Structure of biofilms

Authors: J. William Costerston

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Section I
BIOFILM STRUCTURE AND FUNCTION

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Structure of Biofilms

J. W. Costerton

I. INTRODUCTION

When natural, industrial, and medical ecosystems were examined by direct microscopic methods, it soon became apparent that most of the bacteria in aquatic systems grow in biofilms attached to available surfaces. Some of these biofilms were seen to be thick and highly structured, as in algal "mats" and dental plaque, and some were seen to be simple accretions of microcolonies of bacterial cells embedded in a hydrated exopolysaccharide matrix. This initial realization[1] that biofilms were the predominant mode of bacterial growth in most aquatic systems, including many of great interest and importance to man, came as a profound shock to microbiologists who had, collectively, been studying cells in the planktonic mode of growth for more than 200 years. The importance of the recent American Chemical Society Symposium on Biofouling/Biocorrosion in Water Systems depends, in large measure, on the extent to which bacterial cells in biofilms differ from planktonic cells of the same species floating in the bulk fluid. If biofilm cells and planktonic cells are structurally and functionally identical, the study of biofilms becomes a somewhat esoteric and scholarly exercise. If biofilm bacteria are substantially different from planktonic bacteria, most of the fundamental concepts of microbiology must now be reexamined because these concepts have been based entirely on the study of planktonic cells. If the differences between planktonic cells and biofilm cells are profound, we must expect to be able to explain many microbial phenomena that

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have not yet been rationalized because the old microbiological model — the planktonic cell growing in monospecies culture in a rich medium — does not reflect reality. Because microbiology involves the study of very small organisms, it has necessarily been extrapolative, and extrapolation from a model to reality is only accurate if the model approximates reality.

It is entirely fitting to highlight the importance of choosing models that approximate reality, which was the contribution made by Bill Characklis to the study of biofilms. As an engineer, Bill was inherently free of the preconceptions and conventions of the traditional microbiologist, and he had a natural instinct for direct examination of real systems; therefore, it was to Bill and his precise engineering methods that founding members of the biofilm group turned for quantitation and confirmation of direct observations.

II. THE STRUCTURE OF BIOFILMS

While thin accretions of bacteria on glass surfaces can readily be examined by light microscopy, fully formed biofilms were too complex and too refractile to yield useful images by this method.

For this reason, we relied on electron microscopy for our initial explorations of biofilm structure. Electron microscopy was informative only if the viewer could mentally rehydrate the images, because both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) require the rigorous removal of water from the specimen, and the biofilm matrix is 99% water.[2] However, TEM images of bacterial biofilms (Figure 1) showed condensed residue of the biofilm matrix that invoked the living hydrated structure, and SEM images showed relatively rigid bacterial cells among the condensed amorphous remnants of a biofilm matrix (Figure 2) that could be mentally rehydrated. These images of biofilms, which are collected, described, and interpreted in a 1979 review,[3] allowed one to deduce the presence of bacterial cells and a hydrated matrix, and most biofilm diagrams of that era show these cells randomly distributed through a homogenous hydrated matrix. We guessed that bacteria and algae formed microcolonies within the homogenous matrix (Figure 3), but hard spatial data were lacking.

The modern use of the confocal scanning laser (CSL) microscope has allowed the examination of hydrated biofilms by a totally nonintrusive technique that yields[4] clean, three-dimensional images (Figure 4) of living biofilms in real time. This revolutionary new technique has shown[5] that more of the volume of bacterial biofilms is occupied by matrix ($\pm$75 to 95%) than by bacterial cells ($\pm$5 to 25%) and that these cells may be concentrated either in the lower or upper regions of these biofilms. Detailed spatial examinations[6] have shown that cells of many species

