PRACTITIONERS' SECTION

BACTERIAL BIOFILM FORMATION, PATHOGENICITY, DIAGNOSTICS AND CONTROL: AN OVERVIEW

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ABSTRACT

Bacterial biofilms are complex, mono- or poly-microbialn communities adhering to biotic or abiotic surfaces. This adaptation has been implicated as a survival strategy. The formation of biofilms is mediated by mechanical, biochemical and genetical factors. The biofilms enhance the virulence of the pathogen and have their potential role in various infections, such as dental caries, cystic fibrosis, osteonecrosis, urinary tract infection and eye infections. A number of diagnostic techniques, viz., bright-field microscopy, epifluorescence microscopy, scanning electron microscopy, confocal laser scanning microscopy and amplicon length heterogeneity polymerase chain reaction, have been employed for detection of these communities. Researchers have worked on applications of catheter lock solutions, a fish protein coating, acid shock treatment, susceptibility to bacteriophages, etc., for biofilm control. However, we need to rearrange our strategies to have thorough insight and concentrate on priority basis to develop new accurate, precise and rapid diagnostic protocols for detection and evaluation of biofilm. Above all, the strict compliance to these techniques is required for accurate diagnosis and control.

Key words: Bacterial, biofilm, control, diagnostics, E. coli, pathogenicity, pili, pseudomonas, streptococcus, vibrio

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Biofilms are the colonial way of life of microorganisms. More appropriately, they have been defined as complex microbial assemblages anchored to abiotic or biotic surfaces. This microbial assemblage may harbor single or multiple microbial populations or microcolonies. The cells are embedded in extracellular matrix, where they interact with each other and the environment. This miniature ecosystem provides a safe home for the members of the community, where they are untouched by the counter-defense mechanisms of host immune responses, phagocytosis and antibiotic treatment. Watnick and Kolter rightly called it as City of Microbes.[1] Biofilm formation has been observed by most of the bacteria found in natural, clinical and industrial setups.

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WHY ARE BIOFILMS FORMED?

It would not be absurd to say that the answer to this is still a matter of investigation. The voluminous studies are underway. The new

metabolic interaction, phylogenic grouping and ecological significance of this adaptation are being explored.[2] The mono-microbial or poly-microbial populations of the biofilm tend to live unitedly thereby as a single community which may exhibit a mutually beneficial relationship as evident in glucomannan-mediated pea-rhizobia symbiosis.[3] Contrary to this, there may be a host parasitic interaction with pathogenic manifestations by infectious organism.[4-8]

The biofilm formation has also been documented as survival strategy of pathogens.[9] Some microorganisms in biofilm can even modulate the pathogenic potential of bacteria as evident from cariogenic bacteria in plaque biofilms.

The biofilms have been reported to be less susceptible to antimicrobial agents and have reduced sensitivity to inhibitors, thereby adding to their survival.[10] The findings have shown delayed penetration of ciprofloxacin into *Pseudomonas aeruginosa* biofilms.[11] *E. coli* biofilms exhibited decreased susceptibility to cetrimide.[12] Similar reports are available in *Staphylococcus aureus* exposed to tobramycin.[13] The resistance shown by these biofilms, in general, has been attributed to factors such as poor penetration of antimicrobials, nutrient limitation, accumulation of toxic metabolites and decreased oxygen tension.[14]

The biofilms also act as safe niche for some organisms to survive protozoan grazing. The studies on *V. cholerae* showed that biofilms are the protective agents that enable the organism to survive protozoan grazing. Grazing on planktonic *V. cholerae* was found to select for the biofilm-enhancing rugose phase variant, which is adapted to the surface-associated niche by the production of exopolymers.[15]

HOW ARE THE BIOFILMS FORMED?

Researchers are of the view that the formation of biofilms is mediated by a number of mechanical, biochemical and genetical tools.

