Microbial Biofilms in Ophthalmology and Infectious Disease

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Most bacterial infections involve biofilms. Biofilms are collections of microorganisms encased in a matrix that is often composed of both bacterial and host materials. They form on natural surfaces such as heart valves or abiotic surfaces such as contact lenses or intraocular lenses. The biofilm matrix promotes adherence of the microbe to smooth surfaces as well as to other cells. Biofilms thereby form large 3-dimensional microbial communities of complex architecture through cell-to-cell communication and coordinated multicellular behavior. The biofilm architecture promotes the exchange of nutrients and waste products. The ability of microorganisms to attach to abiotic surfaces and grow in highly stable communities greatly confounds the medical use of implantable devices. Much effort is now being invested to understand the molecular nature of biofilms, with a view toward designing biofilm-resistant implantable devices and more effective antimicrobials.

Arch Ophthalmol. 2008;126(11):1572-1581

" . . . *Tho my teeth are kept usually very clean, nevertheless when I view them in a Magnifying Glass, I find growing between them a little white matter as thick as wetted flower . . . to my great surprise . . . contained very many small living Animalicula, which moved themselves extravagantly . . . hence I conclude, that the Vinegar with which I washt my Teeth, kill'd only those Animalicula which were on the outside of the scurf, but did not pass thro the whole substance of it . . . The number of these Animalicula in the scurf of a mans Teeth, are so many that I believe they exceed the number of Men in a kingdom.*"

Anthony van Leeuwenhoek[1,2]

Evidence of biofilm formation in harsh environments such as hot springs and deep-sea hydrothermal vents[3] suggests that the ability to form biofilms dates back more than 3 billion years to an early period in the evolution of life on Earth.[4,5] The first characterization of medical microbial biofilms described observations made on dental plaque by van Leeuwenhoek.[1] By observing that he could only kill a small fraction of the microorganisms adhering to his teeth, he can be credited as the first to recognize the natural resistance of bacteria in a biofilm to killing by a biocide, acetic acid.[1,2] Of course, it took 3 more centuries for Koch to describe the germ theory for disease. It has taken an additional century for the medical importance of biofilms to be recognized despite its estimated involvement in more than 80% of bacterial infections.[6,7]

Most of what we know about bacterial physiology and behavior stems from studies of bacteria in pure culture, often suspended as individual cells in liquid broth. Yet most bacteria associated with the human body, in health and at sites of infection, are surface-associated. Collections of microorganisms on natural or implanted surfaces are known as biofilms. Biofilms are heterogenous mixtures of bacteria held together by a secreted matrix called extracellular polymeric substances (EPS). They exhibit surprisingly complex multicellular behaviors that are coordinated by cell-to-cell signaling networks (**Figure 1**). Biofilms may consist of cells of several or a single bacterial species interacting cooperatively. Cells within a biofilm are physiologically heterogenous because a variety of microniches occur within the biofilm structure. Cells on the surface of the struc-

