REVIEW ARTICLE

Susceptibility of oral bacterial biofilms to antimicrobial agents

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There is great interest in the use of antimicrobial agents for the prevention and treatment of plaque-related oral diseases and many publications have reported the results of studies in which the minimum inhibitory concentrations of agents for cariogenic and periodontopathogenic bacteria have been determined. However, such data are relevant only to situations where the organisms of interest are in aqueous suspension, whereas in caries and the inflammatory periodontal diseases the target organisms are in the form of biofilms. On the basis of studies with medically important bacteria, it has been established that bacteria in biofilms are invariably less susceptible to antimicrobial agents than their planktonic counterparts. Therefore, in the laboratory assessment of agents which may be suitable for treating plaque-related diseases, the target organisms should be in the form of biofilms. While laboratory evaluation of chemical agents for the prevention of plaque formation has usually employed biofilm-based models, the search for antimicrobial agents effective in the treatment of plaque-related diseases has not. Therefore, there are few data available regarding those characteristics of antimicrobial agents (e.g., their biofilm eliminating concentrations or biofilm killing concentrations) that could be used to judge their suitability for treating plaque-related diseases. In this review the limited information available concerning the antimicrobial susceptibility of oral bacteria in biofilms is presented.

Introduction

Two of the most common diseases of man, caries and inflammatory periodontal disease, result from the accumulation of bacterial biofilms (plaques) on tooth surfaces. The dental profession has, of course, been aware of this for many years and consequently has developed preventive and therapeutic regimens for these diseases based on mechanical removal of the biofilms. However, there has always been some interest in the possibility of using chemicals to replace or augment mechanical preventive and therapeutic procedures. There are obvious attractions to such an approach in that, if it were effective, prevention and therapy would be easier for both the patient and the dental practitioner. Appropriate chemicals could be used to: (i) prevent biofilm formation; (ii) disrupt existing biofilms; (iii) prevent further biofilm growth; or (iv) kill particular organisms in the biofilm. Each of these tasks would require chemical agents with

appropriate, but not necessarily the same, characteristics. Hence, (i) and (ii) could be achieved with chemicals that do not either kill or inhibit the growth of plaque bacteria, whereas the chemicals used in (iii) and (iv) would, generally, be antimicrobial agents. Of necessity, laboratory evaluation of agents that might be useful for achieving (i) and (ii) (generally referred to as anti-plaque agents) has involved the use of biofilm-based models and a number have been developed for this purpose. Such models range from very simple systems consisting of nichrome steel wires immersed in a bacterial suspension [1] to more complex flow cells [2, 3] and “artificial mouths” [4]. In contrast, evaluation of antimicrobial agents for use in (iii) and (iv) have invariably utilised methodologies more appropriate for the selection of agents suitable for treating systemic infections due to planktonic organisms. Such approaches, e.g., determination of the minimum inhibitory concentration (MIC) of the agent, do not take into account the fact that the target organisms are in the form of a biofilm, thereby ignoring the results of extensive studies carried out with non-oral organisms which have established that their susceptibility to

antimicrobial agents is considerably lower when they are in a biofilm than when they are in an aqueous suspension [5, 6]. This decreased susceptibility explains the difficulties encountered in trying to resolve extra-oral biofilm-associated infections with antibacterial agents [7, 8]. Examples of such infections include those associated with intravascular devices [9], extravascular devices [10], bacterial endocarditis [7] and lung infections in cystic fibrosis patients [11]. In recognition of the differing antimicrobial susceptibilities of planktonic and biofilm-grown cells of the same organism, it has been proposed that an antimicrobial agent should be characterised not only by its MIC and MBC (minimum bactericidal concentration) for a particular organism but also by its biofilm eliminating concentration (BEC), i.e., the concentration of the agent required to eradicate the organism when present in the form of a biofilm [12, 13]. However, as this would appear to imply also the removal of the organism (alive or dead), the terms biofilm inhibitory concentration (BIC) and biofilm killing concentration (BKC), analogous to MIC and MBC have also been proposed [14]. Unfortunately, there are as yet no generally agreed experimental protocols for determining these important characteristics of antimicrobial agents.

Despite the tremendous interest currently being shown in the use of antimicrobial agents for treating plaque-related diseases, there are few reports of the antimicrobial susceptibility of oral bacteria when they are in the form of biofilms. Such data are essential to predict the likely ability of an agent to kill bacteria in dental plaque or to prevent further plaque growth. During the last 10 years, more than 200 papers have been published on the antimicrobial susceptibility of bacteria in biofilms, yet fewer than 10 of these have involved oral bacteria. One possible explanation for this is the more technically demanding nature of biofilm-based determinations compared to the simplicity of MIC determinations. This review summarises the data obtained from biofilm-based studies concerning the antimicrobial susceptibility of oral bacteria, grouped according to the type of biofilm model used.

