Dental Plaque as a Microbial Biofilm

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Key Words

Biofilm · Antimicrobial agents · Antimicrobial resistance · Gene transfer · Gene expression · Cell signalling · Dental plaque

Abstract

New technologies have provided novel insights into how dental plaque functions as a biofilm. Confocal microscopy has confirmed that plaque has an open architecture similar to other biofilms, with channels and voids. Gradients develop in areas of dense biomass over short distances in key parameters that influence microbial growth and distribution. Bacteria exhibit an altered pattern of gene expression either as a direct result of being on a surface or indirectly as a response to the local environmental heterogeneity within the biofilm. Bacteria communicate via small diffusible signalling molecules (e.g. competence-stimulating peptide, CSP; autoinducer 2); CSP induces both genetic competence and acid tolerance in recipient sessile cells. Thus, rates of gene transfer increase in biofilm communities, and this is one of several mechanisms (others include: diffusion-reaction, neutralization/inactivation, slow growth rates, novel phenotype) that contribute to the increased antimicrobial resistance exhibited by bacteria in biofilms. Oral bacteria in plaque do not exist as independent entities but function as a co-ordinated, spatially organized and fully metabolically integrated microbial community, the properties of which are greater than the sum of the component

species. A greater understanding of the significance of dental plaque as a mixed culture biofilm will lead to novel control strategies.

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Dental plaque can be defined as the diverse community of micro-organisms found on the tooth surface as a biofilm, embedded in an extracellular matrix of polymers of host and microbial origin. There is a high level of interest in the properties of biofilms and microbial communities across all sectors of industrial, environmental and medical microbiology [Allison et al., 2000]. This is because biofilms express properties not exhibited by the same organisms growing in liquid (planktonic) culture, while bacteria are invariably found in nature as part of a consortium, the properties of which are more than the sum of the component species. This review will focus on the significance to oral bacteria of their adoption of a biofilm and microbial community lifestyle, and highlight areas of current controversy and research activity.

Dental Plaque – Existing Perspective

Research over several decades has provided a solid foundation for current studies of oral biofilms. Numerous cultural studies have reported the diversity of the resident oral microflora, both at the genus and species level in health and disease [Newman and Wilson, 1999]. The

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0008-6568/04/0383-0204\$21.00/0

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development of dental plaque has been described in detail (a) on a clean surface over time, (b) in people of different ages, from different countries and diets, and with precise deficiencies in their host defences (acquired and innate), and (c) following various therapies [Percival et al., 1991; Nyvad, 1993; Marsh, 2000a]. The composition of dental plaque also varies on distinct anatomical surfaces (e.g. fissures, approximal and smooth surfaces, gingival crevice, dentures) due to the prevailing physical and biological properties of each site [Bowden et al., 1975; Slots, 1977; Theilade et al., 1982]. Recognition of these environmental influences on plaque composition has led to concepts on disease prevention that have embraced ecological principles [Marsh, 2003].

Dental plaque accumulates preferentially at stagnant sites that afford protection from the vigorous removal forces that apply in the mouth. Distinct phases of development can be recognized, including:

(a) *Adsorption of host and bacterial molecules to the tooth surface.* This conditioning film (the acquired pellicle) forms immediately following eruption or cleaning [Al-Hashimi and Levine, 1989] and directly influences the pattern of initial microbial colonization. Modern techniques offer the opportunity to more fully explore the distribution and composition of pellicle components [Li et al., 2003]. The conformational changes that may occur following adsorption of molecules, and the impact of this on their properties, are now amenable for study: for example, the molecular structure of glucans changes when glucosyltransferases are adsorbed to a surface [Vacca-Smith et al., 1996; Kopec et al., 2001].

(b) *Passive transport of oral bacteria to the tooth surface.* Weak, long-range physicochemical interactions between the microbial cell surface and the pellicle-coated tooth create a weak area of net attraction that facilitates reversible adhesion [Busscher and van der Mei, 1997]. Subsequently, strong, short-range interactions between specific molecules on the bacterial cell surface (adhesins) and complementary receptors in the pellicle can result in irreversible attachment [Jenkinson and Lamont, 1997; Lamont and Jenkinson, 2000] and can explain microbial tropisms for surfaces. Oral bacteria generally possess more than one type of adhesin on their cell surface and can participate in multiple interactions both with host molecules and similar receptors on other bacteria (co-adhesion).

