The mob response

The importance of biofilm research for combating chronic diseases and tackling contamination

One for all and all for one; strength is in numbers—humans have successfully used the strategy of working together whether as nations, tribes, groups or families. Yet, many other species use the same strategy to equally good effect, whether they are fish or birds that school or flock together to evade and confuse predators, or hive insects, such as bees, that developed communal societies long before humans did. Yet, communalism is not limited to higher animals: even the most basic organisms—bacteria and other microorganisms—have successfully adopted this strategy to colonize and survive many different environments. Since the beginning of the nineteenth century, scientists have known that bacteria and other single-celled organisms are able to form colonies, but the level of sophistication and the importance of these for the whole biosphere has only become apparent during the past 40 years. This new understanding has led to one of the most actively researched areas of modern molecular biology: biofilms and the extraordinary signalling network, called quorum sensing, which underpins them.

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This research is highly relevant for human health because biofilms are implicated in numerous debilitating—and often chronic—diseases, including cystic fibrosis, tuberculosis, orthodontal disease, sinusitis and some forms of heart disease. Biofilms also cause

many problems elsewhere, such as contaminating food and its packaging, and fouling water supplies. On the beneficial side, biofilms have an essential role in breaking down sewage and decontaminating polluted sites, and are vital for the health of ecosystems. These bacterial communities break down organic debris from dead fish and plants in rivers and streams, and absorb heavy ions from water, while biofilms on the surface can break down debris without consuming additional oxygen.

In any case, research on biofilms and quorum sensing is shedding light on the extraordinary complexity of these microbial colonies, which, in addition to different species of bacteria, might also include other microorganisms such as protozoa, microscopic algae, fungi and even small insects. Furthermore, biofilms confer on their constituents some of the advantages that were previously thought to be confined to multicellular organisms—notably, the ability to distribute tasks to different cells within the community, with constituent species performing unique roles.

Biofilms exist on the surfaces of liquids or solids, and one defining characteristic is their ability to bind strongly to a surface. Other crucial properties are the existence of an extracellular protective matrix, almost like a community exoskeleton, and communication between cells by quorum sensing. The latter was the last property of biofilms to be discovered, through research on the marine bacterium *Vibrio fischeri*, in the late 1960s. *Vibrio* begins to produce light when sufficiently large numbers of cells are present in liquid cultures. The initial explanation for this was that the luminescence was an inherent feature of the organism that was inhibited by the culture medium, which

could be overcome by the collective action of many bacteria (Kempner & Hanson, 1968). However, it was soon shown that the luminescence occurred because of the accumulation of an activator molecule now known as an auto-inducer (Nealson *et al*, 1970; Eberhard, 1972).

This was the first published example of quorum sensing in the scientific literature: in each individual, certain genes are up-regulated once the biofilm has reached a critical mass. The activated genes are important for the community to survive as a whole, but not for individual free-form 'planktonic' bacteria. Of course, the immediate question was what advantage such a system conferred on marine biofilms. Although the answer has never been established beyond all doubt, the accepted wisdom is that luminescent bacterial colonies light up to attract 'predators' such as zooplankton. In the nutritionally depleted deep oceanic waters, being ingested by an animal represents an important source of food for the biofilm. Francisco Rodriguez-Valera, Professor of Microbiology at the Universidad Miguel Hernandez in Elche, Spain explained: "[t]he animal gut will act as a recharger for the particle associated biomass [...] [i]f they survive the passage through the animal intestinal tract they will have renovated resources as part of the faecal pellet." By contrast, single free-moving bacteria would gain nothing from being digested by zooplankton and it would therefore be a waste of energy for individuals to expend energy making light.

Since this initial discovery, other examples of quorum sensing have been found; there are now many genes that are known to be activated or repressed by individuals, depending on whether they are free-flowing plankton or part of a biofilm (Conway & Greenberg, 2002). Quorum sensing seems

to be an evolutionary adaptation that allows bacteria to flip between different modes of behaviour, which is induced when the concentration of critical inducers reaches a certain level. The principle is simple: each bacteria—whether free-living or confined within a biofilm—produces and recognizes a particular molecular signal. However, the signal can only be detected when the concentration of the molecule is sufficient—that is, after the biofilm has grown to a certain size. There are now various signalling compounds, such as the N-acyl homoserine lactones, which are known to serve this function. When these auto-inducers bind to receptors on the bacteria, they activate the transcription of certain genes, but the probability of this happening remains very small at low concentrations because the inducer dissipates before reaching its target receptor (Nadell *et al*, 2008).

