Development and application of a polymicrobial, in vitro, wound biofilm model

Authors: J. Woods, Laura Boegli, Kelly R. Kirker, A.M. Agostinho, A.M. Durch, Elinor deLancey Pulcini, Paul S. Stewart, & Garth A. James

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Development and application of a polymicrobial, in vitro, wound biofilm model

J. Woods, L. Boegli, K.R. Kirker, A.M. Agostinho, A.M. Durch, E. deLancey Pulcini, P.S. Stewart and G.A. James

Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA

Keywords
biofilm, chronic wounds, Clostridium perfringens, model, polymicrobial, Pseudomonas aeruginosa, Staphylococcus aureus.

Aims: The goal of this investigation was to develop an in vitro, polymicrobial, wound biofilm capable of supporting the growth of bacteria with variable oxygen requirements.
Methods and Results: The strict anaerobe Clostridium perfringens was isolated by cultivating wound homogenates using the drip-flow reactor (DFR), and a three-species biofilm model was established using methicillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa and Cl. perfringens in the colony-drip-flow reactor model. Plate counts revealed that MRSA, Ps. aeruginosa and Cl. perfringens grew to 7Æ39 ± 0Æ45, 10Æ22 ± 0Æ22 and 7Æ13 ± 0Æ77 log CFU per membrane, respectively. The three-species model was employed to evaluate the efficacy of two antimicrobial dressings, Curity™ AMD and Acticoat™, compared to sterile gauze controls. Microbial growth on Curity™ AMD and gauze was not significantly different, for any species, whereas Acticoat™ was found to significantly reduce growth for all three species.
Conclusions: Using the colony-DFR, a three-species biofilm was successfully grown, and the biofilms displayed a unique structure consisting of distinct lay-ers that appeared to be inhabited exclusively or predominantly by a single species.
Significance and Impact of the Study: The primary accomplishment of this study was the isolation and growth of an obligate anaerobe in an in vitro model without establishing an artificially anaerobic environment.

Introduction

Chronic wounds such as diabetic foot ulcers, venous leg ulcers and pressure ulcers affect c. 6Æ5 million patients in the United States and represent $25 billion in annual healthcare costs (Sen et al. 2009). This burden is rapidly increasing owing to escalating healthcare costs, an ageing population and the rising incidence of diabetes and obesity (Sen et al. 2009). Chronic wounds are often open for a prolonged period of time (Wolcott and Rhoads 2008), which increases the possibility of bacterial infection. The wound bed is an ideal surface for bacterial growth (Falanga 2004), and the deleterious effects of microbial infection on wound healing have been recognized (James et al. 2008). Therefore, controlling and identifying wound bioburden are important aspects of wound management (James et al. 2008).
Micro-organisms colonize chronic wounds as biofilms, and despite the prevalence of biofilms in chronic wounds, their role in chronic wound pathogenesis is not well understood. To that end, many investigators have developed biofilm models to begin to elucidate the effects of biofilms on host tissue (Kirker et al. 2009b; Secor et al. 2011), the efficacy of antibiotics on biofilms (Wolcott et al. 2010; Hammond et al. 2011) or the effect of wound dressings on biofilm growth (Lipp et al. 2010) and viability

(Ammons *et al.* 2009; Thorn and Greenman 2009; Kostenko *et al.* 2010; Ammons *et al.* 2011a). To keep the complexity of the study to a minimum, these models were utilized with a single-species biofilm; however, most chronic wounds are colonized with multiple species of bacteria, including several anaerobic genera (Dowd *et al.* 2008a,b; James *et al.* 2008; Thomsen *et al.* 2010).

The literature is beginning to illustrate the importance of anaerobic bacteria within chronic wound pathogenic biofilms (Dowd *et al.* 2008a), but their identification is seldom achieved in traditional, clinical microbiological analysis owing to the difficulty of culturing strict anaerobes (Bowler *et al.* 2001). Furthermore, the isolation of strict anaerobes from chronic wound samples requires specialized techniques, equipment and environments to encourage anaerobiosis. Therefore, the investigation described herein was designed to isolate obligate anaerobes from chronic wound samples without establishing an artificially anaerobic environment, to establish an *in vitro*, polymicrobial, model wound biofilm capable of supporting bacteria with variable oxygen requirements and to demonstrate the utility of the established biofilm model.

