



Klebsiella aerogenes PgaB orthologue can efficiently hydrolyze *Staphylococcus aureus* biofilms

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Abstract

Staphylococcus aureus, a gram-positive bacterium, is the prevalent cause of numerous infections. Its ability to form biofilms significantly enhances its pathogenicity, resulting in increased antibiotic resistance and evasion of the host immune response. Poly- β -(1,6)-N-acetylglucosamine (PNAG) plays a crucial role in the formation and maintenance of *S. aureus* biofilms. In this study, we heterologously expressed *KaPgaB* from *Klebsiella aerogenes* and evaluated its efficacy in both degrading and inhibiting *S. aureus* biofilm formation. Additionally, we investigated the combined effects of *KaPgaB* with DNase I and papain. Our results demonstrated that *KaPgaB* alone removed up to 81% of biofilm biomass within 4 h when used at a concentration of 0.5 mg/mL. Moreover, when the enzyme was applied sequentially with DNase I, approximately 97% of adhered biofilms were removed. We also observed significant inhibition of biofilm formation across *S. aureus* strains. The findings presented in this study might be useful for the development of enzymatic tools capable of degrading *S. aureus* PNAG-based biofilms.

Keywords Biofilm degradation, *KaPgaB* · PNAG · *Staphylococcus aureus*

Introduction

Staphylococcus aureus is a gram-positive bacterium responsible for a number of clinically important infections, such as skin inflammation, ulcer infections, pneumonia, and bacteremia (Cheung et al. 2021). *S. aureus* is a component of the human microbiota, frequently residing on the skin, in the anterior

nares, nasopharynx, and various mucous membranes (Gehrke et al. 2023). Its pathogenicity is significantly enhanced by its capacity to form biofilms, complex microbial communities encased in a self-produced extracellular polymeric matrix (Sugimoto et al. 2018). This biofilm formation provides *S. aureus* with a protective environment that enables it to evade host immune defenses and withstand antibiotic treatments, thereby increasing its resistance to antibacterial agents by up to 1,000-fold as compared to its planktonic, or free-floating, form (Aniba et al. 2024).

Poly- β -(1,6)-N-acetylglucosamine (PNAG) is a crucial factor in *S. aureus* biofilm formation, with its synthesis, modification, and export regulated by the *icaADBC* operon (Nguyen et al. 2020). PNAG is essential for cell adhesion and contributes significantly to biofilm stability (Lin et al. 2015). In addition to PNAG, the *S. aureus* biofilm matrix may contain proteins such as biofilm-associated protein (Bap) and fibronectin-binding proteins (FnBPs), which facilitate biofilm attachment to surfaces. Extracellular DNA (eDNA) further stabilizes the biofilm matrix, enhancing its structural integrity and cohesion (Parastan 2020; Liu et al. 2021).

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Traditional antibiotic therapy often fails to eradicate biofilms, highlighting the need for alternative treatment strategies (Maya et al. 2007). One of the possible approaches to this problem involves use of specific enzymes to hydrolyze the structural components of biofilms, thus promoting detachment of sessile bacterial cells and converting them into a planktonic state to increase their susceptibility to antibiotics and the host immune system (Ramakrishnan et al. 2022).

Previous studies have demonstrated that glycoside hydrolases (GHs), including dispersin B, cellulase, and PslG, can partially degrade the exopolysaccharide matrices of bacterial biofilms, resulting in partial biofilm dispersion (Gawande et al. 2014; Wang et al. 2023). Poly- β -(1,6)-N-acetyl-D-glucosamine deacetylase (PgaB) is encoded by *ypgABCD* operon, specifically by the *pgaB* gene, and has been shown to degrade PNAG-dependent biofilms formed by species such as *Bordetella* sp., *Bordetella pertussis*, *Escherichia coli*, *Staphylococcus carnosus*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Little et al. 2018; Forman et al. 2019). PgaB is a two-domain bifunctional periplasmic protein, consisting of an N-terminal carbohydrate esterase domain from the CAZy family CE4 and a C-terminal glycoside hydrolase domain, a member of GH153 family (Little et al. 2015, 2018). Notably, PgaB orthologues from *Bordetella bronchiseptica* and *Escherichia coli* have been shown to cleave deacetylated PNAG and disperse their own biofilms (Little et al. 2015, 2018). However, the activity of *Klebsiella aerogenes* PgaB (KaPgaB) against *S. aureus* biofilms has not been characterized. Given the central role of PNAG in *S. aureus* biofilm integrity, evaluation of KaPgaB biofilm-disrupting capacity expands our understanding of PgaB orthologues and supports the development of enzymatic strategies for biofilm control. Combined with available antibiotics, PgaB can potentially be used as an auxiliary enzymatic tool, enhancing treatment of biofilm-associated infections.

In this study, we have successfully cloned and heterologously expressed PgaB from *Klebsiella aerogenes* and evaluated its efficacy against biofilms produced by various *S. aureus* strains, including antimicrobial resistant strains. We explored multiple strategies for delivering the enzyme to *S. aureus* biofilms and assessed their effects when combined with DNase I and papain.

Materials and methods

Alignments and phylogenetic analysis of KaPgaB

The amino acid sequence of KaPgaB from *K. aerogenes* was subjected to multiple alignments with PgaB sequences

obtained from the PDB database (<https://www.wwpdb.org/>). This sequence comparison was performed using the MUSCLE alignment in MEGA 11.0 (<https://www.megasoftware.net/>). The phylogenetic trees were constructed and displayed using the same software.