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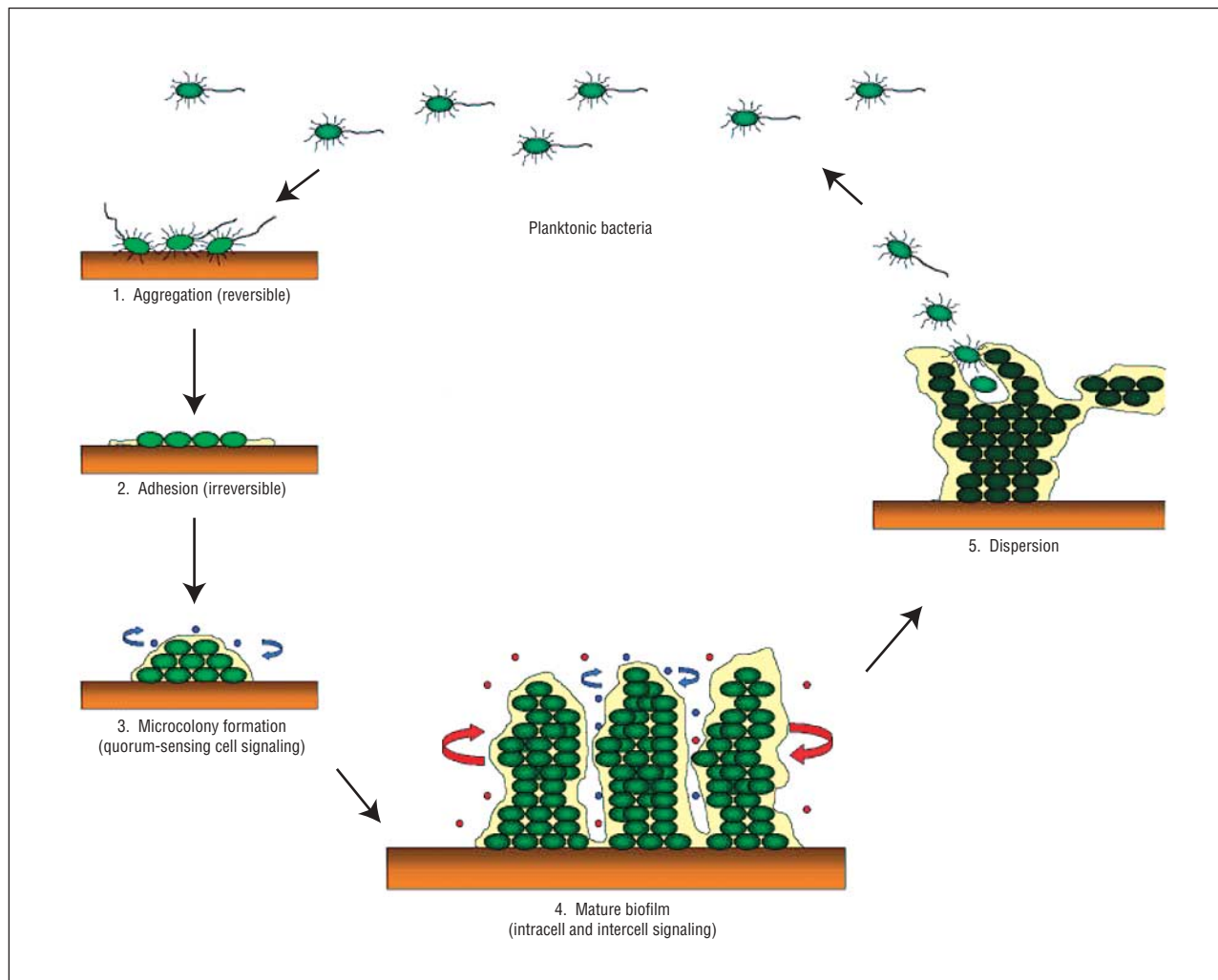
Figure 1. Stages 1 through 5 of biofilm developmental cycle. The various shades of green represent different transcriptional and translational expression levels between the planktonic states and the different developmental stages of a bacterial biofilm.

ture may have ready access to nutrients and be actively metabolizing and dividing. More internal cells may be largely dormant. This concept of physiological heterogeneity within a biofilm is important because, unlike the relative physiological synchrony of bacteria suspended as individual cells in broth culture, this heterogeneity within biofilms results in cells with vastly different susceptibilities to antibiotic. As a result, biofilms may be as much as 1000 times more refractory to antibiotic killing than bacterial cells suspended in broth culture. It is likely that this phenomenon underlies the often-observed disparity between in vitro minimum inhibitory concentration values and ineffectiveness of an antibiotic to eradicate a well-established infection.

INDWELLING DEVICES AND BIOFILM INFECTION

A large percentage of biofilm-related infections are associated with indwelling medical devices. About 1 million cases—an estimated 60% of hospital-associated infections—are due to biofilms that have formed on indwelling devices.[8] Device-related biofilm infections increase hospital stay, on average, 2 to 3 days and add approximately $1 billion per year to US hospitalization costs.[9] Owing to the aging population and the increasing number of implantable medical devices available, infections associated with biofilms are expected to increase. Currently, approximately 2 million fracture-fixation devices are implanted, accompanied by an infection rate of 5%, with an additional cost of $15 000 per case. Approximately 2% of the 600 000 prosthetic joints implanted per year become infected at a cost of $30 000 per patient, not including months of loss of function or income. Mechanical heart valves (85 000/y), vascular grafts (450 000/y), and pacemaker-defibrillators (300 000/y) have rates of infection of about 4% at a cost of $35 000 to $50 000 per infection and significant morbidity and mortality. Some implantable devices, such as ventricular-assist devices, are associated with infection rates as high as 40% despite their short-term use and inclusion of prophylactic antibiotics. These are associated with a cost of $50 000 per infection, with high rates of morbidity and mortality.[8] Indwelling prosthetic devices have been instrumental in saving lives and have enhanced the quality of life for a great many more patients, yet the presence of an indwelling foreign body both predisposes to and greatly complicates the eradication of bacterial and fungal infections.