Materials and methods

Anaerobic micro-organism isolation and identification

The development of the polymicrobial model began with the identification and isolation of anaerobic micro-organisms from chronic wound samples. Pressure ulcer (PU) wound specimens were collected from patients at the Southwest Regional Wound Care Center in Lubbock, TX under a protocol approved by the Montana State University and Western Institutional Review Boards. After collection, tissue samples were immediately placed in Port-A-Cul™ transport jars (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and shipped to the Center for Biofilm Engineering (CBE). The PU specimens received by the CBE were subsequently homogenized under anaerobic conditions, and the homogenate derived from each specimen was used to inoculate an individual channel of a drip-flow reactor (DFR) (Xu *et al.* 1998; Desrosiers *et al.* 2007). The DFR model provides a low shear and high gas transfer environment for growing biofilms on a microscope slide and is an ASTM E2647-08 Standard Test Method (Buckingham-Meyer *et al.* 2007). The DFR was maintained at 37°C and fed with 100%-strength BHI broth supplemented with 5% v/v adult bovine serum (ABS) at 5 ml h^{-1} per channel. After 3 days, the channels were reinoculated with the appropriate homogenate, and the DFR was operated for an additional 4 days for a total of 7 days.

After 7 days of growth, the biofilms were scraped into 10 ml of phosphate-buffered saline (PBS), vortexed for 30 s, sonicated for 2 min, followed by an additional 30 s of vortexing and plated on agar media, including BHI agar, anaerobe colistin-naladixic acid (CNA) agar with 5% sheep blood, anaerobe neomycin 5% sheep blood agar, phenylethyl alcohol agar with 5% sheep blood and Trypticase™ Soy Agar with 5% Sheep Blood (TSA II™) (all from Becton, Dickinson and Company). The plates were incubated anaerobically for 24 h to 2 weeks at 37°C using an anaerobic chamber with a BBL Gas Pak™ (Becton, Dickinson and Company). Unique colonies were then streaked on two blood agar plates, one of which was incubated anaerobically and one aerobically. Colonies demonstrating only anaerobic growth were isolated and identified to the species level using 16S-based molecular amplification.

DNA was extracted from the isolated colonies using MO-BIO Microbial DNA Isolation Kit (MO-BIO Laboratories Inc., Carlsbad, CA, USA). The DNA was PCR-amplified using 16S primers 8 F (3′-AGA GTT TGA TCC TGG CTC AG-5′) and 1492 R (3′-GGT TAC CTT GTT ACG ACT T-5′). Primer reactions and DNA amplification were performed using the following parameters: 94°C for 2 min, 15 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 45 s with a final extension step of 72°C for 7 min. Verification of the presence of DNA was assessed in 1·5% agarose gels. PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA, USA). Plasmids were extracted and isolated from selected white colonies on LB agar plates containing X-gal and ampicillin using the QIA-prep Spin Mini-prep Kit (Qiagen, Germantown, MD, USA). Plasmids were sent to Laragen, Inc (Culver City, CA, USA) for sequencing. Sequences returned to CBE were queried by a Basic Local Alignment Search Tool (Blast) search to identify bacterial species.

Cryopreservation of anaerobic bacteria

Frozen stocks of the isolated strict anaerobes were produced and maintained for further experimental use. Specifically, stock cultures were made using a nontraditional cryopreservation media. A planktonic culture (10 ml) of the isolated micro-organism was centrifuged at 3000 ***g*** for 10 min. The supernatant was then removed, while the precipitate was resuspended in 3 ml of sterile skim milk with 9% peptone and 0·45% thioglycollate. Aliquots (1 ml) of stock solutions were maintained at −70°C until a stationary-phase culture was required. For the experiments described below, stationary-phase cultures were obtained by adding 1 ml of the frozen stock to 10 ml of 100%-strength BHI broth and incubating anaerobically for 24 h at 37°C.

Development of three-species *in vitro* biofilm model

The model system used in this investigation, the colony-DFR, was developed to be a practical method of growing *in vitro* biofilms in a manner that more closely mimics a chronic wound environment (Lipp *et al.* 2010; Agostinho *et al.* 2011). The colony-DFR utilizes the standard DFR with a slide containing a 2·5 cm in diameter absorbent pad over which a 0·2-μm sterile polycarbonate membrane of the same dimension is placed. The membranes are inoculated and fresh, flowing nutrients are supplied. The nutrients are wicked upwards through the pad, feeding the micro-organisms on the membrane, and eventually producing a biofilm.

