Findings about biofilms from George O'Toole's lab have graced the covers of numerous journals, including those pictured behind him here.

Science versus Slime

Text by Amos Esty • Photographs by Jon Gilbert Fox

For a WEB EXTRA with links to more on biofilms and some of O'Toole's papers, see dartmed.dartmouth.edu/f10/we05.

Biofilms are everywhere. These colonies of bacteria and other microorganisms can be found in mundane places, such as shower curtains, sink drains, and toothbrushes, as well as in more exotic locales, from chilly Arctic waters to scalding deep-sea vents.

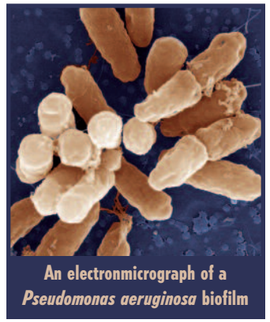
In some cases, biofilms are composed of a single species. Elsewhere—such as on human teeth, where they form plaque—a biofilm may include hundreds of species. And in addition to bacteria, biofilms can include fungi, algae, protozoa, and other organisms. Some biofilms are large enough to be visible to the naked eye, but most remain very small, perhaps 30 to 50 micrometers across—roughly half the width of a human hair. For many microorganisms, joining a biofilm is business as usual. Most bacterial cells spend at least part of their life cycle in a biofilm, and for some bacteria this settled lifestyle makes up the bulk of their existence.

Biofilms' ability to thrive wherever there is moisture, food, and a surface for microbes to attach to has made them a subject of scientific curiosity. But it also makes them a treacherous threat to human health. They are the culprits, for example, in more than half of all hospital-acquired infections, and they form large colonies in the lungs of people with cystic fibrosis, causing chronic infection and, eventually, premature death.

The difficulty scientists and physicians have had combating biofilms might seem surprising, given that one of the great medical advances of the 20th century was the development of antibiotics to fight off bacterial infections. But the problem with biofilms, explains DMS microbiologist George O'Toole, Ph.D., is that although they are composed of organisms that can often be treated effectively in their independent form, when those organisms gather as a biofilm they are suddenly much less vulnerable. Bacteria in biofilms can be 1,000 times more resistant to antibiotics than free-swimming bacteria. This increased resistance is only one of the many ways bacteria in biofilms differ from bacteria outside biofilms. In some ways, biofilms are more like a stand-alone organism than a community of individuals. And the more scientists learn about biofilms, the more complicated, sophisticated, and impressive these microbial aggregations seem.

Scientists have been interested in bacteria for centuries—ever since an amateur Dutch scientist named Antonie Van Leeuwenhoek observed "little animals" through his homemade microscope in the 1670s. But researchers only began to pay serious attention to biofilms three centuries later, in the 1970s, when they realized that bacteria in biofilm form were causing medical problems, such as dental plaque and hospital-acquired infections. They began their research by trying to tease apart the mechanisms that allow biofilms to adhere to different surfaces, thinking that perhaps the bacteria were positively charged and the surface negatively charged, for example. "There wasn't a lot of focus on the microbe," O'Toole says.

O'Toole began reading about biofilms in the late 1980s, when he was a graduate student at the University of Wisconsin. What he learned convinced him to focus on biofilms when he moved on to a postdoctoral fellowship at Harvard. At the time, the mechanical approach to biofilm research made sense, he says,



An electronmicrograph of a *Pseudomonas aeruginosa* biofilm

Biofilms can be as mundane as slime on a shower curtain, or as menacing as antibiotic-resistant bacterial colonies on a medical device or in a human lung. But luckily, scientists are making progress in understanding these mysterious aggregations of microorganisms.

Amos Esty is the managing editor of Dartmouth Medicine.

