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The War on Slime

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I love Yellowstone National Park. Like many of the millions of visitors who travel to Yellowstone every year, I have fallen in love with this place. Since my first visit almost 20 years ago, I have returned many times, more often than to any other place that I have visited. What's so special about this place? I have visited countries as far east as Japan, followed the footsteps of Romans, experienced volcanoes from afar and close up, touched glaciers, admired geysers, and pretended to hold up the leaning tower of Pisa. So what makes me return to Yellowstone? For one, the park is unbelievably beautiful. It offers unparalleled opportunity to view waterfalls, lakes, and hydrothermal features such as hot springs, geysers, mudpots, and fumaroles in large numbers in a relatively small area. And then there are the vivid colors that are so common to many of Yellowstone Park's thermal features!

These very vivid, bright colors of the rainbow is what gets me every time and what makes me come back again — knowing that these colors are produced by bacteria, by millions and millions of tightly packed bacterial cells. While individual bacteria are not visible to the naked eye, here they form communities as far as the eye can see. These vast communities are commonly referred to as microbial mats or biofilms. The term “biofilms” originates with the words “biological films.” But rather than being thin and two-dimensional, these communities are structured, with microbial cells/bacteria sticking to each other, arranged as filaments and in layers. They're capable of forming structures resembling tiny towers. These features are dispersed with water channels and protected by a layer of slimy material. But you don't have to go to Yellowstone to see these biofilms. You may have encountered them as thick and slimy build-up in your drains or pipes, as a ring around your bathtub, as pond scum, as the slippery layer coating rocks and pebbles in streams, or as crud coating ship hulls.

When I see these colorful communities, I see virtual cities of slime, with architectural features not unlike the streets and paths followed by drivers and pedestrians. A pulsing city with blocks and skyscrapers, and streets that are busier than the busiest city streets. We are not talking about Fifth Avenue in New York; we're talking about Shibuya crossing in Tokyo. What's going on inside these virtual cities may first appear to be barely organized chaos — think of the thousands of people crossing the street at Shibuya and walking in all different

directions — that then resolves into orderly lanes as the light changes and the pedestrians make way for cars.

This “organized chaos” is what makes biofilms tick. They communicate and exchange information; they organize their architectural features. There are rules that govern their community living and their behavior, too. When Yellowstone’s bacterial communities “talk” to each other, what first may seem to be cacophonous shouting turns out to have distinct lines of messaging. And therein lies the beauty of it. Once you understand what’s governing the “organized chaos,” you can interfere or intervene or change the flow. My colleagues and I are starting to be able to listen in and, sometimes, break the signal. Breaking the signal is very much like hacking the system. We want to put in a red light where there wasn’t one before, or redirect traffic.

Why do this? These virtual cities of slime, most visible in places with large bacterial formations such as Yellowstone, are replicated on a less extensive scale in industry, elsewhere in nature and on a far smaller scale in our bodies and in medical devices. While biofilms in industrial settings are a real nuisance, in medicine, biofilms are a threat to our health. When bacteria succeed in forming a biofilm within human hosts, they can be the culprits in infections of surgical sites, the urinary tract and chronic and burn wounds, including non-healing diabetic wounds. Biofilms cause lung infections, and they are the cause of chronic colonization of medical devices and implants such as catheters, prosthetic joints, and heart valves. Overall, 65 percent of hospital-acquired infections are due to bacteria growing as biofilms. To put this in perspective, the Centers for Disease Control and Prevention estimates that hospital-acquired infections account for an estimated 1.7 million infections and 99,000 associated deaths each year in American hospitals alone. Taking into account all the infections caused by biofilms, biofilms are estimated to affect millions of people in the developed world each year, and claim as many lives as cancer — a grim statistic indeed.

Why are so many people affected by biofilms and to such detriment? And what does this have to do with Yellowstone Park? Our strategies to combat bacterial infections are geared toward treating infections caused by individual bacteria, not biofilms. Biofilms not only have an extreme capacity for evading our host defenses and immune responses, and fail to respond to vaccines, but often turn out to be untreatable with antibiotics. This extraordinary resilience of biofilms is not linked to bacteria having acquired drug resistance or becoming the “superbugs” that we hear so much about these days. Instead, it has everything to do with biofilms’ cities of slime and that “organized chaos” I mentioned earlier. Picture a heart catheter, which may have one tiny pocket of biofilm. Or remember recurring middle ear infections, which are so common and such a nuisance for children. While in both cases the biofilms are tiny, and although the number of bacteria is so small that it may be tough to locate them, these biofilms are literally the same as those in Yellowstone Park — they are virtual cities of slime, with citizens in communication and governed by “organized chaos.”