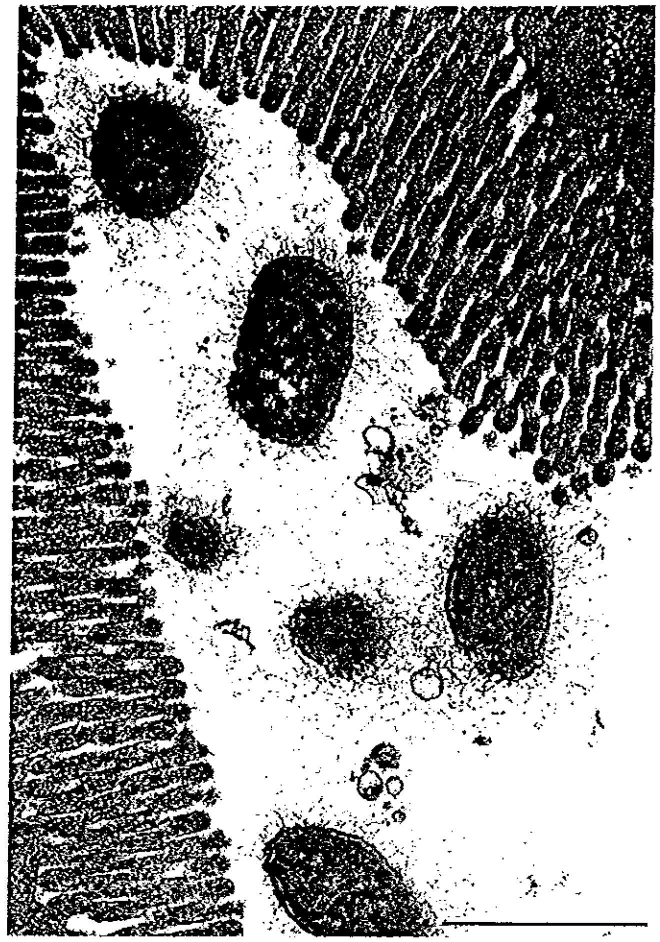

FIGURE 1. Transmission electron micrograph of a ruthenium red-stained preparation of cells of *Escherichia coli* that had formed a biofilm on the microvillar surface of the gut of a calf. Note the dehydration-condensation of glycocalyces to produce radial patterns of fibrils in regions that would be fully occupied by the polysaccharide matrix in its living hydrated state. The bar indicates 1.0 μm.

tend to form microcolonies within the biofilm and that the areas of the matrix between these aggregates of sister cells may be occupied by "water channels" within which the matrix is less dense (Figure 5). Because CSL microscopy allows the continuous observation of living biofilms, Lawrence and colleagues[7] have been able to monitor the initial stages of biofilm formation and to describe how the different post-adhesion "behaviors" of cells of various species produce local aggregates of cells that may become well-defined microcolonies within mature biofilms.

While the study of monospecies biofilms allows us to determine the surface colonization strategies of a particular species, we must remember that single-species surface populations are exceedingly rare in nature. When we examined a wide variety of natural biofilms, we noticed that the microcolonies of particular morphotypes (e.g., curved rods) were

