

MINIREVIEW

Biofilm, City of Microbes

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In most natural environments, association with a surface in a structure known as a biofilm is the prevailing microbial life-style. Surface association is an efficient means of lingering in a favorable microenvironment rather than being swept away by the current. Taken to the extreme, we may view the planktonic or free-swimming microbial phase primarily as a mechanism for translocation from one surface to another.

Genetic studies of single-species biofilms have shown that they form in multiple steps (46), require intercellular signalling (7), and demonstrate a profile of gene transcription that is distinct from that of planktonic cells (35). From this perspective, biofilm formation may be viewed as a developmental process that shares some of the features of other bacterial developmental processes such as sporulation of gram-positive bacteria (9), fruiting body formation in *Myxococcus xanthus* (33, 40, 44), and stalked-cell formation by *Caulobacter crescentus* (13, 19, 24, 37, 48). In natural environments, however, the biofilm is almost invariably a multispecies microbial community harboring bacteria that stay and leave with purpose, share their genetic material at high rates, and fill distinct niches within the biofilm. Thus, the natural biofilm is less like a highly developed organism and more like a complex, highly differentiated, multicultural community much like our own city.

There are several steps that we must take to optimize our lives in a city. The first is to choose the city in which we will live, then we must select the neighborhood in the city that best suits our needs, and finally we must make our home amongst the homes of many others. Occasionally, when life in the city sours, we leave. The same steps occur in the formation of a bacterial biofilm (Fig. 1). First, the bacterium approaches the surface so closely that motility is slowed. The bacterium may then form a transient association with the surface and/or other microbes previously attached to the surface. This transient association allows it to search for a place to settle down. When the bacterium forms a stable association as a member of a microcolony, it has chosen the neighborhood in which to live. Finally, the buildings go up as a three-dimensional biofilm is erected. Occasionally, the biofilm-associated bacteria detach from the biofilm matrix. Micrographs of these steps in biofilm formation by a single bacterial species are shown in Fig. 2. Although these micrographs are static views of the steps in biofilm formation, a biofilm is not a motionless heap of cells. Figure 3 shows the first frame of a real time movie, accessible at <http://gasp.med.harvard.edu/biofilms/jbmini/movie.html>, that documents the activity in a mature biofilm. In this frame, the pillars of a mature biofilm are visible, distributed on top of a

monolayer of surface-associated cells. The associated movie shows that, in addition to fixed cells, there are motile cells that maintain their association with the biofilm for long periods of time, swimming between pillars of biofilm-associated bacteria. The biofilm, therefore, demonstrates a level of activity similar to that of a bustling city.

The genetic basis of the steps in biofilm formation has been investigated for a number of bacterial species, including *Escherichia coli* (34), *Pseudomonas aeruginosa* (31) and *Vibrio cholerae* (46). For these studies, a simple genetic screen was utilized in which random transposon mutants are grown in 96-well plates (5, 16, 32). After removal of the planktonic cells, the remaining biofilm-associated cells are stained with crystal violet. Those wells with no crystal violet staining correspond to mutants that are defective in biofilm formation. These genetic screens for biofilm-defective mutants have shown that the initial interaction with the surface is accelerated by force-generating organelles such as type IV pili and flagella. Once temporary contact with the surface is made, bacteria use either flagella or type IV pili to move along the surface in two dimensions until other bacteria are encountered and microcolonies are formed or enlarged (31, 34, 46). Finally, exopolysaccharide production is necessary to stabilize the pillars of the biofilm (46). Competition studies between wild-type *V. cholerae* and pilus or flagellar mutants show that these structures provide a great advantage in surface colonization (P. I. Watnick and R. Kolter, unpublished results). Thus, speed of attachment may be an important factor in garnering an apartment in the microbial city.

