Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid (PNA) fluorescent *in situ* hybridisation (FISH)

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ABSTRACT

Biofilms provide a reservoir of potentially infectious microorganisms that are resistant to antimicrobial agents and their importance in the failure of medical devices and chronic inflammatory conditions is increasingly being recognised. Particular research interest exists in the association of biofilms with wound infection and non-healing *i.e.* chronic wounds. In this study, fluorescent *in situ* hybridisation (FISH) was used in combination with confocal laser scanning microscopy (CLSM) to detect and characterise the spatial distribution of biofilm-forming bacteria which predominate within human chronic skin wounds (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus* sp. and *Micrococcus* sp.). *In vitro* biofilms were prepared using the Constant Depth Film Fermenter (CDFF) and a reconstituted human epidermis (RHE) model. *In vivo* biofilms were also studied using biopsy samples from non-infected chronic venous leg ulcers (CVLUs). The specificity of Peptide Nucleic Acid (PNA) probes for the target organisms was confirmed using mixed preparations of planktonic bacteria and multiplex PNA probing. Identification and location of individual bacterial species within multi-species biofilms demonstrated that *P. aeruginosa* was most predominant. CLSM revealed clustering of individual species within mixed species biofilms. FISH analysis of archive chronic wound biopsy sections showed bacterial presence and allowed bacterial load to be determined. The application of this standardised procedure makes available an assay for identification of single or multi-species bacterial populations in tissue biopsies. The technique provides a reliable tool to study bacterial biofilm formation and offers an approach to assess targeted biofilm disruption strategies *in vivo*.

INTRODUCTION

Chronic wounds are an important and often unrecognised cause of disease and disability of the elderly population (Davies *et al*., 2007; Howell-Jones *et al*., 2006). These wounds harbour a diverse microflora, which contributes both directly and indirectly to their non-healing phenotype (Stephens *et al*., 2003; Wall *et al*., 2002). Much attention has recently been focussed on the ability of the bacteria within chronic wounds to form and exist within a biofilm (James *et al.*, 2007). Bacterial biofilms consist of a complex microenvironment of single- or mixed species bacteria attached to each other or attached to surfaces; being encased within extracellular polymeric substances (EPS).

The moist chronic wound surface with its proteinaceous substrate, and supply of nutrients, represents an ideal environment for biofilm development. Many researchers have demonstrated that bacteria within the wound environment possess the ability to form biofilms in both acute and chronic wounds (Bjarnsholt *et al.*, 2008; Davis *et al.*, 2008; Mertz, 2003; Percival & Rogers, 2005; Serralta *et al.*, 2001). Such biofilms may play an important role in resistance to host immune responses (Leid *et al*., 2005) and conventional treatment in these wounds (Rhoads *et al*., 2007).

To understand more of the relationship of the biofilm to the disease, there is a need to better characterise the bacterial communities which exist within the wound. As conventional microbiological techniques (using currently available growth media) identify <5% of bacterial species, the development of alternative methods for the identification of bacterial populations and communities within the human disease states has become increasingly important (Amann, 2001; Moter & Goebel, 2000). To this end, molecular microbiological techniques are being increasingly employed in the study of the wound microflora (Andersen *et al.*, 2007; Davies *et al.*, 2001; Davies *et al.*, 2004; Hill *et al.*, 2003). The characterisation of pathogenic bacteria within disease-associated biofilms has become an important area of current research. The effective monitoring of organisms within a biofilm being crucial to assessing possible management strategies (Gu *et al*., 2005).

Studies of oral biofilms have demonstrated the relationship between the presence of particular bacterial species and the absence/presence of dental caries; suggesting that changes in the local oral environment (*e.g*. nutrition, oxygen, pH, long term use of medication) alter gene expression and favour disease-associated organisms (ecological plaque hypothesis; Marsh *et al.* (2003). Dental caries is associated with a lowering environmental pH and an associated increase in the proportion of acidogenic and aciduric bacteria (*e.g*. *Streptococcus mutans*, *S. sobrinus* and lactobacilli) which demineralise the enamel. In periodontal disease, the number of anaerobic bacteria including Gram-negative proteolytic species is increased (Socransky *et al*., 1998). These studies of plaque biofilm highlight the importance of analysing the diversity and distribution pattern of pathogenic organisms in biofilm-associated human infections.

