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# Biofilms

*A new understanding of these microbial communities is driving a revolution that may transform the science of microbiology*

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When we think about bacteria, most of us imagine a watery milieu, with single-celled organisms swimming about. We might envision these solitary entities getting together with some of their brethren now and then to cause some disease or spoil some food, but once the job is done they return to their isolated existence. This image of bacterial existence, it turns out, is not only oversimplified but perhaps misleading as well. In nature, the majority of microorganisms live together in large numbers, attached to a surface. Rather than living as lonely hermits in the so-called planktonic form, most bacteria spend much of their lives in the microbial equivalent of a gated community—a biofilm.

A mature biofilm is a fascinating construction: It can form layers, clumps and ridges, or even more complex microcolonies that are arranged into stalks or mushroom-like formations. The residents of the biofilm may be a single species or a diverse group of microorganisms distributed in various neighborhoods. Their common bond is a matrix made of polysaccharides, DNA and

proteins, which together form an *extracellular polymeric substance*—what many microbiologists just call slime.

It's becoming increasingly clear that the communal life offers a microorganism considerable advantages. The physical proximity of other cells favors synergistic interactions, even between members of different species. These include the horizontal transfer of genetic material between microbes, the sharing of metabolic by-products, an increased tolerance to antimicrobials, shelter from changes in the environment and protection from the immune system of an infected host or from grazing predators. The formation of a biofilm has even been likened to the program by which cells within a multicellular organism differentiate.

An appreciation of the significance of biofilms is a relatively recent phenomenon. Only within the past 15 to 20 years have biologists begun to examine the physiology of these microbial communities. This is an extraordinary state of affairs, given that the Dutch microscopist Antonie van Leeuwenhoek first described biofilms in the late 1600s. Using acetic acid, he had tried to kill a biofilm—the dental plaque on his dentures—but noted that only the free-swimming cells could be destroyed. Despite the early discovery of microbial communities, microbiology departed from these observations to focus primarily on planktonic bacteria.

To be sure, not everyone agrees that biofilms are the predominant form of bacteria in nature. The vast majority of laboratory methods used today examine cultured microorganisms in their

planktonic mode. But we believe that microbiology is experiencing a shift in how bacteria are conceptualized. We predict that this new perspective of how microorganisms live will have fundamental consequences for medicine, industry, ecology and agriculture.

## Biofilms Are Everywhere

Most people are familiar with the slippery substance covering the rocks in a river or a stream. This particular slime is an aquatic biofilm made up of bacteria, fungi and algae. It begins to form after bacteria colonize the rock's surface. These microbes produce the extracellular polymeric substance, which is electrostatically charged so that it traps food particles and clay and other minerals. The matter trapped in the slime forms microscopic niches, each with a distinct microenvironment, allowing microorganisms that have different needs to come together to form a diverse microbial consortium.

A biofilm matrix is considered to be a *hydrogel*, a complex polymer hydrated with many times its dry weight in water. The hydrogel characteristics of the slime confer fluid and elastic properties that allow the biofilm to withstand changes in fluid shear within its environment. So biofilms often form streamers—gooey assemblages of microbes that are tethered to a surface. As running water passes over the biofilm, some pieces may break free and so spread the microbial community downstream. It is believed that bacteria can colonize the lungs of patients on ventilators in this way, causing often-fatal pneumonia in critically ill patients.

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Figure 1. Yellowstone National Park is full of unusual microscopic life, including thermophilic algae and (*at bottom right*) filamentous bacteria. The biofilms that these organisms often form may be obvious to the nature photographer's eye, but they are not well understood. Despite the discovery of microbial biofilms as far back as the 17th century, scientists have largely focused their attentions on the solitary, or planktonic, forms of microorganisms. In nature, however, most microorganisms live together in large communities attached to a surface, a lifestyle that profoundly affects their interaction with other organisms and their resilience as pathogens. New studies of biofilms may change the direction of microbiological research—with the promise of controlling infections by bacteria and other microorganisms. (Photographs courtesy of the National Park Service.)

A microorganism's extraordinary ability to spread explains how biofilms show up in the unlikely places. The steel hull of a ship at sea can be coated with biofilms that increase the drag on the vessel and so compromise its speed. Other biofilms wreak havoc in the oil industry by facilitating the microscopic corrosion of metals and limiting the lifespan of pipelines. Some biofilms, made up of the ancient lineage of prokaryotes (organisms lacking a nucleus) called *archaea*, can even survive the hostile hydrothermal environments of hot springs and deep-sea hydrothermal vents. The aptly named archaeobacterium *Pyrodictium* thrives at the bottom of the sea, growing in a moldlike layer on sulfur crystals in the dark, anaerobic environment of a hydrothermal vent, where temperatures may exceed 110 degrees Celsius.

Perhaps one of the most extraordinary environments where one can find a biofilm is in the belly of a dairy cow. Biofilms are part of the normal complement of microbes in many healthy animals, but the presence of these microbial communities in ruminants pro-

vides a rich example of the interactions within a biofilm.