Growth on agar plates

One of the simplest models of a biofilm consists of a confluent lawn of bacteria growing on an agar plate. The resulting growth resembles a biofilm in that it consists of tightly packed cells embedded in an extracellular matrix, but would, of course, only be representative of a biofilm formed at a solid/gas interface. A number of investigators have used this model to study the susceptibility of bacteria to antimicrobial agents [15, 16]. Although this technique does not appear to have been used for testing the susceptibility of oral bacteria to conventional anti-

microbial agents, it has been used to determine the susceptibility of a number of oral bacteria to killing by light-activated drugs [17]. Biofilms of the periodontopathogenic species *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Actinobacillus actinomycetemcomitans* were grown on agar plates, a solution of a photosensitising agent was added and the biofilms were irradiated with light from a helium-neon laser for various periods. To determine whether killing of organisms in the biofilms had occurred, sterile nitrocellulose membranes were placed over those portions of the plates which had been exposed to the laser light. They were then removed, inverted, placed on the surfaces of fresh agar plates, incubated and examined for bacterial growth. A growth-free zone on the membrane was taken as an indication that at least some of the irradiated bacteria had been killed. This technique was used to test the ability of a number of compounds to act as photosensitisers of the target bacteria. Toluidine blue O was an effective sensitiser of all three organisms and killing was detected after light energy doses of 73 mJ (energy density = 5.5 J/cm²). The laser light had no detectable effect on the viability of any of the organisms in the absence of a photosensitiser. The same approach has been used to demonstrate that light-activated drugs can also be used to kill cariogenic bacteria (*Streptococcus mutans*, *Str. sobrinus*, *Lactobacillus casei* and *Actinomyces viscosus*) in biofilms [18].

Membrane filter-based model

A refinement of the above technique involves growing the test organism on a membrane filter in contact with the agar surface. Once the biofilm has been formed it can then be removed intact and exposed to the antimicrobial agent. This technique has been used for testing the antimicrobial susceptibility of oral bacteria (*Str. sanguis* and a number of periodontopathogens) and *Pseudomonas aeruginosa* [19–21]. In the study of Caufield *et al.* [19] a number of periodontopathogenic bacteria were grown on membranes in contact with enriched trypticase soy agar. Once a biofilm had formed, the membrane was transferred to another agar plate on the surface of which paper disks containing various concentrations of antimicrobial agents had been placed. After being left in contact with the disks for 5 min, the membrane was then transferred to another agar plate containing the redox indicator triphenyl tetrazolium chloride (TTC) and incubated for 12 h. As viable bacteria can reduce the TTC to a red product, a colourless zone on the membrane was taken to indicate a bactericidal effect. In preliminary studies, viable counting of samples taken from such zones confirmed that they did not contain viable bacteria. Therefore, the minimum concentration of the agents required to kill the organisms in the biofilms could be determined by visual inspection. Although the authors refer to these

concentrations as MBCs, the values obtained would in fact represent the BKC of the agents. Four antimicrobial agents (chlorhexidine, iodine, stannous fluoride and sodium fluoride) were tested against a number of oral bacteria by this technique. Unfortunately, the susceptibilities of planktonic cells of the organisms studied were not determined. However, comparison with previously reported values of the MICs and MBCs suggested that the concentrations of the antimicrobial agents required to kill the bacteria in biofilms were far greater than those effective against planktonic cells of the organisms. This technique offers a simple means of determining the susceptibility of oral bacterial biofilms to antimicrobial agents. Unfortunately, there are no reports of it having been used to assess the effects of a number of parameters that could influence the susceptibility of the biofilms to antimicrobial agents, such as biofilm age, length of exposure to the agent and the presence of serum or blood. One potential problem associated with the use of redox dyes as indicators of cell "viability" is that non-viable bacteria may still be able to reduce the dye. Therefore, when used in the manner described above, dye reduction may be apparent on plates containing antimicrobial concentrations greater than those required to kill the organisms. This would lead to an erroneous value for the BKC and an underestimate of the effectiveness of the antibacterial agent [22].

A membrane-based approach has also been used for studying the susceptibility of *Str. sanguis* biofilms to chlorhexidine [20]. The organism was inoculated on to cellulose nitrate membrane filters on the surface of a blood-containing medium and incubated for various periods to provide biofilms of different age, each containing *c.* 10^7 cfu/cm². The membrane-supported biofilms were then removed from the agar plates and exposed to chlorhexidine gluconate (CH) in experiments designed to study the effects of biofilm age, CH concentration, exposure time and the presence of blood on the kills attained. The biofilms retained their integrity (in the absence of agitation) when immersed in Wilkins Chalgren broth with no viable bacteria being detectable in the supernate after a 6-h period of immersion. Older (72 h) biofilms were found to be less susceptible to CH than younger (24 h) ones, the BKC for a 4-h contact time being 200 µg/ml in the case of the former and 50 µg/ml for the latter. The presence of blood was found, not surprisingly, to reduce the effectiveness of CH against the biofilms — the number of viable streptococci in 24 h biofilms being largely unaffected during a 6-h exposure to CH 100 µg/ml in the presence of horse blood 10%. In contrast, no viable streptococci were detectable in similar biofilms exposed to CH 100 µg/ml for 4 h in the absence of blood. The streptococci were also shown to be less susceptible to CH when in intact biofilms than when in disrupted biofilms, the BKC of CH being approximately twice its MBC for the disrupted biofilms.