Fluorescent *in situ* hybridisation (FISH) permits the visualisation and identification of individual bacteria in human disease states *in situ* (Moter & Goebel, 2000). Traditionally labelled DNA probes hybridise to their complementary nucleic acid targets, obeying Watson-Crick base-pairing rules (Moter & Goebel, 2000; Perry-O'Keefe *et al.*, 2001b). Peptide nucleic acid (PNA) probes may also be utilised and are DNA-analogues (pseudopeptides), with an uncharged polyamide backbone instead of conventional sugar phosphates. Compared with traditional DNA probes, PNA probes have superior hybridisation characteristics including higher specificity and improved hybridisation kinetics, which result from the uncharged chemical backbone of the PNA probe. As hybridisation with PNA probes can be performed in a low salt buffer, a decrease in the stability of the rRNA secondary structure can be induced, facilitating the hybridisation of PNA probes to less accessible targets (Perry-O'Keefe *et al.*, 2001b).

The objectives of the current study, were to utilise validated PNA probes in combination with confocal laser scanning microscopy (CLSM) to examine the spatial organisation of chronic wound bacterial species in biofilms generated in a constant-depth film fermenter (CDFF), on human epithelium (*ex vivo*) and in skin biopsies from chronic leg wounds. Through the use of these approaches the aim was to for the first time, to characterise the species distribution of *in vitro* biofilms comprised of chronic

wound bacteria, and to demonstrate the applicability of this technique to tissue from chronic wounds. Such an approach could also in the future be employed to evaluate biofilm management strategies using *in vitro* models and in clinical situations.

METHODS

Bacterial strains. *Staphylococcus aureus* D76, *Streptococcus oralis* B52, *Micrococcus luteus* B81, *and Pseudomonas aeruginosa* D40 isolated from non-infected chronic venous leg ulcers (CVLUs) were used in this study (Davies *et al.*, 2004; Davies *et al.*, 2007; Hill *et al.*, 2003). Previous work from our group had already indicated that *Staphylococcus* and *Pseudomonas* species predominate by culture in these wounds (Davies *et al.*, 2004). The other two strains were selected on the basis of growth rate (to compete in mixed culture) as well as colony morphology (results not shown). Bacteria were routinely subcultured on blood agar No. 2 (BA; Lab M, Bury, UK) supplemented with 5% (v/v) sheep blood and then cultured in Brain Heart Infusion broth (BHI; Oxoid, Basingstoke, UK) for experiments.

PNA probes. The PNA probes and their hybridisation conditions are summarised in Table 1. Two of the probe sequences used (Psaer-FITC and Sta-CY5) were designed to specifically target the 16S rRNA of two bacterial species (*P. aeruginosa*, *S. aureus*) and the third 16S rRNA probe (Bac-Uni1-CY3), was a universal bacterial probe. All probes were manufactured by Boston Probes (Foster City, USA) for Applied Biosystems (Warrington, UK) and were labelled either with fluorescein isothiocyanate (FITC), cyanine 5 (CY5) or cyanine 3 (CY3). Probe sequences were pre-validated using a PNA probe designer software (Applied Biosystems) before synthesis.

Detection of fixed, planktonic bacteria using PNA probes. The specificity of the PNA probes for planktonic bacteria in both single species and mixed bacterial populations was confirmed using all three PNA probes. In these experiments, fixed and unfixed bacteria were tested in a similar method to that previously described by Perry-O'Keefe *et al.* (2001a). Briefly, overnight bacterial cultures were pelleted by centrifugation (16,000 *g*, 5 min) and resuspended in Phosphate Buffered Saline

(PBS; 7 mM Na_2HPO_4, 7 mM NaH_2PO_4, 130 mM NaCl). Bacterial cell suspensions were centrifuged again (16,000 *g*, 5 min), and fixed by re-suspension in PBS with 4% (w/v) paraformaldehyde (Sigma, Poole, UK) for 1 h. Fixed bacteria were rinsed in PBS, re-suspended in 50% (v/v) ethanol and incubated for at least 30 min at -20°C prior to probe hybridisation.

For PNA-FISH, a 100-μl volume of prepared cells was concentrated by centrifugation and the pellet rinsed with PBS and re-suspended in 100 μL hybridisation buffer (25 mM Tris-HCl, pH 9.0; 100mM NaCl; 0.5% (w/v) SDS) containing 150-500 nM PNA probe (Table 1). The cells were incubated at 55°C for 30 min, centrifuged at 16,000 *g* for 5 min and re-suspended in 500 μL wash solution (10 mM Tris pH 9.0, 1 mM EDTA). After a further incubation at 55°C for 10 min, the cells were pelleted by centrifugation. This was repeated twice for a total of three washes. The bacteria were then re-suspended in 100 μL wash solution and 2 μL of the suspension was spread on to a microscope slide and allowed to air-dry. Cells were mounted and visualised using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany).