We begin with the *rumen*, the largest compartment of the bovine stomach, which can hold a liquid volume in excess of 150 liters. It is filled with so many microbes that microbiologists refer to cows as mobile fermenters. Bacteria colonize the digestive tract of a calf two days after it is born. Within three weeks the microorganisms have modified the chemistry inside the rumen, which soon becomes home to a reported 30 species of bacteria, 40 species of protozoa and 5 species of yeast. The cells in this biofilm thrive in the mucous layer of the stomach and grow on the food ingested by the animal. Cows, of course, eat grass, which consists largely of cellulose, a complex carbohydrate that cannot be broken down by mammalian digestive enzymes. But cellulose is a perfect fuel for the bacteria in the biofilm, which convert it into a microbial biomass that in turn supplies the proteins, lipids and carbohydrates needed by the cow.

The heart of this process is a microscopic ecosystem that begins when a

pioneering planktonic bacterium in the rumen, a species such as *Ruminococcus flavefaciens*, gains access to the inner parts of a leaf, perhaps one that might have been broken by the cow's chewing. These bacteria attach themselves to the cellulose in the inner layers of the leaf and proliferate to form a rudimentary biofilm. The microbes release cellulose-degrading enzymes, which produce simple sugars and metabolic by-products that attract other bacteria—anaerobic fermenters such as the spiral-shaped *Treponema byranti*, which ingest the sugars and produce organic acids, including acetic acid and lactic acid.

The acidic metabolites would normally slow the growth of the bacteria by a process of feedback inhibition, but it so happens that other microorganisms join the biofilm community and eat the organic acids. These are the *methanogens*, archaea whose actions accelerate the growth of the bacterial community and prevent the inhibitory feedback. As the name suggests, methanogens produce methane—lots of it. Approximately 15 to 25 percent of the global emission of methane, which totals 7.5

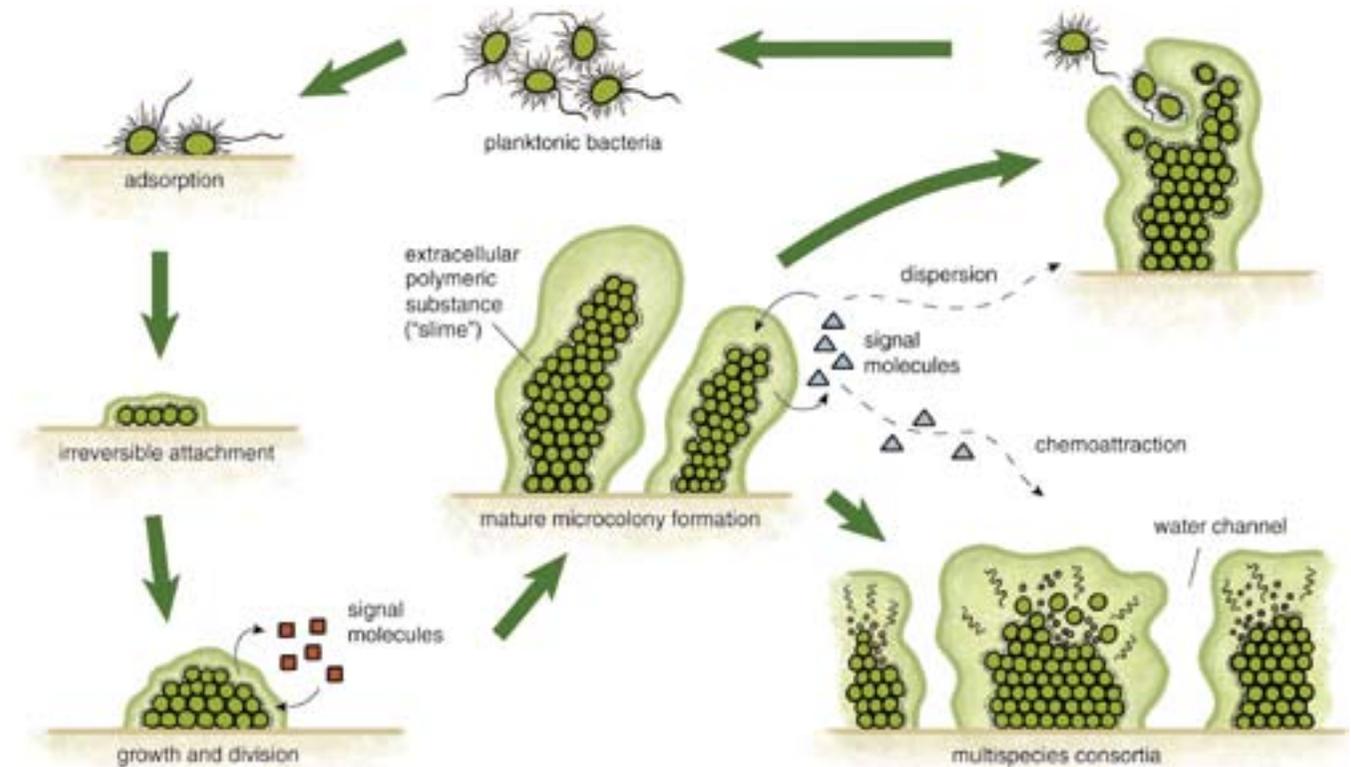


Figure 2. Formation of a biofilm is analogous to the development of a multicellular organism, with intercellular signals regulating growth and differentiation. A typical biofilm forms (follow arrows from upper left) when free-swimming planktonic bacteria adsorb to a biotic or inanimate surface—an association that is initially reversible, but then irreversible. Adhesion triggers the first physiological changes on the path to a biofilm lifestyle. As the bacteria grow and divide, molecular signals passed between the cells provide information on cell density—a process called quorum sensing. In a maturing colony, the microbes produce an extracellular polymeric substance—a matrix of polysaccharides, DNA and proteins that encases the microcolony structure. Planktonic cells may leave the biofilm to establish new biofilm structures. Signals from the collective may also recruit new microbial species to join the consortium.

