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Dental plaque: biological significance of a biofilm and community life-style

P. D. Marsh

Leeds Dental Institute, and Health Protection Agency, Centre for Emergency Preparedness and Response, Porton Down, UK

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Abstract

Background: Most microorganisms in nature attach to surfaces and form matrix-embedded biofilms. Biofilms are highly structured and spatially organized, and are often composed of consortia of interacting microorganisms, termed microbial communities, the properties of which are more than the sum of the component species. Microbial gene expression alters markedly in biofilms; organisms communicate by gene transfer and by secretion of diffusible signalling molecules. Cells in biofilms are less susceptible to antimicrobial agents.

Aim and Materials & Methods: To comprehensively review the literature to determine whether dental plaque displays properties consistent with those of a typical biofilm and microbial community.

Results: Novel microscopic and molecular techniques have demonstrated that plaque has a structured architecture with an extracellular matrix, and a diverse composition (around 50% of cells are unculturable). The constituent species communicate by gene transfer, by secreted peptides (Gram-positive bacteria) and autoinducer-2 (Gram-positive and Gram-negative bacteria). These organisms are functionally organized for increased metabolic efficiency, greater resistance to stress and for enhanced virulence. Plaque formation has direct and indirect effects on gene expression.

Conclusion: Dental plaque displays properties that are typical of biofilms and microbial communities in general, a clinical consequence of which is a reduced susceptibility to antimicrobial agents as well as pathogenic synergism.

Key words: antimicrobial resistance; biofilm; cell signalling; dental plaque; ecology; gene expression; gene transfer; microbial community; review

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What is the Significance of Biofilms?

The vast majority of microorganisms in nature are found attached to surfaces, where they grow to form biofilms. Biofilms have been defined as matrix-embedded microbial populations, adherent to each other and/or to surfaces or interfaces (Costerton et al. 1995). Indeed, the ability to attach to, and be retained at a surface, is a fundamental survival strategy for most prokaryotic organisms. Our understanding of biofilms has been advanced over the last decade by the application of novel techniques. These include non-invasive and non-destructive microscopic techniques (e.g. scanning confocal laser microscopy), the publication of annoted microbial genomes (which has spawned new fields such as functional and comparative genomics, transcriptomics and proteomics), the development of molecular tools (e.g. reporter systems) to determine gene activity in situ, and culture-independent approaches to fully characterize (e.g. by 16S rRNA gene amplification and sequencing) and determine the location (e.g. by fluorescent in situ hybridization (FISH)) of the biofilm microflora. These approaches have shown that biofilms are usually highly structured with channels traversing the depth of the biofilm, creating primitive circulatory systems (Costerton et al. 1995). The component species are not randomly distributed but are spatially and functionally organized, and many natural biofilms have a highly diverse microflora.

Gene expression can alter markedly when cells form a biofilm, resulting in many organisms having a radically different phenotype following attachment to a surface. For example, the genes responsible for alginate synthesis in *Pseudomonas aeruginosa* are up-regulated within 15 min. of the cell's initial contact with a surface (Davies & Geesey 1995). More recently, DNA microarrays have shown that 73 genes (Whiteley et al. 2001) and 50% of the detectable

proteome (Sauer et al. 2002) were differentially regulated in biofilms of *P. aeruginosa* when compared with conventional liquid grown (planktonic) cells. It has been proposed that the environmental heterogeneity that develops in biofilms can accelerate phenotypic and genotypic diversity in bacterial populations and might be a mechanism whereby cells are better prepared to cope with adverse conditions (a form of ''biological insurance'') (Boles et al. 2004).

The binding of bacteria to specific host receptors can also trigger significant changes in host cell patterns of gene expression, as has been demonstrated following the initial attachment of *Escherichia coli* to uro-epithelial cells (Abraham et al. 1998). As the biofilm matures, there is continued synthesis of exopolymers to form an extracellular matrix. The matrix is not only important physically as part of the scaffolding that determines the structure of biofilms, but it is also biologically active and can retain nutrients, water (thereby preventing desiccation) and key enzymes within the biofilm (Allison 2003, Branda et al. 2005).