Construction of CDFF biofilms. Biofilms were prepared in a CDFF (Pratten & Wilson, 1999; Vroom *et al.*, 1999). Wound bacteria (approx. 10^8 cfu) were cultured overnight at 37°C in 10 ml of brain heart infusion (BHI) broth, and 5 mL of each culture was added to 1 L of BHI medium. This inoculum was re-circulated through the CDFF for 24 h to seed the system. The 15 CDFF plugs were recessed to a depth of 400 μm and overlaid with a thin PTFE disk. The CDFF turntable housing the plugs was rotated at 20 revs min^{-1} at 37°C. After the 24 h seeding time, the inoculum was disconnected and fresh medium fed into the CDFF. Fresh medium was delivered at 30 mL h^{-1}, using a peristaltic pump (Watson-Marlow, Falmouth, UK). Biofilm samples were taken for analysis over a period of 7 d every 24 h.

The PTFE disks containing the biofilms were placed in molten agarose (2% w/v; Sigma) and fixed in 2% (v/v) paraformaldehyde solution for 24 h and then embedded in paraffin-wax using standard histological techniques.

Construction of *in vitro* biofilms on a reconstituted human epidermis. Cultivated 0.5 cm^2 reconstituted human epidermis (RHE) was obtained from SkinEthic Laboratories (Nice, France). These tissues had previously been cultured for 17 d in a chemically defined culture medium lacking antibiotics. The RHE comprised of normal human keratinocytes (human foreskin-derived) with a well differentiated epidermis consisting of basal, spinous, granular layers and a stratum corneum.

Bacteria were initially cultured on BA overnight at 37°C. The resultant growth was used to inoculate 10 ml of BHI which was incubated at 37°C for 24 h. Bacterial cells were harvested by centrifugation and washed (×3) with PBS. Pelleted bacteria were re-suspended in RHE chemically defined medium MCDB 153 containing 5 μg mL^{-1} insulin and 1.5 mM calcium chloride with no antibiotics (SkinEthic Laboratories). Two hundred μL of the resulting bacterial suspension was added to the RHE and this was incubated for 24-28 h at 37°C in a humidified atmosphere, enriched with 5% CO_2. A non-infected control was included for comparison. To investigate the effect of epithelial disruption on biofilm formation, experimental skin wounds were created on the surface of the RHE model using a sterile scalpel. After incubation, the entire RHE was fixed in 10% (v/v) formalin for 24 h and then embedded in paraffin wax using standard histological techniques.

Processing of biopsies from chronic wound patients. Wound biopsies were previously obtained with ethical approval and patient informed written consent from non-infected chronic venous leg ulcer patients attending the Wound Healing Research Unit Clinic at the University Hospital of Wales, Cardiff between 1999 and 2002; these biopsies had been maintained at -20°C (Davies *et al.*, 2007). Biopsies were fixed in 10% (v/v) formalin for 24 h and then embedded in paraffin using standard histological techniques.

PNA FISH analysis on processed specimens. Prior to FISH, sections (20 μm) of processed specimens were placed on Histobond+ coated microscope slides (Raymond A Lamb, East Sussex, England), de-waxed and processed through xylene and ethanol to water before probe hybridisation. To hybridise the PNA probes to

bacterial 16S rRNA, sections were directly treated with 100 µL lysozyme (10 mg mL^{-1}; Sigma) and incubated at 37°C for 30 min. Sections were then briefly washed in pre-warmed, wash solution (10 mM Tris pH 9.0, 1 mM EDTA) prior to application of the probe. Pre-warmed hybridisation buffer (150 µL; 25 mM Tris-HCl, pH 9.0; 100 mM NaCl; 0.5% (w/v) SDS) containing 150-500 nM of fluorescently-labelled PNA probe (Table 1), was then added to each biofilm or wound section (20 µm) and placed in a dark, humidified chamber and incubated at 55°C for 90 min. The stringency was adjusted by adding 30% (v/v) formamide to the hybridisation buffer for the *S. aureus* specific probe (Sta-CY5). After incubation, each slide was washed with pre-warmed wash solution using a magnetic stirrer for 30 min.
RHE sections (5 µm) were also Gram-stained to demonstrate bacterial presence on the tissues.

Staining of RHE keratinocytes. For nuclear context, RHE and biopsy sections were counterstained with Hoechst 33258 (2 µg mL^{-1}; Sigma) for 20 min, before washing in wash-solution and mounted using Vectashield® fade-retarding mountant (Vector Laboratories Ltd., Peterborough, UK).