(c) *Co-adhesion of later colonizers to already attached early colonizers.* This stage also involves specific interbacterial adhesin-receptor interactions (often involving lectins) and leads to an increase in the diversity of the bio-

film and to the formation of unusual morphological structures, such as corn-cobs and rosettes [Kolenbrander et al., 2000]. Co-adhesion may also facilitate the functional organization of dental plaque. Bacteria engage in a range of antagonistic and synergistic biochemical interactions [Marsh and Bradshaw, 1999]. The efficiency of metabolic interactions among bacteria in food chains may be enhanced if they are brought into close physical contact. Likewise, the co-adhesion of obligately anaerobic bacteria to oxygen-consuming species can ensure their survival in overtly aerobic oral environments [Bradshaw et al., 1998].

(d) *Multiplication of the attached micro-organisms.* Cell division leads to confluent growth and, eventually, a three-dimensional spatially and functionally organized, mixed-culture biofilm. Polymer production results in the formation of a complex extracellular matrix made up of soluble and insoluble glucans, fructans and heteropolymers. Such a matrix is a common feature of biofilms and makes a significant contribution to the known structural integrity and general resistance of biofilms; the matrix can be biologically active and retain nutrients, water and key enzymes within the biofilm [Allison, 2003]. Further studies are required to fully understand the influence of the matrix on the architecture and properties of dental plaque. When viewed by conventional light or electron microscopy, mature dental plaque appears as a densely packed structure [Listgarten, 1999; Marsh and Nyvad, 2003]; however, the recent application of novel microscopic techniques has demonstrated a more open architecture (see later). Endogenous substrates (derived from saliva or gingival crevicular fluid) are the main source of nutrients for oral bacteria [Beighton et al., 1986], but their catabolism requires the concerted and sequential action of groups of microbes with complementary enzyme profiles [Bradshaw et al., 1994; Marsh and Bowden, 2000], i.e. plaque functions as a true microbial community.

(e) *Active detachment.* Bacteria can respond to environmental cues and detach from surfaces, enabling cells to colonize elsewhere. For example, enzymes produced by sessile bacteria can hydrolyse the specific adhesins that anchor cells to the surface [Cavedon and London, 1993; Lee et al., 1996].

Once established, the resident plaque microflora remains relatively stable over time and is of benefit to the host [Marsh, 2000b]. The resident microflora of all sites plays a critical role in the normal development of the physiology of the host and also reduces the chance of infection by acting as a barrier to colonization by exogenous (and often pathogenic) species ('colonization resis-

Table 1. General properties of biofilms and microbial communities

General property	Dental plaque example
Open architecture	Presence of channels and voids
Protection from host defences, desiccation etc.	Production of extracellular polymers to form a functional matrix
Enhanced resistance to antimicrobials	Increased resistance to chlorhexidine and antibiotics
Neutralization of inhibitors	β -Lactamase production by neighbouring cells to protect sensitive organisms
Novel gene expression	Synthesis of novel proteins; up-regulation of <i>gtfBC</i>
Co-ordinated gene responses	Production of cell-cell signalling molecules (e.g. CSP, autoinducer 2)
Spatial and environmental heterogeneity	pH and O ₂ gradients; co-adhesion
Broader habitat range	Obligate anaerobes in an overtly aerobic environment
More efficient metabolism	Complete catabolism of complex host macromolecules (e.g. mucins) by consortia

tance') [McFarland, 2000]. Mechanisms contributing to colonization resistance include more effective competition for nutrients and attachment sites, the production of inhibitory factors and creation of unfavourable growth conditions by the resident microflora. Thus, treatment should attempt to control rather than eliminate the plaque microflora.