Within biofilms, sophisticated systems of cell–cell communication are used by some bacteria to co-ordinate gene expression. Gram-positive bacteria generally communicate via small diffusible peptides (Sturme et al. 2002), while many Gram-negative bacteria secrete acyl homoserine lactones (AHLs) (Whitehead et al. 2001), the structure of which varies depending on the species of bacteria that produce them. AHLs are involved in quorum sensing whereby cells are able to modulate gene expression in response to increases in cell density. Another system involves the synthesis of autoinducer-2 (AI-2), the structure of which is unknown, but a gene product, LuxS, is required (Federle & Bassler 2003, Winzer et al. 2003). This system may be involved in cross-communication among both Gram-positive and Gram-negative bacteria, as homologues of LuxS are widespread within the microbial world. These changes in global gene expression equip the cell for growth and survival on a surface and enable the formation of biofilm. Research is focussing on generating analogues of signalling molecules used by pathogens in order to manipulate the properties of biofilms, including making them more consistent with health (Stewart & Costerton 2001).

An important clinical consequence of both the structural organization of biofilms and the subsequent altered pattern of gene expression therein is the reduced susceptibility of cells to antimicrobial agents (Gilbert et al. 1997, 2002, Ceri et al. 1999, Stewart & Costerton 2001). Conventionally, the sensitivity of bacteria to antimicrobial agents is determined on cells grown in liquid culture by the measurement of the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC). Numerous studies have shown that the MIC of an organism growing on a surface can range from two- to 1000-fold greater than the same cells grown planktonically (Stewart & Costerton 2001, Johnson et al. 2002). Given the decreased sensitivity of an organism on a surface, it has been argued that it would be more appropriate to determine the ''biofilm inhibitory concentration'' (BIC) of an agent (also described as the ''biofilm eradicating concentration'' or biofilm killing concentration) (Anwar & Costerton 1990, Nichols 1994, Johnson et al. 2002).

The mechanisms behind the increased resistance of biofilms to antimicrobial agents are still the subject of much research and debate (Stewart & Costerton 2001, Gilbert et al. 2002). Cells conventionally become resistant because of mutations affecting the drug target, to the presence of efflux pumps or to the production of modifying enzymes, etc., but even innately sensitive bacteria become less susceptible when growing on a surface. The structure of a biofilm may restrict the penetration of the antimicrobial agent; charged inhibitors can bind to oppositely charged polymers that make up the biofilm matrix (diffusion–reaction theory). The agent may also adsorb to and inhibit the organisms at the surface of the biofilm, leaving cells in the depths of the biofilm relatively unaffected. The matrix in biofilms can also bind and retain neutralizing enzymes (e.g. β-lactamase) at concentrations that could inactivate an antibiotic or inhibitor (Allison 2003). As stated earlier, bacteria growing on a surface display a novel phenotype, and this can result in a reduced sensitivity to inhibitors. Growth on a surface may also result in the drug target being modified or not expressed in a biofilm, or the organism may use alternative metabolic strategies. Bacteria grow only slowly under nutrient-depleted conditions in an established biofilm, and, as a consequence, are much less susceptible than faster dividing cells. In addition, it has also been proposed that the environment in the depths of a biofilm may be unfavourable for the optimal action of some drugs (Gilbert et al. 2002). Furthermore, a recent hypothesis suggests that the increased tolerance of some biofilms to antibiotics is due largely to the presence of a sub-population of ''persister'' organisms that are specialized survivor cells (Keren et al. 2004). At present, it is not clear whether some or all of these effects account for the observed resistance of cells in biofilms.

What is the Significance of Microbial Communities?

Most natural biofilms contain multiple species and are termed microbial communities. Evidence is accumulating that the component organisms are not merely passive neighbours but rather that they are involved in a wide range of physical, metabolic and molecular interactions. Indeed, these interactions may well be essential for the attachment, growth and survival of species at a site, enabling organisms to persist in what often appear to be hostile environments.

This community life-style provides enormous potential benefits to the participating organisms (Caldwell et al. 1997, Shapiro 1998, Marsh & Bowden 2000). These include: (a) a broader habitat range for growth. The metabolism of early colonizers alters the local environment, making conditions suitable for attachment and growth of later (and sometimes more fastidious) species. Thus, the diversity of the microflora increases over time because of microbial succession. (b) An increased metabolic diversity and efficiency; molecules that are normally recalcitrant to catabolism by individual organisms can often be broken down by microbial consortia. (c) An enhanced resistance to environmental stress, antimicrobial agents and the host defences. It has already been cited that bacteria in biofilms are more tolerant of antimicrobial agents, but this effect can be enhanced still further in microbial communities. Neighbouring cells of a different species can produce neutralizing enzymes (β-lactamase, IgA protease, catalase, etc.) that protect inherently susceptible organisms from inhibitors (Brook 1989). A penicillin-sensitive pathogen (*Strep-*

