the extracellular matrix and the effect of bacteriophages on biofilms of *S. aureus* strains sensitive and resistant to cefotaxime and azithromycin.

2. Object and methods of the study

To achieve the aim of the work, 2 film-forming strains of *S. aureus* were selected from the culture collection of the Department of Microbiology, Virology and Biotechnology of the Oles Honchar Dnipro National University. One of them was sensitive to cefotaxime and azithromycin, the other was resistant. The choice of these antibiotics was due to the fact that cefotaxime inhibits cell wall synthesis and can potentially inhibit the synthesis of the polysaccharide component of the matrix, while azithromycin is a blocker of protein synthesis and can potentially inhibit the synthesis of the protein component of the biofilm matrix.

The effect of bacteriophage therapeutic preparations on the film formation process was determined by changes in the number of cells in the biofilm and the optical density of biofilms. To determine the number of cells in the biofilm, 1 ml of the suspension of the studied culture with a cell content of 1×10^4 CFU/ml was seeded into 6-well plates in 1 ml of MPB. Only MPB (2 ml) was added to one of the wells and used as a sterility control. The plates with the test samples were incubated at 37°C for 72 hours. 1 ml of the standard bacteriophage preparation was added to the formed biofilms and incubated for another 24 hours. After incubation, the remains of the nutrient medium were removed from the wells of the plate. The contents of the wells were washed three times with 0.5% isotonic NaCl solution. Then, 1 ml of sodium dodecyl sulfate solution (0.004 mol/dm3) was added to the wells for 15 min to destroy intercellular bonds and the biofilm's bond to the plastic surface. The contents of the wells were collected and placed in sterile plastic tubes and centrifuged three times for 10 min at 5000 rpm. After each centrifugation, the liquid phase was collected and an isotonic NaCl solution was added. To determine the number of cells in the biofilm, 10-fold dilutions were made (0.1 ml of the suspension was added to a tube with 0.9 ml of 0.5% isotonic NaCl solution). 0.1 ml of each dilution was sown on Petri dishes with MPA. The sown areas were incubated for 24 h, the number of CFU in

1 ml of the sample was counted and compared with the number of cells in the biofilm not treated with phages.

To determine the change in optical density under the influence of bacteriophage preparations, a daily culture of the studied strains was diluted in 0.5% physiological solution according to the turbidity standard of 1×10^9 CFU/ml. The resulting suspension in an amount of 50 µl was added to the wells of a 96-well plate containing 150 µl of meat-peptone broth. The plates were incubated in a thermostat at a temperature of 37°C for 72 hours. Phages were added to the formed 72-hour biofilms. 24 hours after the introduction of the phage, the remnants of the nutrient medium were removed from the wells, washed three times with distilled water (200 µl each), fixed with 96° ethyl alcohol (50 µl) and stained with a crystal violet solution (50 µl) for 2 minutes. Then the dye was removed and washed three times with distilled water (200 µl). Optical density was determined on a SUNRISE microplate photometer, Tecan (Austria), in the "Absorption" measurement mode, "Normal" reading mode, and a wavelength of 620 nm using Magellan software.

To study the chemical structure of the extracellular matrix of *S. aureus* biofilms, substances that provide specific destruction of various matrix components were used *(Izano et al., 2008, Liu et al., 2021).* Seeding and incubation were performed as described above. After 72 hours of incubation, the remnants of the nutrient medium were removed from the wells, 50 µl of a solution of enzymes were added: proteinase K (Amresco, USA) and DNase (Fermentas, Lithuania) at a concentration of 250 µg/ml, trypsin (Merck Farma, Germany) at a concentration of 250 µg/ml and 50 µl of a solution of sodium periodate (sodium meta iodate) at a concentration of 40 mM. Biofilms with enzymes were incubated for 60 min at 37°C. Tris-Cl buffer (pH 7.5) without enzymes was used as a control. Biofilms with sodium periodate were incubated for 23 h at 4°C. Distilled water was used as a control. After incubation, the destructors were removed from the wells, washed three times with distilled water (200 µl each), fixed with 96° ethyl alcohol (50 µl) and stained with crystal violet solution (50 µl) for 2 minutes. Then the dye was removed and washed three times with distilled water (200 µl). Optical density was determined on a SUNRISE microplate photometer, Tecan (Austria), in the measurement mode "Absorption", reading mode "Normal" and wavelength 620 nm using Magellan software. The results were expressed as a percentage relative to the control samples.

