In vitro interaction of chronic wound bacteria in biofilms

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Abstract

Biofilms are abundant in nature and represent the most prevalent mode of growth of microorganisms. Wound biofilms are recalcitrant to antimicrobial treatment, evade the host immune response and impair the wound healing process. Consequently, considerable effort has been directed towards increasing our understanding of wound biofilms and methods to aid treatment. The aim of this study was to use *in vitro* biofilm models of wound bacterial isolates and compare the biofilms produced for different combinations of these microorganisms. *In vitro* biofilms generated by *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus oralis* and *Micrococcus luteus* in microtitre plates and a constant depth film fermenter (CDFF) were studied. All the chronic wound bacteria isolates formed biofilms (both individually and in mixed culture) in these models. In mixed species microtitre plate biofilms, both *P. aeruginosa* and *S. aureus* appeared to antagonise biofilm formation by *S. oralis* and *M. luteus*, with *P. aeruginosa* completely inhibiting the growth of these organisms. Similar effects were evident in the CDFF model, when all four bacterial species were added simultaneously with *M. luteus* being 'out-competed' by the other organisms present, and *S. oralis* occurring at numbers at the limits of detection.

Introduction

Chronic wounds are defined as wounds that do not heal and are unable to restore anatomic and functional integrity within a normal and expected time-frame (Schreml *et al.*, 2010). Chronic wounds represent a major world health problem, occurring in 1% of the world's population (James *et al.*, 2007) and exist as biofilm communities (Dowd *et al.*, 2008a; Dowd *et al.*, 2008b; James *et al.*, 2007; Kirketerp-Moller *et al.*, 2008; Percival *et al.*, 2008; Wolcott & Ehrlich, 2008). Indirect and direct evidence from clinical studies has highlighted the importance of bacteria in mediating chronic inflammation and impairing cellular responses within the wound bed (Stephens *et al.*, 2003; Wall *et al.*, 2002).

Whilst cellular and molecular studies have demonstrated that considerable heterogeneity exists in bacterial colonisation of chronic wounds, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are often reported as the predominant wound bacteria (James *et al.*, 2010; Moore *et al.*, 2010; Percival *et al.*, 2010; Werthen *et al.*, 2010), together with anaerobic bacterial species (Davies *et al.*, 2004; Dowd *et al.*, 2008a; Gjodsbol *et al.*, 2006; James *et al.*, 2007; Price *et al.*, 2009). In chronic wound biofilms, pathogenic bacteria appear to become the dominant microflora at the expense of commensal species. Studies have previously shown that *P. aeruginosa* and *S. aureus* can both develop biofilms in animal wound models (Akiyama *et al.*, 2002; Davis *et al.*, 2008; Serralta *et al.*, 2001), and in human wounds aggregates of bacterial micro colonies have been identified that appear to be coated with extracellular polysaccharide substances (EPS) (Bjarnsholt *et al.*, 2008; James *et al.*, 2007; Kirketerp-Moller *et al.*, 2008).

The increasing realisation that bacteria in chronic wounds exist within a polymicrobial biofilm (Dowd *et al.*, 2008a; Sun, 2008; Sun, 2009) highlights the importance of correct treatment strategies against the biofilm bacteria. A recent molecular study by Thomsen *et al*. (2010) detected bacteria that had not previously been identified from wounds and suggested the need to determine their potential pathogenesis and impact on wound healing. To do this, all of the bacteria present in a wound must be identified (Thomsen *et al.*, 2010). Therefore, to further our understanding of wound biofilms, a thorough characterisation of the individual wound bacterial species and their interactions within biofilms is important.

In this study, we used microtitre plate (Djordjevic *et al.*, 2002; Kennedy & O'Gara, 2004; Ramage *et al.*, 2001; Ramage *et al.*, 2005) and constant depth film fermenter (CDFF) models (Allan *et al.*, 2002; McBain *et al.*, 2003; Wilson, 1999). The CDFF has previously been used to model biofilms of bacteria from non-wound environments *e.g.* oral and waste water biofilms and more recently chronic wounds (Hill *et al.*, 2010; Malic *et al.*, 2009). The aim of the study was to establish whether these different species of wound bacteria could influence each other in terms of biofilm development and thus affect the final climax communities that develop in chronic wounds.

