Biofilm formation: A complicated microbiological process.

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ABSTRACT: Bacteria employ certain basic survival strategies one of which is to form in natural and industrial systems biofilms, within which they are protected from antibacterial chemicals, environmental bacteriophages, and phagocytes. In contrast to planktonic form of microorganisms, biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to 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. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems. Biofilms are preferentially formed in very high shear environments.The solid-liquid interface between a surface and an aqueous medium provides an ideal environment for the attachment and growth of microorganisms. The solid surface may have several characteristics that are important in the attachment process. Proximity of cells within the microcolony (or between microcolonies) provides an ideal environment for creation of nutrient gradients, exchange of genes, and quorum sensing. Bacteria within biofilms may be subject to predation by free-living protozoa, bacteriophage, and polymorphonuclear leukocytes. Biofilms present both heterogeneity and a constant flux, as they adapt to changing environmental conditions and the composition of the community and may be dispersed with various mechanisms for will undoubtedly emerge. The key to success for biofilm prevention and control may hinge upon a more complete understanding of what makes the biofilm phenotype so different from the planktonic phenotype.

Key Words: Biofilm, Extracellular polymeric substance, High shear environments, *Solid-liquid interface, Exchange of genes, Quorum sensing.*

INTRODUCTION

Van Leeuwenhoek have described for the first time biofilms when he examined the "animalcules" in the plaque on his own teeth in the seventeenth century, but the general theory of biofilm predominance was not promulgated until 1978[1]. According to this theory , firstly, the majority of bacteria grow in a matrix that encloses biofilms which are adherent to surfaces in all nutrient-sufficient aquatic ecosystems, and secondly, sessile bacterial cells differ profoundly from their planktonic (floating) counterparts[1]. Direct microscopic observations and direct quantitative recovery techniques showed that more than 99.9% of common pathogenic bacteria grow in biofilms on a wide variety of surfaces.

The consensus that bacteria grow preferentially in matrixenclosed biofilms in natural and industrial systems was not immediately accepted in the medical community despite the universal acceptance of dental plaque as a type of biofilm. However, new methods for the direct examination of biofilms soon showed that the organisms that cause many device-related and chronic infections actually grow in biofilms[2].

Bacteria form biofilms in natural and industrial systems. This is a survival strategy, within which they are protected from antibacterial chemicals (including natural antibiotics), environmental bacteriophages, and phagocytes. Biofilms have great significance for public health, because biofilm-associated microorganisms exhibit dramatically decreased susceptibility to antimicrobial agents. For these reasons, chronic biofilm associated infections resist antibiotic therapy and

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are phenomenally resistant to host clearance mechanisms. Many bloodstream infections and urinary tract infections are associated with indwelling medical devices and, therefore, (in most cases) biofilm associated.

Organisms that have successfully survived for millions of years in the environment (e.g. *Pseudomonas* and *Staphylococcus* spp.) are now mounting successful attacks on health care facilities. They are making use of the biofilm strategy that has protected them so well in their native habitats. Compromised individuals, are especially susceptible to these new "environmental" pathogens that have invaded homes and schools just as they are invading our hospitals[3].

In 1976[4] Marshall noted the involvement of "very fine extracellular polymer fibrils" that anchored bacteria to surfaces. Costerton et al.[5] observed that communities of attached bacteria in aquatic systems were found to be encased in a "glycocalyx" matrix that was found to be polysaccharide in nature, and this matrix material was shown to mediate adhesion. Costerton et al., in 1987[6], stated that biofilm consists of single cells and microcolonies, all embedded in a highly hydrated, predominantly anionic exopolymer matrix. Characklis and Marshall in 1990[7] went on to describe other defining aspects of biofilms, such as the characteristics of spatial and temporal heterogeneity and involvement of inorganic or abiotic substances held together in the biofilm matrix. Costerton et al, in 1995[8], emphasized that biofilms could adhere to surfaces and interfaces and to each other. Costerton and Lappin-Scott[9] at the same time stated that adhesion triggered expression of genes controlling production of bacterial components necessary for adhesion and biofilm formation, emphasizing that the process of biofilm formation was regulated by specific genes transcribed during initial cell attachment.

The definition for biofilm must take into consideration not only readily observable characteristics, cells irreversibly attached to a surface or interface, embedded in a matrix of extracellular polymeric substances which these cells have produced, and including the noncellular or abiotic components, but also other physiological attributes of these organisms, including such characteristics as altered growth rate and the fact that biofilm organisms transcribe genes that planktonic organisms do not.