tococcus pyogenes) was shown to be protected during antibiotic treatment in an animal model by β-lactamase produced by a commensal strain (*Moraxella catarrhalis*) and, as a result, was still capable of causing a lethal infection (Hol et al. 1994). Horizontal gene transfer is also more feasible in multi-species biofilms (Molin & Tolker-Nielsen 2003, Wilson & Salyers 2003), and the transfer of resistance genes from commensal to pathogenic strains is well documented. Microbial communities might also afford physical protection from phagocytosis for cells deep within a spatially organized consortium (Costerton et al. 1981, 1987, Fux et al. 2005). Also, on occasions, microbial communities may have (d) an enhanced ability to cause disease. Abscesses are examples of polymicrobial infections whereby organisms that individually cannot cause disease are able to do so when they are present as a consortium (pathogenic synergism) (Brook 1987). Thus, microbial communities display emergent properties, i.e., the properties of the community are more than the sum of its component populations.

What is the Biological Significant of Dental Plaque Being Both a Biofilm and Microbial Community?

Dental plaque has been defined as the diverse *community* of microorganisms found on the tooth surface as a *biofilm*, embedded in an extracellular matrix of polymers of host and microbial origin (Marsh 2004). Many of the novel microscopic and molecular techniques that have recently been developed to investigate environmental biofilms have now been used to explore the properties of dental plaque. These studies have shown that dental plaque behaves as a classical biofilm (see Socransky & Haffajee 2002, Marsh 2004) (Table 1). Some of the most important findings that are changing our established views on dental plaque will be reviewed briefly below.

Plaque structure

In contrast to early studies using electron microscopy, in which plaque appeared as a compacted consortium of microorganisms, confocal laser scanning microscopy has revealed that supragingival plaque can have a structured architecture. Polymer-containing channels or pores have been observed that link the plaque/oral environment interface to the tooth surface (Wood et al. 2000, Auschill et al. 2001, Zaura-Arite et al. 2001). The use of live/dead stains has indicated that bacterial vitality varies throughout the biofilm, with the most viable bacteria present in the central part of plaque, and lining the voids and channels (Auschill et al. 2001). This more open architecture should enable molecules to readily move in and out of plaque, but the presence of a matrix comprised of a diverse range of exo-polymers creates a complex environment for accurately predicting the penetration and distribution of molecules within plaque (Robinson et al. 1997, Thurnheer et al. 2003, Marcotte et al. 2004), including the delivery of therapeutic agents.

Because of difficulties of access, sub-gingival plaque has not been viewed directly by confocal microscopy, and so information on its architecture is limited. Histological sections of human sub-gingival plaque viewed by conventional light microscopy suggest a complex organization of attached microorganisms in which there can exist distinct tooth-associated and epithelial cell-associated biofilms, with the possibility of a less dense zone of organisms between the two (Socransky & Haffajee 2002). These regions may differ in microbial composition (e.g. there may be more putative periodontal pathogens in the epithelial biofilm), physiological state and, consequently, in their response to antimicrobial treatment (see later).

Bacterial metabolism in plaque ensures that gradients develop in parameters that are critical to microbial growth (nutrients, pH, oxygen, etc). These gradients are not necessarily linear; the use of two-photon excitation microscopy coupled with fluorescent life-time imaging demonstrated considerable heterogeneity in pH over relatively short distances in model mixed culture oral biofilms (Vroom et al. 1999). Such environmental heterogeneity will allow fastidious bacteria to survive in plaque, and enable microorganisms to co-exist that would be incompatible with one another in a more homogeneous environment. This explains how organisms with apparently contradictory metabolic and growth requirements (e.g. in terms of atmospheric and nutritional requirements) are able to persist at the same site.

Bacterial composition of dental plaque biofilms

The application of rigorous culture-dependent and culture-independent approaches are only now beginning to reveal the full richness and diversity of the oral microflora, especially from sub-gingival sites. This renaissance in our understanding of the plaque microflora will have a profound impact on our ability to (a) define the causative bacteria in disease, and (b) develop diagnostic microbiological approaches. Molecular approaches based on nucleotide sequence analysis of the16S subunit rRNA gene (16S rDNA) have identified a large number of novel taxa (Kroes et al. 1999, Wade 1999, Dewhirst et al. 2000, Paster et al. 2001) and demonstrated that approximately 50% of cells in plaque cannot as yet be cultured in the laboratory.