Materials and methods

Preparation of bacterial species

Bacterial wound isolates were obtained following written informed consent and local ethical committee approval, from a previous prospective study of 70 patients with chronic venous leg ulcers (Davies *et al.*, 2004; Davies *et al.*, 2007). Selected Isolates were species of the four bacterial genera most frequently encountered, namely: *S. aureus, P. aeruginosa*, *Micrococcus luteus* and *Streptococcus oralis*. These species were routinely cultured on blood agar (BA; Lab M, Bury, UK) supplemented with 5% (v/v) sheep blood. BA plates were incubated aerobically at 37°C for 24 h.

In vitro biofilm formation using a microtitre plate assay

Preliminary studies were undertaken to identify suitable culture media and incubation times that allowed growth and biofilm formation of all isolates. A total of four different culture media were used, including Brain Heart Infusion (BHI; Oxoid, Hampshire, UK) broth, Tryptone Soya Broth (TSB; Oxoid), defined BM medium (McKee *et al.*, 1985) and a simulated wound fluid (Addison *et al.*, 2004).

Micrococcus luteus (B81), *S. oralis* (B52), *P. aeruginosa* (D40) and *S. aureus* (D76) were cultured at 37°C for 24 h in 10 ml of culture medium. Standardised bacterial preparations with an optical density of 0.4 at 600 nm were initially prepared in PBS and this preparation was centrifuged and cells resuspended in the equivalent volume of culture medium, thereby standardising the preparation.

Biofilm formation in 96-well microtitre plates (flat bottomed, Greiner, UK) involved the use of a range of bacterial inocula (using two-fold serial dilutions in appropriate culture medium) of standardised bacterial preparations. A 100-µl volume of bacteria was added to the microtitre plate wells, which were sealed with Parafilm® (American National Can Group, Chicago, USA) and incubated at 37°C for 24-96 h. Single and dual species biofilms (cultured in BHI at 24 and 48 h) were examined in the microtitre plate assay with biofilm formation subsequently measured by crystal violet staining and total viable counts. A broth control was also included.

Crystal violet assessment of biofilm biomass (Djordjevic *et al.*, 2002) involved the removal of the culture medium followed by washing (×3) with 200 µl of sterile dH_2O to remove loosely associated bacteria. Plates were dried at 56°C for 45 min, and each well stained with 150 µl of 1% (v/v) crystal violet solution (in water) for 45 min and then washed (×3) with sterile distilled water. A 200-µl volume of 95% (v/v) ethanol was added to de-stain the cells and after 5 min, 100 µl from each well was transferred to a new microtitre plate and the intensity of the crystal violet present in the de-stain solution measured by absorbance at 595 nm.

Viable cell counts were also performed following removal of the planktonic phase of growth. Firstly, a 100-µl volume of PBS was added to each well to re-suspend the biofilms. Subsequently, the biofilms of 6 wells were pooled and serially diluted in PBS and dilutions (100 µl) plated on to BA plates which were incubated aerobically at 37°C for 24 h. Enumeration of each species was based on differential colony counting.

Chronic wound bacterial biofilms prepared using the CDFF

A sterilised CDFF was fully assembled with PTFE plugs recessed to a depth of 400 µm. The CDFF was incubated at 37ºC with the turntable rotated at 20 revs min^{-1}, and ambient air allowed to enter into the CDFF via sterile air filters. In these CDFF experiments, BHI was the selected growth medium.

Bacterial isolates were tested for their ability to form biofilms in the CDFF using both single and mixed species inocula. Culture inocula were prepared in 10 ml BHI broth for 24 h at 37°C in a shaking incubator. Five ml of each culture was then added to 1 L of BHI broth which was recirculated through the CDFF for 24 h

to ‘seed’ the system. The inoculum was disconnected and fresh medium fed into the CDFF without recirculation at 30 ml h^{-1} using a peristaltic pump (Watson-Marlow, Falmouth, UK). At 24 h intervals over a 7 d period, a single pan holding 5 plugs was aseptically removed from the CDFF for analysis of the biofilm. The 5 plugs were placed in 2.5 ml of PBS, vortexed for 5 min until a homogenous suspension of bacteria was evident which was than serially diluted and 100 µl plated on to BA plates which were incubated aerobically at 37°C for 24 h for determination of viable counts.

