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BIOFILMS IN DENTAL UNIT WATERLINES: ULTRASTRUCTURAL AND CYTOCHEMICAL ANALYSIS

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Abstract

Dental unit waterlines are heavily colonized by bacteria which contaminate the water used to perform intraoral, and sometimes invasive, procedures. There is little information on the nature of the biofilm colonizing the flexible plastic tubing used to supply water to the different handpieces. We have therefore undertaken quantitative microbial analysis and ultrastructural studies of these biofilms by direct counting with the epifluorescent filter technique and by transmission and scanning electron microscopy after staining with ruthenium red and periodic acid-thiosemicarbazide-silver proteinate to visualize the composition and the distribution of exopolysaccharides and intracellular polysaccharides. The biofilm was revealed as a non-uniform structure characterized by an uneven cell distribution in an extracellular matrix. The microbial community of the biofilm was composed of **metabolically active** Gram-negative, short rod and coccoid bacteria that formed microcolonies embedded in a multilayer exopolysaccharides envelope. Our results indicate that even though the biomass of dental unit waterlines biofilms increases with time there is no direct correlation with the concentration of free floating bacteria in the water. It can be concluded that the biofilm structure described herein is responsible for the high level of bacteria in some medical devices. The consequences are of clinical significance as it is known that this extracellular material limits the action of biocides and host defense.

Key Words: Dental unit waterlines, biofilm, ultrastructure.

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Introduction

The formation of biofilms on surfaces can be regarded as a universal bacterial strategy for survival and for optimal positioning with regard to available nutrients (Costerton *et al.*, 1987). In addition, biofilm bacteria are substantially protected from surfactants, biocides and antibiotics (Govan, 1975; Ruseska *et al.*, 1982). Although the mechanisms of this resistance are poorly understood, exopolysaccharides (EPS) are likely to play a role (Hoyle *et al.*, 1990). The matrix formed by EPS of a biofilm also serve to trap diluted nutrients necessary for microbial metabolism (Costerton *et al.*, 1987). It has also been proposed that biofilms could allow the multiplication of microbial pathogens stochastically present in freshwater, as well as providing a mechanism for bioaccumulation of toxic substances (Carpentier and Cerf, 1993; Lyttle and Bowden, 1993; Wolfaardt *et al.*, 1994). The importance of EPS matrix has been emphasized by studies on *Pseudomonas aeruginosa* biofilms where the polyanionic matrix is seen as an ion exchanger that conditions the attack of antibacterial molecules on biofilm bacteria (Costerton *et al.*, 1990).

The planktonic (free floating) population is normally used to quantify the level of contamination of dental unit waterlines as well as to monitor the efficacy of disinfection protocols (Furuhashi and Miyamae, 1985; Mills *et al.*, 1986). Treatment of dental unit waterlines with traditional concentrations of biocides kills planktonic organisms while leaving the biofilm populations (sessile) virtually unaffected, thus giving the false appearance of successful disinfection (Cabot *et al.*, 1971; Fiehn and Henriksen, 1988; Kelstrup *et al.*, 1977). To be effective, it is advantageous to design disinfection protocols based on an understanding of the attachment process and the composition of EPS subsequent to attachment (Hernandez-Mena and Friend, 1993).

Since the first report by Blake (1963) about the microbiological status of dental unit waterlines, there have been many studies on the microbial contamination of waterlines and their outlet water used to perform intraoral treatments (Cabot Abel *et al.*, 1971; Fitzgibbon *et al.*,

1984; Kelstrup *et al.*, 1977; McEntegart and Clark, 1993). The main concern, however, has almost invariably been centered on the concentration of bacterial cells in the water. Bacterial counts can reach levels of up to 10^6 cfu/ml (Barbeau *et al.*, 1996; Robert *et al.*, 1994), which far outnumbers the accepted standard concentration of 500 cfu/ml for potable water (Geldreich, 1986). There seems to be a universal consensus that all dental unit waterlines, even those using sterile water, are heavily contaminated by bacteria. Although yeast cells, algae and protozoa were inconsistently reported, bacteria made up the majority of the inhabitants of dental unit waterlines. Microorganisms identified in dental unit water systems belong to the normal aquatic and soil microbial life forms (Barbeau *et al.*, 1996; Cabot Abel *et al.*, 1971; Fitzgibbon *et al.*, 1984; Kelstrup *et al.*, 1977). However, some opportunistic pathogens like *Pseudomonas aeruginosa* and *Legionella pneumophila* were frequently isolated (Oppenheim *et al.*, 1987).

It has been shown that there is a direct link between fixed bacterial communities and the free microbial counterpart surrounding them (Costerton *et al.*, 1994a), and indeed the microbial composition of the environment is the reflection of the fixed communities that act as reservoirs. In this context, knowledge of the organisation and composition of bacterial communities is crucial to the understanding of these ecosystems. The ecosystems of dental unit waterlines are poorly defined because little is known about the structure of the fixed microbial community and the link between the biofilm and the water contamination (Shearer, 1996). Indeed, the formation of biofilms is the primary culprit in the colonization of dental unit waterlines, and thus a better knowledge of its structure and composition is needed in order to avoid and eliminate this type of contamination. We used the direct epifluorescent filter technique to evaluate the microbial concentration in dental unit waterlines biofilms, and transmission (TEM) and scanning (SEM) electron microscopy following application of histochemical techniques to identify EPS and intracellular polysaccharides (IPS), and their distribution, within the dental unit waterlines biofilms. In SEM, the secondary electron imaging mode is used to reveal the three-dimensional features of the specimens, whereas the backscattered electron imaging mode, coupled with ruthenium red staining, is used to provide information about composition.

