Research Article

Bacteriophage-Derived Peptidase CHAP$_K$ Eliminates and Prevents Staphylococcal Biofilms

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New antibacterial agents are urgently needed for the elimination of biofilm-forming bacteria that are highly resistant to traditional antimicrobial agents. Proliferation of such bacteria can lead to significant economic losses in the agri-food sector. This study demonstrates the potential of the bacteriophage-derived peptidase, CHAP$_K$, as a biocidal agent for the rapid disruption of biofilm-forming staphylococci, commonly associated with bovine mastitis. Purified CHAP$_K$ applied to biofilms of *Staphylococcus aureus* DPC5246 completely eliminated the staphylococcal biofilms within 4 h. In addition, CHAP$_K$ was able to prevent biofilm formation by this strain. The CHAP$_K$ lysin also reduced *S. aureus* in a skin decolonization model. Our data demonstrates the potential of CHAP$_K$ as a biocidal agent for prevention and treatment of biofilm-associated staphylococcal infections or as a decontaminating agent in the food and healthcare sectors.

1. Introduction

Staphylococcal species commonly colonise the skin and mucosal membranes of both humans and animals. They are a significant causative agent of bovine mastitis in dairy herds [1] and are also associated with a number of diseases in humans, ranging from a variety of skin conditions to more serious infections such as septicemia [2]. Staphylococcal food poisoning is among the most common food-borne microbial diseases [3] and contamination of food industrial surfaces with staphylococcal species has been demonstrated to be a considerable risk factor [4–6]. Along with the urgent requirement for novel antibacterials to combat the prevalence of antibiotic/disinfectant resistant staphylococci in food processing, veterinary and healthcare settings, there is an increasing need for effective antimicrobial agents which can prevent and treat staphylococcal biofilm-associated infections [7–11].

Biofilms are multilayered communities of sessile cells protected by an extracellular matrix, which often adhere to food contact surfaces, damaged tissue and indwelling medical devices [12–14]. Once formed, biofilms may be up to 1,000 times more resistant to antimicrobial agents than planktonic cells alone making them particularly difficult to eliminate [15]. This can ultimately lead to increased risk of persistent infections, as is commonly the case with bovine mastitis [16]. In addition, because of their increased levels of resistance, biofilm-associated infections can result in a need for explanation of medical devices in human healthcare settings [17, 18]. Although the precise mechanisms of biofilm antibiotic resistance have yet to be fully resolved, failure to successfully treat infections with conventional therapies necessitates the investigation and development of novel treatment strategies [9, 18, 19].

In recent years, bacteriophage endolysins (phage lysins) have been the focus of research into combatting antibiotic resistance in Gram-positive pathogens [20–24]. These cell wall peptidoglycan hydrolases possess a number of advantages over conventional antibiotics including, rapid lytic activity against bacterial cells, low probability of developing
bacterial resistance, and significantly lower chance of disrupting commensal microflora, due to the enzymes’ specificity [25–28].

Our group previously reported the ability of phage K and modified derivatives to prevent biofilm formation and to reduce established biofilm density [29]. However, endolysins have a number of advantages over using whole phage as antimicrobial agents. In the case of whole phage, resistance arising from either adsorption inhibition, restriction modification and abortive infection have been reported in many genera [30–32]. Bacteriophage and their hosts have coevolved over millions of years. The equilibrium in this relationship has been maintained by continual development of resistance and counter resistance. In contrast, with the use of phage endolysins, there has been no report of bacteria developing resistance to these lytic agents even after extensive growth of the bacterium in the presence of sublethal levels of enzyme [25, 33, 34]. In addition, by using phage endolysins instead of whole phage the risk of horizontal gene transfer of virulence genes is avoided. Phage preparations also have the possibility of containing exo- and endotoxins from their respective bacterial host. By overexpressing an endolysin in well-characterised avirulent laboratory strains this risk is circumvented.

To date a number of phage lysins have been described which demonstrate activity against staphylococci including LysK [35], LysWMY [36], Φ11 lysin [37], MV-L [38], LysH5 [39], LysGH15 [40], PlyV12 [41], SAL-2 [42], and SAL-1 [43]. Producing tailor-made proteins by combining domains from phage lysins with various other domains has also been investigated [28, 44, 45]. Multidomain lysins have previously been examined for control of biofilms namely Φ11 lysin [37] and SAL-2 [42]. This is the first report of a single domain lysin being used for this purpose. Cysteine, histidine dependant amidohydrolase/peptidase CHAPK (18.6 kDa) is a truncated derivative of a phage lysin (LysK, 54 kDa) from the myoviridae staphylococcal phage K [35, 46]. This endopeptidase specifically cleaves the peptide bond between D-alanine and the first glycine in the pentaglycine cross-bridge of staphylococcal cell wall peptidoglycan [47]. We have previously reported that purified CHAPK exhibits stronger activity than the original multidomain lysin (LysK) [46] and displays rapid lytic activity against a variety of pathogenic staphylococcal species including Staphylococcus epidermidis and methicillin-resistant S. aureus (MRSA) strains [46, 48]. Here we examine the efficacy of the phage-derived CHAPK enzyme in eliminating Staphylococcus aureus biofilms and demonstrate its efficacy for removal of S. aureus from skin.

