Biofilms in Medicine

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BIOFILMS: AN INTRODUCTORY OVERVIEW

In 1862 Louis Pasteur introduced the "Germ Theory of Disease." Subsequently the study of microbiology has flourished greatly and its medical significance has continuously grown. Many microbial organisms implicated in disease have been identified and studies. A general science of medical bacteriology has been determined and is widely taught. Such study has been largely based upon the activity of individual free-swimming (planktonic) cells and colonies that they form. However, microorganisms often form communities called biofilms which can have properties that very different from their planktonic predecessors.

Biofilms are mucoid aggregates of microorganisms which tend to grow on surfaces exposed to water. Biofilms are not the only form of microbial cell aggregate but are distinguished from other aggregates by specific properties. Another form of microbial aggregate is the familiar bacterial colony. Colonies tend to feed on their undersurface and utilize the gaseous surface above for gas exchange; they are usually clones of a single preceding cell (Wimpenny 2000). Biofilms are characterized by their locations at phase boundaries (ibid.) and their production of Extracellular Polymeric Substance or EPS. The sliminess of biofilms is due to their enveloping EPS matrix. The phase boundary at which biofilms generally form is Solid: Liquid (there are a few examples of biofilms growing at other phase boundaries but they tend to have industrial or environmental applications). An additional characteristic of biofilms which is

not generally cited in their description is the strong alteration of their cell physiology from that of planktonic cells (Donlan and Costerton 2002).

Biofilms grow on many surfaces, in many environments, and with many different effects. The most common example of a biofilm is dental plaque; however biofilms also grow on many other natural and artificial surfaces within the body for which they have been implicated in many diseases. Biofilms are also prevalent on household surfaces such as cutting boards, counters, toilets, sinks, tubs, and the interior of pipes.

Although biofilms appear to serve only adverse functions, they have many positive users in the environment and industry. For example, biofilms are used in water treatment filters to metabolize organic substances that are within the water. This strategy has been shown to decrease microbial proliferation downstream (Cunningham 2007). Biofilms are also used in "Bioremediation" activities in which they are utilized to metabolize toxic materials that contaminate soil or water into safe (possibly beneficial) byproducts (ibid).

Biofilms tend to have different medical implications than do planktonic cells and thus require different treatments. Treating biofilm infections requires an understanding of biofilms. To truly understand the effects of biofilms we must take a deeper look at their structure and

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physiology.

THE SCIENCE OF BIOFILMS

It is initially important to note that although we may investigate a variety of controlled laboratory-grown biofilms of defined compositions, the biofilms found in natural environments have highly heterogeneous compositions comprised of numerous bacterial species as well as fungal organisms. The composition of a biofilm changes dynamically as some organisms and organic material are incorporated from the surroundings and some are emitted to the surroundings (Wimpenny 2000). The composition is regulated by a complex variety of genetic and environmental factors.

FORMATION AND GROWTH

Ironically, biofilms have been shown to form more easily in high shear environments (where high mechanical pressure, such as the flow of fluid, is applied to the biofilm). Additionally, the biofilms that form in high shear environments are much stronger than those in low shear environments. Research also shows that biofilms form as easily on smooth surfaces as they do on rough surfaces (Donlan and Costerton 2002).

Busscher and van der Mei depict biofilm proliferation in eight semi-sequential steps (Busscher and van der Mei 2000).

1) Deposition of a conditioning film on the substratum surface
2) Mass transport of microorganisms to the substratum
3) Initial adhesion of microorganisms to the substratum

4) Co-adhesion to attached microbes
5) Anchoring by appendages and polymers to the substratum
6) Co-aggregation of planktonic microbes
7) Growth of the biofilm
8) Detachment of biofilm material.

Each of these steps is an independent phenomenon worthy of great research and description.

Colonization by microorganisms is always preceded by the development of a conditioning film of macromolecules that absorb to the substratum surface. Conditioning films form due to the diffusive and ubiquitous properties of macromolecules. The composition of a conditioning film is related to the chemical properties of the substratum surface and the macromolecules within its surroundings. Examples can include salivary matter on dental surfaces, tears on contact lenses, and urinary components on a catheter surface (Ibid).

There are a variety of methods by which microorganisms reach a substratum; these include passive forms of transport such as Brownian motion, convective transport (movement by fluid), and sedimentation (due to difference in the specific gravity of the microbes and the mass fluid surrounding them), as well as active (flagellar) transport. Current research indicates that chemotaxis mediated active transport is not a factor, yet there is only limited research on such a premise (Davies 2000).

