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Potential use of targeted enzymatic agents in the treatment of *Staphylococcus aureus* biofilm-related infections

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SUMMARY

Staphylococcus aureus is a leading cause of healthcare-associated infections. The ability of *S. aureus* to attach and subsequently accumulate on the surfaces of implanted medical devices and in host tissues makes infections caused by this pathogen difficult to treat. Current treatments have been shown to have limited effect on surface-associated *S. aureus*, and may be enhanced by the addition of a dispersal agent. This study assessed the enzymatic agents dispersin B, lysostaphin, alpha amylase, V8 protease and serrapeptase, alone and in combination with vancomycin and rifampicin, against biofilms formed by meticillin-resistant and -susceptible strains of *S. aureus*. The efficacy of both antibiotics was enhanced when combined with any of the dispersal agents. Lysostaphin and serrapeptase were the most effective dispersal agents against all strains tested. These data indicate that combinations of biofilm dispersal agents and antibiotics may extend the therapeutic options for the treatment of *S. aureus* biofilm-associated infections.
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Introduction

The use of medical devices and biomaterials has revolutionized modern health care. However, the colonization of these devices by surface-adhering bacteria, resulting in biofilm formation and subsequent device-related infection, is associated with significant patient morbidity and mortality.[1] Staphylococci, particularly *Staphylococcus aureus* and *Staphylococcus epidermidis*, are a leading cause of healthcare-associated infections due to their ability to attach and accumulate on host tissues or implantable medical devices, causing infections such as surgical site infections, intravascular catheter infections and implant-related infections. Evidence that these common infections are due to biofilm formation is well documented, with up to 80% of human bacterial infections said to involve biofilm-associated micro-organisms such as staphylococci.[2] These biofilms are highly resistant to innate and adaptive immune defence systems, and antimicrobial agents, resulting in

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persistent infections and treatment failure. Amongst staphylococci, *S. aureus* is regarded as the most virulent species due to its wide array of secreted and cell-surface-associated virulence factors, immune evasion mechanisms and toxin production.

Over recent years, understanding of the mechanisms of biofilm formation by *S. aureus* has evolved. It is now clear that *S. aureus* employs multiple mechanisms of biofilm formation; for example, cell wall proteins (e.g. fibronectin binding proteins), surface-associated polysaccharide intercellular adhesin (PIA)/polymeric *N*-acetyl-glucosamine (PNAG) and extracellular DNA are well-characterized mediators of biofilm formation.[3–6] Meticillin susceptibility also influences the biofilm phenotype expressed by *S. aureus* clinical isolates; in meticillin-resistant *S. aureus* (MRSA), the major cell wall autolysin Atl, eDNA and the fibronectin-binding proteins FnBPA and FnBPB play fundamental roles in biofilm production.[7,8] This biofilm phenotype appears to be significantly less prevalent in meticillin-susceptible *S. aureus* (MSSA), which produce PIA/PNAG-mediated biofilm.[9] A serine protease, V8, produced by *S. aureus* has also been shown to play a role in its adhesive phenotype.[10] More recently, the authors' group and others have identified that the production of coagulase by *S. aureus* plays a critical role in biofilm formation under more physiologically relevant conditions, is universal for all *S. aureus* strains (i.e. both MSSA and MRSA strains), and, upon maturation, these fibrin-shielded biofilms or fibrin scaffolds exhibit increased resistance to antimicrobial drugs.[11,12]

Given the clinical significance of these infections, there is a clear need to identify novel antibiofilm therapeutics and to have a greater understanding of these mechanisms of biofilm formation by *S. aureus*, as this is vital in guiding these new approaches.[13] Given the frequency of treatment failure with conventional antimicrobials, enzymatic agents that can depolymerize components of an established staphylococcal biofilm have been the subject of recent research, in which their effectiveness in removing a biofilm from a surface has been investigated.[14,15] Examples of such enzymatic agents that target differing mechanisms of *S. aureus* biofilm formation and which have been studied incompletely include dispersin B, lysostaphin, alpha amylase, proteases and the fibrinolytic agent serrapeptase.