Material and Methods

Specimen collection

The specimens for this study comprised sections of tubing (polyurethane) and water from 28 different waterlines (ten 30 year-old, fourteen one year-old, and four 10-25 day-old, dental unit waterlines) located in the Faculty of Dentistry at Université de Montréal. At the beginning of the work day, 4 ml water samples were collected from each of the waterlines. Pieces of tubing, measuring 4 to 5 cm, were then sectioned at the end of the lines where the air and water syringe had been connected. The part directly connected to the handpiece was discarded. The samples were divided into two groups. For TEM and SEM observations, several pieces of 5 mm were cut longitudinally with a sterile scalpel to expose the biofilms and then processed as described below. For bacterial counts, 2 cm pieces of tubing were cut longitudinally into two pieces and placed in test tubes containing 2 ml of sterile water and processed as described below.

Bacterial counts

Pieces of tubing were treated in an ultrasonic bath (model 8890, Cole-Parmer, Montreal, Canada) at 47 kHz for five minutes to disrupt the biofilm. The resulting suspension was then transferred aseptically in a sterile test tube. Water samples and biofilm suspensions were then processed in the same manner for direct counts according to the method of Hobbie *et al.* (1977). Briefly, 0.2 ml of sterile 0.1% acridine orange solution in water was mixed with 1.8 ml of the samples and incubated at room temperature for two minutes. The samples were then filtered through a sterile polycarbonate black filter (13 mm diameter, 0.2 μm-pore size, Millipore Corp., Bedford, MA, USA) using a sterile 5 ml syringe. The filter was then placed on a microscope slide, and immersion oil and a coverslip was placed on top. Filters were observed at 1000X with a Zeiss Axiophot (Carl Zeiss, Thornwood, NY, USA) equipped for epifluorescence optics. Four fields were examined on each filter and microbial morphotypes and counts were recorded using a micrometric reticule. Biofilm samples were placed on a microscope slide and stained for Gram reaction and acidfast bacteria by the Ziehl-Neelsen procedure (Bartholomew, 1981).

Capsule staining

Water samples taken from the outlet of dental units (2 ml) and nearby tap (50 ml) were concentrated 100X by centrifugation (12,700 X g for 5 minutes). Samples were collected at the beginning of the work day before the dental unit was used. Biofilm suspensions were prepared as stated above. The negative capsule stain of White (Bartholomew, 1981) was used. Briefly, an emulsion of the samples was made in a mixture of Congo red (5%) and horse serum (11%), spread as a thin film on a slide, and dried with gentle warmth. The slides were flood with 0.5% HCl, dried and counterstained with acidulated 1% methylene blue. Slides were observed at 1000X and different fields were photographed using the

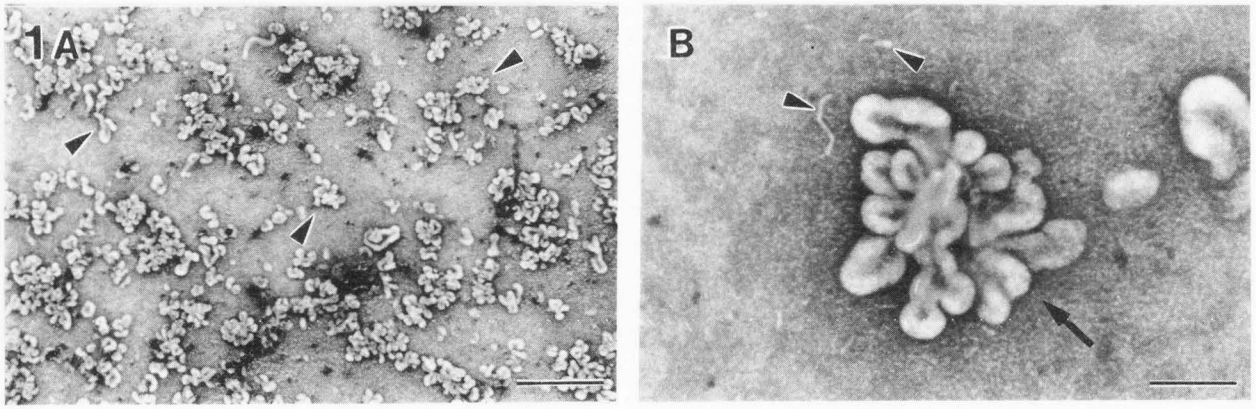

Figure 1. (A) Individual and small clusters of bacteria recovered directly from dental unit waterlines' effluent are covered by a thick glycocalyx (arrowheads). These were never observed in tap water samples. Bar = 10 μm. (B) High magnification of a cluster (arrow). Negative bacteria can be seen in the background (arrowheads). Negative staining with Congo red. Bar = 1 μm.

Nikon Labophot/F-601M (Nikon, Montréal, Canada). Data were recorded qualitatively as absence or presence of capsule. *Klebsiella pneumonia* (ATCC 13883) was used as a positive control.

Electron microscopy

Specimens were prefixed for 30 minutes in 0.2% glutaraldehyde and 0.15% ruthenium red (RR) in 0.1 M cacodylate buffer at pH 7.4, followed by a two hour fixation in 1% glutaraldehyde with 0.05% RR in the same buffer. After several buffer washes, all samples were post-fixed in 1% osmium tetroxide and 0.5% RR for one hour, then processed for SEM or TEM.

Morphological analysis

Scanning electron microscopy (SEM) Following fixation, post-fixation, and dehydration, samples were critical point dried with carbon dioxide in a Balzers CPD 030 critical point dryer (Balzers, Furstentum, Liechtenstein), then mounted with a conductive carbon paint on aluminum stubs and sputtered with gold, or carbon-evaporated in a Bal-Tec MED 020 high vacuum coating system (Bal-Tec Products Inc., Middlebury, CT, USA). The interior of tubing segments were examined with a field emission JEOL JSM 6300F SEM (JEOL, Peabody, MA, USA) operated at an accelerating voltage of 15 kV. The gold-coated specimens were used for the secondary electron imaging (SEI) mode while the carbon-coated ones were visualized by backscattered electron imaging (BEI) mode.