The initial adhesion of microorganisms is by van-der Waals forces while there is even some repulsion due to the corresponding negative electrostatic charges of the substratum and microbial surfaces. Cellular motility appears to be the force that counteracts such repulsion (Davies 2000). Other early factors can be acid base interaction as well as hydrophobic interaction. The degree of relative hydrophobicity between the cell, the substratum and surrounding liquid has been shown to influence initial attachment (Ibid.). This initial adhesion is quite reversible, however it becomes stronger as water is removed from between the interacting surfaces.

Microbial adhesion becomes irreversible when the cells are anchored by EPS polymers and/or cellular appendages such as pili. These macromolecular structures adhere by dipole, ionic, hydrogen bond, and hydrophobic interactions. Research indicates that phenomena such as the secretion of EPS and the protrusion/extension of cellular appendages are activated in response to surface association (Davies 2000).

Adhesion throughout a biofilm is apparently a common theme in the science of biofilms. Two predominant styles of cell-to-cell adhesion are seen in the development of a biofilm; Coadhesion and Coaggregation. Coadhesion refers to the binding of a planktonic cell to a biofilm cell. Coaggregates are planktonic aggregates of microbial cells which can be incorporated as units into biofilms by binding the substratum or by Coadhesion (Kolenbrander et al. 2000).

Following the “early events” of biofilm formation, the biofilm proliferates primarily by cellular growth of the biofilm cells. Research has shown that substrata-attached *Pseudomonas* reproduce at an extremely slow rate, with a generation time inversely relates to the attachment strength, indicating that growth is not an early event (Busscher and van der Mei 2000).

An essential step in the biofilms development cycle is detachment. Biofilms are subject to numerous physical and chemical forces many which can disrupt cell-to-cell interactions causing

detachment by part of the biofilm. If the interaction between the substratum and biofilm cells is broken, complete detachment occurs (Ibid.).

Microbes throughout nature display defense mechanisms towards their competition. Early colonizers of biofilms have been shown to prevent further colonization by other species through the secretion of specific bio surfactants which alter the chemical or physical properties of the surface in ways which prevent attachment of the undesired species (Ibid.).

STRUCTURE OF BIOFILMS

Prior to the use of Confocal Lens Scanning Microscopy (CLSM) in biofilm study, there were many misconceptions of biofilm structure. Biofilms were thought to be homogeneous unstructured accumulations of bacteria on surfaces. CLSM provided accurate images of unprepared live biofilms which contradicted these early beliefs. Prior study had been with electron microscopy which requires dehydration of samples (Donlan and Costerton 2002).

Dispute had raged over the correct biofilm structure with three common models observed. Some believed biofilms to be irregular branched or simple stalked structures, others believed biofilms to be fairly flat and homogeneous structures while others argued that biofilms are composed of mushroom or tulip shaped structures which are internally accessed by pores. Subsequent study has indicated that all three models are correct and depend on the available nutrient resources. The first model had been found in water distribution systems, which are low nutrient environment. The second model appears where nutrient concentration is very high e.g. the human body. The third model is observed in laboratory growth in which media containing moderate nutrition is utilized. Both presence of specific substrates and concentration of those present relate to a biofilm's structure (Wimpenny 2000).

As previously described, biofilms in nature are composed of multiple species and mutants with structural and physiological properties that can be harnessed for the entire "community". Under certain growth conditions P. *aeruginosa* biofilms have been shown to form mushroom shaped structures. Research has shown that the stalks of the mushroom are formed of a specific population and the mushroom caps are formed by a motile subpopulation which travels up the stalk to reside atop it (Parsek and Fuqua 2004).

Biofilms are composed of approximately 15% cells and 85% matrix. The structural units of a biofilm are microcolonies; these structures feature many of the biofilm implied properties such as quorum sensing, antimicrobial resistance, and detachment. Water channels flow between microcolonies of sessile cells. Microcolonies of biofilms in environments with high shear forces have been shown to assume tadpole shapes which oscillate in the bulk fluid. Interestingly, detachment of microcolonies can have dire medical results as they can travel in the planktonic manner while retaining such properties as antimicrobial resistance (Donlan and Costerton 2002).

Considering that the matrix content of biofilms dwarfs that of cells, understanding the EPS which forms the matrix is of great importance.

