

## MINIREVIEW

### Biofilms, the Customized Microniche

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#### INTRODUCTION

At its 1993 annual meeting, the American Society for Microbiology deemed the biofilm mode of growth to be a concept worthy of an extraordinary 4-day colloquium consisting of 52 lectures from invited speakers. Perhaps, immediately following this herculean exercise, it is germane to consider the available evidence for the importance and uniqueness of this mode of growth and to assess the consequences of this concept in modern microbiology. It is, clearly, timely to ask the question "What are the essential differences between a planktonic cell growing in the conventional batch culture and a cell of the same species growing in a natural multispecies biofilm?" This question can be asked with some urgency, because whether we intended it or not, microbiologists have actually extrapolated between laboratory cultures and real ecosystems for hundreds of years and, lately, these extrapolations appear to have become increasingly strained and inaccurate.

#### DIRECT MORPHOLOGICAL DATA

While morphological data suffer the disadvantage of subjectivity, because images must be selected to achieve reasonable economy, they do enjoy the advantage of being direct and therefore unaffected by extrapolation. Microscopic data can readily be linked to the most direct of observations, that by the unaided eye, in many aquatic systems in which a macroscopic slime layer develops on available surfaces. These slime-coated surfaces can be recovered and washed to remove planktonic organisms before they are examined, live and fully hydrated, by increasingly sophisticated nondestructive modifications of light microscopy.

The first direct observation concerning microbial biofilms was that these adherent populations generally contained many more cells than the planktonic population in the bulk fluid of the ecosystem concerned (8). This direct light and electron microscopic observation clearly showed that biofilm bacteria were enveloped in very large amounts of a fibrous, highly hydrated, exopolysaccharide matrix whose chemical composition was species specific (28). Because of the limited resolution of available methods of light microscopy and of the draconian dehydration necessitated by all methods of electron microscopy, except environmental scanning electron microscopy, we perceived that biofilm bacteria were distributed relatively evenly and randomly throughout an abundant exopolysaccharide matrix that mediated their aggregation and adhesion to the colonized surface (5). Ecologists had begun to perceive a

heterogeneity of bacterial cell distribution, but the biofilm matrix was generally conceived as being essentially homogeneous.

This image of the homogeneous biofilm, which dominated all biofilm models and cartoons throughout the 1980s, was shattered by the application of confocal scanning laser (CSL) microscopy to the study of biofilms. This elegant nondestructive tool allowed the examination of successive focal planes within living hydrated biofilms and clearly showed that biofilm bacteria grow in matrix-enclosed microcolonies interspersed with less-dense regions of the matrix that include highly permeable water channels (4, 14). This entirely direct method of examination shows that microbial biofilms, ranging from single-species laboratory simulations to natural river populations, consist of cellular aggregates suspended in complex matrices of variable density. Detailed direct morphological examinations of these living hydrated biofilms confirm the presence of single-morphotype microcolonies within biofilms in simulated and actual aquatic ecosystems (Fig. 1). The somewhat enigmatic water channels that anastomose throughout microbial biofilms and provide direct high permeability access from the bulk fluid to the colonized surface (Fig. 1) have been shown to permit the penetration of large (2,000-kDa) molecules (15). Direct observations of inert particles injected into water channels, reinforced by nuclear magnetic resonance (NMR) visualization of flow patterns, have shown unequivocally that convective flow patterns are operative within the water channels directly observed in microbial biofilms (15a).

In a monospecies biofilm, each individual bacterial cell clearly occupies a unique spatial niche that is typically within a microcolony of sister cells of the same strain (13). The metabolic activity of this hypothetical cell would be controlled by the phenotypic expression of specific genes that are derepressed by adhesion to the surface, by conditions at the surface, or by growth in the biofilm mode (6). The biofilm matrix, which is typically composed of polysaccharides that may contain one or more anionic uronic acids (28), is densely concentrated around the microcolonies of cells that have produced its polymers and less densely distributed in the very extensive spaces between these microcolonies (Fig. 1 and 2). The intercolonial spaces must, in some cases, contain the same polymer found in the dense matrix surrounding the microcolonies (because *Pseudomonas aeruginosa* produces only alginate), but its more sparse distribution produces ill-defined but functional water channels that allow convective flow and rapid molecular and ionic equilibration with the bulk fluid. In essence, individual biofilm bacteria enjoy some of the advantages of multicellular life, in that a primitive "circulatory system" delivers nutrients from the bulk fluid to the microcolonial niche and removes metabolic products by the same process. Inhibitors and other antibacterial agents would have ready access to the water channels (Fig. 2) of the intercolonial