The technique has also been used to study the relative efficacies of CH and cetylpyridinium chloride (CPC), at concentrations recommended for clinical use, against biofilms of *Str. mutans*. As can be seen in Fig. 1, there was no significant reduction in the viable count of 17-h biofilms (2.4×10^8 cfu/cm²) after exposure to either CH 0.2% w/v or CPC 0.05% w/v for 1 min. A 5-min exposure of the biofilms to CPC also had little effect on the viability of the streptococci in the biofilms whereas CH achieved a 2 log₁₀ reduction in the viable count. Three-day-old biofilms (2.2×10^8 cfu/cm²) proved to be less susceptible to both agents with no statistically significant decrease in the viability of the bacteria after a 5-min exposure to either antimicrobial agent. In contrast, exposure to planktonic cells of the organism (10^8 cfu/ml) to either agent resulted in 3 log₁₀ and >4 log₁₀ reductions in the viable count after exposure for only 1 and 5 min, respectively.

The technique proved to be less useful for studying biofilms of *P. gingivalis* as these tended to disintegrate when immersed in a liquid. Nevertheless, the viable count of biofilms containing 6×10^8 cfu (1.2×10^8 cfu/cm²) of the organism showed <1 log₁₀ reduction when exposed to CH 0.2% w/v for 1 min and <3 log₁₀ reduction after exposure for 5 min. In contrast, no viable bacteria were detected when broth cultures (10^8 cfu/ml) of *P. gingivalis* were exposed to the same concentration of CH for 1 min.

Chemostat models

Bradshaw *et al.* [23] have used the chemostat to grow stable communities of up to 10 species of oral bacteria and, more recently, have included hydroxyapatite disks in the culture vessel to allow the formation of multi-species biofilms [24]. The cell density of the resulting biofilms was *c.* 2.5×10^7 cfu/cm² and 60% of the viable bacteria were gram-negative. The disks with their associated biofilms were then removed from the chemostat and exposed for 1 h to triclosan 20 µg/ml, with or without polyvinyl methyl ether maleic acid (PVM/MA) 134 µg/ml, zinc citrate 50 µg/ml or pyrophosphate 333 µg/ml, and the effects on the microbial composition of the biofilms were determined. Triclosan, with or without the PVM/MA, reduced the viable count by ≤ 40% whereas it was reduced by 80% when triclosan plus either zinc citrate or pyrophosphate was used. Interestingly, the reductions in viable counts achieved by the triclosan with either zinc citrate or pyrophosphate were due to an almost complete elimination of the gram-negative bacteria from the biofilms. As the gram-negative bacteria included the periodontopathogenic species *P. gingivalis* and *F. nucleatum*, these results suggest that triclosan, in combination with either zinc citrate or pyrophosphate, may prove useful in the treatment of gingivitis or periodontitis, or both.

Fig. 1. Effect of chlorhexidine gluconate 0.2% w/v (▨) and cetylpyridinium chloride 0.05% w/v (□) on the viability of *Strep. mutans* in (a) 17-h-old biofilms and (b) 3-day-old biofilms. Error bars represent standard deviations. The horizontal broken lines indicate the detection limit of the viable counting method used.

Constant depth film fermentor

A number of devices have been specifically designed to study the physiology and antimicrobial susceptibility of bacteria in biofilms [25,26]. Perhaps the most widely used of these is the modified Robbins device which has been employed in many investigations concerning the susceptibility of non-oral bacteria to antimicrobial agents but has not yet been used for such

studies involving oral bacteria [27]. Another useful device is the constant depth film fermentor (CDFF) which provides a sophisticated means of reproducibly producing large numbers of biofilms for various purposes [28]. The CDFF consists of a glass vessel with stainless steel end-plates, the top one of which has ports for the entry of medium and gas and for sampling, while the bottom end-plate has a medium outlet (Fig. 2). The vessel houses a stainless steel disk

Fig. 2. The constant depth film fermentor. (a) Overall view of the fermentor; (b) details of internal features.