Confocal Laser Scanning Microscopy (CLSM). Sections hybridised with the PNA probes were viewed and analysed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany). The sections were scanned through the full depth using appropriate settings for single, double or triple channel fluorescence recordings of FITC, CY5, CY3 or Hoechst 33258 as detailed in Table 2. For multi-channel recordings, fluorochromes were scanned sequentially to eliminate spectral overlap between probes. Selected images were presented either as single confocal optical sections or maximum intensity type reconstructions.

Quantitative analysis of bacterial populations within tissue sections. In an attempt to assess the bacterial composition of CDFF generated biofilms, a confocal Z series was imported into Image J 1.42l software (NIH, USA; http://rsbweb.nih.gov/ij/) and the images' scale bar used to calibrate the ImageJ area measurement algorithm. Image stacks were separated into their constituent red (Universal Cy-3 probe), green (*P. aeruginosa* FITC probe) and blue channels (*S. aureus* Cy-5 probe) and these

were intensity thresholded to discriminate the labelled bacterial populations from the background. The area of each of the resultant binary threshold masks was quantified for each confocal optical section within the Z-stack. The area occupied by each of the labelled bacterial populations was then expressed as a percentage of the total area of labelled bacteria (*i.e.* the total area occupied by red, green and blue label in composite images).

To assess the number of bacteria within CVLU biopsy section, an automated bacterial cell counting was employed using the ITCN: Image-based tool for counting nuclei plug in; version 1.5. (http://www.bioimage.ucsb.edu/projects/itcn.php). Particles were counted that had a width of 5 pixels; a minimum distance of 2.5 pixels and a threshold value of 10. These threshold values were selected based on typical bacterial sizes.

RESULTS

PNA probe specificity. To verify PNA probe specificity, planktonic preparations of bacteria were first analysed before applying the probe to biofilms and biopsy sections. All bacteria (in mixed culture samples) were successfully detected using the PNA universal probe (Bac-Uni1CY3) (Fig. 1a). Using the Psaer-FITC probe, only a proportion of the population stained green, identifying only *P. aeruginosa* (Fig. 1b). Cells which did not stain with the Psaer-FITC probe clearly exhibited a distinct 'coccus type' morphology. When all the CLSM channels were overlaid, detection of all bacteria was evident with the three different fluorescent markers (Fig. 1c). *P. aeruginosa* cells are represented as yellow in this overlay as a result of hybridising with both the Bac-Uni1-CY3 and with the Psaer-FITC probes. Blue/purple cells represent *S. aureus*, as a result of hybridising with both the Bac-Uni1-CY3 and the Sta-CY5 probes. Red cells in this overlay were either *M. luteus* or *S. oralis* as these hybridised only with the Bac-Uni1-CY3 probe. Multiplex PNA staining demonstrated that these combined probe preparations were effective for species identification.

Analysis of CDFF generated biofilms using PNA FISH. Both single- and multi-species biofilms in the CDFF were successfully detected with the PNA FISH probes. In mixed species biofilms (hybridised with all three PNA probes), distinct zones of

each bacterial population were observed (Fig. 1d-g). These mixed species biofilms appeared heterogeneous with respect to species composition. However, the biofilm mass was composed principally of bacillus-shaped bacteria (*P. aeruginosa)*, with cocci found in isolated pockets. Hence, within these mixed biofilms, *P. aeruginosa* was the predominant organism (Fig. 1e) and was detected throughout the biofilm; *S. aureus* was generally concentrated towards the surface of the biofilm (Fig. 1f). The other bacterial species (*M. luteus* and *S. oralis*) used in this system appeared to be focused in the middle and lower sections of the biofilm (Fig. 1g). The main constituent of these mixed species CDFF biofilms seemed to be *P. aeruginosa*, followed by cocci bacteria. Quantitative analysis of the Z-stacks supported this observation with ~49±15.75% and 5±7.86 of the bacterial population determined as *P. aeruginosa* and *S. aureus*, respectively.

PNA Analysis of RHE biofilms. Biofilms generated on the RHE were successfully processed and Gram stained (Fig 2a and b) or stained with the PNA FISH probes (Fig. 2c). The Hoechst dye used to stain the nuclei of the RHE model also stained the DNA of the bacteria not detected by any of the species-specific PNA probes (i.e. non-*S. aureus* or *P. aeruginosa*). Superficially, the bacteria were present in clusters or aggregates on the surface of the RHE with no evidence of RHE invasion (Fig. 2a). Wounding of the stratum corneum facilitated bacterial invasion, and demonstrated localisation and invasion of bacteria at the site of the wound (Fig. 2b). When a 3-dimensional construction was created from the CLSM data, the bacteria represented a multi-layered community, with bacteria evident within the tissue (Fig. 2c).

