Biofilms: An Evolving and Universal Evasive Strategy of Bacterial Pathogens

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The surfaces of bacterial cells are perhaps the most important structure in bacteria because they are in immediate contact with the external environment. When bacteria are examined in natural environments they exist as suspended growth forms known as "Planktons". Planktonic forms often are highly motile, have unnatural access to nutrients and multiply rapidly. Also they are more susceptible to the effects of antibiotics, environment and host factors. During the conditions of nutrient limitations bacteria may grow more slowly and have restricted mobility. This kind of growth forms are otherwise termed as "Sessile". The growth of many sessile bacteria results in formation of large aggregates which are known as biofilms (Watnick & Kolter, 2000). A biofilm can be defined as "a community of bacteria and their extracellular polymers that are attached to surfaces".

Biofilms have been described in many systems since Van Leeuwenhoek examined the "animalcules" in the plaque of his own teeth in the seventeenth century, but the general theory of biofilm existence was not promulgated until 1978 (Costerton *et al.*, 1978). This theory state that the majority of bacteria grow in matrix-enclosed biofilms adherent to surfaces in all nutrient-sufficient aquatic ecosystems and these sessile bacterial cells differ profoundly from their planktonic (floating) counterparts. The data on which this theory is predicted came mostly from natural aquatic ecosystems, in which direct microscopic observations and direct quantitative recovery techniques showed unequivocally that more than 99.9% of the bacteria grow in biofilms on a wide variety of surfaces.

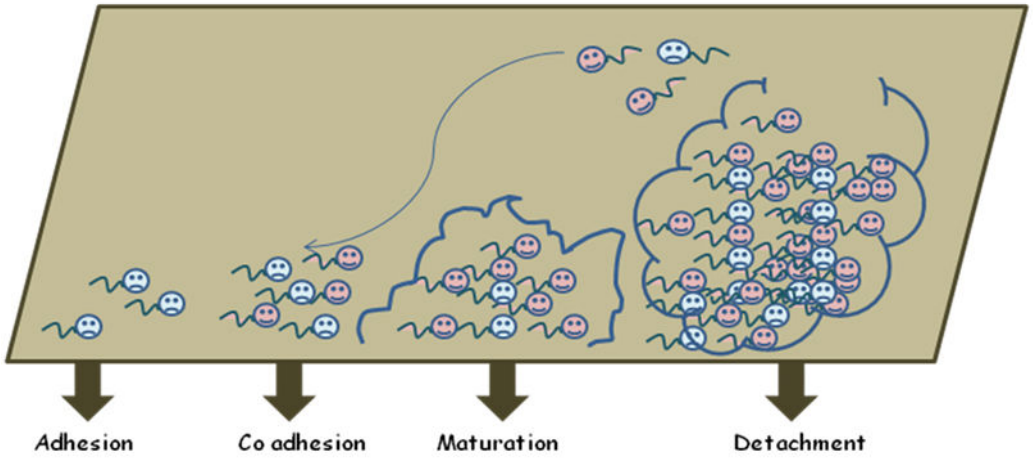
Biofilms constitute a protected mode of growth that allows survival in a hostile environment. The complexity of biofilm structure and metabolism has led to the analogy of biofilms to tissues of higher organisms. It is likely that biofilms evade antimicrobial challenge by multiple mechanisms. Some of the factors that are thought to contribute to the ability of biofilms to tolerate high concentration of antibiotics include:

1) Binding of antimicrobial agents to the extracelluar matrix of the biofilms, thereby limiting their penetration.
2) Inactivation of the antimicrobial agent by enzymes trapped in biofilms matrix.
3) The reduced growth rate of bacteria in biofilms renders them less susceptible to the antimicrobial agent.
4) Altered micro environment within the biofilms can reduce the activity of the agent.
5) Altered gene expression by organisms within the biofilms can result in a phenotype with reduced susceptibility to the antimicrobial agent.

The understanding of biofilms has evolved as the methods for biofilm examination and characterization (Webster *et al*., 2004; Hunter and Beveridge, 2005). Much of the early investigative work on biofilms relied heavily on the scanning electron microscopic studies. This technique utilizes graded solvents (alcohol, acetone, and xylene) to gradually dehydrate the specimen prior to examination, since water of hydration is not compatible with the vacuum used with the electron beam. This dehydration process results in significant sample distortion and artifacts; the extracellular polymeric substances, which are approximately 95% water, appear more as fibers than as a thick gelatinous matrix surrounding the cells.

