



Bacterial Biofilms Resist Key Host Defenses

Once in biofilms, bacterial pathogens resist antibiotics and withstand several host-defense measures, including phagocytosis

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Bacteria have long intrigued, terrified, and baffled humans. Although most microorganisms are not pathogenic to humans, the thought of microbes existing everywhere can have a dramatic effect on the psyche. Once-specialized terms and acronyms, such as Staph, for *Staphylococcus aureus*, MRSA, for methicillin-resistant *S. aureus*, and XDR, for extensively drug-resistant *Mycobacterium tuberculosis*, are now catchphrases in the news media. In light of this and of several recent outbreaks of foodborne illnesses, it is easy to trace the source of some of those psychological effects.

Yet, even though scientists have been studying these pathogens for almost two centuries, more and more researchers believe that it is how these bacteria live that can really matter in terms of the diseases they cause. One key issue is whether they live as single-celled “planktonic” organisms or as complex communities called biofilms that cause chronic infections by withstanding standard antibiotic treatments and escaping the destructive forces of the host. Several bacterial pathogens form biofilms having complex interactions with components of the innate host defense system. Deciphering these interactions could lead to novel, biofilm-specific therapies.

Defining Biofilms and How They Resist Drugs and Host Defenses

Biofilms consist of communities or groups of microorganisms that attach to the surfaces of animate objects such as heart valves, bones, or tissues, or to inanimate

objects such as artificial heart valves, prosthetic implants, or catheters (Fig. 1). One of the best examples of a biofilm on a human tissue is dental plaque, which consists of a community of microorganisms that initially attach to tooth surfaces. If left undisturbed, their products can penetrate the tooth enamel, causing cavities, and eventually progress to soft tissue disease such as periodontitis.

Biofilm communities of microbes are different from their planktonic counterparts in very important ways. First, when microbes live as a community, they become much less susceptible to antibiotics, even if highly susceptible as individual cells. Thus, when microorganisms form a community, they are protected against a variety of antibiotics that clinicians commonly prescribe for their patients. Second, and more the focus of this perspective, these communities of microorganisms resist attack and killing by the host immune system.

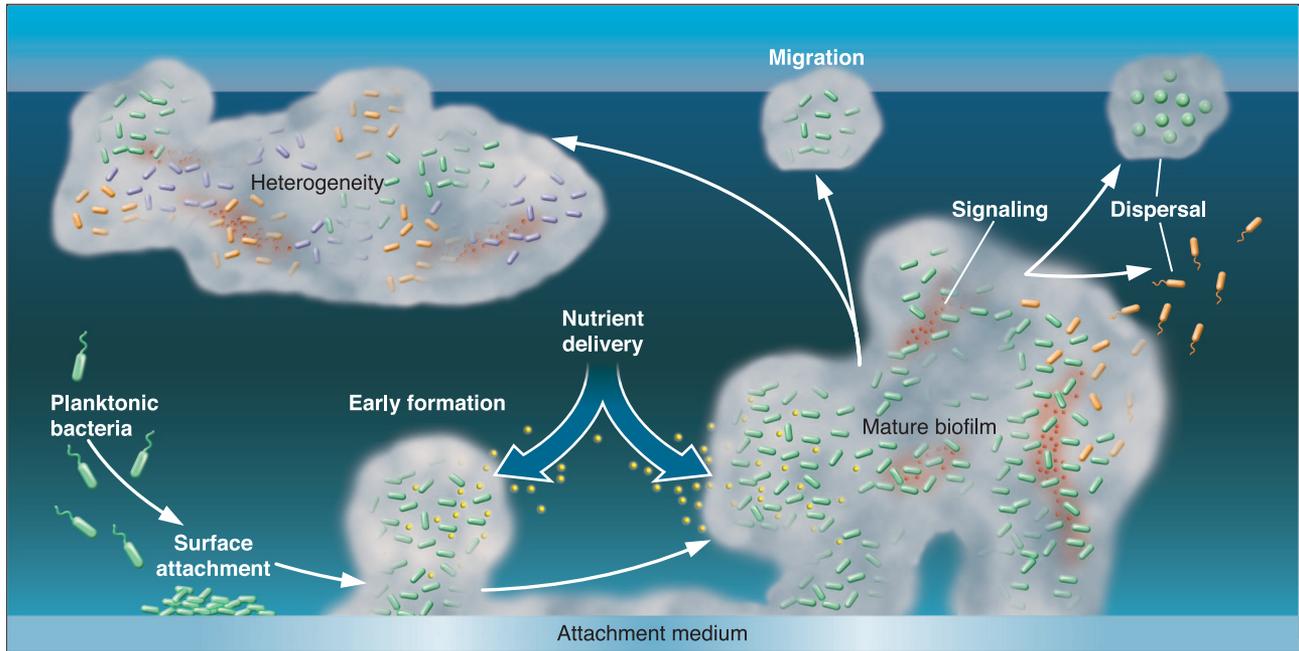
Biofilm communities are found in virtually ev-