Statistical processing of the results was performed for a significance level of 0.05 using Origin Lab Pro 7.0 software.

3. Results of the research and their discussion

When studying the effect of the drug "Staphylococcal bacteriophage liquid" on 72-hour biofilms of the studied strains, it was determined that for the strain sensitive to cefotaxime and azithromycin, a decrease in the optical density of the dye eluted by the 72-hour biofilm was observed by 19.1%, while the number of cells in the biofilm decreased by 253.0 times. For biofilms of the strain resistant to cefotaxime and azithromycin, a greater effect of staphylococcal bacteriophage was determined, it led to a decrease in optical density by 33.9%, while the number of cells decreased by 393.6 times.

Removal of mature biofilms is the main task of therapy, since it is such films that are the basis of the pathogenetic process. The ability of the matrix to actively bind and slow the diffusion of antibiotics through the biofilm leads to incomplete elimination of microorganisms, which in turn contributes to their survival and the formation of chronic processes *(Omwenga et al., 2024).* The effect on staphylococcal biofilms can be aimed at the destruction of the

extracellular polysaccharide matrix. Such treatment, which should affect the structure of the biofilm, is more effective than standard antibacterial therapy. Doolittle et al. *(Doolittle et al., 1996)* in 1996 described that phage progeny spread radially along the entire biofilm, infecting neighboring cells and destroying the biofilm matrix. To do this, phages produce depolymerases that are able to destroy the biofilm matrix *(Topka-Bielecka et al., 2021),* which largely depends on the structure of the polymer matrix. Therefore, an important stage of our research was the study of the biochemical composition of the polymer matrix of staphylococcal biofilms.

The study of the nature of the biofilm matrix was based on the specific destruction of its various components. To determine the proportion of exopolysaccharides in the composition of biofilms, the treatment of the formed 72-hour films with sodium periodate was used. It was found that after it there was a decrease in the optical density of the eluted dye by 47.00±2.87% for the antibiotic-sensitive strain and 59.02±2.10% for the resistant one.

To determine the presence of proteins in the matrix, proteinase K and trypsin were used. It was found that after the addition of proteinase K to the 72-hour biofilm, the optical density of the eluted dye retained by the biofilm decreased by 8.52±1.72% from the control of the 72-hour biofilm without the addition of the enzyme for the antibiotic-sensitive strain and by 3.52±1.11% for the resistant one. When trypsin was added, the decrease in the optical density of the eluted dye was also small – 12.16±1.49% for the antibiotic-sensitive strain and by 7.20±2.12% for the resistant one.

The content of extracellular DNA in the matrix was also determined. For this purpose, the DNase enzyme was added to the already formed 72-hour biofilm. It was shown that the optical density of biofilms decreased by 15.72±2.55% from the control 72-hour biofilm without the addition of the enzyme for the antibiotic-sensitive strain and by 14.50±1.33% for the resistant one.

Comparing the obtained results with known data *(Balducci et al., 2023, Bowden et al., 2024),* it can be assumed that the matrix of the studied staphylococcal biofilms consists mainly of polysaccharide material, which contains DNA and protein impurities. This does not contradict the literature data *(Lamret et al., 2021, Lu et al., 2022, Schilcher et al., 2020),* which indicate the existence of S. aureus strains with different matrix types.

Several studies of phage-biofilm interactions have demonstrated the ability of phage to degrade biofilm exopolysaccharides and infect biofilm cells *(Duarte et al., 2024, Plota et al., 2021).* Adams and Park *(Adams et al., 1956)* proposed the use of phage polysaccharide depolymerases to degrade bacterial exopolysaccharides in 1956. These enzymes are attached to the phage basal lamina in the form of spikes. The capsular material can serve as a secondary receptor and provide phage binding while the polymer is being dissolved, and then the phage is able to reach the outer cell membrane, bind to the receptor, and infect the cell *(Nazir et al., 2023).* Subsequently, a number of authors *(Dicks et al., 2024, Guo et al., 2023)* confirmed that the bacteriophage genome contains genes whose expression leads to the synthesis of specific destructive enzymes – polysaccharide depolymerases, which increases the mobility of bacteriophages upon contact with a polymeric substance. Phages that use extracellular depolymerases destroy a sufficient part of the matrix to make bacteria immersed in biofilms vulnerable.