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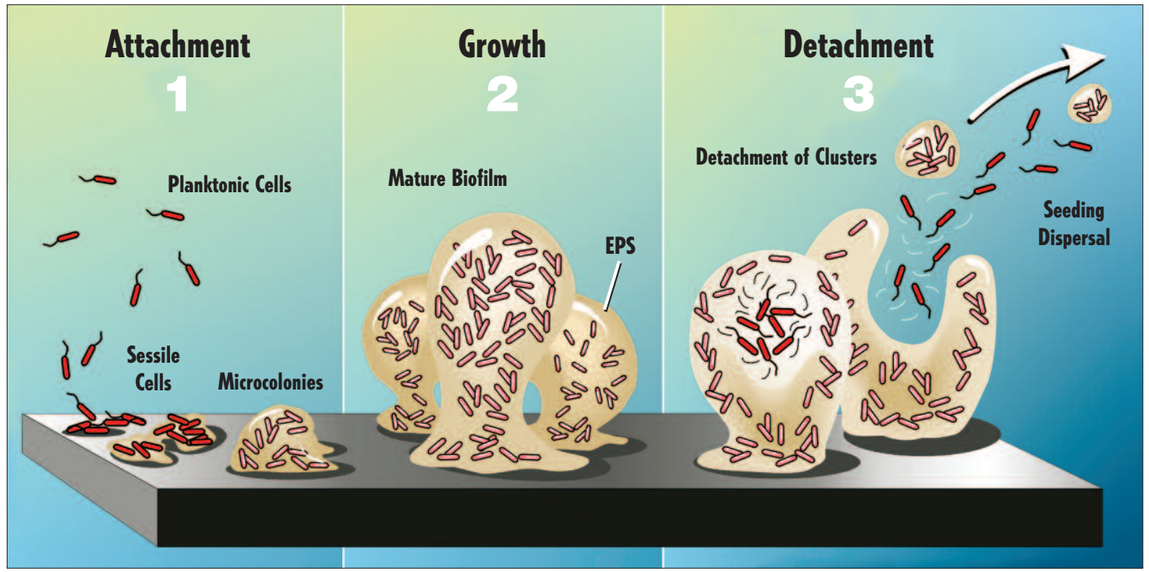


This illustration shows the life cycle of a biofilm. In panel 1, free-swimming (or planktonic) bacteria attach to a surface; those attached (or sessile) cells then create microcolonies. In panel 2, the colony grows and matures as the cells divide and other cells attach to it; EPS stands for extracellular polymeric substances, also called the extracellular matrix. Eventually, in panel 3, some bacteria split off, either individually (seeding) or in clusters, and attach to another surface to start a new colony.

An individual bacterial cell may decide to attach to a surface, usually in a moist environment with a good supply of nutrients. The colony grows as the cell divides and other bacteria also attach to the surface. As the bacteria attach, they secrete a mix of molecules that forms what even scientists often refer to as "slime."

because the study of biofilms really started as an engineering problem. It had long been realized that colonies of microorganisms would form on the hulls of ships, slowing their progress through the water. That led to attempts to figure out what the organisms were and how they were able to stick so effectively to ships and other surfaces—as well as to attempts to develop surfaces that were resistant to these microbial communities.

These studies, however, often overlooked the complexity of the organisms that made up the colonies. "A lot of the time the microbes were treated as inanimate spheres," O'Toole says.

At Harvard, O'Toole started to study the bacterium *Pseudomonas aeruginosa*. The species was already widely used for research because it is possible to manipulate it genetically to decipher which genes are involved in which processes; this made it a good model organism for examining biofilms. Since then, O'Toole has contributed to the growing understanding of two important aspects of biofilms: the molecular details of the attachment process and why bacteria in biofilm form are so resistant to antibiotics. Over the course of his career, he has helped to show that biofilms are more complex than anyone imagined back when he first learned about them.

Depending on the species of microorganisms involved, the process of biofilm formation can be triggered by a number of factors. "It's clear that bacteria actually regulate whether or not they want to attach to a surface," O'Toole says. In other words, these tiny, single-celled organisms take an active role in determining whether to live on their own or settle down and join a biofilm.

If certain environmental conditions are met, an individual bacterial cell may decide to attach to a surface, usually in a moist environment with a good supply of nutrients. The colony grows as the cell divides and other bacteria also attach to the surface. As the bacteria attach, they secrete a mix of molecules that together form what even scientists often refer to as "slime"—or, in more technical terms, an extracellular matrix. This slime is essential for the formation of a healthy biofilm. It surrounds the colony, giving it shape as it grows, helping to protect it from threats such as antibiotics and immune cells, and facilitating the collection of nutrients to feed the bacteria.

