The Biofilm Lifestyle

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THE BIOFILM LIFESTYLE

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It is a distinct pleasure to address the dental research community on the subject of microbial biofilms because it was this same community, more years ago than I care to admit, that inspired many of our initial thoughts in this area. Gibbons and van Houte, of the Forsyth Dental Center, had already addressed that most obvious and troublesome biofilm—dental plaque—in direct observations and elegant experiments, before we began our own oddessey, and our early 1978 article in Scientific American (Costerton *et al.*, 1978) made liberal use of their ideas. Their successors have consistently led the microbial ecology sector of the biofilm research area, because their central biofilm of interest is composed of an especially rich variety of different bacterial morphotypes and bacterial species of different metabolic predilections. In this short review, it is our objective to summarize progress in the general area of biofilm research and to extract from that summary items that may be of particular interest to the dental community.

In a recent review in *Annual Reviews of Microbiology* (Costerton *et al.*, 1995), we defined bacterial biofilms as "matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces". In this same review, we gathered and summarized biofilm research from dozens of specific programs in which subsets of the biofilm research community focus their efforts on biofilms of special interest in certain medical, industrial, or environmental systems. Our objective was to take the same approach as the organisms themselves, which show no obvious regard for anthrocentric points of view and simply make themselves as safe and as comfortable as possible by adhering to available surfaces and forming biofilms in virtually all aquatic systems. Similarly, we have sought to understand the basic advantages of the "biofilm lifestyle" for bacteria growing in any and all aquatic ecosystems.

Key words: Biofilm, bacteria, plaque, periodontitis.

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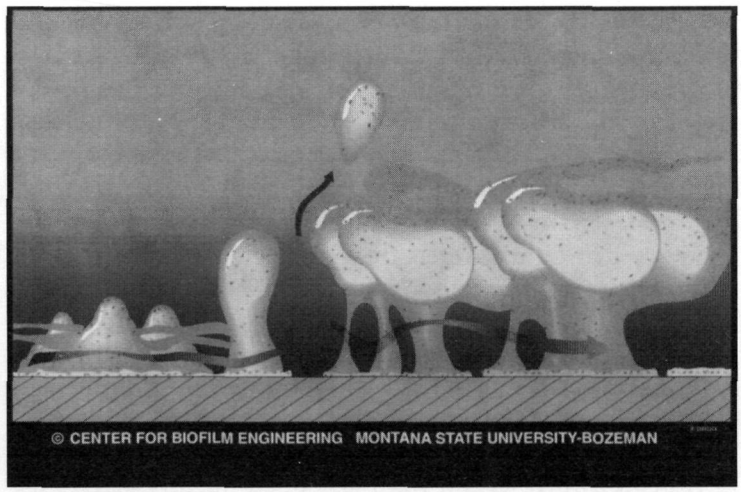
THE STRUCTURE OF BIOFILMS

Direct observations are the "gold standard" in examinations of natural biological phenomena, because preserved specimens have been shown to be severely compromised by dehydration and because extrapolation from culture data has led us astray so often in Microbiology. Accordingly, we have concentrated heavily on the use of the Confocal Scanning Laser microscope (CSLM) in the examination of biofilms (Lawrence *et al.*, 1991). The CSLM allows us to observe living, fully hydrated, bacterial biofilms in real time and with the many advantages of sophisticated image analysis. These examinations of single-species laboratory biofilms, as well as of mixed-species biofilms formed in natural environments, have clearly shown that many if not most bacterial biofilms are composed of microcolonies of cells enveloped in a dense exopolysaccharide (EPS) matrix interspersed with remarkably open water channels. The image presented in the Figure is actually the summary of hundreds of digital images collected in the laboratory and in nature, and it clearly shows that biofilm bacteria actually live in EPS-enclosed microcolonies that may be deformed by fluid forces at the biofilm-bulk water interface and may even detach if fluid forces exceed their tensile strength. Some workers, whose equipment does not yield high resolution, have reported that bacteria grow in "stacks" within biofilms, but they also report seeing ramifying water channels much like the ones we see extending down to the colonized surface and throughout the biofilm itself.

