# COMPLEX ANTIBACTERIAL ACTION OF ENZYMES ACTING ON *STAPHYLOCOCCUS AUREUS* BIOFILMS

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The widespread use of antibiotics in medicine and agriculture has significantly accelerated the emergence rate of bacterial infections showing multiple antibiotic resistance. Since resistance to conventional antibiotics is developed rather quickly, designing alternative antimicrobial drugs with other mechanisms underlying their effects on bacteria is a promising. The enzymes possessing bactericidal activity may be one option for such antibacterial agents. The study aimed to produce the combination recombinant protein-based products active against bacteria and their biofilms. Soluble forms of five recombinant proteins were produced using the genetic engineering approaches. Two of these have a bacteriolytic effect (endolysins LysK and PM9 from the *Staphylococcus aureus* bacteriophages), the other are capable of disrupting extracellular DNA matrix in biofilms (two nonspecific nucleases NucA, as well as the DNA-specific deoxyribonuclease I). It has been shown that natural endolysin PM9 with the truncated catalytic domain shows 4 times lower bacteriolytic efficacy compared to the full-size LysK version. Comparative analysis revealed 1.5–2 timed higher efficacy of nonspecific nucleases in terms of bacterial biofilm disruption compared to the DNA-specific deoxyribonuclease I. It has been shown that simultaneous use of endolysins and nucleases has a synergistic antibacterial effect and disrupts biofilms of the pathogenic bacterium *Staphylococcus aureus*. The findings show the prospects of developing the recombinant protein-based antibacterial drugs.

**Keywords:** endolysin, nuclease, biofilms, *Staphylococcus aureus*

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# КОМПЛЕКСНОЕ АНТИБАКТЕРИАЛЬНОЕ ДЕЙСТВИЕ ФЕРМЕНТОВ НА БИОПЛЕНКИ *STAPHYLOCOCCUS AUREUS*

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Широкое использование антибиотиков в медицине и сельском хозяйстве значительно ускорило темпы возникновения бактерий со множественной устойчивостью к антибиотикам. Поскольку к традиционным антибиотикам довольно быстро возникает резистентность, одним из перспективных направлений является разработка альтернативных антимикробных препаратов, обладающих иными механизмами действия на бактерии. Ферменты с бактерицидным действием могут быть одним из вариантов таких антибактериальных средств. Целью работы было получить комбинированные препараты рекомбинантных белков, активных в отношении бактерий и их биопленок. С помощью подходов генной инженерии были получены растворимые формы пяти рекомбинантных белков. Два из них обладают бактериолитическим эффектом (эндолизины LysK и PM9 из бактериофагов *Staphylococcus aureus*), остальные способны разрушать внеклеточный ДНК-матрикс у биопленок (две неспецифические нуклеазы NucA, а также ДНК-специфичная дезоксирибонуклеаза I). Показано, что природный эндолизин PM9 с усеченным каталитическим доменом обладает меньшей в 4 раза бактериолитической эффективностью по сравнению с полноразмерным вариантом LysK. С помощью сравнительного анализа выявлена в 1,5–2 раза большая эффективность неспецифических нуклеаз для разрушения бактериальных биопленок по сравнению с ДНК-специфичной дезоксирибонуклеазой I. Продемонстрировано, что одновременное использование эндолизинов и нуклеаз оказывает синергичное антибактериальное действие и разрушает биопленки патогенной бактерии *Staphylococcus aureus*. Полученные результаты показывают перспективность разработки антибактериальных препаратов на основе рекомбинантных белков.

**Ключевые слова:** эндолизин, нуклеаза, биопленки, *Staphylococcus aureus*

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Drug resistance of pathogenic bacteria is growing rapidly, turning into a global issue of the world's healthcare system. According to the last data of the World Health Organization, the number of antimicrobial drugs being at various stages of their development has increased from 80 (2021) to 97 (2023). However, there is the question about creating novel, innovative agents for treatment of infectious diseases caused by antibiotic-resistant strains, which might complement the existing antibiotics that lose their efficacy due to widespread use [1].