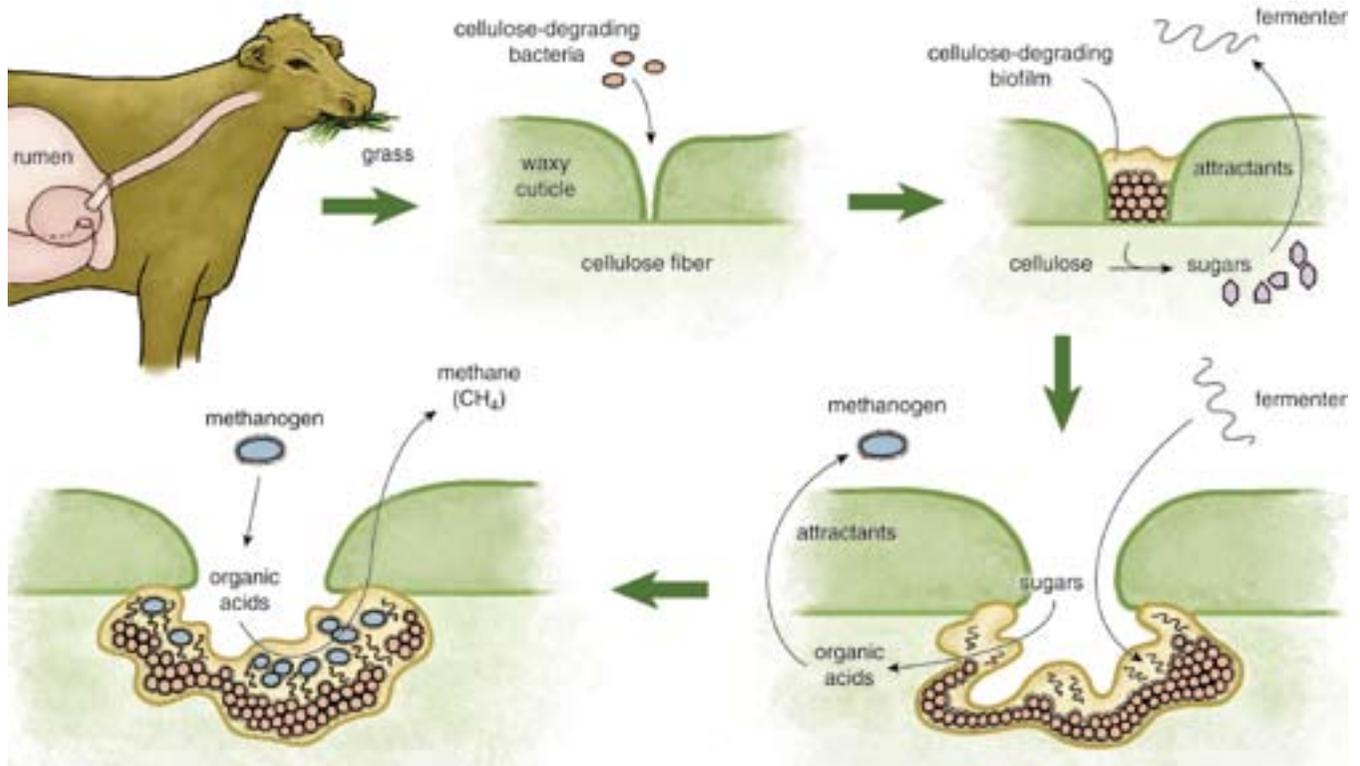


Figure 3. A multispecies biofilm in a cow's rumen provides an example of the intricate relations between the cells in a microbial community, not to mention the roles biofilms play in the nutrition of ruminants and other animals. The colony begins with cellulose-degrading bacteria, which digest the grass eaten by the ruminant. (A cow's cud can be passed between its mouth and rumen several times before these products are passed to its remaining stomachs and intestines.) The simple mono- and disaccharide sugars produced by these cellulolytic bacteria attract fermenting microorganisms, which convert the sugars into organic acids. In turn, the organic acids attract methanogenic microbes that join the biofilm. The organic acids not neutralized by the cow's saliva would normally inhibit further growth in the biofilm, but the methanogens convert these molecules into methane. The entire process produces a protein-rich microbial mass that can be digested by the cow, providing the bulk of the animal's nutrients.

billion kilograms per year, is attributable to the flatulence of ruminants. Because methane traps heat in the atmosphere, the biofilm hidden away in a cow's stomach may play a nontrivial role in global climate change.