The use of transmission electron microscopy and specific polysaccharide stains like ruthenium red allowed researchers both to identify the nature of these extracellular fibers in biofilms and to elucidate their association with the cells in a better manner. Electron microscopy has been used for the examination and characterization of biofilms on medical devices (Raad *et al.,* 1993; Stickler *et al.,* 1998) and in human infections (Nickel and Costerton, 1992). Because of its excellent resolution properties, the electron microscope in spite of its limitations continues to be an important tool for the biofilm scientific community.

Several stages exist in the formation of biofilms and these can be grouped as follows.



Stages of Biofilm Formation

Stage 1: Adhesion

First of all, the bacteria must reach the substratum to which they will ultimately adhere. In the case of non-motile organisms such transport can result from random, brownian motion of the organism which may be carried by the flow of the suspending fluid.

In contrast motile organism may actually "seek out" the surface guided by some chemotactic, aerostatic or phototactic response. Once it has reached the substratum the organism may then adhere to it. It is important to emphasize at this point that, in natural environments, bacteria rarely adhere to substratum itself, invariably this is coated with a layer of molecules known as a "conditioning film" and it is to this film that organism usually adheres.

Stage 2: Co-Adhesion

Adhesion of bacteria is then followed by a colonization stage which involves the synthesis of extra cellular matrix molecules (polysaccharides), multiplication of the attached organisms and attachment of other bacteria to the already adherent cells–a phenomenon known as co-adhesion. The synthesis of matrix molecules is crucial to this stage of biofilm development.

Stage 3: Maturation and Detachment

The stage is now set for the further growth of the attached organism resulting in the formation of the dense bacterial aggregates, a characteristic of mature biofilms. The latter structures are often exposed to strong mechanical and hydrodynamic forces that can result in detachment of the biofilms or parts of it. The detached sections can in course readhere to the substratum and thus constitute an effective means of colonizing a large area of the substratum. The mode of dispersal apparently affects the phenotypic characteristics of the organisms. Eroded or sloughed aggregates from the biofilm are likely to retain certain biofilm characteristics, such as antimicrobial resistance properties, whereas cells that have been shed as a result of growth may revert quickly to the phenotypic type. Detachment of biofilm aggregates may result in blood stream or urinary tract infections or lead to production of emboli.

A number of factors can affect biofilm structure, there is no single, unifying structure that can be said to characterize all biofilms. The key variables include the nature of the organisms, the concentration of nutrients present, the hydrodynamic properties of the environment and the presence of any mechanical forces. Hence, the structure of a biofilm can range from a relatively featureless flat type to one consisting of more complex organization involving mushroom like aggregates separated by water channels. The latter are characteristics of biofilms formed under the conditions of low nutrient concentration, high hydrodynamic shear stress and the absence of mechanical, abrasive and compressive forces.

The progress in microbiology has lead to the better understanding of planktonic bacteria which helped in better design of vaccines as well as effective antibiotics. As we began to gain control over epidemic diseases another type of disease has come in to fore. These diseases are much less aggressive than acute infections, they often persist for months or years. With the advent of new tools of microbiology, it is clear now that the etiological agents of such infections are common microflora with which an individual had daily contact and against which they often had adequate immunity.

Although these environmental organisms appeared to be sensitive to conventional antibiotics in laboratory conditions, but these antibiotics failed to resolve these bacterial infections. Even more puzzling was the observation that, in many cases it was not possible at all to recover any bacteria by traditional culture mechanisms. This led many researchers to posit that these chronic diseases are sterile inflammatory conditions which persisted after eradication of all microorganisms. However, the application of molecular diagnostics demonstrated unequivocally that both bacteria were present and were metabolically active when viewed, as they grow in affected tissues.They were found to be present as matrix enclosed community known as “biofilms”. Direct structural examination of biofilms showed that their component microcolonies, which are composed of cells embedded in matrix material are bisected by ramifying water channels that carry bulk fluid in to the community by convective flow.

The biofilm concept of chronic infections offers at least a partial explanation for a phenomenon that has troubled clinicians for some time. As it has been known that the infecting organisms exist both as biofilms and as individual planktonic, we must expect the planktonic cells to be killed by circulating antibiotics and activated phagocytes. If we grasp the concept that these bacteria are embedded in matrix material, that they have adopted a distinct biofilm phenotype, and that they have formed interactive communities, then we can bring all of the power of biofilm science to bear on chronic infections.

Stoodley *et al,* 2001 made a point that microbial biofilms constitute the most “defensive life strategy that can be adopted by bacterial cells. They made the intriguing suggestion that bacteria may have developed their biofilm phenotype early in the evolutionary process, when survival in a hostile environment was a *sine qua non*. They suggest that the planktonic phenotype, with its genetically “expensive” and very sophisticated chemotactic and motility mechanisms, may have developed later, for the purposes of dissemination and the colonization of new habitats.

