

## BIOFILM OF MEDICAL IMPORTANCE

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**Keywords:** Biofilm, Chronic Infections, Microbiology, Tolerance, Resistance, Wound infection, Otitis Media, Foreign Body Implants, Tissue Fillers

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### **Summary**

Bacteria predominantly grow as either planktonic cells or surfaced attached biofilms. Biofilm structures are highly tolerant to both antimicrobials and immune cells. Due to this tolerance the biofilm mode of growth is an inevitably hallmark of chronic infections. This chapter covers the recent findings and progresses found on the topic of biofilm infections.

### **1. Introduction**

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Bacteria are found in at least two distinct states – either as planktonic or sessile cells. Planktonic cells are classically defined “*as free flowing bacteria in suspension*” as opposed to the sessile state (the so called biofilm): “*a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface*” as stated by Costerton and coworkers (1999). Microbiologists have up until the last decades focused and emphasized the planktonic state over the sessile state – the biofilms. However, the biofilm mode of growth is becoming more and more renowned as better methods to study these sessile bacteria have become available, and hence the

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subsequent accumulation of evidence for its widespread presence. Actually it has turned out that bacteria are predominantly growing as communities rather than as single cells. Bacteria growing as biofilm, are highly responsible for chronic infections in hearts (endocarditis), respiratory tracts of cystic fibrosis (CF) patients, middle ears (otitis media) paranasal sinuses (rhinosinusitis), chronic wounds, osteomyelitis, intravenous catheters, stents, dental diseases, contact lenses and implants.

Biofilms were already discovered by one of the first microbiologist, the Dutch Antonie van Leeuwenhoek in 1650s, but the actual breakthrough of the phenomenon had to wait another 328 years until Costerton and colleagues published their work on “How Bacteria Stick” in 1978. Since 1978 the research within biofilm bacteria has exploded. The inherent high tolerance to antimicrobials and immune cells explain its vast implication in chronic infections. This review will summarize present knowledge of biofilms of medical importance related mainly to the pathogen *Pseudomonas aeruginosa*.

## 2. The Structure of Biofilms

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In the above section sessile growing bacteria were defined as an assemblage of cells embedded “in a self-produced polymeric matrix”. This matrix is very important for the properties of the biofilm, since it offers great structural stability and other beneficial traits such as tolerance to antimicrobials and phagocytes. To understand this phenomenon, one has to look at how a biofilm is established. In this review we will focus on and describe how the model organism *P. aeruginosa* forms biofilms. The *in vitro* process can be divided into at least 4 stages, of which the first stage is where planktonic cells reversibly attach to a surface. Irreversible binding follows this attachment and then multiplication into microcolonies. These microcolonies produce a polymer matrix, which in turn surrounds the colonies. After a couple of days the microcolonies attain tower- or mushroom-like structures measuring up to 50µm. The matrix contains a mixture of polysaccharides, proteins and DNA. When the biofilm grows to a size not beneficial for bacterial survival and growth (e.g. due to nutrient limitations), focal areas of the biofilm are liberated (sloughs off). It is hypothesized this enables the bacteria from these areas to spread and colonize to form a new biofilm. Hence it seems that the biofilm development the model organism *P. aeruginosa* is a very dynamic process constantly renewing it self.

The importance of biofilms in chronic infection recently led Niels Høiby to propose following definition in 2010: “*Biofilm growing bacteria cause chronic infections which are characterized by persisting inflammation and tissue damage. Chronic infections, including foreign body infections, are infections which 1) persist in spite of antibiotic therapy and the innate and adaptive immune- and inflammatory response of the host, and 2) which, in contrast to colonization, are characterized by immune response and persisting pathology*”.

There is no doubt that biofilms are very important in chronic infections. Interestingly, peptide nucleic acid (PNA) fluorescence *in situ* hybridization (FISH) and confocal microscopy of sites of infections has shown that bacteria do not need to be attached to surfaces in order to establish chronic infections. Rather, the bacteria generate non-

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attached microcolonies by aggregating to their fellow bacteria through matrix components, and they seem to put up an impenetrable barrier to the host e.g. phagocytic cells. Figure 1 demonstrates the impenetrable barrier found in the biofilms (Microscopy of sections from **A**) chronic *P. aeruginosa* infected wound **B**) and a chronic *P. aeruginosa* infected CF lung showing a sharp interface between biofilm aggregates and immune cells. This interface is also found in PMN overlaid *in vitro* biofilm **C**).