Dental Plaque – Paradigm Shifts

Novel non-invasive and non-destructive microscopic techniques, the publication of annotated microbial genomes (which has facilitated new fields such as functional and comparative genomics, transcriptomics and proteomics), the development of molecular tools (e.g. reporter systems to determine gene activity, oligonucleotide probes to identify and locate specific bacteria via PCR or fluorescent in situ hybridization) combined with laboratory and in vivo biofilm models are changing our understanding of the biology of dental plaque (table 1). Selected topics of current research activity are highlighted below.

Structure of Dental Plaque

Confocal laser scanning microscopy has confirmed that supragingival plaque has a more open architecture (similar to that of biofilms from other habitats) than was suggested by the earlier electron microscopy reports, with channels traversing from the outside of the biofilm to the enamel surface [Wood et al., 2000; Auschill et al., 2001; Zaura-Arite et al., 2001]. Live/dead stains have suggested that bacterial vitality may vary 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], although the fidelity of such stains for viable/non-viable cells is not 100% [Gelle et al., 2003; Hope and Wilson, 2003]. This more open architecture, combined with the synthesis of a matrix comprised of a diverse range of exopolymers, creates a complex environment for predicting the penetration and distribution of molecules within plaque. Uneven patterns of penetration of radiolabelled fluoride, sucrose and phosphate were found in plaque generated naturally on an in situ biofilm model in volunteers [Robinson et al., 1997], while the diffusion of glucans of increasing molecular size was retarded in laboratory mixed culture biofilms [Thurnheer et al., 2003]. Bacterial metabolism ensures that gradients develop in parameters that are critical to microbial growth (nutrients, pH, oxygen); the gradients in pH are also responsible for enamel demineralization. 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 [Vroom et al., 1999]. Such environmental heterogeneity enables micro-organisms to co-exist in plaque biofilms that would be incompatible with one another in a homogeneous environment; this explains how organisms with contradictory metabolic requirements (e.g. in terms of atmosphere, nutrition) persist at the same site.

Bacterial Composition of Dental Plaque

Approximately 50% of cells in plaque (especially from subgingival sites) cannot as yet be cultured in the laboratory. Molecular approaches based on nucleotide sequence analysis have characterized the full diversity of dental plaque [Kroes et al., 1999; Wade, 1999; Paster et al., 2001] and identified a large number of novel taxa [De-whirst et al., 2000]. Improvements in the taxonomy of plaque isolates based on, for example, the unique DNA sequences of the 16S subunit rRNA (16S rDNA) gene

have resulted in the more valid subdivision of existing species. This can also lead to the finer resolution of species into biovars, genotypes or other subgroups, which facilitates better epidemiological studies [Redmo-Emanuelsson et al., 2003] and the possibility of the closer correlation of particular clonal types with disease, as has occurred with *Actinobacillus actinomycetemcomitans* and early-onset periodontitis [Haubek et al., 2001]. These unique sequences can be used as templates for nucleotide probes which, when coupled with a reporter system and used in conjunction with confocal microscopy, can visualize and quantify individual species in natural biofilms, e.g. by fluorescent in situ hybridization [Thurnheer et al., 2001; Kolenbrander et al., 2002]. Pilot studies of plaque developing on removable materials in deep periodontal pockets 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]. These and similar approaches will enable a more complete description of the plaque microflora in health and disease, and also provide data on the location of, and structural interrelationships among, target species in the biofilm [Thurnheer et al., 2001; Kolenbrander et al., 2002]. The recent application of molecular methods to infected dentine has resulted in the detection and identification of previously uncultured bacteria [Munson et al., 2003].

Biofilm Regulation of Gene Expression

Bacteria in biofilms display a phenotype that is distinct from that exhibited by the same cells growing planktonically. The binding of bacteria to specific receptors can trigger significant changes in both bacterial and host cell patterns of gene expression, e.g. following the initial attachment of *Escherichia coli* to uro-epithelial cells [Abraham et al., 1998]. Similarly, there is up-regulation of genes involved with alginate synthesis when *Pseudomonas aeruginosa* colonizes a surface [Boyd and Chakrabarty, 1995].