Transmission electron microscopy (TEM) Samples were dehydrated in graded concentrations of ethanol and embedded in LR White resin (Marivac, Halifax, NS, Canada), and polymerized at 58°C. Thin sections of the tubing were cut with a diamond knife on a Reichert Ultracut E ultramicrotome ((Leica Canada Inc., Ontario, Canada), recovered on formvar and carbon-coated nickel or gold grids, and processed for histochemical staining as described below. Formvar-coated grids were used to improve the stability of thin sections. Sections were examined with a JEOL JEM-1200 EX TEM operated at an accelerating voltage of 60 kV.

Histochemical procedure Gold grid-mounted sections were treated with 1% periodic acid for one hour, rinsed in distilled water, then reacted for one hour with a solution of 2% thiocarbohydrazide in 20% acetic acid. Sections were then floated for 20 minutes each on drops of 20%, 10%, 5%, and 1% acetic acid solution. The grids were thoroughly rinsed, exposed for 30 minutes in the dark to 1% silver proteinate solution, then washed in distilled water. As controls, the grids were not incubated with the periodic acid oxidation step (Courtoy and Simar, 1974).

Results

Bacterial counts and morphotypes

Observation of biofilm suspensions with the direct epifluorescent filter technique gave excellent results for both morphological analyses and microbial counts. The biofilm suspensions were mainly composed of small coccoid to short rod bacteria and a few filamentous microorganisms. This is in sharp contrast to the morphotypes observed in water samples where the microorganisms were notably larger and mainly composed of rod-shaped bacteria and filamentous microorganisms. The density of microorganisms over the surface of old waterlines (older than 30 years-old) was significantly higher ($6.9 \times 10^7 \pm 1.5 \times 10^7$ microorganisms/cm^2) than in those isolated from waterlines installed within a year ($8.7 \times 10^6 \pm 8.5 \times 10^6$ microorganisms/cm^2). This difference

