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Biofilm: multicellular living of the unicellular bacteria

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Abstract

Unicellular bacterium in nature prefers to gather round to form a surface attached multi-cellular consortium called biofilm rather than living as an isolated planktonic cell. Biofilms comprise of the bacterial cells attached to a biotic or abiotic surface and the extracellular polymeric substances excreted by the participant cells. Many bacteria can detect environmental signals and respond accordingly to form biofilm and to detach from it. Formation of biofilm is crucial for the survival of the bacteria in the environment and for their interaction within and out of the species. Cells within biofilms are distinct from the free swimming planktonic cells – both physiologically and genetically. Such distinctive features are crucial for the maintenance of the biofilm structure. Biofilms provide the bacteria with various survival and metabolic advantages over the planktonic form. Mixed species biofilms better resemble the environmental biofilm consortia where a group of related bacteria gather onto a single surface and interact among them for the betterment of the whole community. This review discusses about the basic steps of biofilm formation and the specialties of this unique bacterial architecture.

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Introduction

Bacterial living system is one of the most primitive and probably the most successful within the biosphere. This success speaks about the special abilities of different bacterium to adapt with the environment, manipulate their own lifestyle and persist for a prolonged time surviving toxic or adverse conditions. Formation of biofilm is central to the idea of most of the advantages that a bacterium can achieve. Biofilm is an aggregate of bacterial population attached to a solid surface consisting of microcolonies of bacterial cells, separated from each other with intervening water-filled channels (Costerton *et al*., 1995; Karatan and Watnick 2009; Shrout *et al.,* 2011). In natural environment, these microcolonies may provide habitats for different bacterial species which reside close together and interact in different stages of their life cycle. Biofilm is the dominant form of bacterial life in the environment and the free swimming planktonic bacterium can be thought as one that has left a biofilm recently and looking to initiate a new one on a suitable surface (Fey and Olson 2010; Costerton *et al.,* 1987). By forming biofilms, bacteria can avail themselves various survival and metabolic advantages, which include survival in a nutrient deprived environment, resistance to environmental stresses, resistance to biocides and antibiotics, better abilities for the acquisition of foreign genes and many others (Brown and Gilbert 1993; Davey and O'Toole 2000; Watnick and Kolter 2000). Within a multiple species biofilm, different bacterial can even share their metabolic abilities and replenish the disabilities of others to gather nutrients from sources that are unavailable to planktonic cells of individual species (Thiele *et al.,* 1988; Lemaire *et al.,* 2008; Schramm *et al.,* 1996). Such abundance, resilience and excellence of biofilm structures have attracted many research works in this field which have brought new lights on the fine structure of biofilms, genetic features of cells within biofilm and communication among different species within a biofilm.

Steps of biofilm Formation

Biofilm is the multicellular organization of unicellular bacterium, which comprises of cells of a single or multiple bacterial species and their exopolymeric substances. This structure is not just a clump of cells, but a well organized architectural setup. Surface attached microcolonies are the basic structural unit of biofilm and the intervening channels are probably the next important feature of the structure (Costerton *et al.,* 1995; Stoodley *et al.,* 1994; DeBeer and Stoodley 1995). Exopolymeric substances are secreted by the cells within the microcolonies and these substances seal the whole biofilm structure (Allison and Sutherland 1987; Allison *et al.,* 1998). Microscopic images of some experimental biofilms are shown in Fig. 1 (a, b).

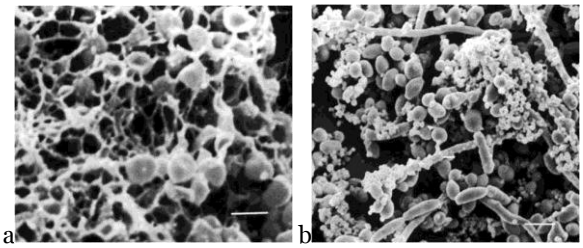


Fig. 1. Scanning electron micrographs of laboratory formed biofilms: single species biofilm of Staphylococcus epidermidis RP62A (a) and mixed species biofilm of Candida albicans and S. epidermidis M7 (b) (Adam *et al.,* 2002).

