Effect of extracellular levanase SacC from *Bacillus subtillis* on two–species *Staphylococcus aureus* – *Pseudomonas aeruginosa* biofilms

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Abstract. The formation of polymicrobial biofilms significantly increases the resistance of bacteria in them to a wide range of antibiotics thus making their eradication challenging in infectious medicine. In recent years, the emergence of a large number of antibiotic-resistant strains has contributed to a significant increase in severe cases of infectious diseases. Natural compounds such as alkaloids, terpenes, bacterial peptides and enzymes have great potential in combating polymicrobial diseases, associated with the biofilm formation. At the same time, the enzymatic treatment of biofilms has a number of advantages, since the enzymes are nontoxic, do not cause resistance and can increase the effectiveness of existing antimicrobials. Here we show that recombinant extracellular levanase SacC can promote the destruction of two-species biofilms *S. aureus – P. aeruginosa* and increase the effectiveness of antibiotics against bacteria in the biofilm up to 16 times.

1 Introduction

Bacterial biofilms are complex communities of microorganisms that are held together by an extracellular polymer matrix [1]. The main function of the biofilm is the protection of bacteria from adverse physical, chemical and biological environmental factors, such as dehydration, ultraviolet radiation, exposure to biocides, humoral and cellular immunity of the host [2]. Bacterial biofilms cause many problems in various areas including food production (biofilm formation by pathogenic microorganisms leads to product spoilage), implantology (biofilms on medical equipment such as intravenous and urinary catheters, cardiac pacemakers, and contact lenses), and surgery as a consequence of chronic diseases [3-6]. Therapy of infections associated with mixed biofilms presents additional problems because antibacterial agents often target only part of bacterial species and is less effective against communities consisting of various microorganisms. Therefore, there is a need to explore alternative strategies to combat mixed biofilms, in addition to antibiotic therapy.

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Staphylococcus aureus and *Pseudomonas aeruginosa* are the most frequently detected species in mixed communities in various infections. It is known that co-infection with these microorganisms slows down wound healing (diabetic ulcers and chronic wounds) and causes inflammation in the host [7]. On the wounds surfaces, *S. aureus* and *P. aeruginosa* engage in symbiotic interactions. Biopsy of wounds has shown that *S. aureus* is typically located in the upper layers of the wound, while *P. aeruginosa* is found in deeper layers of the wound surface, where it produces various virulence factors [8]. In the lungs of patients with cystic fibrosis associated with polymicrobial infections, such microbial representatives as *S. aureus*, *H. influenzae*, *P. aeruginosa* and *B. cepacia* were found, which affect the severity of the disease [9]. Typically, *S. aureus* and *H. influenzae* are the primary colonizers, while *P. aeruginosa* and *B. cepacia* become dominant species at later stages [10].

The extracellular matrix is a complex mixture of organic molecules, including polysaccharides, proteins, extracellular DNA, and phospholipids [11]. The composition of the matrix varies depending on the pathogen, the age of the biofilm, and environmental conditions (pH, oxygen, nitrogen, temperature, nutrient availability) [12]. The extracellular matrix of biofilms acts as a barrier to protect bacteria from antimicrobials. Additionally, it is responsible for maintaining the biofilm cell community in close proximity, thereby facilitating intercellular interactions and horizontal gene transfer [2]. Based on this, the using of enzymes for the matrix components destruction is a promising approach for the biofilm disruption. Thus, the effectiveness of enzymes such as deoxyribonuclease I (DNase I), dispersin B, alginate lyase, protease, cellulase, alpha-amylase, alpha-mannosidase, lysostaphin has already been proven against monospecies bacterial biofilms. [13-18]. However, the effect of enzymes on polymicrobial biofilms remains poorly understood. Xiao et al reported a significant reduction in bacterial biomass and extracellular matrix of polymicrobial oral biofilms after mutanase treatment [19]. Cellobiase and DNase immobilized on chitosan nanoparticles were able to penetrate and promote the destruction of two-species biofilms of *S. aureus* and *C. albicans* [20]. It has also been shown that treatment with Longidase, which is a hyaluronidase cross-linked with a copolymer of 1,4-ethylenepiperazine N-oxide and (N-carboxymethyl)-1,4-ethylenepiperazinium bromide, leads to the destruction of two-species biofilms of *S. aureus – P. aeruginosa* and *S. aureus – K. pneumoniae* [21]. The use of trypsin, β-glucosidase, DNase, and their complexes also contributed to the disruption of dual-species biofilms of *S. aureus – P. aeruginosa* [22].