KaPgaB cloning and heterologous production

The entire coding sequence of KaPgaB from *K. aerogenes* was retrieved from GenBank under the accession code VDZ70154.1. Identification of the signal peptide for secretion was conducted using SignalP 5.0 (Nielsen 2017), and the nucleotides corresponding to the signal peptide were removed. Primers were designed using the HTP-OligoDesigner tool (Camilo et al. 2016) and were added with 5' overhangs for Ligation-Independent Cloning following the method described by Camilo and Polikarpov (2014) (Camilo and Polikarpov 2014). Genomic amplification of KaPgaB was performed by polymerase chain reaction (PCR). The reaction mixture included 5 μ L of 5X Phusion HF Buffer (New England BioLabs), 0.5 μ L of 10 mM dNTPs, and 1.25 μ L each of the forward [5' CAGGGCGCCATGTGCGCGCTTCG C 3'] and reverse [5' GACCCGACGCGTTATCAGTC ATTGTTGGGATACAGTA 3'] primers (10 μ M), 25 ng of genomic DNA (*K. aerogenes* DSM 30053), 0.5 μ L of Phusion DNA Polymerase (New England BioLabs), and volume adjusted to 25 μ L with PCR grade water. PCR cycling conditions were as follows: 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 1 min and 45 s; and a final extension at 72 °C for 5 min. Plasmid amplification (pETTRXA-1a/LIC) and Ligation-Independent Cloning have been reported previously (Camilo and Polikarpov 2014). *Escherichia coli* DH5 α cells were heat-shock transformed with the resulting plasmid, pETTRXA-1a/LIC-KaPgaB, plated in LB agar supplemented with 50 μ g/mL kanamycin, and grown at 37 °C for 16 h. Newly formed colonies were cultivated in LB broth supplemented with 50 μ g/mL kanamycin (Merck Darmstadt, Germany, Catalog number K1637) for 24 h at 37 °C with shaking at 150 rpm. Positive clones were detected by plasmid extraction using the Fast-n-Easy Plasmid Mini-Prep kit (Cellco Biotec do Brasil, Brazil, Catalog number DPK-104 S). The presence of inserts was confirmed using polymerase chain reaction (PCR) with specific primers for KaPgaB and then heat-shock-transformed into *E. coli* BL21(DE3).

Heterologous production of KaPgaB was performed as follows: Positive colonies were cultured in 5 mL of LB medium containing 50 μ g/mL kanamycin at 37 °C/200 rpm. The overnight grown culture (1%) was added to fresh LB broth (1 L) supplemented with 50 μ g/

mL kanamycin at 37 °C/200 rpm until an optical density at 600 nm of 0.6 was reached, and induction of expression was performed at 28 °C/200 rpm for 16 h in the presence of 1 mM isopropyl β -D-1-thiogalactopyranoside. After induction, the cells were harvested by centrifugation at $13,000 \times g$ and 4 °C for 20 min, and cell disruption was performed using an ultrasonic (Branson Branson Analog Sonifier S-450, USA) at 20 Hz (1 cycle of 14 min) in 50 mL Tris-HCl pH 8 containing 150 mM NaCl and 10 mM imidazole. After cell disruption, the protein extract was clarified by centrifugation at $13,000 \times g$ for 20 min, and the resulting supernatant containing recombinant KaPgaB was supplemented with 0.1 mM phenylmethylsulfonyl fluoride (Merck Darmstadt, Germany, Catalog number 10837091001) and stored at 4 °C until purification.

Protein purification

The clarified supernatant enriched with KaPgaB (50 mL) was applied to a 5 mL His-Trap column in an Akta Purifier system (Cytiva, Chicago, USA), and the unbound protein fraction was washed out with 10 column volumes of 50 mM Tris-HCl pH 8 containing 300 mM NaCl and 10 mM imidazole, followed by isocratic elution with 50 mM Tris-HCl pH 8 containing 300 mM NaCl and 500 mM imidazole. Fractions were analyzed using Coomassie blue-stained 10% SDS-PAGE, and those containing the recombinant protein of interest were subjected to diafiltration using a 30 kDa membrane and ultrapure water.

Following the same protocols we also cloned and purified full-length *E. coli* PgaB enzyme (EcPgaB) as a positive control of PNAG-degrading enzyme (Little et al. 2018).

Effects of temperature and pH on KaPgaB activity

To test the effects of temperature and pH on the hydrolytic activity of KaPgaB, synthetic substrate 4-Nitrophenyl N-acetyl- β -D-glucosaminide (pNP- β -GlcNAc, Catalog Number N9376, Sigma-Aldrich, USA) was used. The substrate was dissolved in ethanol absolute at a concentration of 50 mM. The temperature effect assay was performed by adding 0.001 mg/mL of the purified enzyme to 2.5 mM 4-Nitrophenyl acetate (Catalog Number 8.43075, Sigma-Aldrich, USA) in 100 mM HEPES buffer pH 7.0 for 30 min in a thermocycler in the temperature range of 30–80 °C with increments of 5 °C. The reaction outputs were read using a spectrophotometer at 405 nm. The effect of pH was assessed by performing the standard enzymatic reactions described above using 100 mM citrate-phosphate buffer in a pH range of 2 to 8.

Biofilm disruption assays

Bacterial strains

S. aureus ATCC 25,923, a well-characterized clinical isolate, is a well-characterized clinical isolate known for forming PNAG-based biofilms and is widely used as a model strain in exopolysaccharide degradation studies (Samaniego et al. 2023). It demonstrates susceptibility to a broad range of antibiotics across multiple classes. These include penicillinase-resistant penicillins, such as oxacillin; glycopeptides, including vancomycin; and aminoglycosides, such as gentamicin. It is also susceptible to tetracyclines (e.g., doxycycline), and sulfonamides (e.g., trimethoprim-sulfamethoxazole). Additionally, this strain is sensitive to oxazolidinones, such as chloramphenicol (Treangen et al. 2014; Manner et al. 2017; Iglesias and Van Bambeke 2020).

The *S. aureus* strains 01 and 02 were isolated from patients diagnosed with urinary tract infections at the University Hospital of the State University of Ponta Grossa. Biochemical characterization of *S. aureus* CMA 01 revealed it to be positive for coagulase, DNase, lactose, mannitol, trehalose, and Voges-Proskauer test, while it tested negative for urea. This strain exhibited resistance to several antimicrobials, including lomefloxacin, norfloxacin, ampicillin, oxacillin, cefoxitin, cephalothin, penicillin, imipenem, and the combination of ampicillin and sulbactam. In contrast, *S. aureus* CMA 02 was characterized as positive for coagulase, DNase, lactose, mannitol, trehalose, and Voges-Proskauer test, and also positive for urea. This strain demonstrated resistance to lomefloxacin and norfloxacin.