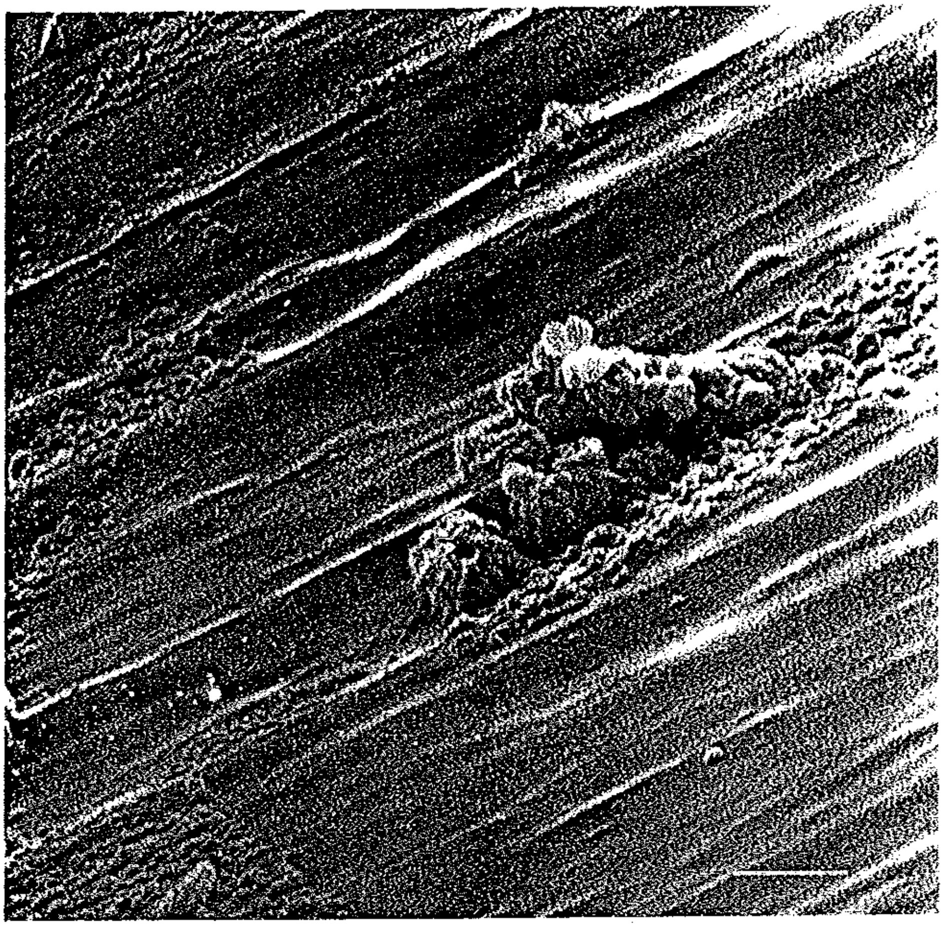

FIGURE 2. Scanning electron micrograph of a critical-point, dried preparation in which a microcolony of cells of *Staphylococcus aureus* had formed on the battery pack of a cardiac pacemaker. Note the dehydration-condensation of the matrix material that would have entirely covered the rigid bacterial cells in their living hydrated state. The bar indicates 5 μm.

often juxtaposed to microcolonies of another morphotype (e.g., a filamentous cyanobacterium) within the mature biofilm. The frequency and intimacy of these associations led us to suggest[8] that cells of one species may associate with those of another species for mutual physiological benefit. Since that time, the concept of microbial consortia has emerged, and this exciting perception is intimately bound up with biofilms because it is only in stable, multispecies aggregates that the spatial relationships necessary for physiological cooperation can be established and maintained. It is obvious that physiological cooperation between planktonic cells of different species floating past each other in the bulk fluid is difficult to sustain, but we can readily visualize how similar cells immobilized in adjacent, or even mixed, microcolonies within a biofilm could initiate and sustain the cooperative exchange of metabolic substrates and products. Two properties of the bacterial cell equip it perfectly

