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Formation of *Propionibacterium acnes* biofilms on orthopaedic biomaterials and their susceptibility to antimicrobials

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Abstract

Failure to treat and eradicate prosthetic hip infection with systemic antibiotic regimens is usually due to the fact that the infection is associated with biofilm formation and that bacterial cells growing within a biofilm exhibit increased resistance to antimicrobial agents. In this in vitro study, we investigated the susceptibility of prosthetic hip *Propionibacterium acnes* and *Staphylococcus* spp. isolates growing within biofilms on polymethylmethacrylate (PMMA) bone cement to a range of antibiotics. All *P. acnes* isolates in the biofilm mode of growth demonstrated considerably greater resistance to cefamandole, ciprofloxacin and vancomycin. In contrast, only four of the eight *P. acnes* isolates demonstrated an increase in resistance to gentamicin. All ten *Staphylococcus* spp. isolates in the biofilm mode of growth exhibited large increases in resistance to gentamicin and cefamandole with eight of the ten isolates also exhibiting an increase in resistance to vancomycin. However, only three of the ten *Staphylococcus* spp. isolates exhibited an increase in resistance to ciprofloxacin. Biofilms were also formed on three different titanium alloys and on PMMA bone cement using *P. acnes*, *Staphylococcus epidermidis* and *Staphylococcus aureus* strains to determine if the underlying biomaterial surface had an effect on biofilm formation and the antimicrobial susceptibility of the bacteria growing within biofilms. Although differences in the rate at which the three strains adhered to the different biomaterials were apparent, no differences in biofilm antibiotic resistance between the biomaterials were observed. In the light of these results, it is important that the efficacy of other antibiotics against *P. acnes* and *Staphylococcus* spp. prosthetic hip isolates growing within biofilms on orthopaedic biomaterials be determined to ensure optimal treatment of orthopaedic implant infection.
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1. Introduction

Although total hip replacement has become commonplace in recent years, bacterial infection remains a significant complication following this procedure. In a recent study, in which we combined mild ultrasonication to dislodge the bacteria growing within adherent biofilms on the surface of the removed prosthesis with the use of strict anaerobic techniques, we cultured bacteria from 26 of 120 (22%) retrieved prostheses [1].

Sixteen of the 26 implants (62%) were infected by the anaerobic bacterium, *Propionibacterium acnes*, either as the single infecting organism (12 implants) or in combination with a Gram-positive coccus (four implants). The results of our study demonstrated for the first time that *P. acnes*, which when previously isolated had been disregarded as a skin contaminant, is associated with chronic low-grade infection of implanted biomaterials as frequently as *Staphylococcus* spp.

Attempts to treat prosthetic hip infections using systemic antibiotics usually fail as the infecting bacteria grow predominantly within a confluent biofilm on the surface of the prosthesis, rendering them resistant to currently employed antibiotics. Although previous studies have examined the antimicrobial susceptibility

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of *Staphylococcus epidermidis* [2,3] and *Staphylococcus aureus* [2,4,5] biofilms formed on orthopaedic biomaterials and determined the antimicrobial susceptibility of planktonically grown *P. acnes* strains isolated from retrieved orthopaedic implants [6], there is a lack of detail with regard to *P. acnes* biofilm formation. To date, no studies have examined either the formation of *P. acnes* biofilms on orthopaedic biomaterials or determined the antimicrobial susceptibility of *P. acnes* strains growing within biofilms.

In this study we investigated *P. acnes* and *Staphylococcus* spp. biofilm formation on polymethylmethacrylate (PMMA) bone cement and titanium alloys and determined the susceptibility of these biofilms to a range of antimicrobial agents.

2. Materials and methods

2.1. Bacterial strains

Fourteen of the 18 clinical prosthetic hip isolates used in this study (HJ1 to HJ14, Table 2) were isolated as described previously [1], with a further four *P. acnes* strains (L671, L149, L1958, CK77) which had also been isolated from prosthetic joints kindly supplied by Dr. Carl Kamme, University of Lund, Sweden.