This study shows the ability of recombinant extracellular levanase from Bacillus subtillis to disrupt mature two–species biofilms of *S. aureus – P. aeruginosa* and increase the effectiveness of antibiotics against both detached cells and cells in biofilms.

2 Materials and methods

2.1 Antimicrobials

Amikacin (Sigma, USA), Ciprofloxacin (Sigma, USA) were used as antimicrobials. Solutions of the test compounds were prepared at a concentration of 10 mg/ml in deionized water.

2.2 Bacterial strains and growth conditions

The antimicrobial effect was evaluated using *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. For the hyperproduction of recombinant saccharose SacC, *Escherichia coli* BL21 strain was used.

Bacteria were cultivated in LB medium. To obtain two-species biofilms, the BM medium was used [23, 24].

2.3 Purification of extracellular levanase SacC

Plasmid pASK-SacCst, carrying the extracellular levanase *sacC* gene, was obtained previously [25]. The SacC protein, carrying a C-terminal strepII-tag sequence, was expressed in *E. coli* BL21 cells and purified using affinity chromatography on Strep-tactin sepharose columns (IBA, Germany) [26]. Samples containing the highest amount of target protein were concentrated by ultrafiltration using VivaSpin columns (Sartorius).

2.4 Evaluation of antimicrobial activity

The minimum inhibitory concentration (MIC) was determined by serial microdilution in 96-well plates according to the EUCAST recommendations with some modifications. [27]. MIC was defined as the lowest concentration of antimicrobials, at which no visible bacterial growth was observed after 24-hour incubation.

2.5 Biofilm assay

The effect of extracellular levanase on mature dual species biofilms was assessed using crystal violet staining [28] with modifications [23]. Bacteria with an initial density of 3×10^7 CFU/ml were seeded in 2 ml of BM nutrient medium in 24-well adhesive plates (Eppendorf) and cultivated at 37 °C for 48 hours, after which the nutrient medium was replaced with a fresh one with the addition of enzyme. Then the culture liquid was removed, the plates were washed twice with phosphate-buffered saline (PBS) pH = 7.4 and dried in air for 2 hours. Then, 1 mL of 1% crystal violet (Sigma-Aldrich) in 96% ethanol was added to each well, followed by incubation for 20 min. Unbound dye was washed off with PBS, and the bound dye was eluted with 1 ml of 96% ethanol. The optical density was measured at 570 nm using a Tecan Infinite 200 Pro microplate reader (Switzerland).

The viability of bacteria in biofilms after combined exposure to hydrolase and antimicrobials was assessed using a metabolic MTT test [29]. To analyze the metabolic activity of detached cells, 100 µl of culture fluid was transferred to a round-bottomed 96-well plate. To assess bacterial viability, the liquid from wells was removed and the biofilm was mechanically destroyed in 0.9% NaCl solution and 100 µl of suspension was transferred to a 96-well plate. The plate was centrifuged for 5 minutes at 3500 rpm. Then, the supernatant was removed and 100 µl of an MTT solution at a concentration of 1 mg/ml in PBS was added. The plate was incubated at 33 °C for 2-4 hours until formazan crystals appeared in the control wells. Subsequently, to precipitate the crystals, the plate was centrifuged for 5 minutes at 3500 rpm. Afterward, the supernatant was removed, and the formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO) for 15 minutes at 33 °C. Then the absorption was measured on a Tecan infinite 200 Pro microplate reader (Switzerland) at 550 nm.

