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REVIEW

Antimicrobial resistance in clinically important biofilms

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Abstract

A biofilm contains a consortium of cohesive bacterial cells forming a complex structure that is a sedentary, but dynamic, community. Biofilms adhere on biotic and abiotic surfaces, including the surfaces of practically all medical devices. Biofilms are reported to be responsible for approximately 60% of nosocomial infections due to implanted medical devices, such as intravenous catheters, and they also cause other foreign-body infections and chronic infections. The presence of biofilm on a medical device may result in the infection of surrounding tissues and failure of the device, necessitating the removal and replacement of the device. Bacteria from biofilms formed on medical devices may be released and disperse, with the potential for the formation of new biofilms in other locations and the development of a systemic infection. Regardless of their location, bacteria in biofilms are tolerant of the activities of the immune system, antimicrobial agents, and antiseptics. Concentrations of antimicrobial agents sufficient to eradicate planktonic cells have no effect on the same microorganism in a biofilm. Depending on the microbial consortium or component of the biofilm that is involved, various combinations of factors have been suggested to explain the recalcitrant nature of biofilms toward killing by antibiotics. In this mini-review, some of the factors contributing to antimicrobial resistance in biofilms are discussed.

Key words: Biofilm; Bacteria; Antimicrobial agent; Medical devices; Nosocomial infections; Resistance

Core tip: Biofilm formation on host tissues and medically implanted devices is a major health problem, and the infections caused by bacteria in biofilms are hard to treat with antimicrobial agents. They are the cause of frequent and recurrent infections after the termination of antimicrobial treatments. The reasons for the recalcitrant nature of biofilms to antimicrobial treatment are varied and have been attributed to different factors, including impermeability of biofilms, slow rates of growth and metabolic activity, and the presence of small colonies and persisters. They have been the subject of many investigations that will be discussed in this minireview.

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INTRODUCTION

Over the past three decades, it has become increasingly clear that microbial biofilms represent the norm and not the exception for microbial life. Most microorganisms reside under diverse environmental stresses, with less than optimal levels of such essentials as nutrients and oxygen, and under a constant threat of physical removal. To combat such a hostile environment, microorganisms form surface-associated communities, embedded in a self-produced extracellular matrix. This is evident in the clinical setting, where the biofilm survival lifestyle affords resistance to high concentrations of antimicrobials and to the host defense system[1,2]. It has been estimated that 60% to 80% of infections in the developed world involve biofilms[1,3]. By definition, biofilms are microbially derived sessile communities characterized by cells that are irreversibly attached to a substratum or interface or each other, are embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription[4]. The bacterial population in biofilms may be comprised of one or more bacterial species that have formed a cohesive matrix, consisting of live bacteria embedded in polysaccharides, proteins, and extracellular DNA, all of which are bacterial byproducts, and may also include fungi and host-related materials[5]. This matrix, which is produced over time, is important for the structural stability of biofilms and the protection of living microorganisms in the biofilm from antimicrobial agents and the immune system[6]. Clinically important microbial biofilm growth may occur on various host tissues and medically-implanted foreign bodies, resulting in a variety of infections[7-9]. Bacteria in the biofilm matrix have a survival advantage, including protection from the host' s immune defense system of antibodies and phagocytic leukocytes, host-derived inhibitory substances, and antimicrobial agents that encompass antibiotics, disinfectants, and germicides[4,10]. Consequently, these biofilm infections tend to be chronic or recurring, even when formed by opportunistic bacterial pathogens[11]. For further reading, we suggest these excellent reviews[12-14].

MEDICALLY IMPORTANT BIOFILMS

Biofilm formation occurs on a variety of surfaces and can be either indwelling medical device-associated[15] or formed on native host tissues[16]. In most cases, formation of a biofilm on a medical device results in failure of the device, requiring removal of the device and/or debridement, which leads to significant morbidity and economic loss[17-19]. These devices include intravenous catheters, biliary and urinary stents, prosthetic heart valves, joint prostheses, peritoneal dialysis catheters, cardiac pacemakers, cerebrospinal fluid shunts, endotracheal tubes, breast implants and urethral catheters[20-24]. It has been estimated that 1.8 billion dollars per year are spent on treating orthopedic implant-related infections in the United States alone[25,26]. Most, if not all, implanted medical devices are susceptible to biofilm formation, as devices are coated by host matrix proteins, such as fibronectin and collagen, which serve as sites for adherence by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)[17,27-31].

In addition to being the cause of local infection, a biofilm on an implanted device may shed bacteria that produce abscesses in other locations or cause systemic infections[32]. The presence of a biofilm may result in blockage of an indwelling medical device, resulting in complication of treatment. Implanted tube devices may be clogged by biofilms, which necessitate their removal[24,33]. In addition to nosocomial and other infections associated with insertion of tubes, catheters and other devices, biofilms also are involved in causing atherosclerosis, sinusitis, otitis media, chronic wound infections, endocarditis, bronchopneumonia, urinary tract infection, cystic fibrosis, osteomyelitis, colitis, dental plaque and gingivitis[6].