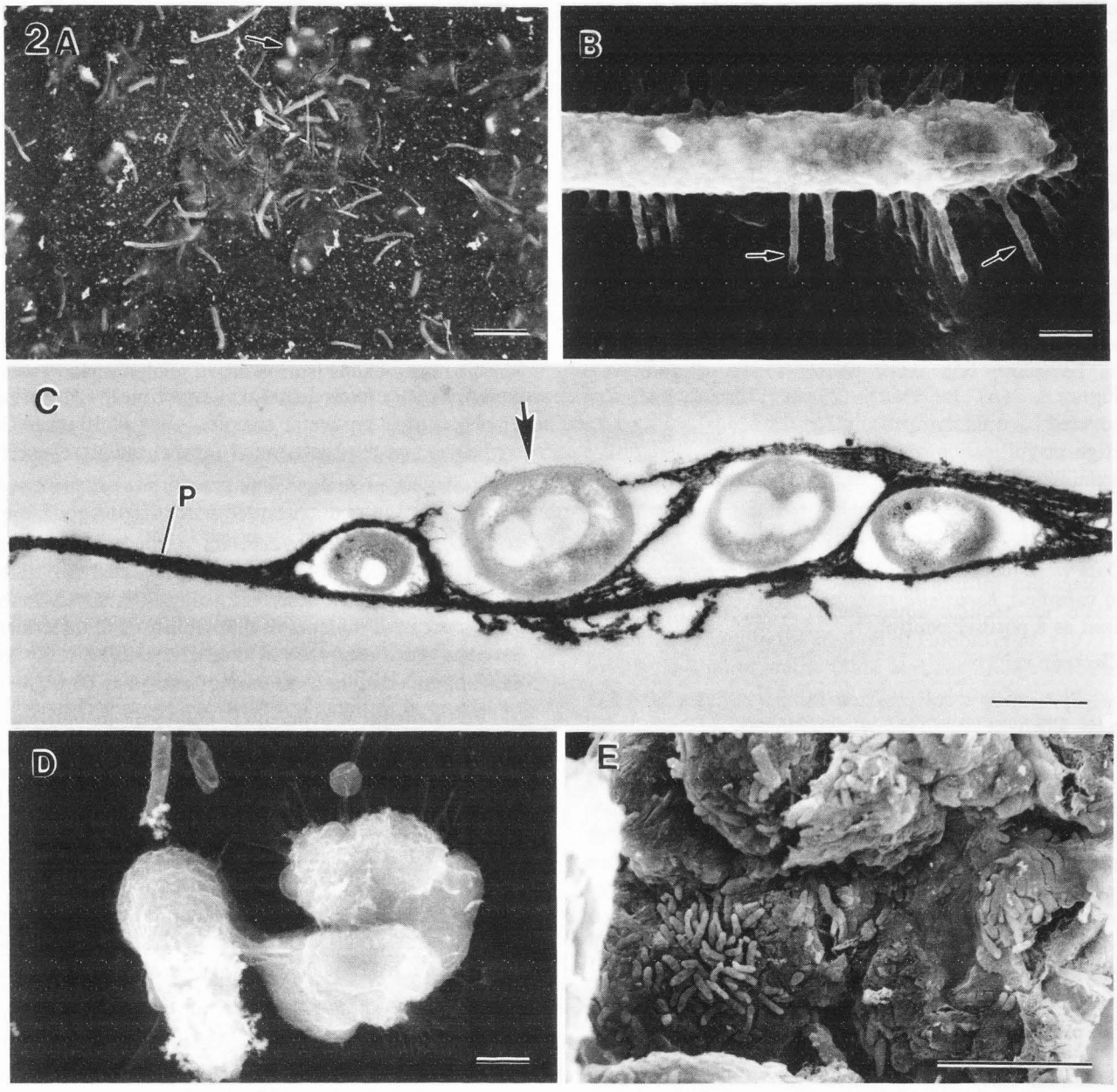

Figure 2. **(A)** Scanning electron micrograph (SEI mode) of the lumenal aspect of a 13-day-old biofilm lining obtained from the tubing of a dental unit waterline showing mainly rod-shaped bacteria. Bacteria embedded in an extracellular matrix can also be seen (arrow). Bar = 10 μm. **(B)** In some bacteria, elements of the glycocalyx appear as small, fibrillar appendages (arrows) from the cell surface (BEI mode). Bar = 0.2 μm. **(C)** Transmission electron micrograph of bacteria (arrow) from the surface of a tubing and held together by a thin layer of exopolysaccharides (P). Bar = 0.5 μm. **(D)** Small groups of bacteria are sometimes trapped under a sheet of anionic material stained with RR and visualized by BEI. Bar = 1 μm. **(E)** Scanning electron micrograph (SEI mode) of a 30 year-old biofilm showing clusters of rod-shaped bacteria enclosed in a abundant polysaccharide matrix. Bar = 1 μm.

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was not correlated with the mean number of microorganisms/ml of water which showed no difference between old and new waterlines (141 X 10^6 $\pm$ 15 X 10^6 and 10 X 10^6 $\pm$ 7 X 10^6, respectively). The majority of microorganisms present in these biofilms were Gram-negative bacilli. Unidentified protozoa and yeast-like microorganisms were observed in biofilm suspensions and represented less than 0.1% of the microorganisms observed, regardless of the age of the waterlines. Acidfast bacilli (after Zielh-Neelsen staining on biofilm

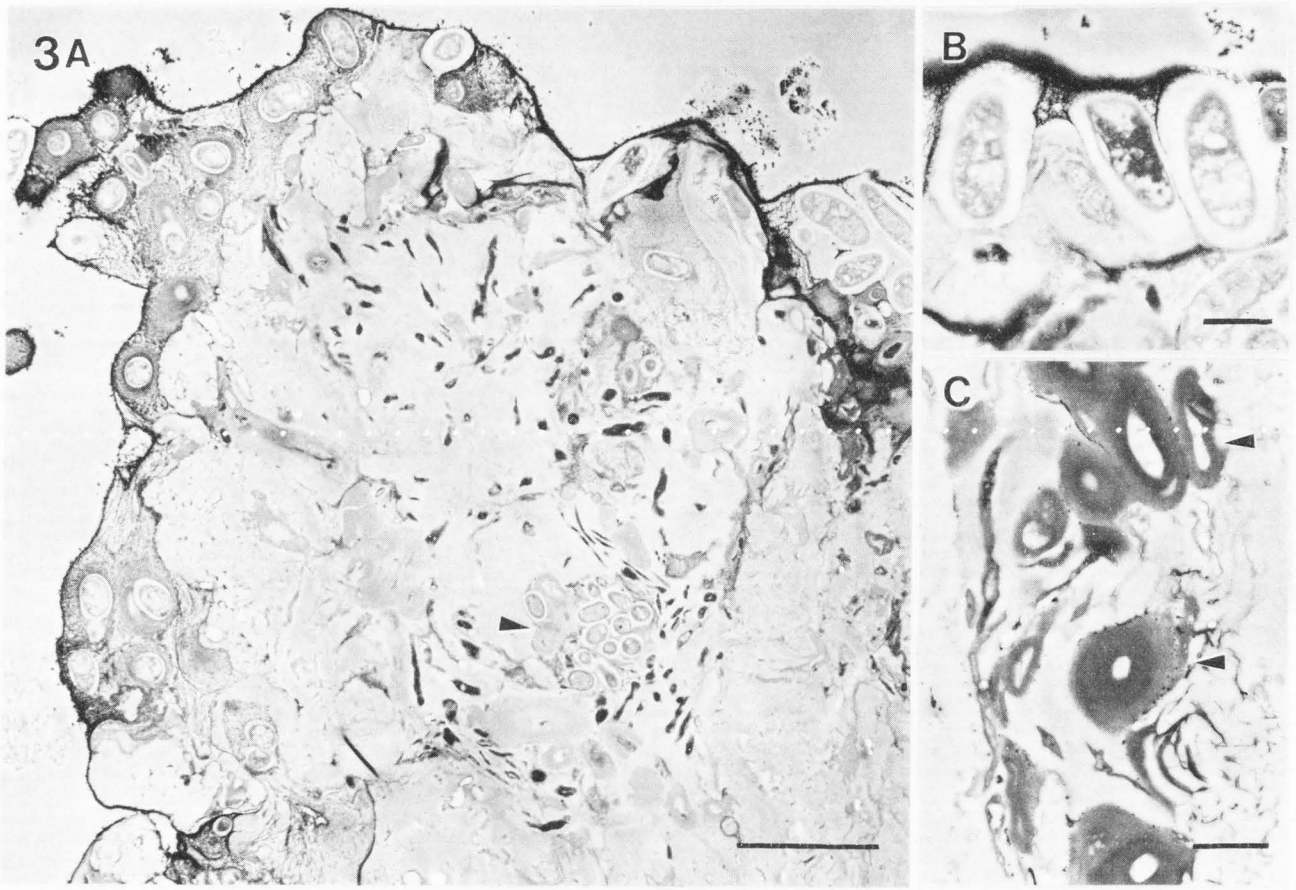

Figure 3. **(A)** Low magnification transmission electron micrograph showing the heterogenecity of a 30 year-old biofilm. Isolated cells, and microcolonies (arrowhead). Bar = 2 μm. **(B)** Dense layer of fibrillar anionic material that reacts strongly with RR at the lumenal surface of the biofilm. The RR stain shows little penetration into the biofilm matrix. Bar = 1 μm. **(C)** TEM appearance of ghost cells (arrowheads). These cells were mainly located in the deeper portion of the biofilm. Bar = 1 μm.

smears) represented less than 1% of the organisms and were found mostly in microcolonies.