Evidence exists that different genes are transcribed in the planktonic and biofilm-associated phases of the bacterial life cycle. This is again reminiscent of a developmental process. Prigent-Combaret et al. performed a screen for genes in *E. coli* that are differentially expressed in biofilm-associated cells, using a library of random insertion mutants generated with a MudX transposon carrying a promoterless *lacZ* gene (35). One interesting finding from this study is that flagellin synthesis is decreased in biofilm-associated cells, while production of colanic acid, an exopolysaccharide made by *E. coli*, is increased. The situation appears to be similar in *P. aeruginosa*. Alginate is an exopolysaccharide that is found in *P. aeruginosa* biofilms (14). Transcription of *algC*, a gene involved in the production of alginate, is increased approximately fourfold in biofilm-associated cells as compared with planktonic cells (6, 15). Furthermore, for many years, researchers have noted that pulmonary isolates of *P. aeruginosa* are mucoid due to production of copious amounts of alginate (14). Recently, Garrett and co-workers noted that flagella are absent from these mucoid isolates (15). In addition, they showed by mutational analysis that while alginate synthesis is positively regulated by the alternative sigma factor σ^{22} , this sigma factor negatively regulates the synthesis of the flagellum. This suggests that when synthesis of

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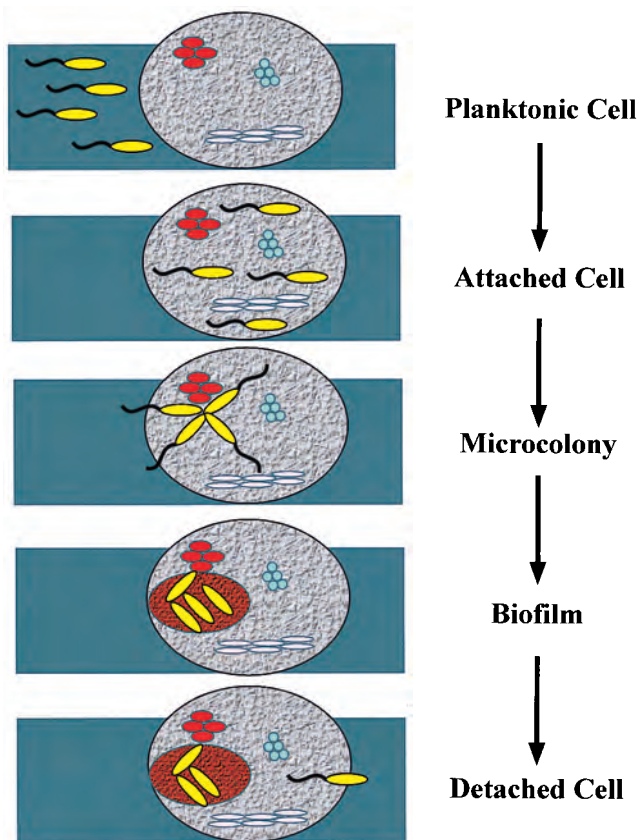


FIG. 1. A schematic representation of the steps a new bacterial species takes in forming a biofilm on a rock previously colonized with multiple species of bacteria. The yellow bacteria represent an aquatic species that swims towards the rock using polar flagella, forms random loose attachments to the rock, migrates over the surface to form a microcolony, and finally produces exopolysaccharide to form a three-dimensional biofilm. When environmental conditions become unfavorable, some of the bacteria may detach and swim away to find a surface in a more favorable environment.

the exopolysaccharide, alginate, is increased in biofilm-associated cells, flagellar synthesis decreases. Thus, to become a productive member of a biofilm community, the bacterium must differentiate into a biofilm-associated cell by repressing synthesis of the flagellum that might destabilize the biofilm and producing exopolysaccharide that will reinforce the biofilm structure.

Some genes may be expressed in response to a specific surface on which the bacterium has chosen to settle. For instance, chitin, a polymer of *N*-acetylglucosamine, is a component of crustacean and insect exoskeletons. Attachment to and degradation of chitin for use as a nutrient source is an important part of survival for many marine *Vibrio* species (3, 21). The structural genes that are important for attachment to chitin differ from those required for attachment to abiotic, nonnutritive surfaces such as plastic and glass (36, 45). Furthermore, although liquid medium that is rich in nutrients primes many bacteria for attachment to any local surface (32, 34, 45), the bacteria will attach to chitin, but not plastic or glass, even when surrounded by a nutrient-poor medium (45). In some marine bacteria, it has been shown that chitinase and chitin-binding genes are expressed selectively in the presence of chitin (29, 42). Thus, when the bathing medium is rich in nutrients, a bacterium will attach to any available surface, while in a nutrient-poor environment the bacterium will attach preferen-

tially to a nutritive surface. This adaptation ensures that the bacterium will maximize access to nutrients in both nutrient-poor and nutrient-rich aqueous environments.