That’s why we need to listen in on the signal and try to hack the system to find new ways to treat biofilm infections. What are the ‘red lights’?? And how are scientists “redirecting traffic” to combat biofilms.

Making sense of the ‘organized chaos’ and controlling the virtual cities using red lights and detours first requires an understanding of how bacteria form biofilms and switch between the two different lifestyles, growing as single, planktonic cells and growing as part of a community, as biofilms (Fig. 1). The first clues that biofilms are not simply an accumulation of cells at a surface but instead are built over time and require a sophisticated regulatory network to develop, were provided in 1998 by O’Toole and Kolter (O’Toole & Kolter, 1998a, O’Toole & Kolter, 1998b). The researchers demonstrated that bacterial attachment and initiation of biofilm formation by the soil bacterium *Pseudomonas fluorescens* not only required the synthesis of new proteins, but also the presence of 24 genes to be able to attach. While some of the genes required for attachment encoded known proteins such as adhesins (flagella and pili), the majority of genes were those of unknown function, suggesting that being an attached cell meant taking on a novel bacterial physiology. Sauer et al. then demonstrated in 2002 that bacterial cells not only adapt upon surface contact but keep changing and adapting, as the biofilm continues to develop from just a few attached cells into the ‘virtual city of slime’, with each biofilm stage depicted in Fig. 1 being represented by a different bacterial physiology, and different sets of proteins being produced (Sauer et al., 2002a, Stoodley et al., 2002). Evidence of biofilm formation being a developmental process and as such, amenable, was provided in 2009, by demonstrating that biofilm formation by the opportunistic pathogen *Pseudomonas aeruginosa* is governed by a network of regulatory proteins that enable the transition from one biofilm stage to the next in a sequential manner (Petrova & Sauer, 2009). These findings suggested that biofilms, like cities, are built from the ground up, with the construction following a master plan one building phase and one city block at a time. Like any city plan, we have learned using the regulatory network underlying biofilm development, to regulate the development of these virtual cities. And at least in the laboratory, we can already allow biofilms to progress in their development, stop them at a particular developmental stage, or similar to demolition, make them revert to earlier developmental stages (Petrova & Sauer, 2009). By doing so, scientists have begun to understand the difference between planktonic and biofilm cells as it extends to motility, slime production, communication, gene expression, protein production, to genes and factors contributing to the extreme resilience of biofilms to antimicrobial agents, the needs of biofilm cells to maintain their structure, to physical factors such as viscosity, elasticity, and diffusion barriers that contribute to the ineffectiveness of current treatment strategies.

Based on our current knowledge of biofilms, what are ‘red lights’ and ideas for ‘redirecting traffic’ to combat biofilms, to make the virtual cities crumble? The following will address some of the ongoing and most promising strategies, starting attachment control and progressing to strategies targeting more developed biofilms.

As the formation of biofilms is initiated by a few planktonic bacteria attaching to surfaces (Fig. 2A), an obvious ‘red light’ is preventing bacterial attachment in the first place. When it comes to controlling attachment, much research has focused on the development of surface materials or coatings capable of killing bacteria upon contact or discouraging bacteria from attaching (Miquel et al., 2016) (Fig. 2A). Killing bacteria upon contact can be accomplished using surface coatings or impregnated surfaces that enable local delivery of antibacterial agents at high concentrations. Several such surfaces are already commonplace

in hospitals and include antibiotic-impregnated sutures, bone cement containing antibiotic-impregnated beads, as well as catheters, wound dressings, and endotracheal tubes coated with colloidal silver or silver nanoparticles. The silver ions kill bacteria upon contact. While the mechanism of killing is not fully understood, silver ions have been shown to cause oxidative damage (oxidation of biomolecules of microorganisms), leading to cell damage and bacterial cell death (Jung et al., 2008, Markowska et al., 2013). In addition to silver, anti-bacterial or growth-limiting factors such as detergents (soap-like substances), metal oxide and metal salts (iron, mercury (Hg), tellurium (Te) zinc, titanium) are being tested for clinical purposes. These antimicrobial metals work by causing discrete and distinct types of injuries to bacterial cells as a result of oxidative stress, protein dysfunction or membrane damage (Lemire et al., 2013).