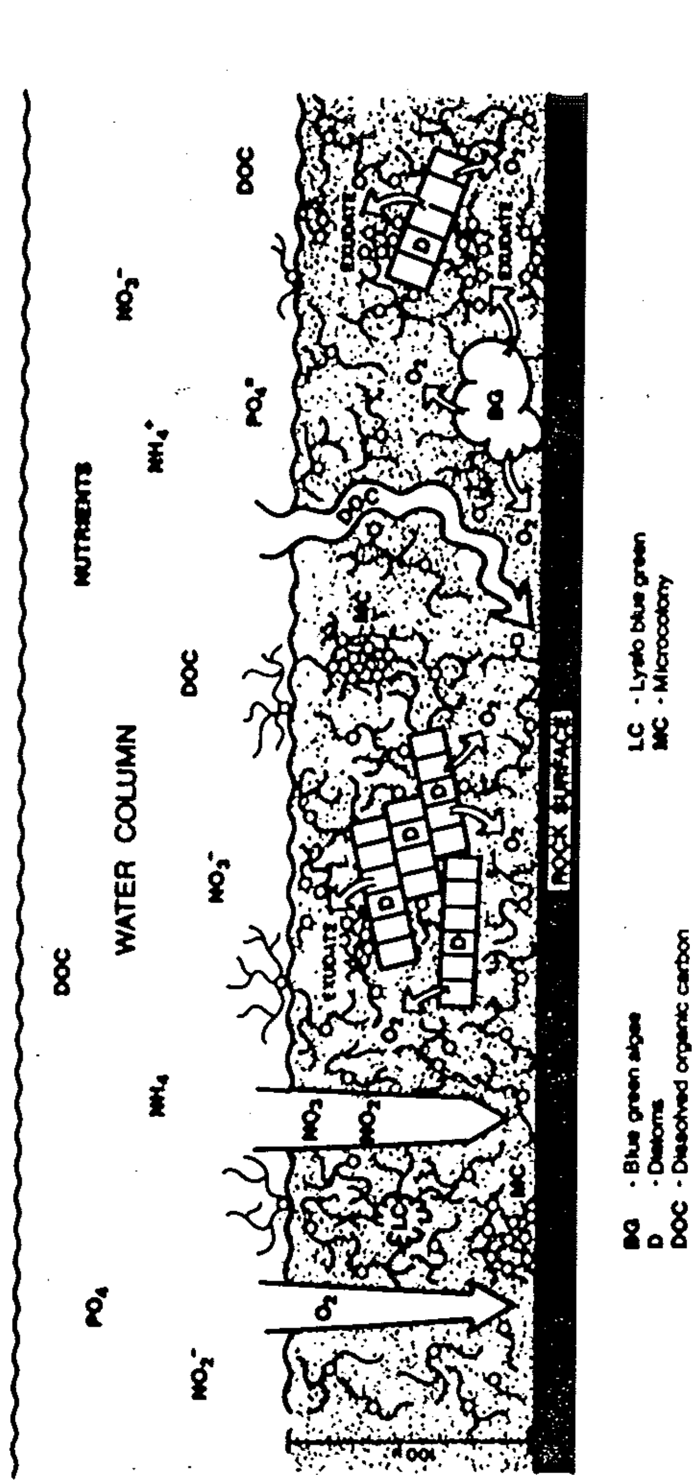

FIGURE 3. Diagrammatic representation of a mixed natural biofilm in which bacteria and algae are present as microcolonies within a homogeneous matrix.

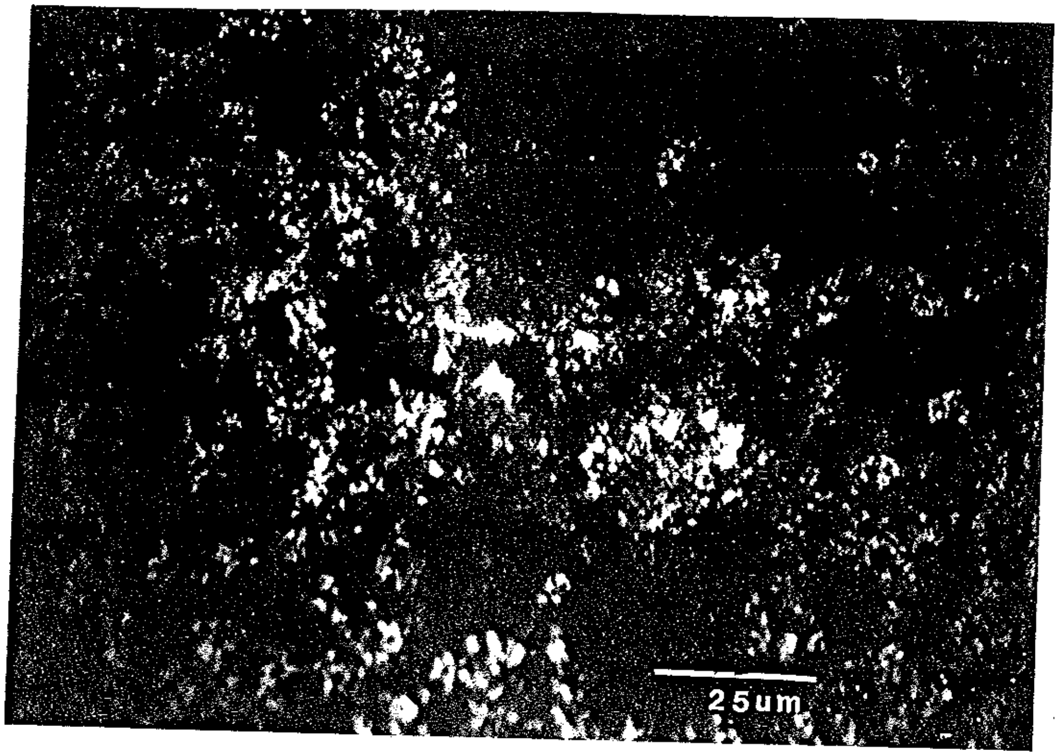

FIGURE 4. Confocal scanning laser micrograph showing the development of microcolonies and water channels within a 24-h biofilm formed by *Escherichia coli*.