containing 15 polytetrafluoroethylene (PTFE) sampling pans and this rotates under a PTFE scraper bar which smears the incoming medium over the 15 pans and maintains the biofilms, once formed, at a constant pre-determined depth. Each sampling pan has six cylindrical holes containing PTFE plugs, on which the biofilms form, with their upper surfaces recessed to a pre-determined depth below the surface on the steel disk. The PTFE plugs may also be used to support disks of hydroxyapatite, enamel, etc, enabling the

formation of biofilms on a range of substrata. The sampling pans can be removed aseptically during the course of an experimental run and can then be exposed to antimicrobial agents. Alternatively, the agent can be delivered directly to the fermentor. It is particularly suited to studies of biofilms of oral bacteria in that it provides an environment similar to that found in the oral cavity, i.e., a biofilm growing on a solid substratum with nutrients being provided in a thin film of liquid, continually replenished, trickling over the

surface of the biofilm. Furthermore, the removal of the surfaces of the biofilms by the scraper blade simulates the continuous removal of the outermost layers of supra-gingival plaque due to mastication and tongue movements. The advantages of the system are: (1) it provides many replicates (up to 90) in a single run, hence permitting good statistical analysis; (2) it allows intermittent pulsing of nutrients or antimicrobial agents; (3) it allows sampling of the biofilm (with large numbers of replicates) at various intervals during the course of a run; (4) pure or mixed cultures or homogenised plaque samples can be used as inoculum (these can be added continuously or intermittently); (5) the system is autoclavable and temperature-controlled; (6) a number of substrata can be investigated, e.g., hydroxyapatite, enamel; (7) it can be used to study the effects of pre-treatment of substrata with antimicrobial agents on subsequent biofilm formation.

We have used the CDFF to investigate the susceptibility of biofilms of a representative plaque organism (*Str. sanguis*) to (a) chlorhexidine and cetylpyridinium chloride and (b) lethal photosensitisation.

Susceptibility of Str. sanguis biofilms to anti-septics

A suspension of *Str. sanguis* in a mucin-containing artificial saliva [29] was recycled through the CDFF for 24 h after which time sterile artificial saliva was delivered at a rate of 0.7 L day⁻¹ — the mean salivary flow rate in man. The PTFE plugs in the sampling pans, on which the biofilms form, were recessed to a depth of 300 µm. Pans were removed 4 days after inoculation and the biofilms (consisting of *c.* 4×10^7 cfu/cm²) were exposed to CH 0.2% w/v or CPC 0.05% w/v for various periods and the survivors were enumerated. Viable streptococci survived in the biofilms even after exposure to these agents for 4 h [30]. In contrast, no viable bacteria were detectable after exposure for 5 min of planktonic cells grown in the same medium. CH achieved kills corresponding to *c.* 2 log₁₀ reduction in the viable count of the biofilms after 5 min whereas the corresponding kills achieved by CPC amounted to *c.* 1 log₁₀ reduction. However, on a molar basis, CPC was the more effective of the two antiseptics. In contrast, MIC determinations on planktonic *Str. sanguis* revealed that CH was the more effective of the two antiseptics. The results of the study clearly demonstrate the unreliability of MIC data for predicting the relative efficacy of these antiseptics against biofilms of *Str. sanguis*.

Susceptibility of Str. sanguis biofilms to lethal photosensitisation

The experimental system employed was that described above except that the biofilms were grown on hydroxyapatite disks pre-treated with the artificial saliva. On removing the biofilms from the CDFF, they

were treated with the photosensitiser toluidine blue O and irradiated with various doses of light from a helium-neon laser [31]. There was a light energy dose-dependent decrease in the numbers of viable streptococci in the biofilms (consisting of 6×10^7 cfu/cm²) with no viable cells being detected after irradiation with a light dose of 19 J (energy density = 96 J/cm²). Although the biofilm-grown streptococci appeared to be less susceptible to lethal photosensitisation than planktonic cells (in terms of the light dose required to kill each cell), it is difficult to ascertain whether this was due to the differing physiologies of the cells (see below) or to differences in the optical properties of the two test systems. Penetration of light to all cells is more likely to occur when they are in a suspension as a result of either Brownian motion or deliberate agitation. However, in a biofilm, light may not penetrate to the innermost cells as the static cells in superficial layers could provide shielding by continuing to absorb light even after they had been killed.

Tubular bioreactor

A biofilm of *Str. mutans* was produced on the internal walls of 6.5-mm diameter Tygon tubing with a sucrose-yeast extract-phosphate medium [32]. Sections of the tubing were then placed in 5-ml samples of a range of oral care products and the number of viable cells was determined after exposure for 5 and 30 min. Commercial formulations containing CH 0.04% w/v or hexetidine 0.01% w/v achieved 5 log₁₀ reductions in the viable count of the biofilms after 5 min. In contrast, only a 1.34 log₁₀ reduction was found when the biofilms were exposed for 5 min to a commercial formulation containing CPC 0.02% w/v. Unfortunately, the initial cell density in the biofilms was not stated and the susceptibility of planktonic cells of the organism to the agents was not determined.

Accounting for the reduced antimicrobial susceptibility of bacteria in biofilms

As mentioned previously, a large number of studies with medically important bacteria have demonstrated that biofilm-grown bacteria are less susceptible to antimicrobial agents than their planktonic counterparts. Similar findings have been obtained in the limited number of studies carried out with biofilms of oral bacteria. The reasons for the differing susceptibilities of bacteria when grown as biofilms and in aqueous suspension are a subject of continuing debate. Several explanations have been suggested which can best be appreciated by first considering the structure of bacterial biofilms.