2. Materials and Methods

2.1. Bacterial Strains. The strains used in this study were S. aureus strain DPC5246 (Moorepark Food Research Centre, Teagasc, Fermoy, Cork, Ireland) and S. aureus strain Xen29 (Caliper Lifesciences, UK). Strain DPC5246 is an S. aureus bovine mastitis isolate [49]. Strain Xen29 is a confirmed biofilm producing pleural fluid isolate derived from the parental strain S. aureus ATCC 12600 [50]. It has been engineered to emit luminescence when metabolically active [51, 52]. Both strains were stored at −80°C and routinely grown on tryptic soya agar (TSA), in tryptic soya broth (TSB) or TSB supplemented with 1% D-(+)-glucose (TSBg) at 37°C. All media was supplied by Sigma-Aldrich.

2.2. Production of CHAPK. CHAPK is comprised solely of the lytic CHAP domain of the anti-staphylococcal bacteriophage endolysin, LysK [35]. In a previous study by our group, the truncated phage lysin gene was cloned untagged into a pQE60 expression vector (Qiagen) and overexpressed in Escherichia coli (E. coli) XL1-Blue [46]. Highly active CHAPK (18.6 kDa) was purified to >90% homogeneity by cation exchange chromatography. The protein was then desalted and concentrated using an amicon ultra centrifugal filter (Mili-pore) with 10 kDa cut-off and subsequently stored at −80°C in 25 mM Tris pH 7. The lytic activity of CHAPK against live planktonic cells of staphylococci including multi-antibiotic resistant strains of clinical origin has been demonstrated previously [46, 53].

2.3. Staphylococcal Biofilm Reduction Using CHAPK

2.3.1. Plate Staining Assay. A modified static microtitre plate assay, based on previous studies [54], was used to analyse biofilm formation and treatment with CHAPK. Briefly, overnight (18–24 hr) colonies of S. aureus DPC5246 from a TSA plate were suspended in sterile ringers to an optical density equivalent to 0.5 McFarland standard and subsequently diluted 1:100 in TSBg to give a starting inoculum of 1.29 × 10^6 CFU mL^-1_. In the biofilm disruption assay, 200 μL volumes of the prepared culture were aliquoted into wells of a sterile 96-well microtitre plate (Sarstedt) and incubated at 37°C for 24 h. After this incubation period, wells were washed three times with 200 μL of sterile ringers using a multichannel pipette (Gilson) to remove media and planktonic cells. Biofilm containing wells were then treated with 200 μL of various concentrations of CHAPK (3.91–500 μg mL^-1_) in sterile 25 mM Tris pH 7 or with 200 μL of sterile 25 mM Tris pH 7 alone (control), at 37°C for 4 h. At the end of treatment all wells were washed again before the plate was inverted and left to dry for 1 h at 60°C. The biofilms were then stained with 200 μL of 0.5% crystal violet solution for 15 min. The stain solution was removed and the wells were gently washed before. The plate was left to dry, after which, 30% acetic acid were added to solubilise the stain. The biofilm disrupting ability of CHAPK was determined by examining the optical density of the wells spectrophotometrically.