2.2. Antimicrobial agents

The following antimicrobial agents were used: Gentamicin sulphate (Sigma Chemical Co. Dorset, UK); cefamandole naftate as Kefadol® (Dista Products Ltd, Basingstoke, UK); ciprofloxacin as Ciproxin® (Bayer plc, Newbury, UK) and vancomycin as Vancocin® (Eli Lilly and Company Ltd, Basingstoke, UK).

2.3. Biomaterials

Palacos R® PMMA bone cement (Schering-Plough, Herts, UK) was prepared in accordance with the manufacturer's instructions by mixing the powdered methylmethacrylate with the liquid monomer in a bowl using a spatula. The cement mixture was immediately placed between two glass plates covered with non-adhesive backing paper (Scotch Pak, 3M, France), which were pressed together to form a sheet of cement approximately 1 mm thick. Following hardening of the cement, $1 \, cm^2$ sections were cut with a sterile scalpel blade and stored under dark, sterile conditions at room temperature. Three different titanium–aluminium–vanadium (Ti–6Al–4 V) alloys with variable surface roughness were provided in the form of alloy discs (3.5 cm diameter) by Johnson and Johnson Medical, Ascot, UK. These were: titanium alloy 1 (TA1), 36 grit AlO_2 blast; titanium alloy 2 (TA2), 36 grit AlO_2 and wet ceramic bead blast; titanium alloy 3 (TA3), 24 grit AlO_2 and wet ceramic bead blast. TA2 was the smoothest of the three surfaces followed by TA1 and TA3 in order of increasing roughness.

2.4. In vitro development of bacterial biofilms

For comparison of biofilm formation on the four different biomaterials, one isolate from each of three major bacterial species which have been previously shown to cause prosthetic hip infection [1] (*P. acnes* [HJ 4], *S. epidermidis* [HJ 5] and *S. aureus* [HJ 9]) was selected. An overnight culture of the *P. acnes* strain which had been grown on anaerobic horse blood agar (ABA) at 37°C in an anaerobic chamber was suspended in pre-reduced cation-supplemented Mueller–Hinton broth (CSMHB, Unipath Ltd, Basingstoke, UK) at an inoculum density of approximately 1×10^8 cfu/ml. The inoculum for *S. epidermidis* and *S. aureus* was prepared by adding an aliquot (10 ml) of an overnight culture to fresh pre-warmed CSMHB (100 ml) and incubating at 37°C in an orbital incubator for 4–6 h until the exponential growth phase had been reached. This culture was then adjusted by spectrophotometric measurement to provide a final inoculum density of approximately 1×10^8 cfu/ml. Bacterial biofilms were formed by adding 5 and 25 ml of the final inoculum for each test strain to bottles containing PMMA sections and titanium alloy discs, respectively. Samples were subsequently incubated at 37°C for 0.5, 1, 2, 3, 4, 6, 8 or 18 h either aerobically (*S. epidermidis*, *S. aureus*) or anaerobically (*P. acnes*). Following biofilm formation the biomaterial samples were removed with sterile forceps and gently washed by the addition of sterile phosphate-buffered saline (PBS) three times to remove any non-adherent bacteria. The biomaterial samples were then placed in PBS and bacteria retained on the biomaterial dislodged by mild ultrasonication (5 min) in a 150 W ultrasonic bath operating at a nominal frequency of 50 Hz followed by rapid vortex mixing (30 s). Serial ten-fold dilutions were performed and viable counts estimated following the Miles and Misra drop plate count method [7]. After overnight incubation, the number of colony forming units on each biomaterial sample were counted and expressed relative to the surface area of the biomaterial sample (cfu/cm²). All experiments were performed in triplicate with three samples of each biomaterial at each time point and the mean values were calculated.