Although a variety of microorganisms are involved in biofilm formation, the bacteria most frequently associated with biofilms are *Staphylococcus epidermidis* (*S. epidermidis*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), and *Enterobacteria*, such as *Escherichia coli* (*E. coli*)[24]. Other more frequently isolated bacteria, depending on the site of infection, are *Haemophilus influenzae*, *Burkholderia cepacia* (*B. cepacia*), *Enterococcus* spp., *Klebsiella* spp., *Proteus* spp., and *Helicobacter pylori*[24,34,35]. Other bacterial genera, including *Bacteroides*, *Clostridium*, *Finegoldia* and *Fusobacterium*, have been shown to form biofilms *in vitro*, and some have been isolated from clinical biofilms[6,36]. Regardless of their location and bacterial makeup, the bacterial populations in biofilms coexist and form a cohesive matrix, which allows them to survive and be protected from the detrimental effects of antibiotics and the immune system[37].

BACTERIAL BIOFILM DEVELOPMENT

The formation of a biofilm occurs in three sequential phases, each involving specific factors - irreversible attachment to the surface, growth and production of an extracellular matrix leading to a mature biofilm, and finally, detachment or dispersal[38]. Abiotic biofilm formation starts with planktonic bacterial cells that attach to the foreign body surface by reversible, specific or nonspecific adhesion[39]. The initial attachment is primarily governed by physicochemical forces, such as hydrophobicity and electrostatic forces, between the surface of attachment and the attaching microorganism[20,21,36]. In addition, bacterial appendages, such as flagella, pili, have been shown to be associated with attachment in the case of *P. aeruginosa*[40,41].

More than likely, the production of different bacterial cell wall-associated proteins that adhere to many of the host matrix proteins, either on tissue surfaces or on the surfaces of medically-implanted devices, plays a more important role in attachment[42]. Curli fibers which are proteinaceous extracellular compounds produced by many *Enterobacteriaceae* and belong to class of fiber called amyloids have been shown to be involved in the bacterial attachment and biofilm formation[43]. In *S. aureus*, the MSCRAMMs have been identified not only as important components of staphylococcal infection but also as adhesins for attachment to host tissues and/or foreign bodies covered with host matrix proteins[44,45]. Human plasma has been shown to enhance the expression of genes encoding these proteins in *S. aureus*[46,47]. These proteins include fibronectin-binding proteins[48,49], fibrinogen-binding proteins[50-52], extracellular matrix binding protein[53], protein A[54], and accumulation-associated protein[55-57].

Once attached, bacteria proliferate and produce an extracellular polymeric substance (EPS) matrix consisting, in the staphylococci, of polysaccharides[58], proteins[59], and extracellular DNA[60]. This EPS, also known as glycocalyx or slime[7,61], surrounds the cells and functions as an intercellular adhesin that leads to the formation of a microcolony, which is irreversibly bound to the surface. In the staphylococci, polysaccharide intercellular adhesin (PIA) or poly-N-acetyl glucosamine (PNAG) is usually the main component of the EPS[62-64], but recent studies indicate that the PIA/PNAG may be less important in methicillin-resistant *S. aureus* than in *S. epidermidis* and methicillin-susceptible *S. aureus*[65-67]. Other microbial components have recently been determined to be involved in biofilm maturation. These include extracellular DNA, which is hypothesized to be released from either small vesicles secreted from the outer membranes[68,69] or released by prophage-mediated cell death[70], in *P. aeruginosa*, or programmed cell death, in *S. aureus*[60,71]. However, the exact role is not known, as this has only been demonstrated under *in vitro* conditions. Other staphylococcal polymers that have been implicated in biofilm formation are the cell wall-associated teichoic acids[72,73]. In the Gram-negative bacterium *P. aeruginosa*, three different polysaccharides are produced[74-77].These are the glucose-rich Pel polysaccharide[78], the mannose-rich PSl polysaccharide[78], and alginate[79-81]. Alginate is a key component in the mucoid phenotype of *P. aeruginosa* and a contributing factor to chronic cystic fibrosis pathology[79,82]. Over time, as bacteria are surrounded by a much larger amount of EPS matrix, the biofilm continues to grow in thickness, and mushroom-like or column-like structures up to 10-100 μm thick have been observed *in vitro*[75]. Detailed analyses of mature biofilms show a heterogeneous structure, in which bacterial biomass exists in a polymeric matrix surrounded with water-filled spaces, thought to be essential for providing nutrients to the deeper layer of sessile bacteria but constituting only 15% of the total volume of the biofilm community[8,83]. It is at this stage of biofilm maturation that the cells are recalcitrant to antimicrobial agents[6].

Bacteria from a biofilm may be released and dispersed, which results in the spread of bacteria and the potential for the formation of new biofilms in other locations. Detached bacteria may produce other types of infections[84]. Dispersion could be either by the release of individual cells or aggregates of cells into the fluid or surrounding substances or by surface dispersal and movement of biofilm structures across a surface as in the case of motile bacteria[7,85]. The biofilm matrix formed in the laboratory may differ from those formed in the body, in which adherent bacteria in biofilms may be associated with molecules of host origin or with fungi[7].