Bacteria within a biofilm can communicate with each other through a process called quorum sensing. They release molecules that act as signals, basically creating a group discussion that helps the bacteria decide when to form a biofilm, how to react to a threat, or when to move on to a new spot if the nutrient level begins to drop.

A successful biofilm doesn't just grow in one place but can also colonize new areas. Some of the bacteria within a biofilm may detach and float downstream, eventually attaching to a new part of the surface or a different surface altogether to create another biofilm.

Once part of a biofilm, individual bacterial cells can change their identity dramatically. They may become larger or smaller, change their shape, or acquire significant differences in their gene expression. In *P. aeruginosa*, for example, the expression of more than 70% of a cell's genes can be affected by whether it's free-swimming or part of a biofilm.

When O'Toole began studying biofilms as a postdoc, he didn't assume that the attachment of a bacterial cell to a surface was merely a mechanical process. He looked at the microbe itself, trying to figure out what was going on inside the cell that might allow it to take the first step in biofilm formation. To do that, he decided to examine whether there was a genetic component to the attachment.

O'Toole created thousands of strains of *P. aeruginosa* that had various genetic mutations, looking for those that could not form biofilms. He found two different strains lacking the ability to form colonies, and when he looked closer he realized that there was a different explanation in each case. *P. aeruginosa* has two methods of moving around, and both are required for a cell to attach to a surface to form a biofilm. One type of movement involves the use of the cell's flagellum, a sort of tail that extends outward from one end of the cylindrical cell. "The flagellum is essentially a bacterial propeller," O'Toole says. When a cell decides to attach to a surface, the flagellum helps it form the initial attachment. Once it's attached, the cell can use the flagellum to move across the surface.

The second kind of movement is called twitching motility, and it involves the use of pili, which are like little hairs on the surface of the bacteria. "You can almost think of it as throwing out a grappling hook and then pulling themselves along," O'Toole explains. "They can extend their pili, the tip of the pili attaches, and then they retract the pili and pull themselves sort of hand over hand, or pili over pili."

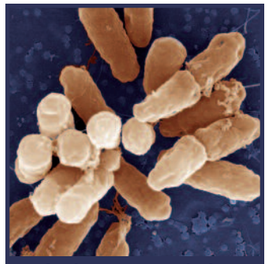
After a bacterial cell uses its flagellum to establish the initial attachment, it uses its pili to form a stronger attachment to the surface. The mutants O'Toole identified in those early studies lacked either flagellar motility or twitching motility, preventing biofilm formation.

"For us, it was exciting to see because it suggests different genes playing a role at different steps in the process," O'Toole says. "That was some of the first evidence that maybe there was a regulated series of steps." In other words, the attachment of a bacterial cell to a surface is not merely the result of mechanical forces, as is the case in the attachment of a magnet to the door of a refrigerator. Instead, the bacteria have to "decide" to attach to a surface.

Alicia Ballok, a graduate student in microbiology, has been a member of O'Toole's lab for the past two years. She is one of five graduate students currently based in his lab.

In recent years, O'Toole and the other researchers in his lab have continued to break down the steps involved in that decision, identifying more genes and proteins involved in the process of biofilm formation. "The unfolding picture of biofilm formation has certainly become quite complex," says Sherry Kuchma, Ph.D., a researcher in O'Toole's lab. "These organisms that we think of as being so simple actually can engage in very complex behaviors." The more scientists learn about attachment and the more steps they identify, the more targets there may be to try to prevent biofilm formation in situations where that can be harmful.

While O'Toole has shown how complicated biofilms are, he has also helped unify the field by providing a framework to study them. In 2000, he wrote an article proposing what he called the developmental model of biofilm formation. His idea, in short, was to compare the growth of biofilms to the development of multicellular organisms.



An electronmicrograph of a *Pseudomonas aeruginosa* biofilm

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Grad student Dae Gon Ha, left, explains the results of a recent experiment to O'Toole; in the background is another of the grad students in O'Toole's lab, Kyle Cady.

