Slimebusters

Bacteria do not always simply float around — more often they grow on surfaces in mucilaginous communities called biofilms. Working out how to block their formation or dismantle them could help treat life-threatening infections, says Marina Chicurel.

Imagine yourself shrunk to the size of a bacterium. William Costerton, director of the Center for Biofilm Engineering at Montana State University in Bozeman, does so frequently. “If you found yourself in a biofilm, you’d be going along a channel full of water, like the canals in Venice, and up from the bottom of the channel, on either side, would be these slime towers,” he says. “The channels would be bringing in oxygen and nutrients, and removing waste. And within each building, so to speak, some of the bacteria would be cooperating with each other, making one compound and passing it along to the next. It’s at least as complicated as a tissue, and possibly as a city.”

Costerton was instrumental in alerting the world to the importance of these biofilms, coining the term in 1978¹. Today, microbiologists have realized that most species of bacteria adjust their biochemistry and behaviour to form slimy microbial cities when conditions allow. Biofilm-dwellers pump out sugar polymers called exopolysaccharides to create a complex, three-dimensional matrix, which typically comprises 85% of the volume of a biofilm. And within the past two years, researchers have made great strides in understanding the triggers that cause free-floating bacteria to form a biofilm and adjust to a sedentary existence.

From this vantage point, many possibilities are emerging, including the potential for designing antimicrobial drugs with fewer side effects that are less likely to promote resis-

In harmony: when a biofilm of *Pseudomonas putida* (blue) and *Acinetobacter* (red) grows on a medium containing benzyl alcohol, the two species can become tightly associated (blue/green). This is because, as *Acinetobacter* metabolizes the alcohol, it excretes benzoate, a major carbon source for *P. putida*.

tance. “Biofilms are a hot topic now because of the recognition that they are medically important,” says Roberto Kolter, a microbiologist at Harvard Medical School in Boston. Up to 60% of hospital-acquired infections involve biofilms², which contaminate implants and catheters. Biofilms also cause diseases ranging from the lung infections that can afflict cystic fibrosis patients to tooth decay^{3,4}. Add to this the fact that they cause billions of dollars’ worth of damage by coating equipment such as cooling systems, plus their role in bioreactors used for such tasks as manufacturing antibiotics, and it is easy to see why biofilm research has moved to the fore.

To create biofilms, says Kolter, microorganisms must be aware of environmental conditions — including the availability of nutrients and oxygen — and take a census of their neighbours, determining their density and characteristics. Many clues to the mechanisms of biofilm formation have come from screening mutants created using transposons — snippets of DNA that insert randomly into chromosomes, disrupting the genes into which they land. Researchers seed hundreds of tiny wells with individual mutants, allow them to attach, and then wash off the free-floating cells. Mutants that fail to leave their mark on the wells can then be studied to map their mutations, in the knowledge that the

affected genes are important for biofilm formation. These studies have shown that there is no universal set of genes involved. “What applies for *Vibrio cholerae* need not apply for *Pseudomonas aeruginosa*,” says Kolter. In addition, a microorganism can use different genetic pathways to build biofilms, depending on the environmental conditions.

In several cases, the first steps of biofilm formation require genes involved in building force-generating appendages^{5,6}. *Pseudomo-*

Lethal layers: biofilms are thought to be behind fatal lung infections in cystic fibrosis patients.

SOREN MOLIN

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Virtual biofilms: computer simulations of the growth of a biofilm containing two species (blue and yellow). When nutrients are scarce and unevenly distributed (left), mushroom-like colonies form. A smooth and compact biofilm forms when the medium is rich in nutrients (right).

nas aeruginosa, for example, uses flagella to swim along surfaces, as if scanning for potential sites to colonize. The early settlers appear to move towards each other to form micro-colonies, using thin, retracting strands of protein called type IV pili to pull themselves across the surface^{7,8}. Other bacteria rely simply on cell division to begin the process⁹.