Biofilm formation is a multi-step process which is initiated with the attachment of a single bacterium onto a suitable surface. Once the free-swimming bacterium reaches a suitable surface, it slows down and eventually sticks onto the surface. This event initiates the development of a microcolony where thousands of cells are attached either to the surface or to other surface attached cells (Shalá *et al.,* 2011; Prüß *et al.,* 2006). Attachment is followed by the overproduction of exopolysaccharides by the attached bacterial cells which helps to immobilize the loosely attached cells and hold the biofilm structure with great strength (Davey and O'Toole 2000; Watnick and Kolter 1999; Yildiz and Schoolnik 1999). A mature biofilm

comprises of a number of such microcolonies separated by void spaces which possibly carry water and nutrients inside the biofilm (Figure 3.b and Fig. 2). The sequential steps of biofilm formation are shown using schematic diagrams in Figure 3.a.

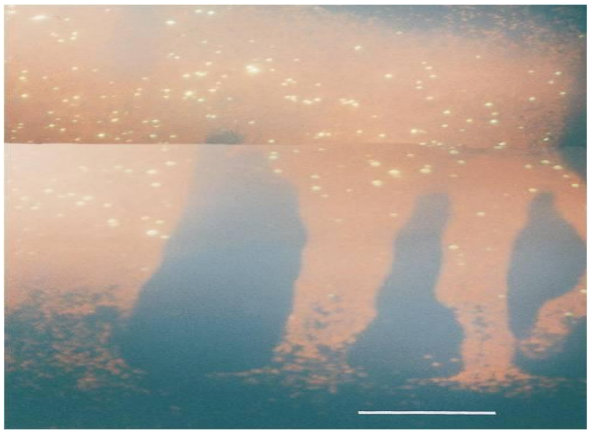


Fig. 2. Photomicrograph of *Klebsiella pneumoniae* biofilm cross section stained for APase activity (Huang *et al.,* 1998).

The physiological and genetic features of the bacterial cells change with the development of biofilm in accordance with the requirements of the developing structure. Some genetic features are crucial to the formation of the biofilm and cells lacking these features are unable to form biofilms. Attachment to surface is elementary to the formation of biofilm and bacteria employ their flagella and/or type IV pili in this purpose (Pratt and Kolter 1998; Watnick *et al.,* 1999). Mutants that lack flagella or type IV pili cannot make stable interactions with a surface and so are unable to form biofilms. In case of *Pseudomonas aeruginosa* such mutants have been termed as surface attachment defective (*sad*) mutants (Pratt and Kolter 1998; O'Toole and Kolter 1998). After the initial attachment has formed exopolysaccharide (EPS) production is essential for a firm and stable biofilm structure and experimental biofilms of EPS mutant bacterial cells have been found to be unstable and less rigid compared to wild type biofilms (Yildiz and Schoolnik 1999).