Summary

- Many bacterial pathogens form complex communities called biofilms that cause chronic infections and withstand standard treatments.
- In natural environments, biofilms likely serve as an important survival factor, enabling microorganisms to adapt to changing conditions collectively instead of as single cells.
- The exopolysaccharide alginate protects *P. aeruginosa* in part through a process called “frustrated” phagocytosis, in which macrophages and neutrophils encounter but can no longer engulf this pathogen.

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FIGURE 1



Depiction of the dynamic nature of a biofilm community. The community starts to form when single cells called planktonic bacteria attach to an animate or inanimate surface that normally is conditioned to enhance attachment. As those individual cells strongly adhere, and expand in number, they are surrounded by an extracellular matrix. As the community matures, partly by cell to cell communication (signaling), parts of the biofilm can disperse, migrate, or the community can continue to develop into a heterogeneous population of cells that are metabolically, physiologically and genetically distinct from one another. If appropriate attachment reservoirs are available downstream from the dispersion event, the entire cycle can start over again.

ery habitat. In natural environments, they likely serve as an important survival factor, enabling organisms to adapt to changing conditions collectively instead of as single cells. Certainly, the role of biofilms in diverse environmental settings has been established over the last four decades by numerous studies.

Establishing the role of biofilms in medicine has proceeded much more slowly, for a variety of reasons. First and foremost, the molecular tools necessary for investigating infections biofilms— high-throughput sequencing, fluorescent in-situ hybridization, and global gene analysis— are only now becoming widely available. As these tools become more commonplace in medicine, so too does our understanding of the role of biofilms in infectious disease. Other factors likely play a role, including limited biofilm-specific resources, limited overall scientific knowledge about microorganisms living as communities, and reluctance to step aside from 160 years of scientific and medical understanding of mi-

crobes as single-celled, planktonic organisms living an individualistic lifestyle.

Some structural attributes are considered universal to biofilms. For example, they contain macro- and/or microcolonies of bacterial cells

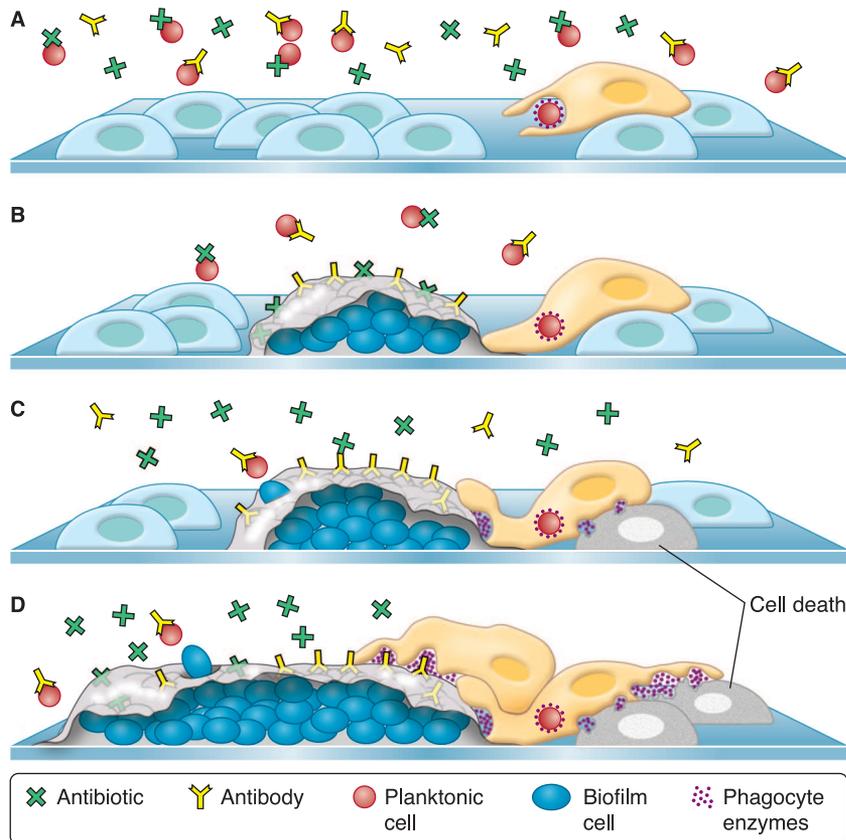
Mechanisms of biofilm resistance to host defenses