Molecular studies using culture-independent approaches (e.g. 16S rRNA amplification; FISH) have shown that the sub-gingival microflora is extremely diverse, even in health. Around 40% of the amplified clones represent novel phylotypes. Human oral TM7 bacteria, of which there are no culturable examples were detected frequently in samples and made up around 1% of the total bacteria in healthy sub-gingival sites (Brinig et al. 2003). A number of spirochaetes were detected, including *Treponema vincentii*, *T. denticola*, *T. maltophilum* and *T. lecithinolyticum*, as well as members of the *Selenomonas*, *Prevotella*, *Capnocytophaga* and *Campylobacter* genera (Paster et al. 2001).

The application of similar molecular methods to characterize the sub-gingival microflora of sites with chronic periodontitis has further emphasized the diversity of bacteria found in these sites. Studies of plaque developing on removable materials in deep periodontal pockets using FISH techniques showed that the deepest zones were colonized mainly by spirochaetes and Gram-negative bacteria, whereas shallow regions comprised predominantly Gram-positive cocci (Wecke et al. 2000). An extensive analysis of $>13{,}000$ sub-gingival samples from nearly 200 adults using a checkerboard DNA–DNA hybridization approach showed that ''complexes'' of bacteria were associated with either health or disease (Socransky et al. 1998, Socransky & Haffajee 2002). While certain groups of bacteria were early colonizers of the tooth surface, the

presence of others, such as members of the ''red complex'' (*Porphyromonas gingivalis*, *T. denticola*, *Tannerella forsythensis*), were associated more commonly with clinical indicators of periodontal diseases, and were rarely detected in the absence of members of other ''complexes'' (e.g. the ''orange complex'', which includes representatives of several genera, including *Peptostreptococcus*, *Prevotella* and *Fusobacterium*) (Socransky et al. 1998, Socransky & Haffajee 2002).

Studies using 16S rRNA gene sequencing have confirmed that an even larger proportion of clones at diseased sites belong to novel phylotypes, many of which also have no cultivable representatives. For example, some studies have detected unculturable examples of *Treponema* spp. (Dewhirst et al. 2000), or members of the Obsidian Pool, OB11, and TM7 phylotypes (Brinig et al. 2003, Ouverney et al. 2003). Within the TM7 group, the oral clone I025 was detected in only 1/18 samples from healthy sites but was found in 38/58 samples from periodontally diseased sites (although these sites were from patients diagnosed with periodontitis, necrotizing ulcerative gingivitis, NUG, and ''refractory periodontitis'') (Brinig et al. 2003). Interestingly, the I025 cells from sites with chronic periodontitis were more abundant and fourfold longer than those from healthy sites (Ouverney et al. 2003). Other strains that have been recovered almost exclusively from diseased sites include *T. socranskii*, *Filifactor alocis*, *Dialister pneumosintes*, *T. forsythensis*, *P. gingivalis* and *P. endodontalis* (Paster et al. 2001). These culture-independent studies are changing our views on the role of bacteria in disease. They have confirmed that complex consortia can be isolated from sites with advanced disease, and that poorly classified organisms that are currently difficult or impossible to grow in the laboratory can predominate in deep pockets. It has yet to be determined whether these organisms are playing an active role in disease or are there as a consequence of tissue destruction. It is likely in the near future that it will be possible to screen for the presence of these disease-associated complexes using probes in DNA microarray or DNA–DNA checkerboard hybridization formats. Ultimately, the outcome of these studies may enable a clearer association of certain consortia with disease to be discerned and contribute to improved diagnosis and facilitate treatment monitoring.

Biofilm regulation of gene expression

Surface-associated changes in gene expression are now being identified in plaque bacteria, although the magnitude of this shift in regulation may be less than that observed in some free-living species because of the absolute dependence of oral bacteria on a biofilm lifestyle (Burne 1998). Most studies have been performed on bacteria that predominate in supragingival plaque (e.g. streptococci). During the initial stages of biofilm formation by *S. mutans* (first 2 h following attachment), 33 proteins were differentially expressed (25 proteins were up-regulated; eight proteins down-regulated) (Welin et al. 2004). There was an increase in the relative synthesis of enzymes involved in carbohydrate catabolism; these might be needed for energy generation, although these molecules are multi-functional and can also act as adhesins when located on the cell surface. In contrast, some glycolytic enzymes involved in acid production were down-regulated in older (3 day) biofilms, while proteins involved with a range of biochemical functions including protein folding and secretion, amino acid and fatty acid biosynthesis, and cell division were up-regulated (Svensater et al. 2001). Of particular significance, novel proteins of as yet unknown function were expressed by biofilm but not planktonic cells. Similarly, genes associated with glucan (*gtfBC*) and fructan synthesis (*ftf*) in *S. mutans* were differentially regulated in biofilms (Li & Burne 2001). There was little influence of surface growth in early biofilm formation (<48 h), but *gtf* expression was markedly up-regulated in older (7 days) biofilms, whereas *ftf* activity was repressed. This findings demonstrate that growth in biofilms can have both a direct (i.e. as a result of attachment) or indirect (i.e. because of the altered environmental conditions within the biofilm, e.g. sugar concentration, pH, etc) effect on gene expression by plaque bacteria. As yet, there have not been in depth studies of the effect of biofilm formation on gene expression by periodontal pathogens.

