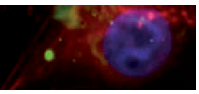
molecular oral microbiology



molecular oral microbiology

Perpetuation of subgingival biofilms in an *in vitro* model

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SUMMARY

This study evaluated the reproducibility of *in-vitro*-grown biofilms, initiated with subgingival plaque from patients with periodontal disease, and continued through several cycles by re-inoculating new biofilms from previously grown biofilms. Subgingival plaque samples from bleeding pockets along with saliva samples were collected from three patients with chronic periodontitis and perpetuated through seven cycles. Calcium hydroxyapatite disks were coated with sterilized saliva inoculated with dispersed subgingival plaque. The biofilms were grown anaerobically at 37°C for 10 days, and at specific intervals total viable bacteria were enumerated and the species present were analysed by DNA–DNA checkerboard hybridization. All cycles of biofilm growth occurred at similar rates and reached steady-state at day 7. No statistically or microbially significant differences were found for viable counts or species present, at the same period of maturation, among the different cycles. This study demonstrated that growth of certain target subgingival periodontal species in this biofilm model was reproducible and could be perpetuated *in vitro* through several cycles. The model could be useful in future studies to characterize different periodontopathogenic properties and biofilm interactions, especially in recolonization studies.

INTRODUCTION

The importance of understanding how bacteria relate and act within biofilms is essential for the proper management of periodontal diseases. It is known that biofilm-grown bacteria constitute a coordinated functional community that is more efficient than mixed populations of planktonic organisms (Costerton *et al.*, 1995). Biofilm-grown bacteria express an increased resistance to physical forces, nutrient deprivation, pH changes, oxygen radicals, antimicrobial agents, and immune defense systems (Jefferson, 2004). The complex structure associated with biofilms helps bacteria to survive in any environment, including the oral cavity. Many of the mechanisms involved are not well understood and require further investigation.

Different models of supragingival biofilms have been developed to understand biofilm properties. Guggenheim and colleagues (2001a, 2001b, 2004) described a multispecies biofilm model to study the composition, structure, and properties of supragingival plaque. Other authors have used different models, such as in-mouth splints (Wood *et al.*, 2000; Auschill *et al.*, 2001; Zaura-Arite *et al.*, 2001) or a constant-depth film fermenter using a plaque inoculum (Dibdin & Wimpenny, 1999), for the same purposes.

It is widely accepted that the development of periodontal disease is marked by an increase in species belonging to the orange and red complexes defined by Socransky *et al.* (1998). The study of subgingival biofilms is essential for the understanding of periodontal disease progression as well as for effective

treatment. Hope & Wilson (2006) have developed a model to study subgingival biofilm on hydroxyapatite (HA) disks in a constant-depth film fermenter using a plaque inoculum and described the structure of viable and non-viable subgingival biofilm composition. Walker & Sedlacek (2007) have developed a very simple, easy to grow, *in vitro* model of subgingival biofilm grown on HA disks in an anaerobic environment. This model supports the growth of the predominant species found in the subgingival environment both in health and disease and appears to be a feasible technique to study the complex subgingival community.

Important features of a biofilm model are its reproducibility and its survival so that its properties can be characterized accurately. In this model we have observed that the biofilm reaches maturity and a steady state of growth around day 7. However, by day 11 or 12, the biofilms started flaking off from some of the HA disks and from then on, the biofilm was no longer reproducible. Hence, to study ongoing biofilm properties, a new sample collection would have to be made for the growth of a new biofilm. As the composition and characteristics of the subgingival biofilm vary from person to person, from site to site, and often within the same site, over time, it would be difficult to standardize the growth of different biofilms even if collected from the same site. It is of extreme interest to be able to model both the consistency and the changes that occur in the subgingival plaque over time.

The purpose of this study was to evaluate the reproducibility and maintenance of *in-vitro*-grown biofilms initiated with subgingival plaque from patients with periodontal disease and continued through multiple cycles by re-inoculation from the previous biofilm. This would permit us to monitor changes that may occur and variations in the subgingival biofilm over extended time.