Development of a biofilm may also be influenced by cell-to-cell communication and quorum sensing (QS), in which certain genes are activated when the concentration of bacteria in a given space in the environment reaches a critical level[86-89]. In the staphylococci, the primary QS system that has been most studied, with respect to biofilms, is the accessory gene regulator (Agr) system[90]. The Agr QS system is comprised of two divergent transcripts, one containing a four-gene operon (*agrA*, *agrB*, *agrC*, and *agrD*) that functions as a sensor/response regulator sensing a secreted self-made autoinducing peptide (the product of the *AgrD* gene). The autoinducing peptide is then sensed by the AgrAC two-component, trans-membrane transduction system that, in turn, generates the effector molecule, RNAⅢ. This effector molecule then regulates the expression of numerous cell wall-associated and secreted proteins in a temporal fashion[91]. In biofilms, activation of the Agr system negatively affects biofilm formation, as the Agr system downregulates many of the MSCRAMMs shown to be involved with adhesion and activates proteases, which are most likely responsible for biofilm maturation by degrading many of the proteins known to be involved with adhesion in staphylococcal strains independent of PIA for the formation of biofilms[92-94]. Also, in the staphylococci, the DNA-binding protein SarA is an important regulator of not only virulence gene expression but also biofilm formation[92,95-97]. SarA does so by Agr-dependent and independent mechanisms that contribute collectively to switching between planktonic and sessile lifestyles[92,95-97].

In Gram-negative bacteria, the QS molecules are N-acyl-L-homoserine lactones[86,98]. For example, in *P. aeruginosa*, there are three QS systems. The Pqs system senses a specific quinolone, referred to as the *Pseudomonas* quinolone signal, the Las system senses 3-oxododecanoyl-L-homoserine lactone, and the Rhl system senses N-butanoyl-L-homoserine lactone[99-101]. The activation of these systems may result in the production of extracellular polysaccharides,and a variety of enzymes, including virulence factors[99,100,102-104]. Collectively, these QS systems regulate the expression

of various genes in a coordinated fashion[105]. Natural and synthetic QS inhibitors are being considered for the treatment of infections caused by bacteria in biofilms[87-89,106].

In *P. aeruginosa*, the cyclic AMP signaling, in addition to regulating other genes, controls biofilm formation, alters cell surface hydrophobicity and signals irreversible attachment[107]. *In vitro* experiments with *E. coli* have shown crosstalk between QS and hormones from the host. Yang *et al*[108] showed that in *E. coli* the *qseC* gene regulates the histidine kinase gene, which senses epinephrine/norepinephrine hormone and stimulates biofilm formation, and found that the addition of these hormones increases the thickness of biofilms. The biofilm thickness and ureolytic activity of *Proteus mirabilis* were shown to increase with the QS signal molecule N-butanoyl-L-homoserine lactone[109].

Other factors that have been shown to be involved with biofilm maturation and dispersal include surfactants. For example, a surfactin is found in *Bacillus subtilis*[110,111] and, in *P. aeruginosa,* a rhamnolipid has been shown to contribute to biofilm maturation[112,113]. In the staphylococci, a group of amphipathic, alpha-helical peptides with surfactant activity, known as the phenol-soluble modulins, have been shown to be involved in biofilm maturation[114,115].

CHARACTERISTICS OF BACTERIA IN BIOFILMS

Biofilm-grown bacteria have different properties from those of free-living bacteria, which affects the diagnosis and treatment of infections caused by biofilms. The gene expression profile in a staphylococcal biofilm is considerably different from the profile of a planktonic culture[96,116,117]. Genes for arginine deaminase and urease are upregulated, probably to maintain a pH homeostatic environment, due to anaerobic growth that results in the formation of acidic by-products. In addition, Scherr *et al*[118] recently reported a significant reduction in gene expression when an *S. aureus* biofilm was exposed to macrophages, but very little change in the biofilm transcriptome when exposed to neutrophils; these are two important cellular components of the innate immune response. An increase in pyrimidine nucleotide biosynthesis is also involved in staphylococcal, *E. coli* and *Streptococcus pneumoniae* biofilm formation[119,120].

Biofilms are formed by heterogeneous bacterial aggregates surrounded by a self-produced matrix, which also may contain host constituents[6]. The physiological heterogeneity of bacteria in biofilms enables the subpopulation of bacteria with specialized activities to tolerate the hostile environment and survive[13,121]. Study of *in vitro* biofilms has shown that the concentration of oxygen may be higher at the surface of a biofilm and lower in the center[122,123]. In *P. aeruginosa*, the oxygen is depleted in mucoid macrocolonies, and in cystic fibrosis, this disease-causing bacterium functions in an anaerobic environment[123,124]. Similarly, there are differences in the concentrations of nutrients and chemicals at the surface and in the center of microcolonies[125,126]. As a result, growth, metabolic activities and protein synthesis are reduced at the center of a biofilm and are higher at the surface[125,126]. There is population diversity within the biofilms; some cells have slow or no growth and other cells are in stationary phase[123,126-129]. There may be also phenotypic variants of regular cells, called persisters, which have reduced cellular activity, are non-growing or dormant, and are tolerant to antimicrobial agents[130-134]. This state of cell growth, which reduces its susceptibility to antimicrobial agents and contributes to relapsing and chronic infections[135], will be discussed later. Also, there is a higher rate of mutation in growing biofilm bacteria, in comparison with free-living bacteria, including mutations in the DNA repair genes[121,136]. Low nutrients, including the lack of some amino acids, and stress responses result in tolerance of *E. coli* biofilms to ofloxacin[121]. The dense population of cells in a biofilm facilitates plasmid transfer, and the frequency of gene transfer is increased in the bacterial population in a biofilm[137]. *In vitro* study of *P. aeruginosa* has shown genetic differences between the planktonic and surface-attached bacteria, which results in differences in biochemical and phenotypic properties, and specific mechanisms to avoid the bactericidal action of antibiotics[138].