Therefore, the determination of the dominant amount of exopolysaccharides in the matrix of the studied biofilms and the information about the encoding by phages of depolymerase enzymes that destroy polysaccharides in the matrix allow us to assume that the detected effect on *S. aureus* biofilms was provoked precisely by this method.

4. Conclusions

It was established that the main component in the matrix of the biofilms of the studied strains is exopolysaccharides, and their amount is 12.02% higher in the antibiotic-resistant strain. In addition, when staphylococcal bacteriophage was introduced into the biofilms of the studied strains, a decrease in the optical density of the eluted dye by 19.1% and the number of cells in the biofilm was noted up to 253.0 times for the antibiotic-sensitive strain and by 33.9% and 393.6 times, respectively, for the resistant strain. We can conclude that the intensity of the effect of bacteriophage preparations on biofilms of the studied *S. aureus* strains depends on the amount of exopolysaccharides in their matrix. The obtained results indicate the effectiveness of using bacteriophage therapeutic preparations to control the growth of biofilms of *S. aureus* strains, the matrix of which consists of a large amount of exopolysaccharides.

References

1. Adams M.H. & Park B.H. (1956). An enzyme produced by a phage-host cell system II. The properties of the polysaccharide depolymerase. Virology, 6 (2). 719-736. Retrieved from https://doi.org/10.1016/0042-6822(56)90054-X

2. Balducci, E., Papi, F., Capialbi, D.E., & Del Bino, L. (2023). Polysaccharides' Structures and Functions in Biofilm Architecture of Antimicrobial-Resistant (AMR) Pathogens. International Journal of Molecular Sciences, 24. Retrieved from https://doi.org/10.3390/ijms24044030

3. Bowden, L.C., Finlinson, J., Jones,B., & Berges, B.K. (2024). Beyond the double helix: the multifaceted landscape of extracellular DNA in Staphylococcus aureus biofilms. Frontiers in Cellular and Infection Microbiology, 14. Retrieved from https://doi.org/10.3389/fcimb.2024.1400648

4. Chang, C., Yu, X., Guo, W., Guo, C., Guo, X., Li, Q., [et al.]. (2022). Bacteriophage-Mediated Control of Biofilm: A Promising New Dawn for the Future. Frontiers in Microbiology, 13. Retrieved from https://doi.org/10.3389/fmicb.2022.825828

5. Dicks, L.M.T., & Vermeulen, W. (2024). Bacteriophage-Host Interactions and the Therapeutic Potential of Bacteriophages. Viruses, 3 (16). Retrieved from https://doi.org/10.3390/v16030478

6. Doolittle, M.M., Cooney, J.J., & Caldwell D.E. (1996). Tracing the interaction of bacteriophage with bacterial biofilms using fluorescent and chromogenic probes. Journal of Industrial Microbiology, 6 (16). 331-341. Retrieved from https://doi.org/10.1007/BF01570111

7. Duarte, A.C., Fernández, L., Jurado, A., Campelo, A.B., Shen, Y., Rodríguez, A., [et al.]. (2024). Synergistic removal of Staphylococcus aureus biofilms by using a combination of phage Kayvirus rodi with the exopolysaccharide depolymerase Dpo7. Frontiers in Microbiology, 15. Retrieved from https://doi.org/10.3389/fmicb.2024.1438022

8. Goltermann, L., Shahryari, S., Rybtke, M., & Tolker-Nielsen, T. (2024). Microbial Primer: The catalytic biofilm matrix. Microbiology, 8 (170). Retrieved from https://doi.org/10.1099/mic.0.001497

9. Guo, Z., Liu, M., & Zhang, D. (2023). Potential of phage depolymerase for the treatment of bacterial biofilms. Virulence. 1 (14). Retrieved from https://doi.org/10.1080/21505594.2023.2273567

10. Izano, E.A., Amarante, M.A., Kher, W.B., & Kaplan, J.B. (2008). Differential roles of Poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms. Applied and Environmental Microbiology. 2 (74). 470-476. Retrieved from https://doi.org/10.1128/AEM.02073-07