Finally, *S. aureus* 03 is a methicillin-susceptible clinical isolate from Brazil, exhibiting a highly mucoid phenotype. It is susceptible to chloramphenicol, cefoxitin, trimethoprim, erythromycin, norfloxacin, and tetracycline, but resistant to rifampin and penicillin G when tested using disk diffusion. Additionally, it is susceptible to vancomycin (MIC=1.5 mg/L) as assessed by Etest (Biomérieux, Marcy-l'Étoile, France), and to gentamicin (MIC=0.25 mg/L) when tested by broth microdilution (data not shown).

Genomes of the three bacterial isolates, *S. aureus* 01, 02 and 03, were sequenced. For that, genomic libraries were prepared using the Illumina DNA Prep (Illumina, USA) and sequenced with the MiSeq Reagent Micro Kit v2 flow cell (Illumina, USA) for 300 cycles, following the manufacturer instructions. Sequencing of their bacterial genomes revealed that all three *S. aureus* strains (01, 02 and 03) have complete *ica* operon, responsible for PNAG production (NCBI PRJNA1266734). Interestingly *S. aureus* 03 strain has a deletion in the *icaR* gene leading to a truncation of its negative regulator IcaR. This fact explains abundant expression of PNAG by this particular strain.

Thermostability and pH tests in biofilm

Four strains (*S. aureus* 01, *S. aureus* 02, *S. aureus* 03, and *S. aureus* ATCC 25923) were evaluated for their biofilm-forming capacity and responsiveness to KaPgaB treatment. Among those, *S. aureus* 03 demonstrated the thickest biofilm formation and the highest sensitivity to the enzyme (data not shown). This strain was therefore selected as the optimal candidate for subsequent thermostability, pH, and antibiotic assay testing. Thermostability and pH effect tests on KaPgaB activity were carried out using preformed 24 h-old *S. aureus* 03 biofilms. Briefly, *S. aureus* 03 was streaked on Tryptic Soy Agar (TSA) plates and cultured at 37 °C for 24 h. Emerging colonies were transferred to 5 mL of Tryptic Soy Broth (TSB) containing 0.75% (w/v) glucose and grown for 24 h at 37 °C under static conditions. After growth the pre-inoculum was diluted 1:100 in TSB containing 0.75% (w/v) glucose and 200 µL of the inoculated medium was transferred to each well of a 96-well cell culture plate (NEST catalog number 701.002). The plate was incubated at 37 °C for 24 h under static conditions to allow biofilm formation. Following incubation the residual medium was removed and the wells were washed twice with saline to remove non-adherent cells. For the thermostability test 0.05 mg/mL of the purified enzyme diluted in 50 mM citrate-phosphate buffer at pH 6 was incubated in a thermomixer for 1 h at temperatures ranging from 30 °C to 90 °C. Subsequently 200 µL of each reaction mixture was added to a well of the microplate containing the growth biofilm and incubated at 37 °C for 4 h under static conditions. To assess the effect of pH on enzymatic activity the reactions described above were prepared in 100 mM citrate-phosphate buffer across a pH range of 2 to 8. The reaction mixtures were then applied to the biofilms and incubated at 37 °C for 4 h under static conditions. After the reactions the remaining enzyme and non-adherent biofilm were removed by washing twice with saline solution. The remaining biofilms were stained with 0.5% (w/v) crystal violet for 5 min after which the non-bound dye was washed thrice with saline solution. To quantify the biofilm degradation it was destained using 30% (v/v) acetic acid and the released crystal violet absorption was measured spectrophotometrically at 595 nm following the protocol described by Samaniego et al. (2024) (Samaniego et al. 2024). Each experiment was conducted in six replicates.

Assessment of the dose-response effect and additive effect of different enzymes in *S. aureus* biofilm dispersal.

To analyze the dose-response and additive effects of different enzymes on KaPgaB activity, performed 24-hour-old

biofilms of *S. aureus* strains 01, 02, 03, and ATCC 25,923 were used. For dose-response experiments, various concentrations of the purified enzyme (0.05, 0.25, and 0.5 mg/mL) were prepared in 50 mM citrate-phosphate buffer at pH 6. These enzyme solutions were then applied to biofilms and incubated at 37 °C for 4 h under static conditions. After the reactions, the remaining enzyme and non-adherent biofilm were removed by washing twice with saline. The biofilm biomass was measured as described above.

Ability of KaPgaB to inhibit the biofilm formation in *S. aureus* ATCC 25,923, 01, 02, and 03 was also evaluated. For the inhibition test, 200 µL of inoculated TSB (as described in the previous section) supplemented with 0.5 mg/mL KaPgaB were incubated at 37 °C for 20 h under static conditions in 96-well plates. After the reactions, the remaining enzyme and non-adherent biofilm were removed by washing twice with saline. The biofilm biomass was measured as described above.

Combined treatments with different enzymes were initiated by the addition of the commercial enzyme DNase Deoxyribonuclease I from bovine pancreas (Sigma-Aldrich, St. Louis, USA) or papain, from papaya latex (Sigma-Aldrich, St. Louis, USA) at a final concentration of 0.5 mg/mL in 20 mM citrate-phosphate buffer at pH 6.0 in the biofilm for 2 h at 37 °C. For reactions with DNase I, 5 mM magnesium chloride was added. After this period, the solution containing the enzymes was removed, and KaPgaB (0.5 mg/mL) was added and incubated again for 2 h with the biofilm. Controls were performed with the addition of only DNase I, papain or KaPgaB- for two hours. The experiments were performed in six replicates for each condition. The experimental conditions were consistent with those described previously, as well as the measurement of remaining biofilm biomass.