Structure of bacterial biofilms

A biofilm growing on a solid substratum in contact

with a liquid consists of bacteria and an extracellular matrix which has an open, porous structure and is filled with liquid. On the basis of the relative proportions of these components, two regions can generally be discerned within this type of biofilm: (i) a base film containing tightly packed cells attached to a layer of adsorbed material coating the surface of the substratum; (ii) a surface film in contact with the liquid phase, in which the cells are less tightly packed and which contains a higher proportion of liquid than the base film. Generally, it has an irregular topography and may be traversed by water channels which may actually penetrate through to the substratum [33–35].

The above description is based on the results of a relatively small number of studies and relates to the structure of an idealised biofilm. It can only be a generalisation, as the structure of a particular biofilm depends on several factors including the chemical composition and physical structure of the substratum, the nature of the constituent organism(s), the flow rate of the liquid with which it is in contact and the presence or absence of shear forces.

Scanning confocal laser microscopy of living biofilms has shown that mono-species bacterial biofilms have similar structures in which the cells occupy only 5–35% of the volume of the biofilm [6, 34, 35]. Little is known concerning the viability of cells within a biofilm. However, Kinniment [36] has shown that in a 300- μm thick biofilm of *Ps. aeruginosa*, maximum viability occurs a few micrometres below the surface of the film with viability decreasing with depth. This might be expected for an obligate aerobe as oxygen is likely to be severely depleted in the depths of the biofilm, but a very different pattern might be obtained for an anaerobic or capnophilic organism or for a multi-species biofilm consisting of organisms with differing atmospheric requirements.

The biofilm matrix is formed from polymeric material secreted by the constituent bacteria and usually consists of negatively charged (or neutral) polysaccharides or proteins, or both [7, 37]. The composition of the fluid within the matrix will not necessarily be the same as that of the fluid surrounding the biofilm as a result of bacterial activity which will deplete some of its constituents and contribute end-products of metabolism.

Penetration of antimicrobial agents into biofilms

To exert any effect on the constituent bacteria of a biofilm, the antimicrobial agent must first of all penetrate the biofilm matrix. One of the reasons often suggested for the reduced susceptibility of bacteria in biofilms to antimicrobial agents is that the copious extracellular matrix protects the bacteria by hindering penetration of the antimicrobial agent by acting as a molecular sieve, by ionic interaction between the

negatively charged matrix polymer and positively charged antimicrobial agents or by chemical reaction between the polymer and the agent. However, this is a highly controversial area and evidence for and against this viewpoint has been reviewed by Hoyle *et al.* [38] and Nichols [39]. From the evidence summarised in these reviews, it is clear that generalisations are difficult to make, as the importance of the matrix as a barrier to penetration by antimicrobial agents is very much dependent on the particular system under investigation. Of particular importance in this respect are the chemical composition of the matrix and how much of it is present, as well as the physicochemical properties and chemical reactivity of the antimicrobial agent. In the case of an agent consisting of chemically unreactive, uncharged, small molecules which are not enzymically degraded by the organism(s) in the biofilm, the matrix would be unlikely to present much of a barrier to diffusion. It would appear that even when there is strong ionic interaction between a negatively charged matrix and a positively charged antimicrobial agent, diffusion of the agent is not hindered to a great extent and, once all of the binding sites have been filled, the matrix would not present any further barrier to diffusion [40]. For example, in a study of the diffusion of the positively charged antibiotic tobramycin through negatively charged alginate (which is very similar to the polysaccharide produced by *Ps. aeruginosa*), Nichols *et al.* [40] found that the alginate decreased the rate of diffusion of tobramycin by less than one-third. It is unlikely that this factor alone could account for the 1000-fold decreased susceptibility of biofilm-grown *Ps. aeruginosa* compared to planktonic cells of the same organism [21].

Growth rate of biofilm-grown bacteria

An alternative hypothesis to account for the decreased susceptibility of biofilm-grown cells to antimicrobial agents has been proposed by Brown *et al.* [41]. They point out that cells in a biofilm grow only very slowly [42, 43] and resemble stationary phase cultures which are also known to be relatively insensitive to antimicrobial agents [44, 45]. The decreased growth rate of cells in biofilms may be attributable to their micro-environment which, because of the high cell density in the biofilm and its extracellular matrix, will tend to be depleted of exogenously supplied nutrients and oxygen but rich in metabolic end-products. Changes in growth rate have a profound effect on the composition of cell surface structures, both qualitatively and quantitatively [46–48]. As uptake of an antimicrobial agent by a bacterium must first of all involve its interaction with cell surface components, any changes in the latter are likely to affect the uptake and hence the effectiveness of the agent. Many examples can be given to illustrate the effect of growth rate and nutrient depletion on the antimicrobial susceptibility of bacteria and these are cited by Brown *et al.* [41].