Another crucial factor for biofilms is the formation of a protective extracellular matrix. Without this structural support the bacterial mass is just a colony, rather than a fully-fledged biofilm, and is much more vulnerable to attack from, for example, phages or antibiotic agents. Molecular details of the matrix remain elusive, mainly because it is translucent in its normal hydrated state and almost invisible to light microscopy. The matrix is also not visible using electron microscopy because the dehydration process involved in sample preparation causes its structure to collapse. However, the use of fluorescence microscopy, for example, to illuminate specimens for optical imaging without having to dehydrate them has allowed scientists to gain insights into the matrix (Bockelmann *et al*, 2002).

This type of analysis has identified some basic properties of the matrix, which comprises a complex assembly of polymers, including polysaccharides, along with smaller amounts of protein and, intriguingly, DNA, which might have a role in binding the components together (Whitchurch *et al*, 2002; Sadovskeya *et al*, 2005). Quorum sensing plays an ongoing role in maintaining the matrix and modifying it to cater for changing environmental conditions.

Furthermore, the matrix confers structural stability and contributes towards the remarkable resistance of biofilms to antibiotics, the immune defences of a host organism or indeed any compound that might be used to remove them from contaminated foods, machinery or medical devices (Fig 1).

Fig 1 | Magnified electron micrograph of a biofilm of *Staphylococcus aureus* bacteria found on the luminal surface of an indwelling catheter. Photo credit: Janice Carr. Content Providers: CDC, Rodney M. Donlan & Janice Carr.

“Biofilm-infected medical devices and catheters must often be removed surgically, resulting in increased trauma and cost,” pointed out Timothy Lu, a bioengineer at Harvard Medical School (Cambridge, MA, USA) who specializes in biofilms. “There is an unmet need for effective treatments against biofilms that cause infection and/or contaminate food/machinery.”

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Complex biofilms are also known to be responsible for the chronic inflammatory conditions associated with cystic fibrosis. Although the disease itself is genetic, it renders the sufferer vulnerable to bacterial infection and, over time, biofilms become established. These bacterial colonies seem to acquire virulence once they have reached a critical size, probably determined by quorum sensing (Greenberg, 2003). The same phenomenon occurs in other bacterial diseases to which many people are potentially vulnerable, possibly including tuberculosis. Graham Hatfull,

a specialist in biofilm research at the University of Pittsburgh, PA, USA, pointed out that the mycobacterium causing tuberculosis is unusually tolerant of antibiotics and can resist the immune system to persist for a lifetime. Hatfull suggests these properties indicate that mycobacteria form biofilms that protect them against drugs and immune mechanisms.

The extracellular matrix also provides some protection against the penetration of toxins into the community, ensuring that at least some cells survive—especially those buried deep within the biofilm. Another protective factor is the genetic and species diversity within the biofilm. According to Tom Battin, Assistant Professor in the Department of Freshwater Ecology at the University of Vienna, Austria, “[h]igh phenotypic plasticity increases the probability that some ‘pockets’ survive the antibiotics, or are simply not reached by them [...] [b]acteria can re-colonize from these pockets.” The antibiotic simply eliminates those bacteria that are susceptible to it from the biofilm community, which then becomes resistant—as happens, for example, in cystic fibrosis, which is characterized by biofilms that include many different species of bacteria. Furthermore, bacteria with antibiotic tolerance can break away and form new

biofilms, leading to increasingly widespread resistance to particular antibiotics.

Another important health aspect of biofilms is their ability to use an infected organism's immune cells for their own ends. Jerry Nick and colleagues at the National Jewish Medical and Research Center in Denver, CO, USA, found that biofilms of the bacterium *Pseudomonas aeruginosa*, which commonly infects cystic fibrosis patients, use the remnants of dead immune cells for the construction and maintenance of their protective matrix. In turn, the matrix then renders neutrophils—white blood cells that fight invading bacteria—impotent. According to Nick, the sheer physical bulk of the biofilm makes it almost impossible for the neutrophils to engulf it, but they continue a futile and counterproductive immune assault. "We believe *P. aeruginosa* biofilms favour environments of intense inflammation, as the bug is able to utilize neutrophil actin and DNA polymers to accelerate its development," he said.

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However, such studies are not easy to conduct because these types of effects are mostly visible in the natural environment rather than under laboratory conditions, which lack any of the immune cells of the host. "My personal bias is that investigators need to look carefully at the niche where their biofilm grows, and try to model as many of the features that are present as possible," said Nick. "In other words, I think if someone wants to study features of medically-relevant *Pseudomonas* biofilms, they need to do it in a stagnant reactor in the presence of massive numbers of neutrophils. I think growing *Pseudomonas* in the absence of eukaryotic cells, in a flow chamber, may have fairly limited applicability to human disease, and the results need to be interpreted with caution."