METHODS

Biofilm preparation

Sampling

Following written informed consent, microbial samples of subgingival plaque and saliva were collected from individuals with generalized chronic periodontitis. Criteria used for selection of the sampled sites included bleeding on probing, a pocket depth ≥5 mm, and an attachment loss ≥4 mm. Subgingival plaque was collected by inserting a sterile absorbent paper point (Henry Schein®, Melville, NY) to the depth of the sulcus and moving it laterally along the surface of the tooth and the sulcular epithelial lining for about 10 s. The paper point sample was immediately placed into a 1-ml aliquot of Amies (1967) transport medium, supplemented with 0.5% gelatin (Fisher Scientific, Ocala, FL) and 0.1% sodium thioglycollate (Fisher Scientific), and stored at 4°C for 2 h. Unstimulated saliva (5 ml) was collected and matched to the same subject as the plaque sample. The saliva was diluted 10-fold in sterile Ringer solution, centrifuged to remove particulate matter and filter-sterilized.

Biofilm development

Biofilms were established using sterile ceramic calcium hydroxyapatite (HA) disks, 5-mm diameter by 2 mm thickness, (Clarkson Chromatography Products, Williamsport, PA) as described by Walker & Sedlacek (2007). In brief, the HA disks were exposed to 10% sterile saliva for 2 h at room temperature, then placed in a tissue culture plate containing trypticase–soy broth (BBL®; Becton, Dickson and Co, Sparks, MD) supplemented with hemin and menadione. Each well was inoculated with 50 μl of sonically dispersed subgingival plaque and incubated in an anaerobic chamber (10% H_2, 5% CO_2, and balance N_2) at 37°C for up to 10 days with change to fresh medium at 48-h intervals.

Biofilm processing

Biofilm-containing disks were removed from the growth medium after 3, 5, 7, and 10 days of growth, and gently rinsed in sterile Ringer solution to remove loosely adherent bacteria. The disks were transferred to 1 ml pre-reduced, anaerobically-sterilized Ringer solution (Holdeman *et al.*, 1977), supplemented with 0.5% Tween-20 (Fisher Scientific), and gently sonicated to disrupt the biofilm matrix and disperse the bacterial cells as previously described (Walker & Sedlacek, 2007). Ten-fold dilutions were made in Ringer solution under anaerobic conditions, then plated onto trypticase–soy agar supplemented with 5% defibrinated sheep blood, 0.005% hemin, and 0.0005% menadione, and incubated anaerobically at 37°C for 5–7 days for total viable counts. Selected target species were detected and monitored by

'checkerboard' DNA–DNA hybridization as described by Socransky and colleagues (1994, 2004).

Pilot study on the re-inoculation period

A pilot study was carried out to verify which day of the initial biofilm growth would enable the best growth for the new biofilms. Plaque samples were collected as described previously from three patients with chronic periodontitis. Ten HA disks were inoculated for each patient. Biofilms were grown and colony-forming units (CFU) were counted as described above. One HA disk was taken out from each group at days 2, 5, and 10 and sonicated. Fifty microliters from each was then used to inoculate a new set of disks. This procedure was repeated in the same manner for the second set as well, totaling three sets of biofilm grown. The first set was grown from the initial plaque sample, the second set was inoculated from the first set, and the third set was inoculated from second set. An additional disk was taken from each of these sets of biofilms at days 5, 7, and 10, sonicated and plated as described previously for viable counts.

Perpetuation of biofilms

For the perpetuation of biofilms, plaque samples were collected from three distinct patients diagnosed with chronic periodontitis as described above. The scheme used for the perpetuation of biofilm growth is shown diagrammatically in Fig. 1. At day 3, a disk from the first biofilm set (set 1) was removed, placed in an aliquot of Ringers solution containing Tween-20, sonicated, and then 50 μl was used to inoculate the new set of disks (set 2), which was again grown for 10 days in anaerobic conditions. The same procedures were then followed for each new biofilm set through to set 7. The 10% saliva used initially to coat the first disks was reserved and frozen at −20°C. At day 3 of each set, the saliva was thawed and used to coat a new set of disks at room temperature for 2 h. The disks were transferred as before to fresh trypticase–soy broth in a tissue culture plate every 48 h. Again, an extra disk was removed at days 5, 7, and 10, sonicated, and plated for counts.

