



Review Article

Pseudomonas Aeruginosa Biofilm: Role of Exopolysaccharides in the Function and Architecture of the Biofilm

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ABSTRACT

More than 90% bacteria live as a biofilm in natural habitats. This surface-attached, sessile mode of life confers many advantages to bacteria and increases their survival. *Pseudomonas aeruginosa*, an opportunistic pathogen, has been extensively studied as a model organism for biofilm studies. Biofilm formation is a stepwise process in which motile bacteria attach to a surface and initiate a cascade of changes. Consequently these bacteria become phenotypically different from their planktonic counterparts. Initially these bacteria attach reversibly to the surface via hydrophobic interactions between the bacterial surface proteins and the substratum. In response to environmental cues, intracellular concentration of a secondary messenger, c-di-GMP, increases in the bacterial cells. This increase in c-di-GMP results in loss of motility, expression of specific adhesins, and irreversible attachment of the bacteria. These bacteria start proliferating while residing in close proximity. The bacterial cells sense the presence of other community members in their close proximity, and start communicating with each other through a mechanism known as quorum sensing. These bacterial cells produce and recruit extracellular polymeric substances (EPS) which encase the bacteria and protect them from unfavourable environmental conditions, predators and biocidal compounds. Components of the EPS include eDNA, proteins, lipids and exopolysaccharides. The exopolysaccharides produced and secreted by the bacteria play a vital role in biofilm formation. *Pseudomonas aeruginosa* produces various types of exopolysaccharides, but three of them are considered very important in biofilm formation; mannose-rich Psl, glucose-rich Pel, and uronic acid-rich alginate. These three exopolysaccharides are synthesized and secreted by the protein-complex encoded by their respective operons. The role played by these exopolysaccharides in architecture and function of biofilm is currently under extensive research. This review presents the mechanism of biofilm formation and the role of exopolysaccharides in biofilm.

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INTRODUCTION

For a long time in the history of microbiology, microorganisms were considered as freely suspended cells in nutritionally rich media and were characterized on the basis of their growth characteristics in the media provided. Heukelekian and Heller (1940) showed for the first time that the presence of a surface for attachment substantially enhanced the bacterial growth. A subsequent study demonstrated that the number of bacteria on the surface were more as compared to their surrounding environment. These indirect evidences provided clues that bacteria can grow as a community after attaching to the surface (Zobell, 1943). This community of surface-attached bacteria is now known as biofilm. However, a detailed study of biofilm was made possible only after the use of electron microscopy. (Jones et al., 1969) used electron microscopy to show that biofilm formed in seawater on a trickling filter was composed of many different kinds of organisms. With the

help of polysaccharide-specific stains, these workers also provided evidence for the presence of polysaccharides in extracellular matrix of bacterial biofilms. In subsequent years it was shown that microbial biofilms in industrial waste water were not only very persistent but also highly resistant to disinfectants such as chlorine (Characklis 1973). Later, a theory was proposed which elucidated the mechanism through which microorganisms attach to a surface and the advantages amassed by the microorganisms from this ecologic niche (Costerton et al., 1978). In the last two decades, the use of scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) and molecular biology techniques to study gene regulation have helped to increase our understanding of architecture and functioning of biofilms.

MICROBIAL BIOFILMS

Biofilm is a consortium of microbes which is irreversibly attached (not removed by gentle washing) to a surface and enclosed in a matrix of extracellular polymeric substances (EPS). The EPS constitute 50– 90% of the total organic mass of the biofilm and is composed of exopolysaccharides, extracellular DNA (eDNA), proteins, lipids and humic substances (Flemming H-C et al., 2000). The extracellular polymeric substances encased microbescells form microcolonies which are separated from each other by the presence of water channels (Lewandowski, 2000). These water channels allow liquid to flow through them which facilitates transfer of nutrients, oxygen, waste metabolites and even antimicrobial agents into the surrounding microcolonies

It is suggested that more than 90% of bacteria live in biofilm mode (Costerton et al., 1995; Geesey et al., 1977). Inside the biofilm, cells living in close proximity can have an increased rate of gene transfer, well developed cooperation and quorum sensing [reviewed in (Donlan, 2002)]. Furthermore, compared to planktonic bacteria, biofilms can better endure the negative consequences of pH changes, nutrient scarcity, free radicals, biocides and antimicrobial agents [reviewed in (Costerton et al., 1995)].

Importance of Microbial Biofilms

Microbial biofilms impact human life in different ways. Bacterial biofilms on medical devices such as catheters, endoscope tubes, contact lenses and implanted prosthetic devices, are a known source of nosocomial infections [reviewed in (Starkey et al., 2009)]. Microorganisms form biofilms on internal and external human surfaces and cause various infections including dental plaques, skin infections, urinary tract infections and chronic lung infections [reviewed in (Costerton et al., 1999)]. Recent studies have shown that chronic lung infections in cystic fibrosis (CF)

patients and some persistent wound infections are due to a biofilm forming microorganism, *Pseudomonas aeruginosa* (Bjarnsholt et al., 2009; DeVault et al., 1990; Harmsen et al., 2010; Kirov et al., 2007).

Biofilm forming microbes also cause infections in animals. Otitis (both, media and externa), a chronic and 'hard to eradicate' infection, is caused by *P. aeruginosa* and *Staphylococcus intermedius* in dogs (Colombini et al., 2000; Petersen et al., 2002). The persistent nature of chronic mastitis in dairy animals is attributed to the ability of the microorganisms to form biofilms [reviewed in (Melchior et al., 2006)]. Biofilms in the food processing industry have also been recognized as a big problem. Bacteria can adhere and grow as biofilm in pipelines. These biofilms are difficult to eradicate due to their resistant nature, and become a continuous source of bacterial contamination [reviewed in (Poulsen, 1999)].

Microbes also form biofilms in the gastrointestinal tract of humans and animals. These biofilms provide an ecological niche where bacteria can interact with each other and with the host cells. These biofilms develop a relationship with the host which may be beneficial or harmful for the host [reviewed in (Macfarlane and Dillon, 2007)].

These diverse effects of biofilms on human and animal lives described above have attracted a lot of attention by researchers. Although many different bacterial species have been used to study the architecture, function, regulation and control of biofilms, a lot of research has been conducted using *P. aeruginosa* as a model organism. Medical relevance, ubiquity, and ability to survive in diverse environments have made *P. aeruginosa* an ideal organism for biofilm study. In this review, salient features of biofilm formation are discussed, focusing on *P. aeruginosa* as the model organism.