Table 1. Examples of Ocular-Associated Infections Relative to Location and Type of Foreign Body

Foreign Body	Location	Infection	Organisms
Penetrating trauma, superficial	Eyelid, periorbital area	Eyelid abscess	*Staphylococcus aureus*
Contact lens	Anterior segment	Keratitis	Gram-positive species (*Staphylococcus* species) and *Pseudomonas* species; rarely, fungi and acanthamoeba
Punctal plugs	Lacrimal duct	Conjunctivitis	*Pseudomonas aeruginosa*
Silicone sponge implants	Conjunctiva and sclera	Posterior segment	*Staphylococcus* species
Intraocular lens	Anterior segment, mainly	Pseudophakic endophthalmitis	*Staphylococcus* species
Penetrating trauma, deep	Crosses anterior to posterior segment	Endophthalmitis, orbital abscess	*Staphylococcus* species, object-associated environmental organisms (*Bacillus* species, fungi)

The nature of an indwelling foreign body—either an implanted device or an object entering through trauma—profoundly influences the host-pathogen dynamic.[10] The mere presence of the foreign body (1) enables a smaller bacterial inoculum to lead to infection, (2) permits a nonpathogenic organism to opportunistically colonize the foreign body and infect, (3) allows a pathogen to persist undetected at the site of the infection, (4) induces a chronic local inflammatory response, and (5) limits the induction and effectiveness of a humoral response in the presence of chronic, persistent infection.[11] Soon after a foreign body enters a host, an inflammatory response occurs. The magnitude of this response depends on the chemical composition, shape, size, mechanical stability, and the location of the object.[12] Most materials are not inert, and there tends to be some surface decomposition with a release of breakdown products resulting in an allergic or irritative response.

Foreign bodies are quickly covered by host matrix molecules. This surface conditioning layer is composed of fibrinogen, fibronectin, and collagen.[13] One of the challenges in the design and construction of implanted medical devices is to control the deposition of these host molecules that form host-conditioned surfaces to limit opportunities for adherence of free-floating (planktonic) bacteria via their matrix attachment adhesins. Bacterial biofilms are observable and have been studied on the surface of explanted medical devices at high resolution by electron microscopy.[14,15] Implant infections are notable for being difficult to diagnose because stable communities of bacteria in biofilms are often walled off by components of the human immune system, resulting in chronic low-grade inflammation. As noted above, because of the physiological heterogeneity of the bacteria within the biofilm, they are also extremely difficult to eradicate using existing antimicrobials. Resolution often only occurs with surgical removal of the device followed by prolonged antimicrobial therapy.

Just as for other anatomical sites, the risk of biofilm-related infection of ocular foreign bodies and medical devices depends on location (**Table 1**). Biofilms have been observed on contact lenses, where they are believed to contribute to the development of microbial keratitis.[16-18] Cataract surgery with intraocular lens (IOL) placement or the introduction of intraocular infusion pumps, glaucoma tubes, stents, keratoplasties, or other ocular prostheses create opportunities for the development of infections involving microbial biofilms.[19,20]

It is estimated that more than 30 000 cases of microbial keratitis occur annually in the United States and 100 000 cases annually worldwide.[21] Nonsurgical trauma and contact lens use are the leading predisposing risk factors for microbial keratitis. The mechanism underlying this increased risk is the subject of much interest. A decrease in corneal epithelial barrier function with contact lens wear may be mechanical in origin, stemming from an accumulation of debris under the lens during closed-eye wear and friction and pressure from normal blinking during open-eye wear. Infections may be caused by bacteria present on the ocular surface, adnexa, or colonizing the lens, and these infections may progress through deeper layers of the cornea through an induced epithelial defect or through microtrauma induced by the foreign object itself.[22] Contaminated contact lenses, cases, and possibly solutions are known to be the source of infection in some instances.[23] In contrast to non–contact lens–associated microbial keratitis, which is more frequently associated with gram-positive organisms, contact lens–related infections in many geographic locations are more commonly associated with gram-negative bacteria (particularly *Pseudomonas aeruginosa*).[18,23] *Pseudomonas aeruginosa* and other environmental organisms also cause conjunctivitis associated with punctal plugs. Because *Pseudomonas* is not a commensal of the ocular surface, it is likely that the abiotic surface of the punctal plug, like the contact lens, provides a site for otherwise transient microbes to attach and form a biofilm, protected from host mucosal defenses while shedding organisms onto the ocular surface, eventually leading to infection.[24,25] The intermittent release of organisms[25,26] or bacterial products such as endotoxin or other bacterial exotoxins from biofilms on the ocular surface may damage the corneal epithelium, rendering it more susceptible to infection.[25,27] The specific role of biofilms in the recent contact lens–associated *Fusarium* keratitis outbreak[28] is as yet unknown, but is suggested by in vitro biofilm modeling systems.[29-31] Lens disinfectant systems are mainly tested against organisms in planktonic broth culture; this is an area of increasing concern.[31]

Most cases of endophthalmitis occurring after cataract surgery are due to bacteria entering the eye at the time of surgery. Gram-positive organisms constitute most culture-positive cases of pseudophakic endophthalmitis,[32] with many cases being caused by coagulase-negative staphylococci.[16,32] Coagulase-negative staphylococcal infection (ie, infection caused by species other than *Staphylococcus aureus*) often occurs as an indolent low-grade process with