2.5. Bactericidal activity of antibacterials against biofilm-grown cells

To test the bactericidal activity of antibiotics against the biofilm cells grown on different biomaterial substrates, the PMMA bone cement and titanium alloy

samples were incubated with the three selected test isolates (*P. acnes* [HJ 4], *S. epidermidis* [HJ 5] and *S. aureus* [HJ 9]) used for the formation of biofilms described above at 37°C for 18 h. The samples were then removed, washed by the addition of sterile PBS three times to remove any non-adherent bacteria and transferred to a series of doubling dilutions of a given antibiotic (8–1024 µg/ml) in CSMHB and incubated at 37°C for 24 h. Antibiotic-free controls were included for each biomaterial. Following incubation, the biomaterial samples were removed, washed, sonicated and the total viable count following antibiotic treatment determined as described previously. Sessile minimum bactericidal concentrations (MBCs) were determined as the lowest concentration of antibiotic giving a 99.9% reduction in viable cells (cfu/cm^2) on a biomaterial compared to the number of cfu/cm^2 on the antibiotic-free control.

The bactericidal activity of the selected antibacterial agents against the remaining 15 prosthetic hip isolates was determined as described above, following the formation of biofilms using PMMA bone cement as the biomaterial substrate.

Planktonic MBCs of all the isolates had been determined previously following evaluation of the planktonic MICs using the broth microdilution method [6,8]. In brief, aliquots from wells in the microdilution trays were plated out and after overnight incubation the planktonic MBC was defined as the lowest antibiotic concentration that produced greater than 99.9% killing of the initial inoculum.

2.6. Statistical analysis

Statistical analysis was performed using two-way analysis of variance to determine if biomaterial surface and time had a significant effect on bacterial adherence. $P < 0.05$ denotes significance (Statview, Abacus Concepts Inc., CA, USA).

3. Results

Adherence of the three selected prosthetic hip isolates to the four different biomaterials are shown in Fig. 1. Analysis of variance of the results obtained showed that both biomaterial surface and time exerted a significant effect on adherence of the isolates to the biomaterials. Significant differences were observed in the adherence of *P. acnes* to each of the biomaterials. *P. acnes* adherence to TA1 was greater than to the other three biomaterials during the initial 3 h period. However, at 4 and 6 h, adherence to PMMA was greater than adherence to the other biomaterials but at 8 and 18 h adherence of *P. acnes* was again greatest to TA1. Significant differences were also observed in the adherence of *S. epidermidis* to each of the biomaterials with *S. epidermi-*

dis adherence to TA1 significantly greater than adherence to the other biomaterials after 18 h. In contrast to the results for *P. acnes* and *S. epidermidis*, where adherence to each of the biomaterials was significantly different, significant differences in adherence of *S. aureus* were only observed between PMMA and the titanium alloys with no differences in adherence observed between the titanium alloys.

The sessile MBCs for the three representative prosthetic hip isolates growing on the four different biomaterial surfaces are shown in Table 1. The planktonic MBCs determined previously [6,8] are also shown for comparative purposes. Differences in the biomaterial surface on which the bacteria were grown did not appear to have an effect on antibiotic resistance with all sessile MBC values for a given antibiotic and test isolate being similar to within one dilution for all four biomaterials.

The sessile MBCs of all 18 isolates grown on PMMA are shown in Table 2. The planktonic MBCs determined previously [6,8] are also shown for comparative purposes. Four of the eight *P. acnes* isolates tested showed no increase in resistance to gentamicin when in the biofilm mode of growth. The remaining four isolates showed either a two- or four-fold increase in resistance. In contrast, all of the *P. acnes* isolates in the biofilm mode of growth exhibited considerably greater resistance to cefamandole, ciprofloxacin and vancomycin. All 10 *Staphylococcus* spp. isolates tested were considerably more resistant to gentamicin and cefamandole when growing in a biofilm. Similarly, eight of the ten isolates also showed an increased resistance to vancomycin when growing in a biofilm. In contrast, seven of the ten isolates tested showed no increase in resistance to ciprofloxacin when growing in a biofilm.