The drugs based on the enzymes possessing antibacterial activity represent one such option. Today, the lytic enzymes of bacteriophages (endolysins) that cause lysis of pathogenic bacteria and can be used as therapeutic molecules are well-known [2]. Endolysins are the enzymes encoded by the bacteriophage genomes and used by bacteriophages for lysis of bacterial cells during viral infection. Since all bacteria have bacteriophages, and bacteriophage diversity is great, their endolysins represent a very promising class of bacteriolytic enzymes. Currently, there is a large amount of scientific research and antibacterial drugs developed based on such enzymes that are through pre-clinical and clinical trials [3]. Endolysins as therapeutic substances often have a broader specificity spectrum, than phages, and their specificity is manifested at the genus or species level. The endolysins' selectivity for bacterial targets is due to the cell wall-binding domain, which recognizes and binds receptors, regardless of certain substrate in the cell wall of the target. Thus, the cell wall-binding domain (CBD-domain) located usually in the C-terminal part of the endolysin molecule is responsible for specific recognition and binding. That is why gram-negative lysins have a broader range of targets, while gram-positive ones often have a narrow host range [4]. The catalytic domain (CD-domain) is responsible for catalytic activity, and its activity can be further enhanced by the presence of amidase domain [5]. The fact that bacteria do not develop resistance and the possibility of influencing various chemical bonds within the cell wall represent one more advantage of bacteriolytic enzymes over conventional antibiotics. However, endolysins cannot effectively disrupt stable biofilms formed by many pathogenic bacteria, which is one of the major challenges of modern antibacterial therapy. Biofilms are complex extracellular structures stabilized by multiple bonds between organic molecules [6]. The enzymes that destroy biofilms also represent a promising class of antibacterial agents that differ in enzyme activity type depending on the target biofilm component [7]. In particular, matrix of extracellular DNA that can be destroyed by nucleases is typical for many bacterial biofilms. It has been earlier shown that deoxyribonuclease I possessed such activity [8]. In this regard, the search for and characterization of novel nucleases possessing antibacterial activity are relevant. The use of bacteriolytic enzymes and their combinations with the enzymes having a destructing effect on biofilms can make it possible to increase the effectiveness of bacterial infection therapy. For example, such cumulative effect has been reported for antibacterial activity against *Mycobacterium tuberculosis* [9]. The enzymes that disrupt bonds within biofilms can become a component of the combination antibacterial drug together with endolysins, having a synergistic antibacterial effect. That is why the development of such antibacterial drugs based on the recombinant proteins showing different catalytic activity is a promising area. Such drugs are most effective against gram-positive bacteria, in particular, methicillin-resistant Staphylococcus aureus (MRSA), the fight against which in clinical settings is a priority [10].

The study aimed to perform comparative analysis of antibacterial effects of several combinations of the enzymes possessing antibacterial activity or activity against bacterial biofilms.

## METHODS

### Constructing plasmids

All the plasmids used in this study were produced by restriction enzyme DNA cloning. DNA sequences of the endolysin genes and DNA nucleases were *de novo* synthesized; this involved codon optimization of nucleotide sequences for *E. coli* (IDT, https://www.idtdna.com/). The nucleotide sequences were taken from the PhaLP (www.phalp.org) and Uniprot (www.uniprot.org) databases. Amplification of all DNA sequences involving the use of the Pfu high-fidelity polymerase (Takara, USA) was performed in accordance with the manufacturer's protocol. The target genes were cloned into the pET21a vector (Novagen, UK), as well as into the set of vectors with auxiliary peptides [11] based on pET28a (Novagen, UK) at the NdeI and NotI restriction sites through the enzyme reaction involving two appropriate restriction endonucleases (SibEnzyme, Russia) for 1 h at 37 °C. After electrophoresis, DNA fragments were purified from agarose gel using the commercially available GeneJet Gel Extraction Kit (Thermo Scientific, USA). Then the purified DNA fragments were mixed and used in the ligation reaction with the T4 DNA ligase (NEB, USA) for 30 min at room temperature. After that the *E. coli* 10G competent cells (Lucigen, UK) were transformed by electroporation (Bio-Rad, USA) in accordance with the protocol of the device manufacturer. Plasmid DNA was extracted from the clones comprising the correct inserts, and correctness was confirmed by Sanger sequencing.

### Recombinant protein production in *E. coli*

Proteins were produced in the following strains designed for recombinant protein production: *E. coli* BL21 (DE3) (Novagen, UK), *E. coli* Rosetta gami 2 (Novagen, UK), *E. coli* SHuffle T7express (NEB, USA). Thus, plasmids comprising the genes encoding nucleases or endolysins were used to transform the selected strain by the heat shock method, the transformation mixture (100 µL) was seeded in liquid LB medium contained in the test tubes (3–5 mL) with selective antibiotics. Incubation was performed for 12–14 h at 37 °C with mixing at 180 rpm. Then the culture grown was passaged at the ratio of 1 : 200 in the flasks containing appropriate amounts of the LB culture medium (100–500 mL) with selective antibiotics. The cell culture was grown to the optical density $OD_{600}$ = 0.5–0.6 on the shaker at 180 rpm and 37 °C. After reaching the target optical density, the culture was cooled on ice for 10 min, supplemented the inducer to the final concentration of 1 mM IPTG, incubated at different temperatures (18 °C and 37 °C) with mixing at 180 rpm for 18 h (18 °C) or 6 h (37 °C). The bacterial cells were then precipitated by centrifugation for 20 min at 4000 g and 4 °C.