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more than 55% of cases occurring more than 1 week after surgery and 20% more than 2 months later.[33] *Propionibacterium acnes* have also long been associated with low pathogenicity and very delayed onset of pseudophakic endophthalmitis,[34,35] yet only recently have biofilmlike deposits been seen on IOLs with polymerase chain reaction confirmation.[36] Contact between the lens and external tissues surrounding the incision has been observed to result in 26% of the lenses becoming colonized.[37] The IOL appears to provide a niche where bacteria may attach to form a biofilm. Biofilms may occur on the IOL, haptic or capsule, and the composition of the lens appears to influence biofilm development.[16,38,39] Because of limited circulation and access to the innate immune system, IOL-associated biofilms are likely stable communities resulting in intermittent release of bacteria and bacterial products. This stability, as well as the prospect that bacteria in a dormant physiology in a biofilm may not readily revert to active growth, may explain the relatively low yield of aqueous and vitreous culture.[40] The aqueous blood barrier is reestablished within 12 weeks after ocular surgery. This limited access may be responsible for restricting secondary seeding of the IOL from transient organisms in the bloodstream (in contrast to foreign bodies with more direct access to the vascular supply).[22]

Other ocular infections associated with abiotic materials and likely involving biofilms include keratitis related to corneal sutures (such as in penetrating keratoplasty)[41,42] and scleral buckle infections, where rates of biofilm formation may be as high as 65%.[43] Chronic inflammatory stimulus of bacteria and antigens released from the biofilm may contribute to tissue damage in these infections.[25,44]

Staphylococcus aureus is the leading cause of all indwelling device infections,[8] and these have become a special concern because of the increasing resistance of these organisms to antibiotics,[45] including the identification of strains resistant to vancomycin.[46] *Staphylococcus aureus* strains vary widely in their ability to form biofilms[47-51]; this appears to be true for ocular isolates as well (I.B. and M.S.G. unpublished data from ongoing study [2007 to present]).

BIOFILM INFECTIONS UNRELATED TO INDWELLING DEVICES

Native valve endocarditis, cystic fibrosis pneumonia, periodontitis, bladder infections, and otitis media are all examples of biofilm-related diseases that do not involve indwelling devices. Damaged endothelium exposes the underlying basement membrane (collagen, laminin, vitronectin, and fibronectin) that provides a substratum for the adherence of organisms transiently passing through the bloodstream. Ensuing inflammation stimulates the clotting cascade, leading to the deposition of fibrin and the creation of an insoluble clot of fibrin and platelets. Endocarditis vegetations adherent to cardiac valves consist of biofilms formed from aggregates of bacterial cells, platelets, fibrin, fibronectin, and collagen. Several bacteria have fibronectin receptors, including *S aureus* and several species of streptococci.[52] Early work demonstrated that *Streptococcus sanguis* adheres to the surface of sterile vegetations when injected into rabbits that have had catheter-induced damage to the vascular endothelium. The bacteria attach and begin to replicate within 30 minutes.[53] Most
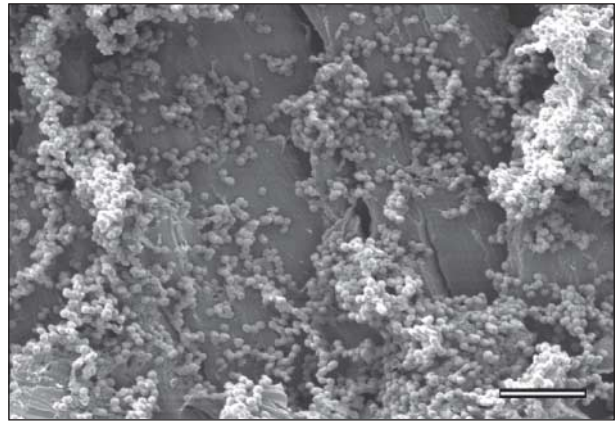

Figure 2. Microcolonies with early biofilm formation. Scanning electron micrograph of a *Staphylococcus aureus* clinical ocular isolate grown in vitro on polymethyl methacrylate. Magnification ×2000; scale bar, 10 µm.

of the metabolic activity of biofilm bacteria occurs on the surface while bacterial colonies deep within the thrombus were relatively inactive. By 2 days, most bacteria in a vegetation have entered metabolic quiescence.[54] As a result, this subacute to chronic infection is recalcitrant to antibiotic therapy.[55,56] Parallels to these processes likely occur in biofilm-related infections at other sites.

