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Overview of microbial biofilms

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As the success of this two-issue special section of the Journal of Industrial Microbiology attests, the study of microbial biofilms is truly burgeoning as the uniqueness and the importance of this mode of growth is increasingly recognized. Because of its universality the biofilm concept impacts virtually all of the subdivisions of Microbiology (including Medical, Dental, Agricultural, Industrial and Environmental) and these two issues incorporate contributions from authors in all of these disciplines. Some time ago we reasoned that bacteria cannot possibly be aware (*sic*) of their precise location, in terms of this spectrum of anthrocentric subspecialties, and that their behavior must be dictated by a standard set of phenotypic responses to environmental conditions in what must seem to them (*sic*) to be a continuum of very similar aquatic ecosystems. In this overview I will, therefore, stress the common features of microbial biofilms that we should bear in mind as we use this simple universal concept to seek to understand bacterial behavior in literally hundreds of aquatic ecosystems traditionally studied by dozens of subspecies of microbiologists reared in sharply different scientific and academic conventions.

Keywords: biofilms; adhesion

Biofilm formation

Between 1936 and 1943, Zobell studied the affinity of marine bacteria for surfaces [26] and the development of a battery of new methods for the direct study of living bacteria at interfaces, coupled with many refinements of image analysis, have made it possible actually to study the behavior of bacterial cells as they adhere to these surfaces. Some bacterial cells approach a surface, adhere rapidly to that surface, initiate glycocalyx (exopolysaccharide) production and form the discrete microcolonies that are the basic organizational units of biofilms. Other cells are seen by direct microscopic methods to adhere to surfaces and then to spread out by rolling or swarming maneuvers to produce an even 'lawn' of glycocalyx-producing adherent cells before microcolony formation is initiated. These adherence behaviors are characteristic of cells of different species and are conditioned by the physiological state of the organisms concerned so that we must anticipate a very significant variety of adherence behavior as more species and more physiological states are examined.

The nature of the surface concerned certainly influences the rate of bacterial adhesion [11], and inert and living surfaces vary through a wide spectrum in the rate at which bacterial adhesion occurs [3], but decades of research have yet to yield an inert surface that is inherently resistant to bacterial colonization. In spite of the huge financial impetus that drives the search for this 'holy grail' in the medical device field, and in spite of hundreds of millions of dollars spent in proprietary corporate research, no inherently colonization-resistant material has yet been discovered. This expensive and futile search was inspired by studies using laboratory strains of bacteria whose phenotypic adaptation to growth *in vitro* had deleted all but a few of their myriad adhesion mechanisms. Hundreds of materials that resisted colonization by bacterial strains modified by thousands of transfers in the laboratory were rapidly colonized when exposed to wild strains of bacteria operating in realistic milieux. In the medical field the use of these putative colonization-resistant materials was further complicated by the extent to which organic molecules in body fluids formed conditioning films on their surfaces and by the fact that a monolayer of adherent bacteria, even if it formed very slowly, constituted a new and very welcoming surface for further bacterial accretion. It is now clear that bacterial adhesion to inert surfaces will be controlled by the incorporation of antimicrobial materials (Ahearn; Keevil, this issue) that kill incoming bacterial cells or by the eventual production of protein-coated surfaces that resemble those of living tissues [14] so closely that they accrete the same surfactants, tissue-bound antibodies, phagocytes, and endothelial cells that protect the surfaces of some living tissues from bacterial colonization.

The use of an especially elegant method of direct observation that enables us to visualize the up regulation of specific reporter genes [7] in living bacteria as they adhere to surfaces has revolutionized the study of biofilm formation. This study produced unequivocal proof that AlgC, the gene that produces the enzyme phosphomannomutase of the alginate synthesis pathway in *Pseudomonas aeruginosa*, is up regulated within 5 min of the adhesion of an individual cell to an inert surface. These direct data can now be linked to very exciting developments in the burgeoning field of biofilm genetics (Chakrabarty; Whitfield, this issue). Deretic's group [23] suggested, on the basis of very strong evidence, that AlgC is one of a large 'cassette' of genes that are up regulated by the production of a sigma factor produced by the AlgU gene and regulated by MucA and MucB [10]. This evidence, which is presented in more detail in a new review of biofilm structure [5] strongly suggests that