In plaque, bacteria bind to many host proteins and co-aggregate with other organisms, and the potential impact of these cues on gene expression is just beginning to be explored. The exposure of *S. gordonii* to saliva resulted in the induction of genes (*sspA/B*) encoding adhesins that can bind to salivary glycoproteins and engage in co-aggregation with *Actinomyces* spp. (Du & Kolenbrander 2000). Similarly, streptococci can engage in a food chain whereby the lactate they produce from carbohydrate metabolism is converted to propionate and acetate by *Veillonella* spp. It has been reported recently that signalling events can occur between these metabolically interacting organisms resulting in increased expression of α-amylase by *S. gordonii* when in co-culture with *V. atypica* (Egland et al. 2004). It remains to be determined whether similar regulatory events occur in the sub-gingival environment following (a) contact between molecules in GCF and putative periodontal pathogens, and (b) co-adhesion between different species in the periodontal pocket.

Cell–cell communication and gene transfer

In addition to the many conventional metabolic interactions (synergistic and anatagonistic) that have been well catalogued to occur among oral bacteria, organisms from plaque have also been shown to communicate with one another in a cell density-dependent manner via small diffusible molecules, using strategies similar to those described for other biofilms (Kolenbrander et al. 2002, Cvitkovitch et al. 2003, Suntharalingam & Cvitkovitch 2005). Again most studies of plaque bacteria have focussed on streptococci. In *S. mutans*, quorum sensing is mediated by a competence stimulating peptide (CSP) (Li et al. 2001). This peptide also induces genetic competence in *S. mutans* so that the transformation frequency of biofilm-grown *S. mutans* was 10–600-fold greater than for planktonic cells (Li et al. 2002b). Lysed cells in biofilms could act as donors of chromosomal DNA, thereby increasing the opportunity for horizontal gene transfer in dental plaque. CSP is also directly involved in biofilm formation; mutants in some of the genes involved in the CSP signalling system (*comC*, *comD*, *comE* and *comX*) produce defective biofilms. This quorum sensing system also functions to regulate acid tolerance in *S. mutans* biofilms (Li et al. 2002a). It has been proposed that *S. mutans*, upon exposure to low pH, could release CSP, and initiate a co-ordinated ''protective'' response among

neighbouring cells to such a potentially lethal stress.

Other communication systems may function between different oral species (see Kolenbrander et al. 2002). *LuxS* genes encode for AI-2, and these have been detected in several genera of oral Gram-positive and Gram-negative bacteria implying that AI-2 may have a broader species range. Mutants of the *luxS* gene that encodes for the AI-2 synthase in *S. mutans* and *S. gordonii* had an impaired ability to produce monospecies biofilms in vitro (Blehert et al. 2003, Merritt et al. 2003). A survey of Gram-negative periodontal bacteria suggests that these organisms do not posses the AHL-dependent signalling circuits detected in other Gram-negative bacteria (Frias et al. 2001), but several periodontal bacteria (*Fusobacterium nucleatum*, *Prevotella intermedia*, *P. gingivalis*, *Actinobacillus actinomycetemcomitans*) secrete a signal related to AI-2 (Fong et al. 2001, Frias et al. 2001). In *A. actinomycetemcomitans*, luxS-dependent signalling induced expression of leukotoxin and a transport protein involved in iron acquisition. The signal could also complement a *luxS* mutation in *P. gingivalis*, suggesting a role for these molecules in intra- and inter-species communication (Fong et al. 2001). In *P. gingivalis*, LuxS-dependent quorum sensing modulated protease (arg-gingipain and lys-gingipain) and haemagglutinin activities, but was not essential for virulence (Burgess et al. 2002).