Animals aren't the only living things that provide a home to biofilms. Microbial colonies have been recognized on tropical plants and grocery-store produce since the 1960s, but it wasn't until the past decade that the term *biofilm* was used to describe bacterial growth on a plant's surface. In this domain, life in a biofilm confers many advantages to the individual cell, including protection from a number of environmental stresses—ultraviolet radiation, desiccation, rainfall, temperature variations, wind and humidity. The biofilm also enhances a microorganism's resistance to antimicrobial substances produced by competing microorganisms or the host's defenses.

Relations between plants and biofilms can be quite varied. In some instances the plant merely serves as a mechanical support, so the biofilm is simply a harmless epiphyte. In other

cases, the plant may provide some nutrients for the microbes, such as the saprophytes that feed on decaying plant matter; these too pose no danger to the plant. But there can be trouble when certain epiphytic populations with the genetic potential to initiate a pathogenic interaction with the host grow large enough to overwhelm the host's defense mechanisms. Then the cells in the biofilm coordinate the release of toxins and enzymes to degrade the plant tissue. What began as an innocuous relationship ends in disease.

Belowground, plants and biofilms may also engage in some fairly elaborate interactions. For example, *Pseudomonas fluorescens* colonizes roots and protects plants from pathogens by producing antibiotics that exclude fungi and other bacterial colonizers. But fungal biofilms can also be beneficial to the plant. Certain mycorrhizal fungi penetrate a plant's root cells while also forming an extensive network in the soil; thus they provide a drastic increase in the surface area that the plant can use for the absorption of water and nutrients.

On the other hand, bacteria of the genus *Rhizobium* fix nitrogen from the atmosphere by converting N<sub>2</sub> gas into ammonia (NH<sub>3</sub>). This process can involve some intricate chemical signaling between the plant and the bacteria that results in the formation of nodules within the root where the bacterial aggregates engage in nitrogen fixation. Perhaps the most intricate relation involves an interaction between the rhizobia, the mycorrhizal fungi and a plant host. The bacteria form a biofilm on the surface of the fungus, which in turn makes its connection with the plant, and so creates a tripartite symbiotic system that relies on the formation of biofilms by two microorganisms. (Unless the soil is alkaline, the system requires another player, nitrifying bacteria to oxidize the ammonia; they live not in the nodule but in nearby soil.)

Finally, let us consider the pathogenic interactions of biofilms within the plant's vasculature. Unfortunately, vascular diseases are currently untreatable and tend to be devastating to many economically important crops. A few pathogenic biofilms have been

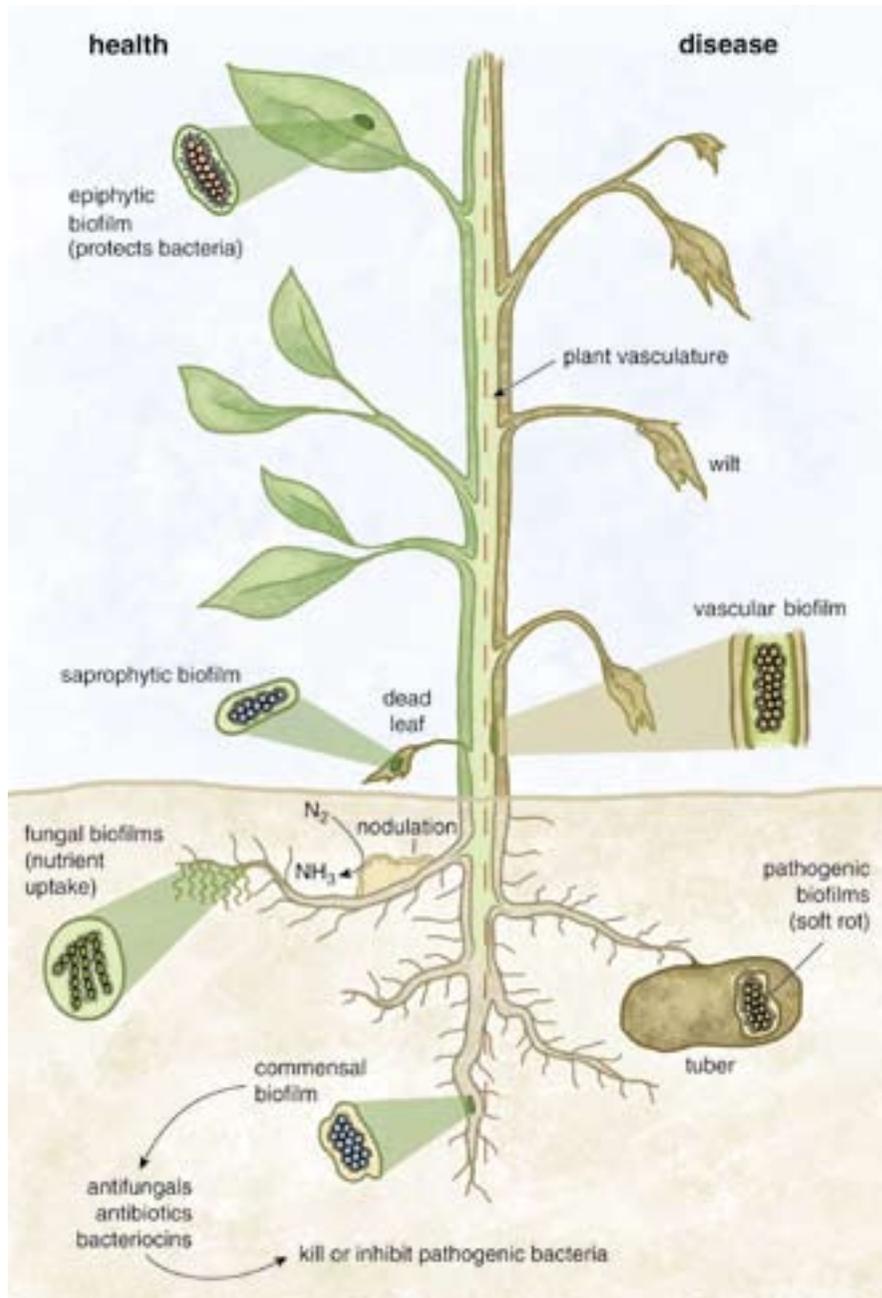
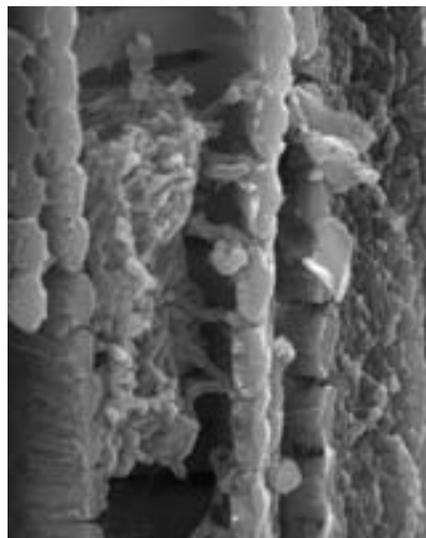