The new definition of a biofilm is *a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to 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*[10]. This definition is useful, because some bacterial populations that fulfille the earlier criteria of a biofilm, which involves matrix formation and growth at a surface, do not actually assume the biofilm phenotype.

BIOFILM CHEMICAL CONSISTENCE AND STRUCTURE

Biofilms are composed primarily of microbial cells and extracellular polymeric substance (EPS). EPS may account for 50% to 90% of the total organic material of biofilms[11]. EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pryruvates confers the anionic property[12]. This property is important because it allows association of divalent cations such as calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm[11]. In the case of some gram-positive bacteria, such as the staphylococci, the chemical composition of EPS may be quite different and may be primarily cationic. Hussain et al[13] found that the slime of coagulase-negative bacteria consists of a teichoic acid mixed with small quantities of proteins.

EPS is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. EPS may be hydrophobic, although most types of EPS are both hydrophilic and hydrophobic[12]. EPS may also vary in its solubility. There are two important properties of EPS that may have a marked effect on the biofilm[12]. First, the composition and structure of the polysaccharides determine their primary conformation. For example, many bacterial EPS possess backbone structures that contain 1,3- or 1,4-β-linked hexose residues and tend to be more rigid, less deformable, and in certain cases poorly soluble or insoluble. Other EPS molecules may be readily

soluble in water. Second, the EPS of biofilms is not generally uniform but may vary spatially and temporally. Different organisms produce differing amounts of EPS and that the amount of EPS increases with age of the biofilm[14]. EPS may associate with metal ions, divalent cations, other macromolecules (such as proteins, DNA, lipids, and even humic substances)[11]. EPS production is known to be affected by nutrient status of the growth medium; excess available carbon and limitation of nitrogen, potassium, or phosphate promotes EPS synthesis[12]. Slow bacterial growth will also enhance EPS production[12]. A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface. Noncellular materials such as mineral crystals, corrosion particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in EPS .

Every microbial biofilm community is unique[15] although some structural attributes can generally be considered universal. The term biofilm is in a way not very correct, since biofilms are not a continuous monolayer surface deposit. In reallity, biofilms are very heterogeneous, containing microcolonies of bacterial cells encased in an EPS matrix and separated from other microcolonies by interstitial channels (water channels)[16]. Liquid flow occurs in these water channels, allowing diffusion of nutrients, oxygen, and even antimicrobial agents. This concept of heterogeneity is descriptive not only for mixed culture biofilms but also for pure culture biofilms common on medical devices and those associated with infectious diseases.

The organisms composing the biofilm may also have a marked effect on the biofilm structure. Biofilm thickness could be affected by the number of component organisms[17]. Pure cultures of either *K. pneumoniae* or *P. aeruginosa* biofilms in a laboratory reactor are thinner, whereas a biofilm containing both species is thicker. This could be because one species enhanced the stability of the other[17].

Structure may also be influenced by the interaction of particles of nonmicrobial components from the host or environment. For example, erythrocytes and fibrin may accumulate as the biofilm forms. Biofilms on native heart valves provide a clear example of this type of interaction in which bacterial microcolonies of the biofilm develop in a matrix of platelets, fibrin,

and EPS[18]. The fibrin capsule that develops will protect the organisms in these biofilms from the leukocytes of the host, leading to infective endocarditis. A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material. Noncellular materials such as mineral crystals, corrosion particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix. Biofilm-associated organisms also differ from their planktonic (freely suspended) counterparts with respect to the genes that are transcribed.

HOW MICROORGANISMS FORM BIOFILMS

Bacteria form biofilms in essentially the same manner in whatever ecosystem they inhabit. The first surprise, for the medical community, was that bacteria form biofilms preferentially in very high shear environments. Planktonic bacteria can adhere to surfaces and initiate biofilm formation in shear forces that assimilate those of heart valves and exceed Reynolds numbers of 5,000[19] (is a dimensionless number describing the turbulent flow of a liquid). Once a biofilm has formed and the exopolysaccharide matrix has been secreted by the sessile cells, the resultant structure is highly viscoelastic and behaves in a rubbery manner[20]. When biofilms are formed in low-shear environments, they have a low tensile strength and break easily, but biofilms formed at high shear are remarkably strong and resistant to mechanical breakage.

Studies of bacterial adhesion with medical laboratory strains of bacteria, indicated that very smooth surfaces might escape bacterial colonization[21]. Subsequent studies with "wild" and fully adherent bacterial strains showed that smooth surfaces are colonized as easily as rough surfaces and that the physical characteristics of a surface influence bacterial adhesion to a different extent[22].

Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems. The variable nature of biofilms can be illustrated from scanning electron micrographs of biofilms from industrial water systems and medical devices, (Figure 1).
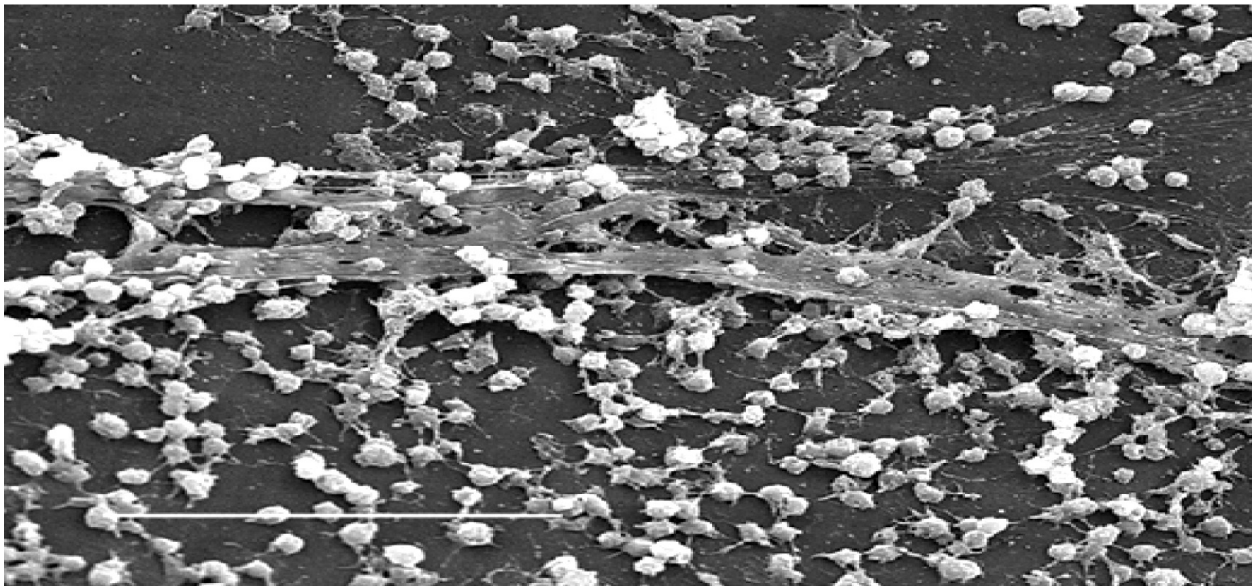
Figure 1. Scanning electron micrograph of a staphylococcal biofilm on the inner surface of an indwelling medical device.

The solid-liquid interface between a surface and an aqueous medium (e.g. water, blood) provides an ideal environment for the attachment and growth of microorganisms[3]. A clear picture of attachment cannot be obtained without considering the effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and various properties of the cell surface.

The solid surface may have several characteristics that are important in the attachment process. Characklis et al.[23] noted that the extent of microbial colonization appears to increase as the surface roughness increases. The physicochemical properties of the surface may also provocate a strong influence on the rate and extent of attachment. Most investigators have found that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces than to hydrophilic materials[24,25,26].

The first that was reported was the formation of conditioning films on surfaces exposed in seawater[27]. The nature of conditioning films is quite different for surfaces exposed in the human host. A prime example may be the proteinaceous conditioning film called "acquired pellicle," which develops on tooth surfaces in the oral cavity. Pellicle comprises albumin, lysozyme, glycoproteins, phosphoproteins, lipids, and gingival

fluid[28]; bacteria from the oral cavity colonize pellicle-conditioned surfaces within hours of exposure to these surfaces. A number of host-produced conditioning films such as blood, tears, urine, saliva, intervascular fluid, and respiratory secretions influence the attachment of bacteria to biomaterials[29].

In theory, the flow velocity immediately adjacent to the substratum/liquid interface is negligible. This zone of negligible flow determines an hydrodynamic boundary layer[30]. Its thickness is dependent on linear velocity; the higher the velocity, the thinner the boundary layer. Cells behave as particles in a liquid, and the rate of settling and association with a submerged surface depends largely from the characteristic velocity of the liquid. As the velocity increases, the boundary layer decreases, and cells will be subjected to increasingly greater turbulence and mixing[31].

Some characteristics of the aqueous medium, such as pH, nutrient levels, ionic strength, and temperature, may play a role in the rate of microbial attachment to a substratum. Several studies have shown a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems[32,33].