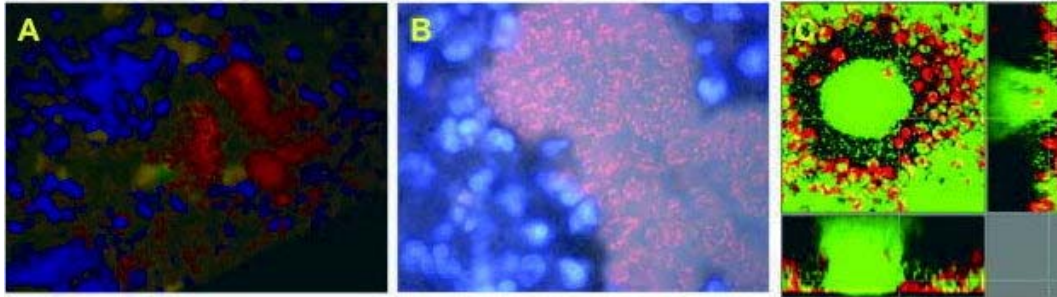


Figure 1. Biofilms are not eradicated by immune cells. Microscopy of sections from **A**) chronic *P. aeruginosa* infected wound **B**) and a chronic *P. aeruginosa* infected CF lung showing a sharp interface between biofilm aggregates and immune cells. This interface is also found in PMN overlaid *in vitro* biofilm **C**). **A**) and **B**) *P. aeruginosa* (red) and PMNs (blue). Reproduced from Alhede *et al* 2009 with permission from Society of General Microbiology.

In addition the authors of this chapter have recently showed that these non-attached aggregates show the same properties (matrix structure and tolerance to antimicrobials) as surface attached biofilms (unpublished data). Therefore the definition of biofilm as a cause of chronic infections could be as suggested by Burmølle and coworkers in 2010 “A coherent cluster of bacterial cells imbedded in a biopolymer matrix, which compared with planktonic cells shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defense”

### 3. Tolerance to Antimicrobials

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Experimental evidence has accumulated over the years showing that biofilms tolerate antimicrobial properties of the immune system, antiseptics and antibiotics. This multifaceted tolerance relies to a certain extent on general resistance mechanisms including efflux pumps and enzymatic modifications in addition to innate tolerances offered by integral structure-functions of the biofilm. Among the features contributing to this are the decreased penetration of phagocytes and antibiotics through the biofilm matrix and reduced nutrient availability ensued by lowered metabolic activity.

Bacteria living within *in vitro* biofilms display highly heterogeneous physiology. For example, *in vitro* biofilms of the model organism *P. aeruginosa* have been shown to accommodate several subpopulations with different susceptibility to antimicrobials. Most conventional antimicrobials interfere with fundamental and basal life processes of the bacteria. Such antimicrobials preferentially kill biofilm cells with high metabolic activity in particular those active in cell division and multiplication. Consequently, the

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top layers of exposed biofilms (which are maintaining high metabolic activities) are killed leaving less active or even dormant deeper layers alive. On the other hand, the stalks of the characteristic mushroom structures can be selectively killed with the antimicrobial peptide colistin and compounds, such as SDS and EDTA, all of which affects membrane integrity.

The bulk of evidence strongly suggests that a single compound is not sufficient to eradicate biofilm infections. Such treatment will leave invulnerable bacteria left to colonize encore. The combination of antimicrobials targeting several biofilm subpopulations has shown its potential as successful treatment strategy. The blends of tetracycline + colistin and ciprofloxacin + colistin have been shown to kill most bacteria in *P. aeruginosa* biofilms *in vitro*. The latter combination has furthermore shown great results, with respect to prevention of chronic colonization, *in vivo* and is recommended by a European Consensus report for treatment of early *P. aeruginosa* infection in Cystic Fibrosis patients.

The mutation frequency in biofilms has also been shown to be significantly higher than planktonically growing bacteria, enabling traditional resistance. The combined resistance and tolerance could explain why bacteria growing as biofilms readily become multi-drug resistant.

Another feature making biofilms extremely tolerant to phagocytic cells is its ability to produce toxins that rapidly kills the incoming immune cells. *P. aeruginosa* has been shown to produce the glycolipid rhamnolipid that rapidly lyse phagocytic cells attacking the biofilm.

#### 4. Lung Infection

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Lungs of cystic fibrosis patients are a favorable milieu for bacteria to establish a biofilm infection. *P. aeruginosa* can grow and establish drug resistant biofilm in the lungs of CF patients, which is thought to be facilitated by the hypersecretion of viscous mucus. The mucus adheres to airway surfaces, and the persistent mucin secretion generates the formation of "thickened" mucus plaques and plugs, which is ideal for bacterial infection. From early childhood the CF patients suffer from acute infections of many different bacteria the most common being *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *P. aeruginosa*, the *Burkholderia cepacia* complex and *Stenotrophomonas maltophilia*. *H. influenzae* and *S. aureus* predominate early in life, but later *S. aureus* and *P. aeruginosa* become the predominant infectious organisms in the CF-lung. Up to 80% of young adults suffering from CF are chronically infected with *P. aeruginosa*.

In 2009, Bjarnsholt and coworkers evaluated the orientation and distribution of *P. aeruginosa* in the conductive and respiratory zones of the lungs of chronic *P. aeruginosa* infected CF patients, using Peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH). The PNA FISH study showed that the bacteria were found embedded in the mucus and that the majority of bacteria were found as aggregates (only a few planktonic bacteria). Interestingly the bacteria were not adhering to the lung epithelia but rather adhering to each other and the mucus as also reported by Worlitzsch

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and colleagues (2002). As with *in vitro* biofilm, also this study showed that the biofilm bacteria are highly tolerant to the phagocytic action of Polymorphonuclear Neutrophilic Leukocytes (PMNs) – leaving the biofilms surrounded by PMNs.