Figure 4. Relations between plants and biofilms run the gamut from healthy (above, left side) to pathogenic (right side). Many biofilms are harmless: Saprophytes merely digest dead leaves, whereas epiphytes often simply use the plant for mechanical support. Some interactions may even be valuable: Bacteria-filled nodules below ground enable a plant to fix nitrogen, and certain fungal biofilms give the plant's roots a greater surface area for the absorption of water and nutrients. Some commensal bacteria release substances that kill potential pathogens. Unfortunately, biofilms may overwhelm the plant's defense mechanisms, causing disease processes that attack the plant from below the ground or even from the vasculature within. *Xylella fastidiosa* biofilms (right, a 25-micrometer-wide segment) are a problem for grape and citrus growers and others. (Micrograph courtesy of the authors.)



described in the water-carrying xylem of plants, but here we'll merely address *Xylella fastidiosa*. This pathogen causes Pierce's disease in grapevines and citrus variegated chlorosis in sweet oranges—diseases that have had a major impact on the wine industry in California and the citrus industry in Brazil, with economic losses exceeding \$14 billion in the past decade. Pierce's disease also limits the development of a wine industry in Florida because the bacterium is endemic in that region.

*X. fastidiosa* is transmitted by xylem-feeding insects, called sharpshooters, that acquire the bacteria while feeding from infected plants. The bacteria form a rudimentary biofilm inside the insect's gut, and this allows them to be sloughed off indefinitely in aggregates sufficient to infect another plant when the insect feeds again. In turn, the biofilms clog the plant's xylem and cause symptoms related to water stress. So the biofilm plays a key role in the colonization of the plant vessels, the propagation of the disease and its pathogenicity.

The appreciation of biofilms' importance in plant disease has only just begun, and it will probably take some time for the idea to be applied in plant microbiology. However, the benefits could be significant. A better understanding of the associations between plants and biofilms may lead to more efficacious and environmentally friendly treatments for disease. It may also lead to the development of commercial applications that could improve the beneficial interactions between plants and microorganisms. Indeed, various rhizobia are now being used on commercial farms as a biotic fertilizer.

### United We Stand

The Centers for Disease Control and Prevention estimates that up to 70 percent of the human bacterial infections in the Western world are caused by biofilms. This includes diseases such as prostatitis and kidney infections, as well as illnesses associated with implanted medical devices such as artificial joints and catheters and the dental diseases—both tooth decay and gum disease—that arise from dental plaque, a biofilm. In the lungs of cystic fibrosis patients, *Pseudomonas aeruginosa* frequently forms biofilms that cause potentially lethal pneumonias. There is a long list of biofilm-related ailments, and many scientists

believe the list will continue to grow as we learn more about the function of these microbial structures.

In almost all instances, the biofilm plays a central role in helping microbes survive or spread within the host. That's because the slimy matrix acts as a shield, protecting pathogenic bacteria from antibodies and white blood cells, the sentinels of the immune system. Biofilms are also notorious for their ability to withstand extraordinarily high concentrations of antibiotics that are otherwise lethal in smaller doses to their planktonic counterparts. In fact, a biofilm can be 10 to 1,000 times less susceptible to an antimicrobial substance than the same organism in suspension.