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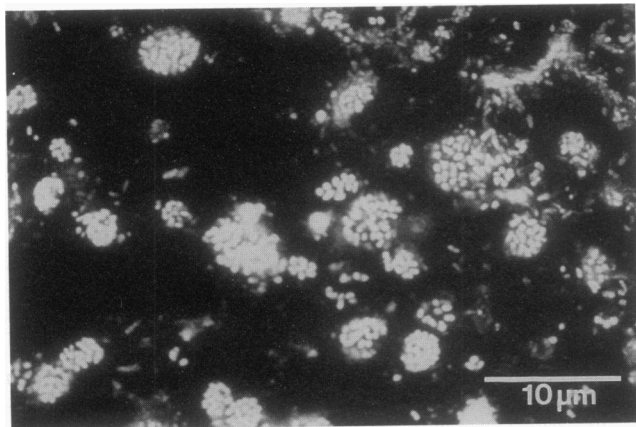


FIG. 1. CSL micrograph of an optical section parallel and close ( $< 3 \mu\text{m}$ ) to a glass surface colonized by a natural mixed living biofilm from the Bow River, Alberta, Canada. Note the distinct microcolonies of morphologically similar bacterial cells and the intervening clear areas designated as water channels.

matrix, but they may still be separated from the inner cells of the microcolonies by an anionic polymeric diffusion barrier. In multispecies biofilms, mixed-species microcolonies are formed by a proportion of the sessile population (20), when cells of metabolically cooperative species are juxtaposed and are thus in a position to benefit from interspecies substrate exchange and/or mutual end product removal. The stationary biofilm mode of growth clearly makes these sustained metabolic cooperations possible, and an individual cell within a mature multispecies biofilm typically lives in a unique microniche where nutrients are provided by neighboring cells and by diffusion, where products are removed by the same processes, and where antagonists may be kept at a distance by diffusion barriers (5). This level of structural organization and of metabolic specialization begins to approach that seen in primitive multicellular eukaryotes, and it goes some way towards explaining the remarkable metabolic efficiency of microbial biofilms (12, 20) and their universal and inherent resistance to antibacterial agents (2, 23).

## PHYSICAL PROBE DATA

The application of CSL microscopy to the study of bacterial biofilms has allowed us to examine fully hydrated living biofilms in real time. We can, therefore, now introduce physical probes into well-defined specific areas within the biofilm and obtain direct chemical and physical data without extrapolation.

Microelectrodes with tip diameters of  $< 10 \mu\text{m}$  are useful in the study of microbial biofilms because they allow the in situ measurement of pH, dissolved oxygen (DO), sulfide, and other chemical species with minimal disturbance of the biofilm structure (19, 25). Because of the obvious structural heterogeneity of biofilms, microelectrode data are more meaningful if these devices are used in conjunction with the CSL microscope so that their tips can be precisely located in relation to microcolonies and water channels. We have used a DO electrode constructed according to the principles delineated by Revsbech and Jorgensen (24) to determine the concentration of DO at various locations within a mixed natural biofilm. This microelectrode was used to penetrate the biofilm at locations monitored by CSL microscopy and to record DO at  $10\text{-}\mu\text{m}$  intervals on the  $z$  axis (7).