To assess solubility of the protein obtained, the cells precipitated were transferred to buffer A for destruction (50 mM Tris-HCl pH 7.5; 0.3 M NaCl; 0.005 M imidazole) and destructed using the Qsonica Q700 ultrasonic homogenizer (Qsonica, USA) until the cell suspension became clear on ice (ultrasonic pulses 3 s, cooling 6 s, 40 cycles). The destructed cells were precipitated by centrifugation (17,000 g) for 20 min at 4 C°. Then supernatant containing the soluble form of the target protein was sampled, and precipitate containing the aggregated form was dissolved in the 2M urea for further analysis by electrophoresis. Proteins were separated by PAGE (10%) electrophoresis under denaturing conditions by standard methods. After the One-Step Blue® staining (Biotium, USA) and washing off the dye, the gels were imaged, and the distribution of soluble and insoluble protein forms was quantified. Densitometry analysis of the gels was performed using the Image Lab application (Bio-Rad, USA).

### Purification of proteins by affinity chromatography

After ultrasonic destruction of biomass and cell debris centrifugation, supernatant was filtered through the membrane

with the pore size of 0.22 μm. Thus the preparation for chromatographic purification was obtained that contained soluble protein fraction in buffer A (50 mM Tris-HCl pH 7.5; 0.3 M NaCl; 0.005 M imidazole) for application onto the IMAC chromatographic sorbent (Bio-Rad® Nuviatm IMAC Resin, USA). Protein was eluted with the chromatographic buffer B (50 mM Tris-HCl pH 7.5; 0.5 M imidazole). When necessary, the preparation obtained by purification involving the use of IMAC was concentrated using the Vivaspin® 500 centrifugal concentrator (Sartorius, Germany) (pore size 3.5 kDa) 30–50 times. If necessary, then the purified protein preparation was treated with the TEV protease in accordance with the manufacturer's instructions overnight at 4 °C; after that chromatographic purification was performed again, but the fraction not bound to sorbent, which contained purified endolysin, was collected. The resulting preparation was used to estimate antibacterial activity.

**Testing of the resulting protein preparation antibacterial activity against *Staphylococcus aureus***

The *Staphylococcus aureus* colony was taken and put in 3–5 mL of liquid LB medium and incubated for 12–14 h at 37 °C with mixing at 180 rpm. Then 1 mL of the overnight culture was transferred to the test tube, centrifuged for 3 min at 4000 g; the cell sediment was resuspended in PBS; the procedure was repeated 3 times. The cells washed were diluted with PBS to 0.1 at $OD_{600}$; 100 μL of the diluted cells were placed in the 96-well plate and added 100 μL of the test protein or 100 μL of PBS in the positive control. The plate was incubated at room temperature for 30 min with constant mixing at 200 rpm. After incubation, the cells were 100-fold diluted with PBS, after which 100 μL of the resulting mixture were seeded on the agar plates without any antibiotic. The plates were incubated overnight at 37 °C. On the next day colonies were enumerated in the control plates and plates with test proteins; antibacterial activity was calculated using the following formula:

$$X = 100 - ((\text{CFU of the sample} \times 100)/ \text{CFU of the control}),$$

where X is antibacterial activity of the test protein in %; CFU of the sample is the number of colony-forming units per plate after incubation of cells with the test protein; CFU of the control is the number of plaque-forming units per plate untreated with the test protein.

To assess anti-biofilm activity of the test proteins, the *S. aureus* colony was placed in 3–5 mL of the liquid TBS medium (glucose free) and incubated for 12–14 h at 37 °C with mixing at 180 rpm. Then the overnight culture was diluted with TBS supplemented with 1% glucose to the value of 0.08 at $OD_{600}$. After that 200 μL of cell suspension were poured in the 96-well plate and incubated at 37 °C without mixing for 48 h. After incubation, the resulting biofilms were triple washed with PBS by pipetting. Thus planktonic cells were eliminated. Then biofilms were added 200 μL of each studied enzyme and incubated for 2 h at 37 °C without mixing. After incubation, the wells were triple washed with PBS by pipetting and dried for 5 min at room temperature. The remaining biofilms were stained by adding 200 μL of the 0.1% crystal violet and incubating for 15 min at room temperature. After staining, the cells were triple washed with PBS by pipetting again and dried for 45 min. Then these were added 200 μL of the 33% acetic acid and incubated for 10 min at room temperature. The analysis performed using the Multiskan SkyHigh system (Thermo Scientific, USA) involved optical density measurement at $OD_{600}$. The effectiveness of biofilm destruction was calculated relative to control.