Cells also ''communicate'' and interact with one another in biofilms via horizontal gene transfer. As discussed above, signalling molecules such as CSP markedly increase the ability of recipient cells in biofilms to take up DNA (Li et al. 2002b). The transfer of conjugative transposons encoding tetracycline resistance between streptococci has been demonstrated in model biofilms (Roberts et al. 2001). The recovery of resident (*S. mitis*, *S. oralis*) and pathogenic (*S. pneumoniae*) bacteria from the naso-pharynx with penicillin resistance genes showing a common mosaic structure confirms that gene transfer can occur in vivo (Dowson et al. 1990, Hakenbeck et al. 1998). Similar evidence suggests sharing of genes responsible for penicillin-binding proteins among commensal and pathogenic *Neisseria* (Bowler et al. 1994). Gene transfer between *T. denticola* and *S. gordonii* has also been demonstrated in the laboratory (Wang et al. 2002). The presence of ''pathogenicity islands'' in periodontal pathogens such as *P. gingivalis* is also indirect evidence for horizontal gene transfer in plaque biofilms, and may explain the evolution of more virulent strains (Chen et al. 2004). These findings suggest that plaque can function as a ''genotypic reservoir'' by harbouring transferable mobile elements and genes. Such genetic exchange could have a wider significance given the number of overtly pathogenic bacteria that appear transiently in the mouth (Loo 2003).

Communication is not just between bacterial cells. Surface components of sub-gingival bacteria are involved in adhesion to epithelial cells at the start of colonization and biofilm formation, and there is also evidence that they are involved in bacterium–host cell cross-talk. Fimbriated *P. gingivalis* cells can induce formation of integrin-associated focal adhesions with subsequent remodelling of the actin and tubulin cytoskeleton in primary gingival epithelial cells (Yilmaz et al. 2003). These authors have argued that these complex interactions reflect a possible evolutionary relationship between *P. gingivalis* and host cells, resulting in a balanced association whereby the organism can survive within epithelial cells without causing excessive harm. *P. gingivalis*-mediated disease may result in part from a disruption of this balance by factors that may trigger virulence or lead to host-immune-mediated tissue damage (Yilmaz et al. 2003).

Antimicrobial resistance

Bacteria growing in dental plaque also display an increased tolerance to antimicrobial agents, including those used in dentifrices and mouthrinses (Marsh & Bradshaw 1993, Kinniment et al. 1996, Wilson 1996, Pratten & Wilson 1999). For example, the BIC for chlorhexidine and amine fluoride was 300 times and 75 times greater, respectively, when *S. sobrinus* was grown as a biofilm compared with the MBC of planktonic cells (Shani et al. 2000). Similarly, it was necessary to administer 10–50 times the MIC of chlorhexidine to eliminate *S. sanguinis* (previously *S. sanguis*) biofilms within 24 h (Larsen & Fiehn 1996). The age of the biofilm can also be a significant factor; older biofilms (72 h) of *S. sanguinis* were more resistant to chlorhexidine than younger (24 h) biofilms (Millward & Wilson 1989). Confocal microscopy of in situ established natural biofilms showed that chlorhexidine only affected the outer layers of cells in 24 and 48 h plaque biofilms (Zaura-Arite et al. 2001), suggesting either quenching of the agent at the biofilm surface or a lack of penetration.

Biofilms of oral bacteria are also more tolerant of antibiotics (e.g. amoxycillin, doxycycline, minocycline, metronidazole) than planktonic cells (Larsen 2002, Socransky & Haffajee 2002, Noiri et al. 2003), although the degree of resistance can vary with the organism, the model system and the inhibitor used. For example, biofilms of *P. gingivalis* tolerated 160 times the MIC of metronidazole that had been determined for planktonic cells (Wright et al. 1997), but other studies using mono-species biofilms of *A. actinomycetemcomitans* or *P. gingivalis* treated with moxifloxacin did not demonstrate such a marked increase in resistance (Eick et al. 2004).

Plaque as a community

The evidence outlined above both on the diversity of the plaque microflora and on the ability of plaque bacteria to interact with neighbouring cells in biofilms provides compelling support for the concept that oral bacteria do not exist as independent entities but rather function as a co-ordinated, spatially organized and metabolically integrated microbial community (Marsh & Bradshaw 1999, Marsh & Bowden 2000) (Fig. 1). Benefits of a community life-style to plaque microorganisms are similar to those described for other microbial communities (Table 1). These include: (a) a broader habitat range for growth, e.g. oxygen-consuming species such as *Neisseria* spp. (together with the accumulation of reduced end products of metabolism) create environmental conditions suitable for colonization in plaque by obligate anaerobes (Bradshaw et al. 1996). Similarly, the heterogeneity of pH, oxygen tension and redox potential in plaque biofilms enables species with a wide range of growth requirements to co-exist. (b) A more efficient metabolism, e.g. many complex host macromolecules, especially glycoproteins such as mucins, can only be degraded efficiently by consortia of oral bacteria (Bradshaw et al. 1994). This process can involve the concerted action of interacting species as well as the sequential breakdown of a substrate with a complex structure