Influence of micro-environmental factors on the antimicrobial agent

Trapping, by the matrix, of antibiotic-inactivating enzymes such as β -lactamases resulting in high local concentrations has been suggested as another reason why bacteria in biofilms are less susceptible to antimicrobial agents than their planktonic counterparts. Lambert *et al.* [11] have shown that *Ps. aeruginosa*, when in a biofilm, is highly resistant to piperacillin and imipenem but is susceptible to these antibiotics when the biofilms are disrupted. High levels of β -lactamase (removable by ultracentrifugation) were detected in the matrix surrounding the cells in the intact biofilm. It is also possible that bacterial waste products secreted into, and trapped by, the matrix could accumulate to levels which render antimicrobial agents inactive due to alteration of the local environment in terms of pH, Eh, etc.

Altered gene expression in biofilms

It has also been suggested that induction or repression of gene expression in organisms constituting a biofilm could result in a phenotype which also happens to exhibit a reduced susceptibility to an antimicrobial agent [26]. Contact with a surface has been shown to induce alginate synthesis in *Ps. aeruginosa* [49] and close proximity to other bacteria can also alter gene expression [50].

Conclusions

Because of the intractable nature of infections associated with bacterial biofilms, the antimicrobial susceptibility of medically important bacteria in biofilms has been the subject of extensive investigation for many years. In contrast, although oral microbiologists have used biofilm-based laboratory models for investigating anti-plaque agents, few studies concerning the antimicrobial susceptibility of oral bacteria within biofilms have been reported. In view of the current interest in the use of antimicrobial agents for the treatment of plaque-related diseases, especially periodontitis, this is surprising. Certainly, some of the models developed for use in assessing anti-plaque agents could easily be employed for assessing the antimicrobial susceptibility of biofilm-grown oral bacteria and it is unfortunate that this has not, as yet, been done. Suitable models would include the chemostat flow cell system [3] and various artificial mouths [51]. Alternatively, a number of models used for determining the susceptibility of non-oral bacteria to antimicrobial agents could be used. These include the modified Robbins device, the perfused biofilm fermentor [52] and the CDFE.

Current knowledge of the susceptibility of oral bacterial biofilms to antimicrobial agents is very sparse and a good deal of work will have to be

undertaken to reach the level of understanding attained by colleagues working with biofilms of bacteria whose normal habitat is not the oral cavity. However, the comparatively large body of data that has been accumulated concerning the antimicrobial susceptibility of non-oral bacteria in biofilms has been derived almost exclusively from studies with mono-species biofilms. While this may be adequate for many extra-oral diseases, oral microbiologists will have to extend the field to include studies of multi-species biofilms in order to enable prediction of the likely outcome of the interaction between an antimicrobial agent and dental plaque *in vivo*. Current hypotheses which explain the reduced susceptibility of biofilm-grown bacteria in terms of slower growth rates due to nutrient depletion and accumulation of end-products of metabolism may be adequate to explain the situation in mono-species biofilms, but are they applicable to multi-species biofilms? While oxygen depletion, depletion of exogenous nutrients and accumulation of metabolic end-products may adversely affect the growth and survival of cells in the depths of a mono-species biofilm, these processes actually provide a variety of micro-environments within dental plaques which enable the survival of a diverse range of species. Hence, in the depths of the multi-species biofilms formed on tooth surfaces the low oxygen content, low redox potential and high concentrations of metabolic end-products may lead to increased growth rates for anaerobic species such as many periodontopathogens. Will such organisms, therefore, have antimicrobial susceptibilities similar to those of their planktonic counterparts?

Clinical trials are an expensive way of evaluating the effectiveness of antimicrobial agents for use in treating plaque-related diseases and represent an enormous waste of resources if the choice of agents to be tested in this manner has been based on inappropriate laboratory assessment. Clearly, the recommendation that biofilm-based models be employed for the laboratory assessment of antimicrobial agents for treating caries and periodontitis is a sensible suggestion [53, 54].

References

1. McCabe RM, Keyes PH, Howell A. An *in vitro* method for assessing the plaque forming ability of oral bacteria. *Arch Oral Biol* 1967; **12**: 1653–1656.
2. Rundegren J, Simonsson T, Petersson L, Hansson E. Effect of delmopinol on the cohesion of glucan-containing plaque formed by *Streptococcus mutans* in a flow cell system. *J Dent Res* 1992; **71**: 1792–1796.
3. Herles S, Olsen S, Afflitto J, Gaffar A. Chemostat flow cell system: an *in vitro* model for the evaluation of antiplaque agents. *J Dent Res* 1994; **73**: 1748–1755.
4. Coulter WA, Russell C. Effect of chlorhexidine on plaque development in an artificial mouth. *Microbios* 1976; **16**: 21–28.
5. Nichols WW. Susceptibility of biofilms to toxic compounds. In: Characklis WG, Wilderer PA (eds) *Structure and function of biofilms*. Chichester, John Wiley and Sons. 1989: 321–331.
6. Anwar H, Dasgupta MK, Costerton JW. Testing the susceptibility of bacteria in biofilms to antibacterial agents. *Antimicrob*