As the majority of bacteria in virtually all natural ecosystems grow in biofilms , microbial challenge to humans often comes from this source. One of the conceptual areas of microbial ecology is that of the area of natural microbial biofilms seen on the surfaces of normal human tissues. These tissues are among the most attractive surfaces in nature because they are homeostatic and rich in simple nutrients, and bacteria have adapted to virtually all types of mammalian tissues with considerable success. Mucus-secreting tissues, like those of the trachea and the intestine, are covered by “mucus blankets,” and this viscous material is often moved across the surface of the tissues by such forces as ciliary beating or by peristalsis.

Bacteria have great difficulty in accessing mucus-covered tissues, especially if the mucus blanket is 200–250 μm in thickness and is moving at a considerable speed across the tissue surface. For this reason, commensal organisms often use “hold fast” mechanisms like flagella to gain access to the tissue surface by disrupting the mucus blanket. The outer surfaces of “mucus blankets” constitute a rich, if somewhat ephemeral, bacterial habitat and many species proliferate on these surfaces.

To analyze the phenomenon of biofilm formation on various tissues of host we have attempted to study microflora of diverse pathobiology like *H.influenzae*, Enteroaggregative *E.Coli* and Group B Streptococcus. The human nasopharynx serves as a major reservoir of microflora. The pattern of nasopharyngeal colonization is a cryptic event in the pathogenesis, and understanding the mechanisms of colonization of the nasopharyngeal mucosa has become a core issue in the study of respiratory tract infections (Raymond *et a*l., 2001). In this context we undertook a prospective study of children under 2 years old to elucidate the dynamics of persistent bacterial colonization in the nasopharynx and to determine the index of association between bacterial persistence and biofilm formation.

The colonization was found to be transitory with loss of most of the strains beyond twelve weeks of colonization. While there was an exception with majority of the strains belonging to nontypeable type which were more persistent in the nasopharyngeal tract for more than twelve weeks. Since *H.influenzae* are known to produce biofilms, their specific involvement in persistence was evaluated. The biofilm formation abilities when measured quantitatively were found to be higher among the strains which showed high persistence. The persistent strains were having higher ability to form biofilms, showing significantly ($p<0.005$) higher values in quantitative biofilm assay. Further in the logistic regression analysis which was conducted to assess the factors contributing towards the persistence of pathogen in nasopharynx demonstrated that biofilm formation was significantly contributing towards persistence (Sekhar *et al.,* 2009).

Several lines of evidence indicate that *H.influenzae* is viable inside host cells, including macrophages and respiratory epithelial cells (St Geme and Falkow, 1990). It is also postulated that *H.influenzae* has adopted the strategy of changing cells from planktonic growth to biofilms in the human respiratory tract as a survival strategy. Further formation of bacterial aggregates and microcolonies is likely to be important in conferring resistance to natural bacteriostatic compounds such as lactoferrin, lysozyme and peroxidase which are present in human respiratory secretions and are the important components of the innate immune system. In addition they may block the access of antibodies to individual organisms, there by impeding antibody-dependent killing and clearance by phagocytosis.

Biofilm formation was also observed in human GBS isolates that have been isolated from different sources like vagina or anorectal region of pregnant women, neonatal skin swabs, diabetic foot, pus and blood samples. Thus biofilm formation could be an important property in infections caused by GBS (Kaur *et al*.,2009). Many studies have shown biofilm formation in GBS isolates from bovine source causing mastitis (Olson *et al*.,2002). Loo *et al.,* (2000) categorized their strains as good biofilm former when the A575 of the CV-stained biofilm was greater than 2.0, where as Mathur *et al.,* (2006) reported O. D. values greater than >0.240 were strong biofilm producers. We characterized the ability of biofilm formation from asymptomatic and symptomatic GBS strains. Both sources tested showed variable biofilm formation in both isolates however formation was observed to be more with asymptomatic source (Murphy *et al*., 2002). The level of biofilm formation was found to be variable amongst the strains as confirmed by Light microscopy and Scanning electron microscopy (Kaur *et al.,* 2009).