In biofilms, "bacteria actually perform many of the behaviors that multicellular organisms can," O'Toole says. "They can talk to each other. They can discriminate self from nonself. They can even specialize."

"It turns out that bacteria actually perform many of the behaviors that multicellular organisms can," O'Toole says. "They can talk to each other. They can discriminate self from nonself. They can even specialize." This last point is important, he adds, because it is part of the definition of multicellular organisms. In humans, for example, the cells that make up the heart, the lungs, and other organs all have their own characteristics and their own functions. But they also all grow out of the same few stem cells that are created after fertilization.

Similarly, O'Toole explains, different cells within a biofilm carry out different tasks, grow at different rates, and live in very different environments within a biofilm. Yet the cells are also individual organisms, even though they coordinate their behavior in a way that benefits the whole community and that may mean some individual bacteria get less food or face more risk than other bacteria within the biofilm. "It is pretty amazing," O'Toole says.

"I think it has been a very useful framework or hypothesis to test," says DMS microbiologist Deborah Hogan, Ph.D., who also works on biofilms (and is married to O'Toole). She adds that this developmental model helped scientists explore and recognize the many ways in which planktonic bacterial cells are very different from the bacteria in biofilms.

One of the most important of those differences is that biofilms are far more resistant to antibiotics. The question is why, and there is still not a complete answer. "From a medical standpoint, trying to increase the efficacy of antibiotics toward biofilm cells is probably the most important problem that needs to be tackled," Hogan says.

When O'Toole arrived at DMS in 1999, he knew that biofilms cause a number of medical problems. But neither he nor anyone else knew for certain how important biofilms are in making cystic fibrosis (CF) such a deadly disease. It is only in the past decade that scientists have learned that bacteria form biofilms in the lungs of people with CF, making treatment difficult. As a result, CF patients live on average, even in the United States, only into their late 30s.

O'Toole has turned much of his attention to CF research because of two factors, he says. For one thing, *P. aeruginosa* is the primary culprit in the death of most people with CF and is found in the lungs of about 80% of adults with CF. For another thing, DMS has a strong cystic fibrosis research group that fosters collaborations among physicians and scientists in a number of disciplines.

P. aeruginosa is found in many places, O'Toole points out. "Chances are you've inhaled a bunch every time you take a shower. But a normal, functioning lung is able to clear those infections."

The genetic mutation that causes cystic fibrosis creates a buildup of mucus in the lungs, which provides a welcoming environment for *P. aeruginosa*. The bacteria form biofilms, creating a chronic infection. "There's reasonably good clinical data that the longer you can prevent chronic *Pseudomonas* infection, the better it is for patients," O'Toole says. "So there's a real drive to figure out ways to prevent infection of *Pseudomonas*, and, if you can't prevent it, to eliminate the colonization once it occurs."

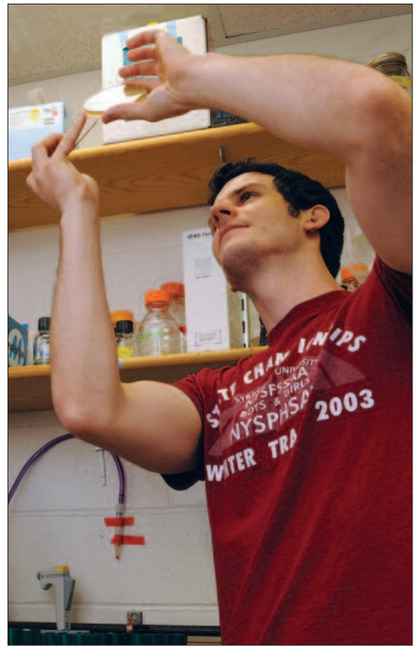
So far, antibiotics alone have not proven effective. But if scientists could figure out what makes biofilms so resistant to antibiotics, they might be able to overcome that resistance. For years, scientists thought that the extracellular matrix—the layer of slime—simply prevented antibiotics from diffusing all the way through a biofilm, protecting at least some of the cells.