City dwellers distribute themselves geographically based on the neighbors and environment that best suits their needs and requirements. Chefs and grocers may settle together in the restaurant district, while musicians may settle near concert halls. The same is true for biofilm-associated cells. Specific coaggregation of oral bacteria is thought to determine the distribution of bacteria within multispecies dental biofilms known as plaque. These interactions are thought to be essential for successful plaque formation (22, 23, 47). Furthermore, the environment in a biofilm is not homogeneous. Microelectrode measurements have shown that the oxygen concentration and pH fall in a biofilm as the substratum is approached (30, 49). In single-species biofilms, the biofilm-associated bacteria alter gene expression to maximize survival in their particular microenvironment (20, 49). In mixed biofilms, which are more representative of biofilms occurring in nature, bacteria distribute themselves according to who can survive best in the particular microenvironment and also based on symbiotic relationships between the groups of bacteria (27, 28, 30). Thus, the bacteria in a multispecies biofilm are not randomly distributed but rather organized to best meet the needs of each.

Villagers establish zoning laws and regulate settlement through communication with each other. Bacteria also communicate with each other. Intercellular communication between bacteria is generally carried out by bacterial products that are able to diffuse away from one cell and enter another cell. It is difficult to envision this as an effective means of communication between planktonic bacteria in natural, aquatic environments, since molecules are likely to be carried off in the aqueous phase with a very small probability of reaching neighboring bacteria. Rather, this method of intercellular signaling seems ideally suited for bacteria in a diffusion-limited environment such as the biofilm. Production of the quorum-sensing molecules known as acyl-homoserine lactones (acyl-HSLs) has been demonstrated in both natural and cultured biofilms (1, 7, 26, 41). The importance of acyl-HSLs in single-species biofilms has been clearly demonstrated. In *P. aeruginosa*, acyl-HSLs are responsible for defining the separations between bacterial pillars in the three-dimensional structure of the biofilm (7). *P. aeruginosa* mutants that do not produce acyl-HSL form biofilms in which the cells are closely packed together and are easily disrupted by sodium dodecyl sulfate. Acyl-HSLs are also mediators of surface attachment in *Pseudomonas fluorescens* (1). Extracellular signals, therefore, enforce the zoning laws in single-species biofilms.

Although little is known of the role of intercellular signaling in multispecies biofilms, we suspect it may differ significantly from that observed in single-species biofilms. We expect these signals to be especially important in favorable environments where surfaces are heavily colonized and competition for attachment to the surface is fierce. We define these signals broadly as any actively or passively transported bacterial products that alter the state of neighboring microbes. These might include bacterial metabolites, acyl-HSLs, secreted proteins, genetic material such as DNA or RNA, or as yet undiscovered bacterial products. These signals might alter the distribution of specific bacterial species in the biofilm, alter protein expression in neighboring cells, introduce new genetic traits into neighboring cells, or lure and incorporate bacteria into the biofilm for subsequent consumption. The last function of intercellular communication in multi-species biofilms is both fascinating and as yet uncharted. There are, however, laboratory models of lethal interspecies bacterial communication (38, 39). *M. xan-*