FACTORS AFFECTING ANTIMICROBIAL RESISTANCE IN BACTERIAL BIOFILMS

The colonization of natural surfaces or medically implanted devices with biofilm-forming bacteria results in the infection of local and surrounding tissues, which if not treated, may result in systemic infection, require prolonged treatment with antimicrobial agents, and may require the removal of infected devices[6,7,12,13]. The bacteria forming a biofilm could be 10-1000 times more resistant to antimicrobial agents than planktonic cells, even when they are formed by *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, *S. epidermidis*, *S. aureus*, and *Enterococcus faecalis*, which are commensal bacteria[6,139]. Various factors contribute to the recalcitrant nature of these bacteria. Vancomycin used for the treatment of methicillin-resistant *S. aureus* was shown to be effective only against planktonic cells, not biofilm cells[135,140].

The mechanisms of resistance to antimicrobials, such as antibiotic-modifying enzymes and efflux pumps, that have long been established for planktonic bacteria, are only marginally involved in resistance of bacteria in a biofilm[141,142]. The role of exopolysaccharides in restricting antimicrobial penetration, which was originally thought to be the key to biofilm resistance, remains uncertain. Additional factors contributing to antimicrobial resistance in biofilms include specific

genetic determinants, such as the *ndvB* gene, which codes for a glycosyltransferase required for the synthesis of a cyclic-β-(1,3)-glucan, which is found in the periplasmic space of *P. aeruginosa* and is thought to be involved with antibiotic sequestration[143]. Given the dynamics of a biofilm lifestyle with cells at various stages of growth, it is not surprising that antimicrobial resistance of a biofilm is multifactorial.

PENETRATION RESTRICTION

Insufficient exposure of bacteria to antimicrobial agents, because of the limitations of transport of antimicrobial agents to the bacteria in a biofilm, contributes to the lack of antimicrobial effectiveness in eradicating bacteria in the biofilm[144]. The exopolysaccharides, proteins and extracellular materials that form the biofilm matrix may prevent the penetration of antimicrobial agents to various layers of the biofilm where the bacteria are vulnerable[144]. The exopolymer matrix of a biofilm may also restrict penetration of antimicrobial agents by binding to the compounds and preventing their diffusion[6]. Reversible or irreversible binding of antimicrobial agents to biofilm components retards antibiotic transport to cells within the biofilm[145]. Gentamicin was shown to penetrate *E. coli* biofilms but not the biofilm with *P. aeruginosa*, which has negatively charged polysaccharides that can bind to gentamicin[146]. Bacterial species and the age of the biofilm affect the retardation of antimicrobial agents[146]. Also, exopolysaccharides and other components of the matrix may present a barrier preventing certain compounds from entering the biofilm and not others[147].

The charge of the matrix also affects diffusion of some antimicrobial agents, and this diffusion barrier is specifically effective against large molecules[6,148]. Aminoglycosides are positively charged and may bind to the negatively charged exopolysaccharide matrix of a biofilm, restricting access to the live cells in the biofilm[149]. Tseng *et al*[150] showed that in *P. aeruginosa* the penetration of tobramycin is limited by the presence of an extracellular matrix.

Some antimicrobial agents, including fluoroquinolones, readily equilibrate across a biofilm and have been shown to be effective. A *P. aeruginosa* biofilm was shown to prevent the penetration and diffusion of piperacillin[151]. However, a biofilm formed by *S. epidermidis* did not prevent the diffusion of vancomycin and rifampicin across the biofilm, indicating that biofilm resistance to antimicrobial agents is not solely because of lack of penetration[152].

Restricted diffusion also may result in the enzymatic degradation of some antimicrobial agents by enzymes produced by the bacteria in the biofilm. The combination of retarded diffusion and enzymatic degradation of antibiotics has an additive effect in rendering antimicrobial agents ineffective for the treatment of bacteria in a biofilm[131,153]. It appears from these reports that whether or not an EPS can serve as a suitable permeability barrier to antibiotics and disinfectants[145,154] depends upon the antimicrobial agent in question and certainly also on the chemical composition of the EPS that surrounds the microorganisms.

INACTIVATION OF THE ANTIMICROBIAL AGENTS

Inactivation of antimicrobial agents by extracellular enzymes also has been suggested to be a contributing factor in the inefficiency of antimicrobial agents in eradicating the cells in biofilms. Extracellular enzymes, like β-lactamases, have been suggested to be involved in the ineffectiveness of β-lactam antibiotics[153]. However, the extent of their involvement in rendering antibiotics ineffective for clinically important biofilms is not known. Vrany *et al*[155] showed that ciprofloxacin and levofloxacin are transported into the *P. aeruginosa* biofilm. Anderl *et al*[147], using ampicillin and ciprofloxacin in biofilms formed by a strain of *Klebsiella pneumoniae* that produced β-lactamase and its mutant lacking β-lactamase, showed that resistance to these antibiotics is not the result of slow diffusion or antibiotic inactivation, and suggested other mechanisms for the resistance of biofilms. In clinical isolates, recurrent infections by *P. aeruginosa* result from persisters, which develop tolerance to a host of antibiotics under a variety of growth conditions and will be discussed further below[156].