Presence of capsule

When directly stained, the bacterial flora of the dental unit water showed the presence of conspicuous thick capsules, surrounding individual or microcolonies of bacteria (Fig. 1A). At high magnification, the capsules appeared well defined (Fig. 1B). This was observed repeatedly with all dental units tested. We made no attempt to quantify precisely the proportion of capsulated bacteria because negative bacteria were difficult to distinguish from the background. However, we estimate this proportion in dental units' water samples to be between 25-50%. Tap water samples never showed these capsules (data not shown).

Early phase of biofilm formation and SEM observations

Morphological observations of 10 to 25-day-old biofilms with the SEM in the SEI mode showed bacterial cells scattered on the inner wall of the tubing (Fig. 2A). In the BEI mode, bacterial cells appeared substantially brighter suggesting reaction of RR with polysaccharides. Bacterial cells were anchored to the tubing by means of polysaccharide projections that looked fibrillar or like small linear projections (Fig. 2B). As seen by TEM, some bacteria were attached to the wall of the waterlines by mean of a thin (25-50 nm) layer of EPS that extended from the surface of the bacteria to the surface of the tubing (Fig. 2C). Groups of bacteria were also trapped under a sheet of anionic material as revealed by SEM. These microcolonies stained strongly with RR as seen in the BEI mode indicating the elaboration of large amounts of EPS (Fig. 2D).

One to four year-old dental unit waterlines were sometimes coated by a pellicle visible to the naked eye (data not shown). Portions of this thin film were sometimes recovered at the output of the tubing after applying

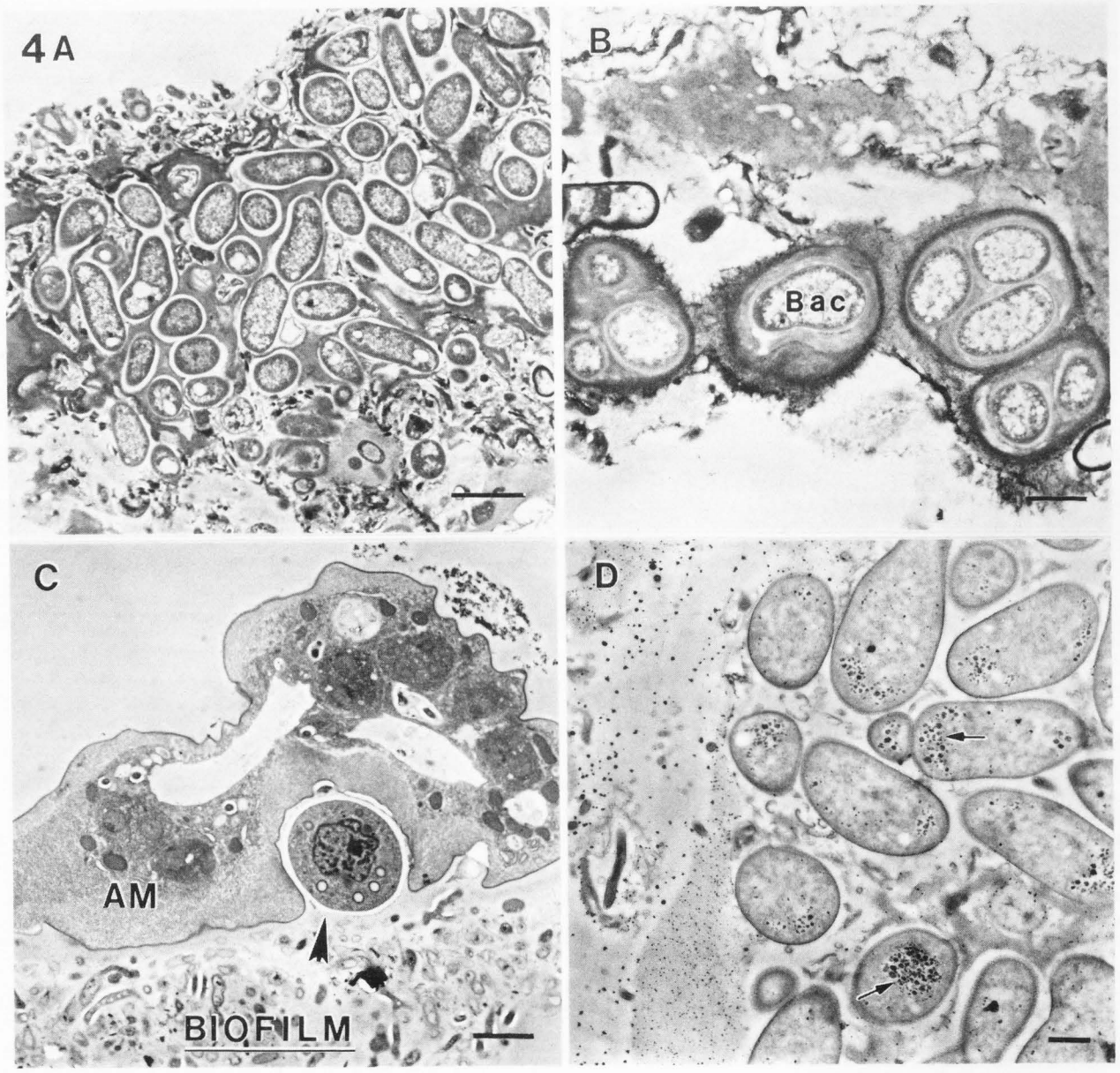

Figure 4. (A) Transmission electron micrograph showing a microcolony of rod-shaped bacteria embedded in RR-stained extracellular matrix. Bar = 2 μm. (B) Transmission electron micrograph at high magnification of structural units containing microcolonies of short rod bacteria (Bac) enclosed in polysaccharide envelopes whose density is enhanced by the anionic EPS stained with RR. Bar = 1 μm. (C) Amoebae (AM) were irregularly found, and when present, were only at the surface of biofilm. Here, the amoebae is in the process of ingesting a yeast-like cell (arrowhead). Bar = 2 μm. (D) Transmission electron micrograph of a biofilm section stained with the periodic acid-thiocarbohydrazide-silver proteinate technique. The polysaccharide composites show particulate staining. IPS granules (arrows) seem to be localized to particular areas of the cells. Bar = 1 μm.

a high pressure water flow through the waterline. However, in some tubing, this coating could not be detached by this method and only scraping was successful in removing it. SEM observations of these older biofilms showed isolated or clustered rod bacteria deposited or embedded in a dense fibrillar, or amorphous, matrix (Fig. 2E).