Once established on a surface, microbial colonists activate genes involved in producing the slimy matrix. Other genes appear to be involved in biofilm growth and in the adaptation to a communal lifestyle. Philippe Lejeune at the Lyon National Institute of Applied Science in Villeurbanne, France, has used the marker gene *lacZ* to track changes in gene expression as *Escherichia coli* forms a biofilm. His studies suggest that the expression of 38% of *E. coli*'s genes are altered¹⁰. But other groups report much more modest changes. In unpublished studies, researchers led by Peter Greenberg at the University of Iowa, working with colleagues at Harvard, have found that fewer than 200 of *P. aeruginosa*'s roughly 5,200 genes significantly change their expression in a mature biofilm.

Molecules called quorum-sensing signals help trigger and coordinate these changes. Bacteria constantly secrete low levels of these signals and sense them through receptors on their surfaces. But the receptors do not trigger

any behavioural changes until there are enough bacteria to allow the signals' concentrations to exceed a critical threshold. Once this occurs, bacteria respond by adopting communal behaviour, such as forming biofilms and, in the case of pathogenic bacteria, deploying virulence factors such as toxins^{11,12}. Illustrating the importance of quorum-sensing signals, Greenberg's team showed in 1998 that a *P. aeruginosa* mutant unable to produce a particular signal formed much thinner and more crowded biofilms¹³.

Exactly how quorum-sensing signals regulate biofilm formation remains a mystery, however. Several studies have revealed that their effects depend on environmental conditions and vary widely between species. In an effort to dissect the signals' effects, Greenberg's team has created a collection of mutants in a strain of *P. aeruginosa* incapable of secreting their own signals, but able to respond to synthetic quorum signals¹⁴. So far, they have found some 70 genes whose expression ramps up in the presence of the signals.

In addition to communicating with members of their own species, bacteria also conduct inter-species conversations. "It's not good enough to be able to only count yourself," says Bonnie Bassler of Princeton University in New Jersey. Bassler has discovered

that a family of genes involved in the synthesis of a particular quorum-sensing signal occur in a wide variety of bacteria¹⁵. In unpublished work, she has isolated the molecule and determined its chemical structure. The signal may form the basis for a bacterial Esperanto, she says, helping bacteria sense the presence of others. Although the mechanistic details remain to be discovered, Bassler suspects that bacteria may compare levels of this signal with those of their own species-specific signals to determine the proportion of their own kind in a growing biofilm.

Bacteria may also rely on signals for disassembling their communities. Kolter, for example, speculates that a lack of food may induce bacteria to produce signals that trigger an exodus from a biofilm.

Food for thought

But quorum-sensing signals are not the entire story. Søren Molin at the Technical University of Denmark in Lyngby has shown that biofilm formation can be shaped by food¹⁶. When fed chlorobiphenyls, species of *Pseudomonas* and *Burkholderia* form mixed biofilms, allowing *Pseudomonas* to feed off the metabolic products generated by *Burkholderia*. But when fed citrate, each species builds its own biofilm, with radically different structures. In addition, if the food source is switched, the bacteria rapidly restructure their biofilms. Molin's results suggest that the structure of biofilms can be explained by bacteria travelling up food gradients, without invoking signalling between cells¹⁶. Computer simulations by Cristian Picioreanu at the Delft University of Technology in the Netherlands suggest that, indeed, nutrients are not evenly distributed within biofilms and may help shape their structure¹⁷.

Work by other researchers has revealed that oxygen is unequally distributed within a biofilm — with bacteria in the deepest reaches often living in almost completely anaerobic conditions¹⁸. "Even small domains of a community may have a composition around them that is quite different from that just a few micrometres away," says Molin. He recently illustrated just how different the lives of individuals in the same biofilm can be by tagging bacteria with fluorescent proteins, and observing their movements⁹. Instead of finding only sedentary bacteria, Molin saw some bacteria swimming around, moving from one micro-colony to another.

A better understanding of biofilms is of high medical importance. Infections mediated by biofilms are tough to eradicate because biofilm inhabitants are up to 1,000 times more resistant to antibiotics than are free-floating bacteria. They are also less vulnerable to the immune system, and their matrix polysaccharides resist enzyme attack. Researchers initially thought the biofilm matrix provided a barrier to the diffusion of antibiotics. But in 1994, Gill Geesey and

his colleagues at the Center for Biofilm Engineering showed that antibiotics can penetrate the depths of a 500-micrometre-thick biofilm within 90 seconds¹⁹.