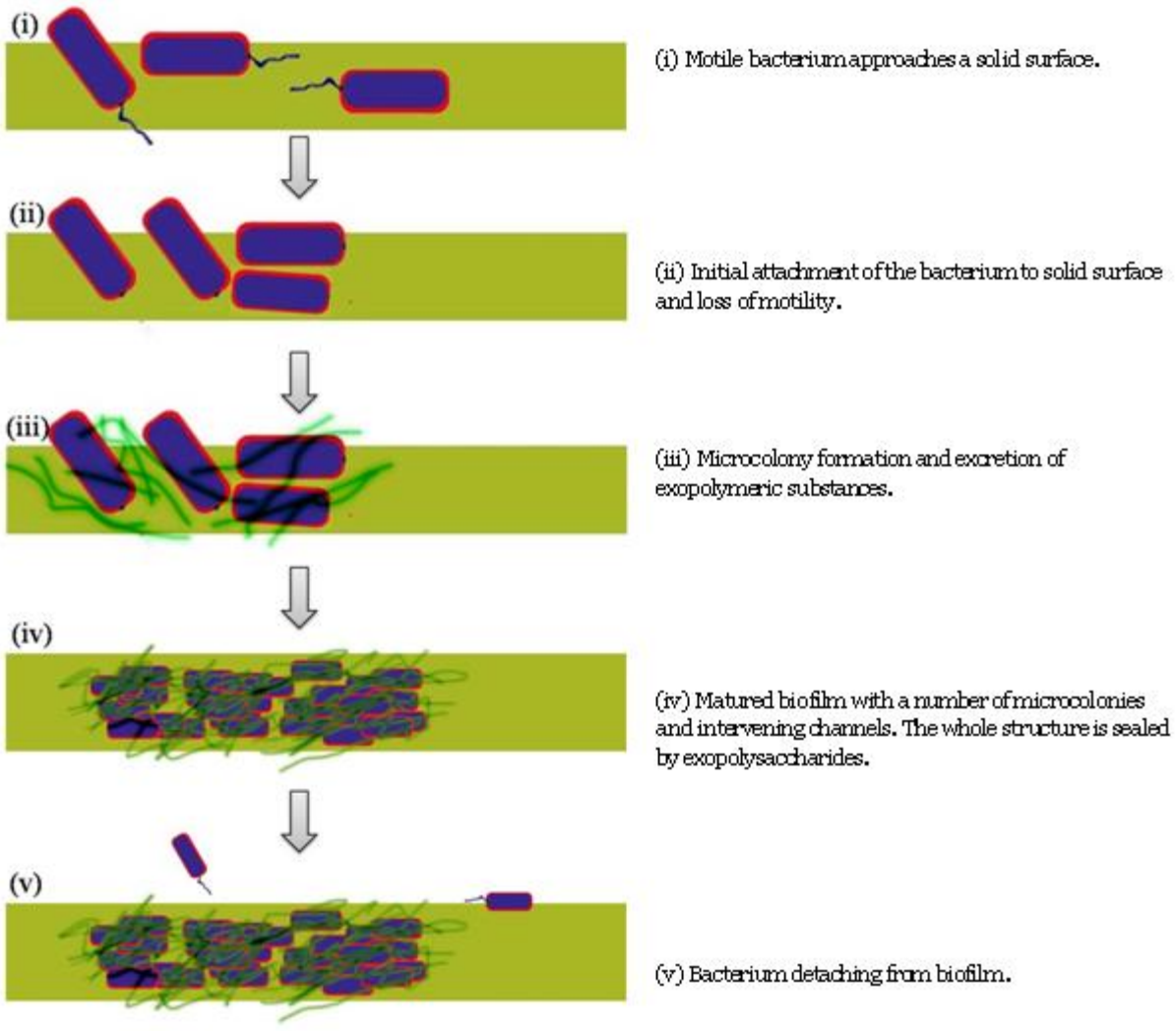
Bacterial cells can even come out of the biofilm if necessity arises. Under starving condition or in response to particular environmental signals, bacterial cells can dissolve the exopolysaccharide and come out of the biofilm, perhaps to look for a nutrient source and form biofilm onto a new surface (McDougald *et al.,* 2012; Fey and Olson 2010; Allison *et al.,* 1998). This particular property of bacteria is crucial for their long term survival in the environment.

Cellular features of biofilm

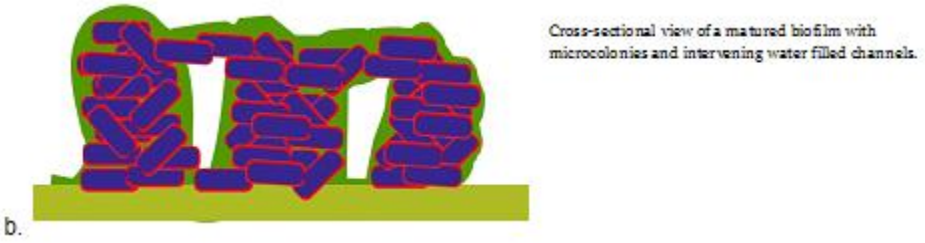
Cells in a biofilm exhibit distinct cellular and genetic properties in comparison to planktonic bacterium. Gene expression profile of the biofilm associated cells is significantly different from that of planktonic cells (Pratt and Kolter 1998; O'Toole and Kolter 1998; Prigent-Combaret *et al.,* 1999; Genevaux *et al.,* 1996). Any alteration in gene expression is expected to contribute a physiological or cellular property that better suits the biofilm associated cells and sums up to strengthen the whole biofilm structure. Decreased flagellin synthesis and increased exopolysaccharide synthesis by the biofilm associated cells are the most common among such alterations (Davies and Geesey 1995; Garrett *et al.,* 1999). Table 1 shows some gene expression alterations that are found in most of the biofilms irrespective of the bacterial species.