Further in a comparative study done to understand the correlation among biofilm formation with respect to GBS serotypes, surface proteins (*c alpha, c beta* and *rib* genes) and antibiotic resistance, it was observed that serotypes v, 1b, iii, nontypeable were distinctly related to biofilm formation than other serotypes. *c alpha* gene, *c beta* and *rib* genes which are the immunoprotective surface proteins were expressed by biofilm forming GBS strains more in comparison to poor biofilm forming GBS isolates there by offering them the innate ability to survive against the host defenses. Thus biofilm formation observed in GBS isolates could also be correlated with the particular serotype (Ia), *c alpha* gene or various antibiotic resistance genes (Kaur *et al* ., 2009).

Besides gram positive bacteria, biofilm forming ability was also observed in diarrhgenic *E.coli* like Enteroaggregative *Escherichia coli* (EAEC).The organism has been implicated as a cause of diarrhoea among children, initially in developing countries but, more recently, in industrialized nations as well (Tompkins *et al*., 1999). Notable epidemiological studies have been made with the association of EAEC with persistent diarrhoeal syndromes (> 2 weeks' duration) (Nataro *et al*., 1998) which is an important clinical syndrome in indigent and immunocompromised populations, and understanding its aetiologies is a high public health priority (Black, 1993).

EAEC strains adhere to the small and large bowel mucosal surface in a thick aggregating biofilm (Vial *et al*., 1988; Tzipori *et al*., 1992). Thereupon, the pathogen secretes one or more enterotoxins, including the *Shigella* enterotoxin 1 (ShET1), the plasmid-encoded toxin (Pet) and/or the enteroaggregative ST-like toxin (EAST) (Czeczulin *et al*., 1999). Upon passage through the stomach, EAEC would experience conditions that are abruptly conducive to proliferation, including favorable pH, high available sugar concentration and appropriate osmolarity. Under such conditions, the bacteria would enter exponential growth phase, which induces the expression of genes involved in adhesion like AggR and AAF/II finally leading to biofilm formation. Thus it can be interpreted that the ability of biofilm formation influences the outcome of persistent diarrhea in a significant way.

Numerous bacterial species such as *Staphylococcus. aureus*, *Stereptococcus. epidermidis, Streptococcus. mutans, Pseudomonas aeruginosa, Salmonella spp., Pasteurella multocida* etc are also capable of producing biofilms (Miller and Bassler, 2001). *Pseudomonas aeruginosa* is remarkable in that it can cause both acute and chronic infections. Current concepts propose that biofilm formation is a key factor in chronic *Pseudomonas* airway infection in cystic fibrosis and bronchiectasis and chronic urinary tract and device-related infections (Parsek and Singh, 2003). It has been showed that *P. aeruginosa* also acquires biofilm-like features after entering epithelial cells (Fux *et al.,* 2005). The bacteria formed dense, pod-like aggregates within epithelia similar to those seen in mouse *P. aeruginosa* lung and *E. coli* bladder infection models (Anderson *et al.,* 2003). After entry, one paradigm of *P. aeruginosa* pathogenesis is that programmed cell death rapidly commences, leading to ejection of the epithelial cell filled with bacteria into the lumen followed by prompt removal by professional phagocytes (Pier, 2000). In summary, it can be said for early pathogenesis of *P. aeruginosa* epithelial cells provide a sanctuary for bacterial proliferation into biofilm-like communities and thus biofilm formation is a decisive factor in the outcome of Pseudomonas infections.

Hence, biofilms can be said as cellular communities with an ordered structure, circulatory system, displaying different physiological states within different regions and have a form of intercellular communication. They can resist noxious chemicals and other threats from their environment and are involved in dissemination of disease within body of infected individuals, so can be termed as niduses of infection. It's the rheostat of colonization and infection which if tipped in favor of overt infection, favors biofilm mode of growth of bacteria over planktonic growth. The universality of biofilm phenotype has been now equivocally accepted and efforts are now on to elucidate the genes specifically expressed by biofilms and evaluating various control strategies for either preventing or remediating biofilm colonization. Although the road ahead in understanding of biofilms looks thorny as many milestones are yet to achieve but this voyage is definitely worthsome as it gives us a chance to decipher one of the basic nuances of biology.

References

1)Watnick P & Kolter R. Biofilm, city of microbes. J.Bacteriol. 2000.182:2675-2679.
2) Costerton JW, Geesey GG, Cheng KJ. How bacteria stick. Sci Am. 1978; 238: 86-95.
3) Webster P, Wu S, Webster S, Rich KA and McDonald K. Ultra structural preservation of biofilms formed by non-typeable *H.influenzae*. Biofilm. 2004; 1: 165-82.