to simpler products by organisms operating in food chains (Carlsson 2000). (iii) Increased resistance to stress and antimicrobial agents. As discussed previously, a sensitive organism can be rendered as being apparently ''resistant'' to an antibiotic if neighbouring, non-pathogenic cells produce a neutralizing or drug-degrading enzyme (''indirect pathogenicity''). In the mouth, GCF can contain sufficient β-lactamase to inactivate the concentrations of antibiotic delivered to the site (Walker et al. 1987, Herrera et al. 2000). (iv) Enhanced virulence – for the development of periodontal diseases, sub-gingival bacteria must adhere, gain nutrients from the host and multiply, overcome or evade the host defences, invade, and induce tissue damage. A diverse range of virulence traits are required for particular stages of the disease process, and it is highly likely that each will require the concerted action of a consortia of interacting bacteria (''pathogenic synergism''; van Steenbergen et al. 1984). Likewise, it is possible that certain species could have more than one role in disease, while different species could perform identical functions in consortia with a distinct composition at other sites. This would contribute to the explanation that communities with varying bacterial composition have been found at sites with similar disease, and would be consistent with the concept of ''complexes'' associated with health and disease (Socransky & Haffajee 2002). Evidence for pathogenic synergism has come from abscess models in animals, in which different combinations of oral bacteria display increasing pathogenicity and tissue damage (Sundqvist et al. 1979, Fabricus et al. 1982, Baumgartner et al. 1992).

As discussed earlier, sub-gingival microbial communities have a diverse composition, and the component species interact and communicate extensively. The predominant organisms differ between healthy and diseased sites, and a major challenge has been to explain how these shifts in plaque composition occur. In most ecosystems, there is a direct relationship between the environment and the diversity and abundance of species present. This relationship is dynamic so that a change in a key environmental factor can alter the competitiveness of individual species, leading to the potential enrichment of a minor component of the community or the loss of a previously dominant organism. Thus, the shift towards communities containing increased proportions and numbers of anaerobic and proteolytic bacteria, as seen in periodontal disease, could be explained by the response of sub-gingival biofilms to changes in local environmental conditions and host responses. During the inflammatory response to plaque accumulation, there is an increase in the flow of gingival crevicular fluid; this not only delivers components of the host defences but also provides an array of novel nutrients (proteins and glycoproteins) that favour the growth of organisms with an asaccharolytic metabolism. A further consequence of this metabolism is a rise in local pH and a reduction in redox potential. Collectively, these changes in environment will selectively enrich for the proteolytic organisms associated with inflamed sites. This relationship in which a change in local environmental conditions drives a deleterious shift in the composition of sub-gingival plaque has been captured in the ''ecological plaque hypothesis'' (Marsh 1994, 2003). Implicit in this hypothesis is that disease could be prevented not only by targeting the causative organisms but also by interfering with the driving forces responsible for their selection.