This challenge, with its grave implications for the fight against pathogens, has been the focus of our research group's investigations. We have developed and licensed to a Canadian start-up company a technology (the Calgary Biofilm Device, now called the MBEC Assay) that can be used to rapidly screen biofilms for their sensitivity to antimicrobials. A pharmaceutical laboratory testing a potential drug to fight pneumonia or catheter-related infection can now find out whether a drug that is effective against free-floating pathogens will be successful in eradicating the same organisms in a biofilm.

During the development of this technology, we have learned some remarkable things about biofilms. We have moved on to exploring some pathogenic "co-biofilms" of unrelated species living together, along with specific mechanisms that may be important in drug development. For example, biofilms' resistance to high metal concentrations makes them useful in removing toxic metals from the environment. But a detailed understanding of how the films handle metal toxicity may also open the door to antimicrobial treatments targeted at biofilms.

We and other investigators have learned that part of the extraordinary resilience of bacteria arises from the remarkable heterogeneity inside the biofilm. Microbes closest to the fluid that surrounds the biofilm have greater access to nutrients and oxygen compared with those in the center of the matrix or near the substratum. As a result, the bacteria in the outer layers of the community grow more quickly than those on the inside. This comes into play as a defense mechanism because many antibiotics are effective only against

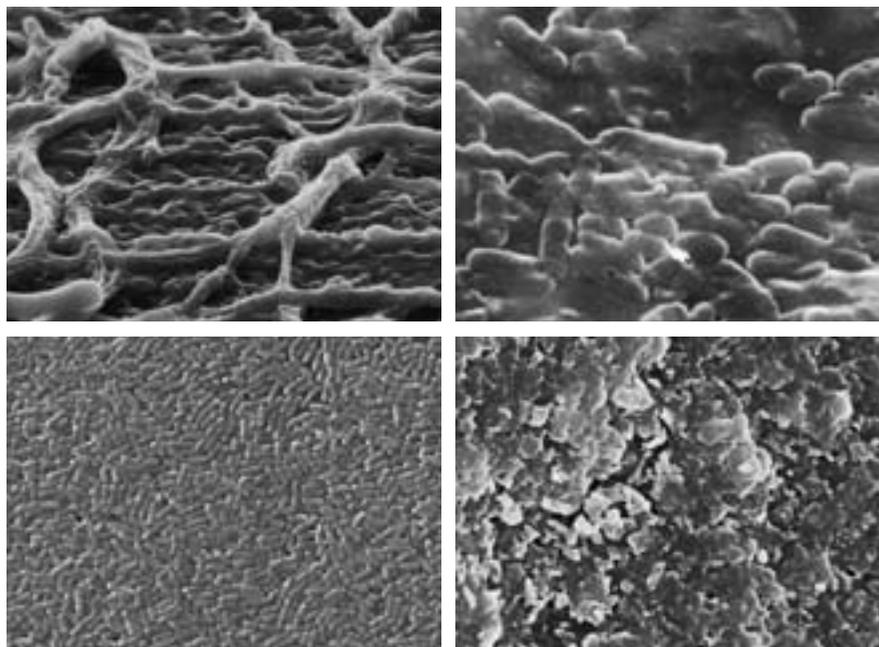


Figure 5. Many biofilms can cause disease and discomfort in human beings. The fungus *Aspergillus fumigatus* (top left) causes potentially lethal lung infections. The opportunistic pathogen *Pseudomonas aeruginosa* (bottom left) can be fatal to patients with cystic fibrosis. Bacterial biofilms growing on contact lenses (top right) or catheters (bottom right) can cause serious infections. (Micrographs courtesy of the authors, Merle Olson and Liz Middlemiss, University of Calgary. Image-area widths range from 14 [contact lens film] to 66 micrometers [*A. fumigatus*].)