2.3.2. Viability Plate Count Assay. A 96-well microtitre plate/peg-lid assay, based on the method used by Moskowitz et al. [55] was used to investigate if CHAPK can completely eliminate a staphylococcal biofilm. A peg-lid plate was used in order to ensure that the maximum number of cells were removed from experimental wells with the same efficiency. This method permits removal of the biofilm matrix by centrifugation prior to plating. Briefly, an overnight colony (18–24 h) of S. aureus DPC5246 was suspended in sterile...
Ringers to an optical density equivalent to 0.5 McFarland standard and subsequently diluted 1:100 in TSBg to give a starting inoculum of $1.29 \times 10^6$ CFU/mL. 200 $\mu$L of the TSBg cell suspension was transferred to the wells of a 96-well plate. As a negative control for biofilm formation, 200 $\mu$L of TSBg was used. A peg lid was added and the plate was incubated statically for 24 h at 37°C [56]. After incubation the peg lid was removed and washed three times by placing it in a 96-well plate containing sterile ringers for 30 sec each time. 200 $\mu$L of CHAPK, at concentrations ranging from 125–1000 $\mu$g mL$^{-1}$ (diluted in 25 mM Tris pH7) was added to treatment wells. 200 $\mu$L 25 mM Tris pH 7 was added to the control wells. The biofilm peg lid was placed on the antimicrobial challenge plate and incubated for 24 h at 37°C. After incubation the peg lid was washed three times in sterile ringers as before. Finally the lid was placed in a plate containing 200 $\mu$L of TSBg with 1% D (+)-glucose for 24 h at 37°C. After incubation the peg lid was washed three times in sterile ringers before.

2.4. Biofilm Prevention by CHAPK

2.4.1. Plate Staining Assay. In order to investigate the ability of CHAPK to prevent the formation of S. aureus biofilms on artificial surfaces, the staining assay, as described previously for the biofilm reduction assay, was carried out with the following modification. At the beginning of the assay 100 $\mu$L of CHAPK, at concentrations ranging from 0.78 to 125.0 $\mu$g mL$^{-1}$, were added to 100 $\mu$L of TSBg with 1.3 $\times 10^6$ CFU mL$^{-1}$ of DPC5246 cells, in a sterile 96-well microtitre plate and incubated for 24 h at 37°C.

2.4.2. Viable Plate Count Assay. The ability of CHAPK to prevent biofilm formation was also investigated using a method similar to the viable plate count method described previously with the following changes. At the beginning of the assay 100 $\mu$L of CHAPK, at concentrations ranging from 0.78 to 125.0 $\mu$g mL$^{-1}$, were added to 100 $\mu$L of TSBg with 1.3 $\times 10^6$ CFU mL$^{-1}$ of DPC5246 cells, in a sterile 96-well microtitre plate.

2.5. Skin Decolonization Assay. This study was carried out using a modified version of the spray test of Hoopes et al. [57]. Briefly, three individual areas, 25 cm$^2$ in size, were marked out on a section of porcine skin (obtained fresh from an abattoir). Each area was disinfected with 70% isopropyl alcohol wipes and allowed to dry at room temperature for up to 30 min. All three marked areas were then seeded with 100 $\mu$L of $6.2 \times 10^7$ CFU mL$^{-1}$ (2.5 $\times 10^7$ CFU cm$^{-2}$) of S. aureus DPC5246 by pipette, distributed evenly within each area with a sterile plastic spreader (Sarstedt) and allowed to dry for 30 min. CHAPK (200 $\mu$g mL$^{-1}$ in sterile H$_2$O) was then misted 20 cm above one of the 25 cm$^2$ areas, in two passes. The two remaining sections served as controls where one 25 cm$^2$ area was misted with sterile H$_2$O and the other was left untreated. The skin was then left to dry at room temperature for 30 min. Sterile cotton tipped swabs (Deltalab sterile swabs, Fisher Scientific, Ireland) were moistened in sterile Ringer’s solution and used to sample each section of skin by rotating and rubbing the swab, in a zigzag pattern, and repeating at right angles. The tips of each swab were placed in 10 mls of Ringers solution and vigorously mixed using a vortex mixer to dislodge cells. The suspensions were serially diluted and plated on Baird Parker agar supplemented with egg yolk tellurite for enumeration of surviving cells. The work was also similarly done using the bioluminescent producing S. aureus Xen29 strain.

3. Results

3.1. Staphylococcal Biofilm Reduction Using CHAPK

3.1.1. Plate Staining Assay. A strong biofilm of S. aureus DPC 5246 was routinely formed when the strain was grown in TSB supplemented with 1% D (+)-glucose for 24 hr at 37°C. This is represented by the strong staining seen in the untreated well in Figure 1. Solubilising of crystal violet stain and subsequent measurement of OD$_{590}$ nm allowed accurate quantification of staining and comparison between control and enzyme-treated wells. The data shown in the bar chart in Figure 1 represents the OD$_{590}$ nm of triplicate wells ± standard error. Mature biofilms (24 h) were treated with enzyme at concentrations ranging from to 3.91–500 $\mu$g mL$^{-1}$, for 4 h at 37°C. The OD$_{590}$ nm data for the biofilm disruption staining assay demonstrated that at all concentrations tested, CHAPK treatment reduced biofilm formation relative to the untreated control well (Figure 1). A one-way ANOVA indicated that CHAPK treatment caused a statistically significant change in biofilm formation ($P < 0.001$). It is clear from the graph in Figure 1 that CHAPK successfully disrupted the S. aureus biofilms in a concentration dependant manner. Visual inspection of the degree and intensity of staining in the CHAPK-treated wells compared to untreated biofilm wells indicated that even at a concentration as low as 3.91 $\mu$g mL$^{-1}$, CHAPK caused a visible reduction in biofilm mass. At a concentration of 62.5 $\mu$g mL$^{-1}$ CHAPK there was little or no visibly detectable staining of the wells (Figure 1).