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biofilm cells are profoundly different from planktonic cells of the same species because of very comprehensive phenotypic changes that are regulated by a sigma factor similar to those that regulate sporulation, starvation survival, and rough–smooth phase variations. Preliminary evidence (Yu and Costerton, unpublished data) indicates that the cell envelope fractions of biofilm and planktonic cells of the same species (*P. aeruginosa*) differ profoundly in the dozens of proteins that can be resolved by modern gel chromatography and this suggests that the cassette of genes regulated by the sigma factor triggered by adhesion may include several that affect cell wall permeability. This sweeping sigma factor-regulated, adhesion-dependent, phenotypic change would be reversed when cells leave the biofilm, perhaps with the aid of the lyase enyzme [1]. This genetic concept must now be added to the consideration of observed differences in the susceptibility of planktonic and biofilm cells to antimicrobial agents (Allison and Gilbert, this issue) because adhesion-dependent phenotypic changes may be as important as diffusion barriers [15], or growth rate-dependent changes [13] in this important phenomenon. Further, these data raise the specter of a phenotypic change in the cell wall of biofilm cells of a given species of bacteria that makes these cells inherently resistant to a particular antibiotic agent, virtually all of which were developed against specific targets in planktonic cells. On a more positive note, a new and aggressive program of antibiotic development using biofilm cells as targets could quickly develop a new class of agents that specifically inhibit the unique metabolic activities of biofilm cells.

Biofilm structure

The lure of very high resolution caused many of us, including this author, to embrace electron microscopy for the examination of bacterial biofilms even though we know that we paid a high price in dehydration artifacts. While we conceded that the expolysaccharide glycocalyx of biofilm bacteria was radically condensed during dehydration, we did not imagine that this virtual collapse of the biofilm matrix profoundly altered a very elaborate biofilm structure. The recent application of the confocal scanning laser microscope (CSLM) to the study of microbial biofilms has produced a whole series of revelations. This elegant CSLM system, coupled with modern techniques for image analysis, allows us to examine living hydrated microbial biofilms [20]. Extensive CSLM studies of biofilms formed by pure cultures of Gram-negative [8] and Gram-positive (Sanford, this issue) bacteria and of natural mixed species biofilms, have allowed us to deduce certain common structural features of these adherent mcirobial populations [19] and to begin to reevaluate our conceptual models (Shea, this issue) of biofilm architecture.

The bacterial microcolony is clearly the basic structural and functional unit of the microbial biofilm (Figure 1). Microcolonies may be composed of cells of a single species or of cells of several species, but they are clearly delineated by their exopolysaccharide matrix which holds them in stable juxtaposition and regulates their effective contact with the fluid phase. Each microcolony consists of the progeny of the cells whose stimulated growth established the

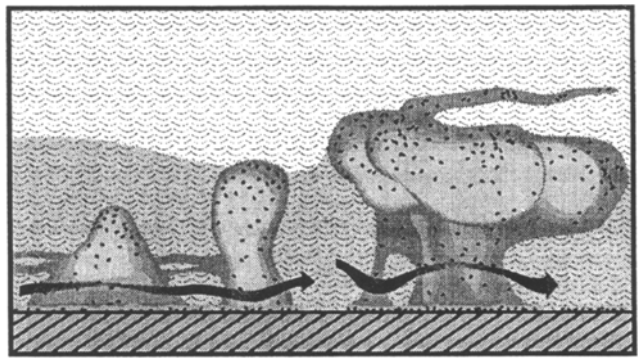

Figure 1 Diagrammatic representation of a microbial biofilm showing the organization of this adherent population, in terms of microcolonies and water channels, and the newly discovered convective flow within these channels

microcolony and therefore many must necessarily be 'sister' cells embedded in an exopolysaccharide matrix of their own creation. Simple proliferation would produce mound-like microcolonies on the colonized surface but direct CLSM observations reveal a preponderance of mushroom-shaped microcolonies in some biofilms (Figure 1) and this complex shape presupposes a measure of growth control by quorum sensing [12] or by complex cell–cell communication [16] similar to that seen in the formation of fruiting bodies by myxobacteria. It is already apparent that many factors can affect biofilm structure (Lappin-Scott, this issue) but the direct demonstration of complex microcolonial structures such as those depicted in Figure 1 demands that we attribute to these basic biofilm units the capability of growth control to produce a complex biofilm architecture. Present direct evidence allows us to conclude that biofilm bacteria live in glycocalyx-enclosed mircrocolonies whose location, size, and shape are determined by nonrandom species-specific factors. Each biofilm cell, therefore, lives in a spatially distinct microniche [4] whose characteristics govern cellular behavior.