Isolating a clinical strain of *Clostridium perfringens* using the DFR provided a source of an anaerobic clinical isolate. Using clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and the *Cl. perfringens*, a three-species biofilm model that included a facultative anaerobe, a strict aerobe and strict anaerobe was attempted. MRSA and *Ps. aeruginosa* were obtained from a chronic wound using methods previously described (James *et al.* 2008) and in accordance with a protocol approved by the Montana State University Institutional Review Board. Frozen stocks of MRSA and *Ps. aeruginosa* were stored in tryptic soy broth (TSB) with 20% glycerol in 1 ml aliquots and stored at −70°C. For experiments described below, stationary-phase cultures were obtained by adding 1 ml of the frozen stock to 10 ml of 100%-strength Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company) and incubated for 24 h at 37°C.

To initiate the three-species biofilm growth, individual stationary-phase cultures of MRSA, *Ps. aeruginosa* and *Cl. perfringens* were made from frozen stocks as described. Next, 1 ml each of the MRSA and *Cl. perfringens* cultures were mixed with 1 ml of a 1 : 10 dilution of the *Ps. aeruginosa* culture. The membranes were then inoculated with 10 μl of the mixture and allowed to dry for 15 min. The reactor was then placed in a 37°C incubator and attached to the medium reservoir. The medium (100%-strength BHI broth supplemented with 5% v/v ABS) was pumped through the system at 5 ml h^{-1} per channel. After 3 days, each membrane and the associated biofilm were then either sampled for plate-count enumeration or cryopreserved for microscopy. Three-species biofilm growth was repeated multiple times using separate inoculum mixtures ($n = 7$).

Plate counting

Viable cell counts for each species within the original inoculum mixture and in the established biofilms were determined using selective agar. The inoculum mixture was serially diluted in PBS and drop-plated (Herigstad *et al.* 2001) on *Staphylococcus* Medium 110™, *Pseudomonas* Isolation Agar™ and SFP Agar Base™ with 370 μg ml^{-1} D-cycloserine (http://www.corn.org/wp-content/uploads/2009/12/VIII-A.pdf, 2010) (all from Becton, Dickinson and Company) to isolate and enumerate MRSA, *Ps. aeruginosa* and *Cl. perfringens*, respectively. The *Pseudomonas* Isolation and *Staphylococcus* Medium agar plates were incubated in a standard aerobic incubator, while the SFP agar plates were incubated anaerobically using described methods. All plates were incubated at 37°C for 24 h. Afterwards, the plates were counted, and the number of colony forming units (CFU) in the original inoculum mixture was calculated for each micro-organism.

After 3 days of growth, the membrane biofilms were aseptically placed into 50-ml conical vials containing 10 ml PBS, vortexed for 30 s, sonicated for 2 min and vortexed for an additional 30 s. The biofilm suspension was then serially diluted in PBS and drop-plated on selective agar as described. After 24 h, the plates were counted, and the number of CFU per membrane was calculated for each micro-organism.

Light microscopy

Microscopy was utilized to determine the location of each species within the biofilm. Imaging of the biofilms was accomplished by Gram-staining frozen biofilm sections. To begin, the membranes and their associated biofilms were embedded in Optimum Cutting Temperature (OCT; Sakura Finetek, Torrance, CA, USA), frozen on dry ice and stored at −70°C in preparation for cryosectioning. The samples were then sliced into 5 μm cross-sections using the Leica CM 1850 Cryostat (Leica, Wetzlar, Germany), placed on Superfrost Plus slides (Fisher Scientific, Pittsburg, PA, USA), stained using the Thermo Scientific Richard-Allan Scientific Gram Stain Tissue Kit (Fisher Scientific) and examined using a Nikon Eclipse E800 microscope with Nikon (Melveill, NY, USA) objectives. Images were taken with an Olympus Q-color 5 camera (Olympus, Center Valley, PA, USA). A minimum of two biofilm samples was analysed by light microscopy and representative images are presented.

Evaluation of dressings using the three-species biofilm model

The three-species biofilm model was used to evaluate the efficacy of wound dressings. Stationary-phase cultures of the individual three species were prepared and mixed as described. The membranes were inoculated with 10 μl of

the mixed-species solution, allowed to dry for 15 min and then a 2 cm × 2 cm piece of Curity™ AMD gauze, (Covidien, Mansfield, MA, USA), Acticoat™ Absorbent dressing (Smith & Nephew, London, UK), or sterile gauze (control dressing) was placed on top of the dried inoculum. The reactor was placed in a 37°C incubator and attached to the medium reservoir. The medium (100%-strength BHI broth supplemented with 5% v/v ABS) was pumped through the system at 5 ml h^{-1} per channel. After 3 days of growth, the membrane biofilm and the associated wound dressing were removed together, placed in 10 ml of Difco Dey/Engley (D/E) Neutralizing Broth™ (Becton, Dickinson and Company), disaggregated, diluted and plated on the three selective agars using the described methods. The number of CFU per sample for each micro-organism was then determined. A minimum of ten dressing samples of each type was tested.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analysis for significance was determined using an ANOVA with a Tukey's HSD *post hoc* test with $\alpha = 0{\cdot}5$ and $P \leq 0{\cdot}05$ considered to be significant.