The aerobic biofilm examined in this study was clearly composed of microcolonies of bacterial cells enclosed in dense exopolysaccharide matrix material and of much less dense water channels (Fig. 3A and C). When the microelectrode was advanced from the bulk fluid through the bulk fluid-biofilm interface (at  $100$  to  $120 \mu\text{m}$ ) and into a bacterial microcolony (Fig. 3A), the data from the DO sensor showed that DO values decreased at the interface and reached almost totally anaerobic levels in the center of the microcolony (Fig. 3B) and at the colonized surface ( $0 \mu\text{m}$ ). When the probe was changed to a position where it would be advanced from the bulk fluid through the bulk fluid-biofilm interface and into an adjacent water channel (Fig. 3C), significant levels of DO were seen at all depths (Fig. 3D), including the colonized surface ( $0 \mu\text{m}$ ). These data clearly show that the structural heterogeneity of the biofilm predicated a corresponding heterogeneity in an important physiological parameter, i.e., DO. The water channels appear to transport oxygen into the biofilm (Fig. 2), but diffusion limitations and oxygen utilization produce very low

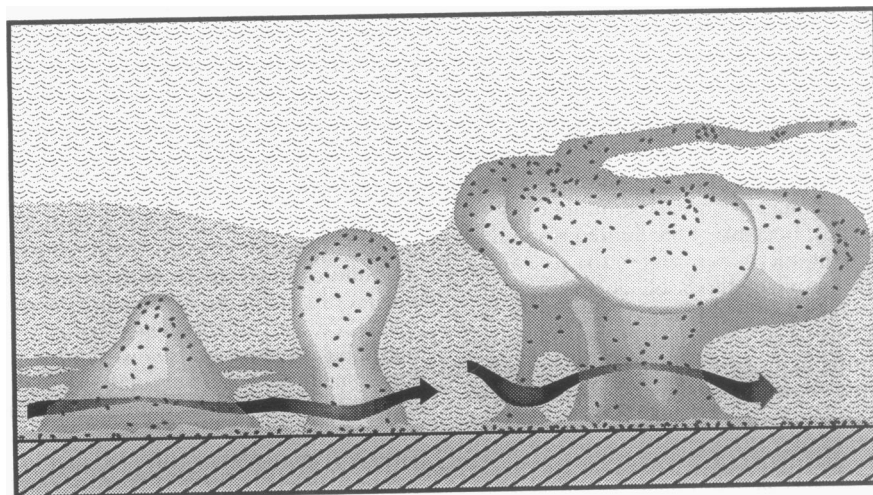


FIG. 2. Diagrammatic representation of the structure of the hypothetical bacterial biofilm drawn from CSL microscopy examinations of a large number of monospecies and mixed-species biofilms. Note the relatively open water channels between discrete microcolonies in which bacterial cells are enclosed in a dense exopolysaccharide matrix. The arrows indicate convective flow within the water channels.