Checkerboard DNA–DNA hybridization

The bacterial strains used as DNA probes (Table 1) were grown planktonically in pre-reduced, anaerobically-sterilized peptone–yeast–glucose broth (Holdeman *et al.*, 1977) until reasonable turbidity (approximately 10^7 CFUs) was present. DNA from both planktonic and biofilm-grown cells was extracted using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI).

Detailed procedures for the checkerboard assay were reported previously (Walker & Sedlacek, 2007). Briefly, whole genomic DNA probes were labeled using the BrightStar® Psoralen-Biotin non-isotopic labeling kit (Ambion®, Austin, TX). Although different labeling reagents and buffers were used, the basic concept of Checkerboard DNA–DNA hybridization was performed as described by Socransky *et al.* (1994, 2004) and Wall-Manning *et al.* (2002). DNA

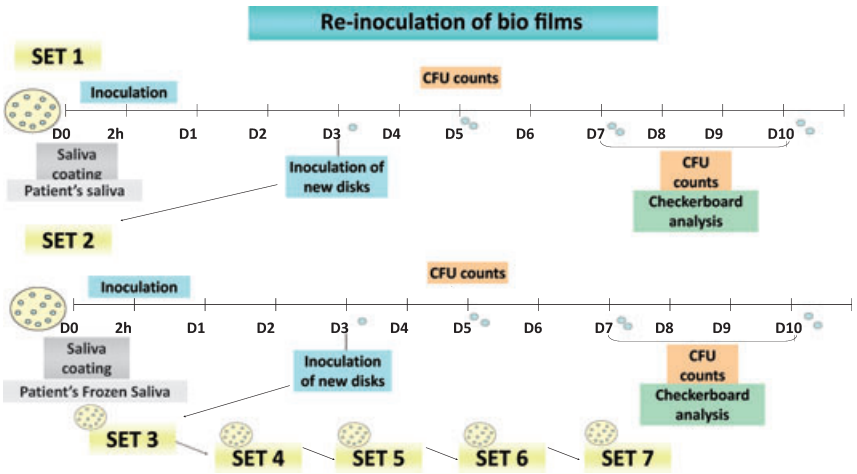

Figure 1 Diagram showing re-inoculation of different sets of biofilms. Small blue circles indicate hydroxyapatite disks that were removed from suspension for sonication and re-inoculations (day 3) and for colony forming unit (CFU) and checkerboard analysis (days 5, 7, and 10).

Table 1 Bacterial species and strain number used for construction of DNA probes

	Source/strain
Health-associated species	
Actinomyces oris	ATCC 12102
Streptococcus salivarius	ATCC 27945
Streptococcus sanguinis	ATCC 10556
Disease-associated species	
Aggregatibacter actinomycetemcomitans	ATCC 29523
Fusobacterium nucleatum ss *nucleatum*	ATCC 25586
Porphyromonas gingivalis	ATCC 33277
Tannerella forsythia	ATCC 43037

ATCC, American Type Culture Collection.

samples (500 ng in a total volume of 5 μl) were mixed with 45 μl sterile de-ionized water. The DNA samples and DNA standards were equivalent to 10^7, 10^6, 10^5, and 10^4 cells of the strains used as labeled probes. Samples were loaded and fixed onto a nylon membrane (Ambion) using a Minislot® Vacuum Manifold (Immunetics®, Cambridge, MA). The membrane was pre-hybridized and incubated for 2.5 h at 37°C. Five microliters of each DNA probe was mixed with 155 μl hybridization buffer, boiled for 5 min, and cooled on ice for 5 min. The membrane was then placed in a 45-channel Miniblotter® (Immunetics). The labeled DNA probes were applied to the membrane; the MiniBlotter, with the membrane sealed in a plastic bag, was incubated overnight at 42°C.