Many in-vitro studies investigating potential treatment of *S. aureus* biofilm with enzymatic agents have been limited in that they do not mimic features such as physiologically relevant shear (e.g. host blood flow) and the deposition of serum proteins on surfaces. Moreover, they do not consider the role of meticillin susceptibility or the more recent evidence on the role of the fibrin scaffold produced by *S. aureus* under physiologically relevant conditions.[11] Therefore, this study used MSSA and MRSA strains within an in-vivo mimicking model of *S. aureus* biofilm infection to investigate a number of targeted enzymatic agents (alone and in combination with conventional antibiotics) to disrupt and treat *S. aureus* biofilm, with the aim of highlighting future novel therapeutic options for these infections.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study were MSSA strain SH1000 and MRSA strain USA300 JE2. Strains were grown with aeration at 37°C in tryptone soya broth (TSB; Sigma-Aldrich, St Louis, MO, USA) overnight.

Treatment agents

Treatment agents were prepared to the following final concentrations: dispersin B 0.125–4 μg/mL (Kane Biotec Inc, Winnipeg, Canada), lysostaphin from *S. saprophyticus* 0.5–16 μg/mL (Sigma-Aldrich), alpha amylase 3.12–100 μg/mL (Sigma-Aldrich), endoproteinase from *S. aureus* strain V8 2–64 μg/mL (Sigma-Aldrich), serrapeptase 3.12–100 μg/mL (iHerb, Moreno Valley, CA, USA), vancomycin 5 mg/mL (Sigma-Aldrich) and rifampicin 5 mg/mL (Sigma-Aldrich). Enzymatic treatment agents were examined alone and in combination with either vancomycin or rifampicin.

Static biofilm formation and treatment

S. aureus biofilm was formed as described previously.[11,16] Platelet-poor plasma was obtained from healthy volunteers. Plasma was diluted to 20% (v/v) in carbonate buffer (pH 9.6), and used to precondition the wells of a 96-well microtitre plate (Nunc, Roskilde, Denmark) at 37°C for 2 h.[11] This was removed and an overnight culture of the test organism, grown in TSB, was diluted 1:100 to an OD600 of 0.2 in prewarmed TSB. From this suspension, 100 μL was inoculated into the microtitre plate wells and incubated at 37°C for 24 h. Following the initial incubation and washing, 100 μL of the treatment agent was added to each test well at 37°C for 2, 6 or 24 h and then washed twice with sterile distilled water; a negative control was included that did not contain bacteria. Treatment agents were used at the concentration range described above. Media was held in the well during incubations, and washing steps were performed as above. After treatment, the plates were washed three times with distilled water to remove unattached bacteria, and dried for 1 h at 56°C, as described previously.[3,17] The absorbance of the adhered, stained biofilms was measured at A_{492} using a microtitre plate reader.

Measurement of static biofilm viability

Biofilms were formed and treated as described above. Treatment agents included enzymatic agents and antibiotics alone and in combination. After treatment, biofilms were harvested from the wells of 96-well plates using TrpLE (1X) (Gibco, Thermo-fisher, Waltham, MA, USA), a dissociation reagent, as described previously.[11] Bacterial numbers were determined using a broth dilution and spread plate method on tryptone soya agar (Fannin, LIP, Galway, Ireland). Log_{10} transformation of colony-forming unit data was used to ensure normal distribution of the data.

Measurement of antibiofilm activity under flow

A flow cell and pump (Cellix Ltd, Dublin, Ireland) was used to create a microfluidic model as described previously.[16] Vena8 Fluoro+ flow chambers were coated with 100% plasma for 2 h at 37°C. Exponentially growing *S. aureus* cultures were adjusted to an OD600 0.2 in TSB and injected into each chamber to attach for 1 h at 37°C on an inverted microscope. The pump was activated, and TSB was infused through chambers of the chip for 24 h at a shear rate of 6.25 dynes (200 μL/min). Treatment agents, dispersin B (1 μg/mL), lysostaphin (1 μg/mL), alpha amylase (64 μg/mL), V8 (64 μg/mL) and serrapeptase (6.25 μg/mL), were injected into each chamber and allowed to treat the

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