GROWTH RATE AND PRESENCE OF PERSISTERS

All antimicrobial agents are more effective in killing rapidly growing cells; decreased growth rates reduce the efficacy of antimicrobial agent killing[131,132]. Several subpopulations of *P. aeruginosa* found in biofilms have different susceptibilities to antimicrobial agents. During treatment, antimicrobial agents preferentially kill the metabolically active cells in biofilms. However, the less active dormant cells that are covered with various substances within the biofilm layers are protected[148]. Considering the heterogeneous nature of microbial subpopulations in a biofilm, multiple resistance mechanisms may be involved in the protection of the different subpopulations[157]. Treatment with a single antimicrobial agent, while bactericidal for part of the population, is not enough to eradicate the infection. In addition to slow growth, induction of stress response genes also could contribute to resistance of bacteria in biofilms[132]. The physicochemical structure of the biofilm components also may eliminate the biocide from the microbial community. Gilbert *et al*[158] showed that sensitivities of *P. aeruginosa*, *E. coli* and *S. epidermidis* in planktonic cultures to tobramycin and ciprofloxacin increased with increasing rates of growth, but the

slow rate of growth of cells in a biofilm protected the cells from antimicrobial action. However, although both planktonic and biofilm cells of *P. aeruginosa* are resistant to ciprofloxacin at slower growth rates, only the planktonic cells of *P. aeruginosa* become more susceptible to ciprofloxacin when the growth rate increases[159]. Other factors in the biofilm, in addition to the slower rate of growth, must contribute to antimicrobial resistance. Desai *et al*[160] found that *B. cepacia* cells in biofilms were 15 times more resistant to antibiotics than planktonic bacteria, and that the growth phase and mode of growth affect the susceptibility of *B. cepacia* to antimicrobial agents. Factors affecting resistance may differ for different antibiotics[161]. Low nutrients, including lack of some amino acids, and stress responses result in tolerance of biofilms to ofloxacin[121].

The majority of cells in a biofilm are killed within the clinically achievable concentration range of antimicrobial agents; however, after the initial 3-4 log drop in the bacterial number, further addition of antimicrobial agents has no effect on bacterial killing[131]. This indicates that a small fraction of the cells persisting in the biofilm are the source of antimicrobial resistance and account for the failure of antimicrobial agents to eradicate bacteria in the biofilm[135].

It is now thought that the main contributor to increased antimicrobial resistance of biofilms is a subset of cells known as persisters[131,162]. By definition, persisters are "small subpopulations of bacteria that survive lethal concentrations of antibiotics without any specific resistance mechanisms"[162]. These bacteria represent a small percentage (0.1%-10%) of the entire population and appear to be the product of a non-heritable phenotypic switch rather than a result of antimicrobial pressure[130,163].

Biofilm exopolymers shield bacteria from the assault of immune factors. During therapy with antimicrobial agents, most of the planktonic cells shed from a biofilm are eliminated and only a small fraction of persisters remains after the symptoms disappear and treatment is discontinued[131,132]. The persisters eventually start shedding new planktonic cells, resulting in the relapse of symptoms. This dynamic cycle of decrease in planktonic bacteria and bacterial shedding from existing biofilms explains the need for lengthy antibiotic therapy and continuous recurrent infection following termination of the use of antimicrobial agents[131]. Although the persisters are not necessarily resistant to antimicrobial agents, being shielded from the effect of the immune system enables them to survive and initiate recurring infections. The persisters cause recurrent meningitis by *S. pneumoniae* and *Helicobacter pylori,* both of which are shielded from the immune system, and cause relapse of infections following therapy[132]. Persisters in a biofilm can be assayed using antimicrobial agents. If the biofilm restricts penetration of antimicrobial agents across the exopolymers, the bulk of bacteria in the biofilm should survive drugs like aminoglycosides, which have restricted penetration and diffusion, and be killed by fluoroquinolones, which can diffuse and penetrate into a biofilm[131].

Both *in vitro* and *in vivo* studies of Gram-positive and Gram-negative bacteria support the theory of persisters in biofilms[37,164-166]. The difficulties in the eradication of persisters in biofilms have been shown for different bacteria and antimicrobial agents[133,135,167,168]. After exposure to a high concentration of ciprofloxacin, a small number of *E. coli* cells insensitive to this drug remain in a biofilm[131,169]. Similarly, the effects of amoxicillin and clindamycin on *Lactobacillus acidophilus*, and those of erythromycin and metronidazole on *Gardnerella vaginalis,* reach a plateau in a biofilm after initial bacterial killing[170].