Analysis of wound biopsy sections using PNA FISH. Biopsy sections from a non-infected CVLU patient were stained with the universal bacterial PNA probe (Fig. 3). CLSM demonstrated that colonising bacteria could be detected within the biopsy sections, where the detected bacteria appeared to be present both as individual cells and in larger aggregates. Quantification analysis indicated a total bacterial count of 1525 per 375 mm^3 of tissue.

DISCUSSION

Sophisticated molecular techniques are increasingly being used in many areas of microbiology. However, in the case of biofilms, application of such methods often destroys biofilm morphology or architecture due to the DNA/RNA extraction process. Traditional microscopical analyses and bacterial stains are useful, but limited, as they often rely on prior cultivation of the bacteria. Whilst Gram staining provides information on the type of bacterial cell wall it does not allow for species identification (Moter & Goebel, 2000). Since cultivation techniques tend to work only for a minority of species (only 2-3% of bacteria are thought to be cultivable using currently available media), the development of alternative methods for the identification and visualisation of bacteria has become a necessity (Amann, 2001; Moter & Goebel, 2000).

The impact of microorganisms on wound healing is poorly understood but there is strong evidence to suggest that bacteria within chronic wounds delay the wound healing process (Edwards & Harding, 2004; Percival & Bowler, 2004; Percival & Rogers, 2005). The production of destructive enzymes and toxins by bacteria results in tissue damage and indirectly promotes an inflammatory state (Percival & Rogers, 2005; Stephens *et al.*, 2003). Moreover, removal of bacteria by host defence mechanisms and antimicrobial therapy is difficult in wound environments where biofilms have been established. In this present study, FISH in combination with CLSM was used to identify the presence of specific bacteria and to examine their spatial organisation within *in vitro* biofilms established using the CDFF and RHE models, as well as *in vivo* in chronic wound biopsy sections.

The PNA - FISH methodology was developed and validated for three PNA probes, to identify and visualise the polymicrobial population of fixed biofilms and clinical specimens. The bacteria used in the described *in vitro* models were all originally isolated from chronic wounds and included *P. aeruginosa* and *S. aureus* that have been shown by culture to be predominant species in many wounds (Davies *et al.*, 2004). In addition, *Streptococcus oralis* and *Micrococcus luteus* were also used. Both of these species had previously been shown to exhibit high growth rates and a propensity to produce biofilms in *in vitro* models (results not shown). These species,

whilst not recognised as primary wound pathogens, are regularly isolated from acute and chronic wounds and in immunocompromised patients can cause opportunistic infection.

Initially, the use of PNA probes in this bacteria-specific FISH protocol permitted the simultaneous identification of mixed bacterial species in planktonic suspension. Hybridisation was carried out under low salt concentration, high-temperature and high pH conditions. Surprisingly, the *S. aureus* specific PNA probe showed very weak fluorescence compared to both the universal PNA bacterial and the *P. aeruginosa* specific PNA probes. However, this has also previously been observed in other studies (Hartmann *et al.*, 2005; Lefmann *et al.*, 2006; Wellinghausen *et al.*, 2007). To improve the fluorescence intensity of this probe, it was necessary to expose biofilms containing the Gram-positive organisms to lysozyme pre-treatment for 15 min at 37°C and to add 30% formamide to the hybridisation buffer. Thurnheer *et al*. (2004) found that a 1 h paraformaldehyde fixation treatment was sufficient to permeablise Gram-positive cells although this did not prove effective in the present study.

In RHE sections, few bacteria were detected on the surface and only a sparse biofilm was evident, indicating poor invasiveness of bacteria in this intact tissue. However, In scratch wounded RHE sections, it was apparent that bacteria started to penetrate the tissue and to migrate beneath the damaged surface layer. This suggests that once the integrity of the epithelial barrier has been disrupted (as would be encountered in a wounding situation) the bacteria can invade the tissue.

A possible limitation of the RHE system was the lack of specific host immune responses, although proinflammatory cytokine responses by *in vitro* epithelial cell-lines in a similar reconstituted oral epithelium model have been reported (Schaller *et al.*, 2002; Villar *et al.*, 2005). Hence, although the RHE has some drawbacks, it is currently the most suitable *in vitro* model of human skin epithelium.