The abbreviation of EPS has remained a staple term in biofilm study, however it has multiple long forms associated with it. They include extracellular polysaccharides, exopolysaccharides, exopolymeric substances, exopolymers, and extracellular polymeric substances (Flemming et al. 2000).

Although confusion in determining the correct long form of the term "EPS" may be quite trivial, there is practical confusion in determining the composition of EPS. This is primarily due to the dynamic properties of this substance. The composition of EPS differs based on which organisms are present, and by their surrounding environment (Parsek and Fuqua 2004).

While polysaccharides such as alginate were believed to be the primary components of EPS, many studies have shown proteins and nucleic acids to prevail in quantity. In addition to polysaccharide, protein and nucleic acid components, EPS also contains lipids and phospholipids as well as humic substances (Flemming et al. 2000).

EPS is formed primarily of polymers bearing charged functional groups such as phosphate, carboxylate, and sulfate groups. Alginate which has been found in large concentrations within P. aeruginosa biofilms is formed of mannuronate and guluronate monomers; the carboxyl groups of these moieties are anionic. Other anions prevalent in EPS are proteins and nucleic acids. The charged functional groups of these polymers relate to biofilm structure (Flemming et al. 2000).

UNRAVELING THE "BIOFILM PHENOTYPE"

As described above, the behavior of cells embedded within a biofilm, differs greatly from that of planktonic cells. Julian Wimpenny states in regard to the behavior of biofilms as communities that "the sum of its activities is greater than the sum of all the activities of is constituent members," and that "…a community might have *emergent* properties" (Wimpenny 2000). Theses distinctions are both genetic and environmental in source.

In terms of generic expression, there is a definite deviation in the physiology of the bacterial cells of a biofilm. In regard to the environmental influence of phenotype, there are apparent differences in the collective action of the varied multitude of microbial cells that form a biofilm and their planktonic analogues.

Environmental differences can be attributed to the presence of the surrounding EPS matrix and to the heterogeneity of the biofilm population. The variety of species and mutants within a biofilm can be though to act together as a single multicellular unit which utilizes different cell types for the differing functions for which they are optimally suited. However, the genetic basis for the biofilm phenotype is a much more complex matter requiring a more complex explanation.

Molecular explanation of the biofilm phenotype is related to two cell density-dependent mechanisms; quorum sensing and gene transfer.

As its name implies. Quorum Sensing (QS) is a microbial cell to cell (pheromone) signaling system which is dependent upon cell concentration. Signal molecules are secreted by some cells; if the cell density is low they diffuse providing a minimal effect. If the cell density is high, a sufficient (threshold) quantity of signal molecules is present to activate the receptors of other cells in the vicinity inducing a signal transduction cascade which activates the expression of a number of genes (Stoodley et al. 2000).

Quorum sensing signals induce a multitude of properties including the development of genetic competence (the ability to genetically transform), synthesis of antibiotics, and even virulence (Cvitkovitch et al. 2003).

Such quorum sensing activities occur frequently when there is a high concentration of cells. Biofilms always indicate a high concentration of cells and are thus probable locations for quorum sensing to occur. Additionally, quorum sensing pathways have been shown to induce biofilm, development (Stoodley et al. 2000).

Quorum sensing is also implicated in bacterial dispersions from biofilms. Such dispersion occurs by expression of density- dependent genes which code for enzymes that degrade EPS matrix thus freeing cells from it (Davies 2000).

There are various specific quorum sensing pathways utilized. Gram negative bacteria such as P. aeruginosa primarily on Acyl-Homoserine Lactones (AHLs) as inducers. Gram positive species, such as the various streptococci, have their own variety of inducers molecules. For example, many streptococci utilize molecules classified as Competence Stimulating Peptides (CSPs) which act by QS to activate cascades leading to genetic regulation of numerous properties which are likely to include those which influence the "biofilm phenotype" (Cvitkovitch et al. 2003).

During horizontal gene transfer (transformation and conjugation), fragments of genetic material are transferred among microbial populations conferring a variety of phenotypic properties to non-descending cells.

Cells must be in a state of genetic competence to accept DNA by gene transfer. Competence stimulating peptides are so-names due to their induction of competence in their recipient cells. This implies that high cell density, as is found within biofilms, greatly increases the level of transformation. Gene transfer is also increased within biofilms due to the presence of an "abundant extracellular gene pool" (Cvitkovitch et al. 2003).

Conjugation rates also appear to be higher for surface associated cells than for cells within liquid culture (Ehlers 2000).

