Review

Antibiotic resistance of bacteria in biofilms

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Bacteria that adhere to implanted medical devices or damaged tissue can encase themselves in a hydrated matrix of polysaccharide and protein, and form a slimy layer known as a biofilm. Antibiotic resistance of bacteria in the biofilm mode of growth contributes to the chronicity of infections such as those associated with implanted medical devices. The mechanisms of resistance in biofilms are different from the now familiar plasmids, transposons, and mutations that confer innate resistance to individual bacterial cells. In biofilms, resistance seems to depend on multicellular strategies. We summarise the features of biofilm infections, review emerging mechanisms of resistance, and discuss potential therapies.

Bacteria that adhere to implanted medical devices or damaged tissue can become the cause of persistent infections.[1,2] These bacteria encase themselves in a hydrated matrix of polysaccharide and protein, forming a slimy layer known as a biofilm. Direct microscopic examination of colonised surfaces shows dense aggregates of bacteria held together by diffuse extracellular polymers (figure 1). Biofilm formation is important because this mode of growth is associated with the chronic nature of the subsequent infections, and with their inherent resistance to antibiotic chemotherapy.

Periodontitis and chronic lung infection in cystic fibrosis patients are examples of diseases that are generally acknowledged to be associated with biofilms.[3,4] Various nosocomial infections such as those related to the use of central venous catheters,[5] urinary catheters,[6] prosthetic heart valves,[7] and orthopaedic devices[8] are clearly associated with biofilms that adhere to the biomaterial surface. These infections share common characteristics even though the microbial causes and host sites vary greatly. The most important of these characteristics is that bacteria in biofilms evade host defences and withstand antimicrobial chemotherapy.

Even in individuals with competent innate and adaptive immune responses, biofilm-based infections are rarely resolved. In fact, tissues adjacent to the biofilm might undergo collateral damage by immune complexes and invading neutrophils.[9] Susceptibility tests with in-vitro biofilm models have shown the survival of bacterial biofilms after treatment with antibiotics at concentrations hundreds or even a thousand times the minimum inhibitory concentration of the bacteria measured in a suspension culture.[10] In vivo, antibiotics might suppress symptoms of infection by killing free-floating bacteria shed from the attached population, but fail to eradicate those bacterial cells still embedded in the biofilm. When antimicrobial chemotherapy stops, the biofilm can act as a nidus for recurrence of infection. Biofilm infections usually persist until the colonised surface is surgically removed from the body.

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As an example of sequelae of biofilms, let us consider the case of a patient with pacemaker endocarditis.[11] A man aged 56 years was admitted with a 4-day history of nausea, vomiting, and shaking chills. Physical examination showed a temperature of 39·2°C and tenderness in his upper right quadrant. *Staphylococcus aureus* grew from blood cultures. He was treated intravenously with 12 g cloxacillin daily for 4 weeks. 1 week after discharge he developed nausea, vomiting, fever, and sweating. Again, *S aureus* grew from blood cultures. For 6 weeks he was treated with 12 g intravenous cloxacillin daily and with 600 mg oral rifampicin daily. There were no signs of endocarditis. He promptly responded to antibiotic therapy, but was readmitted a third time 9 days after discharge with the same symptoms. Once again, *S aureus* grew from blood cultures. The entire pacing system was removed and intravenous cloxacillin was continued for 4 weeks. He remained well thereafter. Swabbing of the infected pacemaker lead recovered *S aureus*, and examination by electron microscopy showed localised accretions of coccoid bacteria.

Bacteria in biofilms persist in the body by a strategy that might be characterised as tenacious survival as opposed to aggressive virulence. Biofilm infections can linger for months, years, or even a lifetime. Although they compromise quality of life, these infections are rarely fatal and are often traced to species of bacteria, such as *Pseudomonas aeruginosa* or *S epidermidis*, that are ubiquitous in water, air, soil, or skin. These are opportunistic pathogens that persist because they are adept at forming biofilms, in which they are protected.