Combined effect of KaPgaB and antibiotics on biofilm cell viability

Preformed 24-hour-old *S. aureus* 03 biofilms were treated with solutions containing gentamicin, tetracycline, or chloramphenicol at concentrations ranging from 10 to 500 µg/mL, with or without the addition of 0.05 mg/mL KaPgaB in TSB containing 0.75% (w/v) glucose. Biofilms were incubated for 20 h at 37 °C. After incubation, the biofilms were washed with PBS and the wells were filled with 100 µL of PBS. The biofilms were carefully resuspended by pipetting and subsequently analyzed for cell viability using the resazurin assay. For this assay, 40 µL of the resazurin solution (0.15 mg/mL) was added to 200 µL of the PBS+detached biofilm solution. The mixture was incubated at 37 °C for 2 h and fluorescence was measured at excitation/emission wavelengths of 550/590 nm using an Infinite 200 M PRO

microplate reader (Tecan, Hombrechtikon, Switzerland) (Samaniego et al. 2023, 2024).

The experiments were performed in triplicate. Biofilm viability and distribution were also analyzed by confocal laser scanning microscopy (CLSM), as detailed in the Supplementary material.

Statistical analysis

All experiments were conducted in six replicates ($n=6$) for each condition. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with p -values less than 0.05 considered statistically significant.

Results

Heterologous expression and biochemical characterization

KaPgaB is a two-domain protein with 54.04% and 47.33% identity to the previously characterized *PgaB* proteins from *Escherichia coli* (Little et al. 2018) and *Bordetella bronchiseptica* (Little et al. 2015), respectively (Supplementary Figures S1 and S2). Following induction of *E. coli* BL21(DE3) containing the pET-TRXA-1a-*KaPgaB* plasmid, a protein band corresponding to 6xHis-TRX-tag-*KaPgaB* was observed in the soluble fraction (Supplementary Figure S3a). After purification, *KaPgaB* was obtained as a soluble pure protein (Supplementary Figure S3b). The purified protein had an approximate molecular weight of

107 kDa, of which near 33 kDa corresponds to the expression tags. This molecular weight is consistent with those reported for other *PgaB* proteins (Little et al. 2015, 2018).

After heterologous expression and purification of *KaPgaB*, its biochemical properties were evaluated and the maximum activity against pNP- β -GlcNAc was observed at pH 6 and 50 °C (Fig. 1a & b).

The effect of pH was also evaluated against plate-adhered *S. aureus* biofilms. The maximum activity against this natural substrate was also observed at pH 6 (Fig. 1c). The residual activity of *KaPgaB* remained relatively constant between 30 °C and 60 °C (Fig. 2a), indicating that the enzyme maintains its activity within this temperature range.

However, a progressive decline in residual activity was noted at 70 °C, culminating in 80% activity loss after 20 min of incubation at this temperature, and complete inactivation between 80 and 90 °C. These results show that *KaPgaB* hold a potential for biofilm-degrading biomedical applications, since its thermal stability makes it more feasible to be transported and administered, particularly when compared to other proteins known to hydrolyze PNAG-based biofilms such as GH20 Dispersin B (Tan et al. 2015).

Notably, *KaPgaB* ability to hydrolyze the synthetic substrate pNP- β -GlcNAc differs from that of previously characterized *PgaB* enzymes described in the literature (Little et al. 2015, 2018). Both *Bordetella bronchiseptica* and *Escherichia coli* *PgaB* lack hydrolytic activity toward pNP-glycoside substrates and short PNAG. Little et al. (2018) (Little et al. 2015) demonstrated that *PgaB* from *B. bronchiseptica* and *E. coli* can completely disperse their own PNAG-dependent biofilms within a short

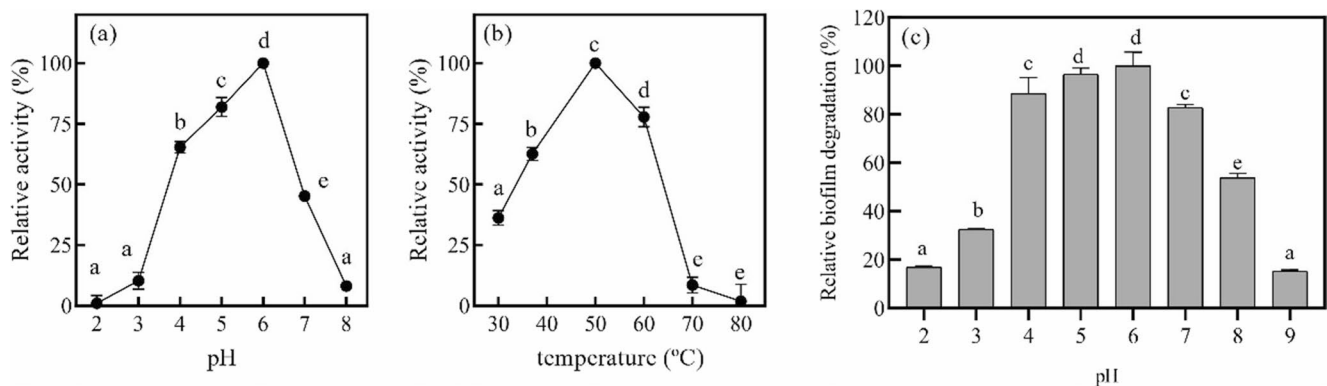


Fig. 1 Effects of pH and temperature on *KaPgaB* activity. **(A)** Optimum pH for *KaPgaB* activity. The activity was determined by incubating 0.001 mg/mL of the purified enzyme at 40 °C for 30 min at pH values ranging from 2 to 8 (100 mM citrate-phosphate buffer), using a synthetic substrate, 4-Nitrophenyl N-acetyl- β -D-glucosaminide. **(B)** Assay of the effect of temperature on *KaPgaB* enzymatic activity. Enzyme activity was tested under the standard assay conditions described above, varying the temperature from 30 to 80 °C for 30 min at pH 7.0 (100 mM HEPES buffer). **(C)** Enzymatic degradation assay

on 24-hour-old *S. aureus* 03 biofilms as a function of pH. The assay was performed by adding *KaPgaB* at a final concentration of 0.5 mg/mL in 20 mM citrate-phosphate buffer at pH 6.0. The cells were incubated for 4 h at 37 °C. The control experiment was performed in the absence of the enzyme, using only 20 mM citrate-phosphate buffer. Narrow vertical bars represent the standard deviations of six replicates. The rows and columns labeled with different letters represent statistically significant differences ($p < 0.05$), whereas equal letters indicate non-significant differences ($p > 0.05$)