Statistical analyses of biofilm biomass

Statistical analyses were performed on data obtained from the crystal violet microtitre plate assays. The Friedman test and the Wilcoxon signed rank test were used to examine any differences in biofilm formation when different starting inocula were used. The Kruskal-Wallis test is an extension of the Wilcoxon rank sum test and was used for the comparison of more than two groups. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 16 software (SPSS Inc. Chicago, USA).

Results

Comparison of biofilm biomass in microtitre plates using crystal violet staining

Of the culture media examined, limited biofilm biomass was generated in both BM and simulated wound fluid media. Hence, the majority of subsequent experiments were therefore undertaken in BHI or TSB media. Results of biofilm formation by wound isolates in BHI and TSB media for different starting inocula are presented in Figures 1a-d. No statistical differences in the final biofilm levels attained were observed for the different inocula used.

Single species biofilm formation exhibited marked variation for the growth media over 24-48 h. Using BHI, all isolates formed biofilms, although after 48 h (results not shown) the extent of biofilm formation decreased below an OD_{595} of 0.075, presumably as a result of nutrient limitation and cell death. The hierarchy

of biofilm formation in BHI over 24-48 h, was *M. luteus* > *S. oralis* ≥ *S. aureus* > *P. aeruginosa* (Figs. 1a, 1b and 2a).

In TSB (Figs. 1c, 1d and 2b), consistent levels of biofilm growth were again evident over 24-48 h. After 48 h (results not shown), the biofilm levels again reduced dramatically. Furthermore, the relative hierarchy of TSB biofilm formation was found to differ compared to that in BHI, with *P. aeruginosa* producing the most abundant biofilm growth in TSB.

Subsequent microtitre experiments compared the growth of mixed species biofilms after 24 h, to investigate whether synergistic or antagonistic interactions occurred (Fig. 2c-2f). Interestingly, when *M. luteus* and *P. aeruginosa* were combined (Fig. 2c), a biofilm level intermediate to that generated by the respective single species occurred. Interestingly, in other mixed species combinations, biofilm levels decreased to that of the slower growing partner (Figs 2d-f)

To determine the relative contribution of bacterial species in the composition of the mixed biofilms, differential viable counts were determined at 24 and 48 h (Table 1). These showed that when grown individually for 24 or 48 h, all species reached 10^7-10^8 colony forming units (cfu) ml^{-1}. In contrast, *M. luteus* did not survive in biofilms when either *P. aeruginosa* or *S. aureus* were present, and only grew in mixed species biofilms with *S. oralis*. *Streptococcus oralis* also did not compete with *P. aeruginosa*, with growth of the former not evident in these mixed biofilms, although it grew well in pair wise culture with the other two wound isolates.

Comparison of biofilm formation by test species using the CDFF

All wound bacterial species produced biofilms in single culture in the CDFF (Fig. 3a). In terms of bacterial counts, the highest growth occurred with *S. aureus* and *P. aeruginosa* over the 7 d incubation period (~10^9 cells ml^{-1}), with lower growth detected for both *S. oralis* and *M. luteus* (~10^7 cells ml^{-1}).

In mixed species biofilms (Fig. 3b), with all 4 species present similar numbers of viable *S. aureus* and *P. aeruginosa* were detected as previously encountered in single species experiments (*i.e.* 10^8-10^9 cfu ml^{-1}). However, there was an apparent increase in the numbers of *S. oralis* compared with its single

culture equivalent. In contrast, *M. luteus* was detected only inconsistently in these mixed species biofilms, at levels near or below the limits of detection.

Discussion

Recently, attention has focused on the ability of bacteria within chronic wounds to form biofilms (Bjarnsholt *et al.*, 2008; Davis *et al.*, 2008; Dowd *et al.*, 2008a; Dowd *et al.*, 2008b; James *et al.*, 2007; Percival *et al.*, 2008; Wolcott & Ehrlich, 2008). An important feature of such growth in the clinical context is the well-documented enhanced resistance of biofilms to antimicrobial agents and host immune defences (Costerton *et al.*, 1999; Stewart & Costerton, 2001).