EPS matrix composition was also found to be different in biofilm associated cells. Accordingly, lyase activity – the machinery that biofilm associated cells employ to cut loose from the biofilm, is also different in biofilm derived and planktonic cells (Allison *et al.,* 1998). Another interesting observation was the increased expression of catalase and superoxide dismutase gene – which confer resistance to oxidative stress. Experimental H_2O_2 exposure revealed that wild type biofilms were 14-fold more resistant to such stress than wild type planktonic cells (Hassett *et al.,* 1999).

Within the biofilm the bacterial cells maintain a uniform gene expression pattern and act in unison.



a



b.

Fig. 3. Basic steps of biofilm formation are shown with a schematic diagram (a). Here the blue bacterium approaches a solid surface and forms biofilm after some sequential steps. Small appendages on the bacterium represent the flagella. Green meshworks represent the exopolymeric substances. A cross-sectional view is also shown for better understanding of biofilm architecture (b).

Table 1. List of the genes with altered expression in biofilm formed cells.

Genes upregulated	Genes downregulated	Function of the genes
	flagellin	Structural unit of flagellum that is responsible for bacterial locomotion.
EPS		Polymers of various carbohydrate and non-carbohydrate chemicals and found external to the bacterial cell.
Catalase		An antioxidating enzyme that catalyzes the decomposition of hydrogen peroxide.
Superoxide dismutase		An antioxidating enzyme that clears superoxide radicals.

Bacterial cells synthesize small molecules that can diffuse away and initiate a defined set of responses when received by a neighbouring cell – a process which is known as quorum sensing and better understood for single species biofilms (Waters and Bassler 2005). Quorum sensing is vital for three dimensional packing and maturation of biofilms, as the lack of this pathway results in the loss of integrity and proportion from the biofilms (Shrout *et al.,* 2011; Hentzer *et al.,* 2002; McLean *et al.,* 1997; Stickler *et al.,* 1998). Quorum sensing pathway mutants can attach to and gather onto inert surfaces, but such biofilms are much thin compared to that of wild type cells. Also they lack the characteristic three dimensional structures of microcolonies and intervening water-filled channels. These mutants can be easily dispersed and removed from a surface by the treatment of common biocides or detergents, while removal of wild type biofilms require much higher concentrations of the same biocides (Davies *et al.,* 1998).

Bacterial biofilms and environment

Biofilm has been suggested by many research works to be the dominant form of bacterial living in natural habitats (McDougald *et al.,* 2012). A number of different bacterial species have been found to exist as biofilm formed cells in the environment. Such biofilms ensure persistence of the bacterium in the environment and have great influence upon the environmental and medical complications associated to the species. Every bacterium responds to some environmental stimulus to cycle between biofilm and free swimming forms (Karatan and Watnick 2009; Walther *et al.,* 2002). Some bacteria even have their unique set of properties for their biofilms and the stimulus they respond to. We will now focus on the *Vibrio cholerae* biofilm formation and their interaction with the environment. Both toxigenic and nontoxigenic strains of *V. cholerae* are natural inhabitants of aquatic environments, both marine and freshwater that provides the environmental reservoir of virulent *V. cholerae* strains (Colwell 1996; Colwell and Huq 1994). A number of studies in recent past have demonstrated the surface-associated biofilms on zooplankton, phytoplankton and other suspended particulates as an important strategy in the life cycle of *V. cholerae* for the persistence and accumulation in nature (Huq *et al.,* 1983; Tamplin *et al.,* 1990). Biofilms generally have been proposed to constitute an environmental refuge for *V. cholerae* as well as a number of other bacterial pathogens and to provide an adaptive advantage promoting their environmental persistence (Hall-Stoodley and Stoodley 2005; Kierek and Watnick 2003; Parsek and Singh 2003). The central role of biofilm formation in the persistence of *V. cholerae* in natural habitats raises the question how and by what mechanisms environmental factors select for biofilm-associated phenotypes.