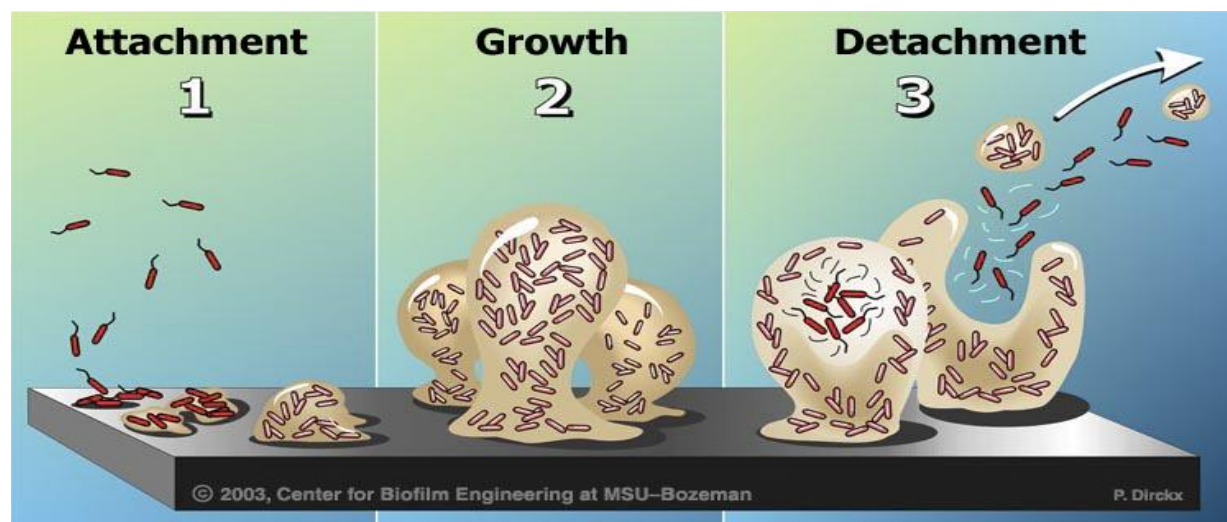


Figure 1: Schematic diagram of biofilm development. Reproduced with permission (Stoodley and Dirckx 2003)

The Model Organism: *Pseudomonas Aeruginosa*

P. aeruginosa is a gram negative, rod shaped bacterium which has an ability to adapt to a wide range of environments. In the presence of water the bacterium can colonize both biotic and abiotic surfaces. *P. aeruginosa* is an opportunistic

mammals (Hendrickson El Fau – Plotnikova et al., 2001; Sibley et al., 2008; Tan et al., 1999). A variety of virulence factors produced by *P. aeruginosa* help it to survive in wide range of hosts. In addition, *P. aeruginosa* increases the chance of its survival by living both planktonic and sessile

modes of life and can switch from one mode of life to the other in response to changing environmental conditions. The ability of *P. aeruginosa* to cause acute and chronic infections in immuno-compromised patients can be linked with planktonic and sessile modes of life respectively. Both modes of life are governed by various transcriptional regulators produced by the bacterium (Stover et al., 2000). During planktonic life, motile bacteria produce numerous virulence factors which help the bacteria to cause acute infections in the host. These factors include type IV pili (West et al., 1994; Wolfgang et al., 2003), the Type III secretion system (T3SS) (Wolfgang et al., 2003), the Type II secretion system (T2SS) secreted toxins and proteases. On the other hand, *P. aeruginosa* can also become non-motile and starts developing a biofilm which protects bacteria from host response and increases its chance of survival inside the host. This sessile mode of life is a major cause of chronic infections in human and animals. A better understanding of this bacterial mode of life will lead us to develop better treatment and control strategies.

BIOFILM FORMATION

Biofilm formation is a stepwise, complex and dynamic process which involves many physiological, chemical, genetic and biological processes. Biofilm formation can be roughly divided into the following steps: reversible and irreversible attachment to substratum surface, followed by microcolony and biofilm formation with the help of EPS (produced and recruited by constituent bacterial cells), and finally dispersal of biofilm. A schematic representation of biofilm formation in *P. aeruginosa* is presented in Figure 1.

Attachment to the Surface

Biofilm formation is initiated by attachment of free living cells to a surface. With the help of Brownian movement and flagellar-dependent motility, bacterial cells reach to an appropriate surface described below.

The Role of Substratum in Attachment

Compared to growth in liquid, attachment to a surface provides several advantages to the bacterial cells. First, surfaces provide a good nutritional supply because most of the macromolecules are adsorbed to the surface (called "conditioning" of the surface) [reviewed in (Donlan, 2002)]. Second, laminar flow velocity and shear forces are minimal just above the surface at the liquid-solid interface, which allows settlement of the bacterial cells on the surface [reviewed in (Donlan, 2002; Korber et al., 1989)]. Physical and chemical properties of the surface may play an important role in attachment, as rough surfaces provide an extended surface area and friction for strong attachment (Characklis, 1990). Similarly, hydrophobicity and hydrophilicity of the surfaces also play a role in bacterial attachment. Previous studies suggest that microorganisms readily attach to hydrophobic surfaces, such as Teflon and plastics, as compared to hydrophilic surfaces, such as glass and metals (Bendinger et al., 1993; Fletcher and Loeb, 1979; Pringle and Fletcher, 1983).

Environmental Cues for Attachment

Bacterial attachment to surfaces is initiated in response to some environmental cues. Scarcity of nutrition is one of the important environmental cues in response to which many

bacterial species initiate attachment (Allegrucci et al., 2006; Costerton et al., 1995; Humphrey et al., 1983). Limited supply of inorganic phosphate (P_i) is sensed by the bacteria for initiation of biofilm, but a concentration of P_i below a certain threshold level impacts negatively and results in impaired attachment in *Pseudomonas aureofaciens* PA147-2 (Monds et al., 2001). In *Escherichia coli* K-12, different nutritional sources were tested and it was shown that carbon sources which were metabolized to produce acetyl coenzyme A, acetyl phosphate, and acetate resulted in initiation of biofilm formation (Průš et al., 2010). The presence of monosaccharides (the building blocks of extracellular polysaccharide) in the growth media is sensed by *Vibrio cholerae* as a cue to form biofilm (Kierek and Watnick, 2003). In addition, osmolarity, divalent cations and pH are also environmental factors which are sensed as cues by many bacterial species to initiate attachment and begin biofilm formation [reviewed in (Petrova and Sauer, 2012)].