Results

Anaerobic micro-organism isolation and identification

A unique approach to isolating anaerobic bacteria was taken in this investigation. A DFR was inoculated with wound tissue homogenates and supplied with nutrients to encourage natural flora of the specimen to propagate. After 3 days, biofilm growth was evident, and a second inoculation was performed in an effort to isolate anaerobic bacteria that might have required an established biofilm for integration. After 7 days of growth, gaseous, mucoid and multicoloured biofilms developed (Fig. 1a). The biofilms, which exhibited copious amounts of extracellular polymeric substance (EPS), grew to the size of the glass slide within the reactor (2·5 cm × 7·5 cm). Disaggregating and plating the biofilms on to several selective media led to the isolation of several, unique, anaerobic colonies. PCR amplification yielded long segments of DNA that covered conserved and variable regions of 16S rDNA and allowed for species-level identification of bacteria. The unique colonies were identified with 100% matching sequences as *Bacteroides thetaiotaomicron* (GenBank: AE015928.1), *Clostridium clostridioforme* (GenBank: HM008264.1), *Clostridium bolteae*, (GenBank: NR025567.1) and *Cl. perfringens* (GenBank: CP000246.1). Additional clones returned as uncharacterized *Bacteroides* spp.

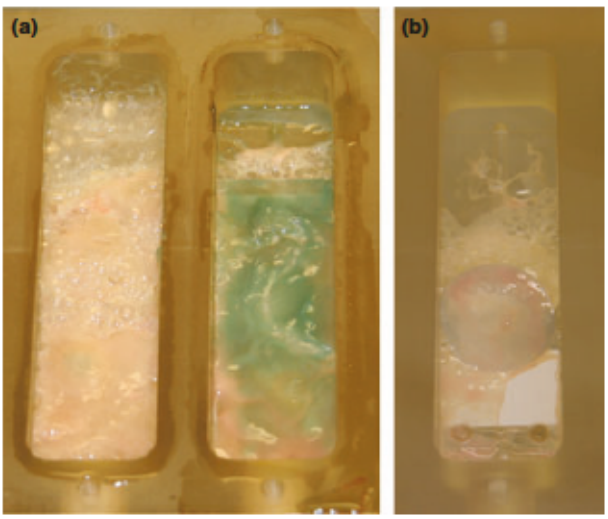

Figure 1 Images of multispecies biofilms. (a) Image of two chambers of a DFR inoculated with wound tissue homogenates and cultivated for 7 days. (b) Image of a three-species biofilm grown using the colony-DFR.

Three-species *in vitro* biofilm model

Clinical isolates of MRSA, *Ps. aeruginosa* and *Cl. perfringens* were chosen as the components of the three-species model for their different aerotolerances and common prevalence in chronic wounds (Bowler 1998; Bowler *et al.* 2001; Dowd *et al.* 2008a; Thomsen *et al.* 2010). The colony-DFR model was selected for the development of the three-species model because it more closely mimics the environmental conditions of the chronic wound by growing the biofilms at a liquid/solid/air interface (Fig. 1b). Furthermore, nutrients are wicked from below to feed the biofilm, much like a chronic wound.

The biofilms were inoculated with a solution containing a mixture of all three species. The mixture was comprised of 1 ml each of the MRSA and *Cl. perfringens* stationary-phase cultures, and a 1 ml of a 1 : 10 dilution of the *Ps. aeruginosa* stationary-phase culture. Previous investigations demonstrated that if the inoculum of *Ps. aeruginosa* was higher (undiluted in a 1 : 1 : 1 ratio with the other species), it resulted in a biofilm dominated by *Ps. aeruginosa* with little to no recovery of the other species (data not shown), an occurrence also observed by other investigators (Dalton *et al.* 2011). The experimental inoculum was 5·54 ± 0·18 log CFU per membrane for MRSA, 4·22 ± 0·79 log CFU per membrane for *Cl. perfringens* and 4·54 ± 0·03 log CFU per membrane for *Ps. aeruginosa* (Fig. 2). After inoculation, the biofilms were fed continuously for 72 h with 100%-strength BHI broth supplemented with 5% ABS at 5 ml h^{-1} per channel. Media formulas with a lower concentration of BHI (10 and