In the present study, the growth of four bacterial species prevalent in chronic wounds was studied both in single and mixed species biofilms. In this way, any synergistic or antagonistic influences of interactions on growth could be determined. This is important, as it provides information on how communities characteristic of a pathogenic wound biofilm develop and also offers insight into whether modulation of these communities could be beneficial to wound healing.

The microtitre plate assay is a frequently used method to assess the biofilm-forming ability of bacteria (Allegrucci & Sauer, 2007; Hall-Stoodley *et al.*, 2008; Kennedy & O'Gara, 2004; Lizcano *et al.*, 2010; Munoz-Elias *et al.*, 2008). In this present study, this assay was used to examine biofilm formation by chronic wound bacterial species and in particular, to directly compare the development of single and mixed wound bacterial biofilms.

Throughout the study, consistent species differences in biofilm levels in the microtitre plate assay were evident. The hierarchy of biofilm formation also varied with the medium used, which highlights the importance of the environmental milieu and is an observation consistent with those presented by other authors (Allegrucci & Sauer, 2007; Hall-Stoodley *et al.*, 2008; 2004; Lizcano *et al.*, 2010; Munoz-Elias *et al.*, 2008). The observed inter-species differences in biofilm-forming ability may reflect different growth rates or the relative adherence of the species to the microtitre plate surface. Previous work has indeed shown that bacteria adhere more favourably to some materials than others (Simoes *et al.*, 2007; 2008; Stepanovic *et al.*, 2000).

It was interesting to note that in mixed species biofilms, both *P. aeruginosa* and *S. aureus* appeared to antagonise the growth of both *S. oralis* and *M. luteus*. Such an effect could arise for a variety of reasons, including the occurrence of inter-species competition for surface attachment sites, competition for nutrients within the growth medium or the production of less stable biofilm architecture. The fact that the microtitre plate was a 'closed' system that would allow accumulation of inhibitory bacterial products (Lizcano *et al.*, 2010) might enhance antagonism for certain species in this model. Since, the CDFF allows culture medium to be constantly replenished and waste products removed, such a situation might have been expected to be less pronounced in the CDFF. Results from the CDFF again showed that individually, all species were able to form biofilms. Interestingly, using BHI medium, *S. aureus* produced the highest biofilm level, followed by *P. aeruginosa, S. oralis* and *M. luteus.* This was in contrast to the microtitre plate assay, where the hierarchy of biofilm formation was *M. luteus* > *S. oralis* > *S. aureus* > *P. aeruginosa*. The explanation for these differences could relate to the batch conditions of the microtitre plate assay or the differences in the actual surface for attachment of the two models, which would influence initial adherence and subsequently the relative biofilm growth of the species.

CDFF experiments combining all four bacterial species showed that *S. aureus* and *P. aeruginosa* biofilms were greater in terms of cell numbers than those of *M. luteus* and *S. oralis*, which mirrors the findings of the microtitre plate assay. In the present study, only inconsistent detection of *M. luteus* was evident in the CDFF, although Hill *et al*. (Hill *et al.*, 2010) have previously shown that *M. luteus* can survive in mixed species CDFF biofilms, albeit at lower levels than *P. aeruginosa* and *S. aureus*. It is possible that *M. luteus* can colonise the substratum used and as a non-motile organism, provides a base for the maturing biofilm. Given the expected limitations in terms of nutrients and oxygen at such locations, it is possible that a dormant state or even a complete loss of viability might result, making the species less amenable to detection by culture alone (Hill *et al.*, 2003). Furthermore, a motile organism like *P. aeruginosa* could readily be envisaged as having an advantage over non-motile species within a community environment, being able to migrate to areas of the biofilm that are more beneficial for its growth.