Reversible Attachment

Attachment can either be reversible or irreversible. The reversible attachment is a transitory phase and bacterial cells can detach from the surface at any time to become freely swimming cells again. Reversibly attached bacterial cells adhere to a surface via a single pole using a flagellum, fimbriae or other adhesins produced by the bacterium [reviewed in (Petrova and Sauer, 2012)]. This attachment is initiated when bacterial cells make first contact with the surface and overcome the repulsive electrostatic forces. A repulsion is expected, due to net negative charges both on bacterial and other surfaces [reviewed in (Goulter et al., 2009; Pratt and Kolter, 1999)]. However, these repulsive forces are overcome by the hydrophobic interactions between adhesins present at the bacterial surface and the attachment surface (Corpe, 1980). The role of fimbriae in bacterial attachment to surfaces has been reported by many studies (Bullitt and Makowski, 1995; Rosenberg et al., 1982) and most fimbriae have been shown to contain high numbers of hydrophobic amino acid residues and may contribute to cell surface hydrophobicity (Rosenberg et al., 1982). Flagella have also been shown required for attachment.

In *P. aeruginosa*, mutations in flagellar biosynthesis and type IV pili genes rendered the mutants defective in attachment to the surface (O'Toole and Kolter, 1998). It was speculated that this defect in attachment was due to inability of the mutants to swim and reach the surface. However, later studies proposed that FilD, a flagellar cap protein, interacts with the mucin and may be required for attachment in the lungs of cystic fibrosis (CF) patients (Arora et al., 1998). Recent studies have shown that attachment is reduced in the absence of structural flagellar proteins and defects in flagellar motility also results in impaired attachment and reduced biofilm growth. *P. aeruginosa* have a single flagellum and one motor but two stators, MotAB and MotCD, which provide energy for flagellar rotation. Although the presence of a single stator was sufficient for flagellar movement, both were shown to be essential for attachment and biofilm development. Using beads coated with purified Type IV pili, a recent study has demonstrated that type IV pili are essential and sufficient for maximal binding to the surface of polarized epithelial

host cells (Bucior et al., 2012). In addition, a chaperone-usher pathway (Cup) which synthesizes and assembles the fimbriae also has been suggested as an essential component for attachment to abiotic surfaces (Ruer et al., 2007).

Irreversible Attachment

Irreversibly attached bacteria remain fixed to the surface and form a monolayer. In the case of irreversible attachment, cells are adhered to a surface along their long axes and show reduced flagellar movements [Reviewed in (Petrova and Sauer, 2012)]. A few factors have been identified in some bacterial species which are suggested to be involved in the transition from reversible to irreversible attachment state.

The transition from reversible to irreversible attachment in *P. aeruginosa* has been linked with SadB/SadC/BifA protein complexes. These proteins control swarming motility and biofilm formation [reviewed in (Petrova and Sauer, 2012)]. In many bacteria transition from planktonic to sessile mode of life is controlled by a secondary messenger known as cyclic diguanylate monophosphate (c-di-GMP) which is synthesized from two molecules of GTP. The group of proteins which synthesize c-di-GMP are known as diguanylate cyclases (DGCs) and the group of proteins which are involved in the degradation of this secondary messenger are known as phosphodiesterases (PDEs) (Hengge, 2009). In *P. aeruginosa*, SadC is a DGC which synthesizes c-di-GMP in a localized area and, as a result of that, motility is reduced and biofilm development is increased (Merritt et al., 2007; Merritt et al., 2010). In contrast, BifA, a PDE, c-di-GMP degrading enzyme, increases motility of bacterial cells, decreases attachment and reduces biofilm development (Merritt et al., 2007; Merritt et al., 2010). SadB is suggested to control the transition from reversible to irreversible by decreasing flagellar reversal rate and increasing exopolysaccharide production (Caiazza et al., 2007; Caiazza and O'Toole, 2004).

In *Pseudomonas fluorescens*, a large protein, LapA, is secreted which is associated with the cell surface and helps the bacterial cell to attach permanently to surfaces (Hinsa et al., 2003). Similarly, another large protein, LapF, secreted by *Pseudomonas putida* is required for attachment and colonization on the roots of the plants (Martinez-Gil et al., 2010). Interestingly no orthologues of *LapA* and *LapF* are found in *P. aeruginosa* (Fuqua 2010) but presence of large protein, CdrA (c-di-GMP-regulated protein A), has been suggested to be required to form stabilized biofilm structures by interacting with Psl (Borlee et al., 2010). In *E. coli*, biosynthesis and secretion of poly- δ -glutamate (PGA) is proposed as an important adhesin required for permanent attachment (Agladze et al., 2005). In *Caulobacter crescentus* transition is initiated by removal of flagellum with the help of protease (Kanbe et al., 2005) and an exopolysaccharide 'holdfast' is produced which is composed of oligomers of N-acetylglucosamine (Janakiraman and Brun, 1999; Smith et al., 2003). The holdfast acts as an adhesin and firmly attaches the bacterium to the surface (Tsang et al., 2006).

Microcolony and Biofilm Formation

In some microbes, transition from reversible to irreversible attachment is mediated by the presence of EPS. After attachment, the bacterial cells start proliferating to form

localized microcolonies and EPS production increases within the microcolonies. Eventually a hydrated extracellular matrix encases the immobilized bacterial cells, where bacterial cell division continues inside the matrix and results in formation of a heterogeneous biofilm [reviewed in (López et al., 2010; O'Toole et al., 2000)]. Increase in heterogeneity of biofilm allows the development of physical and chemical microgradients inside the biofilm, including those of nutrients, pH and oxygen [reviewed in (Stewart and Franklin, 2008)]. For instance, when biofilm attains a thickness of 10–25 μ m, the microenvironment near the attachment surface changes and becomes anaerobic (Hamilton, 1987). This decreased oxygen supply may result in altered gene expression in the cells. Many subpopulations may develop within the biofilm with differential gene expression in different subpopulations of the biofilm. For instance, a study showed that enzymatic activity of an oxygen-dependent alkaline phosphatase was high at the surface and low at the base of *P. aeruginosa* biofilm (Xu et al., 1998). This was related to less oxygen supply near the base compared to that on the surface. Similarly, protein and DNA synthesis was observed in different subpopulation of *S. aureus* biofilm. The cells located near the surface with ample supply of oxygen showed high metabolic activity, whereas the cells close to agar were metabolically less active (Rani et al., 2007). This presence of sub-populations of cells within biofilm is an indicative of division of labour. This division of labour between cells in the biofilm also influences the architecture of the biofilm (Barken et al., 2008).