2.6 Statistical analysis

All experiments were performed in triplicate with three technical replicates per experiment. The data was analyzed and graphically visualized using GraphPad Prism version 6.00 for Windows (GraphPad Software, USA, www.graphpad.com). In each experiment,

comparisons with negative controls were made using the nonparametric Kruskal-Wallis test of variance. Significant differences with control were considered at $p < 0.05$.

3 Results

3.1 Assessment of hydrolysis of a two–species biofilm of *S. aureus* – *P. aeruginosa* by extracellular levanase SacC

It was shown previously that levanase SacC is able to destroy mature biofilms of *P. aeruginosa* and thus increases the effectiveness of ciprofloxacin [25]. The composition of the extracellular matrix of polymicrobial biofilms can change significantly compared to monomicrobial communities of the same species, and the amount of each component can increase or decrease [30].

To determine the optimal concentration and exposure time of the enzyme, the destruction of biofilms was assessed using crystal violet staining. Bacteria with an initial density of 3×10^7 CFU/ml were seeded in 2 ml of BM medium and cultivated at 37 °C for 48 hours, after which the culture liquid was removed and fresh medium containing the enzyme at final concentrations of 500 and 1000 μg/ml was added and incubated in for 7, 30 and 120 minutes. It is known that the studied levanase exhibits maximum activity at pH=5.0, therefore, the BM medium had an appropriate pH. As shown in Figure 1, the enzyme had the greatest effect at a concentration of 500 μg/ml, both on *P. aeruginosa* biofilm and the dual-species community of *S. aureus – P. aeruginosa.* Moreover, incubation for 120 minutes led to the destruction of the two-species biofilm by more than 40% (Figure 1).

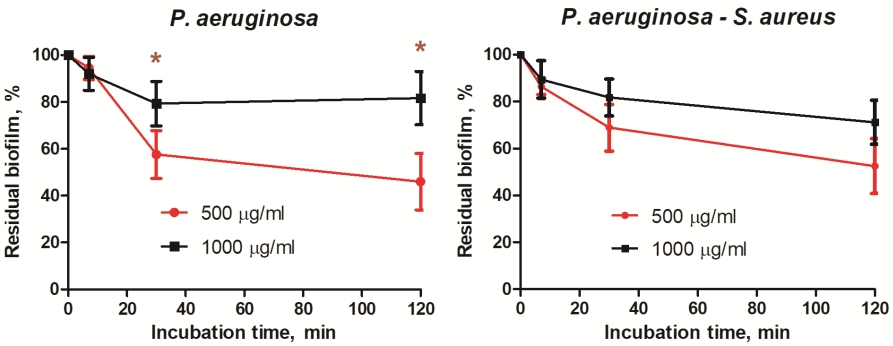


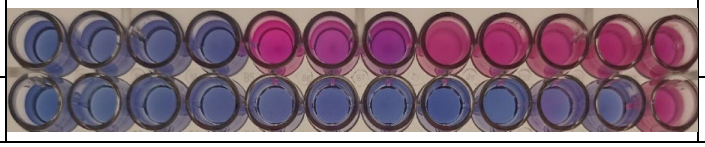
Fig.1. The effect of extracellular levanase SacC on mature biofilms of *P. aeruginosa* and *S. aureus – P. aeruginosa* for 7, 30 and 120 minutes. Evaluation of the residual biofilm was carried out by crystal violet staining. Wells without enzymatic treatment were taken as 100%.

3.2 Assessment of the combined action of extracellular levanase SacC and antimicrobials on dual-species biofilm *S. aureus – P. aeruginosa*

The extracellular matrix provides the cells in the biofilm increased resistance to antibiotics and biocides. When the components of the extracellular matrix are destroyed, the permeability of the biofilm for antimicrobials increases, leading to a significant enhancement of their effectiveness against the bacteria within the biofilm.