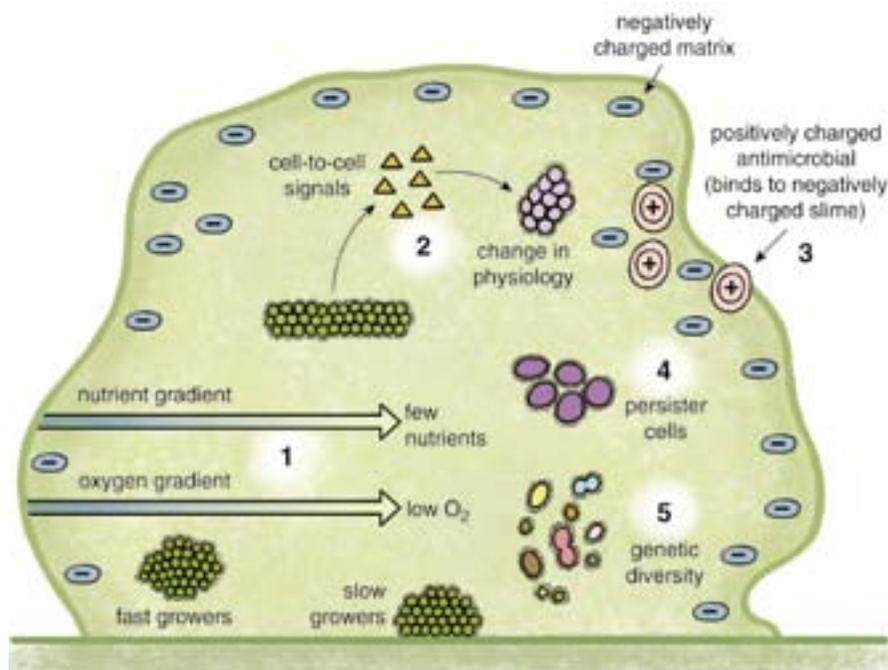


Figure 6. Biofilms derive their extraordinary tolerance to antimicrobial compounds from several factors. Bacteria near the center of a microcolony grow very slowly because they are exposed to lower concentrations of oxygen and nutrients (1). They are thus spared the effects of antibiotic drugs, which are much more effective against fast-growing cells. Intercellular signals (2) can alter the physiology of the biofilm, causing members to produce molecular pumps that expel antibiotics from the cells and allow the community to grow even in the presence of a drug. The biofilm matrix is negatively charged (3) and so binds to positively charged antimicrobials, preventing them from reaching the cells within the colony. Specialized populations of persister cells (4) do not grow in the presence of an antibiotic, but neither do they die. When the drug is removed, the persisters can give rise to a normal bacterial colony. This mechanism is believed to be responsible for recurrent infections in hospital settings. Finally, population diversity (5), genetic as well as physiological, acts as an "insurance policy," improving the chance that some cells will survive any challenge.

## A New Way to Look at Microorganisms

The conventional way to grow bacteria is to inoculate a flask that contains a broth of nutrients. If you stir the broth constantly, the cells will have plenty of oxygen and a homogeneous distribution of food. Under these optimal growth conditions, you'll get a nice batch of planktonic bacteria floating in the solution.

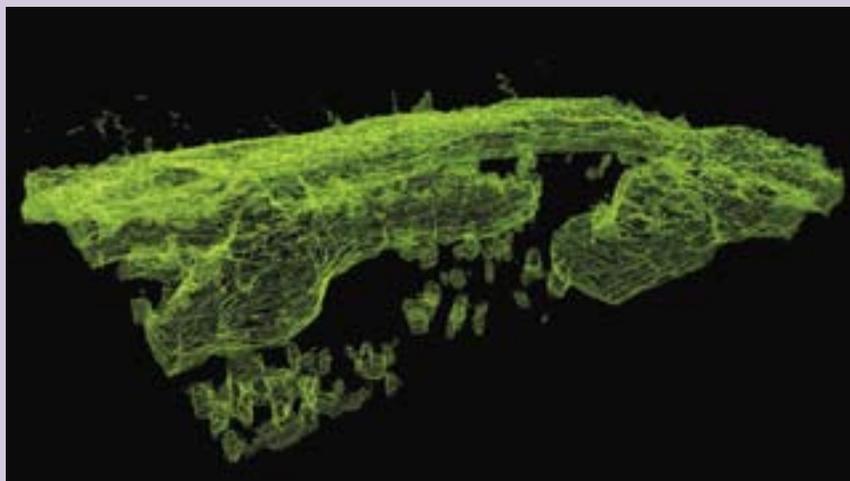
Of course, nature rarely provides such a perfectly uniform environment. Bacteria in a biofilm grow in a matrix of heterogeneous microenvironments that vary in oxygen content, nutrient distribution and countless other chemical vagaries. The bacteria that stick to the sides of the laboratory flask form mature biofilms. Ironically, until recently these were largely ignored or destroyed.

Several new technologies have been explicitly developed to grow and examine biofilms in the laboratory. One method uses a rotating disk inside an inoculated broth. The shear force caused by the rotation encourages the formation of a biofilm on the disk. Our laboratory group has also recently developed a biofilm-based assay for examining the effectiveness of antimicrobials in a high-throughput fashion—that is, the device allows us to create 96 statistically equivalent biofilms, and it can also be used to test various dilutions of antimicrobial compounds with a standard microtiter plate, the MBEC assay. We are currently using this tool to discover new substances that may be effective against biofilms.

Another device, called a flow cell, consists of a chamber and an optically transparent surface, such as a glass coverslip. A growth medium is pumped through the chamber, promoting the formation of a thick biofilm on the glass surface. This method allows scientists to examine microbial communities in a confocal laser-scanning microscope (CLSM). Specialized computer software can be used to assemble images captured by CLSM to create a three-dimensional view of a biofilm.

CLSM might be considered as a complement to scanning electron microscopy (SEM). SEM can achieve magnifications that are 10 times greater than CLSM and so can be used to examine the shape and arrangement of single cells, whereas CLSM provides an overview of the biofilm's structure. SEM also kills the microbial community, whereas CLSM is not as invasive. Sequences of images can be compiled into movies that show how microorganisms live and die in a biofilm.

Finally, new methods in proteomics and transcriptomics allow scientists to examine the distribution and patterns of proteins and gene expression in biofilms. The development of these techniques has opened the door to a new view of how microorganisms live.



Images assembled from "slices" created by confocal laser-scanning microscopy can provide a detailed look at a microbial biofilm's structure. This is a biofilm of *Escherichia coli* that has been grown in the laboratory and made visible by splicing a gene for a fluorescent protein into its DNA. A close-up appears on the magazine's cover. (Image courtesy of EDM Studio.)