The direct demonstration of an anastomosing network of water channels [20] that penetrate to all levels of the biofilm and bring the bulk fluid phase amongst and even behind (Figure 1) the bacterial microcolonies, was a profound revelation. This remarkable feature of microbial biofilms has now been explored in some detail [5,17] and convective fluid flow has been demonstrated within these water channels [25]. This convective flow, which maintains the same directions as the bulk fluid flow, has been quantitated within living biofilms and its discovery must revolutionize our conception of mass transfer in the adherent populations. We suggest [5] that turbulence caused by elements of the biofilm that protrude into the bulk fluid [6] may increase connective flow within these water channels, some of which are sufficiently open to permit the passage of 0.3-μm polystyrene beads [9]. The direct measurement of dissolved oxygen in living biofilms using oxygen-specific microelectrodes [21], showed that this labile nutrient penetrates through the water channels as far as the colonized surface even though the water channels are lined with respiring bacteria in microcolonies. Because the water channels of the biofilm are kept open, even near the

colonized surface (Figure 1), we must conclude that the development of bacterial microcolonies in biofilms operates under a system of elaborate controls that prevents the occlusion of these water channels. We cannot, of course, conclude that all microbial biofilms exhibit the architecture depicted in Figure 1, but our observation that mixed species biofilms at several locations in a fast flowing river display this structure [5], suggests that it is representative of biofilms in many natural ecosystems.

When we consider the elaborate architecture of microbial biofilms we are moved to suggest that this sessile mode of growth represents the highest phenotypic expression of the bacterial genome. Within biofilms bacterial replication and exopolysaccharide production are regulated so that an open system of microcolonies and water channels is produced and maintained. This property of biofilm bacteria differs profoundly from the uncontrolled replication of planktonic cells and suggests the sophistication of multicellular eukaryotic organisms whose component cells replicate under the control of lectins and hormones to produce elaborate tissues. The reward (*sic*) to the individual component cells is the same in both cases in that they gain a protected niche [4] within which they have a measure of homeostasis while keeping contact with the primitive analogue of a circulation system that delivers nutrients and removes wastes.

Another consequence of bacterial growth in structural biofilms is the opportunity for metabolic cooperation within consortia of cells of different species whose juxtaposition is stabilized within the microcolonies of the biofilm (James *et al*, this issue; [19]). Metabolic cooperation between bacteria growing in the planktonic mode of growth is difficult and must operate via the bulk fluid. However, several species may cooperate effectively in such complex activities as methane generation [22] or cellulose degradation [18] when cells of metabolically cooperative species are stimulated by each other's presence to form highly structured mixed microcolonies. Within these stable microcolonies interspecies cross-feeding is facilitated and fastidious organisms can be maintained in a microenvironment (eg complete anaerobiosis) that allows them to contribute their metabolic activity (eg methane generation) to the overall activity of the consortium (eg degradation of organic molecules). Lewandowski and his colleagues have used direct microelectrode studies of living biofilms [8] to show that completely anaerobic loci can be detected within microcolonies of a biofilm that developed in an aerobic environment and similar specific microenvironments have been detected within biofilms by the use of specific fluorescent probes [17]. These data add another dimension to the developing perception of the biofilm as the highest expression of the bacterial genome in that we see the complexity that is considered to be a distinguishing characteristic of multicellular organisms. As more direct examinations reveal more instances of complex biofilm architecture and of sophisticated cell–cell interactions within microcolonies a new perception of the phylogenetic position of bacteria in the living world begins to emerge. The planktonic cells that we have studied so assiduously during the 15 decades since the pioneering work of Koch and Pasteur may represent a simple mode of growth specialized to accomplish dispersal and the colonization of new habitats. The biofilms that have been neglected during all but the past decade may constitute a higher and much more complex mode of bacterial growth that has effective homeostasis, a primitive circulatory system, and a measure of cellular specialization. What we microbiologists have done during these 15 decades is somewhat similar to a study of plants and animals that has been confined to the examination of their spores, gametes, seeds, and other propagules.