RESULTS

Production of the enzyme-based combination antibacterial drugs is a promising method to combine different enzyme activities against bacterial pathogens in the same medicinal product. This makes it possible to not only increase the efficacy of antimicrobial activity, including against biofilms, but also reduce the drug dose. To produce single components of such drugs, it is necessary to produce homogenous protein preparations for each enzyme. In this study we produced different recombinant protein preparations and then tested their antibacterial activity against single *Staphylococcus aureus* cells and biofilms. The general scheme of the experimental study is provided in Fig. 1. The first phase of the study involved production of the genetic constructs encoding the following proteins: 1) endolysin LysK of the staphylococcal phage K; 2) not previously annotated endolysin PM9_074 of the staphylococcal phage PM9 with the truncated catalytic domain; 3) human deoxyribonuclease I (DNAse I); 4) nonspecific nuclease NucA1 from *Serratia marcescens*; 5) nonspecific nuclease NucA2 from *Anabaena sp.*

All the nucleotide sequences were codon optimized for expression in *Escherichia coli* and encoded the histidine tag at the N-terminus for further purification by affinity chromatography. After *de novo* synthesis of genes these were cloned into the pET21a plasmid vector for further expression of the genes encoding recombinant proteins. The genetic constructs obtained were used to transform cells of the *E. coli* BL21 (DE3) strain designed for recombinant protein production. After induction and incubation, we estimated the extent of target protein production in the soluble and insoluble forms. Protein production at 37 °C and 20 °C failed to yield positive results, since all the target enzymes formed insoluble aggregates (inclusion bodies). Replacement of the strain with two alternative ones (*E. coli Rosetta gami 2*, *E. coli SHuffle T7express*) yielded no positive results (Table 1).

That is why later is was decided to turn to production involving auxiliary polypeptides potentially increasing protein solubility and ensuring their correct folding in space in order to obtain soluble forms of proteins [12]. The nucleotide sequences encoding the selected endolysins and nucleases were cloned into a number of the earlier constructed vectors comprising various auxiliary polypeptides. We had earlier used this approach in practice to produce the homogenous ribonuclease inhibitor preparation in the soluble form [13]. The system represents the set of plasmids, each of which contains certain helper polypeptide and the TEV protease cleavage site at the N-terminus of the target gene nucleotide sequence. Production of chimeric protein using such vector increases the likelihood of obtaining the soluble protein preparation. The following auxiliary polypeptides were used in the system: MBP, GST, TIG, YrhB, PpiB, TRX, TSF, SUMO, FH8. Verification of recombinant protein production using this set of plasmids was also performed in the *E. coli* BL(DE3) strain at two temperatures, as earlier reported. Protein production at 37 °C, regardless of the presence of partner polypeptide, yielded mainly insoluble aggregates, in contrast to protein production at 18 °C, where certain auxiliary polypeptides considerably increased yield of the target protein soluble form (Fig. 2, Table 2).

Thus, the most productive "auxiliary polypeptide-target protein" combinations were obtained; we also managed to select appropriate production conditions that allowed us to produce target proteins in the soluble form. The highest yield of endolysins and nucleases was reported when using auxiliary polypeptides MBP and TIG (Table 2).

Then we assessed antibacterial activity of the enzymes obtained, as well as their ability to destroy the bacterial

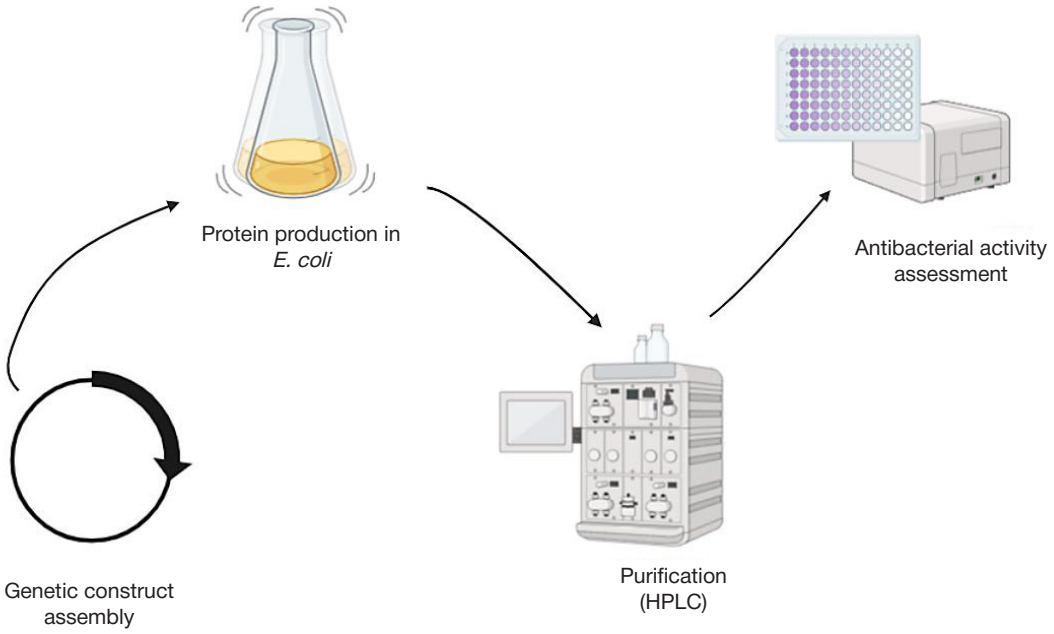


**Fig. 1.** Scheme of the experimental study involving production of enzybiotic drugs and testing their antibacterial activity