4) Hunter RC, Beveridge TJ. Application of a Ph sensitive fluoroprobe (C-SNARF- for pH microenvironment analysis in *Pseudomonas aeruginosa* biofilms. Appl Environ Microbiol. 2005; 71: 2501-10.
5) Raad I, Costerton W, Sabharwal U, Sacilowski M, Anaissie E, Bodey GP. Ultra structural analysis of indwelling vascular catheters: a Quantitative relationship between luminal colonization and duration of placement. J Infect Dis. 1993; 168: 400-07.
6) Stickler D, Morris N, Moreno MC, Sabbuba N. Studies on the formation of crystalline bacterial biofilms on urethral catheters. Eur J Clin Microbiol Infect Dis.1998; 17: 649-52.
7) Nickel JC, Downey J, Costerton JW. Movement of *Pseudomonas aeruginosa* along catheter surfaces. A mechanism in pathogenesis of catheter-associated infection. Urology. 1992; 39: 93-98.
8) Stoodley P, Wilson S, Hall-Stoodley L, Boyle JD, Lappin-Scott HM, Costerton JW. Growth and detachment of cell clusters from mature mixed –species biofilms.Appl Environ Microbiol.2001;67:5608-5613.
9) Raymond J, Armand-Lefevre L, Moulin F, Dabernat H, Commeau A, Gendrel D, Berche P. Nasopharyngeal colonization by *Haemophilus influenzae* in children living in an orphanage. Pediatr Infect Dis J. 2001; 20: 779-84.
10) Sekhar S, Kumar R, Chakraborti A. Role of biofilm formation in the persistent colonization of Haemophilus influenzae in children from northern India. J Med Microbiol.2009; 58:428-432.
11) St Geme JW III and Falkow S. Haemophilus influenza adheres to and enters cultured human epithelial cells. Infect Immun 1990; 58: 4036-4044.
12) Kaur H, Kumar P, Ray P, Kaur J, Chakraborti A. Biofilm formation in clinical isolates of group streptococci from north India. Microb Pathog. 2009; 46:321-327.
13) Loo CY, Corliss DA, Ganeshkumar N.Streptococcus gordoni biofilm formation: identification of genes that code for biofilm phenotypes.J Bacteriol. 2000; 182:1374-1382.
14) Mathur T, Singhal S, Khan S, Upadhay DJ, Fatma T, Rattan A. Detection of biofilm formation among clinical isolates of Staphylococci: an evaluation of 3 different screening methods. Indian J Med Microbiol. 2006; 24: 25-29.
15) Murphy TF, Kirkham C. Biofilm formation by nontypeable *Haemophilus influenzae*: strain variability, outer membrane antigen expression and role of pili. BMC Microbiol. 2002; 2: 7.
16) Olson ME, Ceri H, Morck DW, Buret AG, Read RR. Biofilm bacteria: formation and comparative susceptibility to antibiotics.Can J Vet Res. 2002; 66:86-92.
17) Nataro JP, Steiner T and Guerrant RL. Enteroaggregative E.Coli. Emerg.Infect.Dis.1998; 4: 251-261.
18) Black RE. Persistent diarrhea in children of developing countries.Pediatr.Infect.Dis.1993; 12: 751-761.
19) Vial PA, Robins Browne R, Lior H, Prado V, Kaper JB, Nataro JP, Maneval D etal. Characterization of enteroadherent-aggregative E.coli, a putative agent of diarrheal disease. 1988. J.Infect.Dis; 158:70-79.
20) Tzipori S, Montanaro J, Robins-Browne RM, Vial P, Gibson R and Levine MM. Studies with enteroaggregative E.Coli in the gnotobiotic piglet gastroenteritis model.1992.Infect Immun ; 60: 5302-5306.
21) Czeczulin JR, Whittam T, Henderson I, Navarro-Garcia F and Nataro JP. Phylogenetic analysis of virulence genes in enteroaggregative and diffusely adherent E.Coli. Infect Immun.1999; 67: 2692-2699.
22) Miller MB and Bassler BL. Quorum sensing in bacteria. Annu Rev Microbiol.2001; 55:165-199.
23) Parsek, MR and Singh PK**.** Bacterial biofilms: an emerging link to disease pathogenesis. Annu. Rev. Microbiol. 2003; 57:677-701.
24) Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms .Trends Microbiol.2005 ; 13 :34 - 40.
25) Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, and S. Hultgren SJ.Intracellular bacterial biofilm-like pods in urinary tract infections.Science.2003; 301**:**105-107.
26) Pier GB. Role of the cystic fibrosis transmembrane conductance regulator in innate immunity to *Pseudomonas aeruginosa* infections. Proc Natl Acad Sci. USA. 2000; 97**:**8822-8828.