Microbial composition

The biofilms were characterized by a highly variable

cell density (Fig. 3A). The vast majority of bacteria identified were rods ranging from 2 μm to 10 μm in length. Cocci were seldom encountered. All samples, obtained from the various clinics at Université de Montréal, showed roughly the same organization and composition. The cellular elements were unevenly distributed throughout the biofilm. Bacteria were found to be either isolated or organized in microcolonies (Figs. 3A and 4A) dispersed among extensive cell-free areas. The extracellular matrix formed the bulk of the biofilm structure in all specimens. In all samples observed, we noted the presence of numerous microcolonies of tightly packed bacteria embedded in a common dense anionic envelope (Fig. 4B). These microcolonies contained a mean of three to nine coccoid and short rod Gram-negative bacteria. Many ghost cells and bacterial cells in the process of lysis were observed, and their number increased in the deeper layer of the biofilms (Fig. 3C).

Yeast-like cells and amoebae (Fig. 4C) were irregularly observed and, when present, were only seen at the outermost portion of the biofilm, embedded in the matrix. Occasionally, some degenerating, unidentified eucaryotic cells were observed trapped in the biofilm matrix (data not shown).

EPS distribution and structure

Based on the intensity of staining with RR, the distribution and structure of EPS showed considerable variation within the biofilms, being more concentrated around bacterial cells and microcolonies and less densely distributed in spaces between these microcolonies. The water side of the biofilm (lumenal side) was generally isolated by a thick (100-500 nm) and very dense layer of fibrillar anionic material as seen after RR staining (Fig. 3B).

IPS distribution

Histochemical staining disclosed the intrapolysaccharides (IPS) granules in the biofilms. Control specimens did not react to the staining. The majority of cells showed intrapolysaccharides granules in their cytoplasm. However, the amount and the size of these granules varied among the different cell types and within the same bacterial cell (Fig. 4D). Bacterial cells enclosed in microcolonial units showed relatively few IPS granules in comparison to the majority of the inhabitants of the biofilm.

Discussion

It has been demonstrated by many studies that water from dental unit waterlines is highly contaminated with microorganisms (Barbeau *et al.*, 1996; Cabot Abel *et al.*, 1971; Fitzgibbon *et al.*, 1984; Kelstrup *et al.*, 1977; Robert *et al.*, 1994). As a result, potentially pathogenic microorganisms may come in contact with oral tissues and surgical wounds, thus increasing the risk of infection (Martin, 1987). The high levels of microorganisms is thought to arise from the gradual formation of a biofilm inside the small bore tubing of the dental unit (Mayo *et al.*, 1990). However, little is known about the composition of this biofilm.

Bacterial counts and morphotypes

There was a 10-fold difference in the microbial counts of biofilm between old and new waterlines. This finding is in agreement with the known dynamics of bacterial biofilms where there is a gradual increase in biomass over time as a result of cell division and new recruitment of bacteria from the planktonic phase (Costerton *et al.*, 1987). The lack of correlation between the mass (or thickness) of the biofilm and the concentration of planktonic bacteria in the water is interesting. It can be postulated that waterflow will carry bacteria detaching from the outermost layer of the biofilm. This process is irrelevant to the thickness of the full layer. Accordingly, the age of a tubing would have marginal effect on water contamination and on the bacterial counts in the water. Bacterial counts of 2.2×10^4 bacteria/cm^2 in 180 days old dental unit waterlines biofilms were reported (Tall *et al.*, 1995). The differences between these counts and ours can be explained in two ways. First, we used ultrasound to disrupt the biofilms, and this may have freed more microorganisms from the biofilm. Second, the fluorescence direct count method permits the enumeration of bacteria that cannot grow on normally used media or are viable but non-culturable (Barbeau *et al.*, 1996).

Microbial organization in biofilms

Colonization of the waterlines occurs rapidly after a new dental unit is run for the first time, even if no patient is being treated (Whitehouse *et al.*, 1991). This is in accordance with the fact that oral microorganisms do not constitute a significant source of water contamination (Blake, 1963; Fitzgibbon *et al.*, 1984; Gross *et al.*, 1976; Kelstrup *et al.*, 1977; Martin, 1987; Mills *et al.*, 1986). In the natural environment, biofilms forms very rapidly within a few hours (Eighmy *et al.*, 1983).

EPS seem to play a role in the initial attachment of microorganisms. A thin pellicle of RR-positive material was detected on the wall of the tubing beyond any bacterial cell. When concentrations of nutrients and substrates in a liquid medium are low, they become absorbed on solid surfaces allowing bacteria to stick to the surface (Costerton *et al.*, 1987; Karamanev, 1991).