Besides this, certain physiochemical interactions such as cell surface hydrophobicity (long-range noncovalent interactions, defined as the attraction among apolar or slightly polar cells or other molecules immersed in an aqueous solution), charge, roughness and chemical constitution of the material have also been studied to mediate bacterial adhesion to the surface during biofilm formation.[16]

The studies on *Staphylococcus epidermidis* indicated that its adherence was to a higher extent to silicone substrate than to acrylic. This behavior has been attributed to higher surface hydrophobicity and roughness of silicone as compared to acrylic. [16]

The roughness of polymeric surfaces has also been implicated to some extent in promoting the adhesion of bacterial cells due to increased surface area and protection from shear forces during colonization.[17] The formation of biofilm on polyvinyl chloride (PVC), polyethylene (PE) and stainless steel surfaces has also been studied. It was observed that in general, the accumulation of biofilm on surfaces of different materials was quite similar. However, the cell volume was recorded to be slightly higher on PE surface than on PVC surface.[18]

Further, recent studies on *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* suggested that adhesion was dependent in pyrolytic carbon surface free energy and roughness. Thus, the improvement of pyrolytic carbon physicochemical properties has been suggested as a feature leading to reduction in valvular prosthetic infections.[19] However, the biofilms are formed preferentially at high shear locations which are even as small as heart valves.[20]

MECHANICAL TOOLS OR SURFACE FACTORS

The pili and flagella are generally involved as adhesive structures to help in attachment to the biotic or abiotic surfaces.[21,22] The role of attachment factor, cellulose fiber and lipopolysacchride (LPS) interactions to maintain strength and integrity in biofilm making in *Pseudomonas fluorescens* SBW25 has also been studied.[23] The requirement of type IV pili has been implicated in maximal biofilm formation by *Clostridium perfringens.*[24]

Biochemical tools

Biofilm formation appears to be influenced by large-scale changes in protein expression over time. There is an increased production of proteins involved in attachment, resistance and virulence as the biofilm develops. The evidence is available on characterization of temporal protein production in *Pseudomonas aeruginosa* biofilms.[25] Scientists are of the view that a novel histone-like nucleoid structure–like protein is involved in the formation of lateral flagella and that it has a role in biofilm formation in *Vibrio parahaemolyticus.*[26] Moreover, the soluble colonization factor, TcpF, in different serotypes of *Vibrio cholerae* has also been studied as a tool in biofilm formation.[27] Some amino acid residues have also been identified to have a role in the plague biofilm Hemin storage (Hms) proteins.[28] Moreover, the roles of proteins exported via the PrsD-PrsE Type I secretion system; and RbmA, a novel protein, have been well documented in biofilm formation in *Rhizobium leguminosarum* and *V. cholerae,* respectively.[15,29] There exists interplay between cyclic AMP–cyclic AMP receptor protein and cyclic di-GMP–signaling biofilm formation in *Vibrio cholerae.*[30] A cyclic-di-GMP phosphodiesterase has been found to inversely regulate biofilm formation in *Pseudomonas aeruginosa.*[31] The role of HtrA gene in surface protein expression and biofilm formation by *Streptococcus mutans* has also been studied.[32]

Genetical tools

Biofilm formation is said to be under genetic control. A number of workers have worked on genetics of biofilm formation, especially in medically important bacteria.[33,34] The biofilm formation in *Bordetella,* especially *B. pertussis,* the causal organism of whooping cough, has attracted the attention of medical fraternity due to evidences of high antimicrobial tolerance and contribution to persistent infections.[35] In detailed studies on *Bordetella,* a gram-negative bacterium harbored in respiratory tract of humans and animals, it has come to light that the biofilm development is regulated by BvgAS signal transduction system.[35] This regulatory system is said to regulate the expression of almost all known or suspected colonization and virulence factors currently associated with infection of the said organism.