- Agents Chemother* 1990; **34**: 2043–2046.
7. Costerton JW, Cheng NJ, Geesey GG *et al.* Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 1987; **41**: 435–464.
 8. Wilcox MH. Treating medical device associated infections. In: Wimpenny J, Nichols W, Stickler D, Lappin-Scott H (eds) Bacterial biofilms and their control in medicine and industry. Cardiff, Bioline. 1994: 155–158.
 9. Elliott TSJ. Intravascular-device infections. *J Med Microbiol* 1988; **27**: 161–167.
 10. Dickinson GM, Bisno AL. Infections associated with indwelling devices: infections related to extravascular devices. *Antimicrob Agents Chemother* 1989; **33**: 602–607.
 11. Lambert PA, Giwerzman B, Hoiby N. Chemotherapy of *Pseudomonas* infections in cystic fibrosis. In: Wimpenny J, Nichols W, Stickler D, Lappin-Scott H (eds) Bacterial biofilms and their control in medicine and industry. Cardiff, Bioline. 1994: 151–153.
 12. Anwar H, Costerton JW. Enhanced activity of combinations of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1990; **34**: 1666–1671.
 13. Costerton JW, Khoury AE, Ward KH, Anwar H. Practical measures to control device-related bacterial infections. *Int J Artif Organs* 1993; **16**: 765–770.
 14. Nichols WW. Biofilm permeability to antimicrobial agents. In: Wimpenny J, Nichols W, Stickler D, Lappin-Scott H (eds) Bacterial biofilms and their control in medicine and industry. Cardiff, Bioline. 1994: 141–149.
 15. Kurian S, Lorian V. Discrepancies between results obtained by agar and broth techniques in testing of drug combinations. *J Clin Microbiol* 1980; **11**: 527–529.
 16. Al-Hiti MMA, Gilbert P. A note on inoculum reproducibility: solid culture versus liquid culture. *J Appl Bacteriol* 1983; **55**: 173–176.
 17. Dobson J, Wilson M. Sensitisation of oral bacteria in biofilms to killing by light from a low-power laser. *Arch Oral Biol* 1992; **37**: 883–887.
 18. Burns T, Wilson M, Pearson G. Sensitisation of cariogenic bacteria in biofilms to killing by low power laser light. Proceedings of the 4th International Conference on Lasers Dentistry in 1994; 66.
 19. Caufield PW, Allen DN, Childers NK. *In vitro* susceptibilities of suspected periodontopathogenic anaerobes as determined by membrane transfer assay. *Antimicrob Agents Chemother* 1987; **31**: 1989–1993.
 20. Millward TA, Wilson M. The effect of chlorhexidine on *Streptococcus sanguis* biofilms. *Microbios* 1989; **58**: 155–164.
 21. Nichols WW, Evans MJ, Slack MPE, Walmsley HL. The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. *J Gen Microbiol* 1989; **135**: 1291–1303.
 22. Stewart PS, Grieve T, Srinivasan R *et al.* Comparison of respiratory activity and culturability during monochloramine disinfection of binary population biofilms. *Appl Environ Microbiol* 1994; **60**: 1690–1692.
 23. Bradshaw DJ, McKee AS, Marsh PD. Prevention of population shifts in oral microbial communities *in vitro* by low fluoride concentrations. *J Dent Res* 1990; **69**: 436–441.
 24. Marsh PD, Bradshaw DJ. Microbiological effects of new agents in dentifrices for plaque control. *Int Dent J* 1993; **43**: 399–406.
 25. Nichols WW. Sensitivity of bacteria in biofilms to antibacterial agents. In: Denyer SP, Gorman SP, Sussman M (eds) Microbial biofilms: formation and control. Society for Applied Bacteriology Technical Series no. 30. Oxford, Blackwell Scientific Publications Ltd. 1993: 187–200.
 26. Brown MRW, Gilbert P. Sensitivity of biofilms to antimicrobial agents. In: Quesnel LB, Gilbert P, Handley PS (eds) Microbial cell envelopes: interactions and biofilms. Oxford, Blackwell Scientific Publications. 1993: 87–97.
 27. Lappin-Scott HM, Jass J, Costerton JW. Microbial biofilm formation and characterization. In: Denyer SP, Gorman SP, Sussman M (eds) Microbial biofilms: formation and control. Society for Applied Bacteriology Technical Series no. 30. Oxford, Blackwell Scientific Publications Ltd. 1993: 1–12.
 28. Wimpenny JWT, Peters A, Scourfield MA. Modeling spatial gradients. In: Characklis WG, Wilderer PA (eds) Structure and function of biofilms. Chichester, John Wiley and Sons. 1989: 111–127.
 29. Wilson M, Kpendema H, Noar JH, Hunt N, Mordan NJ. Corrosion of intra-oral magnets in the presence and absence of biofilms of *Streptococcus sanguis*. *Biomaterials* 1995; **16**: 721–725.