Detection was performed using the BrightStar® BioDetect® non-isotopic detection kit (Ambion). The membrane was then exposed to imaging film (X-OMAT, Eastman Kodak Co, Rochester, NY) overnight at room temperature. The resulting images were semi-quantified by comparing the different intensity values obtained for the four standards of each probe with the intensity value obtained for each sample, if present at detectable levels, using a standard curve (ChemiDoc XRS hardware and Quantity one, 4.4.1; Bio-Rad Laboratories, Inc., Hercules, CA).

Statistical analysis

Log numbers were transformed and the differences among biofilm sets either for total counts or for different species present were tested using either analysis of variance or its non-parametric version, the Kruskal–Wallis test (both for the pilot study and for the second perpetuation study). A $P \leq 0.05$ was considered statistically significant. For analysis of different species, a mean was calculated for the groups of disease and health species and this mean was compared among sets of biofilms.

RESULTS

Serial reinoculation

The results of the pilot study revealed that regardless of which day of growth was used for the re-inoculation (day 2, 5, or 10), the new biofilms would grow similarly and reach maturity (climax) and steady growth around the same time (Fig. 2). Statistical

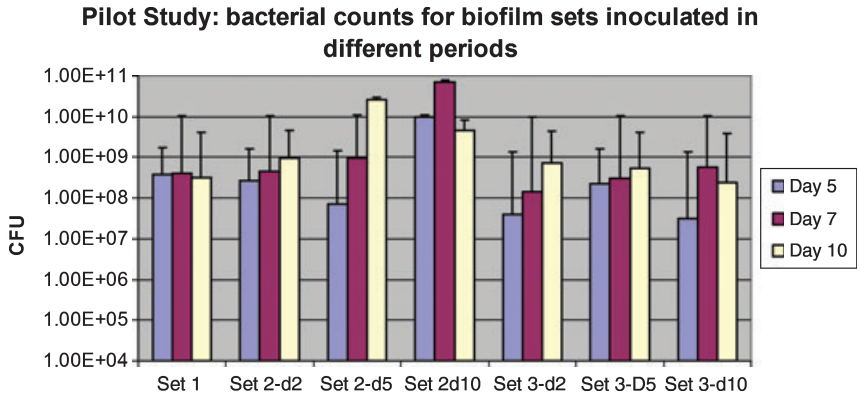

Figure 2 Mean colony-forming unit (CFU) counts (and standard deviation bars) showing different sets of biofilms grown from different inoculation days. On *x*-axis the day numbers following the different sets of biofilms indicate the inoculation day from the previous set (e.g. Set2-d2 indicates that set 2 was inoculated with day 2 biofilm from set 1; Set3-d5: indicates that set 3 was inoculated with day 5 biofilm from set 2; etc.). Statistical analysis (Kruskal–Wallis) showed no difference in CFU counts among same days of different sets ($P = 0.1813$).

analysis showed no difference among the sets ($P = 0.1813$). The CFUs (mean ± SD) are given in Fig. 3 for the three subjects at different days of growth for each of the seven sets. No statistically significant differences were detected among the sets for any particular day. Different biofilms were observed to show slightly different rates of growth up to day 7; however, these differences were not significant ($P = 0.224$ for day 5, $P = 0.891$ for day 7). The biofilms of all sets reached a steady state of similar growth at day 10 ($P = 0.461$) at a level of approximately 10^8 cells.

Serial re-inoculation study: analysis of health and diseased-related species

Fig. 4 shows means for disease-related species in different sets of subgingival biofilms. No significant differences were found among the different sets of biofilm for any of these species ($P > 0.05$). The target species detected were grouped into species generally associated with health (*Actinomyces oris*, *Streptococcus sanguinis*, and *Streptococcus salivarius*) or disease (*Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Aggregatibacter actinomycetemcomitans*). The total relative counts (mean ± SD) calculated for the species associated with health and disease are given in Fig. 5 for the seven sets of mature biofilms at day 10 of growth. There were no significant differences among the sets for species associated with either health or disease ($P > 0.05$).