A number of factors are responsible for the biofilm formation at different stage. Flagella and fimbriae are necessary for the initial adherence to the solid surface

(Schembri *et al.,* 2003; Whiteley *et al.,* 2001). However exopolysaccharides (EPS) is the prerequisite for vibrio biofilms and its synthesis occurs after the attachment to solid surface and microcolony formation. Three distinct signaling pathways are known so far for EPS production in *V. cholerae*. Each may operate via discrete signals and/or microenvironments, including (i) a quorum-sensing pathway, where the transcriptional regulator HapR is the main key and its absence results in enhanced EPS synthesis and biofilm formation (Hammer and Bassler 2003; Zhu and Mekalanos 2003; Vance *et al.,* 2003); (ii) a flagellum-dependent pathway, where nonflagellated cells induce the expression of EPS synthesis and biofilm formation (Lauriano *et al.,* 2004; Watnick *et al.,* 2001); and (iii) a phase variation pathway that yields two distinct morphological variants termed smooth and rugose (wrinkled) (Yildiz *et al.,* 2001). Rugose variants have a better aptitude to produce EPS (Yildiz and Schoolnik 1999). EPS synthesis is encoded by the *Vibrio* polysaccharide (*vps*) genes (Watnick *et al.,* 2001) and its expression is associated with virulent factors which are regulated by HapR (Hammer and Bassler 2003). Therefore, *hapR* mutants are rugose (Jobling and Holmes 1997), leading to thicker biofilms and enhanced production of virulence factors (e.g., cholera toxin) (Zhu and Mekalanos 2003; Miller *et al.,* 2002).

As a waterborne pathogen *V. cholerae* is known to transit between host intestine and aquatic environment during epidemics of cholera (Faruque *et al.,* 1998) and biofilms have been proposed to influence the transmission (Faruque *et al.,* 2006). Biofilms also enhance the formation of conditionally viable environmental cell (CVEC) (Kamruzzaman *et al.,* 2010) which is known as non-culturable form of *V. cholerae* (Colwell and Huq 1994) but can revive into fully culturable and virulent form when inoculated into the ileal loops of adult rabbit (Faruque *et al.,* 2006). It reflects the potentiality of CVEC to cause diseases even by remaining in undetectable state by conventional culture method.

Advantages of living within biofilm

Living within biofilm facilitates the bacteria with a number of advantages over the planktonic form. Attachment to surfaces enables the bacterium to persist within a favourable environment - having sufficient nutrient supply and better survival conditions, because of the reduced chance of getting carried away by the flow of air or water as happens for planktonic bacterium. Biofilm ensures prolonged survival of the bacterium in any environment – both by altering the metabolic profile of the bacterium and providing protection from environmental adversities. EPS matrix of the biofilm structure can help the bacterium survive various environmental stresses, such as UV radiation, acid-base imbalance, osmotic shock etc. and the altered physiology of the cells in biofilm enables them to live significantly longer than planktonic cells.

Biofilm associated cells also exhibit increased resistance to antibiotics and biocides – which are commonly used to eradicate bacterial contamination. EPS matrix can limit the access of such components into the biofilm, protecting the cells within the biofilm from associated toxicity. In an experiment with the dual species biofilm containing *P. aeruginosa* and *Klebsiella pneumoniae*, Cl^- concentration within the biofilm was found to be only 20% or less of the bulk liquid concentration (DeBeer *et al.,* 1994). Increased gene acquisition capability is another important advantage of biofilm living. Bacterial conjugation is a common method of gene transfer within and between related species, and within biofilm the rate of conjugation is much higher, rising as much as 1000 times that of planktonic cells (Hausner and Wuertz 1999). This is probably a result of stable cell-to-cell contact within the biofilm microenvironment (Angles *et al.,* 1993). Accelerated gene acquisition may

eventually lead to the emergence of new strains with novel degradative, virulent and survival capabilities.