*Staphylococcus aureus* biofilms both in the form of monodrugs and in the form of enzyme activity combinations. First, we compared activity of two endolysins against bacterial cells (Fig. 3). The identical recognition domains of both endolysins allow one to compare catalytic activity of these molecules in the direct experiment. The findings demonstrate that both enzymes possess antibacterial activity and can cause lysis of the Staphylococcus aureus cells, but endolysin of bacteriophage PM9 with the truncated catalytic domain yet shows lower activity (almost four times lower relative to endolysin LysK). Presumably, such variants are less evolutionarily advantageous for viruses, so their number in the genome is significantly lower compared to the number of full-size endolysin genes. It is possible that the genes encoding such molecules represent "evolutionary waste" in bacteriophage genomes, but their exact biological role is poorly understood. These data suggest that it is reasonable to select endolysins with the full-fledged catalytic domain (or several molecules with the catalytic domains showing different specificity) to create effective antibacterial drugs.

In the next phase we tested three nucleases obtained for the ability to disrupt *Staphylococcus aureus* biofilms via destruction of the matrix consisting of extracellular DNA. It was earlier shown that deoxyribonuclease I could destroy bacterial biofilms [8], however, no such research was conducted for nonspecific nucleases. All three nucleases, we have isolated in the form of recombinant protein preparations, specifically human deoxyribonuclease I, nonspecific nuclease NucA1 from *Serratia marcescens*, and nonspecific nuclease NucA2 from *Anabaena sp.,* can degrade bacterial biofilms (Fig. 4). The results obtained show that both nonspecific nucleases show higher biofilm destruction effectiveness, which can be due to their mechanism of action. Their higher nuclease activity has led to broader use of nonspecific nucleases for solving scientific and biotechnology tasks, specifically for the

**Table 1.** Estimation of the recombinant endolysin and nuclease production in *E. coli* strains

| Temperatures of protein biosynthesis | *Escherichia coli BL21 (DE3)* | *Escherichia coli Rosetta gami 2* | *Escherichia coli SHuffle T7express* |
|---|---|---|---|
| pET21-LysK | | | |
| 37 °C (6 h) | I | I | I |
| 18 °C (16–18 h) | I | I | S+ |
| pET21-PM9 | | | |
| 37 °C (6 h) | I | I | I |
| 18 °C (16–18 h) | I | S+ | S+ |
| pET21-DnaseI | | | |
| 37 °C (6 h) | I | I | I |
| 18 °C (16–18 h) | I | I | I |
| pET21-NucA1 | | | |
| 37 °C (6 h) | I | I | I |
| 18 °C (16–18 h) | I | I | I |
| pET21-NucA2 | | | |
| 37 °C (6 h) | I | I | I |
| 18 °C (16–18 h) | I | I | S+ |

**Note:** S — soluble, I — insoluble, "+" — extent of soluble protein production.

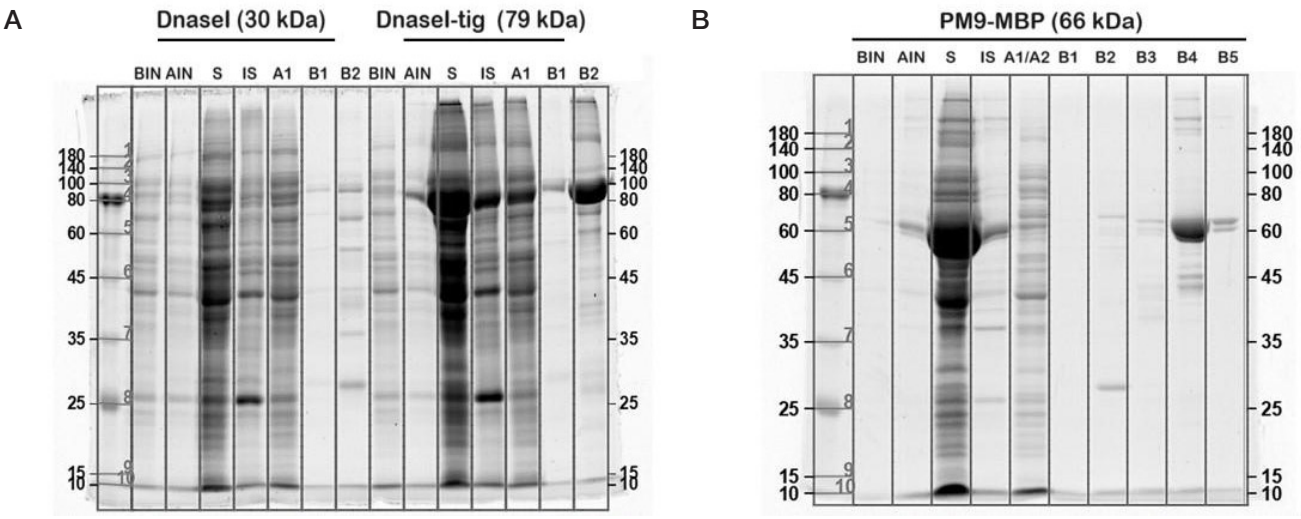


**Fig. 2.** Strategy of obtaining recombinant proteins prone to aggregation via production in the form of the chimeric molecule, in which there is an auxiliary polypeptide increasing the target protein solubility at the N-terminus. In particular, PAGE electropherogram demonstrates the examples of the TIG auxiliary polypeptide effects on the DNAse I solubility **(A)** and MBP auxiliary polypeptide effects on the endilysin PM9 solubility **(B)**