4. Discussion

This study is the first to describe the formation of *P. acnes* biofilm on different orthopaedic biomaterials and to determine the susceptibility of biofilm-grown *P. acnes* to a range of antimicrobial agents. The three titanium alloys compared were of the same chemical composition, but all possessed a different surface finish and, therefore, a different surface roughness. The differences in surface finish of the alloys were based on different regions of the femoral component of a prosthetic hip, which are related to aiding tissue integration and the biocompatibility of the prosthesis. *P. acnes*, *S. epidermidis* and *S. aureus* strains that had been isolated from retrieved prosthetic hip implants formed biofilms on PMMA and on the three titanium alloys. With the exception of *S. aureus*, which adhered in similar numbers to each of the three titanium alloys, differences in the biomaterial surface did have an effect
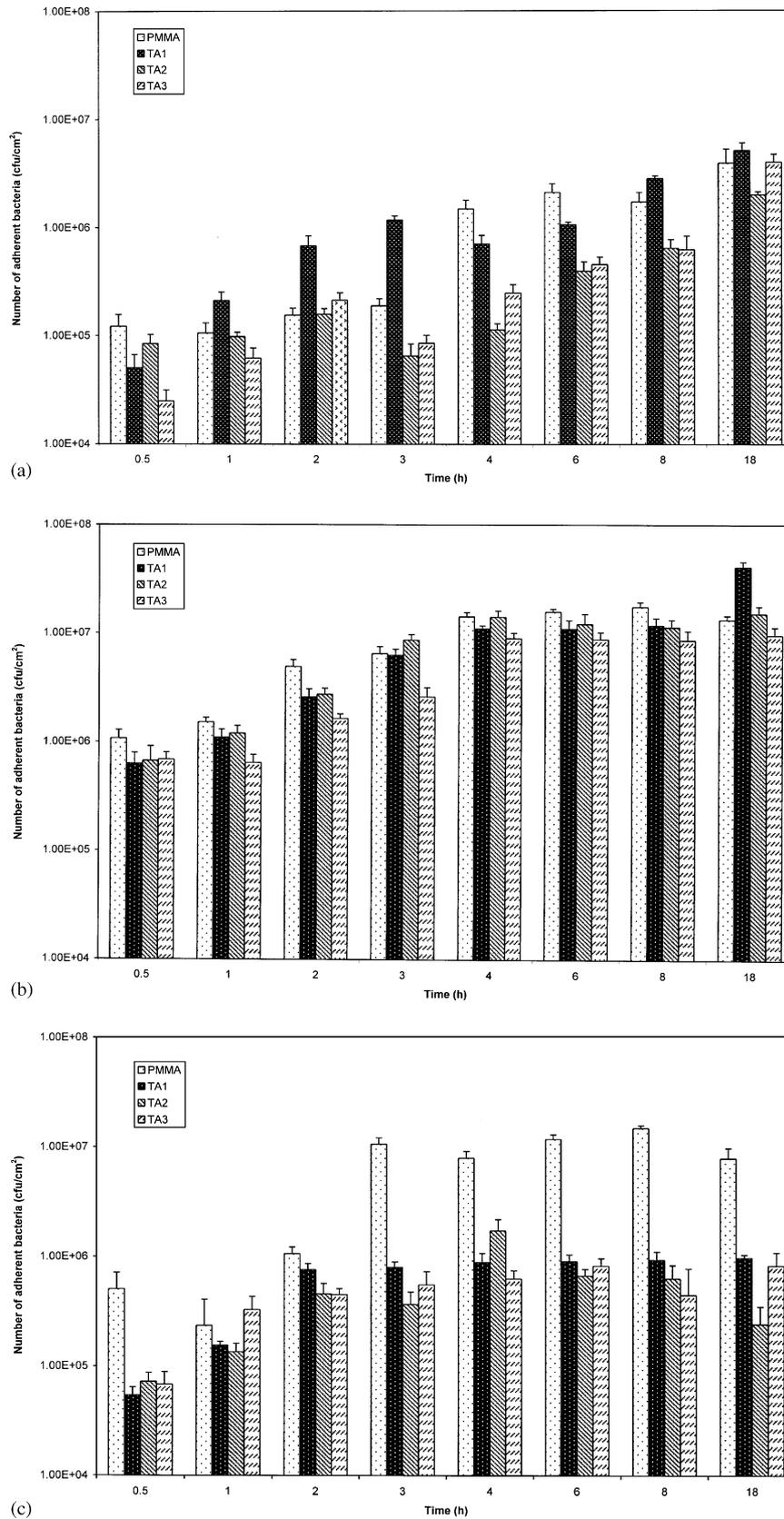
Fig. 1. Adherence of prosthetic hip isolates to biomaterials: (A) *P. acnes*; (B) *S. epidermidis*; and (C) *S. aureus*.