- Limited penetration of leukocytes and their products into the biofilm^a
- Global response regulators and quorum sensing that protects biofilm bacteria
- Decreased phagocytic capacity of host cells against biofilm bacteria
- Genetic switches that increase resistance of biofilm bacteria^b
- Suppression of leukocyte effector function, including magnitude of respiratory burst

^aData from many groups have shown this mechanism is not likely a major factor in biofilm resistance.
^bExamples could include the *flgK* and *ndvB* genes in *P. aeruginosa*.



FIGURE 2



Cartoon schematic of frustrated phagocytosis occurring as phagocytes respond to biofilm organisms. (A) Representation of a human phagocyte responding to a planktonic infection (red bacteria). Host antibodies, antibiotics, and the phagocyte cell are well equipped to kill these types of cells. As a biofilm develops (B–D), antibodies, antibiotics and even host phagocytes gain access to the bacteria within these communities, but the host phagocytes cannot engulf and kill the bacteria. Nonetheless, the biofilm pathogens are recognized by the phagocyte, which induces the release of enzymes and other toxic compounds, causing death of the healthy host cells (C–D). The necrotic debris can then become additional substrate for the biofilm community to expand.

that are separated from other microcolonies by interstitial voids. Liquid flow within these voids, or channels, allows nutrients, gases, and antimicrobial agents to diffuse throughout the biofilm. While biofilms have common structural features, their architecture is constantly changing in response to internal and external processes. Moreover, the individual organisms themselves are heterogeneous within each biofilm community. Proximity of cells within or between microcolonies is ideal for exchanging extrachromosomal plasmids, nutrients, and quorum-sensing molecules (communication molecules). Biofilm-associated microorganisms have dramatically

reduced susceptibility to antimicrobial agents and host immune defenses. One of the hallmark mechanisms of resistance within biofilms is that bacteria in such communities regulate gene expression in a coordinated fashion that is mediated by bacterial communication.

Bacteria in biofilms resist antibiotics via several mechanisms, including (i) decreased penetration or diffusion of antimicrobial agents into biofilms, (ii) increased activity of multidrug efflux pumps, (iii) involvement of quorum sensing systems, (iv) starvation or stress responses, and (v) genetic switches that turn susceptible planktonic cells into antibiotic-resistant persisters. Meanwhile, the mechanisms that enable bacteria in biofilms to resist host defenses are less well characterized, but include (i) limited penetration of leukocytes and their bactericidal products into the biofilm, (ii) global response regulators and quorum sensing activities that increase resistance to leukocytes, (iii) decreased ability of leukocytes to engulf biofilm bacteria, (iv) genetic switches that increase resistance of bacterial cells in biofilms to the immune system, and (v) suppression of leukocyte activity through effector regulation (see table).

Treatment options for clinicians fighting biofilm infections are limited. One of the only effective tools is excision or removal of infected tissues. This strategy is successful only if all the infected tissue is removed and the surrounding tissue is kept healthy while it

regenerates. The recolonization of this new tissue by commensal flora biofilms is likely an important attribute that facilitates the healing process. Besides developing novel biofilm-specific antimicrobials, a better understanding of how biofilm communities evade host immune responses is critical.