But in 2003, O'Toole identified an entirely different mechanism that protects bacteria in biofilms. His lab created mutant strains of *P. aeruginosa* that were able to form biofilms but were not as resistant to antibiotics as normal strains. O'Toole reasoned that if the extracellular matrix surrounding a biofilm was perfectly fine but the biofilm still could not resist an antibiotic, then there must be other factors involved in protecting biofilms.

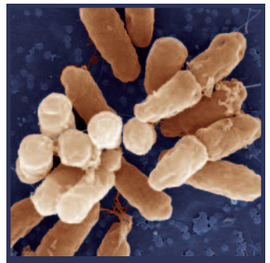
His lab found that a strain of *P. aeruginosa* lacking the gene *ndvB* was able to form normal biofilms but was less resistant than it should have been to tobramycin, an antibiotic often used against the bacterium in people with CF. Looking closer, the researchers realized that *ndvB* causes the production of molecules called glucans that trap antibiotics before they can reach their target.

A glucan, O'Toole explains, is a cylindrical sugar molecule. "You can think of it almost as a barrel, and it can hold molecules inside of that barrel," he says. Tobramycin works by targeting the ribosomes of bacterial cells, which are found in the cytoplasm. O'Toole discovered that glucans bind tobramycin while it is still in the periplasm—which is outside the cytoplasm—thus preventing the drugs from reaching the ribosomes.

This finding led to a more accurate picture of how antibiotic resistance works but also made it clear that biofilms are more devious than had been previously thought. They have mechanisms other than simply a layer of slime to protect themselves from drugs and other threats.



Jack Hammond, a technician in O'Toole's lab for the past three years, is entering DMS's Ph.D. program this fall.



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Dartmouth has turned out to be a good place for O'Toole to try to figure out ways around that resistance. He is now the associate director of the CF Research Development Program at DMS, one of only a few sites in the U.S. funded by the Cystic Fibrosis Foundation to carry out CF research. When he arrived at DMS 12 years ago, notes O'Toole, perhaps 10 people would show up at meetings of the CF group. Now, he says, it's closer to 35 or 40 people, including cell biologists, immunologists, microbiologists, and physicians.

Over the past several years, O'Toole has worked closely with Bruce Stanton, Ph.D., a physiologist at DMS. Together, they and other researchers in their labs have developed a model of the cells found in the lungs of people with CF. There is no good animal model that can be used to explore treatments for CF, so the cellular model developed by Stanton and O'Toole filled a major gap and is making it possible to get a more realistic look at CF's effects.

But one of the first findings from this model was daunting. The researchers found that biofilms

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grown on human airway cells were even more resistant to antibiotics than were biofilms grown on the plastic surfaces often used in experiments with bacteria. So the problem they were trying to solve was even harder than they'd figured.

But there has been good news as well. The failure of individual antibiotics against biofilms led the DMS researchers to try various combinations of therapies, hoping to simultaneously address some of the multiple ways that biofilms are able to escape the effects of individual antibiotics.

In 2009, O'Toole, Stanton, and postdoctoral researcher Sophie Moreau-Marquis, Ph.D., reported that they had been able to eliminate 90% of the bacteria in *P. aeruginosa* biofilms using a combination of tobramycin and iron chelators—chemicals that bind to iron and prevent it from being used by cells. And happily, the two iron chelators they tested in the study had already been approved by the Food and Drug Administration for use in humans, making the results very clinically relevant.

Since then, O'Toole says, they have been testing different combinations of antibiotics to find even more effective treatments, and they hope soon to be able to start clinical trials using a combination of iron chelators and antibiotics.

"We don't understand why iron is important and why it seems to be more important particularly to the cells in the center," says O'Toole. "But the bacteria die really quickly, so it's likely not a growth issue. There's something critical about that iron, and when you bind it up, the cells become hypersensitive to treatment with antibiotics."

O'Toole didn't anticipate at the beginning of his career that he would focus on CF, but he's pleased that his work could have implications for the treatment of this difficult disease. "I think we have the possibility of contributing to improving patient care," he says. "A lot of that has . . . been influenced by the colleagues that are here and their willingness to collaborate."

It's a good thing O'Toole is still energized by the scientific challenge. For despite his progress, much remains to be learned about the mysterious lives of biofilms. ■