The architecture of hydrated biofilm was impossible to study using light or electron microscopy, but became evident using CLSM. Many studies have demonstrated that biofilm are composed of clustered cells, encased in an extracellular matrix. On the basis of the shape of these cell clusters, biofilm architecture can be designated as flat, filamentous, cylindrical or mushroom-like [reviewed in (Flemming et al., 2007; Flemming and Wingender 2010)]. Architecture of the biofilm is also influenced by availability of nutrients. The wild-type *P. aeruginosa* PAO1 usually forms mushroom-like microcolonies. However, when genes required for the iron binding protein pyoverdine were inactivated, the mutants formed very thin and unstructured biofilms even in the presence of iron in the media (Banin et al., 2005). Increase in iron supply above a threshold level also proved to be harmful for microcolony formation in wild type *P. aeruginosa* PAO1 (Yang et al., 2007).

Extracellular Matrix Formation

EPS make a meshwork of polymers which contribute to cell to surface attachment, cell to cell attachment, and provide a scaffold for the architecture of the biofilm. The EPS hold the cells together and help them to live in close proximity where cell to cell communication is made possible; this is essential for the functioning of the biofilm. EPS protect bacterial cells from desiccation, biocidal agents, protozoal grazing, heavy metals, free radicals and the host immune system [reviewed in (Flemming and Wingender, 2010)].

The Matrix is composed of EPS, metal ions and water. Initially the extracellular polysaccharides were thought to be the only polymeric substances present in the biofilm matrix, but many other components have subsequently been identified. Components of the EPS are eDNA, proteins,

lipids, polysaccharides and humic substances [reviewed in (Branda et al., 2005; Flemming and Wingender, 2010)].

Extracellular DNA

Previously, eDNA was considered as a bi-product of lysed cells, but recent studies have shown that eDNA is a very important polymer in the matrix of biofilms formed by both Gram-positive and Gram-negative bacteria (Allesen-Holm et al., 2006; Qin et al., 2007). eDNA helps to maintain the architecture of the biofilm and acts like glue to stabilize the cell to cell connections (Whitchurch et al., 2002). A role of eDNA has also been suggested in protection of bacterial cells in biofilm against the harmful effects of cationic antibacterial polypeptides (CAP) (Mulcahy et al., 2008). In bacterial biofilms, eDNA is discharged by various mechanisms including prophage-mediated cell death (Kirov et al., 2007; Webb et al., 2003), quorum sensing regulated DNA release (Allesen-Holm et al., 2006), and by outer membrane vesicles (OMV) produced by living cells (Kadurugamuwa and Beveridge, 1995; Schooling and Beveridge 2006). In *P. aeruginosa*, eDNA plays an important role in architecture and function of the biofilm (Whitchurch et al., 2002). Treatment of *P. aeruginosa* biofilms with DNase at early stages of biofilm development dissolved the biofilm. However, DNase treatment at later stages did not prove detrimental (Whitchurch et al., 2002). In a young two days-old *P. aeruginosa* biofilm, eDNA was located on the surface of microcolonies, whereas it was found around the stalk of mushroom-like biofilm structures in the mature biofilm (Allesen-Holm et al., 2006). Recently eDNA has been shown as important component for the motility of the *P. aeruginosa* cells. It acts as trails and allows cells to move on it and this helps bacterial biofilms to organise their cells in the required way to develop the architecture of the biofilm (Gloag et al., 2013).

The role of eDNA has been studied in various other bacterial biofilms. In *Haemophilus influenzae* biofilms, eDNA forms a dense meshwork of fine threads like a 'thick rope' that span water channels (Jurcisek and Bakaletz, 2007). Members of the genus *Rhodovulum* form self-flocculating, aggregating structures with the help of exopolysaccharides, proteins and eDNA. Interestingly, DNase treatment of these clumps resulted in breakdown of the structures, whereas protein and polysaccharide lysing enzymes showed no impact on biofilm architecture, suggesting eDNA as the main polymer involved in biofilm formation in *Rhodovulum* (Watanabe et al., 1998).

Proteins

Proteins are also important structural components of the extracellular matrix in biofilm. In addition, some enzymes are also held in EPS which make an external digestive system for bacterial cells.

In *P. aeruginosa*, twogalactose-specific lectins, LecA and LecB, have been shown important for the maintenance of the biofilm architecture (Diggle et al., 2006; Tielker et al., 2005). Biofilm was completely disrupted when LecB was targeted with high-affinity ligand (Johansson et al., 2008). Role of another polymeric protein, CdrA, has also been demonstrated for *P. aeruginosa* biofilm. CdrA binds with exopolysaccharide Psl, and links Psl with Psl or Psl with cell (Borlee et al., 2010). In addition, Cup fimbriae (Vallet et al.,

2001) and type IV pili (Klausen et al., 2003) are also required for architecture of the *P. aeruginosa* biofilm. Similarly, TasA in *B. subtilis* (Branda et al., 2006) and Bap in *S. aureus* (Lasa and Penades, 2006) are two more examples of proteins which are required to form biofilms by the respective bacteria species.

Exopolysaccharides

Many bacteria produce extracellular polysaccharides that play important roles in bacterial virulence. These extracellular polysaccharides can be classified into two subclasses: (a) capsular polysaccharides, which remain associated with the cells after harvesting the bacteria by centrifugation, and (b) exopolysaccharides which remain in the supernatant. This distinction can be well defined for bacteria grown in shaken cultures, but it is difficult to discriminate between the two when studying structured communities such as biofilms. Nevertheless, many studies strongly support the notion that biofilm architecture is mainly defined by these extracellular polysaccharides produced by the biofilm-forming bacterial species [reviewed in (Branda et al., 2005)].

Cellulose is one of the most studied exopolysaccharide regarding biofilm formation. *Gluconacetobacter xylinus* is the first species shown to produce extracellular cellulose (Ross et al., 1987). Cellulose has also been reported in some other bacterial species, for instance *Salmonella typhimurium* and *Escherichia coli*. (Zogaj et al., 2001). Comparative genome analysis of many other species has revealed the presence of genes homologous to the *G. xylinus* BCS (Bacterial Cellulose Synthesis), indicating their capability to produce cellulose (Ross et al., 1991). Cellulose is shown to be involved in biofilm formation in such bacterial species. It forms gel which retains water and consequently protects the residing bacteria from desiccation (Yamanaka et al., 1989).