Infectious crystalline keratopathy may occur in normal or diseased corneas, corneal grafts, or around sutures.[16,57,58] In pathology specimens from patients with chronic microbial keratitis, bacteria growing in an apparent biofilm were seen in infectious crystalline keratopathy.[59,60] Fixation with ruthenium red demonstrated bacteria within an exopolysaccharide matrix predominantly in patients with infectious crystalline keratopathy, but not in other patients with chronic keratitis. Infectious crystalline keratopathy is believed to relate to biofilm formation because of the prolonged clinical course, poor antibiotic responsiveness, and difficulty culturing individual organisms.[25] A bacterial biofilm concretion has been reported in a patient who had prior pterygium surgery for necrotizing scleritis that required a scleral patch graft 7 years earlier.[61] In this case, a more permissive environment for biofilm formation appears to have been provided by the presence of abnormal tissue with impaired mucosal defenses.[25]

WHAT WE KNOW ABOUT BIOFILM STRUCTURE AND DEVELOPMENT

Structure of Biofilms

Biofilms are mostly water. Because of the extensive hydration and thickness of the EPS in which the microbes are imbedded, they are challenging to image at high resolution by traditional microscopic techniques. Despite the effect of dehydration on structure, scanning electron microscopy (**Figure 2** and **Figure 3**) has been used to visualize the fine details of biofilm surfaces and transmission electron microscopy has provided a detailed understanding of deeper structures.[62] Advances in cryotechniques, particularly freeze-substitution, which rapidly freezes biofilms in noncrystalline or nanocrystalline ice, have provided much better structural detail of biofilms.[63] Advances in confocal scanning laser microscopy
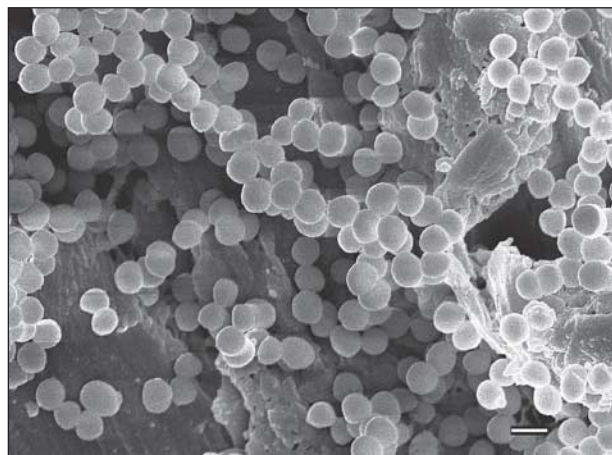
Figure 3. Mature biofilm with associated extracellular polymeric substances and architectural structure. Scanning electron micrograph of a *Staphylococcus aureus* clinical ocular isolate grown in vitro on polymethyl methacrylate. Magnification ×7000; scale bar, 1 µm.
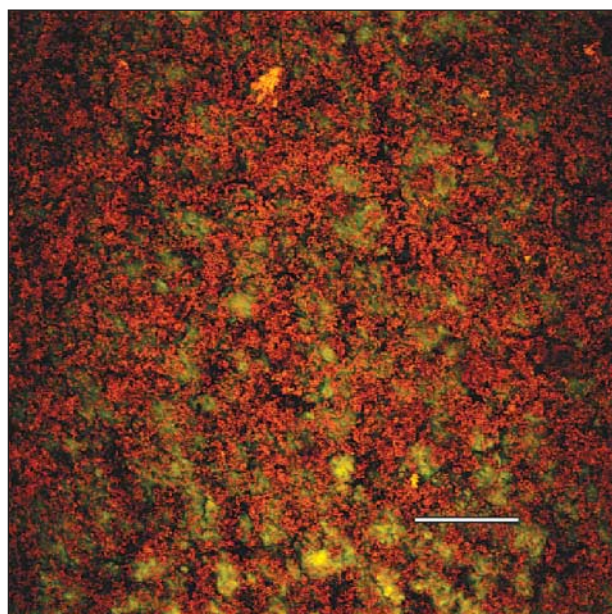

Figure 4. Mature biofilm. Laser scanning confocal micrograph of a *Staphylococcus aureus* clinical ocular isolate grown in vitro on polymethyl methacrylate and stained with acridine orange. Magnification ×1000; scale bar, 40 µm.

(**Figure 4**) have permitted in situ, high-resolution, real-time study of biofilm formation and structure in native fully-hydrated form.[64,65] Using this tool, biofilms have been revealed to be highly organized and surprisingly dynamic communities of microorganisms, densely packed in the often heterogeneous matrix of extracellular polymeric substances, with open water channels (Figure 1).