development of the commercially available *Benzonase* drug by *Merck*. Thus, nonspecific nucleases can be appropriate for the creation of antibacterial drugs capable of destroying bacterial biofilms. That is why it is reasonable to focus on the physical and chemical properties of such enzymes, as well as on the results of pre-clinical trials during further development of the combination antibacterial drug. Despite the fact that the DNAse I enzyme shows lower activity, the advantage is its origin, which potentially minimizes the drug immunogenicity. It should be also noted that there are already commercially available drugs based on this recombinant enzyme (Dornase Alfa and analogues), which are used for treatment.

The last fundamental objective of this study was to test the antibacterial activity effectiveness of the combination drug combining at least two different catalytic activities against the *Staphylococcus aureus* biofilms. Thus, we planned to test various combinations of two endolysin molecules and three nucleases. In our situation the best results were reported for the combinations of full-size endolysin LysK and nucleases, which can be explained by higher lytic activity of this enzyme compared to endolysin PM9 with the truncated catalytic domain (Fig. 4). All three nucleases used were active in the combination drug, but the best results were shown by nonspecific nucleases. This was possibly due to experimental conditions, and DNAse I could show comparable efficacy in case of optimal reaction buffer selection. However, the difference in the enzyme activity mechanisms can play a major role during practical use of drugs. Thus, nonspecific nucleases can be used in the recombinant protein-based combination drugs or can potentiate the effects of antibiotics on bacterial biofilms.

Summarizing the findings, we selected conditions for production and chromatographic purification of two endolysins (LysK and PM9) possessing activity against *Staphylococcus aureus*, as well as of three nucleases (NucA1, NucA2, and DNAse I) capable of destroying the bacterial biofilm DNA matrix. For that we used a screening approach to production of enzymes in combination with helper polypeptides. Then we demonstrated antibacterial activity of these enzymes, as well as their complex activity against the *Staphylococcus aureus* biofilms.

**Table 2.** Estimation of the recombinant endolysin and nuclease production in *E. coli* strains

| Temperatures of protein biosynthesis | Protein solubility degree | Yield of protein after purification (mg/L) |
|---|---|---|
| pET28MBP-LysK | | |
| 37 °C (6 h) | I | |
| 18 °C (16–18 h) | S+ | 2.55 |
| pET28MBP-PM9 | | |
| 37 °C (6 h) | I | |
| 18 °C (16–18 h) | S++ | 4.95 |
| pET28TIG-DnaseI | | |
| 37 °C (6 h) | I | |
| 18 °C (16–18 h) | S+++ | 7.4 |
| pET28MBP-NucA1 | | |
| 37 °C (6 h) | I | |
| 18 °C (16–18 h) | S+++ | 6.7 |
| pET28TIG-NucA2 | | |
| 37 °C (6 h) | I | |
| 18 °C (16–18 h) | S++ | 4.8 |

**Note:** S — soluble, I — insoluble, "+" — extent of soluble protein production.

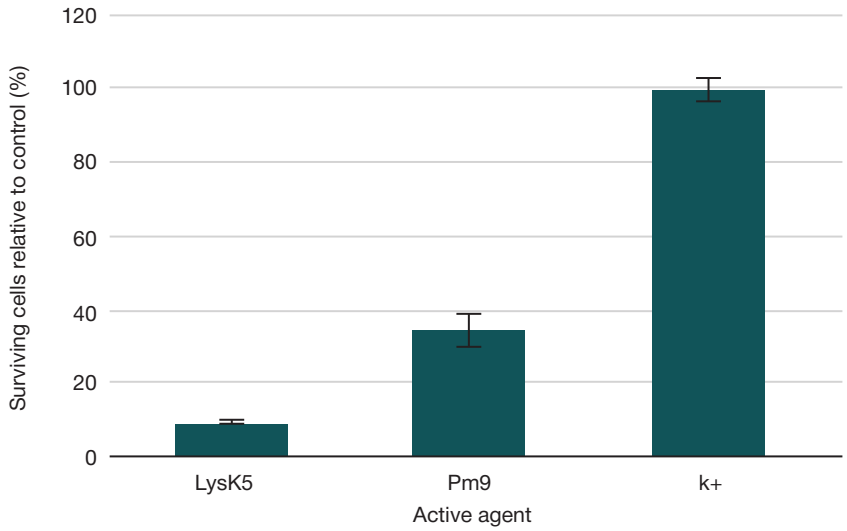


**Fig. 3.** Catalytic activity of two endolysins against the *Staphylococcus aureus* bacterial cell wall. Both recombinant enzymes possess antibacterial activity disrupting the cell wall. Endolysin LysK having a full-fledged catalytic domain possess significantly higher antibacterial activity against the gram-positive *Staphylococcus aureus* pathogen compared to endolysin PM9 having a truncated catalytic domain