The predominant microorganisms in chronic wounds have been shown to be *P. aeruginosa* and *Staphylococcus* spp., which were found in 34.8% (surface), 31.8% (tissue), and 71.2% (both surface and tissue) of patients with chronic wounds (Davies *et al.*, 2007). The isolates used in this study came from four different wounds which all had a polymicrobial composition (>5 species per wound) (Davies *et al.*, 2004; 2007). Furthermore, all four species have also been found to occur simultaneously within the same wound (unpublished data). However, it is unclear whether the relative proportions of these species in the current *in vitro* studies reflect those found within a wound environment. Extrapolating *in vitro* results to wounds might however suggest that *P. aeruginosa* would predominate over organisms such as *M. luteus.*

Davies *et al.* (2007) in a study examining the relationship between bacterial load and wound status, quantified both *S. aureus* and *P. aeruginosa* from the same group of chronic wounds and showed that the relative proportions of these two species was variable and patient dependant. Similarly, *Micrococcus* sp. were found in 40.9% (surface) and 45.5% (tissue) of chronic wounds, thus highlighting the possible role of other factors, including the presence of other species and host defence processes, in wound biofilm composition. It could be hypothesised that *M. luteus* represents a wound contaminant from the skin, with only transient occurrence in chronic wounds. However, its high incidence in chronic wound patients (Davies *et al.*, 2007) might indicate that this species is a true coloniser of chronic wounds. In contrast, the presence of *P. aeruginosa* and/or *S. aureus* could be indicative of a pathogenic state, and in this regard, treatment options targeting *P. aeruginosa* would be advocated. The findings in this *in vitro* study could support the functional equivalent pathogroups (FEPs) concept by Dowd *et al*. (Dowd *et al.*, 2008b). They hypothesised that certain bacterial species may not be capable of maintaining a chronic infection on their own, unless they acted synergistically to create a pathogenic group (Dowd *et al.*, 2008b). Another study by Percival *et al.* (2010) suggested targeted therapies against specific bacteria in the wound could act to convert a pathogenic biofilm into a commensal one. Further work is still needed on clinical samples to confirm such a hypothesis.

It is tempting to speculate that in chronic wounds the introduction of either *P. aeruginosa* or *S. aureus* to the environment may cause replacement of harmless skin commensal organisms such as *M. luteus,* thus causing an ecological shift to an infected state. This situation has indeed previously been postulated to occur in plaque mediated diseases (Marsh, 2003). To confirm this hypothesis, there is a need for more in-depth cultural analysis of the chronic wound environment and identification of the bacterial species involved. Potentially, if such a microbial succession does occur, then there may be a possible role for probiotic therapy in chronic wounds that could reverse the microbial ecology to one of a 'healthy state'. Indeed, succession studies in biofilm formation represent an area that appears to be currently neglected by researchers. One possibility for future work would therefore be to add the species of interest in a successive manner, *e.g.* by addition firstly of potential commensal species followed by subsequent addition of pathogenic organisms. This could give a better understanding of how biofilms form *in vivo*, and offer insight into how best to treat them.

The present study has shown the value of the CDFF as an effective and reliable model to generate sustainable *in vitro* growth of multispecies biofilms from chronic wound bacteria. Species differences in terms of biofilm formation were evident for specific growth media in both closed and open model systems, whilst a far more complex picture was demonstrated for mixed species biofilms, with distinct species antagonism observed. This apparent antagonism of pathogenic species over 'commensal' ones might provide insight into the development of the composition of a 'non-healing' wound biofilm, which could be further utilised for the development of defined anti-biofilm therapies.

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FIGURE LEGENDS

Fig. 1. Comparison of biofilm formation (OD_{595}) for different inocula of individually grown wound bacteria using the microtitre plate assay and BHI medium for (a) 24 and (b) 48 h and in TSB medium for (c) 24 and (d) 48 h. Error bars represent standard deviation of 8 replicates.

Fig. 2. Relative biofilm development (OD_{595}) for individually grown wound bacteria in the microtitre plate assay for 24 h in (a) BHI and (b) TSB medium; and for individual and pairwise-growth of wound bacteria for 24 h in BHI (c) *M. luteus* and *P. aeruginosa*; (d) *S. oralis* and *S. aureus*; (e) *S. aureus* and *P. aeruginosa*; (f) *S. oralis* and *P. aeruginosa*. The box represents the data distribution between the 25th and 75th percentiles. The thick black horizontal line is the median. The median is described as the number separating the higher half of a sample from the lower half.

Fig. 3. Relative biofilm formation in the CDFF for 7 d in single species biofilms (a); relative biofilm formation of four bacterial species in the CDFF for 7 d when cultured together as a biofilm (b). *Micrococcus luteus* was inconsistently detected in the mixed species biofilms.