Another interesting finding from this study was that in aggressively treated CF patients, the bacteria were predominantly found in the conductive zone of the lung (versus only a few in the respiratory part of the lung). This was in contrast to a non-intensively treated CF patient where the respiratory zone was also filled with aggregating bacteria and PMNs. Thus it is believed that the intensive antibiotic therapy of the CF patients, confines the bacteria in the conductive zone leaving the respiratory zone healthy for a longer period.

Another interesting feature of *P. aeruginosa* lung infection is its conversion from non-mucoid to mucoid over the course of the infection. The mucoid mutant phenotype produces an exopolysaccharide/alginate that makes its colonies mucoid. This exopolysaccharide/alginate greatly increases the bacterial resistance to phagocytosis however contradicting data is found on its tolerance to antibiotics. It is clear that the mucoid *P. aeruginosa* that cannot be eradicated by antibiotics, becomes predominant with age, predicts shortened survival of the CF patient, and is associated with increased lung function decline.

In spite of intensive treatment and endobronchial accumulation PMN during chronic *P. aeruginosa* lung infections, in CF patients, the bacteria persist. The fact that *P. aeruginosa* forms biofilms in the lung, is most likely the cause of its tolerance to antibiotics and immune cells. As a consequence of the chronic infection, the lung encounters a vast degradation of lung tissue. This degradation is partly caused by the toxins produced by the biofilm (e.g. rhamnolipid, pyocyanin, proteases and elastases), but also by the enduring inflammatory processes initiated by the persistent biofilm (e.g. reactive oxygen species produced by PMNs).

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### Biographical Sketches

**Morten Alhede** born in 1981. Studied Biotechnology at the Technical University of Denmark (DTU). Graduated 2006 with a M.Sc. in Civil Engineering. Spent one year at the Rigshospitalet doing research on autoimmune diseases. In 2007 he started his Ph.D. project at DTU on host interactions of the bacterium *Pseudomonas aeruginosa*. The project is specially focused on how biofilms evade immune cells. During his Ph.D. project, Morten spent 8 months in Alice Prince's lab at Columbia University, NY, USA., where he investigated how *P. aeruginosa* interacts with epithelial cells and if these cells are more prone to infection in cystic fibrosis patients. Morten will graduate for his Ph.D. in November 2010.

**Peter Østrup Jensen** born in 1962, studied biology at the University of Copenhagen, where he received his M.Sc. in 1991. Peters research has been focused on the innate immune defense and flow cytometry. In 2003 he received his PhD based on research on the innate immune response during the lung infection in patients with cystic fibrosis. Today he is the head of research and head of the phagocyte laboratory at the Department of Clinical Microbiology at Copenhagen University Hospital. Peter has within the last years contributed significantly to the understanding of the interplay between biofilms in chronic infections and the host response both in the clinic and in experimental settings.

**Michael Givskov** born in 1954 in London, England. Studied Biochemistry and Cell Biology at the University of Southern Denmark where he graduated (MSc) in 1983. He received his PhD in Microbiology from the University of Copenhagen in 1988 and worked as postdoctoral fellow at department of Microbiology at the Technical University of Denmark (DTU). At 1996 he was hired as Associate Prof of Microbiology, Department of Microbiology, DTU. From 1996 till 2004, he was inventor and shareholder in Biosignal Pty. Ltd., Australia, member of the faculty board, Biocentrum-DTU, Founder of, inventor, and shareholder in QSI-Pharma A/S, Denmark and Vice-president of QSI-Pharma A/S, Denmark. In 2003 he became head of the Centre of Biomedical Microbiology, BioCentrum-DTU, was promoted full professor in 2004, and rewarded the Statoil research price in 2004. In 2006 he received the Doctorial degree in Technical Science (Dr techn) for his work on quorum sensing and its exploitation as a novel antimicrobial drug target. In 2008 he was employed as Professor of Biomedical Microbiology, ISIM, Panum Institute, University of Copenhagen and in 2009 he became Director for the Center for Antimicrobial Research, Panum Institute, University of Copenhagen

**Thomas Bjarnsholt** born in 1975, studied microbiology at the Technical University of Denmark (DTU), where he graduated as civil engineer in 2000. During his further work at DTU he studied the role of biofilm and quorum sensing of *P. aeruginosa* in the lungs of patients with cystic fibrosis. In 2005 he received a PhD based on this research. Today he has a combined position at University of Copenhagen, Faculty of Health Sciences (associate professor) and Copenhagen University Hospital, Department of Clinical Microbiology (head of molecular bacterial diagnostics). With this experience Thomas has a vast knowledge of both scientific and applied approaches of the role of biofilms in chronic infections.