Importantly, both models (CDFF and RHE) revealed heterogeneous biofilm structures with discrete clusters of bacterial species, with *P. aeruginosa* as the predominant organism. In the case of the former, this was confirmed using quantitative image analysis of the CLSM Z-stack data set.

It is likely that the bacterial species within the biofilms compete with each other for space, attachment and nutrients. Two possible factors for the predominance of *P. aeruginosa* in these mixed biofilms are its twitching and flagella-mediated motility, allowing *P. aeruginosa* to migrate to optimal growth localities within the biofilm as it matures, as well as its faster growth rate.

Clearly, these experiments do not exactly reflect the *in vivo* situation within a chronic wound. *In vivo,* different microbial species could well be expected to colonise at different times, with specific species acting as pioneer or primary colonisers. In our experiments, all four bacteria species were added to the RHE simultaneously. It would therefore, be interesting to add the species in a successive manner. For instance, *P. aeruginosa* could be added to the model first to form a base for the biofilm, with other species added subsequently to determine if they were able to grow to a greater extent on the biofilm surface. Conversely, since it was observed in this study that *P. aeruginosa* appeared to 'out-compete' the other organisms, it might also be of interest to see whether staging of the infection times allows the other species to grow at higher levels, before *P. aeruginosa* is added to the system.

Further research could include incubating the infected RHE for longer than 24 h to see if this would result in greater infiltration of the bacterial cells within the keratinocyte wound model. The use of different culture media that promote bacterial growth as opposed to the tissue maintenance medium could also be assessed. In addition, detection of any extracellular polysaccharide matrix generated by the chronic wound bacterial biofilms may be possible, for example using calcofluor white, after completion of the FISH probing (Perry-O'Keefe *et al.*, 2001b; Thurnheer *et al.*, 2004).

Analysis of chronic wound biopsy sections by FISH using the universal bacterial PNA probe did reveal the presence of colonising bacteria. Routine haematoxylin and eosin staining (data not shown) as well as PNA FISH detection confirmed that bacteria were present in these biopsy sections.

PNA FISH was shown to be a rapid and versatile tool for research purposes and potentially for clinical microbiology diagnostics when used in conjunction with CLSM and quantitative image analysis. CLSM has been established as a valuable method for obtaining high-resolution images and three-dimensional reconstructions of fluorescently labelled biofilms and biological samples (Lopez *et al.*, 2005; Sunde *et al.*, 2003; Thurnheer *et al.*, 2004; Wagner *et al.*, 2003). The continued application of this technique to clinical biofilms from infected tissues or indwelling medical devices could facilitate the identification and estimation of the relative proportions of bacteria within a biofilm. This approach could be used to assess biofilm management strategies or evaluate the effectiveness of antimicrobials against members of the biofilm consortium. Importantly, this technique could potentially be applied to clinical samples for both identifying and estimating the proportion of bacterial species present.

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Table 1. PNA probes and their conditions of use.

Probes	Target	Nucleotide sequence	Hybridisation conditions: temp. [°C] formamide [%]	Fluorescent label	Working Conc. [nM]	Excitation wavelengths [nm]	References
Psaer	**PNA** *P. aeruginosa*	5'-OOAACTTGCTGAACCAC-3'	55°C no formamide	FITC	300	488	(Coull & Hyldig-Nielsen, 2003)
Sta	**PNA** *S. aureus*	5'-OOGCTTCTCGTCCGTTC-3'	55°C 30% formamide	CY5	500	633	(Perry-O'Keefe *et al.*, 2001b)
Bac-Uni1	**PNA** Universal probe (Eubacteria)	5'OOCTGCCTCCCGTAGGA-3'	55°C no formamide	CY3	150	561	(Perry-O'Keefe *et al.*, 2001b)

Table 2. Wavelength scan parameters used for the simultaneous excitation and detection of the four probes used for confocal laser scanning microscopy.

Fluorochrome	**Laser Excitation Line (nm)**	**Emissions Detected (nm)**
Hoechst 33258	405	410-485
FITC	488	498-540
CY3	543	550-610
CY 5	633	635-700

FIGURE LEGENDS

Fig. 1. Confocal laser scanning microscopy images showing PNA FISH of: (a-c) mixed planktonic bacteria labelled using three PNA probes. (a) Universal bacterial probe (Bac-Uni1CY3) showing all bacteria stain red. (b) *Pseudomonas aeruginosa* probe (PsaerFITC) showing that a proportion of the population stain green. (c) Multiplex PNA staining of *P. aeruginosa* (yellow bacteria showing hybridisation with both universal and *P. aeruginosa* specific probes), *S. aureus* (purple bacteria hybridised with both universal and *S. aureus* specific). *Micrococcus luteus* and *Streptococcus oralis* (red bacteria showing hybridisation with the universal probe only). (d-g) Mixed bacterial CDFF biofilms labelled using three PNA probes (d) universal bacterial probe, red. (e) *P. aeruginosa*-specific probe (green). (f) *S. aureus*-specific probe (blue). (g) multiplex PNA staining.