The ramifying water channels of the biofilm are really the structural reciprocal of the microcolonies themselves, and we have recently discovered (DeBeer *et al.*, 1994a, b) that bulk fluid enters this channel system and moves, by convective flow, throughout its very considerable extent. Polystyrene beads (0.3 microns) that become entrained in water channels move rapidly and smoothly by convective flow, and it is clear that no structural component of these open passages constitutes a barrier to their passage. We give special currency to these direct observations of living biofilms, and we conclude that some form of the microcolony/water channel structure is seen in most, if not all, bacterial biofilms. It is not obvious that water channels would ramify throughout the very considerable depth of dental plaque, but some workers report seeing open channels, and studies of

these structures will help to relate dental biofilm research to biofilm research in other disciplines. The microcolony/water channel architecture of biofilms suggests the possibility of very pronounced structural heterogeneity within these sessile microbial communities, and recent work by Lewandowski's group (Lewandowski *et al.*, 1994) extends this concept to physiological heterogeneity. DeBeer *et al.* (1994a, b) have measured dissolved oxygen concentrations at various locations within biofilms, using a very small (10 micron) dissolved oxygen microelectrode, and they have discovered that bacteria in the center of a microcolony may live in complete anaerobiasis, while other bacteria only 100 microns away and at the edges of adjacent water channels may live aerobically. Clearly, access to nutrients and the removal of waste products also depend on the position of a given cell within the biofilm, and this structural heterogeneity provides a whole series of "customized microniches" within which cells of very different physiological persuasions can live happily in the same biofilm (Costerton *et al.*, 1994).

Growth within a biofilm also provides a stable juxtaposition between cells of physiologically cooperative bacterial species within biofilms (Wolfaardt *et al.*, 1994) and thus facilitates metabolic cooperation and interspecies substrate transfer. We believe that metabolically cooperative microbial consortia predominate in natural biofilms, and we have suggested (Costerton *et al.*, 1987) that the complex structure of these sessile communities may both accommodate and reflect the cellular juxtapositions necessary to facilitate these interactions. It is interesting to reflect on these matters in the context of dental plaque, and of bacterial biofilms in peridontal pockets, in that we isolate a bewildering array of different bacterial species from these biofilm communities. When we have studied equally complex biofilms living in equally rich nutrient environments, such as the bovine rumen (Costerton *et al.*, 1987), we have found that virtually all of the bacteria in these systems are functional members of highly coordinated physiological consortia. If dental biofilms are not fundamentally different from complex biofilms in other nutrient-rich ecosystems, we would expect that most cells are members of consortia in which their metabolic activities are facilitated by juxtaposition with cooperative cells that provide suitable nutrient substrates and/or remove waste products. When this happens in rumen biofilms, metabolic processes such as cellulose degradation proceed at rates as much as 100X those seen in the absence of consortia (Costerton *et al.*, 1987), and very steep gradients of organic acids develop because their production exceeds their diffusion. This metabolic acceleration, and this local concentration of organic acids and other corrosive products, may account in large measure for the role of biofilms in the focused attack of micro-organisms on insoluble substrata (Costerton, 1992). We have studied this process of focal bacterial attack on many substrata, from stainless steel to wool, and we find that biofilms provide the mechanism for the concentration of corrosive chemicals at a surface because their consortia can produce these molecules faster than they can diffuse through the biofilm matrix and because the biofilm matrix holds these



molecules close to the surface being attacked. Bacteria growing free in the bulk fluid really have no means of focusing an attack on a surface. They may eventually change the pH of the bulk fluid sufficiently to cause the erosion of surfaces, or they may gradually build up enzyme concentrations to a point where generalized surface degradation occurs, but they cannot attack with any degree of efficiency. Biofilms, on the other hand, develop internal "concentration cells", develop local concentrations of corrosive molecules and/or protons, and produce enzymes in direct contact with the substratum to be attacked (Costerton, 1992). It is therefore not at all surprising that bacteria within biofilms are capable of degrading substrata as resilient as stainless steel by a local attack that characteristically produces "pits. Parallels in the dental field are obvious.