In addition to cellulose, there are other exopolysaccharides known to be involved in the formation of extracellular matrices. For example, the exopolysaccharide intercellular adhesin (PIA), produced by enzymes encoded by the *ica* locus in staphylococcal species (Götz 2002). PIA synthesized by *Staphylococcus epidermidis* and *S. aureus* is also known as poly-N-acetyl glucosamine (PNAG) polymer. These polymers act as adhesins, and are essential for biofilm architecture (Mack et al., 1996; Mack et al., 1994; Maira-Litrán et al., 2002). PIA-like polymers were also reported to be synthesized in other bacterial species. For example in *E. coli* MG1655, a polymer similar to PIA has been shown to play a role in biofilm formation (Wang et al., 2004). Similarly, in *Yersinia pestis*, synthesizes a polymer similar to PIA which is required for the biofilm formation inside the mouth of parasite host which results in an irritation in parasite and the parasite bites the human host. Consequently results in spreading of plague (Darby et al., 2002). Colanic acid is another sugar polymer produced by *E. coli*, composed of galactose, fructose and glucose, and commonly found in extracellular matrix of biofilms formed by *E. coli* (Danese et al., 2000).

Most strains of the Gram-positive bacterium *B. subtilis* are capable of synthesizing two polymers: the exopolysaccharides, EPS and PGA, which play important role in the process of biofilm formation (Branda et al., 2006;

Stanley and Lazazzera, 2005). The role played by each polymer depends on the strain and environment surrounding the biofilm. For instance, the wild strain NCIB3610 needs exopolysaccharide EPS¹ but not PGA to form the biofilm (Branda et al., 2006). PGA overproducing mutants formed mucoid colonies and biofilm which lost its structure. In some wild strains like RO-FF-1 and the laboratory strain JH642, PGA is naturally produced and is crucial for surface-attached biofilm formation (Stanley and Lazazzera, 2005).

Teichoic acid is another exopolysaccharide which constitutes the biofilm matrix in *S. epidermidis* (Sadovskaya et al., 2004). *S. epidermidis* synthesizes two types of teichoic acid: one which is linked with the bacterial membrane, and the other which is secreted into extracellular milieu. Secretion of the extracellular teichoic acid increases viscosity of the colony [reviewed in (Lembre et al., 2012)].

P. aeruginosa has been extensively used as a model organism to study biofilms. Three exopolysaccharides important in biofilm formation in *P. aeruginosa* are Psl, Pel and alginate. Psl is a mannose-rich exopolysaccharide, which has been shown essential for the initial attachment and biofilm architecture (Ma et al., 2007). The Role of Pel, likely to be glucose-rich exopolysaccharide (Friedman and Kolter, 2004), is not well understood yet. Alginate is an anionic polymer of manuronic acid and gluronic acid and plays a very important role in biofilm architecture and function (Hay et al., 2009).

All above mentioned polysaccharides play key role in biofilm formation. A detailed understanding of these exopolysaccharides will help us to understand the 'building blocks' of biofilm. A detailed account of the exopolysaccharides produced by *P. aeruginosa* and their role in biofilm formation and function is given in the sections to follow (for Psl and Pel in chapter I, section 2, and for alginate in chapter II).

Dispersal of Biofilm

After maturation, the biofilm continues its life cycle by dispersing bacterial cells. After leaving the mature biofilm, these planktonic cells again attach to a new surface and start the development of another microcolony. Many studies have identified that the dispersal is initiated in response to various external environmental cues. Changes in availability of the following nutrients have been identified as signals that trigger dispersal: a) carbon sources, b) oxygen, c) nitric oxide (NO), and d) iron. In addition, many signalling molecules including the quorum sensing (QS) signalling molecule AHL, diffusible fatty acids, peptides and D- amino acids have been found responsible for initiation of the process [reviewed in (McDougald et al., 2012)].

In *P. aeruginosa*, and other *Pseudomonas* species, active dispersal of the bacteria from biofilm is initiated by localized cell death in a subpopulation (Tolker-Nielsen et al., 2000; Webb et al., 2003). These dead cells are used as nutrients by other bacterial cells which provide these cells with sufficient energy to become 'dispersal cells' [reviewed in (McDougald et al., 2012)]. These active cells leave the biofilm through 'local break out points' and swim away to start another lifecycle (Purevdorj-Gage et al., 2005). In *P. aeruginosa*, localized cell death is also thought to be a source of digestive enzymes which might play a role in the

digestion of the exopolysaccharides and allow the release of bacterial cells [reviewed in (McDougald et al., 2008)].

The intracellular level of c-di-GMP controls the mode of life in bacteria. Decrease in concentration of this secondary messenger leads to changes which result in switching from sessile to planktonic life again (Barraud et al., 2009). Nitric Oxide has been identified as a signal in *P. aeruginosa* which activates the PDEs. Activated PDEs can lead to a decrease in c-di-GMP level in bacterial cell. Consequently the bacterial cells become motile and ready to disperse (Barraud et al., 2009).

REGULATION OF BIOFILM FORMATION

Two important molecules involved in regulation are discussed below.

Cyclic Diguanylate Monophosphate

In the bacterial cell c-di-GMP is synthesized by DGCs from two molecules of GTP and degraded to 5'-phosphoguanlylyl-(3'-5')-guanosine (pGpG) or guanosine monophosphate (GMP) by PDEs (Hengge 2009). DGCs and PDEs are characterized by the presence of the conserved domain GGDEF (Paul et al., 2004) and EAL / HD-GYP (Christen et al., 2005) (Ryan et al., 2006), respectively. Proteins containing GGDEF/EAL domains are mostly multi-domain proteins that contain some regulatory domains (Galperin et al., 2001). These regulatory domains respond to environmental stimuli and consequently induce the DGC or PDE activity of the protein. In response to increased activity of DGCs and PDEs, localized concentration of c-di-GMP is changed. The effector proteins, containing specific c-di-GMP-binding domains, bind to c-di-GMP (Ryan and Dow, 2010). The effectors become activated or inactivated in response to binding with c-di-GMP and regulate motility, attachment, exopolysaccharide biosynthesis, biofilm formation and dispersal of the biofilm [reviewed in (Hengge, 2009)].