Extracellular polymeric substances consist of polysaccharides, proteins, nucleic acids, and lesser amounts of lipids and other polymers.[66-69] In addition, bacterial outer-membrane vesicles, flagella, phages, pili, host matrix material, and lysed cell debris may also be present.[63] Biofilms consist of approximately 15% bacteria and 85% EPS by volume. Extracellular polymeric substance is highly hydrated and consist of 97% water by mass.[70] Although highly hydrated, diffusion within the biofilm is not unrestricted, and there is substantial evidence of the occurrence of mi-

croniches that vary substantially in oxygen content, osmolarity, pH, and other parameters.[66,68,71,72] Water channels facilitate nutrient uptake and waste exchange.[72-74]

Biofilm Development

It is increasingly clear that the microorganisms undergo specifically programmed developmental changes when they transition from free-swimming individual organisms (planktonic) to a sessile surface-associated community (biofilm). The environmental signals that promote biofilm formation include nutritional content, temperature, osmolarity, pH, iron, and oxygen, and vary among species. For example, most species of *Pseudomonas* will form biofilms under many conditions that are permissive to growth[75] while the pathogen of food-borne diarrheal outbreaks, *Escherichia coli* 0157:H7, forms biofilms only in nutrient-poor media.[76]

One of the most thoroughly studied medically relevant organisms with respect to biofilm formation is *P aeruginosa*. Based on confocal microscopic observations of structural development as well as observed changes in gene expression patterns, a 5-stage life cycle for biofilm formation by *Pseudomonas* has been proposed[60] (Figure 1). Stage 1 involves the initial weak nonspecific association of cells to the surface. Stage 2 involves conversion to firm adherence with initial EPS production. Stage 3 occurs with bacterial cell division and aggregation of cells to one another in a microcolony, with further EPS production. Stage 4 results in maturation of the biofilm architecture with vertical growth and formation of water channels dividing bacterial communities. The final stage in the cycle, stage 5, involves dispersion of single cells from the biofilm. Protein expression patterns of dispersing single bacterial cells closely resemble those of planktonic cells rather than those of cells in the aging biofilm structure. Microcolonies of the biofilm may slough, particularly as the EPS degrades. Similar stages of development have been proposed for biofilm formation by other organisms, and the development of multispecies biofilm involving interspecies cross-talk may contribute to variation in the developmental cycle depicted (Figure 1).

Mechanisms of Aggregation and Attachment. Stage 1 represents a reversible association of bacteria with a surface, but at this point they are not committed to biofilm formation and may leave the surface to resume a planktonic lifestyle. On association with the surface, bacteria may exhibit species-specific motility such as rolling, pilus-mediated twitching, gliding, tumbling, and aggregate formation before they begin to exude exopolysaccharide and adhere irreversibly.[77] For motile bacteria such as *Pseudomonas*, an event that occurs immediately prior to irreversible attachment to a surface is a switch from flagella-based motility to a lateral surface-associated motility termed *twitching motility*.[75,78,79] This twitching motility allows redistribution of attached cells enabling bacteria to colonize adjacent areas of the surface. Swarming, another mechanism of motility, is also used by some strains.[80]

Nonmotile staphylococci appear to attach to cells of the corneal epithelium through interactions between a fibronectin-binding protein and fibronectin bound to in-

tegrins on the cell surface,[47,49] followed by a growth-dependent accumulation of cells into a microcolony. Initial attachment of staphylococci to abiotic surfaces appears multifactorial. *Staphylococcus aureus* mutants lacking either teichoic acids[81] or autolysins[82-84] show altered biofilm formation. In *Staphylococcus epidermidis*, an autolysin (or peptidoglycan hydrolase), AtlE, contributes to binding vitronectin (a component of the host extracellular matrix [ECM]) and to hydrophobic surfaces such as polystyrene.[83] Mutants of AtlE are less virulent in animal models of catheter-associated infection.[84] *Staphylococcus aureus* mutants lacking an enzyme that adds d-alanine to teichoic acid (*dltA*), thereby regulating the surface charge of the organism, are reduced in biofilm formation. Other complex surface polymers and membrane lipids (eg, lysylphosphatidylglycerol) also play a role in attachment of *S aureus* to biomaterials.[85]

Host matrix factors significantly affect bacterial adhesion to indwelling devices.[86] Host extracellular matrix components, which naturally support association of host cells in forming tissues and organs, also serve as ligands for pathogenic microorganisms.[87] This specific bacterial binding of the host matrix is mediated by bacterial surface protein adhesins. *Staphylococcus aureus* expresses on its surface a family of protein adhesins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). These MSCRAMM proteins specifically recognize host matrix components. Fibronectin, a large multifunctional host glycoprotein found either as a soluble form, particularly in plasma,[85] or as an insoluble form in the extracellular matrix, appears to be a major binding target. Invasive strains of staphylococci have been reported to bind fibronectin more avidly than commensal strains.[88,89] Fibronectin binding may explain the tropism of *S aureus* for traumatized tissues, blood clots, and abnormal heart valves. Two fibronectin adhesins (fibronectin binding proteins A and B [FnBPA and FnBPB]), or MSCRAMMs, have been identified on strains of *S aureus*.