Fig. 2. Microscopical analysis of mixed species biofilms formed *in vitro* on RHE tissue.
(a) Intact RHE section showing Gram stained bacteria on the surface. (b) Artificially wounded RHE section showing Gram stained bacteria within the tissue. (c) PNA FISH using the *P. aeruginosa*-specific (green bacteria) and the *S. aureus* specific probes (red bacteria) and with the epithelial cell nuclei counterstained with Hoechst dye (blue).

Fig. 3. Microscopical analysis of a section from a chronic venous leg ulcer using PNA FISH and showing total bacteria labelled with the universal bacterial PNA probe (red bacteria) and Hoechst dye for epithelial cell nuclei (blue).

Fig. 1.

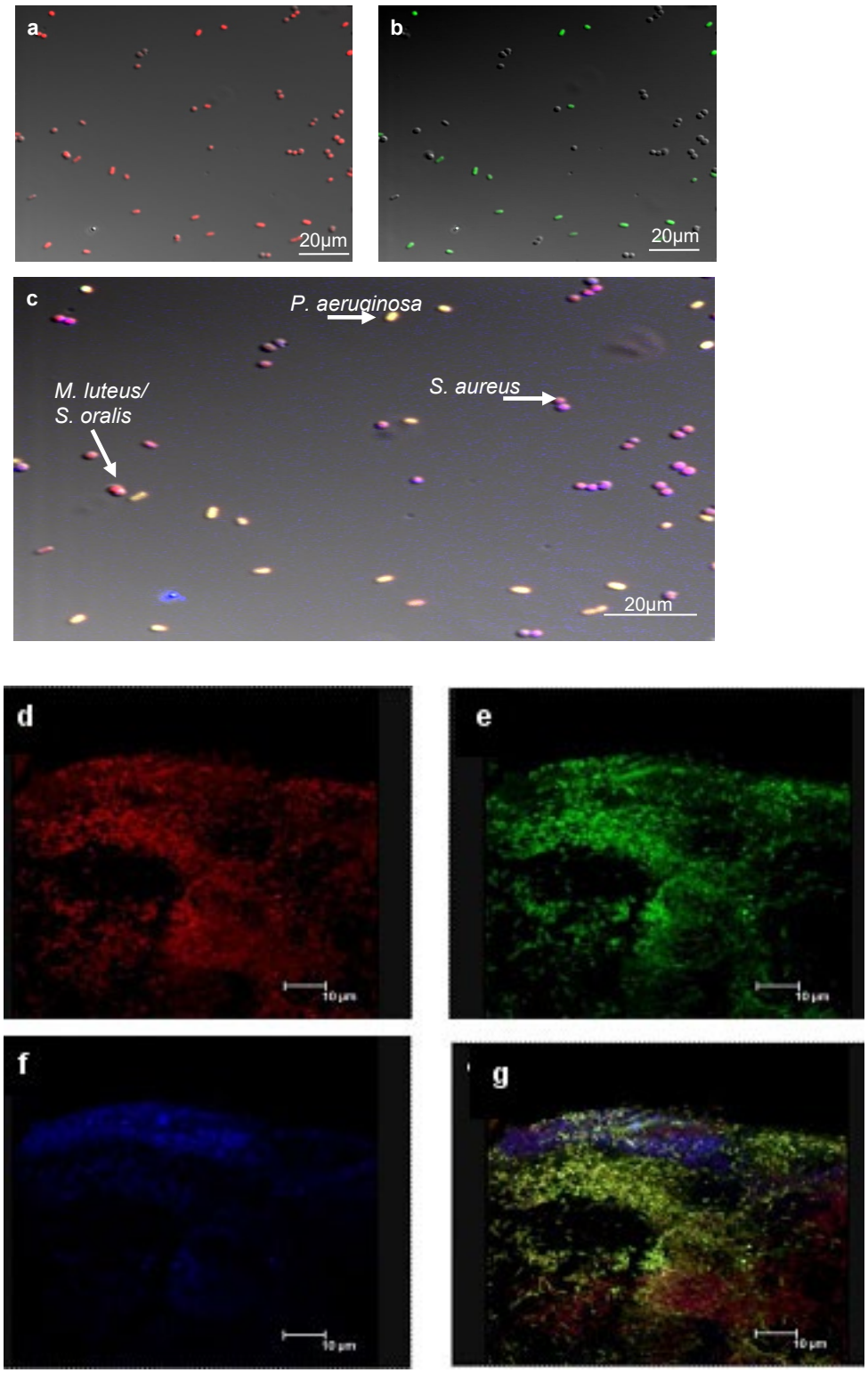
a
20µm
b
20µm
c
P. aeruginosa
M. luteus/
S. oralis
S. aureus
20µm
d
e
f
g

Fig. 2.