In *P. aeruginosa*, c-di-GMP plays its regulatory role in all steps of biofilm development, from attachment to dispersal. Flagellar and Type IV pili, important structures for initial attachment, are regulated by GGDEF/EAL-containing proteins such as MorA and FimX. MorA mutants showed an inability to form biofilm whereas FimX mutants lost polarity of pili expression (Choy et al., 2004; Huang et al., 2003). As mentioned above (Page No. 8), SadC increases c-di-GMP, this results in the transition from reversible to irreversible attachment (Merritt et al., 2007; Merritt et al., 2010). C-di-GMP controls the biosynthesis of exopolysaccharide at transcriptional and translational levels. FleQ represses the transcription of *pel* operon after binding to its promoter but, in the presence of c-di-GMP, FleQ is inactivated and the promoter is derepressed (Baraquet et al., 2012). Pel biosynthesis is controlled at post-transcriptional level by c-di-GMP-binding protein, PelD (Lee et al., 2007). C-di-GMP also regulates the exopolysaccharide alginate biosynthesis in *P. aeruginosa* (Hay et al., 2009). An important protein involved in alginate biosynthesis, Alg44, binds with c-di-GMP for its activity (Merighi et al., 2007).

Quorum Sensing

After irreversible attachment, the bacterial cells residing close to each other start forming microcolonies. Bacterial cells within these microcolonies communicate with each other and develop coordination amongst the members of the community. This signalling is transmitted through specific chemicals called autoinducers and this phenomena is called quorum sensing (QS) (Venturi 2006). QS is now widely recognized as an essential mechanism in many bacterial species. Most Gram negative bacteria regulate QS by production of autoinducer, *N*-acyl-L-homoserine lactones (AHLs) [reviewed in (Camilli and Bassler, 2006)]. This mechanism was first identified in a marine bacteria *Vibrio fischeri* which lives in symbiotic associations with many marine organisms and produces bioluminescence. The bioluminescence was seen only when the bacterial population-density was above certain threshold level. The bioluminescence was found to be associated with an increase in concentration of an autoinducer – (3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL, 3) produced by *V. fischeri* cells [reviewed in (Dunlap 1999)]. It is now known that *luxI/luxR* circuit controls this mechanism. In bacteria LuxI, an autoinducer, synthase, catalyzes synthesis of AHL. The receptor protein, LuxR, usually remains inactive and is continuously degraded in the cells. When AHL binds with LuxR, it becomes activated and activated LuxR binds to DNA and starts transcription of the target genes. This QS mechanism has been found essential for exopolysaccharide biosynthesis, virulence, biofilm formation, and biofilm related antibiotic resistance [reviewed in (Rutherford and Bassler, 2012)].

In *P. aeruginosa* two sets of *luxI/luxR* type systems have been identified. The *lasI/lasR* regulated QS system produces and senses *N*-(3-oxododecanoyl)-L-homoserine lactone (Passador et al., 1993), and the *rhlI / rhlR*-based system synthesizes and responds to *N*-butyryl homoserine lactone (Ochsner and Reiser, 1995). In addition *P. aeruginosa* has a third QS system, PQS which synthesizes 2-heptyl-3-hydroxy-4-quinolone, and regulates both the *las* and *rhl* QS systems (Diggle et al., 2006).

It has been shown that *P. aeruginosa* biofilm accumulates high concentrations of AHL molecules, i.e the concentration of *N*-(3-oxododecanoyl)-L-homoserine lactone recorded in biofilm was 630µM, whereas in effluent only 14 µM was found (Charlton et al., 2000). This high concentration is enough to initiate QS based responses in biofilm (Pearson et al., 1994). Many studies involving wild type and QS mutants have shown the effect of QS on biofilm development, architecture and exopolysaccharide biosynthesis (Bjarnsholt et al., 2005; De Kievit et al., 2001; Sakuragi and Kolter, 2007). Interestingly, a recent study has shown a link between c-di-GMP, QS and exopolysaccharide biosynthesis in *P. aeruginosa*. The study showed that AHL signals activate the protein TpbA which dephosphorylates TpbB, a PDE, and consequently leads to decreased c-di-GMP production (Ueda and Wood, 2009). This reduction in intracellular concentration of c-di-GMP, negatively impacts on exopolysaccharide production and leads to reduced biofilm formation.

THE IMPORTANT EXOPOLYSACCHARIDES FOR *PSEUDOMONAS AERUGINOSA* BIOFILM FORMATION

Three exopolysaccharides produced by *P. aeruginosa* have been identified as important for biofilm formation, architecture and function: Pel, Psl and alginate. Their detail is given below.

Molecular Structure of Pel

The structure of Pel has not been resolved yet. A comparison of sugar composition of the matrix from *P. aeruginosa* strain PA14 and Pel-deficient mutant revealed that the matrix of the Pel-deficient mutant contained less glucose than the Pel-producing strain (Friedman and Kolter, 2004). The study indirectly suggested that Pel is made of glucose. In contrast, a recent study provided an analysis of the total polymeric substances produced by PA14 and showed that the most abundant extracellular carbohydrates present in a PA14 culture were O-antigen lipopolysaccharide and glycerol-phosphorylated cyclic β-(1,3)-glucans. They did not detect any other glucose rich exopolysaccharide (Coulon et al., 2010).

Pel Biosynthesis

Pel synthesis and export machinery is encoded by *pel* operon which consist of seven genes: *pelA*, *pelB*, *pelC*, *pelD*, *pelE*, *pelF* and *pelG* (Friedman and Kolter 2004) and all these genes have been shown to be essential for Pel biosynthesis (Vasseur et al., 2005). These *pel* genes are conserved in most Gram-negative bacteria and a study showed that a mutation in a homologue of *pelG* in *Ralstonia solanacearum*, *ragG*, resulted in defective in adherence of the bacteria to a solid surface (Vasseur et al., 2005). A little experimental work has been done around the structure and function of the proteins encoded by the *pel* genes. Only two proteins, PelC and PelD, have been experimentally examined to date (Kowalska et al., 2010; Lee et al., 2007; Ueda and Wood 2009; Vasseur et al., 2007). The function and structure of other proteins encoded by the *pel* operon have been suggested on the basis of sequence homology (Franklin et al., 2011; Keiski et al., 2010; Vasseur et al., 2005). Using these experimental evidences and bioinformatics analysis of the proteins encoded by the *pel* operon, hypothetical models for Pel exopolysaccharide biosynthesis has been proposed by different researchers (Franklin et al., 2011; Vasseur et al., 2007).