Similar surface-associated responses are now being identified in plaque bacteria, although the magnitude of this shift in gene expression may be less than that observed in free-living species because of the absolute dependence of oral bacteria on a biofilm lifestyle [Burne, 1998]. The exposure of *Streptococcus 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 and Kolenbrander, 2000], implying similar changes may occur during colonization. Changes in protein profile fol-

lowing attachment have been identified in *Streptococcus mutans* using a whole-cell proteomic approach [Svensater et al., 2001]. Proteins involved in a range of biochemical functions including protein folding and secretion, amino acid and fatty acid biosynthesis, and cell division were up-regulated. 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 and 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-day) biofilms, whereas *ftf* activity was repressed. This was interpreted as an indirect effect of biofilm growth on gene expression, i.e. the altered phenotype was probably due to changes in local environmental conditions within the biofilm (e.g. sugar concentration, pH) rather than due to attachment per se [Li and Burne, 2001]. Thus, biofilm growth can have both direct and indirect influences on gene expression by oral bacteria.

Cell-Cell Communication

In addition to the conventional biochemical and metabolic interactions that have been well catalogued, cells have also been shown to communicate with one another in biofilm communities via small diffusible molecules. Many bacterial species have evolved cell-cell signalling systems (quorum sensing) that help them to adapt and survive various environmental stresses in a cell-density-dependent manner and regulate the expression of genes that also influence their ability to cause disease. In *S. mutans*, quorum sensing is mediated by a competence-stimulating peptide (CSP) [Li et al., 2002b]. This peptide also induced genetic competence in *S. mutans* so that the transformation frequency of biofilm-grown *S. mutans* was 10- to 600-fold greater than for planktonic cells [Li et al., 2001]. 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 [Li et al., 2002b]. 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. CSPs are specific for cells of the same species, but other communication systems may function between different taxa [Kolenbrander et al.,

2002]. Genes encoding autoinducer 2 have been detected in several genera of gram-positive and gram-negative bacteria so that autoinducer 2 may have a broader species range, although its role in plaque remains to be determined. However, mutants of the *luxS* gene that encodes for the autoinducer 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].

Future research will identify more of these sophisticated communication networks, and it has been suggested that analogues of the signalling molecules could be used as novel therapeutic agents to manipulate the properties of biofilms.

Gene Transfer

Cells also communicate 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 in model biofilms has been demonstrated [Roberts et al., 2001]. The recovery of resident (*S. mitis*, *S. oralis*) and pathogenic (*S. pneumoniae*) bacteria from the nasopharynx 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]. 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].

Antimicrobial Resistance

A major finding of clinical relevance with respect to micro-organisms growing on a surface is their increased resistance to antimicrobial agents [Gilbert et al., 1997, 2002; Ceri et al., 1999]. For example, *P. aeruginosa* growing on urinary catheter material can be 500–1,000 times more resistant to antibiotics than the same cells growing in liquid culture. Conventionally, the sensitivity of bacteria to antimicrobial agents is determined on cells grown in liquid culture by the measurement of the minimum inhibitory concentration or minimum bactericidal concentration. Given the decreased sensitivity of an organism on a surface, it has been argued that it would be more appro-

priate to determine the 'biofilm inhibitory concentration' (also described as the 'biofilm eradicating concentration' or biofilm killing concentration) [Anwar et al., 1990; Nichols, 1994; Shani et al., 2000; Johnson et al., 2002]. As yet, however, these proposals have not been widely accepted, and there are no generally agreed standardized methods by which these concentrations could be determined.

Bacteria growing in dental plaque also display increased resistance to antimicrobial agents, including those used in dentifrices and mouth rinses [Marsh and Bradshaw, 1993; Kinniment et al., 1996; Wilson, 1996; Pratten and Wilson, 1999]. For example, the biofilm inhibitory concentration for chlorhexidine and amine fluoride was 300 and 75 times greater, respectively, when *S. sobrinus* was grown as a biofilm compared with the minimum bactericidal concentration of planktonic cells [Shani et al., 2000]. Similarly, it was necessary to administer 10–50 times the minimum inhibitory concentration of chlorhexidine to eliminate *S. sanguinis* (previously *S. sanguis*) biofilms within 24 h [Larsen and 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 and Wilson, 1989]. Confocal microscopy of in situ established natural biofilms showed that chlorhexidine only affected the outer layers of cells in 24- and 48-hour plaque biofilms [Zaura-Arite et al., 2001]. Biofilms of oral bacteria are also more resistant to antibiotics (e.g. amoxycillin, doxycycline, metronidazole) [Larsen, 2002; Larsen and Fiehn, 1996].