The fact that the distribution of microorganisms was non-uniform seems to be a characteristic of biofilms found in aquatic environments (Massoldeya *et al.*, 1995; Stewart *et al.*, 1995). The majority of inhabitants of the waterline biofilms were Gram-negative rods as reported

for other aquatic systems (Eighmy *et al.*, 1983). This is in agreement with the bacteriologic analyses of the water sampled from the waterlines where the great majority of bacterial species isolated were *Pseudomonadaceae* and other Gram-negative bacilli (Barbeau *et al.*, 1996; Cabot Abel *et al.*, 1971; Fitzgibbon *et al.*, 1984; Kelstrup *et al.*, 1977; Robert *et al.*, 1994). Certain bacterial species showed an organized structure of EPS layers enclosing single-morphotype microcolonies. This microcolony mode of growth was reported in wastewater biofilms (Eighmy *et al.*, 1983) and *Pseudomonas sp. in vivo* and is thought to be partly responsible for the great resistance of *Pseudomonas* biofilms to the action of biocides and desiccation (Costerton *et al.*, 1990). Hence, it can be postulated that this organization would provide an extra-resistance of the dental unit waterlines biofilm inhabitants to the action of disinfectants as already reported (Furuhashi and Miyamae, 1985; Mills *et al.*, 1986).

EPS composition of dental unit waterline biofilms

A biofilm consists of single cells and microcolonies of sister cells all embedded in a highly hydrated, predominantly anionic matrix (Sutherland, 1977). RR is generally used to demonstrate the presence of polyanionic proteoglycans and mucopolysaccharides among which the alginate secreted by *Pseudomonas sp.* can be found (Costerton *et al.*, 1987). EPS were the most abundant element of the dental unit waterlines biofilms observed here. Our observations showed the distribution and the structure of the EPS to be irregular throughout individual biofilms. The overall organization of the biofilm EPS may be seen as individual cell glycocalyces or capsules and microcolony envelopes embedded in the matrix of the biofilm. This seems to be a feature of wastewater, freshwater and marine biofilms (Eighmy *et al.*, 1983). The fact that the water side of the biofilm is generally isolated by a thick and dense layer of polysaccharides may be a reflection of the heterogeneity of bacterial species which form the biofilm. It can not be excluded that this layer is produced in order to increase the physical barrier towards the external environment. This may have important implications in regard to disinfection regiments. On the other hand, since extracellular exopolysaccharides are highly hydrated gels they may collapse during dehydration and fixation (Handley, 1991). It can not be excluded that the heterogeneity observed in the distribution of exopolysaccharides was in part due to this phenomenon.

It is difficult to determine the origin of the intercellular biofilm matrix since EPS could also be observed in areas devoid of any bacterial cells. This was also reported in experimental biofilms of *Ps. aeruginosa* and *K. pneumoniae* (Stewart *et al.*, 1995) and in wastewater biofilms (Eighmy *et al.*, 1983) and it was postulated that the spaces between microcolonies in biofilms correspond to water channels (Costerton *et al.*, 1994a).

The fact that free-floating bacteria or microcolonies in water samples are covered by a thick capsule indicates that shedding of fixed bacteria may be involved in the generation of the free-floating bacterial community. The absence of this capsule in tap water microflora is, however, difficult to explain. It can be postulated that water stagnation and reduced waterflow in dental unit waterlines may be propitious for the elaboration or preservation of capsule by planktonic bacteria. The presence of capsulated bacteria in water should be stressed here as dental personnel and their patient are exposed to bacterial aerosols (Grenier, 1995), small enough to reach lung alveoli. It is well known that bacterial capsule offers challenge to the immune system as it reduce phagocytosis (Costerton and Irvin, 1981). If *Ps. aeruginosa*, for example, is inhaled as a single cell suspension, every cell will be phagocytized by alveolar macrophages but if encapsulated bacteria (microcolonies or pieces of biofilm) are inhaled, the lung immune system may not be able to deal with the infection that eventually becomes chronic as shown in animal models (Costerton *et al.*, 1990). Since *Ps. aeruginosa* can be recovered from 2.9-50% of dental unit water samples (Barbeau *et al.*, 1996, Jensen *et al.*, 1997), this finding can have significant clinical implications for some individuals like cystic fibrosis patients.

Intracellular polysaccharides

The presence of IPS seems to be a generalized feature of the bacterial communities of dental unit waterlines biofilms. In thick biofilms, IPS can account for up to 50% of the cell volume and appear to be the principal form of carbon storage (Eighmy *et al.*, 1983). It thus appears that bacterial cells within biofilms are capable of synthesizing polysaccharides in the apparent low-nutrient environment of dental unit waterlines. With a mean concentration of 1.5 mg/l, organic compounds may often be a growth-limiting nutrients in the drinking water (LeChevallier *et al.*, 1991) in dental unit waterlines.

Within the limits of our study, two mutually non-exclusive explanations can be proposed to explain the reported high level of water contamination in dental unit waterlines (Barbeau *et al.*, 1996). On the one hand, it can be postulated that most of the water contamination originates from the multiplication and shedding of biofilm-associated microorganisms. Some data suggest that biofilm-associated bacteria have more active reproduction and general metabolism than corresponding planktonic cells (Costerton *et al.*, 1987). On the other hand, the metabolic activity of the biofilm could release metabolites locally and thus create a nutrient-rich interface that

planktonic bacteria present in water could use to multiply inside the closed circuit of dental unit waterlines. It has been shown that freshwater bacteria grow best in a low nutrient medium at the temperature reached in clinical settings (Reasoner and Geldreich, 1985).

In conclusion, our results indicate that dental unit waterlines biofilms are ecosystems where different microbial species elaborate specific EPS structures that may contribute to their resistance to the action of biocides and disinfectants. In the biofilm mode of growth, bacteria can be 500-to-1000 times more resistant to biocides than their free-floating (planktonic) counterpart (Costerton *et al*, 1994b). The goal of the American Dental Association is to lower bacterial counts in water below 200 CFU/ml by the year 2000. We thus recommend that disinfection protocols aimed at eliminating the bacterial contamination of dental unit waterlines should target the biofilm that is the source of the high bacterial load in water and not only the free-floating bacteria.