From cystic fibrosis patients, a high-persister mutant of *P. aeruginosa* has been isolated. Its presence has been speculated to be the main reason for the recalcitrant nature of this disease to antimicrobial therapy[132]. In *E. coli*, the stress response results in the formation of persisters. The stress response activates the overexpression of TisB, which is a membrane-acting dipeptide, and decreases the ATP level and proton motive force in the cell, leading to cell dormancy and persister formation[132]. Persisters also occur in planktonic cells; most of what is known of persisters has been done with planktonic cells[132]. Whether biofilm bacteria produce more persisters than planktonic cells is not known; however, planktonic cells are subject to elimination by the immune system[6,132].

Clearly, the generation of persisters is multifactorial and most likely involves environmental cues and the expression of genes in response to those cues. It is beyond the scope of this review to discuss all of these factors. For an in-depth discussion, the reader is encouraged to peruse the recent review by Lebeaux *et al*[14]. Many factors have been shown to be involved in the generation of persisters, such as nutrient limitations and the induction of the stress response and stringent responses. However, what is known of persisters from planktonic growth conditions does not necessarily explain the occurrence of persisters under biofilm conditions[121]. Because the environmental cues are numerous, the response to such cues involves several molecular mechanisms and pathways that are overlapping and may actually "cross-talk". Given the dynamic community of a biofilm, the generation of subsets of persisters is essentially endless. While, in general, the phenotypic switch that results in the formation of persisters is transient, exposure of these subsets of cells to continuous antibiotic treatments may lead to a greater genetic diversity with time due to mutational changes. In the staphylococci mutability in the biofilm lifestyle is significantly higher than that in the planktonic lifestyle[171].

In addition to persisters and the ability to hyper-mutate, many Gram-positive and Gram-negative bacteria form small-colony variants (SCV)[172]. These include *S. aureus*[173], methicillin-resistant *S. aureus*[174],

S. epidermidis[175], and *P. aeruginosa*[176]. All of these bacteria are also known to be involved in biofilm-associated infections, and they have been the most studied. This is rightfully so, as *P. aeruginosa* is notorious for causing severe chronic infections in patients with cystic fibrosis[177,178]. The staphylococci represent the leading cause of hospital-acquired infections on indwelling medical devices[179,180]. Both *P. aeruginosa* and *S. aureus* also coexist in the lungs of cystic fibrosis patients and in chronic wounds[181,182]. These same bacteria have also been isolated exhibiting the SCV phenotype in clinical samples that include blood, abscesses, skin and soft tissues, bones and joints, and the respiratory tract[172].

Whereas many alterations in metabolic activity can yield small, slow-growing colonies[183], thus far only a limited number of defects have been associated with clinical strains[172]. In clinical isolates of the staphylococci, the primary determinants of SCV are a reduction in electron transport and thymidine biosynthesis[172,184]. These determinants have been linked to defects in menadione, hemin, and thymidine biosynthesis by using staphylococcal laboratory strains and generating mutations within the *men, hem,* and *thy* operons, respectively[172]. However, a recent study examining the whole genomes of five different clinical isolates of *S. aureus* (four were either hospital-acquired or community-associated United States 300 MRSA strains) demonstrated that all five contained a single-nucleotide polymorphism in one of the menadione biosynthesis genes, specifically *menC*, *menE*, or *menF*[185], thereby identifying the genetic basis for the staphylococcal SCV phenotype.

In addition to exhibiting a small colonial, slow-growing morphology, SCV cells of *S. aureus* produce less pigmentation and exhibit a reduction of hemolytic and coagulase activity as well as other virulence factors. The SCV are slow-growing and tend to have increased resistance to antibiotics, which is strikingly similar to bacteria growing in a biofilm, especially when one considers that the diseases with which SCV and biofilms are most closely associated (endocarditis, pneumonia, soft-tissue infection and osteomyelitis) are persistent, recurrent, and tend to be resistant to most classes of antibiotics[186-188]. The SCVs of *S. epidermidis* and *S. aureus* have an increased ability to form biofilms, which is partly due to increased expression of PIA[189,190]. Similarly, SCVs of two different strains of *S. pneumoniae* were shown to emerge in the initial stages of biofilm formation[191,192]. These SCVs had increased attachment capabilities to solid surfaces and formed mature, three-dimensional[191,192] biofilm structures but had decreased capsules[191,192]. Likewise, in *P. aeruginosa*, both an increase in hydrophobicity[193,194] and EPS production[195,196] have been observed in SCV.

The clinical and laboratory standards institute (CLSI) performance standards for antimicrobial susceptibility testing have recommendations (Fifteenth informational supplement. CSLI/NCCLS document M100-S15.2005) for measuring the susceptibility of bacteria in a biofilm, quantified as the minimal biofilm eradication concentration (MBEC). After exposure to antibiotics, samples are transferred to fresh medium. The MBEC value is the lowest concentration of an antibiotic that prevents regrowth. The recommended MBEC for an antibiotic is defined as the concentration of antibiotic that causes a > 99.9% drop in cell number.

In a clinical setting, increased production of persister cells in biofilms is one reason for the recurrence of infection following discontinuation of treatment[132,135]. Although the small population of persister planktonic cells will be eliminated by the immune system following antimicrobial treatment, antibiotics are not effective for eliminating the persisters in a biofilm because they are protected by the biofilm matrix. The remaining persisters are involved in regrowth after cessation of antimicrobial treatment[135].