Resistance mechanisms

The familiar mechanisms of antibiotic resistance, such as efflux pumps, modifying enzymes, and target mutations,[12] do not seem to be responsible for the protection of bacteria in a biofilm. Even sensitive bacteria that do not have a known genetic basis for resistance can have profoundly reduced susceptibility when they form a biofilm. For example, a β-lactamase-negative strain of *Klebsiella pneumoniae* had a minimum inhibitory concentration of 2 μg/mL ampicillin in aqueous suspension.[13] The same strain, when grown as a biofilm, was scarcely affected (66% survival) by 4 h treatment with 5000 μg/mL ampicillin, a dose that eradicated free-floating bacteria.[13] When bacteria are dispersed from a biofilm they usually rapidly become susceptible to antibiotics,[14,15] which suggests that resistance of bacteria in biofilms is not acquired via mutations or mobile genetic

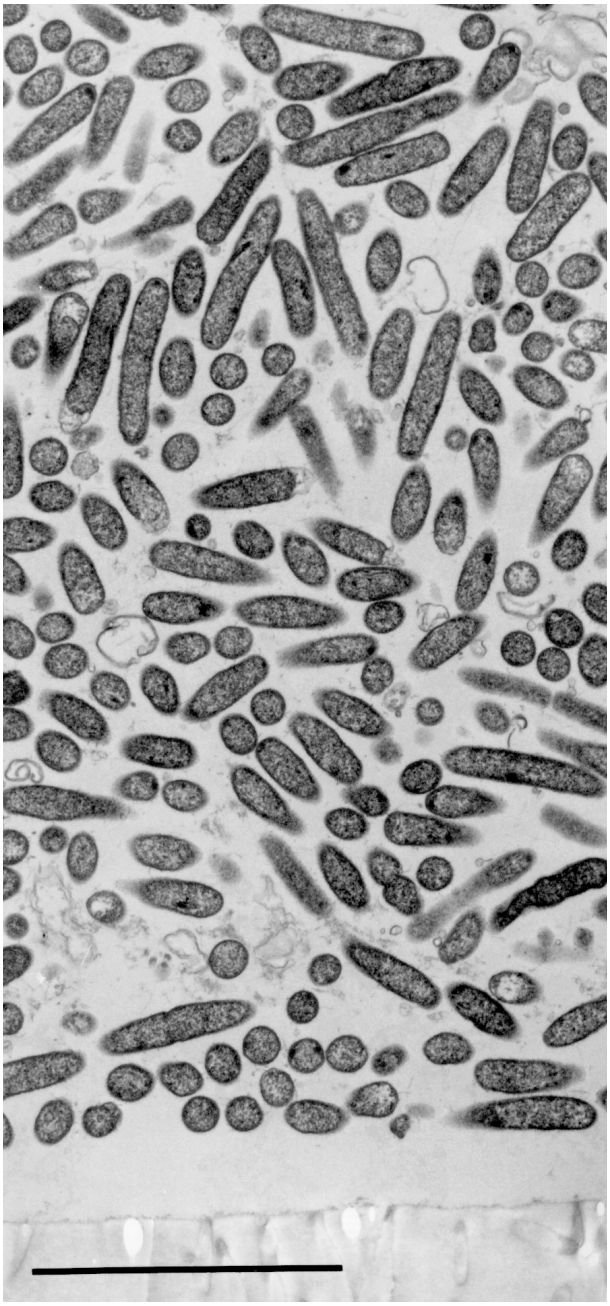

Figure 1: ***Pseudomonas aeruginosa* biofilm**
Electron micrograph of a laboratory-grown *Pseudomonas aeruginosa* biofilm. Bacteria live in multicellular clusters with individual cells in close proximity. The biofilm was grown on a plastic substratum (bottom). Bar=5 μm.

elements. Few studies have investigated the effect of genes encoding multidrug efflux pumps, such as the multiple antibiotic resistance (*mar*) locus.[16] These studies did not show any important role for these genes in mediating biofilm resistance. The *mar* operon is not induced during biofilm growth of *Escherichia coli*, and mutants without *mar* have a resistance to ciprofloxacin similar to strains with *mar* when grown in biofilms.[17,18] In biofilms, strains of *P aeruginosa* that do not have the MexAB-OprM multidrug resistance pump also remained resistant to ciprofloxacin.[19] Preliminary evidence indicates that conventional antibiotic resistance mechanisms are not sufficient to explain most cases of antibiotic-resistant biofilm infections. This evidence does not exclude the