Table 1

Sessile minimum bactericidal concentrations of three prosthetic hip isolates grown on four different biomaterials

Strain and antibiotic	MBC[a] (µg/ml) for planktonic bacteria	MBC (µg/ml) for bacteria adherent to			
		PMMA	TA1	TA2	TA3
P. acnes (HJ 4)					
Gentamicin	16	32	16	16	32
Cefamandole	<0.5	>1024	>1024	>1024	>1024
Vancomycin	32	>1024	>1024	>1024	>1024
Ciprofloxacin	16	512	1024	1024	1024
S. epidermidis (HJ 5)					
Gentamicin	128	>1024	>1024	>1024	>1024
Cefamandole	1	1024	512	1024	512
Vancomycin	32	32	32	32	32
Ciprofloxacin	32	32	32	32	32
S. aureus (HJ 9)					
Gentamicin	32	64	64	64	64
Cefamandole	64	>1024	>1024	>1024	>1024
Vancomycin	16	>1024	>1024	>1024	>1024
Ciprofloxacin	8	256	512	512	512

[a] Minimum Bactericidal Concentration.

Table 2

Minimum bactericidal concentrations of prosthetic hip isolates grown on polymethylmethacrylate

Strain	Gentamicin		Cefamandole		Vancomycin		Ciprofloxacin	
	PMBC[a] (µg/ml)	SMBC[b] (µg/ml)	PMBC (µg/ml)	SMBC (µg/ml)	PMBC (µg/ml)	SMBC (µg/ml)	PMBC (µg/ml)	SMBC (µg/ml)
P. acnes strains								
HJ 1	32	32	1	>1024	32	>1024	8	512
HJ 2	32	32	<0.5	512	8	512	16	512
HJ 3	32	32	<0.5	>1024	16	>1024	16	256
HJ 4	16	32	<0.5	>1024	32	>1024	16	512
L671	32	128	4	>1024	8	>1024	16	512
L149	16	64	2	>1024	8	>1024	4	1024
L1958	32	64	1	256	1	>1024	8	512
CK77	32	32	1	>1024	32	>1024	4	512
S. epidermidis								
HJ 5	128	>1024	1	1024	32	32	32	32
HJ 6	1	128	1	64	16	>1024	16	16
HJ 7	32	>1024	128	>1024	32	1024	1	32
HJ 8	128	>1024	4	>1024	32	32	0.5	0.5
S. aureus (HJ 9)	32	64	64	>1024	16	>1024	8	256
S. hominis (HJ 10)	2	16	1	8	16	512	32	32
S. capitis (HJ 11)	64	256	16	64	32	64	16	16
S. haemolyticus (HJ 12)	256	512	64	>1024	1	>1024	4	4
S. sciuiri (HJ 13)	16	>1024	1	>1024	32	1024	32	64
Micrococcus sp. (HJ 14)	32	128	8	>1024	1	>1024	2	2

[a] Planktonic minimum bactericidal concentration.
[b] Sessile minimum bactericidal concentration.

on adherence of the isolates. These results are similar to the results of previous studies, which also reported differences in adherence of *S. epidermidis* and *S. aureus* to orthopaedic biomaterials [9,10]. Differences in both the adhesins expressed by the different bacteria and biomaterial surface characteristics such as surface energy, hydrophobicity, roughness and chemical composition can affect bacterial adherence to a biomaterial

and may be responsible for the differences in adherence described in this study. However, despite the fact that bacterial adherence to each of the biomaterial surfaces was significantly different, differences in biomaterial surface did not have an effect on the antimicrobial resistance of the three isolates growing within a biofilm, with few differences in sessile MBC apparent between different biomaterials. These results which indicate that once the initial biofilm has formed, the surface characteristics of the underlying biomaterial does not influence antibiotic resistance contrast with results from previous studies which reported that bacterial biofilm resistance to antibiotics was biomaterial specific [11–13].