In case this peculiar misplaced emphasis is dismissed as an obtuse philosophical matter, it is important to consider the considerable impact of this modern biofilm concept on practical areas in industry and in medicine. The biofilm bacteria that cause befouling in industry and device-related infections in medicine (Khardori, this issue) have been shown to be inherently resistant even to very high levels of antimicrobial agents (Khardori; McFeters; Allison and Gilbert, this issue). The general protection of biofilm cells from antibacterial agents extends to surfactants (Busscher, this issue), heavy metals (Ahearn, this issue), and antibiotics (Hoiby, [19]) and even to protection from phagocytic predators [5]. Early in our studies of microbial biofilms, when our working hypothesis visualized essentially planktonic bacterial cells embedded in a homogeneous intercellular matrix [3], we suggested that this matrix might impose a diffusion limitation that protected biofilm cells. Detailed studies of diffusion [24] contradicted this concept and this hypothesis was essentially replaced by an hypothesis invoking the reduced growth rate of biofilm cells [2]. Now it is apparent that biofilm bacteria are profoundly phenotypically different from the planktonic bacteria that were the targets in virtually all of the design and screening programs that produced our vast armamentarium of modern biocides and antibiotics. We can now anticipate that, when biofilm bacteria replace their planktonic counterparts as targets for these design and screening programs, new classes of antibacterial agents will be developed that will be truly effective in killing bacteria within biofilms (McFeters, this issue). Perhaps the most useful aspect of our new understanding of biofilms is the ability to use probes of biofilm architecture and of metabolic activity to monitor the actual killing of bacteria in spatial terms (McFeters, this issue). These very specific probes, including polyanionic TRITC dextran probes for cationic matrix components [5], have even been used to determine the effects of the exposurse of biofilms to the DC fields that enhance the efficacy of antibacterial agents to produce the bioelectric effect (Jass and Lappin-Scott, this issue).

It is axiomatic that sharp expansions of perception based on direct observations tend to rationalize observations of natural systems that have previously been controversial. A case in point is microbially-influenced-corrosion (Arrage and White, this issue) which can now be understood in terms of the effect on a conductive surface of colonization by a biofilm that incorporates aerobic and anaerobic loci, and regions with sharply different metal-binding capabilities, within a few hundred microns of each other. Now that biofilm architecture is more accurately understood the somewhat enigmatic process of MIC can be rationalized in terms of classic oxygen concentrations and metal concen-

tration 'cells'. Similarly, we can conceive of 'engineered' biofilms in reactor systems in which fastidious anaerobes can operate in systems that are open to air and in which changing nutrient feeds can support different bacterial populations within the same areas of the reactor surface.

Conclusion

At the outset of this seminal two-issue exploration of biofilm microbiology it is important that we clearly state the paradigm shift that is now implicit in the use of the term 'biofilm'. The examination of a wide variety of living single species and natural multispecies biofilms by direct nondestructive techniques has clearly shown an architecture in which slime-enclosed microcolonies are interspersed between relatively open cell-free water channels that penetrate all regions of these adherent populations. Convective flow, and the passage of 0.3-μm polystyrene beads, has been shown by direct measurement in the water channels of several biofilms. The detailed genetic analysis of one almost ubiquitous biofilm organism, *Pseudomonas aeruginosa*, has revealed that adhesion to a surface triggers a sigma factor-directed phenotypic change in a large number of cell envelope genes some of which regulate alginate synthesis. Direct examination of living biofilms by the use of chemical or of physical probes clearly indicates that adjacent regions of these microbial biofilms may vary sharply in the concentration of metal ions or of nutrients (eg oxygen) in adjacent areas. We can therefore conclude that many gradients exist within biofilms, perhaps especially between the microcolonies where the bacterial cells live and the ramifying water channels that carry the bulk fluid throughout the biofilm. Highly structured microcolonies have been described in natural multispecies biofilms within which metabolically cooperative organisms are juxtaposed so as to facilitate complex processes like cellulose digestion or methane formation from organic compounds. Taken together these data indicate that the highly structured biofilm mode of growth provides bacteria with a measure of homeostasis, a primitive circulatory system, a framework for the development of cooperative and specialized cell functions, and a large measure of protection from antibacterial agents. These advantages of the biofilm mode of growth, which have led to its functional predominance in most natural aquatic ecosystems, are not available to cells of the same species growing in the planktonic mode. It is, perhaps, unfortunate that we know the structure and function of planktonic cells in exquisite detail but that we are just beginning to study bacterial cells in biofilms.

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