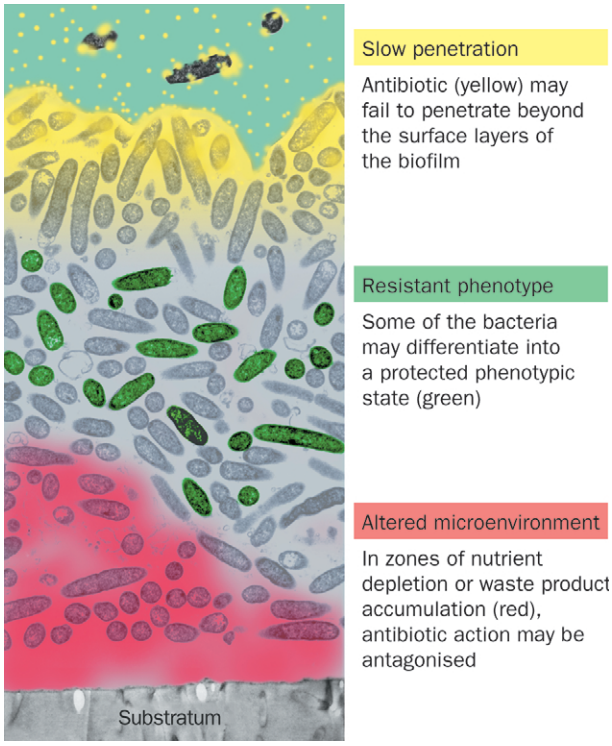

Figure 2: **Three hypotheses for mechanisms of antibiotic resistance in biofilms**
The attachment surface is shown at the bottom and the aqueous phase containing the antibiotic at the top.

possibility that conventional resistance mechanisms, such as drug pumps, are expressed in biofilms and contribute to antibiotic resistance in the attached mode of growth. However, we should look beyond conventional mechanisms to understand biofilm resistance. Conventional antibiotic resistance can develop in biofilms treated repeatedly or for a long time—stable derepression of chromosomal β-lactamase contributes to the persistence of *P aeruginosa* biofilm infections.[20]

The mechanisms of resistance to antibiotics in bacterial biofilms are beginning to be elucidated;[21] figure 2 shows three main hypotheses. The first hypothesis is the possibility of slow or incomplete penetration of the antibiotic into the biofilm. Measurements of antibiotic penetration into biofilms in vitro have shown that some antibiotics readily permeate bacterial biofilms.[22] There is no generic barrier to the diffusion of solutes the size of antibiotics through the biofilm matrix, which is mostly water.[23] However, if the antibiotic is deactivated in the biofilm, penetration can be profoundly retarded. For example, ampicillin can penetrate through a biofilm formed by a β-lactamase-negative strain of *K pneumoniae* but not a biofilm formed by the β-lactamase-positive wild-type strain of the same micro-organism.[13] In the wild-strain biofilm, the antibiotic is deactivated in the surface layers more rapidly than it diffuses. Antibiotics that adsorb into the biofilm matrix could also have a retarded penetration, which might account for the slow penetration of aminoglycoside antibiotics.[24,25] These positively charged agents bind to negatively charged polymers in the biofilm matrix.[26,27]

The second hypothesis depends on an altered chemical microenvironment within the biofilm. Microscale gradients in nutrient concentrations are a well known feature of biofilms. Findings from studies with miniature

electrodes have shown that oxygen can be completely consumed in the surface layers of a biofilm, leading to anaerobic niches in the deep layers of the biofilm.[28] Concentration gradients in metabolic products mirror those of the substrates. Local accumulation of acidic waste products might lead to pH differences greater than 1 between the bulk fluid and the biofilm interior,[29] which could directly antagonise the action of an antibiotic. Aminoglycoside antibiotics are clearly less effective against the same micro-organism in anaerobic than in aerobic conditions.[30] Alternatively, the depletion of a substrate or accumulation of an inhibitive waste product might cause some bacteria to enter a non-growing state, in which they are protected from killing. Penicillin antibiotics, which target cell-wall synthesis, kill only growing bacteria.[31] This alternative possibility is strengthened by direct experimental visualisation of metabolically inactive zones within continuously fed biofilms.[32] Additionally, the osmotic environment within a biofilm might be altered, leading to induction of an osmotic stress response.[33] Such a response could contribute to antibiotic resistance by changing the relative proportions of porins in a way that reduces cell envelope permeability to antibiotics.