Classic Examples of Biofilm Infections

A classic example of biofilm infections in humans is *P. aeruginosa* associated with the lungs of cystic fibrosis (CF) patients. By the time patients are 16–20 years old, this pathogen (among others) will have colonized the lungs of

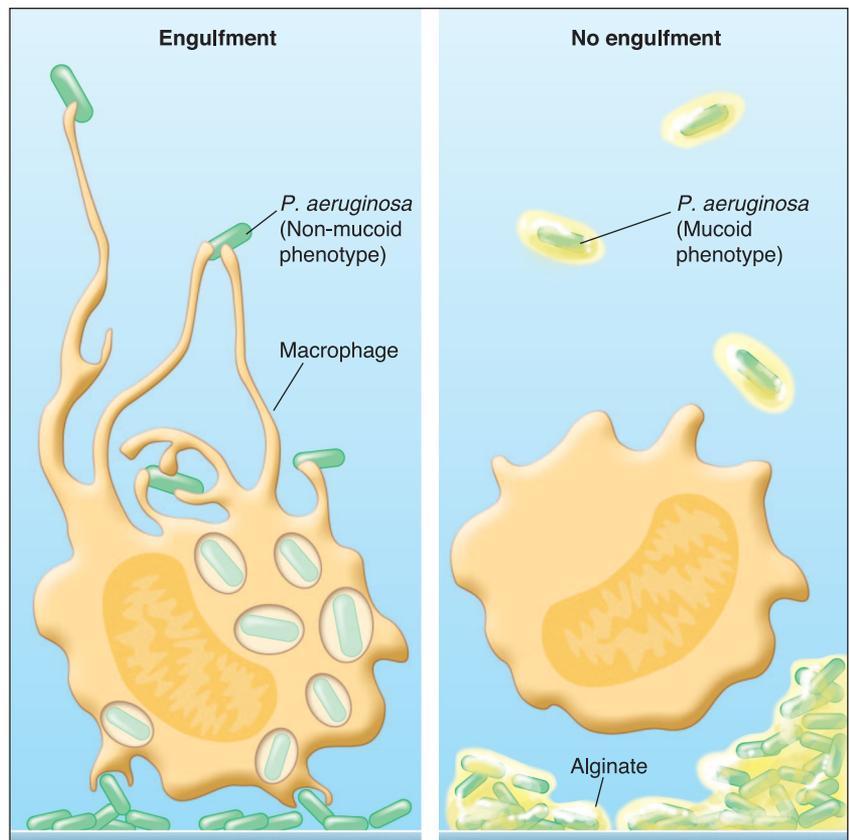
~90% of them, accounting for chronic morbidity and an increased risk of early mortality. More than four decades ago, J. William (Bill) Costerton, who is now at the Allegheny-Singer Research Institute in Pittsburgh, Pa., reported that *P. aeruginosa* biofilms form in the lungs of such patients. Recent studies by Pradeep Singh and his colleagues at the University of Washington School of Medicine have cemented this original observation by demonstrating the molecular signatures of bacterial communication in the lungs of CF patients. They also note that lactoferrin, an iron-binding protein, can prevent *P. aeruginosa* biofilm development in laboratory models of infection.

P. aeruginosa biofilms also form when this pathogen infects the cornea, the skin of patients with burn wounds, or implanted medical devices, and in those also infected with HIV. In all these cases, the host immune system is marginally or severely compromised. One key antibacterial mechanism within the innate immune system depends on phagocytes, including neutrophils and macrophages, engulfing and killing microorganisms. This defensive mechanism is very effective against many types of pathogens when they live as planktonic, individual organisms.

However, this process is less effective when phagocytes encounter bacteria in biofilms—a phenomenon called frustrated phagocytosis (Fig. 2). When such “frustrated” macrophages and neutrophils encounter but cannot engulf bacteria in biofilms, they are activated and secrete toxic compounds that damage nearby healthy host tissues.

In some cases, biofilms modulate the effectiveness of those effector molecules. When neutrophils encounter *P. aeruginosa* biofilms, they produce less superoxide compared to when they encounter the planktonic form of this pathogen. Other oxygen-dependent (nitric oxide) and oxygen-independent host-neutrophil responses (lysozyme, lactoferrin) are also reduced in magnitude in response to *P. aeruginosa* biofilms. Although the mechanisms behind these reductions are not fully understood, biofilm organisms in general modulate the

FIGURE 3



In *Pseudomonas aeruginosa* biofilms, the polysaccharide alginate protects biofilm bacteria from macrophage engulfment and killing. When macrophages encounter *P. aeruginosa* biofilm bacteria that lack alginate, they can be engulfed and destroyed. However, if the biofilm bacteria transition to a mucoid phenotype, where alginate is present, the macrophages still respond to the presence of the pathogen community, but are no longer able to engulf and kill these organisms.

host response so that it is either reduced in magnitude or nonproductive against the bacterial community.