Bacteria with serious defects do not survive but undergo programmed cell death (PCD). It is hypothesized[131,197] that persisters may have disabled their PCD to allow survival of a few cells if the antimicrobial agent reaches the whole population. Lewis[131] hypothesized that production of persisters by bacteria is a lifestyle for bacterial perseverance. Persisters survive challenge by factors that kill planktonic bacteria; the rate of development of persisters is 10-10000 fold higher than the rate of development of mutants[197]. This process of variation in lifestyle insures cell survival.

OXIDATIVE STRESS

Differences in the expression of phenotypes between planktonic cells and cells in biofilms may also include differences in sensitivity to antimicrobial agents. In a mature biofilm, bacteria have slower growth than those in the planktonic state[4]. Changes in growth rate in the biofilm, which may be accompanied by limitation in nutrients, may affect the components of the bacterial cell envelope. Both growth rate and low nutrients affect antibiotic efficacy and tolerance[121,198-200]. The constituent of proteins, polysaccharides, extracellular enzymes, fatty acids, phospholipids and metal cations in the bacterial cell envelope are affected by low nutrients, which in turn affect the bacterial susceptibility to antimicrobial agents[121,200,201]. By growing in an environment with lower nutrients, the bacteria also avoid oxidative stress, which results from the effect of bactericidal antibiotics, the generation of harmful reactive oxygen species (ROS) and the production of cytotoxic hydroxyl radicals (•HO), which damage cellular macromolecules[202].

The interactions of β-lactams, fluoroquinolones and aminoglycosides with the target cells induces •HO formation in bacteria. It is suggested that the production of •HO depends on the activity of the tricarboxylic acid cycle (TCA)[12]. Drug-target interaction results in the oxidation of NADH that is produced during the TCA

cycle. Conversion of NADH to NAD^+, which generates ATP, results in the production of superoxide (O_2^-). The superoxide damages the iron-sulfur cluster in proteins and releases Fe^{2+}. The released Fe^{2+}, in combination with H_2O_2, produces highly reactive •HO, which is damaging to macromolecules. The production of •HO is prevented in the bacteria in biofilms that have low metabolic activities[12]. Roles of oxidative stress and •HO in cell death have mainly been established in planktonic cells. However, Battán *et al*[203] showed that for the induction of ROS production in *Pseudomonas* in biofilms, a higher concentration of piperacillin and ceftazidime is required than for planktonic cells. Also, although ciprofloxacin induces ROS production in *P. aeruginosa* biofilms, in the areas of biofilms with low metabolic activities, •HO production is prevented[202]. A study of the effect of the aminoglycoside tobramycin on a *Burkholderia cenocepacia* biofilm showed that although the production of ROS increased in treated cells, 0.1% of the cells survived the treatment[204]. The genes for the glyoxylate cycle, which allows the cells to utilize simple carbon compounds as carbon sources rather than glucose, were upregulated in the surviving cells in comparison with the untreated cells of a biofilm[204]. However, the genes for the TCA cycle and electron transport were downregulated, avoiding the production of reactive oxygen intermediates. Similarly, low number of persisting cells were produced in catalase mutants[204]. Khakimova *et al*[205] showed that the stress response in *P. aeruginosa* regulates catalase, which is important in protecting biofilm bacteria from antibiotic-mediated killing.

Biofilm bacteria are also exposed to ROS from activated polymorphonuclear leukocytes. If there is a deficiency in the antioxidant system, the production of ROS increases in a biofilm[206]. The increase in oxidative burden and decrease in antioxidant defense results in oxidative stress in biofilms[6,178]. The oxidative stress affects the bacterial DNA break repair mechanism and is a contributing factor in the increased mutability of bacteria. This may lead to the diversity and adaptability of a biofilm community[127]. Boles *et al*[127] showed that in *P. aeruginosa*, endogenous oxidative stress in biofilms promotes antimicrobial resistance and that the addition of antioxidants reduces the occurrence of diversity.

EFFLUX PUMP

Induction of the biofilm phenotype is another suggested reason for the resistance of bacteria in biofilms to antimicrobial agents. It is hypothesized that a sub-population of bacteria in the biofilm expresses an active mechanism to avoid the bactericidal effect of antimicrobial agents[157,207,208]. The multidrug-resistant efflux pump MexAB-OprM may be involved in the resistance of *P. aeruginosa* to ofloxacin, and biofilms lacking this pump are more susceptible to ofloxacin[103,161], but resistance of *P. aeruginosa* to ciprofloxacin is not attributed to this pump[161]. Another efflux pump reported in *P. aeruginosa* is PA1874-1877, which confers resistance to ciprofloxacin, gentamicin and tobramycin[209]. Other efflux pumps in biofilms that contribute to drug resistance are *MacABCsm*, an ABC-type tripartite efflux pump in *Stenotrophomonas maltophilia,* which also is involved in oxidative stress response and biofilm formation[210].