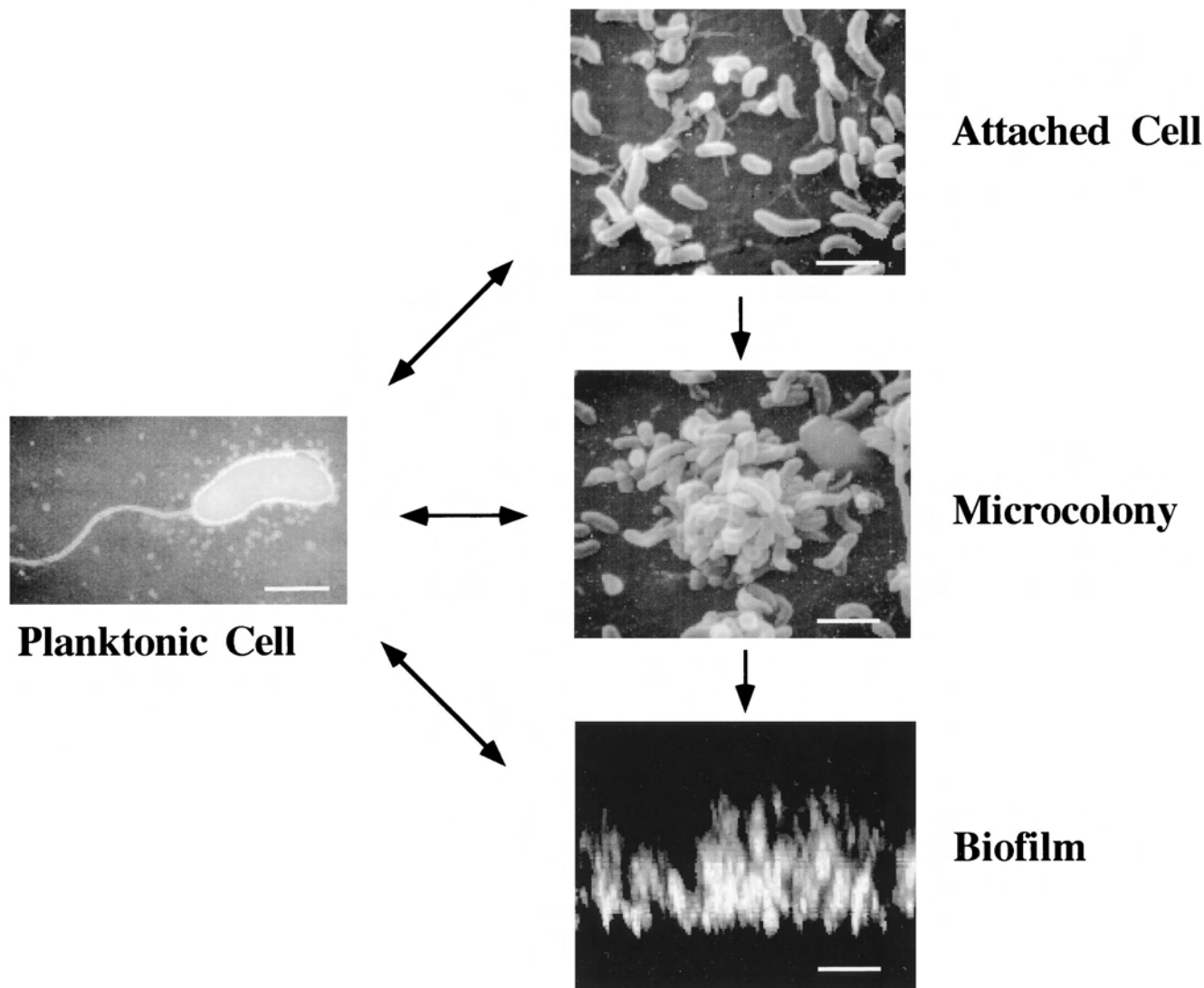


FIG. 2. A microscopic study of the steps in biofilm formation by *V. cholerae*. The planktonic bacterium was visualized by transmission electron microscopy (bar = 1 μ M), the attached cells and microcolony were visualized by scanning electron microscopy (bar = 2 μ M), and the biofilm micrograph represents a vertical section through a 20- μ m biofilm taken by confocal scanning laser microscopy (bar = 10 μ M).

thus, for instance, is known to prey on *E. coli*. On soft agar plates, *E. coli* moves towards *M. xanthus*. Its chemotaxis machinery is required for this directed movement. The hypothesis is that *M. xanthus* secretes a signal that lures *E. coli* to its death (39). The bacteriocins are another example of cell-cell signals that result in lethal interspecies interactions. These are bacterially derived antibacterial proteins that act against closely related species (38). In fact, mathematical models predict that bacteriocin production would be most advantageous in a spatially structured environment such as a biofilm (10, 12), suggesting that these secreted proteins may have evolved specifically for the biofilm environment. The impact of intercellular communication on multispecies biofilms is potentially far reaching, and we predict that intercellular signalling, whether beneficial or detrimental to the recipient, will be a critical factor in the diversity and distribution of bacteria in a biofilm.

The thick biofilm is like a densely settled area. The buildings are back to back, and they are filled with people. It is difficult to imagine how bacteria can divide in such an environment.