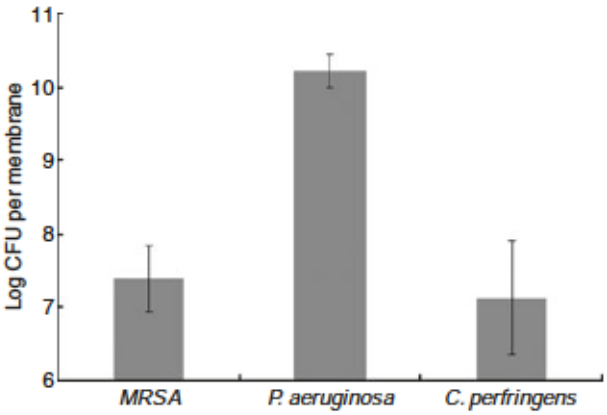

Figure 2 Log CFU per sample for the three-species biofilm growth on the colony-DFR. Data represented as mean ± SD.

50%-strength) proved to be insufficient for the growth of *Cl. perfringens* within the mixed-species biofilm. The resulting biofilms were slightly mucoid and with multiple gas bubbles (Fig. 1b). Drop-plate counts of the disaggregated biofilms allowed for the quantification of the number of viable cells of each bacterial species within the biofilm. MRSA grew to 7·39 ± 0·45 log CFU per membrane, and *Cl. perfringens* grew to 7·13 ± 0·77 log CFU per membrane. *Pseudomonas aeruginosa* consistently had the highest cell count with 10·22 ± 0·22 log CFU per membrane (Fig. 2).

Light microscopy

The location of each species within the biofilm was investigated using light microscopy. Transmitted light microscopy of Gram-stained cryosections revealed a number of distinct architectural features within the biofilms (Fig. 3a). The biofilms displayed a unique structure consisting of distinct layers that appeared to be inhabited exclusively or predominantly by a single species. The topmost layer (air interface) consisted of a layer that was spongy in appearance and appeared to be inhabited exclusively by Gram-negative bacilli consistent in appearance with *Ps. aeruginosa* (Fig. 3b). In the middle of the biofilm was a central and highly dense layer of putative extracellular polymeric substance (EPS) without distinguishable cells (Fig. 3c). A specific stain for EPS was not used. The deepest layer of the biofilm, where the biofilm grew on the membrane, seemed to be the interface