Properties of the Surfaces

The attachment of microorganisms to surfaces is a very complex process, with many variables affecting

the outcome. In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface "conditioning" films[34]. An increase in flow velocity, water temperature, or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels. Properties of the cell surface, specifically the presence of fimbriae, flagella, and surface-associated polysaccharides or proteins, also are important.

Cell surface hydrophobicity, presence of fimbriae and flagella, and production of extracellular polymeric substance (EPS) all influence the rate and extent of attachment of microbial cells. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing nonpolar nature of one or both surfaces involved. Fimbriae, contribute to cell surface hydrophobicity because contain a high proportion of hydrophobic amino acid residues[35] and play a role in cell surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum[36].

Motile cells attach in greater numbers and attach against the flow (backgrowth) more rapidly than do nonmotile strains[37]. Nonmotile strains also do not recolonize or seed vacant areas on a substratum as motile strains, resulting in slower biofilm formation by the nonmotile organisms. Flagella apparently play an important role in attachment in the early stages of bacterial attachment by overcoming the repulsive forces associated with the substratum[37]. Flagella are important in attachment also, although their role may be to overcome repulsive forces rather than to act as adsorbents or adhesives.

Other cell surface properties may also facilitate attachment. Several studies have shown that treatment of adsorbed cells with proteolytic enzymes causes a marked release of attached bacteria[38,39] providing evidence of the role of proteins in attachment. For most strains tested, adhesion is greater on hydrophobic materials. The O antigen component of lipopolysaccharide (LPS) has also been shown to confer hydrophilic properties to gram-negative bacteria[40].

Beech and Gaylarde[41] found that lectins inhibit but do not prevent attachment. Lectins preferentially bind to polysaccharides on the cell surface or to the EPS.

Binding of lectins by the cells would minimize the attachment sites and affect cell attachment if polysaccharides were involved in attachment.

BIOFILM COMMUNITY

The basic structural unit of the biofilm is the microcolony. Proximity of cells within the microcolony (or between microcolonies) provides an ideal environment for creation of nutrient gradients, exchange of genes, and quorum sensing. Since microcolonies may be composed of multiple species, the cycling of various nutrients (e.g., nitrogen, sulfur, and carbon) through redox reactions can readily occur in aquatic and soil biofilms.

Cell-to-cell signaling (Quorum Sensing) has recently been demonstrated to play a role in cell attachment and detachment from biofilms[42]. For example ccording to Davies et al. two different cell-to-cell sigsignaling systems in *P. aeruginosa*, *lasR-lasI* and *rhlR-rhlI,* are involved in biofilm formation[42]. At sufficient population densities, these signals reach concentrations required for activation of genes involved in biofilm differentiation. Mutants unable to produce both signals (double mutant) are able to produce a biofilm, but unlike the wild type, their biofilms are much thinner, cells are more densely packed, and the typical biofilm architecture is lacking. In addition, these mutant biofilms are much more easily removed from surfaces by a surfactant treatment.

Biofilms provide an ideal niche for the exchange of extrachromosomal DNA (plasmids).The mechanism of plasmid transfer occurs at a greater rate between cells in biofilms than between planktonic cells[43,44,45]. Human pathogenic bacteria that contain conjugative plasmids more readily develop biofilms[45].

Plasmid-carrying strains have also been shown to transfer plasmids to recipient organisms, resulting in biofilm formation; without plasmids these same organisms produce only microcolonies without any further development. The probable reason for enhanced conjugation is that the biofilm environment provides minimal shearand closer cell-to-cell contact[46]. Since plasmids may encode for resistance to multiple antimicrobial agents, biofilm association also provides a mechanism for selecting for, and promoting the spread of, bacterial resistance to antimicrobial agents.

Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells, detachment as a result of nutrient levels or quorum sensing, or shearing of biofilm aggregates (continuous removal of small portions of the biofilm) because of flow effects[47]. It is emphasized the importance of physical forces in detachment, stating that the three main processes for detachment are erosion or shearing (continuous removal of small portions of the biofilm), sloughing (rapid and massive removal), and abrasion (detachment due to collision of particles from the bulk fluid with the biofilm)[48.] The rate of erosion from the biofilm increases with increase in biofilm thickness and fluid shear at the biofilm-bulk liquid interface[49]. Sloughing is more random than erosion and is thought to result from nutrient or oxygen depletion within the biofilm structure[48]. Sloughing is more commonly observed with thicker biofilms that have developed in nutrient-rich environments[49]. Detachment is probably species specific; *P. fluorescens* disperses and recolonizes a surface (in a flow cell) after approximately 5 h, *V. parahaemolyticus* after 4 h, and *V. harveyi* after only 2 h[50]. This process probably provides a mechanism for cells to migrate from heavily colonized areas that have been depleted of surface-adsorbed nutrients to areas more supportive of growth.