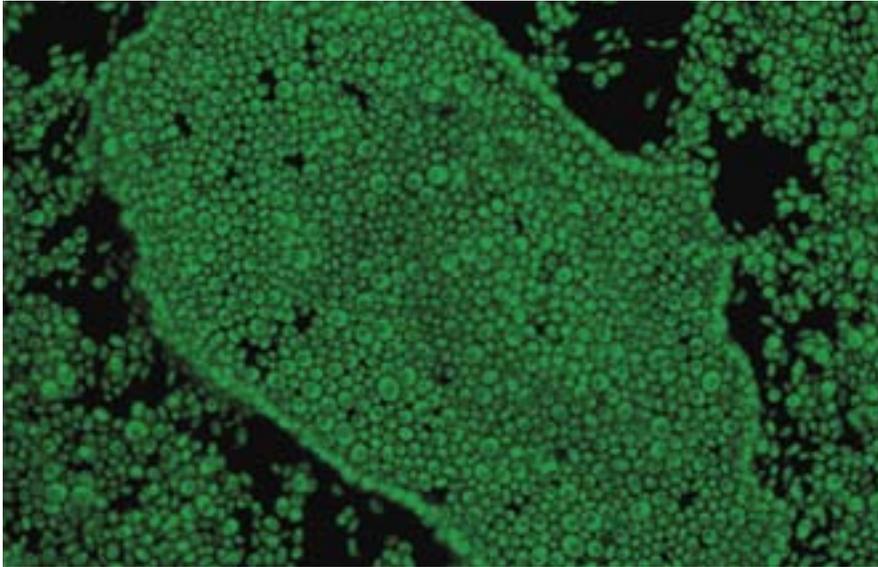
fast-growing cells, so the slow growers within the biofilm tend to be spared. Moreover, the cells in the center of the community are further protected from the environment because the biofilm matrix is negatively charged. This restricts the entry of positively charged substances, such as metal ions and certain antibiotics.

One of the most intriguing defense mechanisms enabled by the formation of a biofilm involves a kind of intercellular signaling called *quorum sensing*. Some bacteria release a signaling molecule, or inducer. As cell density grows, the concentration of these molecules increases. The inducers interact with specific receptors in each cell to turn on "quorum sensing" genes and initiate a cascade of events, triggering the expression or repression of a number of other genes on the bacterial chromosome. Some bacterial strains seem to rely on quorum sensing more than others, but anywhere from 1 to 10 percent of a microbe's genes may be directly regulated by this process.

Quorum sensing is known to affect the production of enzymes involved in cellular repair and defense. For example, the enzymes superoxide dismutase and catalase are both regulated by quorum sensing in *P. aeruginosa*, which forms mucoidal clusters of bacterial cells embedded in cellular debris from the airway epithelial layer in the cystic fibrosis patient's lung. The first enzyme promotes the destruction of the harmful superoxide radical ( $O_2^-$ ), whereas the second converts the equally toxic hydrogen peroxide molecule ( $H_2O_2$ ) into water and molecular oxygen. These enzymes help the biofilm survive assaults not only from disinfectants, but also from the cells of a host's immune system that typically kill bacteria by unleashing antimicrobial agents, including reactive oxygen species.

Quorum sensing may also be involved in the defense against antibiotic drugs. Here the mechanism increases the production of molecular pumps that expel compounds from the cell. These so-called *multidrug efflux pumps* reduce the accumulation of the antibiotics within the bacterium and even allow the microbe to grow in the presence of the drugs.

There is also heterogeneity among the cell types in the biofilm that contributes to antimicrobial tolerance. Specialized survivor cells, called "persisters," are slow-growing variants that exist in



**Figure 7.** *Candida tropicalis*, a yeast that causes vaginitis, thrush and cardiac infections, forms biofilms that are highly resistant to antifungal and antimicrobial treatments. The image was created using a confocal laser-scanning microscope, a new device that provides a snapshot of microbial microcolonies, which make up a biofilm (see discussion on facing page). (Image courtesy of the authors.)

every bacterial population. They are genetically programmed to survive environmental stress, including exposure to antibiotics. Although persisters do not grow in the presence of an antibiotic, they also do not die. Persisters are not mutants; even in a genetically uniform population of cells a small portion undergo a spontaneous switch to the persistent form. This past year Kim Lewis of Northeastern University demonstrated that persisters generate a toxin, RelE, that drives the bacterial cell into a dormant state. Once antibiotic therapy has ceased, the persisters give rise to a new bacterial population, resulting in a relapse of the biofilm infection.

The use of persister cells as a defense mechanism may have evolved early in the history of life. In this post-genomics era, scientists have learned that many related genes are present in a variety of distantly related bacteria, suggesting that similar genes were present in the primeval common ancestors. Yet the reduced growth rate of the persisters poses a paradox because slowed cell division decreases the fitness of a population. Edo Kussell and his colleagues at Rockefeller University recently proposed that bacterial persistence may have evolved as an “insurance policy” against rare antibiotic encounters. If so, in attempting to overcome bacterial antibiotic tolerance, scientists are battling an ancient mechanism that may have been refining itself for billions of years. If we are ever to succeed in controlling

bacterial infection, more research efforts need to be focused on biofilms rather than the comparatively vulnerable planktonic form.

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