## DISCUSSION

The current antibiotic-resistance crisis caused by the widespread use of antibiotics in medicine and agriculture makes it necessary to seek for alternative therapeutic agents to fight against pathogenic bacteria. Such drugs might be used to support therapy with conventional antibiotics or as monotherapy, precluding the use of antibiotics in certain cases. Developing antibacterial drugs based on the recombinant proteins possessing bacteriolytic activity is a promising area [14]. The most interesting are endolysins, the bacteriolytic proteins used by bacteriophages to lyse infected bacterial cells during viral infection. Endolysins have a modular structure and usually comprise two or three domains responsible for recognition of certain bacteria, as well as for catalytic activity against the cell wall components. To date, these molecules are rather well understood; attempts are made to develop antimicrobial drugs, enzybiotics, based on these molecules [14]. As genomes of bacteriophages are discovered and described, the data on the uncharacterized viral proteins, including endolysins, are accumulated. Some of these endolysins have truncated catalytic domains, but biological meaning of this phenomenon is poorly understood. In this study we compared two endolysins encoded by two *Staphylococcus aureus* bacteriophages, K and PM9. One of these is well-known, it has a full-fledged three-domain structure, including catalytic domains (peptidase and amidase), as well as the recognition domain [15], while the second represents an uncharacterized endolysin with the significantly truncated catalytic domain and the binding domain with the identical sequence. When produced in the *E. coli* heterologous system, both endolysins aggregate and form the inclusion bodies. To obtain functional preparations of such proteins possessing activity, we used a methodological approach, in which solubility of the target recombinant protein is ensured by auxiliary polypeptide located at the N-terminus of the chimeric protein molecule (Fig. 2, Table 2). After successful production of chimeric molecules in the soluble form, the auxiliary peptide was cleaved using the TEV protease, and the proteins were used to conduct tests for antimicrobial activity against the *Staphylococcus aureus* cells. Our findings suggest that the phage PM9 endolysin with the truncated catalytic domain exerts significantly lower activity against the *Staphylococcus aureus* cells (Fig. 3). Furthermore, both endolysins have the recognition domains that are completely identical based on the amino acid compositions, which balances the differences when the molecules bind to receptors of bacterial cells. The use of such enzymes is unjustified due to low activity, regardless of their greater compactness and some beneficial differences in physical and chemical properties from full-size molecules. That is why it is necessary to select variants with full-size domains for the development of endolysin-based

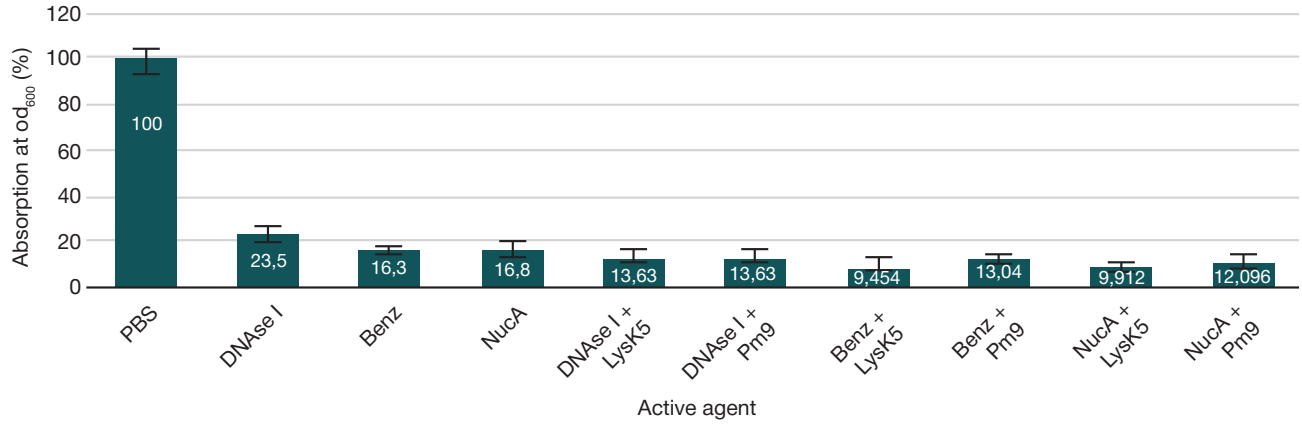


**Fig. 4.** Destruction of the *Staphylococcus aureus* bacterial biofilms with the recombinant nuclease drugs and the combination drugs combining different catalytic activities (endolysin + nuclease). Nonspecific nucleases show higher activity in terms of bacterial biofilm disruption compared to DNAse I. Combining the enzymes showing different specificity in the same drug enhances antibacterial effects