To assess the combined effect of antimicrobials, their minimum inhibitory concentration (MIC) against *P. aeruginosa* cells was determined. As a result, the MIC of amikacin was 16 μg/ml, and the MIC of ciprofloxacin was 0.25 μg/ml (Table 1).

Table 1. Minimum inhibitory concentration of antibiotics against *P. aeruginosa* cells. Cell viability in the presence of antibiotics was assessed using the resazurin test. The blue color of the wells indicates cell death

Compound	Concentration, μg/ml												MIC, μg/ml
	256	128	64	32	16	8	4	2	1	0.5	0.25	0	
Amikacin													16
Ciprofloxacin													0.25

Next, the effect of the complex SacC with antibiotics was assessed against cells in the dual-species biofilm of *S. aureus – P. aeruginosa*. Bacterial culture with an initial density of 3×107 CFU/ml was seeded into BM medium in 2 ml wells and incubated for 48 hours without shaking at 37 °C. Then the culture liquid was removed and fresh BM medium additionally contained SacC (500 μg/ml) and antibiotics (amikacin, ciprfloxacin) in concentrations corresponding to their 1×, 4× and 16× MIC was added. After 2 hours of incubation, the culture liquid was collected to assess the viability of detached cells. After, to assess the viability of cells into biofilms the wells were washed with a sterile NaCl solution, 100 μl of physiological solution was added and the biofilms were mechanically removed. To assess the viability of bacteria, a metabolic MTT test was performed.

It was found that the introduction of levanase led to an increase in the effectiveness of antimicrobials. However, this effect was more pronounced when using the amikacin+SacC complex, in the presence of which a twofold decrease in the total metabolic activity of cells in the biofilm was observed at 1× MIC of the antibiotic, whereas the use of one amikacin led to a similar effect only at 16×MIC (Figure 2). In the case of ciprofloxacin, the effect was less pronounced.

4 Discussion

Various hydrolytic enzymes, such as proteases, DNase and glycoside hydrolases, can contribute to the destruction of individual components of the extracellular matrix of biofilms. It has been shown that proteolytic enzymes like ficin, papain, and bromelain can disrupt biofilms of *S. aureus* and *S. epidermidis* [23, 31, 32]. *In vitro* studies have shown that enzymes such as alginate lyase and DNase more effectively destroy the biofilm of *P. aeruginosa*, whereas non-specific enzymes such as glycoside hydrolases, cellulases and α-amylases do not significantly change the mechanics of the biofilm [33]. However, only a limited number of studies currently demonstrate high efficiency in enzymatic treatment of polymicrobial biofilms [19-21].

This study has demonstrated that the treatment of a mature two-species biofilm of *S. aureus – P. aeruginosa* with extracellular levanase SacC for 120 minutes leads to a decrease in residual biofilm by more than 40% of the control at enzyme concentration of 500 μg/ml. Meanwhile, for the biofilm of *P. aeruginosa* under the same conditions, the hydrolase exhibited higher activity, and the residual biofilm was less than 50% (Figure 1). Probably, less effect is due to the specificity of the enzyme's action on the polysaccharide component of the biofilm, and the action of the enzyme in a two-species biofilm does not extend to polysaccharides produced by *S. aureus* cells. However, the low effective concentration of the enzyme against dual-species biofilms makes it a promising tool of

combating them, especially when compared to other previously described hydrolytic enzymes such as trypsin, ficin, and papain, which required concentrations of 1000 μg/ml against monospecies biofilms [23, 31, 34].