CONCLUSION

Bacterial cells have grown in the biofilm phenotype for billions of years, as a part of their successful strategy to colonize most of the environment. We have only recognized this distinct phenotype as the predominant mode of bacterial growth and as important phenotype in persistent infections, the last decades. Researchers in the fields of medical, food, water, and environmental microbiology have begun to investigate microbiologic processes from a biofilm perspective. As the pharmaceutical and health-care industries embrace this approach, novel strategies for biofilm prevention and control will undoubtedly emerge. The key to success may hinge upon a more complete understanding of what makes the biofilm phenotype so different from the planktonic phenotype.

Δημιουργία βιομεμβράνης: Ένα πολύπλοκο μικροβιολογικό φαινόμενο.

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ΠΕΡΙΛΗΨΗ: Τα βακτήρια χρησιμοποιούν ορισμένες βασικές στρατηγικές επιβίωσης μία εκ των οποίων είναι η δημιουργία βιομεμβράνης εντός της οποίας προστατεύονται από την δράση αντιβακτηριακών χημικών ουσιών, από βακτηριοφάγους και φαγοκύτταρα. Η βιομεμβράνη είναι *μια άμισχη κοινότητα που αποτελείται από μικροβιακά κύτταρα που είναι αμετάκλητα συνδεδεμένα σε ένα υπόστρωμα ή μία διεπιφάνεια ή το ένα στο άλλο, είναι ενσωματωμένα σε μια μήτρα εξωκυττάριας βλεννώδους ουσίας που τα ίδια παράγουν, και εμφανίζουν διαφορετικό φαινότυπο σε ότι αφορά τον ρυθμό ανάπτυξης και τη μεταγραφή γονιδίων σε σχέση με τα πλαγκτονικά κύτταρα.* Η βιομεμβράνη μπορεί να σχηματιστεί σε μια μεγάλη ποικιλία επιφανειών, συμπεριλαμβανομένων των ζωντανών ιστών, των μόνιμων ιατρικών συσκευών, των βιομηχανικών ή πόσιμου νερού συστημάτων σωληνώσεων ή σε φυσικά υδάτινα συστήματα. Οι βιομεμβράνες δημιουργούνται κατά προτίμηση σε σημεία υψηλής διατμηματικής τάσης. Η στερεού-υγρού διεπιφάνεια μεταξύ μιας επιφάνειας και ενός υδάτινου μέσου προσφέρει ένα ιδανικό περιβάλλον για την προσκόλληση και την ανάπτυξη των μικροοργανισμών. Η στερεή επιφάνεια μπορεί να προυσιάζει ορισμένα χαρακτηριστικά που είναι σημαντικά για την διαδικασία προσκόλλησης. Η εγγύτητα των κυττάρων εντός της μικροαποικίας (ή μεταξύ μικροαποικιών) αποτελεί ένα ιδανικό περιβάλλον για τη δημιουργία διαβαθμίσεων θρεπτικών υλικών, ανταλλαγή γονιδίων, και διεπικοινωνία. Τα βακτήρια στη βιομεμβράνη μπορεί να υπόκεινται απειλές εξάληψεις από ελεύθερα πρωτόζωα, βακτηριοφάγους και πολυμορφοπύρηνα λευκοκύτταρα. Η βιομεμβράνη παρουσιάσει τόσο ετερογένεια όσο και σταθερή ροή, καθώς προσαρμόζεται στις μεταβαλλόμενες συνθήκες του περιβάλλοντος και τη σύσταση της κοινότητας και μπορεί να διασκορπίζεται στο περιβάλλον με διάφορες μηχανισμούς. Το κλειδί για την επιτυχή πρόληψη και τον έλεγχο της δημιουργίας των βιομεμβρανών μπορεί να κρύβεται σε μια πιο ολοκληρωμένη κατανόηση του τί κάνει την βιομεμβράνη τόσο διαφορετική από τον πλαγκτονικό φαινότυπο.

Λέξεις Κλειδιά: Βιομεμβράνη, Εξωκυττάρια βλενώδης ουσία, Υψηλή διατμηματική τάση, Στερεού-υγρού διεπιφάνεια, Ανταλλαγή γονιδίων, Διεπικοινωνία.

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