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Discussion with Reviewers

J.W.T. Wimpenny: The technique for dispersing microbial biofilm could be quantified in a couple of useful ways. I have found that treating cell aggregates to disperse them should be done as a time course. If one plots the log number of colony forming units for example there will be a significant increase followed by a line with a negative slope as cells die due to treatment. If this line is plotted and extrapolated back to zero treatment, we have an accurate estimate of the number of cells originally present. In addition, some simple transmission or scanning electron micrographs of the treated population will give an assessment of frequency of single cell pairs.

Authors: Our quantitative data in this paper were based on direct observation and counts of bacterial cells on membrane under light microscopy. The technique is described in **Materials and Methods**. Counts were based on observation of multiple fields at a magnification of 1000X. In these microscopic fields, bacterial cells were isolated or grouped in small clumps. Bacteria could be easily counted. Of course, tests on cell dispersion would have been included if bacterial culture had been used to quantitate bacteria. This was not the case.

J.W.T. Wimpenny: Nutrient availability is an interesting problem. What do the authors think was the contribution of the waterline itself to nutrient status? For example, a good biofilm can be found on the inside of plastic distilled water containers, presumably plasticisers from the latter are responsible.

Authors: Biofilm formation is a strategic way to trap and extract nutrients from oligotrophic environments like potable water. With a mean concentration of 1.5 mg/l, organic compounds may often be a growth-limiting nutrients in the drinking water in dental unit waterlines. However, we have found that waterborne bacteria isolated from dental unit waterlines can be best recovered using low nutrient media, like R2A medium (Barbeau *et al.*, 1996). These bacteria are, thus, well adapted to survive and multiply in waterlines. It seems that the majority of bacteria isolated from dental unit waterlines belongs to *Pseudomonodaceae* (Barbeau *et al.*, 1996). These bacteria are well known for their ability to use several chemical compounds as carbon sources. For instance, (Nakajima-Kambe *et al.* (1995) showed that *Comamonas acidovorans* can utilize polyester-type polyurethanes as sole carbon and nitrogen sources. We have isolated *C. acidovorans* from some dental unit water samples (Barbeau *et al.*, 1996). It cannot be excluded that bacteria inside biofilms can use synthetic polymers and plasticizers found in plastic hoses as an alternative nutrient source. However, it is likely that once a mature biofilm has formed, the recycling of dead cells components provides a significant source of nutrients.

M. Lavoie: The fact that the observed intracellular polysaccharides seem to be localized inside the cells (Fig. 4D) is very interesting. Would the authors have any explanation?

Authors: Imbalance in the availability of nitrogen or carbon can trigger the storage of reserve material. In our observations here, intracellular granules were frequently observed localized at the poles of bacterial cells. One explanation would be that the physico-chemical properties of intracellular polysaccharides favor their grouping inside the cell. We think the same kind of phenomenon is observed with procaryotic DNA that is condensed inside the cell. Another explanation would be that reserve material may be needed at a particular site inside the cell to be used for cell division. However, in order to determine the exact reason of the observed phenomenon, more research is needed.

T.A. Fassel: Please discuss the impact on infection rate in dental procedures due to dental waterline contamination.

Authors: The controversy over dental unit water contamination is related to the question of whether or not a health risk exists. It would be hard to evaluate the impact of biofilm formation and the high bacterial level in dental unit waterlines on infection risk in dental procedure. To our knowledge, only two cases of infections originating from dental unit water have been reported (Martin *et al.*, 1987) although anecdotal reports and strong suspicions exist (Williams *et al.*, 1996). The risks are very low for most dental clinic patients. However, immuno-compromised patients, the chronically ill and the elderly have an increased susceptibility to opportunistic pathogens. Cases of patients who claimed they became extremely ill from contaminated water in dental units have already been the object of lawsuits in the United States (Clappison *et al.*, 1997). In our opinion, four major opportunistic pathogens that we and other investigators have identified so far are to be considered: *Ps. aeruginosa*, *L. pneumophila*, non-tuberculous mycobacteria, and some amoebas like *Acanthamoeba spp.* Some authors (see e.g., Jensen *et al.*, 1997) found that acquisition of *Ps. aeruginosa* during dental treatment could be a relevant source of infection for cystic fibrosis patients. Aerosolization of *Ps. aeruginosa*-bearing water droplets during the use of high-speed drill or ultrasonic scaler would favor the inhalation of the pathogen by patients. Dental personnel develop significantly higher levels of *Legionella* antibodies than the general population, pointing to chronic exposure and increased risk of infection (Reinthaler *et al.*, 1988). Through the generation of water aerosols or splatters, opportunistic amoebas could potentially represent a risk for ocular infections. Besides infections, the impact of dental unit waterline colonization on the dental profession is very important. Infection control in dentistry represents substantial costs for the clinic and the patients. Dental unit waterline disinfection will undoubtedly become an integral part of infection control protocols and may contribute to increasing expenses directed to infection control.

T.A. Fassel: What may be the consequences of this study for the development of future disinfection protocols?

Authors: Not only some bacterial species are intrinsically resistant to high temperatures and/or biocides, but biofilms can contribute to this resistance (Costerton and Lewandowski, 1995), thus protecting pathogenic bacteria. We think that using inadequate disinfection protocols may have the opposite effect of what is desired, opening the way to colonization by more resistant and more pathogenic bacteria, and increasing their concentrations to critical levels that cannot be detected by routine monitoring (Barbeau and Nadeau, 1997). Disinfection regimens will have to be developed to eliminate both the bacteria and the biofilms that act as reservoirs. Weak disinfectants may leave us with a false sense of security by killing the most vulnerable members of the bacterial population, which unfortunately may have the lowest virulence potential. It seems that few disinfectants can penetrate and destroy biofilms. For instance, we have found that even though they are good bactericides, glutaraldehyde, ethyl alcohol, sodium

hypochlorite, phenols and chlorhexidine gluconate have marginal effects on biofilm inside waterlines (unpublished observations).

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