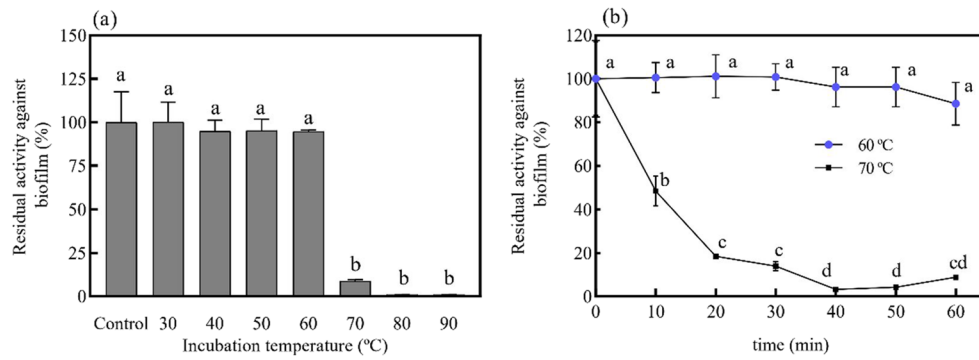


Fig. 2 Residual KaPgaB activity on 24-hour-old *S. aureus* 03 biofilms (A) KaPgaB was pre-incubated for 1 h at a final concentration of 0.5 mg/mL in 20 mM citrate-phosphate buffer pH 6.0, across a temperature range of 30–90 °C. Following pre-incubation, the enzyme was used in biofilm degradation assays for 4 h at 37 °C. (B) Residual activity was assessed under standard conditions described previously, with

pre-incubation at 60–70 °C for 1 h. Aliquots were taken every 10 min and residual activity was measured. The experiments were conducted in six replicates for each condition. Narrow vertical bars represent standard deviations of six replicates. Rows and columns labeled with different letters represent statistically significant differences ($p < 0.05$), whereas equal letters indicate non-significant differences ($p > 0.05$)

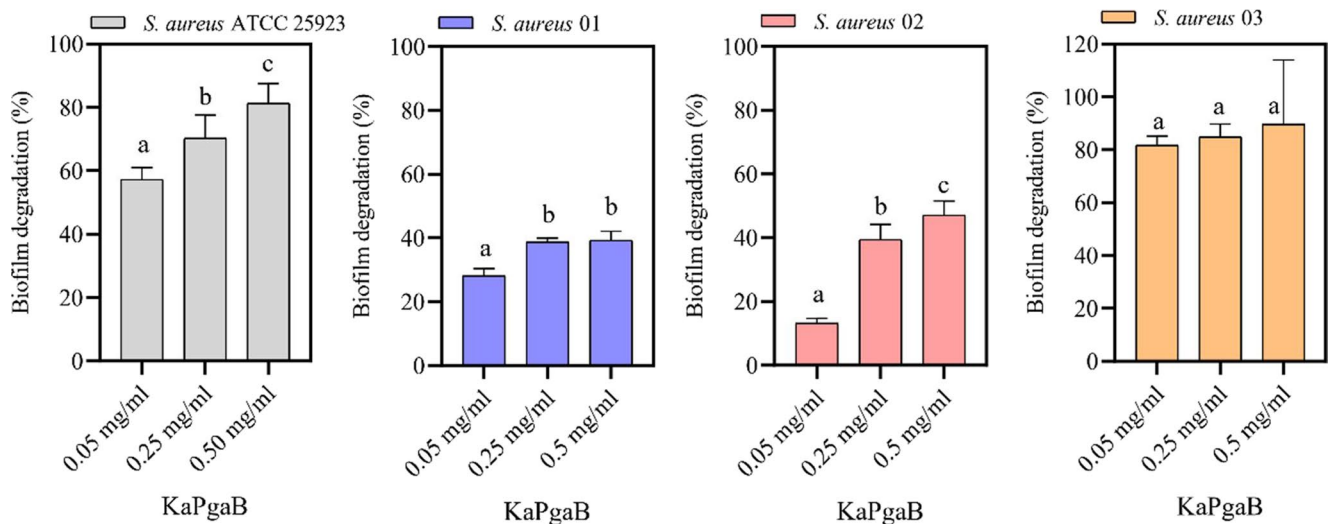


Fig. 3 Eradication of biofilms of 24-hour-old *S. aureus* strains ATCC 25,923, 01, 02, and 03 after treatment with different doses of KaPgaB. The test was carried out with the addition of KaPgaB at final concentrations of 0.05, 0.25, and 0.5 mg/mL in a 20 mM citrate-phosphate buffer at pH 6.0 for 4 h. The experiments were conducted in six repli-

cates for each condition. A control experiment was conducted without the addition of enzyme. Vertical bars represent the standard deviations of six replicates. Columns labeled with different letters represent statistically significant differences ($p < 0.05$), whereas equal letters indicate non-significant differences ($p > 0.05$)

incubation period, highlighting their activity against natural polymeric substrates despite lacking activity toward pNP-glycoside substrates and short PNAG. Additionally, these enzymes exhibit maximum activity at pH 7.0 and 37 °C (Little et al. 2018). Even though KaPgaB has demonstrated maximum activity at the temperatures above 40 °C, it has a high activity at 37 °C. Furthermore, although KaPgaB is capable of hydrolyzing synthetic substrate pNP- β -GlcNAc, the observed activity is quite low, and almost hundred-times smaller than that observed for GH20 dispersin B (Tan et al. 2015). This indicates that polymeric substrates are the preferred substrates for KaPgaB, similar to what was reported for *E. coli* PgaB (Little et al. 2015).

Degradation and Inhibition of biofilm formation

Dose-response effect

The degradation of *S. aureus* strains ATCC 25,923, 01, and 02 biofilms increased with the concentration of KaPgaB, reaching degradation levels of 81%, 38%, and 45%, respectively. For *S. aureus* 03, degradation was greater than 80% at all doses tested (Fig. 3).