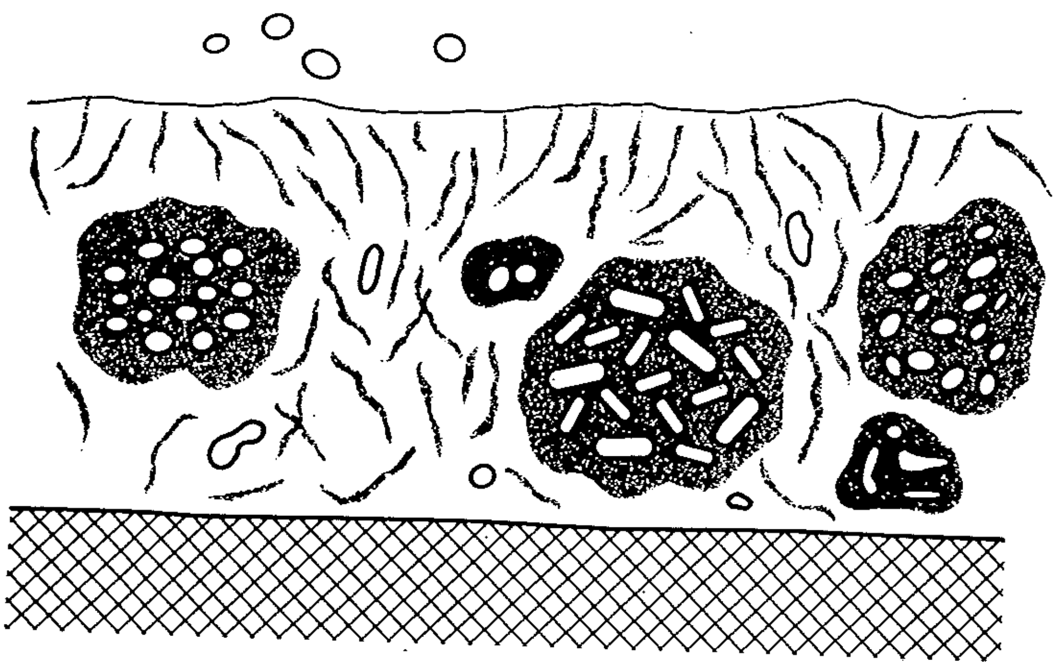

FIGURE 5. Diagrammatic representation of the confocal scanning laser microscope data showing that most biofilm bacteria grow in microcolonies surrounded by dense matrix material interspersed between water channels within which the matrix material is less dense.

for life in the complex, highly structured, physiologically cooperative world of the biofilm — binary fission and phenotypic plasticity.

III. BINARY FISSION AND PHENOTYPIC PLASTICITY

Organisms that reproduce exclusively by sexual process are essentially restricted by their environments, and a shallow pond that freezes to near the bottom may offer poor fishing even though the ecosystem teems with trout food on a brilliant summer day. A finite number of propagules survive the least permissive state of the environment and these survivors can fatten in response to positive changes in the environment, but they cannot increase their number until the next breeding cycle. In contrast, bacteria can respond to positive changes in their environment in a matter of minutes, and reproduction by asexual binary fission persists until the environment again becomes limiting. Oligotrophic aquatic systems are heavily populated with ultramicrobacteria[9] that are dormant because of nutrient limitation. When nutrients suddenly enter the ecosystem, the dormant bacteria resuscitate[10] and adhere avidly to this source of organic nutrients, eventually forming biofilms of macroscopic proportions. At the cellular level within a biofilm, a bacterial cell that finds itself in a nonpermissive niche simply persists as a single cell, while another identical cell that encounters permissive conditions proliferates to produce a microcolony. Figure 6 illustrates the response of two identical cells of a hypothetical bacterium that find themselves in different microenvironments. In part A, the cell persists in spite of nutrient limitation, while in part B, the cell is provided with an essential organic substrate by its proximity to a neighboring microcolony of cooperative organisms and proliferates, favoring the nutrient gradient, until it forms a functional consortium within the biofilm. An extensive morphological survey of the highly structured biofilms found in natural and industrial aquatic systems clearly indicates that the vast majority of sessile bacteria grow within microcolonies that are juxtaposed to microcolonies of cooperative organisms or to the surface of a nutritive substrate.[8] It is the property of rapid reproduction by binary fission, in response to favorable changes in the microenvironment, that builds the remarkably efficient consortia seen in biofilms.