drugs. Research and development in the field of combinatorial engineering of endolysins show the potential of such proteins used as antibacterial agents [4, 14]. Endolysins with the full-fledged catalytic domain predictably possess higher antibacterial activity compared to variants with the truncated catalytic domain. Furthermore, the binding domain of such truncated endolysin variants is fully functional, which targets these molecules towards cellular receptors of bacteria during infection, but biological role of this phenomenon is poorly understood. It can be assumed that such forms of endolysin molecules represent either evolutionarily transitional variants, or rudimentary enzymes, on which the life cycle of the virus no longer depends very much, but they are preserved in the viral genome.

However, it should be noted that antibacterial activity of endolysins against bacterial biofilms that are often formed by pathogenic bacteria is limited. Since the structure of bacterial biofilms is stabilized by extracellular matrix consisting of various bioorganic molecules, the bonds between molecules represent a potential target for biofilm destruction [6]. Extracellular DNA forming sustainable structures that stabilize biofilms represents one component of such biofilm matrix [16, 17]. It has been earlier shown that catalytic activity of deoxyribonuclease I (DNAse I), hydrolyzing phosphodiester bonds between the nucleic acid subunits, can ensure disruption of bacterial biofilms formed in the presence of infection [8]. We decided to test two more nucleases possessing nonspecific activity against DNA and RNA (nuclease NucA from the bacterium *Serratia marcescens* and nuclease NucA from *Anabaena sp.*) for similar activity and compare their efficacy with that of human DNAse I. For that we produced all three nucleases in the form of homogenous protein preparations; since these proteins also aggregate when produced in *E. coli*, we conducted screening and selected appropriate auxiliary proteins for production of nucleases in the soluble form (Fig. 2, Table 2). The proteins isolated show activity against the *Staphylococcus aureus* biofilms, and both nonspecific nucleases, NucA1 from *Serratia marcescens* and NucA2 from *Anabaena sp.*, show the highest activity among three in the biofilm destruction test (Fig. 4). Thus, any nucleases could potentially be used to develop antibacterial drugs; in this case, it is possible to focus on more suitable physical and chemical properties of enzymes and the safety profile based on the results of preclinical trials. Perhaps, human DNAse I is preferable in such preparation, despite its lower catalytic activity, because the immune response to it would be negligible, in contrast to foreign recombinant proteins. Moreover, there are already commercially available drugs based on this enzyme to alleviate the symptoms of cystic fibrosis, as well as bacterial complications of viral infections [18].

Combining two (or more) catalytic activities in the same antibacterial drug makes it possible to target different bacterial biofilm structures during infection [19]. Thus, the enzymes destroying extracellular structural components of biofilms can provide access to the internal layers of bacterial cells for bacteriolytic enzymes and improve the efficacy of their activity against distinct bacterial cells [9]. Then we tried to combine endolysins with the nucleases obtained in our study. We assessed the effects of these combination drugs on the *Staphylococcus aureus* biofilms and estimated their lytic activity against cells of this gram-positive bacterial pathogen. The predictably best results in case of joint action were yielded by the pairs, were endolysin LysK functioned in combination with one of three nucleases (Fig. 4). The combinations of endolysin with the truncated catalytic domain and nucleases did not yield such good results, which was predictable based on testing antimicrobial activity of this endolysin against the *Staphylococcus aureus* cells (Fig. 3). It can be concluded that the use of two (or more) catalytic activities ensured by different enzymes in the same antibacterial drug is promising for treatment of bacterial infections complicated by biofilm formation. It seems feasible to use nucleases (both nonspecific and DNA-specific) in such drugs, since their activity significantly degrades bacterial biofilms, ensuring potentiating effect of antibacterial activity of both endolysins and conventional antibiotics.

## CONCLUSIONS

The findings demonstrate the possibility of producing the combination antibacterial enzyme drugs based on the bacteriophage endolytic enzymes and the enzymes that degrade bacterial biofilms. We have shown that endolysin PM9 with the truncated catalytic domain exerts lower bacteriolytic activity compared to endolysin LysK with the full-fledged catalytic domain, while nonspecific nucleases possess higher destructive activity against bacterial biofilms. We have also conducted comparative analysis of efficacy of the activity of several enzyme combinations against the *Staphylococcus aureus* bacteria and their biofilms. The results show synergistic antimicrobial effect of the purified preparations of recombinant endolysins LysK and PM9, as well as of nonspecific (NucA1 and NucA2) and DNA-specific (DNAse I) nucleases. In the future, this approach to engineering combination drugs that combine two or three different types of catalytic activity will help to produce therapeutic agents with enhanced antibacterial properties against multidrug-resistant microorganisms that are prone to biofilm formation during infections.

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