Surface coatings and impregnated surfaces, however, have an Achilles heel, as the antimicrobial agents aimed at killing bacteria eventually runs out. This combined with the concerns of overuse of drugs and compounds aimed at killing bacteria (including silver) and the consequent emergence of bacterial resistance, has spawned the development of new surface materials that control attachment in the absence of antimicrobial compounds. The surfaces are inspired by nature, emulating the texture of shark skin, the superhydrophobicity of self-cleaning textures found in lotus leaves, or the chemical functionalities used by mussels to repel bacteria. Bio-inspired surfaces do not prevent bacterial attachment per se, but prevent protein adsorption, as the layer of adsorbed protein on surfaces serves as a platform for cell attachment, and subsequent bacterial colonization that leads to the formation of bacterial films. Inhibition of protein adsorption is accomplished by steric hindrance to the approaching protein molecules. Steric hindrance is induced by modulation of surface topography, in particular, the surface roughness (Fig. 2C), by adding nanostructures such as brushes, crystals, and tubules made of hydrophilic polymers including polyethylene glycol (PEG, inspired by mussels), amphiphilic fluoropolymers, or zwitterionic polymers (Inspired by the anti-biofouling properties of blood cells). The nanostructures can be arranged at varying distances to reduce modulate bacterial attachment and combined with anti-bacterial surface coatings for greater impact. Some of these bio-inspired surfaces have now been widely used in clinics, others are still at the proof-of-concept stage, with hurdles such as manufacturing limitations (anchoring of polymers, limited commercial availability of zwitterionic polymers, etc.), toxicity issues, and eventually randomized clinical trials determining which of these new surfaces will make it to the clinical setting.

Despite the promise of anti-biofilm surfaces, additional strategies are needed for the treatment of biofilms to eliminate existing biofilms as in the case of lung and wound infections or treat those that succeeded in overcoming the attachment barriers and managed to form biofilm. There are several different strategies available to target existing biofilms including getting rid of the slime (matrix) surrounding biofilms. The matrix, composed of polysaccharides (long strands of sugar molecules), proteins, and DNA, plays a role in attachment and subsequent biofilm formation, and thus, serves several purposes. For one, the matrix functions as a protective layer encasing the biofilm, acting as a diffusion barrier that prevents or restricts diffusion for some antibiotics and antibodies into the biofilm. Additionally, the matrix served as a structural framework that glues the community together

and to the surface. Thus, removal of the matrix or the architectural framework of the ‘virtual city of slime’ can result in loss of structural integrity and subsequent collapse, apparent by the disaggregation of the bacterial community into smaller cell clusters or individual cells. Matrix degradation, however, does not kill biofilm cells. Instead, matrix degradation is used in combination with antibiotics, to enhance the penetration and delivery of antibiotics into the biofilm upon removal of the diffusion barrier or by more efficiently killing cells that disaggregated from the biofilm into smaller cell clusters or individual cells. Matrix degradation is accomplished by enzymes (proteins that accelerate, or catalyze, chemical reactions). Unfortunately, the composition of the matrix, more specifically the type of proteins and polysaccharides present in the matrix, vary greatly among bacteria, with each type requiring a specific degradative enzymes. This means that treatment strategies focusing on the degradation of proteins and polysaccharides will need to be tailored to the type of bacterium forming biofilms. In contrast, the DNA present in the matrix appears to be ‘universal’ and susceptible to degradation by one enzyme alone, the enzyme DNase I. It is thus not surprising that several clinical trials have focused on the use of DNase I. Not only is DNase I (dornase alfa) being evaluated for the treatment of chronic middle ear (otitis media) and other biofilm infections in combination with antibiotics, DNase I is already used in the treatment of Cystic Fibrosis patients with early lung disease, with treatment coinciding with significantly improved lung function (although here, dornase alfa seems to be reducing the viscoelasticity of the sputum, thus enhancing lung clearance and antibiotic efficacy, rather than inducing a collapse of the biofilms).

A strategy of a different nature has been gleaned from the residents of biofilms and the way in which biofilms form and develop. Based on our current understanding, bacteria can not only form biofilms, they can also disassemble the biofilm structure they have built via a process called dispersion (Fig. 1). Dispersion occurs when bacteria escape from the biofilm if resources become exhausted, biofilms become too overcrowded, or in response to fluctuations in the environment, and serves as a means of survival and dissemination to spawn new communities at other locations (and also the reason why patients with chronic infections have flares of reoccurring sepsis/septicemia or signs of bacterial infections in general, whenever bacteria disperse *en masse* from the biofilm). Revisiting the scenario of Tokyo and Shibuya crossing, dispersion is comparable to a mass exodus, with the vast majority of its inhabitants taking off all at once, leaving behind an empty, depopulated ‘virtual city’. While at first glance this may not sound as a good strategy (as this creates conditions comparable to septicemia), bacteria leaving the biofilm are akin to single, planktonic cells. And after all, these are the kinds of bacteria we are good at treating, as our treatment strategies are geared toward treating infections caused by single, planktonic bacteria (Fig. 4).