The important question is how to dismantle biofilm structures when treating disease or contamination, and the best source of inspiration might be the natural defences that other living organisms have evolved. "Plants and animals have various

methods for controlling biofilm infections that are now beginning to be understood," Lu commented. "For example, brominated furanones [compounds that interfere with auto-inducers] may be used to disrupt quorum-sensing molecules that are important in the development of biofilms. Therefore, disrupting quorum sensing with synthetic molecules may be a promising method of combating biofilms" (Defoirdt *et al*, 2006).

According to Jeremy Webb, Research Fellow at the University of Southampton, UK, another promising anti-biofilm agent is nitric oxide: "There is much recent interest in the finding that nitric oxide, a signal molecule of interest across a range of biological systems, can control biofilm development and dispersal," he said. "This presents an exciting mechanism to regulate biofilms and we are very excited about its potential as a non-toxic and low-dose method to reverse biofilm formation and its associated antibiotic tolerance."

There is also a great deal of interest in the use of phages to combat biofilms, particularly in the context of contamination. Phages are able to break through a biofilm, but they first have to overcome the biofilm's defences against viral attack. Lu and colleagues have engineered a phage that expresses enzymes to degrade crucial components of the matrix, and then to infect and kill its component bacteria (Lu & Collins, 2007). "This strategy sets up a positive feedback loop that combines the advantages of bacteriophage therapy, which include self-replication and rapid killing, and those of enzymatic treatment of biofilms, which include targeted disruption and high effectiveness," Lu said. At the same time the disadvantages of each are mitigated, including the phages' inability to penetrate biofilms effectively, and the difficulties in producing pure therapeutic enzymes and delivering them to their target. As promising as this strategy is, such phages are unlikely to be used against disease in the foreseeable future because of the great risks involved—the phage could integrate into the bacterial genome or transfer virulence genes among the bacteria.

In addition to this health-related research, there is also a growing basic interest in the complex relationships between the species within biofilms, but these are early days. However, it is clear that many types of relationship can exist and not always co-operative ones. A corollary of this diversity is that biofilms are highly tuned to their

environment and often show great variations in their population over very small distances. Evidence of these traits was provided by researchers at the Universidad Autónoma de Campeche, Mexico, who studied biofilms on rocky shores in the Gulf of Mexico (Narváez-Zapata *et al*, 2005). Their analysis of such biofilms, by polymerase chain reaction, identified many bacteria from four main taxonomic ranks, of which 41% were cyanobacteria that produce primary carbohydrates on behalf of the whole community. Some of these had thick sheaths typical of bacteria inhabiting zones between high and low tides that are subject to strong desiccation. This highlighted one of the great adaptive advantages of biofilms: the ability to accommodate various individuals in hostile environments where they could not survive on their own, whereby some members protect the community against external assaults.

But perhaps the most interesting finding of the Mexican study was the great spatial diversity of certain bacteria over distances as small as 1 cm, within which there were no changes in external conditions such as salinity or temperature. The authors suggested that microscopic changes in surface properties of the rock, along with varying biological interactions either within the community or with microorganisms from outside, accounted for this variation.

Practically focused researchers are now trying to harness this enormous diversity and engineer biofilms to remove or break down environmental pollutants. In various cases, biofilms are already replacing earlier bacterial reactors to operate more efficiently, more sustainably and with less maintenance. For example, mills that grind olives into oil now remove toxic phenols by using a bed of carbon granules encrusted with biofilms that comprise a range of archaea and bacteria (Bertin *et al*, 2004); the carbon granules trap the pollutants, which the biofilm degrades. One advantage of this biofilm-based approach is that the community attunes itself to the environment in a highly specific way and shows great local differences in composition. The biofilm—if correctly engineered—will thus comprise sufficient genetic diversity to adapt to the specific environment within which it operates.

While some biofilms can be highly diverse, others are very homogenous—the advantage then is mere strength in numbers—as in the

case of bioluminescence, which Webb describes as an example of the 'mob response' in evolutionary terms. Not surprisingly, there are also examples of biofilms that combine the mob response with phenotypic diversity. In any case, as though the enormous diversity of microorganisms were not enough, microbiologists are also facing the interesting and challenging task of identifying, classifying and analysing the myriad different biofilms that exist in nature, as well as developing countermeasures against those that cause disease and contamination.

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