Biosynthesis of polysaccharide starts with polymerization of sugar molecules which are available as activated nucleotide-sugars (Rocchetta et al., 1999; Samuel and Reeves 2003; Whitfield and Roberts 1999). These activated sugars are usually synthesized by the enzymes encoded by genes from the polysaccharide biosynthesis operon. Interestingly, the *pel* operon lacks any such genes suggesting that precursors for Pel biosynthesis are taken from the main pool of nucleotide-sugars (Franklin et al., 2011). Recently it has been shown that AlgC, is involved in biosynthesis of glucose-1-phosphate, and *algC* negative mutants were not able to form Pel (Ma et al., 2012). It is possible that AlgC may be a common enzyme required for precursor synthesis for Pel, alginate and Psl. The nucleotide-sugars required for Pel biosynthesis are not known. On the basis of sugar composition analysis of

exopolysaccharides produced by *P. aeruginosa* PAK strains, it is suggested that Pel is made up of glucose. This suggests that an activated-glucose may be used towards Pel biosynthesis. These activated sugars are transferred onto growing polymer by the enzymes called glycosyltransferases (GT). According to bioinformatics analysis, PelF is the only glycosyltransferase encoded by the *pel* operon (Franklin et al., 2011). On the basis of sequence similarities it is predicted that PelF belongs to the GT-4 family (according to the classification of CAZy(carbohydrate-active enzymes database) and members of this family are retaining glycosyltransferases (Coutinho et al., 2003). The retaining glycosyltransferases transfer sugar residues onto the acceptor molecule by retaining the anomeric configuration of the sugar as it was in the donor substrate. No transmembrane (TM) helices are predicted in PelF which is suggestive of a cytosolic subcellular localization. It is predicted that a cytosolic PelF synthesizes Pel on the cytosolic face of the inner membrane. It is not known how this newly synthesized polymer is transported across the plasma membrane. The *pel* operon contains only one glycosyltransferase, PelF which suggests that, like alginate, Pel is a homopolymer. Therefore, it is likely that Pel translocation occurs via a Wzy-independent pathway. In addition, the *pel* operon lacks a gene for an undecaprenyl-phosphate glycosylphosphotransferase which is required in undecaprenyl-phosphate dependent translocation of polysaccharide from the cytosol to the periplasm [reviewed in(Franklin et al., 2011)]. The newly polymerized Pel is likely to be translocated to the periplasm by PelD or PelE or PelG, because all of these proteins are predicted to be located in the inner membrane and contain multiple TM domains [reviewed in(Franklin et al., 2011)]. PelD binds with c-di-GMP and regulates the Pel biosynthesis (Lee et al., 2007). The role of c-di-GMP can be linked with Pel production because when the intracellular concentration of c-di-GMP was manipulated by deletion of tyrosine phosphatase, TpbA, the amount of Pel production and pellicle formation was also changed (Ueda and Wood, 2009). PelG is predicted to be an inner membrane protein which contains 12 TM domains (Krogh et al., 2001). On the basis of structural homology, PelG has been shown to be similar to NorM from *V. cholerae* (PDB ID: 3MKU). NorM belongs to the multidrug and toxic compound extrusion (MATE) family of proteins (He et al., 2010). Members of this family of proteins are involved in extrusion of molecules across the inner membrane. PelG could be involved in export of Pel polysaccharide from the cytoplasmic face of the inner membrane to the periplasm. PelE, the third inner membrane protein, is predicted to have two transmembrane domains which orient the protein in such a way that the C-terminus becomes localized to the periplasm. The C-terminus of the protein is predicted to contain 4–6 copies of tetratricopeptide-like repeats (TPR), the protein-protein interaction motif. Previously it has been shown TPR motifs are involved in the assembly of large protein complexes (Blatch and Lassle 1999; D'Andrea and Regan, 2003), therefore, PelE, may be involved in interaction with other TPR-containing proteins in the periplasm. The homology modelling suggests that the N-terminus of PelB forms a domain which has similarity with TPR-containing anaphase-promoting complex/cyclosome subunit Cdc 16/Cut9 [PDB ID: 2XPI; (Zhang et al., 2010)] and is

predicted to contain TPR motifs. It can be proposed that the C-terminus of PelE and N-terminus of PelB are involved in protein interactions to make a scaffold. PelC has been proposed as a lipoprotein in which Cysteine-19 is lipidated and contains a C-terminal amphipathic α -helix which is inserted in the outer membrane (OM) in a Wza-like manner. This C-terminus domain has been shown to be essential for Pel biosynthesis. PelA is a large 105 kDa protein in which an N-terminal domain is structurally similar to glycoside hydrolases (PDB ID: 2AAM) and a C-terminal domain shows similarity to carbohydrate esterase [PDB ID: 2VYO; (Urch et al., 2009)]. The glycosidic hydrolase activity of PelA suggests that PelA may play a role similar to AlgL. AlgL hydrolyses alginate which escapes into the periplasm (Bakkevig et al., 2005). Therefore, it is possible that any Pel that escapes into the periplasm is hydrolyzed by PelA.

Pel biosynthesis can be summarized as PelF polymerizing a homopolymer of glucose which is transferred from the cytoplasm to the periplasm by PelE /PelD/ PelG.

Molecular Structure of Psl

Previous studies suggests that Psl is composed of galactose, D-mannose, D-glucose, and L-rhamnose (Ma et al., 2007; Ma et al., 2009)Recently the molecular structure of Psl is revealed (Byrd et al., 2009). The study showed that Psl exopolysaccharide is composed of pentasaccharides repeating units and each unit is composed of three D-mannose, one D-glucose, and one L-rhamnose.

Psl Biosynthesis

Biosynthesis of Psl in *P. aeruginosa* is under the control of the *pepsl* operon which contains 15 co-transcribed genes: *pslA-pslO*(Friedman and Kolter 2004). Interestingly, only 11 of the 15 gene products of the *pepsl* operon were found to be essential for Psl biosynthesis. The product of *pslB* has redundant function with WbpW, an enzyme which has similar activity to that predicted for PslB (Byrd et al., 2009). Deletion of the last three genes of the operon, *pslMNO*, has shown no effect on the ability of *P. aeruginosa* to produce Psl (Byrd et al., 2009).

Although the Psl biosynthesis mechanism has not been established experimentally, the repeating unit structure of Psl and bioinformatics analysis of the proteins encoded by the *psl* operon suggest that Psl biosynthesis bears a resemblance to the undecaprenyl diphosphate-based biosynthesis of *E. coli* group-1 capsular and extracellular polysaccharides. Therefore the Psl biosynthesis pathway resembles a Wzy-dependent pathway. This hypothesis is further strengthened by the finding that three proteins encoded by the corresponding genes of the *psl* operon PslA, PslD and PslE show sequence similarity with WbaP, Wza, and Wzc, respectively(Franklin et al., 2011). The role of gene products of *psl* operon is presented in a recent review (Franklin et al., 2011).