Fibrinogen, the blood plasma coagulation protein, also occurs in the ECM. It is the major blood protein deposited on implanted cardiovascular devices such as vascular grafts, catheters, kidney dialyzers, and cardiac assist devices.[87,88] *Staphylococcus aureus* also expresses 2 fibrinogen-binding proteins that belong to the MSCRAMM family known as clumping factors A and B. Clumping factor A has also been shown to be important in the binding of *S aureus* in adhesion to both polyethylene and polyvinyl surfaces.[90] Additional bacterial adhesins bind other host proteins including collagen and laminin (major glycoprotein found in human basement membrane).

Stage 2 is characterized by the production of the biofilm matrix, EPS. One such EPS is alginate, which is produced by some strains of *P aeruginosa*. The genes responsible for alginate production by this bacterium are upregulated within 15 minutes of attachment. Production of alginate is also triggered by nitrogen limitation, membrane perturbation induced by ethanol, and high osmolarity.[91-94] Alginate has been found in large quantities in mucoid strains of *P aeruginosa* from the lungs of patients with cystic fibrosis. Mucoid strains of *P aeruginosa* become the predominant pathogen with time and parallel a worsening clinical prognosis.[95] Alginate appears to scavenge free radicals and protect bacterial cells from phagocytic clearance. Despite the development of antialginate antibodies, they are ineffective in triggering opsonic killing in patients with cystic fibrosis.[95-99] Overproduction of alginate leads to architectural changes in the biofilm, with resultant increased resistance to antimicrobials and innate immune system.[100-104]

Making the issue more complex, it has recently been shown that alginate production is not the only mechanism for biofilm formation by *P aeruginosa* strains.[98,104,105] Some strains produce other extracellular polymers (Psl)[100,106] that contribute to cell-to-surface and cell-to-cell adherence.[100,107] Not only is Psl required for adherence to mucin-coated surfaces and airway epithelial cells, but it is important for maintenance of the biofilm scaffold.[108] Another *Pseudomonas* polysaccharide-encoding gene is *pel*, which is involved in biofilm formation.[109] Both Psl and Pel are highly conserved in *Pseudomonas* strains and their genes are regulated in response to biofilm formation.[110-113]

Adherent cells of staphylococcal species will produce an extracellular polysaccharide, polysaccharide intercellular adhesin for *Staphylococcus epidermidis*[114-117] or poly-N-acetylglucosamine for *S aureus*.[51,118] These similar polymers are synthesized by enzymes encoded by a group of closely related operons termed the intercellular adhesin (*ica*) operon.[51,119] The *ica* operon is more commonly associated with invasive than noninvasive strains of *S epidermidis*[120] and contributes to foreign body infection in animal models.[84,120-122] The relationship between virulence and the *ica* operon for *S aureus* is less clear. The *ica* operon is found in most *S aureus* strains,[51,123,124] and a clear role in virulence has been difficult to demonstrate.[125] However, it is clear that the gene products of the *ica* operon contribute to biofilm formation and are tightly regulated.[126-137] In addition to the carbohydrate polymers synthesized by the *ica* locus, a cell wall protein, accumulation-associated protein, has been implicated in biofilm formation by coagulase-negative staphylococci on polymer surfaces.[138] Accumulation-associated protein, containing a novel 5-glycine domain (G5 domain), is proposed to play a role in anchoring polysaccharide intercellular adhesin or poly-N-acetylglucosamine to cell surfaces. Accumulation-associated protein mutants produce polysaccharide intercellular adhesin or poly-N-acetylglucosamine that is only loosely attached to the cell surface.[139-141] A second protein, biofilm-associated protein,[142] is expressed by both *S aureus* and coagulase-negative staphylococci and permits these organisms to form biofilms independently of polysaccharide intercellular adhesin and poly-N-acetylglucosamine.

Maturation of the Biofilm. Biofilm stage 3 is characterized by the growth of surface-attached microcolonies that progress to a mature biofilm architecture in stage 4 with increased synthesis of EPS. The mature biofilm is characterized by complex architecture that includes channels, pores, and even a redistribution of the bacteria farther away from the substratum. Growth generally occurs as the result of binary division of attached cells spreading daughter cells upward and outward from the attached surface to form cell clusters.[143] However, recruitment of cells from the bulk fluid to the aggregating microorganisms has also been observed.[144] There is physi-

Table 2. Differences Between Biofilm and Planktonic Bacteria

Planktonic Bacteria	Biofilm Bacteria
Suspended cells; single cells	Aggregated cells; multiple cells at an interface
Little capsular matrix	Surrounded by EPS matrix
Physiologically homogeneous and active	Physiologically heterogeneous
Intracellular signaling not critical for cell division	Intracellular signaling critical for growth and higher-order architecture formation
Antibiotic-susceptible physiologically active cells	10-1000 times increased antibiotic resistance
Individual cells recognized and effectively targeted by host immune response	Matrix-embedded cells are inaccessible to the host response and resistant to antimicrobial agents

Abbreviation: EPS, extracellular polymeric substance.

ological adaptation of bacteria in the biofilm that includes increased resistance to antibiotics as a more protective environment develops.