Thus, zero population growth may be the norm because the spatial constraints are such that cell division is impeded by surrounding exopolysaccharide. Such a situation may be akin to that of the polymer-encased bacteria that are used for biocatalytic engineering applications (25). Although these bacteria do not divide, they are viable and culturable once freed from the plastic encasement. Thus, one of the dictates of planktonic bacterial life, that consumed nutrients are funneled into procreation, may not apply to biofilm-associated cells. One possibility is that cell division is infrequent in a mature biofilm, and instead excess energy is used to make exopolysaccharide, an edible scaffold, that the cell can digest and use in time of need. As an example of this, production of an exopolysaccharide lyase has been shown for *P. fluorescens* under starvation conditions (1). This enzyme degrades the biofilm-associated exopolysaccharide for consumption and frees cells from the biofilm scaffold to seek more favorable environments. Both these functions seem adaptive during times of starvation.

One advantage of biofilm living is the ability to acquire

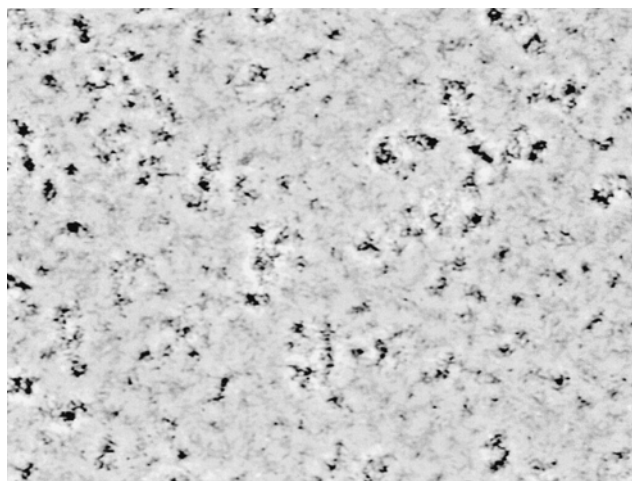


FIG. 3. The first frame in a movie taken of the activity in a mature *V. cholerae* biofilm. The dark collections of bacteria represent pillars in the biofilm, while a monolayer of cells is seen between the pillars. The corresponding movie, which demonstrates the activity associated with a mature biofilm, is accessible at <http://gasp.med.harvard.edu/biofilms/jbmini/movie.html> both in gray scale and in color to accentuate moving bacteria.

transmissible, genetic elements at accelerated rates. There are many reports of accelerated rates of conjugation in bacterial biofilms (2, 18). This suggests that evolution by horizontal transfer of genetic material may occur rapidly in a biofilm, making it the perfect milieu for emergence of new pathogens by acquisition of antibiotic resistance, virulence factors, and environmental survival capabilities.

There are other advantages to living in a city. People live together because this is advantageous in times of adversity. Similarly, biofilm-associated cells are more resistant to many toxic substances such as antibiotics, chlorine, and detergents (4). There is evidence that decreased diffusion into the biofilm (8, 43), decreased bacterial growth rate in a biofilm (11), biofilm-specific substances such as exopolysaccharide (50), and the quorum-sensing specific effects (7, 17) may be reasons for this resistance. This property of biofilms, thus, is most likely multifactorial.

If the bacteria were unable to escape the biofilm, the biofilm would, like an old apartment building, become a death trap when the nutrient supply was exhausted, environmental conditions became unfavorable, or an unfriendly neighbor entered the community. Once the bacterium is encased in exopolysaccharide, however, abandoning the biofilm becomes a significant task. At such times, a polysaccharide lyase may provide the bacterium with an escape (1). This product hastens detachment of biofilm-associated cells. Thus, the cycle of attachment shown in Fig. 1 is completed.

We liken the multispecies bacterial biofilm to a city where bacteria settle selectively, limit settlements of new bacteria, store energy in exopolysaccharide, and transfer genetic material horizontally all for the good of the many. A genetic and biochemical understanding of the interactions between species in a biofilm, complex though they may be, is critical to our understanding of how the biofilm city functions and survives. We predict that in multiple-species biofilms many different types of soluble biofilm-specific signals will be discovered whose influence on dissimilar bacterial neighbors will be sometimes helpful and sometimes detrimental or even fatal. When conditions in the biofilm change, such interactions may determine which cells survive, which perish, and which move on. An

understanding of the relationships among species in the biofilm city is essential to our appreciation of the benefits of biofilm-associated living.

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