3.1.2. Viable Plate Count Assay. Purified CHAPK ranging in concentration from 125–1000 $\mu$g/mL, was used to treat a 24 h staphylococcal biofilm. The results of the viable plate counts are summarised in the bar chart in Figure 2, where each bar represents the average of triplicate plate counts ± standard error. After treatment with 125 $\mu$g mL$^{-1}$ a 2-log decrease was seen in the number of cells in the biofilm matrix on the pegs. The average plate count from the wells with untreated DPC5246 biofilms was $2.7 \times 10^6$ CFU mL$^{-1}$. The average viable plate count for the wells treated with 125 $\mu$g mL$^{-1}$ was $2.2 \times 10^5$ CFU mL$^{-1}$. After treatment with CHAPK at concentrations of 500 $\mu$g mL$^{-1}$ or higher there was complete eradication of the biofilm which corresponded to a 4-log drop in CFU mL$^{-1}$ when compared to the untreated control wells.
3.2. Biofilm Prevention by CHAPK

3.2.1. Plate Staining Assay. To investigate the capacity of CHAPK to inhibit the formation of S. aureus biofilms, various concentrations of the enzyme were incubated with strain DPC5246 for 24 h at 37°C in a microtitre plate assay. After staining and subsequent solubilisation of stain, OD₅₉₀ nm measurements were recorded and used to assess the ability of CHAPK to prevent biofilm formation. This data is presented in Figure 3. A one-way ANOVA indicated that CHAPK treatment caused a statistically significant change in biofilm formation (P value < 0.001). Increasing degrees of biofilm prevention were evident in the presence of increasing concentrations of enzyme. At 15.63 μg mL⁻¹ a considerable decrease in optical density is seen when compared with the untreated control wells. A concentration of 31.25 μg mL⁻¹ indicated complete prevention as the mean OD₅₉₀ nm value (0.14) is exactly the same as that of the control medium.

3.2.2. Viable Plate Count Assay. To confirm that CHAPK is able to completely prevent biofilm formation a plate count assay was performed on wells in which S. aureus was grown in TSBg at various concentrations of CHAPK. The bar chart in Figure 4 represents averages of triplicate values ± standard error. Growth of bacteria in the presence of 7.8 μg mL⁻¹ CHAPK caused a 2-log drop in biofilm formation and at 15.63 μg mL⁻¹ a 4-log reduction was evident. Complete prevention corresponding to a 6-log drop was achieved when the DPC 5246 was incubated with CHAPK at a concentration of 31.25 μg mL⁻¹ or higher.

3.3. Removal of S. aureus from Skin Using CHAPK. The potential of CHAPK as a skin decolonization agent was assessed by incorporation of the enzyme into a spray. Sections of porcine skin (25 cm²) were seeded with 2.5 × 10⁵ CFU cm⁻² of S. aureus strain DPC5246 and misted with 200 μg mL⁻¹ solution of CHAPK for two seconds. Water-treated and untreated skin sections had similar CFU values when enumerated after 30 minutes. This contrasted with a significant reduction in CFUs on the CHAPK-treated skin, which received approximately 60 μg of enzyme. CHAPK treatment was found to be sufficient to remove >99% of S. aureus DPC5246 in 30 min when compared with treatment with water, i.e., reduced from 3.7 × 10⁵ CFU mL⁻¹ when treated with water to 1.7 × 10¹ CFU mL⁻¹ when treated with the CHAPK solution. Similar results were achieved when CHAPK was employed against the bioluminescent strain S. aureus Xen29 (Figure 5).
Concentrations of enzyme ranging from 7.8 µg mL⁻¹ were incubated with S. aureus DPC5246 in TSBg at 37°C for 24 h. The wells were stained with crystal violet (1%) and subsequently the stain was solubilised with acetic acid. Optical density readings at 590 nm (OD590) of all wells were recorded in a microtitre plate reader and displayed on the bar chart. Assays were carried out in triplicate and OD590 data was expressed as the mean ± SE.