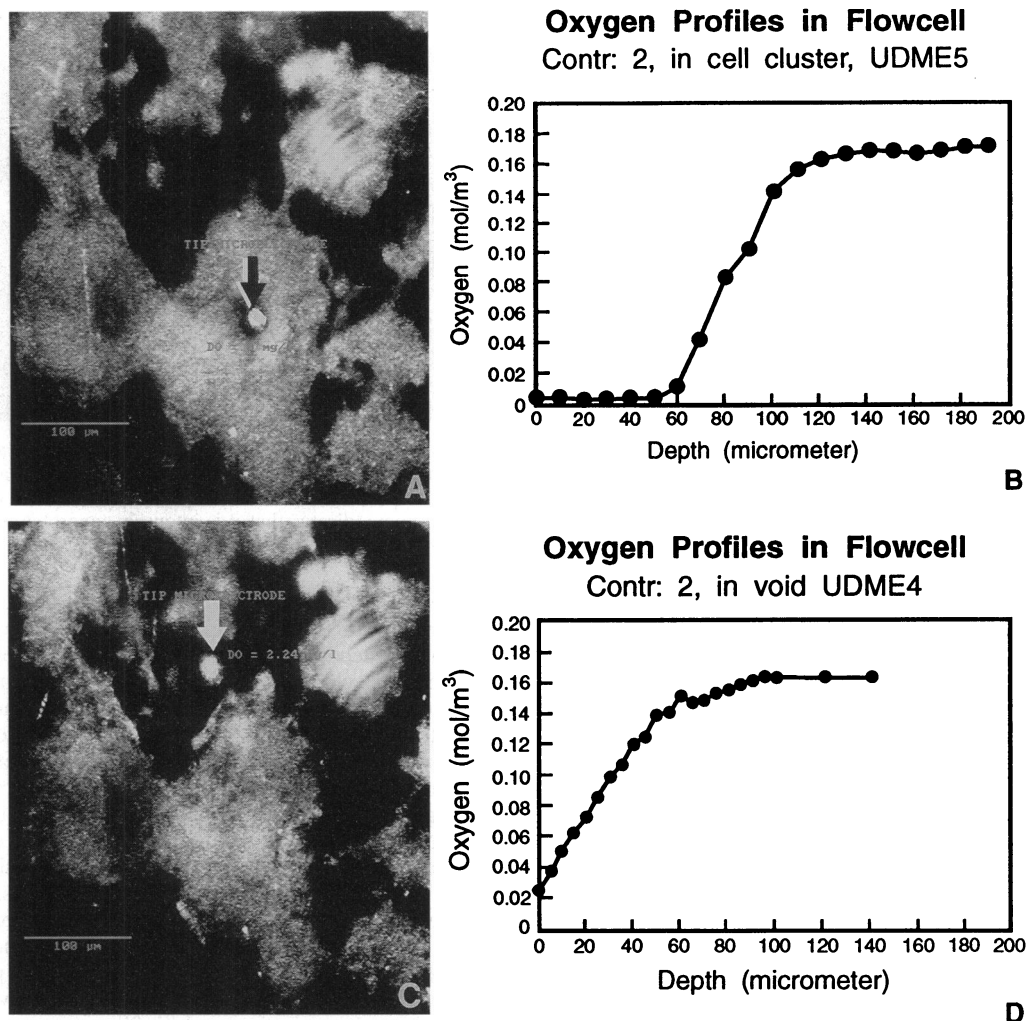


FIG. 3. CSL micrographs and DO data obtained during the examination of a living *P. aeruginosa* biofilm with a DO-sensitive microelectrode. The DO probe was positioned in the bulk fluid and advanced through the bulk fluid-biofilm interface into the center of a large microcolony (A) to produce a trace of DO readings at 10- $\mu$ m intervals (B). The colonized surface is at depth 0, while the biofilm-bulk fluid interface is between 100 and 120  $\mu$ m. Note the anaerobiosis seen within the microcolony. The probe was then advanced from the bulk fluid into a water channel (C), where DO readings were substantially higher (D). These data indicate that water channels may conduct DO within biofilms. The arrows indicate the positions of the DO probe.

oxygen levels at the centers of cellular microcolonies. These direct observations of living biofilms may explain the existence, and even the physiological activity, of fastidious anaerobes within mixed biofilms in aerobic environments.

Because a bacterial biofilm consists of viscoelastic biopolymers and because its surface is soft and sometimes filamentous, flow is very difficult to characterize at a biofilm-colonized surface. However, the discovery of water channels within the biofilm (Fig. 1 and 2) suggests that convective flow may occur within this population of adherent organisms. We have used NMR imaging to study flow velocities at biofilm-colonized surfaces and within living biofilms. A versatile set of these noninvasive NMR techniques has been developed to study chemical and physical properties of small samples (1) and to allow spatial imaging of larger systems (22). Liquid velocity can be determined from spin-lattice relaxation time-weighted NMR data (21), and our determinations have shown that the flow velocity does not reach zero at the biofilm surface (16, 17) and that fluid flow can be detected within biofilms (18). These

data indicate that convective flow occurs within the water channels (Fig. 2) of living biofilms.

#### CHEMICAL PROBE DATA

During the examination of eukaryotic tissues by CSL microscopy, a large number of fluorescent chemical probes have been developed (9). These probes can be introduced into fully hydrated living bacterial biofilms and their fluorescent emissions can be monitored for location and intensity to yield very valuable direct data concerning chemical and physical conditions in virtually all parts of these complex matrix-enclosed adherent populations. For example, fluorescent probes may be used to define the penetration and chemical interaction of defined molecules (e.g., fluoroconjugated lectins or dextrans), cellular physiological conditions (e.g., cell viability, membrane potential, or membrane permeability), microzonal variations in biofilm chemistry (e.g., pH,  $E_h$ , or ion concentration), or efficacies of antimicrobial agents (4, 10, 11).