DISCUSSION

This *in vitro* model of biofilms grown on HA disks (Walker & Sedlacek, 2007) is reproducible and the species are consistent with those *in vivo* (Haffajee & Socransky, 1994; Socransky & Haffajee, 2005). A limitation of the model was the longevity of the biofilm

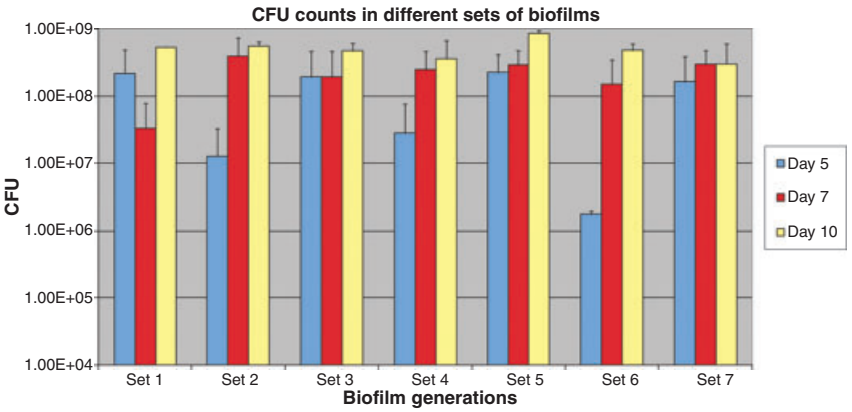

Figure 3 Mean colony-forming unit (CFU) counts and standard deviations for different maturity stages from seven sets of biofilms. Each set of hydroxyapatite disks containing biofilm was inoculated with 50 μl of biofilm from the previous set and grown for 10 days (set 1 was inoculated from initial patient sample). There were no differences when comparing the same days among the sets of biofilms using Kruskal–Wallis test ($P = 0.224$ for day 5, $P = 0.891$ for day 7, and $P = 0.461$ for day 10).

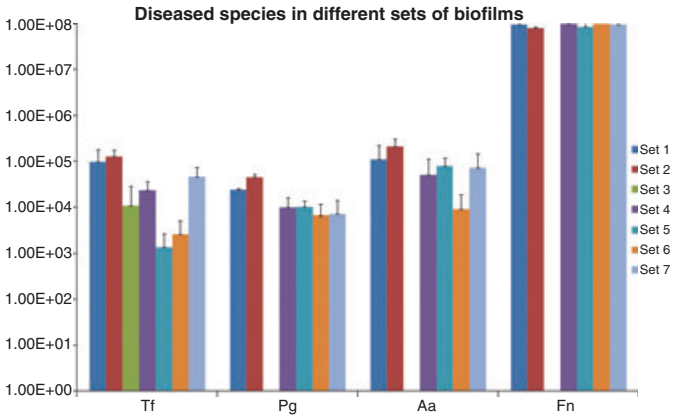

Figure 4 Mean relative counts and standard deviations for disease species analysed in the different sets of mature biofilms. No differences were observed among the sets for any of the disease species ($P > 0.05$). Tf, *Tannerella forsythia*; *Aa*, *Aggregatibacter actinomycetemcomitans*; *Pg*, *Porphyromonas gingivalis*; *Fn*, *Fusobacterium nucleatum*.