A third and still speculative mechanism of antibiotic resistance is that a subpopulation of micro-organisms in a biofilm forms a unique, and highly protected, phenotypic state—a cell differentiation similar to spore formation. This hypothesis is lent support by findings from studies that show resistance in newly formed biofilms, even though they are too thin to pose a barrier to the penetration of either an antimicrobial agent or metabolic substrates.[34,35] Additionally, most bacteria in the biofilm, but not all, are rapidly killed by antibiotics.[19,36] Survivors, which might consist of 1% or less of the original population, persist despite continued exposure to the antibiotic. The hypothesis of a spore-like state entered into by some of the bacteria in a biofilm provides a powerful, and generic, explanation for the reduced susceptibility of biofilms to antibiotics and disinfectants of widely different chemistries.

Multicellular nature of biofilm defence

All three main hypotheses of biofilm resistance to antibiotics depend on the multicellular nature of biofilms.[37] An antimicrobial agent cannot slowly or incompletely penetrate the biofilm unless the micro-organisms form aggregates that affect its diffusion. Local variations in the concentrations of microbial substrates and products develop only when a cluster of cells reaches a critical size and the bacteria exert their combined metabolic activity. The small population of cells that differentiate into a dormant and protected state depend on their growing neighbours to propagate the genome, and their neighbours depend on them to reseed the community in the event of catastrophic killing. The fact that all these antibiotic resistance mechanisms are inherently multicellular helps to explain why bacteria dispersed from biofilms rapidly revert to a susceptible phenotype.

Researchers investigating bacterial biofilms are beginning to discuss biofilm formation in terms of developmental biology. Recent results lend support to the idea of biofilm formation as a multicellular developmental process. We now know that specific gene products are required for the initial association of bacteria with a surface. Dozens of new genes are turned on and others are turned off as bacteria move onto a surface, suggesting a pathway of differentiation. Motility seems to be critical in the early stages of biofilm formation. Coordinated by unknown cues, bacteria use flagellar, twitching, and gliding motility mechanisms to grow together in nascent clusters. The further organisation of the biofilm into complex structures is regulated by the exchange of chemical signals between cells in a process known as quorum sensing. Add to these observations the capacity for bacteria in biofilms to collectively withstand antimicrobial treatments that would kill a lone cell, and the case for multicellularity in biofilms is compelling. The recognition of biofilm formation as a multicellular developmental process is important because this insight will allow new approaches for treatment of the persistent infections stemming from biofilms.

Potential for new therapies

More work is needed to fully elucidate antibiotic resistance mechanisms in biofilms and develop new therapeutic strategies, but we have enough evidence to make some observations and suggestions. Clearly, there are multiple resistance mechanisms that can act together. Antibiofilm therapies might have to thwart more than one mechanism simultaneously to be clinically effective. Heterogeneity is a common theme of these resistance mechanisms; micro-organisms in a biofilm exist in a broad spectrum of states. First, cells might be exposed to different concentrations of antibiotic depending on their spatial location. Second, gradients in the concentration of microbial nutrients and waste products crisscross the biofilm and alter the local environment, which leads to a broad range of growth rates of individual microbial cells. Third, a small proportion of cells in a bacterial biofilm might differentiate into a highly protected phenotypic state and coexist with neighbours that are antibiotic sensitive. The proliferation of states that arises when these three types of heterogeneity are crossed means that any given antimicrobial agent might be able to kill some of the cells in a biofilm, but is unlikely to effectively target all of them. Most or all the antibiotics in current use were identified on the basis of their activity against growing cultures of individual cells. New screens of existing and potential antibiotics that select for activity against non-growing or biofilm cells might yield antimicrobial agents with clinical efficacy against biofilm infections. As genes that mediate biofilm resistance to antibiotics are identified and their gene products characterised, these will become targets for chemotherapeutic adjuvants that could be used to enhance the effectiveness of existing antibiotics against biofilm infections.

Because biofilm resistance depends on aggregation of bacteria in multicellular communities, one strategy might be to develop therapies that disrupt the multicellular structure of the biofilm. If the multicellularity of the biofilm is defeated, the host defences might be able to resolve the infection, and the efficacy of antibiotics might be restored. Potential therapies include enzymes that dissolve the matrix polymers of the biofilm,[38] chemical reactions that block biofilm matrix synthesis,[39] and analogues of microbial signalling molecules that interfere with cell-to-cell communication, required for normal biofilm formation.[40] As the genetic basis for biofilm development emerges, the gene products identified as required for multicellular colony formation will become a potential target for chemotherapy. In other words, we believe that treatment strategies will target the formation of multicellular structures rather than essential functions of individual cells. We will learn to treat the persistent infections associated with biofilms when the multicellular nature of microbial life is understood.

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