The genetics of biofilm formation in *Pseudomonas* and *E. coli,* the important human pathogens of otitis media and urinary tract infection, has also been documented. A three-component regulatory system in *Pseudomonas aeruginosa* and the transcriptional antiterminator RfaH in *Escherichia coli* have been found to regulate and repress biofilm formation, respectively.[36,37]

Biofilm formation has also been attributed to the quorum-sensing system. Quorum-sensing is a cell-to-cell signaling which allows the bacteria to react to environmental changes in order to survive and thrive. AlgR repression of the Rhl quorum-sensing system in a biofilm-specific manner has also been stated in *Pseudomonas aeruginosa.*[38] The rapA gene controls the antibiotic resistance of biofilms of *Escherichia coli,* thereby assisting in survival of the organisms in this mode.[39] In depth studies have shown that cell density–dependent regulator *hapR* controls the production of the factor in biofilms. Researchers have also focused their attention on gene expression within a catheter-associated urinary tract infection biofilm model.[40] The studies on *Staphylococcus aureus,* an important biofilm former on medical implants and host tissues, showed that the quorum-sensing system was turned on by auto-inducing peptides (AIPs). It has been reported that the *agr* quorum-sensing system of this organism modulates the expression of virulence factors in response to AIPs. Further to it, it has been demonstrated that repression of this system forms the biofilm, and reactivation in established biofilms disperses the cells.[41] The dispersal or detachment in staphylococcal biofilms has been studied as a protease-mediated process, where the extracellular protease production increased as a result of activation of quorum sensing. Thus, manipulating the protease gene and using quorum sensing as a tool have been suggested to modulate the treatment of *S. aureus* biofilms.[41]

BIOFILMS AND PATHOGENICITY

It has been well documented that biofilms add to the virulence of the pathogen. It has been estimated that the frequency of infections caused by biofilms, especially in the developed world, lies between 65% and 80% as per reports from Centres for Disease Control and Prevention (CDC) and National Institutes of Health (NIH), respectively.[42] Many food-borne pathogens such as *E. coli, Salmonella, Yersinia enterocolitica, Listeria, Campylobacter* form biofilms on the surface of food or storage equipments. Moreover, the potentially pathogenic bacteria, viz., *Staphylococcus aureus, Enterococcus faecalis, Streptococcus, E. coli, Klebsiella, Pseudomonas,* tend to grow on catheters, artificial joints, mechanical heart valves, etc. Thus, these organisms can lead to persistent infections as a result of periodic release from the said focus.[20,42] In *Pseudomonas aeruginosa,* the localized depletion of nutrition in a biofilm has been hypothesized as inducer for release or detachment of cells from the biofilm.[43] However, in general, factors such as microbially generated gas bubbles, cross-linking cations, growth status, contact surface material, shear stress, quorum sensing and activation of lytic bacteriophages have been considered to be important contributors in biofilm detachment.

The biofilm activity has been recorded in various infections, viz., dental caries, cystic fibrosis, osteoradionecrosis, urinary tract infections, native valve endocarditis, otitis media and eye infections.

The pathogenic and commensal isolates of *Histophilus somni* have been characterized for biofilm.[44] The studies have shown association of *E. coli* and *Proteus mirabilis,* important uropathogens, biofilms in patients with complicated catheter-associated urinary tract infections.[45] The recurrent epidemics of cholera have been explained as the bacterial ability to form biofilms with biotic and abiotic surfaces of aquatic ecosystem.[46] The different studies evidenced distribution of bacterial proteins and greater disease burden attributable to biofilm formation by *Haemophilus influenzae* in cases of otitis media.[47,48] The biofilm formation has been observed in clinical isolates of *Staphylococcus aureus.*[49]

Dental caries has also been potentially attributed to the plaque biofilms.[50,51] Recent studies have focused on the role of biofilms in eye infections.[52] The biofilms in such cases have been generally associated with corneal infection through contact lens.