As a negative control we used the buffer at the absence of the enzyme and a heat-inactivated KaPgaB (Supplementary Figure S4a). No *S. aureus* 03 biofilm degradation was observed, which strongly contrasts with the

application of enzymatically-active KaPgaB, thus supporting a notion that the biofilm degradation is caused by KaPgaB hydrolysis of PNAG. Furthermore, we applied recombinant *E. coli* EcPgaB which was previously proven to degrade PNAG-rich biofilms, including the ones from *S. aureus* (Little et al. 2018). Again, “no-enzyme” buffer and heat-inactivated EcPgaB did not promote degradation of *S. aureus* 03 biofilm, whereas active EcPgaB caused strong degradation of the biofilm in a dose-dependent manner (Supplementary Figure S4b). Overall, levels of the enzymatic degradation of *S. aureus* 03 biofilms by these two enzymes were very similar (Supplementary Figure S4a&b).

Additionally, KaPgaB inhibited biofilm formation of all tested *S. aureus* strains (Fig. 4). For strains 01 and 02, the highest inhibition rates of 65% and 55%, respectively, were observed at the maximum enzyme dosage. In contrast, for strains ATCC 25,923 and 03, inhibition rates of 62% and 96%, respectively, were observed at the minimal enzyme dosage. Notably, the degradation and inhibition rates of KaPgaB in the biofilms of ATCC strains 25,923 and 03 were higher than those of dispersin B in *S. aureus* biofilms (Sugimoto et al. 2018; Izano et al. 2008; Hogan et al. 2017; Poilvache et al. 2021).

The differences observed in the degradation of biofilms formed by various *S. aureus* strains likely result from several factors, including the inherent variability of biofilms and differences in the composition and proportion of extracellular components such as exopolysaccharides, proteins, and extracellular DNA. Even when biofilms are formed by strains of the same species, considerable variation in biofilm formation is expected (Avila-Novoa 2022). Moreover, differences in biofilm composition can be key determinants of a strain pathogenicity and resistance to antimicrobial agents, further reinforcing the role of biofilms in the development of multidrug-resistant strains. PNAG appeared to be

a predominant component in the biofilms of *S. aureus* strains ATCC 25,923 and 03, as indicated by the high degradation rates achieved by KaPgaB.

We hypothesized that the addition of enzymes targeting other biofilm components, such as extracellular DNA (eDNA) and proteases could further enhance degradation rates, particularly for the *S. aureus* strains 01 and 02.

Sequential addition of the enzymes

In a first attempt to evaluate the additive effects of different enzymes on *S. aureus* biofilms, KaPgaB was incubated with DNase I or papain at a final concentration of 1 mg/mL in 20 mM citrate-phosphate buffer at pH 6.0 for 4 h. However, after the incubation period, there was no increase in the biofilm degradation rate compared with the action of KaPgaB alone (data not shown). In contrast, when the enzymes were added sequentially, an increase in the degradation rate of *S. aureus* strains 01, 02, and 03 biofilms was observed (Fig. 5).

Sequential treatment with DNase I followed by KaPgaB resulted in degradation rates of 70%, 71%, and 97% for *S. aureus* strains 01, 02, and 03, respectively. The sequential addition of papain followed by KaPgaB yielded degradation rates of 65%, 65%, and 90% for the same strains. The results for *S. aureus* strain ATCC 25,923 were consistent with the initial observations, showing no increase in biofilm dispersion using tested combinations of the enzymes.

Antibiotic susceptibility

Next, we undertook investigation into combined action of KaPgaB treatment associated with three different antibiotics: chloramphenicol, tetracycline and gentamicin. At the absence of antibiotics, KaPgaB did not have any effect on

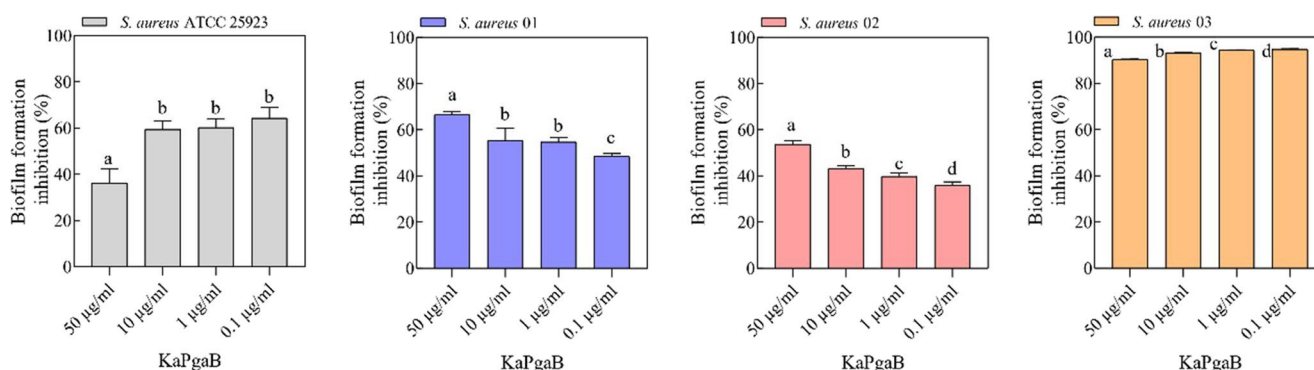


Fig. 4 Inhibitory effect of KaPgaB on biofilm formation by *S. aureus* ATCC 25,923, *S. aureus* 01, *S. aureus* 02, and *S. aureus* 03 strains. The experiment was conducted using KaPgaB concentrations of 50 µg/ml, 10 µg/ml, 1 µg/ml, and 0.1 µg/ml in 20 mM citrate-phosphate buffer at

pH 6.0, for 24 h. Vertical bars represent the standard deviations of six replicates. Columns labeled with different letters represent statistically significant differences ($p < 0.05$), whereas equal letters indicate non-significant differences ($p > 0.05$)