Mixed-Species biofilms

Most of the knowledge regarding bacterial biofilms are obtained through laboratory cultured biofilms, which are more often single-species set ups. Mixed-species biofilms are more likely and more resembling of a natural setting, because of the number of different species in the environment and natural competition and interaction among them (Hansen *et al.,* 2007; Yamada *et al.,* 2005; Wolfaardt *et al.,* 1994). Overall structural features are similar for both laboratory cultured single-species biofilms and natural mixed-species biofilms. But the later involves some additional considerations resulting from both competition and cooperation among the participating species (Moller *et al.,* 1996; Moller *et al.,* 1998). Within mixed-species consortia, spatial distribution and relative abundance of different species can be altered in response to various signals and such alterations are most usually designed for the better functioning of the whole consortia (Moons *et al.,* 2009; West *et al.,* 2006; Huang *et al.,* 1998).

Relative abundance of a particular species within a mixed-species biofilm depends on its metabolic ability, growth rate on the available nutrients, colonizing ability and behaviour of the other participating species. Consequently, alteration of the available nutrient source might lead to an altered pattern of species abundance. In an experiment, sulphate reducing bacteria was found to be increased from 15% to 30-40%, when sulphate was added to an anaerobic biofilm that was generated using glucose as the sole energy source (Hansen *et al.,* 2007). Colonizing ability is a prerequisite for all the biofilm forming species, but it is particularly crucial for those with lower growth rates. For example, in a condition that favours much higher growth rate of *K. pneumoniae*, *P. aeruginosa* can co-exist in a dual-species biofilm only because of its excellent colonizing ability (Stewart *et al.,* 1997). When toxic substance is present in the medium, the species with resisting ability will account for most of the biofilm mass. The sensitive strain will survive only under protection from the resistant strain – either by physical shielding or detoxification of the substance (Tait and Sutherland 2002; Rao *et al.,* 2005; Moons *et al.,* 2006).

Community level processes: Metabolic cooperativity is an outstanding feature of some particular mixed-species biofilms. There are many complex chemical molecules which are not degradable and so cannot be utilized as a source of energy by common bacterial species. Some of these molecules can be degraded and utilized as energy source by a consortium of interacting species, while no single member of the consortium was able to degrade the molecule alone (Palmer *et al.,* 2001; Lappin *et al.,* 1985). Such multi-species consortiums are required for the degradation of many xenobiotic compounds, like chlorinated herbicides, alkylbenzene sulfonates, naphthalene derivatives and nitrate esters (Jiménez *et al.,* 1991; Hoefel *et al.,* 2006; Wolfaardt *et al.,* 1995; Breugelmans *et al.,* 2008). Biofilms comprising of such consortium distribute the populations so as to facilitate sufficient interactions among them, like interspecies substrate exchange (Nielsen *et al.,* 2000; Christensen *et al.,* 2002).

Conclusion

Surface attached biofilms have been described as the preferred bacterial living system for natural settings. This particular lifestyle has been noted to be advantageous for the bacterium in various aspects – including prolonged survival in minimal supply of nutrients, resistance to adverse conditions and better abilities to transfer genes between cells. With all these advantages biofilm plays a very critical role to the environmental and clinical complexities associated with many different bacterial species. To better understand the life cycle, environmental existence and pathogenesis of these bacteria knowledge of their biofilms would be very useful. Until now, most of the

research works regarding biofilms have been concentrated on laboratory derived single species biofilms. More researches targeting the multi-species environmental biofilms are required to further elucidate this bacterial hallmark and for designing possible preventive measures against the pathogenic biofilms. Mixed species metabolism can be another interesting field for study. Elucidation of the interactions among different bacterial species within environmental mixed-species biofilms may bring out novel characteristics to be exploited as beneficiary to environment or human health. It is true that a good number of researchers have worked on different bacteria and their biofilms, and their works have enlightened many of the mysteries regarding bacterial life. But for the ultimate appreciation of the bacterial life and their contribution towards the environment and human health, bacterial biofilms need to be more explored.

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