The mechanisms behind the increased resistance of biofilms to antimicrobial agents are the subject of much research and debate [Gilbert et al., 2002]. Cells can become resistant due to mutations affecting the drug target, the presence of efflux pumps or to the production of modifying enzymes etc., but even innately sensitive bacteria become resistant when growing on a surface. The structure of a biofilm may restrict the penetration of the antimicrobial agent; some charged inhibitors can bind to oppositely charged polymers that make up the biofilm matrix (diffusion-reaction theory). The agent may also bind to and inhibit the organisms at the surface of the biofilm, leaving cells in the depths of the biofilm relatively unaffected. As stated earlier, bacteria growing on a surface display a novel phenotype, and this can result in a reduced sensitivity to inhibitors, while the transfer of resistance genes can occur more readily in biofilm communities such as dental plaque. 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]. The matrix in biofilms can also bind and retain neutralizing enzymes (β -lactamase, IgA protease; see above) [Allison, 2003]. At present, it is not clear whether some or all of these effects account for the observed resistance of cells in plaque biofilms.

Plaque as a Community

The evidence outlined above 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 and Bradshaw, 1999]. Benefits of a community lifestyle to micro-organisms include: (a) a broader habitat range for growth, e.g. oxygen-consuming species create environmental conditions suitable for obligate anaerobes; (b) a more efficient metabolism, e.g. complex host macromolecules can only be degraded by consortia of oral bacteria; (c) increased resistance to stress and antimicrobial agents, and (d) enhanced virulence ('pathogenic synergism') [Caldwell et al., 1997; Shapiro, 1998; Marsh and Bowden, 2000] (table 1). Microbial community effects can render a sensitive organism as apparently 'resistant' to an antibiotic if neighbouring, non-pathogenic cells produce a neutralizing or drug-degrading enzyme ('indirect pathogenicity'). This has been demonstrated in animal models where a penicillin-sensitive pathogen (*S. pyogenes*) is protected by a β -lactamase-producing commensal strain (*Moraxella catarrhalis*) and, as a result, is still capable of causing a lethal infection [Hol et al., 1994]. In the mouth, gingival crevicular fluid can contain sufficient β -lactamase to inactivate the concentrations of antibiotic delivered to the site [Walker et al., 1987; Herrera et al., 2000].

Future Developments

A greater understanding of the significance of dental plaque as a mixed species biofilm will have the potential to impact significantly on clinical practice. Novel areas for future research include:

(a) the development of inhibitors and antiplaque agents that are more effective against surface-associated micro-organisms, coupled with more effective delivery systems for targeting specific bacteria and for improving the retention of agents in the mouth; this will require the development and use of high throughput biofilm models to screen novel compounds not only for their ability to kill or inhibit sessile cells, but also to promote biofilm detachment;

(b) interference with communication networks that co-ordinate or regulate microbial activities within biofilms; in other areas of microbiology (e.g. *P. aeruginosa* and cystic fibrosis), attempts are being made to block signalling molecules that induce a shift in the host to a more virulent phenotype;

(c) preventing colonization of selected organisms (e.g. by interfering with attachment by modifying the conditioning film or by 'replacement therapy', whereby organisms are deliberately implanted to prevent subsequent colonization by more pathogenic species) [Hillman, 1999; Tagg and Dierksen, 2003];

(d) affecting biofilm architecture, for example, by the use of enzymes that can degrade the exopolymers that comprise the plaque matrix;

(e) the neutralization of parameters that select for the species that are implicated in disease [Marsh, 2003]; thus, strategies that reduce the pH response to dietary carbohydrates will help prevent the enrichment of acidogenic and aciduric bacteria;

(f) the identification of pathogenic clones could also improve diagnosis and might predict sites that are more susceptible to disease.

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