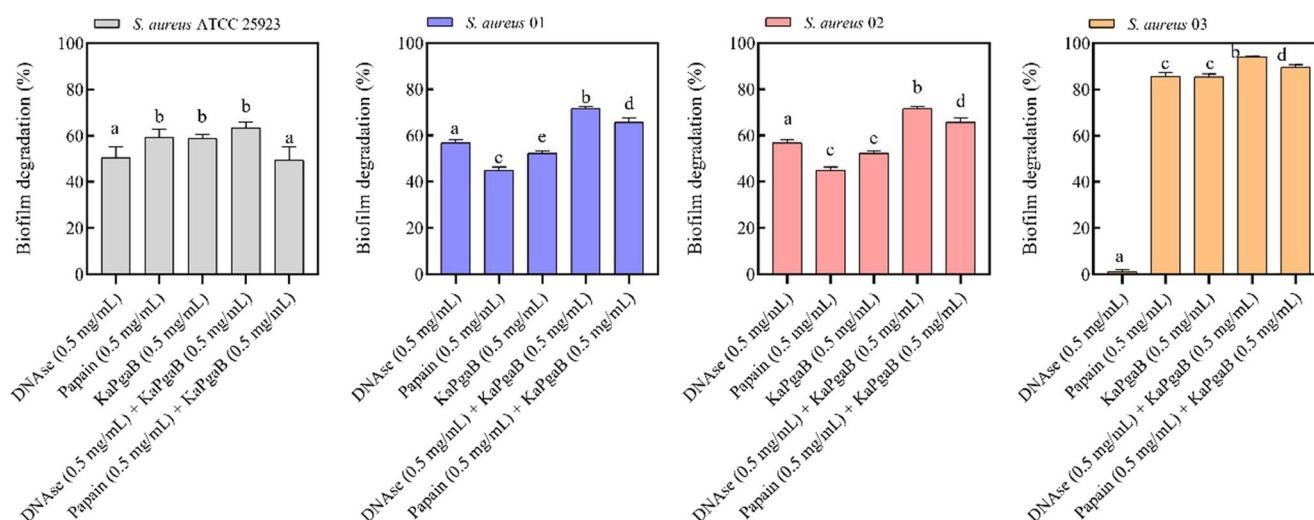


Fig. 5 Biofilm dispersion test of *S. aureus* strains ATCC 25,293, 01, 02, and 03 with sequential addition of DNase I or Papain, followed by *KaPgaB*. Initially, biofilms were treated with DNase I (deoxyribonuclease from bovine pancreas) or papain (from papaya latex) at a final concentration of 0.5 mg/mL in 20 mM citrate-phosphate buffer at pH 6.0 for 2 h. Following this initial treatment, the enzyme solution was

removed, and *KaPgaB* was added at a final concentration of 0.5 mg/mL. Control experiments were performed using DNase I, papain, and *KaPgaB* alone for 2 h. Vertical bars represent the standard deviations of six replicates. Columns labeled with different letters represent statistically significant differences ($p < 0.05$), whereas equal letters indicate non-significant differences ($p > 0.05$)

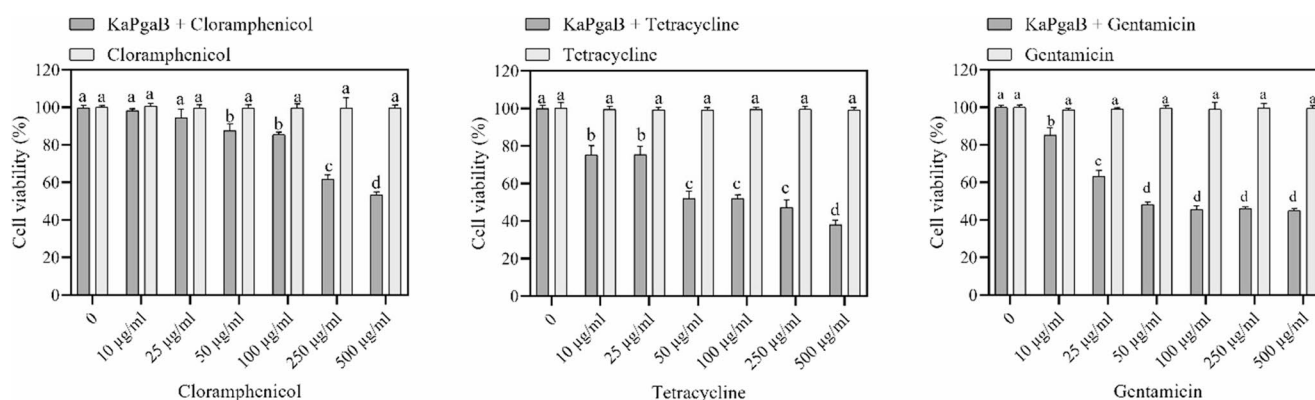


Fig. 6 Assessment of the susceptibility of *S. aureus* 03 biofilms to antibiotic treatment. The test was conducted with different concentrations of gentamicin, tetracycline or chloramphenicol (10–500 µg/mL), in the presence or absence of 0.05 mg/mL of *KaPgaB*, in TSB containing 0.75% glucose. The first point on the X-axis corresponds to the condition without any given antibiotic, but in the presence of the enzyme. That is the gray bar at the ‘zero’ point represents the ‘enzyme-only’ condition, without the addition of any antimicrobial agent. Subsequent

points reflect increasing concentrations of antimicrobial agents with a constant load of the enzyme. After 20 h of incubation at 37 °C, the biofilms were washed and subjected to cell viability assay using resazurin. Vertical bars represent the standard deviation of three replicates. Columns labeled with different letters represent statistically significant differences ($p < 0.05$), whereas equal letters indicate non-significant differences ($p > 0.05$)

S. aureus bacterial viability (Fig. 6, gray bar at the ‘zero’ point, which represents the ‘enzyme-only’ condition). Furthermore, under experimental settings of our study, treatment with antibiotics alone also did not affect the *S. aureus* biofilm cells viability (Fig. 6). However, *KaPgaB*-induced biofilm degradation increased the susceptibility of the biofilms to all tested antibiotics. Combined with *KaPgaB*, chloramphenicol at 500 µg/mL reduced the cell viability to 38%, and tetracycline at the same concentration reduced the cell viability to 50%. Additionally, 50 µg/mL gentamicin decreased cell viability to 45%.