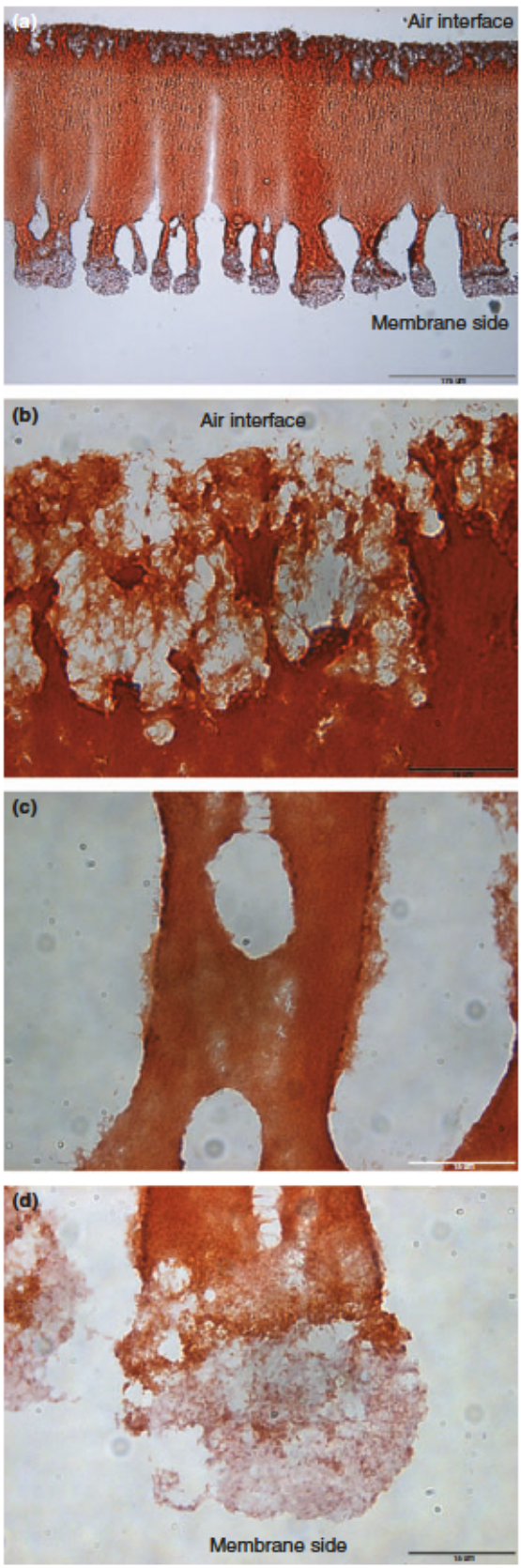

Figure 3 Light microscopy images of a three-species biofilm cross-sectioned and Gram-stained. (a) Full cross-section of three-species biofilm, scale bar = 175 μm; (b) Magnified view of the top of the biofilm, scale bar = 15 μm; (c) Magnified view of the middle of the biofilm, scale bar = 15 μm; (d) Magnified view of the bottom of the biofilm, scale bar = 15 μm.

between MRSA and *Cl. perfringens* (Fig. 3d). The facultative anaerobe MRSA was expected to be distributed throughout the biofilm. However, MRSA appeared to be located only in a discrete band near the bottom of the biofilm at an interface with *Cl. perfringens*.

The most striking microscopic features of the biofilms studied were the projections and associated interstitial voids found extending top to bottom within the biofilms. Such projections and interstitial voids were most prevalent in the thick middle portion of the biofilms, and *Cl. perfringens* was located on the bottom and lower sides of these projections (Fig. 3a–d).

Evaluation of dressings using the three-species biofilm model

The final objective for this study was to utilize the three-species model to test various wound treatments. Therefore, the efficacy of two types of antimicrobial dressings, Curity™ AMD and Acticoat™, against biofilm growth was evaluated and compared to a control dressing, gauze. After 3 days, only the Acticoat™ dressing was able to significantly prevent biofilm growth compared to the other dressings (Fig. 4). Despite inconsistent results of the Acticoat™ dressing for both *Staphylococcus aureus* and *Ps. aeruginosa*, the counts were still significantly lower than the Curity™ AMD dressing ($P < 0{\cdot}0001$) and gauze ($P < 0{\cdot}0001$). However, the Acticoat™ results were constant for *Cl. perfringens*, with no growth detected. This result was also significantly different from both the Curity™ AMD dressing and gauze ($P < 0{\cdot}0001$). The limit of detection in this experiment was one CFU ml^{-1}. After accounting for the initial dilution of the dressing sample and taking the log transformation, the limit of detection was 1 log CFU per sample. The results from the Curity™ AMD dressing were statistically equivalent to the gauze for all three micro-organisms: $P = 0{\cdot}963$ for MRSA, $P = 0{\cdot}9994$ for *Ps. aeruginosa* and $P = 0{\cdot}8313$ for *Cl. perfringens*.

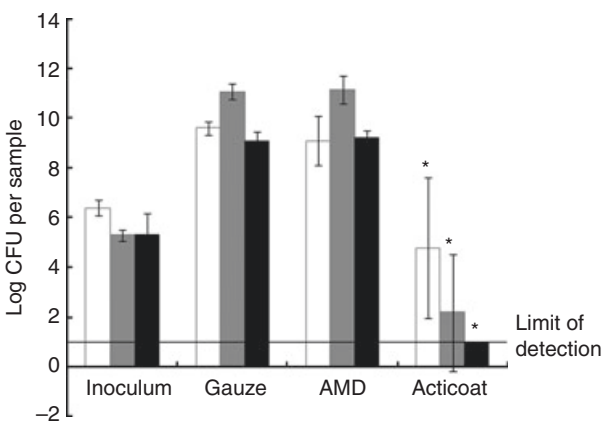

Figure 4 Log CFU per sample for the membrane/dressing pair. Counts for each species on each type of dressing are shown. Data represented as mean ± SD. *Significantly different from the other dressings at $P < 0{\cdot}0001$. (□) MRSA; (▩) *Pseudomonas aeruginosa* and (■) *Clostridium perfringens*.