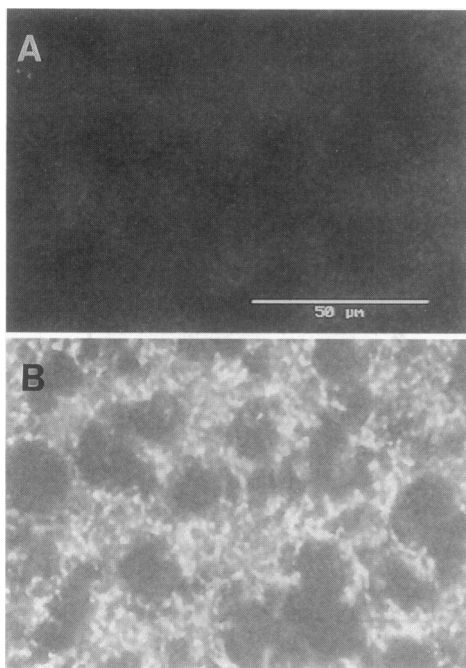


FIG. 4. CSL micrographs of a living biofilm of *P. aeruginosa* reacted with an anionic chemical probe (70-kDa TRITC-dextran). The biofilm showed very little affinity for this anionic probe in its natural state (A), but it bound very large amounts of the probe after it was exposed to a DC electric field to produce the bioelectric effect (B). These DC fields appear to alter the charge pattern of the cells and the matrix of this biofilm.

Direct examinations using fluorescein-coupled dextrans of various molecular masses have shown that 2,000-kDa dextrans readily penetrate the water channels (Fig. 2) of living bacterial biofilms. Polyanionic dextrans (e.g., 70-kDa tetramethyl rhodamine isocyanate [TRITC]-dextrans) have been used to show that the matrix of a mixed-species biofilm, which is generally supposed to be anionic, actually varies regionally in charge density (15). Direct observations of living biofilms of *P. aeruginosa*, using these same fluorescent polyanionic dextrans, have shown limited binding of the probe to matrix components (Fig. 4A), unless the biofilm has been exposed to direct current (DC) fields (Fig. 4B). This observation may contribute to an explanation of the bioelectric effect (3), in which biofilm cells become much more sensitive to antimicrobial agents, because this directly observed change in matrix charge may indicate that cationic sites in the biofilm matrix have been created or exposed by the DC field.

Fluorescent chemical probes can be used to detect biogenic microchemical gradients within biofilms, and Fig. 5 shows intense fluorescence at the center of a microcolony within a biofilm of *P. aeruginosa*. In this living biofilm, this  $E_h$ - and pH-sensitive probe (fluorescein) detects an anaerobic zone at the center of this microcolony, indicating a very low level of DO. If any of the cells at the center of this microcolony were carrying out acidogenic aerobic metabolism, this fluorescent signal would have been quenched. These chemical probe data confirm the physical probe data described above (Fig. 3) and lead us to conclude that the centers of the microcolonies constitute an anaerobic microniche in a generally aerobic biofilm.

A number of fluorescent chemical probes in CSL microscopy

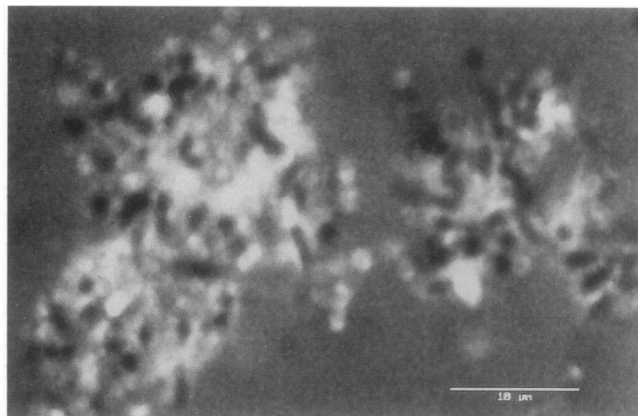


FIG. 5. CSL micrograph of a microcolony within a living *P. aeruginosa* biofilm that had been treated with an  $E_h$ - and pH-sensitive chemical probe. The fluorescence of the center of this complex microcolony indicates that it is intensely anaerobic. Bar, 10  $\mu\text{m}$ .