Discussion

Application of *KaPgaB* alone led to a significant degradation of *S. aureus* strain biofilms, reaching degradation levels of over 80%. Furthermore, *KaPgaB* showed inhibition of biofilm formation for the same strains (Fig. 4), reaching over 95% being higher than those reported for dispersin B (Sugimoto et al. 2018; Izano et al. 2008; Hogan et al. 2017; Poilvache et al. 2021). This shows a clear promise in using of *KaPgaB* alone for *S. aureus* biofilm degradation.

Carbohydrates, proteins, and nucleic acids are essential components of the *S. aureus* biofilm matrix. The combination of enzymes that degrade these polymers represents a promising strategy for increasing the susceptibility of bacterial cells to antimicrobial agents. Previous studies have demonstrated that PgaB, DNase I, and papain improve biofilm dispersal in bacterial biofilms (Little et al. 2018; Song et al. 2020; Lin et al. 2024). PgaB plays a crucial role in PNAG biosynthesis and modification. This enzyme specifically targets partially deacetylated PNAG. Its N-terminal deacetylase domain partially removes acetyl groups, followed by the C-terminal glycoside hydrolase domain that cleaves β -1,6 glycosidic bonds. This reduces the adhesive properties of the biofilm matrix and weakens its structure (Little et al. 2018). DNase I is an endonuclease that primarily targets double-stranded DNA. Divalent cations, such as Ca^{2+} and Mg^{2+} , are required for its catalytic activity. The enzyme cleaves DNA between the 3'-oxygen atom and the adjacent phosphorus atom, producing 3'-hydroxyl and 5'-phosphoryl termini on DNA fragments. DNase I disrupts cell-surface and cell-cell interactions by acting on eDNA in biofilms, thereby compromising the integrity of the biofilm (Hartmann 2017; Li et al. 2020). Finally, papain is a cysteine endopeptidase that preferentially cleaves peptide bonds containing basic amino acids, such as arginine or lysine. The enzyme targets biofilm proteins, including extracellular matrix binding proteins (Emp), surface G protein (SasG), and autolysins (AtlA). These proteins are crucial for biofilm adhesion, stability, and development (Valle et al. 2012; Rosenthal 2014; Tacias-Pascacio et al. 2021). Notably, sequential application of DNase I or papain followed by *KaPgaB* effectively disrupted and dispersed *S. aureus* biofilms, specifically in strains 01, 02, and 03. As expected, the combinations of the enzymes were more effective than the use of individual enzymes. In the case of *S. aureus* strain 03, the combined application of DNase I and *KaPgaB* resulted in a notable increase in biofilm degradation (Fig. 4), leading to ~97% elimination of adhered biofilms. In contrast, treatment with DNase I alone had no effect. This might suggest that DNase I may have cleaved a portion of the eDNA; however, the extent of cleavage was insufficient for biofilm dispersal. Ellis and Rowley (Ellis and Rowley 2024) previously reported that the effect of the sequential addition of pepsin followed by a commercial mixture of degradative enzymes (Zymolyase 20 T) significantly improved the dispersal of *S. aureus* biofilms compared to application of the enzymes separately. Our results highlight the potential of combined enzyme treatment to improve the effectiveness of biofilm disruption strategies. Further testing with additional enzymatic activities is needed for the *S. aureus* ATCC25923 strain to increase its biofilm degradation rates.

S. aureus cells within a complex biofilm matrix exhibit resistance to antimicrobial agents and host immune responses (Gehrke et al. 2023; Aniba et al. 2024). The use of dispersant agents alongside antibiotics represents a promising strategy for managing biofilm-associated infections (Wang et al. 2023). In the clinical treatment of *S. aureus* infections, some of the commonly used antibiotics include chloramphenicol, tetracycline, and gentamicin, which function by binding to ribosomal subunits and inhibiting protein synthesis (Vestergaard et al. 2019; do Canto Canabarro et al. 2022; Tuon et al. 2023). *KaPgaB* used in this study proved to be an effective dispersal agent, enhancing the exposure and susceptibility of bacterial cells to chloramphenicol, tetracycline, and gentamicin. These results align with those of previous studies that highlighted the effectiveness of PNAG-degrading enzymes, such as PgaB and dispersin B, in increasing antibiotic activity against bacterial biofilms (Little et al. 2018; Hogan et al. 2017; Donelli et al. 2007). Thus, we hypothesize that *KaPgaB* could be applied to prevent and/or disperse biofilms on medical devices, such as catheters and implants, and potentially be used for topical wound treatment, aiding biofilm removal and enhancing antibiotic efficacy. Although further studies are needed to evaluate the safety and cytotoxicity of *KaPgaB*, another PNAG-active enzyme, Dispersin B, has demonstrated a favorable safety profile in preclinical studies, showing no cytotoxicity, genotoxicity, or systemic toxicity. Furthermore, Dispersin B applied to wounds was not detected in systemic circulation, indicating a low risk of systemic exposure (Kaplan et al. 2024). These findings support a notion of a potential safety of PNAG-degrading enzymes for surface and wound applications. However, because biofilm disruption can release planktonic bacteria, to prevent bacterial dissemination an application of *KaPgaB* in combination with antibiotics is strongly recommended.

Conclusions

Here, we demonstrate efficiency of *KaPgaB* in *S. aureus* biofilm degradation. Notably, the sequential addition of *KaPgaB* after DNase I or papain treatment significantly increased degradation of *S. aureus* biofilms. Furthermore, simultaneous application of *KaPgaB* with antibiotics considerably improves their efficiency and efficacy, thus highlighting the potential of combined enzymatic-antibiotic strategy for treating persistent biofilm-associated infections. These findings suggest that *KaPgaB* can be a promising candidate for developing enzymatic tools for degrading pathogenic bacterial biofilms.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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