The detailed account of alginate is reviewed elsewhere in the review article (Hay et al., 2010)

ROLE OF EXOPOLYSACCHARIDES IN BIOFILM

Role of Pel in Biofilm

Pel is synthesized in most *P. aeruginosa* strains but strains with intact *psl* loci depend more on Psl contributing to biofilm formation and architecture. However, *P. aeruginosa* PA14 relies mainly on the Pel exopolysaccharide because it lacks Psl (Friedman and Kolter, 2004). This strain produces biofilm at the air-liquid interface (A-L) and it was observed that a *pel* transposon insertion mutant failed to produce A-L biofilm (Friedman and Kolter 2004). This finding led to further detailed investigation of the role of Pel in biofilm formation. Pel has been shown to be required for adherence to abiotic surfaces especially at the later stages of the biofilm in PA14 (Colvin et al., 2011). Recent studies have suggested a redundant role for Pel in *P. aeruginosa* PAO1 biofilm, especially in the presence of Psl. However, in the absence of Psl, Pel has a clearer role in biofilm formation (Colvin et al., 2012). In contrast, a recent study has demonstrated a distinct role for Pel in biofilm formation and suggested that, in addition to Psl, Pel was required for structural stability of mature biofilms (Yang et al., 2011).

Pel has also been identified as an extracellular matrix component which mitigates the effects of antibiotics and may play a vital role in protection of the bacteria (Colvin et al., 2011). Studies demonstrated that Pel-producing PA14 strains showed increased resistance to aminoglycoside antibiotics when compared to Pel-deficient strains (Colvin et al., 2011). In contrast, other authors have reported contradicting results which showed that Pel-deficient mutants were more resistant to the aminoglycoside, tobramycin when compared to wild type PA14 strains (Khan et al., 2010). The two studies used different methods and conditions to assess antibiotic resistance which might have affected the results of their experiments. (Colvin et al., 2011) grew biofilms on polycarbonate filters for 48 hours and then the filter was transferred to solid medium containing respective antibiotics and incubated for 24 hours. Viable bacterial cells were counted to see the effect of antibiotics. It was found that absence of Pel renders PA14 cells more susceptible to aminoglycosides when grown as biofilm. Whereas, (Khan et al., 2010) grew cells in 96-well microtiter plates in liquid medium for 24 hours. These one day old biofilms were washed and treated with respective antibiotics for 24 hours. It was found that in absence of Pel PA14 cells were more resistant to aminoglycosides. This difference of media conditions and duration of growth might have affected the exopolysaccharide production and distribution in biofilm formation. Owing to the significance of Pel in antibiotic resistance and architecture of the biofilm, it is imperative to further investigate its regulation, biosynthesis and role in *P. aeruginosa* biofilm.

Role of Psl in Biofilm

Psl is found in two forms: a larger form associated with cells and a smaller soluble form. It is not yet clear how the smaller form of Psl is produced. Possibly, it may be produced by cleavage or breakdown of the longer cell-associated Psl. Alternatively, there could be another unrecognized mechanism to generate this form of Psl. The long cell-associated form of Psl forms blebs of Psl on the cell surface which can be observed by scanning electron microscopy (SEM) (Byrd et al., 2010). Psl has been shown to be important for initial attachment of non-motile cells to

both biotic and abiotic surfaces (Ma et al., 2006; Byrd et al., 2009; Byrd et al., 2010). Psl aids structural stability of the biofilm and is required for the typical architecture of the biofilm (Ma et al., 2009). Psl also plays an important role in protection of bacterial cells from innate immune effectors, complement and neutrophils (Mishra et al., 2012). Recently it has been shown that Psl also stimulates the production of c-di-GMP in surrounding bacterial cells suggesting a regulatory role of Psl in biofilm formation and maintenance (Irie et al., 2012).

Role of Alginate in Biofilm

Most of the early studies investigating *P. aeruginosa* biofilms were conducted using mucoid (alginate overproducing) strains (Ohman and Chakrabarty, 1982; Pedersen et al., 1992; Sa-Correia et al., 1987). Mucoid strains isolated from chronically infected lungs of cystic fibrosis patients were able to produce copious amount of alginate when grown on agar plates in the laboratory. Mucoid colonies, encased in matrix made of alginate, appear more slimy when compared to non-mucoid strains (Hogardt and Heesemann, 2010). Alginate has been shown to be a very important matrix component for retention of water due to its gel-forming ability and protects the bacterial cells from the harmful effects of desiccation. Although direct evidence of the amount of water retained by alginate is not known, a similar exopolysaccharide, hyaluronic acid, can bind up to 1 kg of water per gram of the polysaccharide (Sutherland, 2001). The degree of acetylation of alginate also plays an important role in its ability to retain water. High degrees of O-acetylation of the residues present in alginate increase its solubility by reducing the chance of interaction between alginate and cations. Alginates without O-acetyl residues show increased binding of divalent cations and decreased polysaccharide solubility (Sutherland, 2001). Alginate protects bacterial cells from the host immune response and antimicrobial drugs (Simpson et al., 1988; Simpson et al., 1989; Simpson et al., 1993). Most of the host immune cells such as neutrophils and activated macrophages kill bacterial cells by releasing free radicals. Alginate can protect bacterial cells by scavenging the free radicals (Simpson et al., 1988; Simpson et al., 1989).

Role of Exopolysaccharides in the Architecture of the Biofilm

The production and quantity of exopolysaccharides influences the architecture of biofilm. Studies show that mutants lacking exopolysaccharides produce biofilms which are structurally different from those produced by the wild type strains. For example, *V. cholerae* deficient in exopolysaccharides (Watnick and Kolter, 1999) and *E. coli* lacking colanic acid (Danese et al., 2000) failed to form 3D biofilm architecture. The fruiting body-like biofilms formed by wild type *B. subtilis* were absent in the mutants lacking exopolysaccharides and the secreted protein TasA (Branda et al., 2006). Less is known about the effects of polysaccharides on the architecture of *P. aeruginosa* biofilms. Some studies have been carried out to investigate the role of alginate in biofilm architecture (Franklin and Ohman 1993; Hay et al., 2009; Wozniak et al., 2003). Although it has been suggested that alginate is not necessary for *P. aeruginosa* biofilm formation (Wozniak et al., 2003), however, biofilm architecture is influenced in the presence of alginate. Some

recent studies have shown the impact of Psl in biofilm architecture (Overhage et al., 2005; Ma et al., 2006; Starkey et al., 2009). Further investigations are required to decipher the role of Pel in the architecture of *P. aeruginosa* biofilms.

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