can be used to indicate the metabolic state of individual bacteria, because actively metabolizing cells produce reducing power (reduced coenzymes) within their cytoplasm (26). Redox-sensitive chemical probes (e.g., resofurin and fluorescein) or fluorogenic substrates (e.g., fluorescein diacetate) can be used to detect metabolic activity in the laboratory (11) and in field samples (27), and most chemical probe examinations of bacterial biofilms detect a majority of metabolically active cells throughout the microcolonies (5). When metabolic activity is indicated by independent parameters, such as cell elongation (29), data obtained from the use of redox-sensitive probes on living biofilms appears to yield accurate results.

The use of chemical probes and CSL microscopy to study living bacterial biofilms has, therefore, confirmed most of the perceptions gained from morphological and physical probe data. Most biofilm cells are metabolically active, even within anaerobic microcolonies, and biofilms are traversed by water channels that penetrate an exopolysaccharide matrix whose anionic nature may vary locally or in response to external factors such as DC fields.

## THE SEQUELAE

We are now able to examine fully hydrated living bacterial biofilms and to collect chemical and physical data, without extrapolation, in many locations within these adherent communities. These direct and therefore unequivocal data allow us to make valid comparisons between bacteria living in single- or mixed-species biofilms and their planktonic counterparts living in pure (single-species) cultures.

Because adhesion to the surface or conditions at the surface have been shown to trigger the expression of at least two (*algC* and *algG*) and probably many more bacterial genes and to affect bacterial behavior (6), we can conclude that biofilm bacteria are phenotypically different from planktonic cells of the same species. Some biofilm bacteria are fixed in a stable juxtaposition to cells of the same species in monospecies biofilms and to cells of several species in multispecies biofilms by virtue of their growth in microcolonies within stable exopolysaccharide matrices. For this reason, unlike planktonic cells, whose spatial relationships with other cells are essentially transitory, biofilm bacteria live in microbial communities where the availability of nutrients and the concentrations of

end products depend in part on the metabolic activity of neighboring cells. Diffusion and convection can readily carry nutrients to and end products from planktonic cells, but biofilm bacteria live in large numbers of matrix-enclosed microenvironments whose properties depend on limited diffusion and on the metabolic activities of adjacent cells. Water channels and convective flow carry bulk fluid components into the biofilm, but diffusion barriers still separate this primitive circulation system from the microenvironments of the microcolonies. The formation of physiologically cooperative bacterial consortia (20) is only really possible in biofilms or in equivalent cellular aggregates. We can therefore conclude that the physiology of biofilm bacteria is profoundly different from that of their planktonic counterparts and that they live in large numbers of different microniches within these adherent populations.

However, it is in the area of ecology where biofilm bacteria differ most radically from planktonic cells. Primary surface colonizers are selected from mixed planktonic populations by their ability to adhere to a specific surface, and they then constitute a spatially limited stable population with the capacity to promote or to preclude secondary colonization and succession by other organisms. These primary colonizers must select microniches that are favorable for their physiological requirements, and recolonization and resuccession will occur if the properties of the colonized surface (e.g., mammalian tissue) are altered. While planktonic bacteria are exquisitely sensitive to antimicrobial agents, because of ready access by diffusion within the bulk fluid, biofilm bacteria are notably resistant to all of these agents (5) and they therefore contribute stability and continuity in natural microbial ecosystems. We can conclude that biofilm bacteria are clearly distinct from their planktonic counterparts.

Direct observations of a wide variety of natural ecosystems have established that the vast majority of bacteria in most aquatic environments grow within matrix-enclosed biofilms. Direct examinations of biofilms have shown that the component bacteria grow in a very large number of microniches whose properties are dictated in part by biofilm structure, diffusion, and the physiological activity of neighboring organisms. Except in rare instances where the properties of a particular biofilm niche happen to correspond to the arbitrary properties of a culture medium, bacteria have never been studied in the conditions that exist in microniches. Because bacteria are protean creatures whose metabolic activities are profoundly influenced by their environment, we are confident that direct studies of biofilm bacteria by a developing battery of modern methods will produce exciting insights into how bacteria actually grow in natural and pathogenic ecosystems.

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