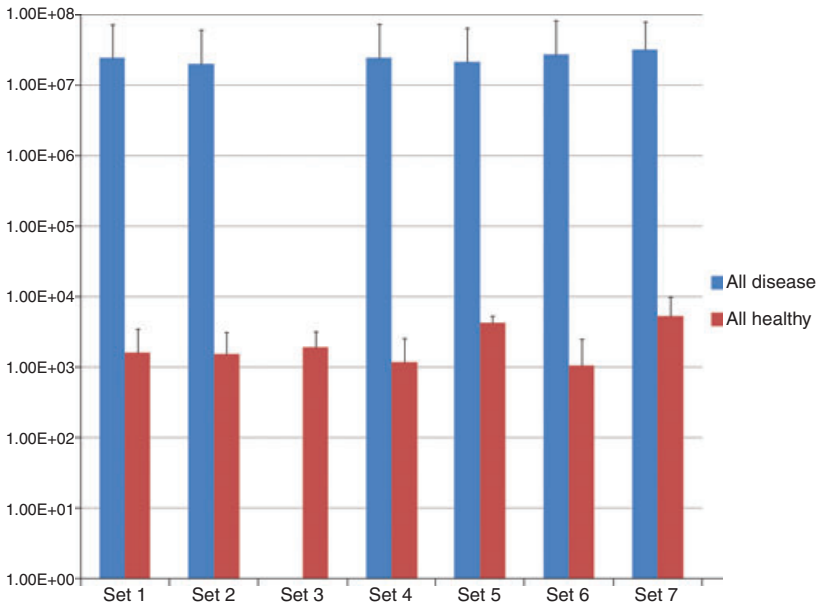

Figure 5 Mean relative counts and standard deviations for disease and health species in the different sets of mature biofilms (day 10). No differences were observed among the sets of disease-related ($P = 0.402$) or health-related ($P = 0.457$) species. Set 3 for disease-related species was excluded from analysis because three out of the four disease-related species analysed could not be quantified.

on the disks. Following biofilm peak maturity, some biofilms would begin detaching from the disks. This would normally happen between day 11 or 12. Some biofilms would separate from the disks a little earlier (day 9) and some would survive until day 13 or 14. Therefore, studies that would need survival of the same biofilm for longer periods would not be feasible, unless the same patient would provide their biofilm and saliva again for the inoculation of new disks This is not only difficult but also could introduce considerable variation into the proportions and composition of the subgingival sample because it is likely that the patient's health or disease status may change over time.

The present study was designed to determine the feasibility and to evaluate the reproducibility of the subgingival model through a series of biofilm cycles. The purpose behind this investigation was to determine if this model might be applicable to long-term studies involving the subgingival microbiota associated with disease processes. It has been previously reported that saliva from the same source as the plaque sample was necessary to obtain maximum biofilm formation and growth (Walker & Sedlacek, 2007). Often biofilms either failed to form or else failed to reach climax phase if the saliva was from a different donor. In this study, the 10% filter-sterilized saliva was reserved and stored at −20°C in individual aliquots. This saliva was then used to coat additional HA disks to perpetuate the biofilms through a total of seven cycles. This allowed us to test for differences in the CFUs obtained and in the proportion of the target bacteria present in each of the seven biofilm sets. The results of this study showed that the different sets of biofilm reached peak levels around the same time (day 7) and the proportions of health-related or disease-related species evaluated were very similar among the different generations. Some sets of biofilms showed somewhat slower growth than others (observed around day 5 in Fig. 2). However, the differences were not statistically and microbiologically significant because they all reached similar peak levels on counts and proportions of species at around day 7.

A possible limitation of this study was the evaluation of only a few species with the checkerboard. This technique allowed verification of up to 40 species. Other methods, such as the 16S ribosomal DNA probes, can also verify uncultivable species that might be present. In addition, the semi-quantification method used here only allowed us to have some standards for comparison (minimum 10^4 and maximum 10^7). However, it provided an idea of relative amounts of a variety of species within the biofilms. A better technique for quantification of bacteria would be the use of real-time polymerase chain reaction or 16S ribosomal DNA species-specific and universal probes. Another limitation of this methodology would be possible mutations developed by the microorganisms in prolonged *in vitro* growth.

Walker and Sedlacek (2007) demonstrated that saliva from different individuals may result in a distinct biofilm growth and the percentage of recovered viable bacteria was <10% when compared with biofilm grown with saliva from the same individual. In the present study therefore we used diluted filter-sterilized saliva from the same patient for all sets of biofilms. The thawing of saliva seemed not to be a problem for the attachment and growth of new biofilms. However, to grow biofilms for longer periods of time, larger amounts of saliva would be needed. We intend to evaluate whether there are significant differences between biofilm growth with saliva from the same patient and artificial saliva to check the feasibility of longer diseased biofilm maintenance.

Conclusion

The present study demonstrated that the growth of certain subgingival periodontal species in an *in vitro* biofilm model is relatively reproducible and can be continued *in vitro* for at least seven generations. This means that a standardized inoculum consisting of a complex bacterial population in similar proportions can be perpetuated *in vitro* for at least 70 days. This model could be useful in future studies to characterize host response, different periodontopathogenic properties, and biofilm interactions.

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