It is apparent that the increased level of gene transfer within biofilms provides a source of phenotype distinction from planktonic microbes. As noted other factors are quorum sensing, and environmental distinctions such as the protective Eps matrix and the communal interaction of differing species and mutants.

BIOFILM INFECTIONS

Above, numerous physiological and structural features of laboratory studied biofilms are described. It is now appropriate to discuss the biofilms that grow within the human body. Biofilms have been found to grow extensively on a number of medical and anatomical surfaces within the human body. In fact the NH indicates that more than 60% of microbial infections are of the biofilm type (Hentzer and Givskov 2003).

Is it important to note the many specific biofilms observed within the body, as well as to truly understand their implications and their distinctions from planktonic flora.

BIOFILMS AND CHRONIC INFECTIONS

As previously mentioned biofilms are phenotypically distinct from suspended microbes; yet the primary methods microbial research have been studies of planktonic cell cultures. This is unfortunate as a multitude of human infections are biofilm based.

Previous bacterial epidemics were planktonic cells that could be easily eliminated with antibiotics and by increasing immune function by vaccination. These infections acted in the acute

manner. However, with such conditions quite controlled, a newer breed of infections has appeared. These infections are not as invasive yet persist for prolonged periods of time with sporadic flare-ups. These diseases also appeared to be caused by common organisms for which the victims were perceived to possess immunity. When the organisms were cultured and tested for antibiotic susceptibility they were deemed sensitive to the conventional drugs; yet patient treatment with the antibiotics failed. These chronic infections, resistant to traditional antimicrobial elements were determined to be of the biofilm, type. Biofilms are noted to be the most defensive prokaryotic "life strategy" (Costerton et al. 2003).

Progressing in the field of medical microbiology requires acknowledgment of the distinct biofilm phenotype, research methods altered for biofilm study, and realization that biofilms induce chronic infections requiring altered treatment mechanisms (Ibid).

SURVEY OF HUMAN BIOFILM INFECTIONS

Below are some examples of common biofilm growth on the natural surfaces of the human body, as well as biofilms which colonize implanted medical devices.

1) *Dental Biofilms and Implications*

The most common example of biofilms in the body, and possible the most studied, is that of dental plaque.

The initial event in plaque formation is the development of an acquired pellicle on the enamel surface of teeth. An acquired pellicle is a protein rich conditioning film derived from saliva. Pellicle formation is followed by colonization by normal oral flora. In the days following colonization, a biofilm matrix begins to appear. These events directly follow cleaning of the enamel surface (Donlan and Costerton 2002).

If proliferation of the biofilm is undistributed for a period of 2-3 weeks, a biofilm with a depth of 50-100μm is observed. This biofilm is termed plaque. If the plaque becomes mineralized by calcium and phosphate ions it becomes calculus or tartar. Eventually plaque colonizes the lateral surfaces of teeth as well as the gingival sulcus (between the tooth and gingival surface); such plaque masses induce periodontal disease and dental caries (erosion of the teeth) (Ibid.).

2) *Native Valve Endocarditis*

Vascular injuries commonly induce a form of endocarditis termed Nonbacterial Thrombotic Endocarditis (NTBE).

A potentially fatal biofilm infection of the body that is linked to bacterial Native Valve Endocarditis (NVE). NVE is caused by adhesion of microorganisms of the endothelial surfaces of the cardiac valves which are damaged by NTBE.

In regions of NTBE a high level of fibronectins are secreted by the cells, platelets, and fibroblasts present. Several bacteria feature receptors and bind the fibronectins; formation of microcolonies follows (Ibid.).

Research shows that these bacteria/fibronectin unions develop so that the bacteria are encapsulated within fibronectins which protects them from phagocytosis. Fibronectins also bind leukocytes and can thus hinder their motility (Ibid.).

Biofilms on heart valves can directly damage the underlying tissues. These biofilms can also disseminate fragments by detachment which can form emboli. Fungal

biofilms are found to be the predominant culprit of theses emboli as their biofilms can be thick (Ibid.).

3) *Biofilms and Otitis Media*

Otitis Media (OM) is a bacterial induced inflammation of the middle ear tissues. Common among children is Chronic Otitis Media with Effusion (COME). COME is a chronic condition in which a viscous fluid is found within the middle ear. Biofilms have been found on the mucosal surface of the middle ear of COME patients (Costerton et al. 2003).