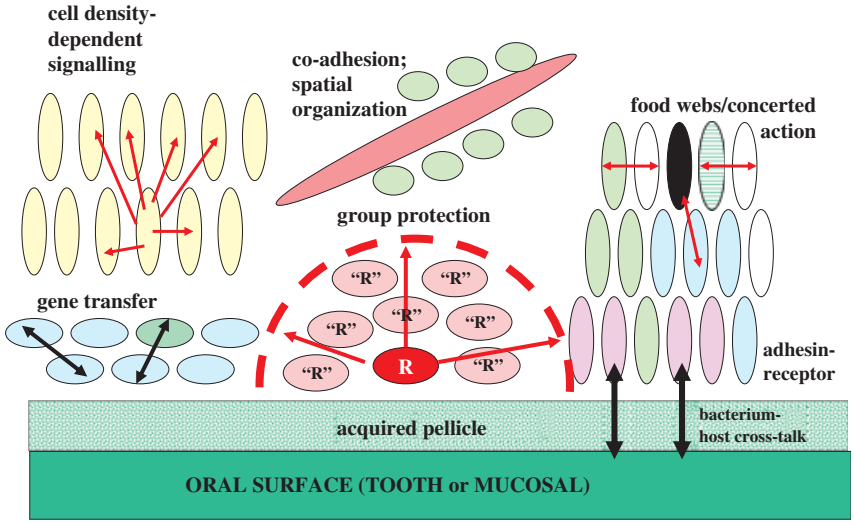

Fig. 1. Schematic representation of the types of interaction that occur in a microbial community, such as dental plaque, growing as a biofilm. Adapted from Marsh & Bowden (2000). Bacteria adhere by adhesin–receptor interactions either to a conditioning film (the acquired pellicle) or to already attached cells (co-adhesion). Bacteria interact synergistically to metabolize complex host molecules, and food webs can develop, enabling the efficient cycling of nutrients. Bacteria communicate via diffusible signalling molecules and by gene transfer; bacteria can also engage in cross-talk if in contact with host cells. Cells in biofilms are less susceptible to antimicrobial agents and the host defences; this may be because of physical properties of the biofilm or to protection from neighbouring cells, e.g. because of the secretion of neutralizing enzymes to making sensitive cells appear resistant (''R''), or following horizontal gene transfer (Marsh & Bowden 2000). The environmental heterogeneity generated within biofilms encourages genotypic and phenotypic diversity, which enhances their ability to persist in the face of assault from the innate and adaptive immune responses, from antimicrobial attack, and from environmental stress (Boles et al. 2004, Costerton 2004).

Concluding remarks

Microbial communities are ubiquitous in nature and usually exist attached to a surface as a spatially organized biofilm. Recent studies suggest that the environmental heterogeneity generated within biofilms promotes accelerated genotypic and phenotypic diversity (even in mono-species biofilms of *P. aeruginosa*) that provides a form of ''biological insurance'' that can safeguard the ''microbial community'' in the face of adverse conditions, such as those faced by pathogens in the host (Boles et al. 2004). This diversity can affect several key properties of cells, including motility, nutritional requirements, secretion of products, detachment, and biofilm formation; this diversity better equips

Table 1. General properties of biofilms and microbial communities

General property	Dental plaque example
Open architecture	Presence of channels or poors
Protection from host defences, dessication, etc.	Production of extracellular polymers to form a functional matrix; physical protection from phagocytosis
Enhanced tolerance to antimicrobials	Reduced sensitivity to chlorhexidine and antibiotics, gene transfer, community effects
Neutralization of inhibitors	β-lactamase production by neighbouring cells to protect sensitive organisms
Novel gene expression*	Synthesis of novel proteins upon attachment; up-regulation of *gtfBC* in mature biofilms
Co-ordinated gene responses	Production of cell–cell signalling molecules (e.g. CSP, AI-2)
Cell–cell signalling	Production of CSP, AI-2, etc.; cross-talk with host epithelial cells
Spatial and environmental heterogeneity	pH and O_2 gradients; co-adhesion
Broader habitat range	Obligate anaerobes in an overtly aerobic environment; environmental heterogeneity in plaque
More efficient metabolism	Complete catabolism of complex host macromolecules (e.g. mucins) by consortia; food webs
Enhanced virulence	Pathogenic synergism in abscesses and periodontal diseases

*A consequence of altered gene expression can also be an increased tolerance of antimicrobial agents.

an organism or community to survive an environmental stress.

Dental plaque represents a classic example of both a biofilm and a microbial community, in that it displays emergent properties, i.e. plaque displays properties that are more than the sum of its constituent members (Table 1). Biofilm formation can have direct and indirect effects on gene expression, organisms can communicate via cell–cell signalling strategies and horizontal gene transfer, and cells display a reduced susceptibility to antimicrobial agents and the host defences. Culture-independent approaches are demonstrating for the first time the complete diversity of the microflora from sites in health and disease and are proving that biofilms provide a heterogeneous environment conducive to the growth of the most fastidious of microorganisms.

The knowledge from these contemporary studies is having a huge impact on our attempts to define the microbial aetiology of plaque-mediated diseases and is challenging current practices for treatment and diagnosis. A paradigm shift away from concepts that evolved from many conventional medical infections, with a simple and specific aetiology, will be needed if we are to (a) fully understand the relationship between plaque bacteria and the host in health and disease, and (b) develop more effective control strategies.

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Address:
P. D. Marsh
Leeds Dental Institute, and Health Protection Agency
Centre for Emergency Preparedness and Response
Porton Down SP4 0JG
UK
E-mail: phil.marsh@hpa.org.uk

Clinical relevance

A review of the scientific literature has shown that dental plaque displays properties typical of a biofilm and a microbial community. Thus, plaque bacteria are bound tenaciously to oral surfaces, and attached cells synthesize extracellular slimes, making them difficult to remove and treat. In biofilms, conditions are conducive for diverse bacterial types to colonize and interact, and these consortia display properties not expressed by the individual species. Two further clinical consequences of plaque being a biofilm and a microbial community are (a) plaque is less susceptible to antimicrobial agents, and (b) the virulence of weakly pathogenic organisms is enhanced (pathogenic synergism).

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