PHENOTYPIC CHANGE

One of the most profound discoveries in biofilm research in the past decade was not anticipated in the literature, and it is still not widely understood outside of the microbial genetics community. Using reporter gene constructs from Chakrabarty's and Deretic's laboratories, Davies *et al.* (1993) and Hoyle *et al.* (1993) were able to show that cells of *Pseudomonas aeruginosa* "up-regulate" certain genes in the alginate (EPS) synthesis pathway as they adhere to surfaces. This finding was intuitive, because EPS is used by the bacteria to adhere and to form biofilms, and Davies et al. were able to follow individual cells by CSLM as they adhered and began to produce EPS in preparation for biofilm construction. What was not intuitive was the finding by Deretic's group that this surface-associated up-regulation of EPS synthesis genes was only a small part of a major sigma-factor-directed phenotypic change (Deretic *et al.*, 1990, 1994; Martin *et al.*, 1994) that alters as many as 60 cell envelope proteins in *P. aeruginosa* and may profoundly affect cell permeability as well as biofilm development. This new development in biofilm research is very disturbing to those of us who rely on extrapolation from laboratory cultures to mixed-species biofilms in natural environments. What this new finding means is that a planktonic bacterial cell in a

single-species pure culture is profoundly phenotypically different from a cell of the same genotype growing in a biofilm on a surface. The cell itself will have different enzymes and different cell wall proteins. They are "apples" and "oranges". They must be expected to have different metabolic activities, different adhesion properties, and different sensitivity to antibacterial agents. In fact, the genotype can be recovered from the living wild biofilm by traditional culture methods, and all of the genes will be present and intact. When the cells are grown as planktonic cells in culture, certain genes will be expressed. When the cells are grown in single-species biofilms in a defined medium, a totally different set of genes will be expressed, and some of these genes will be the same as those expressed in oral biofilms. When the cells grow in mixed-species biofilms in the mouth, a complex set of interspecies stimulations will come into effect, and specific gene expression can be detected only by combining specific probes and reporter systems with CSLM. Let us consider a hypothetical situation in which researchers have developed a compound that binds to a certain cell wall protein and kills cells of a particular bacterial species implicated in dental problems. This compound is effective in *in vitro* tests with planktonic cells of pure cultures of several species of common dental bacteria, and several millions of dollars have been spent. The compound has no effect at all on cells of the same genotype growing in biofilms in the laboratory or in the mouth, and we find that the cell wall protein in question is simply not produced in these bacteria after they adhere and adopt the biofilm phenotype. Perhaps it is time to begin, in dental research, by studying important dental organisms in biofilms rather than in the traditional planktonic cultures! Even more may be gained if we study whole natural dental biofilm communities using the modern tools of specific chemical and physical probes and CSL microscopy.

RESISTANCE TO ANTIBACTERIAL AGENTS

Now that we understand the structure of bacterial biofilms to some extent, and now that we realize that biofilm bacteria are profoundly phenotypically different from their planktonic counterparts, we are less surprised by the remarkable resistance of cells in these sessile communities to a wide variety of antibacterial agents. When this resistance was first documented (Nickel *et al.*, 1985), and shown to be from 500 to 1500X that of planktonic cells of the same species, it seemed intuitive to attribute it to diffusion resistance (Costerton *et al.*, 1987). However, this position was naïve, in that measured diffusion resistance values are not nearly high enough to account for this level of resistance (Nichols *et al.*, 1988), and now it seems logical to explain this really phenomenal resistance by invoking the altered phenotypic state of biofilm cells and differences in growth rates among the different bacteria in different "niches" within the structurally complex biofilm. The Center for Biofilm Engineering at Montana State University has developed a truly innovative method for the study of the killing of biofilm bacteria by antibacterial agents. This method is based on direct observation, in that a fluorescent probe (CTC) is used to distinguish respiring from non-respiring bacteria, and we are able to follow cell death within a biofilm during disinfection using antibacterial chemicals such as biocides (Yu and McFeters, 1994).

The use of a similar fluorescent probe (70-kDa polyanionic TRITC-dextran) has shown that the penetration of antibacterial agents is radically enhanced by the application of DC fields to produce the "bioelectric effect" (Blenkinsopp *et al.*, 1992), which allows these agents to kill biofilm bacteria completely at very low concentrations. Limitations on the use of color micrographs preclude the presentation of these data here, but both data and methods were presented at the American Society for Microbiology biofilm meeting in Snowbird, Utah (1996).

We believe that these direct methods for the direct visualization of bacterial killing within biofilms will be especially valuable in the field of Dental Microbiology.

CONCLUSION

In this review, we have attempted to adapt material published in the *Annual Reviews of Microbiology* (Costerton *et al.*, 1995) to the interests of the dental research community. The reader is referred to the original review for the data on which our conclusions are based, and for more detail concerning the phenomenal structural complexity of bacterial biofilms.

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