One mechanism utilized to treat COME is the insertion of tympanostomy tubes into the ear to alleviate pressure buildup. Unfortunately many of these tubes have been demonstrated to provide a new surface for biofilm colonization. However, silicone tympanostomy tubes bombarded with ions have been shown to remain uncontaminated (Donlan and Costerton 2002).

4) *Biofilms and Cystic Fibrosis*

Cystic fibrosis (CF) is a recessive genetic disorder found predominately among Caucasians of European decent. CF is primarily characterized by respiratory infections as well as other abnormalities throughout the body.

During infancy and early childhood, the lungs of CF patients are infected by organisms such as *Staphylococcus* aureus and *Haemophilus influenzae* which can cause tissue damage. Such damage to the epithelia increases the adhesion of *Pseudomonas aeruginosa* cells. *P. aeruginosa* subsequently become the primary colonizers and induce chronic infection. Chronic *P. aeruginosa* infection is the predominant cause of respiratory dysfunction and subsequent death in CF patients (Lyczak et al. 2002).

An important factor in the *P. aeruginosa* infections of the CF patient's airway is it growth as a biofilm. Electron microscopy has demonstrated the presence of *P. aeruginosa* biofilms in CF lungs. As explained above, growth in the resistant biofilm phenotype is implicated as a cause of chronic disease. Additionally, research has related the quorum sensing regulation of *P. aeruginosa* virulence to that of biofilm growth (Lyczak et al. 2002).

5) *Biofilms and Central Venous Catheters*

Central Venous Catheters (CVCs) are utilized to administer substances into large veins of the neck chest or groin. Many pathogenic micro-flora colonize the lumen and external surfaces of CVCs. Biofilms of multiple species have been found growing on CVC surfaces (Murga et al. 2001).

An early event in biofilm growth on CVC surfaces is the development of a conditioning film of blood on the catheter surface. Although catheters are flushed after blood is drawn through them, it is assumed many serum proteins remain on the surface. The blood proteins fibronectin (described above in regard to NVE pathogenesis) and fibrinogen have been shown to affect surface attachment of microbes, inducing attachment of many organisms (Murga et al. 2001).

6) *Biofilm Growth on Urethral Catheters*

Urinary tract infections are extremely common among patients with long-term urinary catheters installed. Clinical evidence shows that is it quite complicated to eliminate such infections while the catheter is present (Stickler et al. 1998).

Permanent urinary catheters can go unchanged for as long as 3 months allowing infected urine to circulate within them. Biofilms have been shown to grow on the interior of such catheters, often to densities which impede urinary out-flow (Stickler et al. 1998).

Biofilms within urinary catheters have been observed in-vivo and in-vitro by scanning and transmission electron microscopy (Donlan and Costerton 2002).

ANTIBIOTIC RESISTANCE OF BIOFILMS

Numerous clinical scenarios and research studies have been demonstrated the inherent resistance of biofilms to conventional antimicrobial agents. Generally biofilms cannot be eradicated by the same antibiotic regiments that can eliminate their planktonic constituents (Costerton et al. 2003).

It is accepted that the resistance of biofilm enveloped microbes to antimicrobial agents cannot be related to a single factor but is the result of multiple factors which are tied to the biofilm phenotype (Parsek and Fuqua 2004).

RESTRICTION PENETRATION

The initial factor considered for antimicrobial resistance in biofilms is the penetration restriction of their matrix. Restricted penetration is caused by two major mechanisms; the action of the matrix as a diffusion barrier, and the binding of matrix polymers to antimicrobial particles.

Consideration of the restricted penetration model has demonstrated its ability to hinder influx of large molecules such as lysozymes; however it does not eliminate the entry of small antimicrobial molecules. Penetration restriction has been shown to merely slow the penetration of such drugs. Such retarted diffusion can however protect biofilms from degradable antimicrobials as it presents the opportunity for degradation factors such as beta-lactamases to act. the synergistic cooperation of diffusion restriction and antimicrobial destruction/modification is a highly effective resistance mechanism (Lewis 2001).

ALTERED GROWTH RATE

Another factor commonly cited in the discussion of antimicrobial resistance of biofilms is the relationship between growth rate and the killing effect of antimicrobials. Biofilm cells have substantially decreased growth rates; many antimicrobials require cells to be growing for any efficacy. Most other antibiotics (e.g. advanced beta-lactams such as cephalosporins and fluorouqinolones) feature decreased efficacy with decreased growth rates (Lewis 2001).