The susceptibility of a number of additional *P. acnes* and *Staphylococcal* spp. prosthetic hip isolates growing in a biofilm on PMMA to a range of antimicrobial agents was also determined. The antibiotics studied were gentamicin, which is currently mixed with bone cement used for prosthesis fixation at the time of revision surgery, cefamandole, which is routinely used peroperatively during revision surgery and ciprofloxacin and vancomycin, which were shown to be most effective against the prosthetic hip isolates growing planktonically [6]. The high levels of gentamicin resistance determined previously [6,8] amongst planktonically grown *Staphylococcus* spp. strains may be due to the fact that gentamicin bone cement was used to fix in place the majority of implants from which the strains were isolated. Following the initial release of high levels of gentamicin from the bone cement, the subsequent low-level release of sub-inhibitory concentrations of gentamicin could have caused an increase in resistance to gentamicin amongst strains. In general, the results of this study which showed that bacteria growing within biofilms were highly resistant to the antibiotics tested are in accordance with results from previous studies examining the antimicrobial susceptibility of *S. aureus* and *S. epidermidis* biofilms [14,15]. All *Staphylococcus* spp. isolates tested exhibited large increases in resistance to gentamicin when growing in a biofilm. In contrast, biofilm-grown *P. acnes* exhibited either no or a minimal increase in resistance to gentamicin. Gentamicin resistance of sessile *Staphylococcus* spp. has also been previously observed by Chuard et al. [14] who demonstrated that sessile *S. aureus* isolates were considerably more resistant to gentamicin than the same isolates growing planktonically. Both the *P. acnes* and *Staphylococcus* spp. isolates demonstrated greatly increased resistance to cefamandole and vancomycin when growing in a biofilm. Previous studies have reported that vancomycin is unable to eradicate *S. epidermidis* growing within a biofilm [16] and suggested that this may be because as the drug enters the biofilm it is trapped in the glycocalyx and, therefore, does not reach the cell. Ciprofloxacin was the most effective antibiotic

tested against *Staphylococcus* spp. prosthetic hip isolates, with seven of the ten isolates tested showing no increase in resistance to ciprofloxacin when growing in a biofilm. Earlier studies have reported that ciprofloxacin can be used effectively to eradicate bacterial biofilms [17,18]. It may, therefore, be a suitable pre-, per- and post-operative systemic antibiotic, both for prophylaxis and in the direct treatment of prosthetic joint infection caused by *Staphylococcus* spp. However, eradication of established *P. acnes* biofilms with ciprofloxacin is more problematic with all of the *P. acnes* isolates showing an increase in resistance when growing in a biofilm. At present, there is insufficient information available regarding the antimicrobial susceptibility of planktonic and biofilm-grown *P. acnes* and further studies are required in this area.

In conclusion, this in vitro study has shown for the first time that *P. acnes* is capable of forming bacterial biofilms on orthopaedic biomaterials and that the antimicrobial susceptibility of representative prosthetic hip isolates growing within a biofilm is independent of the underlying biomaterial substrate. It has also shown that no single antibiotic is effective against both *P. acnes* and *Staphylococcus* spp. isolates, both of which have been shown to be causative pathogens in prosthetic hip infection [1]. Although ciprofloxacin was effective against biofilm-grown *Staphylococcus* spp. and gentamicin effective against biofilm-grown *P. acnes*, it is important that the efficacy of other antibiotics, for example rifampicin which has been shown to have good antistaphylococcal activity and penetrative properties, be determined to ensure optimal treatment of orthopaedic implant infection.

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