Discussion

The primary accomplishment of the experiments described herein was the isolation and growth of an obligate anaerobe in an *in vitro* model without establishing an artificially anaerobic environment. The role of strict anaerobes has been overlooked in chronic wounds because they are difficult to culture, which is likely due to the need for specific environmental and nutritional conditions that are poorly understood and not provided with traditional culture methods. The utilization of the DFR in combination with wound homogenates allowed for the innate flora of the sample to thrive and to establish a biofilm. From those, gaseous, mucoid and multicoloured biofilms (Fig. 1a), a few strict anaerobes were isolated and identified: *Bacteroides fragilis*, *Bact. thetaiotaomicron*, *Cl. boltei* and *Cl. perfringens*. The ability to culture some pathogenic anaerobes without the use of anaerobic chambers represented not only an increase in convenience but also a more successful approach. Previous attempts to cultivate anaerobes from chronic wound specimens using standard microbiology techniques were unsuccessful.

Although wounds are often colonized by polymicrobial biofilm communities (Dowd *et al.* 2008a,b; James *et al.* 2008; Thomsen *et al.* 2010), much wound microbiology research has focussed on single species of bacteria. The second goal of this investigation was to develop a mixed-species biofilm composed of three species of bacteria, one facultative anaerobe, one aerobe and one strict anaerobe. The three micro-organisms selected for this investigation were clinical isolates of *Staph. aureus*, *Ps. aeruginosa* and *Cl. perfringens*. *Staphylococcus aureus* is a facultative anaerobe and is a predominant component of chronic wound biofilms (Bowler 1998; Dowd *et al.* 2008a; Thomsen *et al.* 2010). In addition, *Staph. aureus* has one of the highest incidence rates in traumatic, surgical, burn and other wound infections (Bowler *et al.* 2001). *Pseudomonas aeruginosa* is an obligate aerobe commonly found in chronic wound biofilms (Bowler 1998; Dowd *et al.* 2008a; Thomsen *et al.* 2010) and burn wounds (Rezaei *et al.* 2011). *Clostridium* spp. are common anaerobic wound pathogens (Dowd *et al.* 2008a), and *Cl. perfringens* is the most the common cause of gas gangrene infections.

Using the colony-DFR, a three-species biofilm was successfully established (Fig. 1b), and the presence of each micro-organism was verified using plate counts and microscopy. After 3 days of growth, *Ps. aeruginosa* was

consistently found to have the highest cell count, followed by MRSA and *Cl. perfringens* (Fig. 2). Microscopic analysis of the three-species biofilms demonstrated that each species appears to inhabit a distinct niche within the biofilm. The layer at the air interface was inhabited exclusively by *Ps. aeruginosa*, while the deepest layer of the biofilm, the side closest to the membrane, was the interface between MRSA and *Cl. perfringens* (Fig. 3a–d). Such observations suggested that the biofilms had discrete microenvironments, which accommodated the individual oxygen requirements of the three species. Microelectrode investigations have demonstrated that oxygen is only able to penetrate microns into biofilms, resulting in highly reduced microenvironments capable of harbouring strict anaerobes (Rasmussen and Lewandowski 1998). Therefore, in this model, oxygen consumption in upper layers of the biofilm by *Ps. aeruginosa* likely contributed to the establishment of anaerobic microenvironments found at the bottom of the biofilm, which were inhabited by MRSA and *Cl. perfringens*. The ability of *Cl. perfringens* to sporulate may have also contributed to its ability to survive at the ambient atmosphere until a reduced environment could be established. This survival pattern may also be relevant to chronic wound pathogenesis. Wounds initially infected with aerobic bacteria may form a biofilm of significant thickness to produce an anaerobic environment within the wound bed. The biofilm could then be secondarily infected with a strict anaerobe such as *Cl. perfringens*. Another remarkable feature of the model wound biofilm was the presence of interstitial voids that extended the entire thickness of the biofilm (Fig. 3a–d). The interstitial voids were likely formed by the production of gases by the *Cl. perfringens*, which is known to produce copious amounts of H_2 and CO_2 (Shimizu *et al.* 2002).

Finally, the three-species model was used to evaluate common wound dressings, a principal component of wound care. Many modern wound dressings possess a variety of attributes that are designed to create a supportive wound healing environment, including absorbing exudate, providing optimum moisture balance at the wound surface, preventing maceration of surrounding tissue and controlling bacterial colonization (Lipp *et al.* 2010). Recent *in vitro* studies illustrated that bacteria can grow unchallenged within a traditional dressing environment; however, an antimicrobial dressing can limit bacterial growth (Ammons *et al.* 2009; Kirker *et al.* 2009a; Lipp *et al.* 2010; Ammons *et al.* 2011a). The dressings in those investigations were challenged using only a single species, and most chronic wounds are contaminated with multiple species of bacteria (Dowd *et al.* 2008a; James *et al.* 2008; Thomsen *et al.* 2010). Therefore, in this investigation, two common antimicrobial dressings, Curity™ AMD gauze and Acticoat™ Absorbent, were challenged using the three-species biofilm model and compared to a traditional dressing, gauze.