ALTERED PHENOTYPE

A predominant factor cited in the discussion of biofilm resistance to antimicrobials is the “biofilm phenotype”. It is believed that biofilms feature physiology that is distinct from that of planktonic cells. As described above such distinction are the results of quorum sensing, increased gene transfer and possible surface association. These factors affect the expressed genotype of cells. An altered phenotype can result in alterations to antimicrobial absorption and efficacy (Parsek and Fuqua 2004).

PERSISTER CELLS

Kim Lewis describes the presence of subpopulation of "persister cells" within the biofilm which are not easily eradicated. It is believed that even with the aforementioned mechanisms of antimicrobial resistance most of the biofilm cells can be eliminated with certain antibiotics; however these persister cells are not eliminated.

Such subpopulations of persisters are found within planktonic populations but are believed to be eliminated by the immune system after antibiotics eliminate the vast majority of the cells. Lewis hypothesized that the biofilm matrix protects the persister cells from immunity factors thus maintaining persistence (Lewis 2001).

The hypothesis that biofilms protect persists from immunity factors may require review. Parsek and Fuqua quote Jeff Leid to have reported at the Biofilms 2003 meeting that human leukocytes do penetrate biofilms (Parsek and Fuqua 2004). It is likely that persisters are responsible for increasing the resistance of biofilms by a different mechanism.

TREATMENT AND PREVENTION OF BIOFILM INFECTIONS

Numerous biofilms relevant in medicine have been discussed as well as their highly resistant nature. It is now essential to propose strategies for preventing, eradication, and treating, biofilm infections. There is not a single target for such strategies but a multitude of targets which must be considered.

PREVENTION OF BIOFILM GROWTH

Prior to exploration of strategies for the treatment of existing biofilms, it is wise to consider some methods by which biofilm growth can be initially prevented.

The general steps in biofilm formulation described above indicate mechanisms by which biofilm formation can be inhibited.

Initially, primary colonizers must adhere to the substratum surface. If adherence to the substratum can be decreased, biofilm growth can also be decreased. One approach to prevent biofilm growth on medical devices is to alter the surface properties of the biomaterials. This hypothesis is supported by J. Chandra and associates who demonstrated that chemical modification of biomaterials influenced the ability of *C. albicans* to form biofilms on them (Chandra et al. 2005).

In addition to material alterations, adjustment of clinical procedures and standards can also decrease biofilm formation on medical devices. For this to occur it is important that the medical community recognizes the existence of biofilms as well as their dire implications. Modifications should be made in the scheduling and methods by which medical devices are installed and replaced (Costerton et al. 2003).

There are also occasions when it is appropriate to utilize prophylactic antibiotic therapy to eliminate planktonic populations to prevent colonization of anatomical and medical device surfaces. Acknowledging the patterns of human biofilm infections can help to identify such occasions.

ELIMINATING BIOFILMS

While there is no central solution, many mechanisms have been proposed to eliminate biofilms. Most of the proposed methods will require much more intensive research prior to any

clinical relevance. However, many biofilm infections can be eliminated utilizing current antimicrobial agents by unique regimens which are depicted simple laboratory susceptibility tests. A prevalent theme is the administration of various antibiotic combinations. Saginur and associates as well as Slinger and associates found that specific combinations of antimicrobials are effective in treating biofilms. These same antibiotics are ineffective against biofilms when administered independently (Saginur et al. 2005, Slinger et al. 2006).

Assuming that persister cells are the predominant basis of biofilm resistance, Kim Lewis presents a possible treatment regimen. Lewis suggests administering a bactericidal antibiotic, withdrawing from treatment, and then re-administrating to the agent.

The first administration is to eliminate the majority of cells; the normal cells. The withdrawal period is to allow growth of the remaining (persister) cells. During this time the vast majority if the population will lose their persister phenotype leaving a relatively insignificant population of persisters. These "normal" cells will now be eliminated by the second drug administration. This mechanism is proposed primarily for cases of direct antibiotic application; where drug delivery is controlled. For example: administration of antibiotics via aerosol directly to the Cystic Fibrosis airway (Lewis 2001).

Many new antibiotic targets have been proposed for the treatment of biofilms. One such methods in the inhibition of quorum sensing. Theoretically, quorum sensing can be inhibited in three ways: inhibition of signal generation, inhibition of signal dissemination, and inhibition of signal reception (Hentzer and Giskov 2003).

Interestingly, efficacy of antibiotics against biofilms has been shown to increase in the presence of ultra sound or low-strength electric fields (Donlan and Costerton 2002). These phenomena are not yet clinically significant.

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