Because the small size and mode of growth of bacterial cells generally deny them homeostasis, the cells must respond to rapidly changing environments in order to survive. The extreme phenotypic plasticity exhibited by bacteria remained unknown by microbiologists for centuries because of the custom of removing organisms from complex natural biofilms and growing them as Pasteur did, as planktonic cells in monospecies cultures in rich media. Brown and Williams[11] showed that they

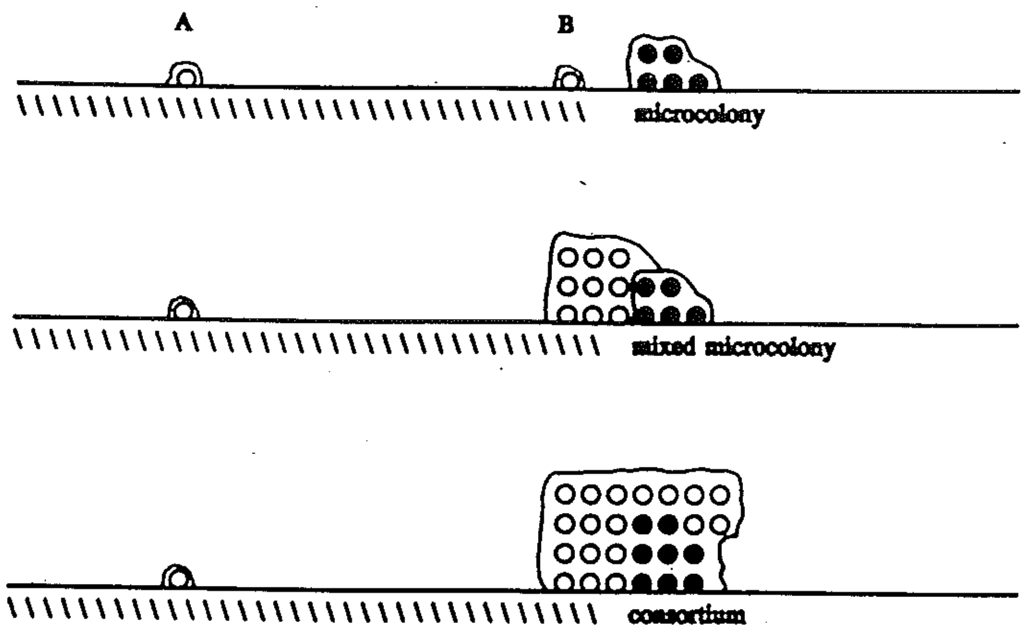

FIGURE 6. Diagrammatic representation of the different responses of two identical, newly adherent cells that find themselves in (A) a nutrient-poor surface area and (B) a surface area supplied with nutrient by a microcolony of a physiologically cooperative organism. The progeny of the cell at B eventually form a mixed microcolony, and then a functional consortium, with the co-operative organism.

could take virtually any microbial genotype, including the redoubtable *Escherichia coli* K12, and alter most of its physiological processes and even the structure of its cell wall by altering its environment within a chemostat. This remarkable plasticity in response to changing environmental conditions is achieved by rapid, directed, phenotypic adaptation, in contrast to slow random changes in the genotype, and this property of bacteria serves them very well in growth within biofilms. Marshall's group[12] has now shown that adherence to a surface itself constitutes an environmental change that depresses certain genes and triggers the production of specific gene products, and Brown et al.[13] have noted profound phenotypic differences between planktonic and biofilm cells of the same genotype.

These two bacterial properties — reproduction by binary fission and phenotypic plasticity — introduce a phenomenal, inherent heterogeneity into bacterial biofilms that stands in stark contrast to the relative homogeneity of planktonic cells floating or swimming in a bulk fluid. Biofilm bacteria are so responsive to changes in their microenvironments that some cells of a particular genotype (species) may be effectively dormant, while other cells of the same genotype living only a few microns away may be proliferating wildly (see Figure 6) and assuming phenotypic forms that differ profoundly from their dormant counterparts and even more profoundly from planktonic cells of the same species. These statements are not extrapolative, but are based on direct structural and physiological observations of living natural systems. We can now begin to question the usefulness of continuing conventional microbiological studies of planktonic bacteria in monospecies cultures in rich media when the intent of the researchers is to extrapolate their data to explain microbial processes in natural, industrial, and medical ecosystems.

IV. DIFFERENCES BETWEEN BIOFILM CELLS AND PLANKTONIC CELLS

The direct demonstration that most bacteria in aquatic systems live in highly structured biofilms and the perception that bacteria are capable of reacting very precisely to microenvironments within these complex sessile communities suggest that biofilm bacteria may differ very substantially from planktonic organisms of the same genotype. As in all sweeping generalizations, it may be useful to examine individual factors that contribute to these potential differences and to attempt a correlation of their cumulative effects in a summary.