Alginate Is One Key Virulence Determinant in *P. aeruginosa* Biofilms

One of the most intensely studied virulence determinants of *P. aeruginosa* is the viscous exopolysaccharide alginate, which many, but not all, biofilm-producing strains generate. This important extracellular component of mucoid strains of *P. aeruginosa* scavenges hypochlorite, reduces polymorphonuclear chemotaxis, inhibits activation of complement, and decreases phagocytosis by neutrophils and macrophages. When produced in high levels, alginate can even



protect this pathogen against grazing protozoans.

The expression of alginate is a tightly regulated genetic process. Although antibodies that are directed against alginate aid in cell-mediated killing of *P. aeruginosa* biofilms, comparable antibodies from CF patients fail to do so. Subsequently, we found that the alginate polysaccharide network protects biofilm bacteria against phagocytosis and killing by human macrophages (Fig. 3). Meanwhile, George O'Toole and his collaborators at Dartmouth Medical School, Hanover, N.H., and at the Center for Biofilm Engineering at Montana State University in Bozeman showed a direct link between antibiotic resistance in biofilm bacteria and biofilm-specific genetic regulation.

Alginate also can influence human cytokines that help to direct the response of the immune system. For clarity, scientists have divided cytokines into a variety of categories. Th1 cytokines direct cell-mediated responses, while Th2 direct humoral or antibody-mediated responses. Microorganisms often are able to regulate this host response to their advantage. For instance, alginate upregulates two Th1-type cytokines, interleukin-12 (IL-12) and tumor necrosis factor- α (TNF- α). However, even though alginate elicits the host to produce higher levels of these Th1 cytokines, the biofilm bacteria have mechanisms that block or inhibit cell-mediated killing. Understanding these resistance mechanisms is one of the keys to developing antibiofilm therapeutics.

Additional Host-Resistance Mechanisms in *P. aeruginosa* Biofilms

Flagellum expression is regulated when individual *P. aeruginosa* cells attach to surfaces while forming biofilms. As they begin to attach, these bacteria express flagella, which can help the cells in a variety of ways. Later, as the biofilm matures and the community becomes increasingly heterogeneous, flagellum expression is dramatically downregulated. However, when bacteria within a biofilm prepare to disperse, flagella production is once again up-regulated, presumably to enhance the motility of single cells as

well as their ability to evade host immune defenses. Experiments suggest that flagella also regulates susceptibility of biofilm organisms to host defenses. In *P. aeruginosa* biofilm cells that lack flagella, neutrophil-secreted lactoferrin kills these bacteria.

Further studies suggested that mononuclear cells (lymphocytes and monocytes) and neutrophils were required for killing of these biofilm bacteria and that cytokines from these cells were important in the production of bactericidal lactoferrin. Although killing of the bacterial biofilms did not involve phagocytosis, appropriate cytokines were vital to generate a killing response. Both interferon- γ (IFN- γ) and TNF- α were required for optimal killing, with the latter cytokine especially important for eliciting neutrophil-mediated killing by inducing release of lactoferrin. Not only can this antibacterial compound prevent biofilms from forming, it also is directly bactericidal—but only on cells lacking flagella.

The human innate immune system is complex yet beautifully simple. Two of the main cellular components, neutrophils and macrophages, display both chemical and physical means for defending the body against pathogens. On the physical side, these cells engulf and kill bacteria. On the chemical side, these cells secrete oxygen-dependent agents such as superoxide or other oxygen-independent enzymes such as lysozyme and lactoferrin. The goal of these products is the destruction of foreign invaders while keeping the body free from the burden of pathogens.

Several of these defenses are extremely lethal against planktonic, single-celled microbes, and effectively keep a host healthy, without reliance on the adaptive immune system. However, when microbes evade these components of the host defense system and then attach to surfaces to form heterogeneous communities, those defenses are no longer effective. These persistent communities can then lead to debilitating chronic infections in humans and sometimes death. In order to improve patient health and survival, understanding the complex interactions between the biofilm communities and the host defenses is essential.