So how can we ‘redirect’ biofilm bacteria to switch their lifestyle to escape from the biofilm, by inducing dispersion? While little is known about the changes that occur upon induction of dispersion, researchers have identified several triggers capable of inducing the lifestyle switch. For one, bacteria seem to have their own way of telling members in the biofilm community to disperse. Research on *Pseudomonas aeruginosa*, a model bacterium for biofilm formation and the causative agent of a large number of hospital-acquired and chronic infections, has shown that this bacterium produces a small diffusible molecule, more

specifically a fatty acid named *cis*-2-decenoic acid (*cis*-DA) (Davies & Marques, 2009) (Fig. 4) that enables this bacterium to signal to its community members that it's time to disperse. This means that *cis*-DA is a way of redirecting traffic and laboratory experiment have also shown that *cis*-DA can induce biofilms formed by other bacteria - that are not *P. aeruginosa* - to disperse from the biofilm. Overall, *cis*-DA has been shown to induce biofilm dispersion by at least five other bacterial species and yeast (Davies & Marques, 2009, Marques et al., 2015). Additionally, *P. aeruginosa* is not the only bacterium that self-produces a dispersion signal. There is evidence of other bacteria producing different version of this fatty acid meaning that biofilm bacteria may use 'dialects' to trigger dispersion (Petrova & Sauer, 2016, Marques et al., 2015). While still at the laboratory stage of testing, using such communication molecules in combination with antibiotics may very likely represent an up and coming future treatment strategy.

Additional triggers for dispersion are based on dispersion being an escape strategy in response to changing environmental conditions. Biofilms disperse when the going is tough such as when exposed to toxins (heavy metals, nitric oxide), too low/high oxygen levels, starvation conditions, or high abundance of nutrients, etc. While these triggers are quite diverse, they all induce dispersion by lowering the level of a universal intracellular signaling molecule named cyclic di-GMP. Levels of cyclic di-GMP determine the stickiness of bacteria to surfaces, with high levels being linked to surface associated biofilm growth and matrix production, and low levels linked to bacteria growing as single, planktonic cells. While dispersion and thus, modulation of cyclic di-GMP levels, in response to these triggers has been linked with 80 percent or more of the biofilm biomass being removed (Petrova & Sauer, 2016) (Fig. 4), not all triggers are suitable for use in medical settings. However, the colorless gas nitric oxide has emerged as a candidate for future treatment options. Our own immune system makes use of nitric oxide to fend off bacterial invaders, and its used medically to improve oxygenation in patients with various forms of pulmonary hypertension (e.g. COPD). Laboratory studies have shown that nitric oxide mediates biofilm dispersal across a broad range of bacteria, indicating that nitric oxide likely affects biofilms in a universal manner. Nitric oxide alone reduces biofilms on average by 63 percent, and when combined with antimicrobial compounds, was found to almost completely remove biofilms in pre-clinical trials (Barraud et al., 2009, Barraud et al., 2012) (Fig. 4). Despite the promising results, nitric oxide poses some clinical challenges including its potential cytotoxicity from systemic exposure, is delivery as a gas to the site of infection, and its lack of specificity in targeting biofilm infections. To better address some of the concerns, several formulations and devices have been developed. An example is cephalosporin-3'-diazoniumdiolates (Barraud et al., 2012). Cephalosporin-3'-diazoniumdiolates is a combination drug composed of an antibiotic (cephalosporin) and a nitric oxide producing agent, that has been developed to selectively deliver nitric oxide to bacterial biofilms. The drug is designed to only release nitric oxide upon contact with bacterial cells that are capable of inducing cleavage of the cephalosporin β -lactam ring via bacterial β -lactamases. Bacteria resistant to antibiotics such as penicillin and ampicillin typically harbor β -lactamases.