A. Adhesion Events

Many microbiologists have attempted to model the process of bacterial adhesion using physical principles that apply to the adhesion of inert colloidal particles to surfaces, but these data have not been useful in understanding the adhesion of wild strains of bacteria in natural ecosystems. This is because wild bacteria are not regular spheres and rods, but are "hairy" cells whose actual surfaces are composed of carbohydrate (glycocalyx) and protein (pili and flagella) appendages, and because bacteria are capable of "sensing" the surface and responding by phenotypic change and by specific behaviors.[4] The responses of bacteria during the process of adhesion range from the activation of the algC gene by *Pseudomonas aeruginosa*[14] to the complete loss of flagella and the initiation of stalk formation by *Caulobacter crescentus*. Furthermore, in the short period immediately following adhesion, bacterial cells carry out lateral movements[7] involving specific patterns of exopolysaccharide secretion and which are species specific and clearly related to their new microenvironment.

B. Matrix Effects

The chemical nature of the biofilm matrices produced by bacteria varies widely between species,[2] but most are carbohydrate in nature and many are highly anionic, uronic, acid-containing polymers. The production and extrusion of these polymers is a very exergonic process that involves many genes whose clustering and conservative patterns of regulation suggest that their cumulative product — the matrix — is extremely important to the survival and success of the species. Planktonic cells may be surrounded by relatively small exopolysaccharide glycocalyces, but cells of most species have been shown to elaborate very large amounts of this highly hydrated exopolymer during and immediately following adhesion.[7] Within minutes of the initiation of colonization, adherent bacteria can be seen to be surrounded by matrix material and, within hours, matrix-enclosed microcolonies are formed that eventually coalesce to produce confluent biofilms.

The loss of Brownian movement is one of the first visible signs that a bacterial cell has adhered to a surface or has been entrapped in a biofilm. The immobilization within an ionic polymer radically changes the state of the surface of a cell that has heretofore been free-floating in bulk fluid. Instead of the relatively free diffusion of molecules and ions to and from the cell surface of a mobile cell, the biofilm cell experiences the effects of a matrix that closely resembles an ion-exchange resin. The biofilm cell is stationary within a fibrous matrix that extends in all directions within which large amounts of ions (including divalent cations) and structured water are associated. Molecules and ions that would readily approach planktonic cells must satisfy all of the spatial and chemical requirements of the matrix in order to even approach the biofilm cell. Similarly, molecules and ions produced by the biofilm cell must satisfy the same criteria to diffuse away from the cell, and some of these molecules and ions may be retained as a "halo" around the biofilm cell by virtue of their size or charge density. The direct observation of a ± 0.5-μm zone of reduced pH around living cells in a biofilm[6] may indicate that protons extruded by these bacterial cells form a "halo" in the vicinity of the biofilm cells, whereas they would be lost to the menstruum by planktonic bacteria. In this sense, a biofilm cell can be considered to live in a microniche that is defined by the biofilm matrix and partially conditioned by its own metabolic activity. Further heterogeneity is introduced into the matrix component of the microniche in which biofilm bacteria grow by the fact that the physical nature of the matrix may change from a sol to a gel when the concentration of Ca^{2+} in the matrix-associated water is raised.[15] This Ca^{2+}-mediated physical change in the alginate matrix of biofilms of *P. aeruginosa* radically alters the penetration of tobramycin into these biofilms, indicating that the diffusion limitation imposed by the matrix varies with changes in the concentration of this divalent cation. These data suggest that growth within a structured exopolysaccharide matrix makes the microenvironment of biofilm cells radically different from the bulk-fluid environment of their planktonic counterparts, and that these differences produce many important physiological differences between cells in these very different modes of growth.