The true explanation for antibiotic resistance may be physiological: the lower metabolic rates of sedentary cells might make them less susceptible to drugs; the membranes of biofilm bacteria might be better equipped to pump out antibiotics before they can cause damage; or biofilm-dwellers may produce fewer of the proteins that are targeted by conventional antibiotics. In addition, biofilm bacteria exchange DNA much more readily than do free-floating bacteria, which might accelerate the transfer of antibiotic-resistance genes²⁰.

Thanks to the newly acquired knowledge on biofilms, these challenges may soon be overcome. Some believe that interfering with quorum-sensing signals holds great clinical promise. Researchers led by Michael Givskov at the Technical University of Denmark and Niels Høiby of the University of Copenhagen, for example, have infected rats' lungs with mutants of *P. aeruginosa* defective in two quorum-sensing systems. Clearing the microbes faster, the rats suffered much less tissue damage than controls²¹.

So the search is on for drugs that bind but fail to activate the receptors of quorum-sensing signals, molecules that interfere with signal synthesis, and enzymes that degrade the signals¹². "If you could trick bacteria so they couldn't count each other, and therefore they never knew when they had reached a high cell number, they wouldn't turn on all these virulence factors," says Princeton's Bassler. And as such drugs would target microbial pathways, they would be less likely to cause harmful side effects. In addition, because they would not kill bacteria, the selective pressure to develop resistance mechanisms should be weaker than with traditional antibiotics.

Slime management

Applying this approach to target a wide variety of bacteria, Quorex Pharmaceuticals in Carlsbad, California, is designing small molecules to inhibit the broad-spectrum signalling pathway discovered by Bassler. Screening their molecules' abilities to interfere with the production of virulence factors and biofilms, the researchers have identified several promising candidates to move into animal experiments.

Inspired by a natural inhibitor of quorum-sensing signals, researchers at the University of New South Wales in Sydney have also developed promising, broad-spectrum inhibitors. In the early 1990s, marine biologist Peter Steinberg was intrigued by the ability of the seaweed *Delisea pulchra* to remain slime-free despite living in bacterium-infested waters. Isolating compounds coating the seaweed's surface, he traced the antifouling activity to molecules called furanones. When

Film-struck: the mushroom structures of a *Pseudomonas aeruginosa* biofilm (top) do not form if bacterial signalling is blocked (bottom).

Steinberg's microbiologist colleague Staffan Kjelleberg saw the furanones' chemical structures, he had a flash of recognition: "They were basically exactly the same thing as a bacterial signalling system — acyl homoserine lactones."

Kjelleberg found that the furanones displaced the bacterial quorum-sensing signals from their receptors²². Working with researchers at the Centre for Marine Biofouling and Bio-Innovation and the company Biosignal, also in Sydney, Kjelleberg has created almost 100 synthetic variants of the seaweed furanones, hoping they will solve a wide range of biofilm problems. Initially, the researchers are developing coatings for keeping aquaculture equipment biofilm-free. Working with Givskov, Kjelleberg is also testing the furanones' effects in mouse models of human disease. "All I can say is that it looks promising," says Givskov. Again, drug resistance is not likely to be a problem. "*Delisea* has been using a signal blocker in nature for millions of years and no resistant bacteria have developed," says Costerton.

Focusing on more specific targets, Greenberg's team recently licensed a method for screening potential inhibitors of the quorum-sensing signals of *P. aeruginosa* to Quorum Sciences in Iowa City — now part of Aurora Biosciences in San Diego, California. The method is based on monitoring the expression of a subset of genes regulated by the signals. Greenberg is also developing drug-screening methods based on measuring ratios of quorum-sensing molecules. In a paper published in *Nature* last month, his team reported that the ratio of signals produced by *P. aeruginosa* living in biofilms differs from that produced by free-floaters²³. Indeed, by measuring these ratios in sputum, the team bolstered the hypothesis that many

of the *P. aeruginosa* that colonize the lungs of cystic fibrosis patients inhabit biofilms.

One key to developing further strategies for combating the formation of such biofilms may be to do as Costerton does, and try to consider these microbial cities from a bacterial perspective. "Microbes tend to be really smart," says Peter Hecht, chief executive officer of Microbia, a company in Cambridge, Massachusetts, that holds commercial rights to Kolter's work. "I guess that's why we want to think like them."

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