DIAGNOSTICS OF BIOFILM

Accurate diagnosis is the key to better understanding the biofilm, harness its beneficial effects and curb deleterious after effects. Despite the potential benefits of biofilm formation, the thrust is on the detrimental effects of this adaptation. The identification of biofilms in persistent infections may assist in deciding suitable therapies. A large number of techniques are being used to study biofilms. The diagnosis starts with establishing the surface-associated biofilms using bright-field microscopy, epifluorescence microscopy, scanning electron microscopy. Confocal laser scanning microscopy (CLSM) has further made it easy to carry out *in situ* examination of biofilms using lower magnification.[20] Activity of destructive and nondestructive biofilms is measured by employing radioisotopic and nonradioisotopic methods. Radioisotopic methods are cumbersome and require trained personnel and safe handling.[53] The introduction of molecular diagnostic methods has linked bacterial biofilms to many infections. Investigations have been carried out on assessment of diversity of the microbial community in biofilms by using Amplicon length heterogeneity polymerase chain reaction.[54] Further, differential expression of proteins in biofilms also offers a reliable opportunity for identifying the biofilm-specific proteins as basis of diagnosis and treatment. The extracellular matrix proteins may also be useful detection targets for diagnosis of biofilms.

CONTROL OF BIOFILMS

Attempts have been made to devise fruitful strategies to control biofilms. The acid shock treatment on proteins expression and upregulation of stress-responsive proteins during acid tolerance in biofilm cells of Streptococcus mutans has been documented.[55,56] The acid is said to affect the physiology of biofilm cells of Streptococcus mutans.[57] The blocking of bacterial biofilm formation using catheter lock solutions in staphylococcal biofilm formation on abiotic surfaces, by a fish protein

coating and synergistic activity of dispersin B and cefamandole nafate in inhibition of staphylococcal biofilm growth are some of the important works carried out in this field.[58-60]

Recent advances focus on bacteriophages as specific and effective therapeutic agents with lytic action against target bacteria. Thus, combination of antibiotics and bacteriophage application has been suggested as a valuable approach for biofilm control. The phage philBB-PF7A showed 63% to 91% activity for biomass removal in *Pseudomonas fluorescens,* an important food spoilage pathogen.[61] Phage specific for *Enterobacter* was demonstrated to control biofilm by depolymerase activity on polysaccharide. Similarly, in *Pseudomonas aeruginosa,* depolymerase enzyme reduced the viscosity of alginates and the EPS of the organism, thereby leading to dispersal of biofilm.[62] The dual approach of impregnation of medical devices with phages and incorporation of phages in hydrogel coating of catheter has proven its efficacy, especially in *Staphylococcus epidermidis.*[62] The vulnerability of pathogenic biofilms to *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus* attack has been documented.[63,64] However, recent studies have shown the dispersal of films by using genetically engineered bacteriophages.[65]

It has been suggested that further understanding of the composition and function of extracellular matrix proteins may hold the key to controlling biofilm infections and that proteins specifically expressed by biofilm bacteria may be useful targets of therapeutic interventions.

POSSIBLE BASIC APPROACH

Biofilms have attracted the attention of the entire science fraternity. Undoubtedly, progressive efforts have been made for better understanding of this adaptation. Some of the key investigations focus on pathways for transition from solitary to biofilm mode, the biochemistry and genetics involved and the efficacy of antimicrobials in biofilm dispersal. However, the basic areas also need to be addressed more emphatically to devise successful methods to control its detrimental effects. Biofilm accumulation has multifactorial control determined by its balance of attachment, proliferation and detachment processes and that the biofilms resist antimicrobial action and host defenses

In routine laboratory medicine practices, careful correlation of various parameters such as persistent infections, co-infections, unresponsiveness to antimicrobials, incremental release of microorganisms from the foci and repeated contaminating sources, to biofilm formation may act as a key tip-off for timely diagnosis and subsequent control of the biofilms.

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