In *E. coli*, acrAB (AG100-B) protects biofilms from 0.004 mg/L, but not 0.1 mg/L, of ciprofloxacin, indicating that biofilm resistance to ciprofloxacin in *E. coli* is not the result of multiple antibiotic resistance operons (*mar*) and the multidrug efflux pump *acrAB*[10]. In *E. coli*, a putative multidrug resistance pump, yhcQ, may be responsible for resistance to penicillin[211]. In *E. coli*, the *emrD*, *emrE*, *emrK*, *acrD*, *acre* and *mtd* genes, which encode the proton motive force multidrug efflux pump, also contribute to the formation of biofilms[212]. In *Salmonella enterica*, serovar Typhimurium there is a link between the multidrug resistance efflux pump and biofilm formation[213]. In the mutants that lack a functional multidrug resistance efflux pump AcrB and TolC the transcription of proteinacous materials of amyloid class (curli) biosynthesis is repressed[213].

Inactivation of the NfxB negative regulator of the MexAB-OprM efflux pump affects the resistance mechanism of *P. aeruginosa* in a biofilm[214].

OTHER FACTORS

In vitro study of *P. aeruginosa*, *E. coli* and *S. epidermidis* has shown genetically based differences between planktonic and surface-attached bacteria, which results in differences in biochemical and phenotypic properties. The bacteria in a biofilm use a specific mechanism to avoid the bactericidal action of antibiotics[138,215]. Mah *et al*[138] detected a periplasmic glucan produced from the *ndvB* locus in the biofilm-forming, antimicrobial-resistant strains that interacted with tobramycin. They suggested that the interaction of the glucose polymer with tobramycin may prevent the antibiotic from reacting with the target.

A biofilm-specific *BrlR* gene product, contributes to a high level of drug tolerance in *P. aeruginosa*[216]. BrlR has similarity to the MerR family of transcription regulators, which function as multidrug transporter activators. MerR transcription regulators activate the expression of multidrug transporters in *B. subtilis* and *Streptomyces lividans*[216]. In *P. aeruginosa*, BrlR activates the multidrug efflux pump operons *mexAB-oprM* and *mexEF-oprN*, which are involved in resistance to a variety of antibiotics[216].

Other genes are also implicated in conferring resistance to *P. aeruginosa* in biofilms. Gupta *et al*[217] found that in *P. aeruginosa*, biofilm development and tolerance to antimicrobial agents are linked. They found a two-component hybrid, SagS that regulates the transition attachment of *P. aeruginosa* during biofilm development and also regulates the tolerance to

antimicrobials. The inactivation of SagS resulted in an increase in susceptibility of biofilm cells to bactericidal compounds, more than that of the planktonic cells, and also contributed indirectly to *BrlR* activation. *SagS* functions upstream of *BrlR*, so its inactivation correlates with reduction of the level of BrlR in biofilms[217]. The activities of the QS system also appear to affect antimicrobial resistance in some bacteria. Unlike their wild types, QS deficient mutants of *P. aeruginosa* are susceptible to kanamycin[218]. By *in vitro* quantitation of biofilm formation in wild type and three QS deficient mutants of *P. aeruginosa*, they showed that the quorum sensing Las system plays an important role, not only in biofilm formation thickness, but also in the production of *ampC* lactamase[219]. A further complication of biofilm infections is the potential for horizontal gene transfer[220]. Both conjugation and plasmid transformation have been shown to occur at a higher frequency in *E. coli in vitro* biofilms[221-223]. Clinically speaking the serious implications of such transfer are the generation of either more virulent bacterial strains and/or bacterial strains having acquired antibiotic resistance determinants. In addition, biofilms and horizontal gene transfer may very well promote and maintain an environment for bacterial heterogeneity. In addition, biofilm may also provide a communal environment where transformation of mobile genetic elements is more conducive not only between cells of the same species but between cells of different species, thus providing a mechanism of evolutionary change[220].

CONCLUSION

Clinically important microbial biofilms are formed on host tissues and medically-implanted devices, resulting in a variety of hard-to-treat infections. Biofilm formation is a major health problem, as microorganisms within biofilms are difficult to eradicate with conventional therapeutic treatments. Bacteria in biofilms persist under less than the optimal conditions that would be required for growth and survival of planktonic bacteria. The sessile bacteria in a biofilm are embedded in a self-produced extracellular matrix that shields them from the host's immune system. The population of bacteria in a biofilm exhibits a heterogeneous physiology that enables the sessile community to survive environmental stresses and allows them to escape the bactericidal activities of antimicrobial agents. After termination of treatment with antimicrobial agents, biofilms may shed bacteria and cause recurrent infections. A variety of mechanisms have been investigated in the last several decades to elucidate the reason for the recalcitrant nature of bacterial biofilms; in this literature review, we have attempted to highlight some of these mechanisms. Various studies have implicated as possible causes the inaccessibility of antibiotics to bacteria because of the protective matrix, the production of antimicrobial-degrading enzymes and efflux pumps, and the lack of oxidative stress. Some of these mechanisms are known to be involved in resistance in planktonic cells. Other studies have shown hypermutability of the cells, as well as the existence of persisters and small colony variants that are characteristic of biofilms of several clinically important bacteria, as contributors to the recalcitrant nature of biofilms to high concentrations of antimicrobials. The reason for the recalcitrance appears to be multifactorial, which challenges the development of strategies for the prevention and treatment of biofilm related diseases.

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