The formation of complex higher-order architectures is coordinated through a system of communication between bacteria in the biofilm. Many bacterial species, including *Pseudomonas* species and *S aureus*, have communication systems that enable them to produce factors involved in cellular repair, defenses, and virulence in a coordinated way. Expression of these traits usually occurs once a threshold of bacteria are present, signaling the occurrence of a suitable site for colonization. This cell density–dependent communication is known as *quorum sensing*. Anywhere from 1% to 10% of the genes of a bacterium are regulated by this process. *Pseudomonas aeruginosa* possess several overlapping quorum-sensing systems.[144-151] *Pseudomonas aeruginosa* cells deficient in one of these genes, *lasI,* produce biofilms that are 20% as thick as wild-type and grow as continuous undifferentiated sheets without evidence of EPS. In the biofilm, LasI is maximally expressed in those *P aeruginosa* cells located nearest the substratum.[147]

Biofilm-associated organisms possess distinct characteristics from planktonic bacteria, such as increased resistance to antimicrobial agents. Using microarray technology, Whiteley et al[94] compared mature *P aeruginosa* biofilms with planktonic bacteria and found 73 genes that were differentially expressed. Genes expressed at higher levels in mature biofilms included those encoding proteins involved in translation, metabolism, membrane transport and/or secretion, and gene regulation. Similarly, adaptive stress-response functions are upregulated in a *P aeruginosa* biofilm.[79,94,152] Proteomic analysis has revealed that more than 50% of the detectable proteome (more than 800 proteins) demonstrated a greater than 6-fold difference in expression, with more than 300 proteins detected in the *P aeruginosa* mature biofilm that were undetectable in extracts from planktonic bacteria. These proteins were involved in metabolism, phospholipid and lipopolysaccharide biosynthesis, membrane transport and secretion, and adaptation and protective mechanisms.[150]

Detachment. Detachment, the release of bacteria from a biofilm, appears to be a physiologically regulated event (Figure 1, stage 5). After biofilm maturation, EPS matrix levels appear to go down, perhaps owing to metabolism, with subsequent detachment of clumps and individual cells.[153,154] Increased concentration of inducer molecules may be responsible for the release of matrix polymer–degrading enzymes that results in detachment of cells from biofilms.[155] Alginate lyase causes degradation of alginate in *P aeruginosa* biofilms. Other mechanisms may involve density-dependent regulation of the release of matrix-degrading enzymes.[153] Inducing biofilm disaggregation would appear to be a promising area for further research.

Selective Advantage of Biofilm Adaptation

It is becoming increasingly clear that communal life within a biofilm offers multiple advantages (**Table 2**). The close physical proximity of other bacterial cells favors synergistic interactions, even between members of different species. These include the horizontal transfer of genetic material between microbes, the sharing of metabolic byproducts, an increased tolerance to antimicrobials, protection from environmental stresses (ultraviolet radiation, temperature changes, wind, environmental extremes from dryness to humidity), and protection from the host immune system or from predator microorganisms in the environment. Much of this protection is conferred by the EPS matrix, which enables a variety of microscopic niches to form in the biofilm, each with its own distinct environment, fostering the existence of a collection of microorganisms in vastly different physiological states.

Biofilms are notorious for withstanding concentrations of antibiotics 1000 times higher than levels lethal to planktonic cells. The EPS matrix may provide some shield, but it appears that the heterogeneity of bacteria inside the biofilm, including some cells that are nearly dormant, accounts for this difference. Microbes closest to the fluid that surrounds the biofilm have greater access to nutrients and oxygen compared with those in the center of the matrix or near the substratum. Many antibiotics target rapidly dividing or metabolizing bacteria, so the slow-growing or stable bacteria within the biofilm community tend to be spared. The negative charge of the biofilm matrix may initially affect the permeability of antibiotics, but most research indicates that the high level of antibiotic resistance exhibited by biofilms does not stem from a permeability barrier.

CONCLUSIONS

There is a compelling need to understand the nature and role of biofilms in infection. We only now appreciate that microbes in a biofilm behave differently than their planktonic counterparts and, importantly, respond differently to antibiotics. Most antibiotics were discovered because of their ability to inhibit bacterial growth in vitro, but this ability may or may not be related to their effect on cells in a biofilm in vivo. At a time of diminishing options because of the spread of antibiotic resistance, it is imperative that our patients receive the greatest benefit from the antibiotics that we have until new ones are discovered. Understanding how bacteria evade the activity of antibiotics by encapsulating themselves in the co-

coon of a biofilm and gaining the knowledge to overcome that will be critical to improving patient outcome for most bacterial infections.

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