Another strategy to combat biofilms, although unrelated to the regulation of biofilm development, is to cause a lethal infection. People are susceptible to viruses. Like people,

bacteria are susceptible to viruses referred to as bacteriophages or phages. However, while people are not affected by phages, bacteria are with phages either permanently infecting or killing bacteria. Since the first studies in 1996 examining the interaction of bacteriophage with biofilms (Doolittle et al., 1996), research has aimed at identifying phages capable of killing bacteria within a biofilm. Additionally, bacteriophages that infect (enter) but not kill bacterial cells are being used to deliver cargo to each and every bacterium within the biofilm, with the cargo being an antibiotic or a matrix-degrading agent (e.g. DNase I). While phage therapy is currently being used outside the US in the treatment of biofilm-derived lung infections by cystic fibrosis patients, phage therapy comes with its own set of problems. For one, bacteriophages infect and kill bacteria with high specificity meaning that a particular phage only infects a specific bacterium. Additionally, similar to antibiotics, bacteria can become resistant to bacteriophage attack, rendering killing and cargo delivery ineffective. To overcome potential resistance to cargo delivering strategies, researchers have developed liposomes lipid-enclosed vesicles (Rukavina & Vani, 2016) (Fig. 3A). Also referred to as nanoparticles due to their small size, the idea behind these kind of mini transporters is to deliver compounds that kill biofilm cells or destabilize biofilms such as antibiotics, anti-bacterial compounds, or matrix-degrading enzymes directly to where they are needed, into the biofilm (Fig. 3B) or to the bacteria itself (Fig. 3C). The delivery mechanism is based on lipids present in the liposomes mimicking the lipids present on the bacterial membranes surrounding the bacterial cells. And here lies the beauty of the delivery system. The matching lipids enable liposomes to fuse with the bacteria, and, consequently, enable the transport system to directly spill its cargo into the bacterium (Fig. 3C) in a manner similar to an injection. An advantage of this strategy is the targeted approach, with anti-bacterial and anti-biofilm compounds only being delivered to the biofilm and nowhere else. Liposomes are considered to be the most widely used antimicrobial drug-delivery nanosystem (Rukavina & Vani, 2016).

In addition to the ones described here, there are numerous ongoing research efforts, some at early stages of development, some at the clinical stage, aimed at preventing and controlling biofilms. The approaches mentioned here barely scratch the surface of the sheer number of strategies that are being tested in laboratories worldwide, which indicates the importance of biofilm control as well as how challenging it is to truly get a handle on biofilms. One of the challenges is that all biofilms are not the same. Each species makes and escapes from biofilms in slightly different ways, using different slime, using different or variations of communication molecules, producing different proteins, etc. Edison once said that “I have not failed 1,000 times. I have successfully discovered 1,000 ways to NOT make a light bulb.” While in the end, Edison figured out ONE way to make a light bulb, it is unlikely there will be just one treatment strategy to defeat biofilms.

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Figure 1. Schematic drawing of the biofilm developmental process.

The formation of biofilms occurs in discernable stages, with single, planktonic cells attaching first in a reversible manner after which bacterial cells either leave the surface or become irreversibly attached. Once attached, bacteria start producing a slimy layer referred to as extracellular matrix (beige color) that enables them to cement themselves to the surface. Continued surface associated growth results first with the formation of aggregates (maturation-1 developmental stage) and then larger structures such as microcolonies (maturation-2 developmental stage). Dispersion describes the biofilm developmental stage at which single cells are released from the biofilm. Based on research described in (Sauer et al., 2002b, Stoodley et al., 2002, Petrova & Sauer, 2009).

Figure 2. Mechanism controlling attachment.

(A) Under normal conditions, bacteria become permanently attached to surfaces via the Once bacteria make contact with surfaces, the production of extracellular matrix material (slime, beige color) is induced. (B) Biomaterials coated with antimicrobial agents do not prevent surface association of bacteria but instead either kill bacteria (shown by bacteria turning red, marked by X) or prevent further growth of surface attached bacterial cells. Arrows indicate release of anti-bacterial compounds. (B) Biomaterials with altered surface topography repel bacteria by preventing bacteria to permanently attach to surfaces. Instead, attachment of cells is only temporary, with the majority of cells leaving the surface.

Figure 3. Liposomes and their mode of delivering cargo.

(A) Schematic drawing of liposome, lipid-enclosed vesicles. The lipids are composed of a hydrophilic head and a hydrophobic tail. The interior of liposomes can harbor various components (cargo) including antibiotics, enzymes etc. Image taken from Wikipedia. (B) Liposomes (red circles) penetrate the biofilm structure to deliver their cargo (indicated in red). (C) Liposomes can deliver their cargo directly to bacteria by fusing with them.

Figure 4. Biofilm control strategy based on biofilm escape.