Because the extent to which the biofilm matrix constitutes a barrier to diffusion has been called into question,[16] it may be useful to carefully separate speculation from data and to designate two unequivocal examples of diffusion limitation. The diffusion of oxygen into biofilms is limited to the extent that fastidious anaerobes grow very vigorously in the depths of well-developed biofilms in aerobic systems.[8] Bacterial cells within biofilms are notably resistant to very high concentrations of biocides and antibiotics,[17] but these cells return to exquisite antibiotic sensitivity just 2 to 3 min after these biofilms have been scraped from the colonized surface and dispersed by vortex mixing and ultrasonication. Brown and colleagues[18] have suggested that growth rate has a profound effect on the sensitivity of bacterial cells to various antibiotics, and they have also suggested that bacteria within a biofilm exhibit a very wide range of growth rates, depending on their local microenvironments. It is clear that the matrix of the biofilm excludes both antibodies[19] and phagocytic cells,[20] and biofilm bacteria are protected from these host defense mechanisms, even when a developing population of adherent bacteria consists only of a few scattered glycocalyx-enclosed microcolonies.[20]

C. Microcolony Effects

The direct examination of monospecies bacterial biofilms by CSL microscopy has clearly shown that the cells of most species tend to form microcolonies.[4] Dextran penetration studies have shown that these microcolonies are surrounded by dense matrix material and are separated by "water channels" where the matrix material is less dense (see Figure 5). The existence of these water channels was confirmed by Fourier transform infrared spectroscopy studies of antibiotic penetration,[21] which showed very rapid penetration of these agents to the bottom of the biofilm without significant killing of the bacteria in well-protected microcolonies. This predominance of microcolonies within monospecies biofilms has been demonstrated by direct morphological and physiological examinations, and it provides a very useful model for the understanding of the essential differences between planktonic and biofilm bacteria. Microcolonies are formed when individual cells within a biofilm find themselves in a permissive microenvironment, and these monospecies aggregates are really the basic units of sessile life. They have access to growth substrates from the water channels and from neighboring microcolonies (see Figure 5), and they have the corporate ability to generate large amounts of enzymes, which remain in the microenvironment[8] and digest available nutrient substrata (e.g., cellulose). The growth of a successful microcolony is limited only by the tendency to reduce the access of individual cells to essential nutrients if large size creates diffusion limitations. A very wide range of physiological cooperations has clearly been recorded within established biofilms in natural and industrial aquatic ecosystems, and each of these cooperations involves the interaction of bacteria in microcolonies that may even reach macroscopic size. A case in point is the methanogenic granules that form spontaneously in wastewater treatment systems[22] and consist of macroscopic microcolonies of methanogens surrounded by concentric layers of acetogenic and heterotrophic organisms. These natural microbial consortia carry out organic degradation and methanogenesis at truly phenomenal rates. Similarly, the microbial digestion of cellulose to produce volatile fatty acids and biomass in rumen systems is dependent on bacterial microcolonies within highly structured biofilms.[23] In the biofilms that form on the cellulose, the rate of cellulose digestion is dependent on the development of consortia in which molecular products (butyrate) are removed from the primary cellulolytic organisms to prevent feedback inhibition and promote maximum rates of digestion.[24] A survey of very large numbers of mixed biofilms formed in natural systems leads us to conclude that most of their component bacteria live in microcolonies within which they are juxtaposed to specific substrates (e.g., cellulose) and/or to microcolonies of physiologically cooperative bacteria. All of the studies of monospecies biofilms confirm that bacteria have evolved the adhesion mechanisms and the rapid plastic responses to favorable environments that enable them to fit themselves into the highly structured physiological mosaic of functioning developed biofilms. Biofilms in the form of algal mats, soil crusts, and aquatic biofilms cover much of the surface of the earth, often in very challenging ecosystems. If these adherent populations were multicellular organisms, which they often resemble in their structural and physiological complexity, they would be considered to be phenomenally successful.

V. CONCLUSION

A biofilm cell is different from its planktonic counterpart because it lives in a microniche that limits its contact with the bulk fluid. It is protected from phagocytic cells, bacteriophage, and antibodies, and nutrients and antagonists must reach it via a *de facto* ion-exchange resin that limits their diffusion. Molecules and ions produced by the cell are similarly diffusion limited and the microniche may be heavily conditioned by protons, organic molecules, and bacterial enzymes. Because each cell lives in a distinct microenvironment, even within a microcolony, adjacent cells of the same species may have very different growth rates and very different phenotypic characteristics in response to environmental conditions in its own microniche. Biofilms are seen to be very highly structured, and we can begin to understand how these very plastic and responsive cells can develop together to form multispecies consortia within which each individual cell is provided with nutrients and stimulated by end-product removal. None of the thousands of microenvironments within a mature biofilm would be expected to approximate that of a planktonic cell, and we must therefore consider biofilm cells and planktonic cells to be profoundly different.

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