Curity™ AMD gauze sponges are treated with 0·2% polyhexamethylene biguanide (PHMB). PHMB is a broad spectrum antimicrobial agent used in a variety of products, including contact lens cleaning solutions, skin disinfectant solutions and wound dressings. The antimicrobial activity of PHMB is attributed to its disruption of the bacterial cell wall; it reacts with acidic membrane lipids and induces aggregation, leading to increased membrane fluidity and permeability and eventual organism death (Kirker *et al.* 2009a). PHMB has also been reported to bind bacterial DNA, alter its transcription and cause lethal DNA damage (Allen *et al.* 2006). The efficacy of the PHMB dressings against Gram-positive and Gram-negative bacteria as well as yeast and fungi has been demonstrated in several *in vitro* and *in vivo* and studies (Cazzaniga *et al.* 2002; Lee *et al.* 2004; Motta *et al.* 2004; Gallant-Behm *et al.* 2005; Kirker *et al.* 2009b).

Acticoat™ Absorbent dressing is a calcium alginate dressing with a nanocrystalline silver coating. The biocidal activity of silver is attributed to the silver ion (Ag^+), which reacts strongly with molecules containing sulfur, nitrogen or oxygen (Schierholz *et al.* 1998). Upon reaction with the silver cation, the molecules are rendered nonfunctional, thereby depriving the bacteria of the necessary molecules and eventually leading to death. The silver cation also binds to DNA, preventing replication and transcription. The antimicrobial effects of silver have been used for centuries; however, only recently has silver been used in wound dressings, and its efficacy is summarized elsewhere (Leaper 2006; Vermeulen *et al.* 2007; Lo *et al.* 2009).

Populations within the three-species biofilms were found to have differing tolerances to the dressing treatments (Fig. 4). Acticoat™ was found to be effective against *Cl. perfringens*. In fact, no *Cl. perfringens* growth was detected in biofilms that were treated with Acticoat™. The Acticoat™ dressing consists of an absorbent alginate coated with nanocrystalline silver, and a number of phenomena could account for the potency of silver ions against biofilm *Cl. perfringens*. The low G+C content of *Cl. perfringens* DNA (28·6%) may make this organism particularly susceptible to the destabilizing effect of silver ions on DNA (Shimizu *et al.* 2002). It may also be possible that silver ions have a particularly deleterious effect upon enzymatic activity within *Cl. perfringens* (Guggenbichler *et al.* 1999). While not as consistent, Acticoat™ also had an inhibitory effect on *Staph. aureus* and *Ps. aeruginosa* as well (Fig. 4). This result is consistent with a previous investigation (Ammons *et al.* 2011b).

Surprisingly, the Curity™ AMD gauze and the gauze control performed equivalently (Fig. 4). Previous investigations demonstrated that PHMB-based antimicrobial

dressings successfully inhibited the growth of Gram-positive and Gram-negative bacteria as well as yeast and fungi (Cazzaniga *et al.* 2002; Lee *et al.* 2004; Motta *et al.* 2004; Gallant-Behm *et al.* 2005; Kirker *et al.* 2009b); however, these experiments were conducted with one individual species. Perhaps, the complexity of the three-species biofilm limited the efficacy of the PHMB.

Chronic wounds colonized with polymicrobial biofilms are especially tolerant to treatment. This may be due to varied microenvironments within the biofilm and the range of bacterial oxygen requirements. Additionally, the EPS of biofilms may provide a physical barrier to antimicrobials, mechanical removal and the immune response. To better understand the increased resistance of wound biofilms, it is imperative to conduct *in vitro* studies that simulate the conditions found within a chronic wound. This investigation describes the development of a polymicrobial biofilm wound model composed of microorganisms with different oxygen requirements. Such a model will allow for investigations into the complex interactions between the differing bacterial species, the different microenvironments and the varying antimicrobial tolerances found within chronic wound biofilms. The care of chronic wounds poses enormous material and patient costs. If a fraction of these cases stem from biofilm infection, identifying and understanding the wound bioburden may have a significant impact on wound care.

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