4. Discussion

Biofilms are recognised as a significant problem in the food industry. Biofilm-forming bacterial strains are generally much more difficult to kill than their planktonic counterparts. They survive in sub-optimal environmental conditions, display widespread resistance to antibiotics and disinfectants and often lead to persistent infections such as is commonly seen with bovine mastitis [16]. Biofilms may also interfere with various processes in food technology and engineering. For example, biofilms can impede liquid flow and heat transfer and lead to increased corrosion rates which can lead to economic losses [58].

This study demonstrates that the phage-derived peptidase, CHAPK, can completely remove a mature staphylococcal biofilm in under 4 h and can also prevent establishment of a staphylococcal biofilm. In the biofilm context it is likely that CHAPK rapidly lyases sessile staphylococcal cells with an efficiency that brings about destabilization of the biofilm matrix leading to their subsequent detachment from solid surfaces. For formation of a mature staphylococcal biofilms a 24 h incubation period is commonly used [37, 56, 59, 60]. In the results presented in this study the untreated wells in the biofilm prevention assay show that bovine mastitis isolate DPC5246 is capable of forming a mature biofilm of 3.9 × 10⁶ CFU/mL in under 24 h.

Disruption of staphylococcal biofilms by phage lysins has previously been reported by Sass and Son [37, 42]. While these studies involved the complete endolysin protein with multiple domains, CHAPK is a truncated form of a natural endolysin (Lys K). It contains one lytic domain and only 33% of the original protein. Due to its lower molecular weight, CHAPK is predicted to have a lower chance of inducing a humoral immune response [46]. Also, because it is smaller CHAPK is more efficiently over expressed in the recombinant E. coli strain compared with the full endolysin; problems of aggregation, which have been encountered with over-expression of cow udders full endolysins are greatly reduced. Lysins with just one catalytic domain, such as CHAPK, may run the risk of being more susceptible to development of host resistance, than a protein with multiple catalytic domains. However, because of the position at which CHAPK cleaves the peptidoglycan (between the characteristic S. aureus pentaglycine bridge and the D-alanine of the tetrapeptide crossink [47]), the possibility of developing resistance seems unlikely but cannot be ruled out. In the earlier studies which reported staphylococcal biofilm disruption by phage lysins, biofilm staining was the sole method used to estimate the efficacy of the enzymatic treatment. Our study combines the staining approach with viable plate counting in order to more accurately represent the effect of the lysin on biofilm forming cells.

At a concentration of 31.25 µg mL⁻¹, CHAPK completely prevented the formation of S. aureus biofilms. This result demonstrates the potential of applying CHAPK as a spray for decontamination of food contact surfaces or of cow udders as a preventative measure for bovine mastitis CHAPK could also be employed as a coating agent on medical implants such as catheters to prevent the adherence of staphylococci and subsequent biofilm formation and infection. Previous studies have shown that coating medical implants with antibacterial agents can be effective in preventing formation of biofilms [61–63]. Previous work by our group demonstrated that CHAPK is also effective as a biocidal agent against several pathogenic species of Staphylococcus including the...
well known biofilm former *S. epidermidis* and all known clonal types of MRSA, and thus can also be considered as a useful antimicrobial agent for prevention or treatment of infections caused by these species. A previous publication by our group on the characterisation of CHAP\(_K\) demonstrated that the lysin is active over a broad range of temperatures and pH and was not seen to be susceptible to degradation by multiple freeze thawing steps [48]. The robustness of the lysin makes it attractive for commercialisation and utilisation as a decontaminating agent.

The two main reservoirs of *S. aureus* on animals are the skin and mucosal membranes. Infection can often originate from commensal flora, especially in veterinary and hospital settings, as is the case with both bovine and human mastitis [64, 65]. The present study demonstrated the potential of CHAP\(_K\) as a decolonisation agent for the removal of *S. aureus* from the surface of mammalian skin. When applied as a spray, CHAP\(_K\) eliminated 99% of *S. aureus* DPC5246 from skin in 30 min. The results of the experiment suggested that CHAP\(_K\) could be included in bovine teat-dip solution for reduction of mastitis causing staphylococci on the udder prior to and after milking in dairy farms. In addition, treatment of human skin with CHAP\(_K\) prior to surgery may help prevent serious nosocomial infections.

In conclusion, our data demonstrates the potential of a novel but natural anti-staphylococcal agent to prevent economically important veterinary infections, nosocomial staphylococcal infections and also reduce biofilm formation in processing systems.

**Authors’ Contribution**

M. Fenton and R. Keary contributed equally to this work.

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