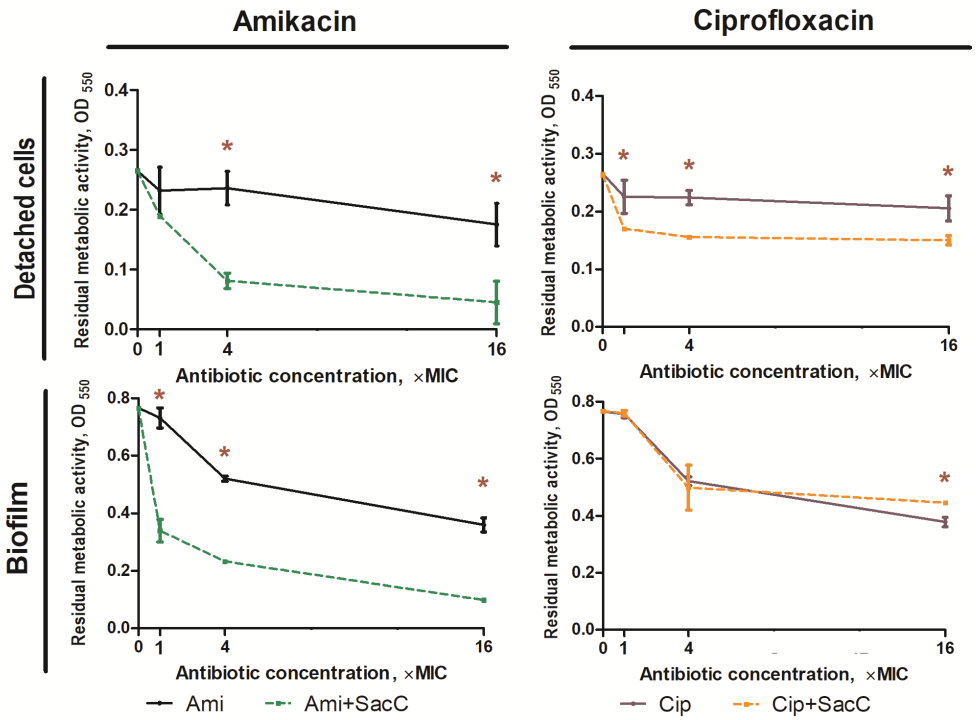


Fig. 2. Assessment of the metabolic activity of cells in a dual-species biofilm of *S. aureus* – *P. aeruginosa* community under the combined action of antimicrobials and the extracellular levanase SacC.

In mixed biofilms, bacteria become more resistant to the action of antimicrobials due to changes in the biochemical composition of the extracellular matrix, which can alter its permeability to antibiotics [30]. By disrupting components of the extracellular matrix, its permeability to antimicrobial agents can be increased. Thus, the combined use of proteases with broad-spectrum antibiotics increased the effectiveness of the latter up to 16 times [23, 31, 34]. In addition, it was shown that the combination of cellulose with ceftazidime led to the inhibition and eradication of *P. aeruginosa* biofilms [35]. Thus, the ability of levanase to destroy two–species biofilms *of S. aureus – P. aeruginosa* can become the basis for the complex use of the enzyme with antimicrobials to increase the effectiveness of the latter against bacteria in a mixed community. The effectiveness of amikacin in the presence of the enzyme increased more than 16-fold compared to the use of antibiotic alone, both against detached cells and within biofilm (Figure 2). At the same time, the combined effect of ciprofloxacin and SacC was less pronounced and the effectiveness of the antibiotic increased only against detached cells. Perhaps a significant increase in the effectiveness of amikacin is due switching of *S. aureus* cells into persistence state in presence of aminoglycosides, when the metabolic activity of cells is significantly reduced, as a result its detection of viability in the MTT test is difficult [34]. However, the demonstrated decrease in the metabolic activity of cells in a dual-species community of *S. aureus – P. aeruginosa* in the presence of a complex of levanase and antimicrobials may indicate the promise of this approach in combating infections associated with mixed bacterial biofilms.

5 Conclusion

Thus, our data indicate that extracellular levanase SacC can contribute to the disruption of dual-species biofilms of *S. aureus – P. aeruginosa*, the major pathogens in various external infections such as burn wounds, ulcers, and dominant in respiratory infections. At the same time, the combined use of saccharase with various antibiotics helps to increase the effectiveness of therapy, which opens up the possibility of using this approach in the treatment of various external and internal infections of a polymicrobial nature.

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