11. Joo, H., Wu, S.M., Soni, I., Wang-Crocker, C., Matern, T., Beck, J.P., [et al.]. (2023). Phage and Antibiotic Combinations Reduce Staphylococcus aureus in Static and Dynamic Biofilms Grown on an Implant Material. Viruses, 2 (15). Retrieved from https://doi.org/10.3390/v15020460

12. Lamret, F., Varin-Simon, J., Velard, F., Terryn, C., Mongaret, C., Marius, C., [et al.]. (2021). Staphylococcus aureus Strain-Dependent Biofilm Formation in Bone-Like Environment. Frontiers in Microbiology, 12. Retrieved from https://doi.org/10.3389/fmicb.2021.714994

13. Liu, J.J., Madec, J-Y., Bousquet-Mélou, A., Haenni, M., Ferran, A.A. (2021). Destruction of Staphylococcus aureus biofilms by combining an antibiotic with subtilisin A or calcium gluconate. Scientific Reports, 11. Retrieved from https://doi.org/10.1038/s41598-021-85722-4

14. Lu, Y., Cai, W-j., Ren, Z., Han, P. (2022). The Role of Staphylococcal Biofilm on the Surface of Implants in Orthopedic Infection. Microorganisms, 10 (10). Retrieved from https://doi.org/10.3390/microorganisms10101909

15. Muhammad, M.H., Idris, A.L., Fan, X., Guo, Y., Yu. Y., Jin, X., [et al.]. (2020). Beyond Risk: Bacterial Biofilms and Their Regulating Approaches. Frontiers in Microbiology, 11. Retrieved from https://doi.org/10.3389/fmicb.2020.00928

16. Nazir, A., Song. J., Chen, Y., & Liu, Y. (2023). Phage-Derived Depolymerase: Its Possible Role for Secondary Bacterial Infections in COVID-19 Patients. Microorganisms, 2 (11). Retrieved from https://doi.org/10.3390/microorganisms11020424

17. Nguyen, H.T.T., Nguyen, T.H., & Otto, M. (2020). The staphylococcal exopolysaccharide PIA – Biosynthesis and role in biofilm formation, colonization, and infection. Computational and Structural Biotechnology Journal, 18, 3324-3334. Retrieved from https://doi.org/10.1016/j.csbj.2020.10.027

18. Omwenga, E.O., & Awuor, S.O. (2024). The Bacterial Biofilms: Formation, Impacts, and Possible Management Targets in the Healthcare System. Canadian Journal of Infectious Diseases and Medical Microbiology. Retrieved from https://doi.org/10.1155/cjid/1542576

19. Pinto, R.M., Soares, F.A., Reis, S., Nunes, C., & Van Dijck, P. (2020). Innovative Strategies Toward the Disassembly of the EPS Matrix in Bacterial Biofilms. Frontiers in Microbiology, 11. Retrieved from https://doi.org/10.3389/fmicb.2020.00952

20. Plota, M., Sazakli, E., Giormezis, N., Gkartziou, F., Kolonitsiou, F., Leotsinidis, M., [et al.]. (2021). In Vitro Anti-Biofilm Activity of Bacteriophage K (ATCC 19685-B1) and Daptomycin against Staphylococci. Microorganisms, 9 (9). Retrieved from https://doi.org/10.3390/microorganisms9091853

21. Schilcher, K., & Horswill, A.R. (2020). Staphylococcal Biofilm Development: Structure, Regulation, and Treatment Strategies. Microbiology and Molecular Biology Reviews, 3 (84). Retrieved from https://doi.org/10.1128/mmbr.00026-19

22. Topka-Bielecka, G., Dydecka, A., Necel, A., Bloch, S., Nejman-Faleńczyk, B., Węgrzyn, G., [et al.]. (2021). Bacteriophage-Derived Depolymerases against Bacterial Biofilm. Antibiotics, 2 (10). Retrieved from https://doi.org/10.3390/antibiotics10020175

23. Xia, Y., Hu, Z., Jin, Q., Chen, Q., Zhao, C., Qiang, R., [et al.]. (2025). Structural characteristics, functions, and counteracting strategies of biofilms in Staphylococcus aureus. Computational and Structural Biotechnology Journal, 27, 488-500. Retrieved from https://doi.org/10.1016/j.csbj.2025.01.021