 30. Wilson M, Patel H, Fletcher J. Susceptibility of biofilms of *Streptococcus sanguis* to chlorhexidine gluconate and cetylpyridinium chloride. *Oral Microbiol Immunol* 1995 (in press).
 31. Wilson M. Lethal photosensitisation of *Streptococcus sanguis* biofilms. In: Wimpenny J, Handley P, Gilbert P, Lappin-Scott H (eds) Life and death in microbial biofilm. Cardiff, Bioline. 1995: 143–147.
 32. Le Magrez E, Jacquelin LF, Carquin J, Brisset L, Choisy C. Antiseptic activity of some antidental plaque chemicals on *Streptococcus mutans* biofilms. *Pathol Biol* 1993; **41**: 364–368.
 33. Rogers J, Lee JV, Dennis PJ, Keevil CW. Continuous culture biofilm model for the survival and growth of *Legionella pneumophila* and associated protozoa in potable water systems. In: Morris R, Alexander P, Wynn-Jones P, Sellwood J (eds) Health-related water microbiology. London, IAWPRC. 1991: 192–200.
 34. Caldwell DE, Korber DR, Lawrence JR. Confocal laser microscopy and computer image analysis in microbial ecology. *Adv Microb Ecol* 1992; **12**: 1–68.
 35. Caldwell DE, Korber DR, Lawrence JR. Analysis of biofilm formation using 2D vs 3D digital imaging. *J Appl Bacteriol* 1993; **74**: 52S–66S.
 36. Kinniment SL. Cryosectioning of biofilm. In: Wimpenny J, Nichols W, Stickler D, Lappin-Scott H (eds) Bacterial biofilms and their control in medicine and industry. Cardiff, Bioline. 1994: 53–56.
 37. Characklis WG, Cooksey KE. Biofilms and microbial fouling. *Adv Appl Microbiol* 1983; **29**: 93–138.
 38. Hoyle BD, Jass J, Costerton JW. The biofilm glycocalyx as a resistance factor. *J Antimicrob Chemother* 1990; **26**: 1–5.
 39. Nichols WW. Biofilms, antibiotics and penetration. *Rev Med Microbiol* 1991; **2**: 177–181.
 40. Nichols WW, Dorrington SM, Slack MPE, Walmsley HL. Inhibition of tobramycin diffusion by binding to alginate. *Antimicrob Agents Chemother* 1988; **32**: 518–523.
 41. Brown MRW, Allison DG, Gilbert P. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J Antimicrob Chemother* 1988; **22**: 777–780.
 42. Evans RC, Holmes CJ. Effect of vancomycin hydrochloride on *Staphylococcus epidermidis* biofilm associated with silicone elastomer. *Antimicrob Agents Chemother* 1987; **31**: 889–894.
 43. Prosser BLT, Taylor D, Dix BA, Cleeland R. Method of evaluating effects of antibiotics upon bacterial biofilm. *Antimicrob Agents Chemother* 1987; **31**: 1502–1506.
 44. Brown MRW. Nutrient depletion and antibiotic susceptibility. *J Antimicrob Chemother* 1977; **3**: 198–201.
 45. Brown MRW, Williams P. Influence of substrate limitation and growth phase on sensitivity to antimicrobial agents. *J Antimicrob Chemother* 1985; **15** Suppl A: 7–14.
 46. Ellwood DC, Tempest DW. Effects of environment on bacterial cell wall content and composition. *Adv Microb Physiol* 1972; **7**: 83–117.
 47. Brown MRW, Williams P. The influence of environment on envelope properties affecting survival of bacteria in infections. *Annu Rev Microbiol* 1985; **39**: 527–556.
 48. Costerton JW. Structure and plasticity at various organization levels in the bacterial cell. *Can J Microbiol* 1988; **34**: 513–521.
 49. Davies DG, Chakrabarty AM, Geesey GG. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 1993; **59**: 1181–1186.
 50. Williams P, Stewart GSAB. Cell density dependent control of gene expression in bacteria—implications for biofilm development and control. In: Wimpenny J, Nichols W, Stickler D, Lappin-Scott H (eds) Bacterial biofilms and their control in medicine and industry. Cardiff, Bioline. 1994: 9–12.
 51. Donoghue HD, Perrons CJ. Establishment of defined mixed bacterial plaques on teeth in a laboratory microcosm (model mouth). *Microb Ecol Health Dis* 1988; **1**: 193–200.
 52. Gilbert P, Allison DG, Evans DJ, Handley PS, Brown MRW. Growth rate control of adherent bacterial populations. *Appl Environ Microbiol* 1989; **55**: 1308–1311.
 53. Wilson M. Laboratory assessment of antimicrobial agents for the treatment of chronic periodontitis. *Microb Ecol Health Dis* 1993; **6**: 143–145.
 54. ten Cate JM, Marsh PD. Procedures for establishing efficacy of antimicrobial agents for chemotherapeutic caries prevention. *J Dent Res* 1994; **73**: 695–703.