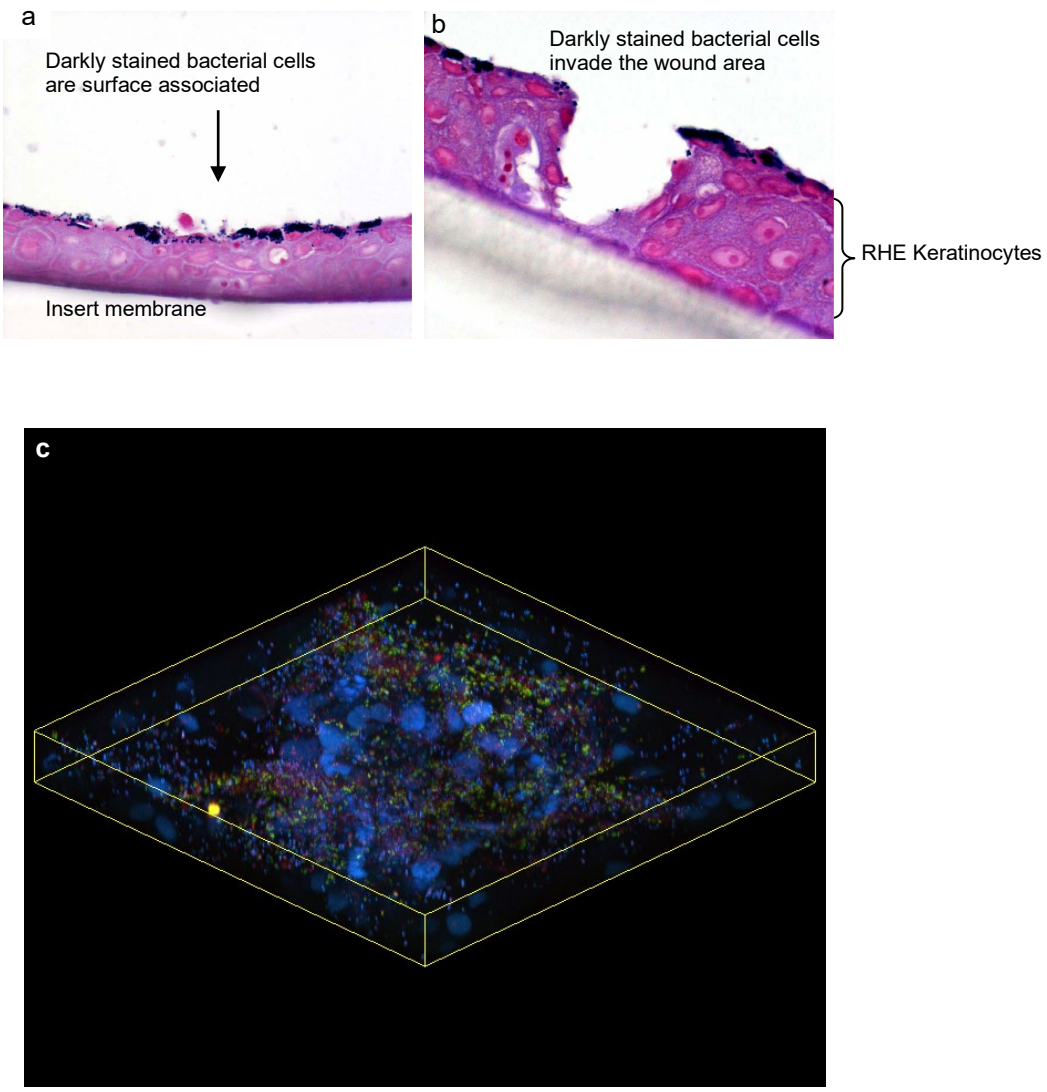
a
Darkly stained bacterial cells
are surface associated
Insert membrane
b
Darkly stained bacterial cells
invade the wound area
RHE Keratinocytes
c

Fig. 3