Microbial Biofilm Development – A Discussion of Two Cases

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Summary: Bacteria are often associated with surfaces – biotic as well as abiotic. The molecular mechanisms by which single suspended cells attach to surfaces and initiate development of elaborate biomass structures have been studied intensely during the past five to10 years, and we now have some general understanding of the processes involved from several examples. This review focuses on factors playing an important role in microbial biofilm development and in particular addresses whether it is possible to extrapolate information about *Escherichia coli (E.coli)* or *Pseudomonas aeruginosa (P.aeruginosa)* flow-chamber biofilms to how these microorganisms live in the human body in connection with the various infections they represent in patients. The major challenge for the coming years is to assess the relevance of the obtained information about laboratory systems for interesting cases of *in vivo* biofilms, and preliminary data suggest that this may not be as easy as one might hope for.

Key words: structural development, molecular mechanism, bacterial life styles

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INTRODUCTION

The past decade in microbiology has to some extent been dominated by the 'biofilm' concept based on the assumption that bacteria in natural and many artificial environments live as surface-associated populations. In addition, the biofilm concept has gained a strong foothold in relation to many infectious diseases, including many associated with the oral cavities. In text books, review papers and on websites we find with increasing frequencies statements claiming that '60-80% of all infections are associated with biofilm development', 'the chronic state of *P. aeruginosa* lung infections in cystic fibrosis (CF) patients is a biofilm', and others like this. These conclusions may be based on very indirect evidence, but it is obvious that they stimulate a general interest in biofilms and specific interests in those organisms, which are associated with the corresponding infections. Combinations of molecular and microscopic approaches have produced considerable information about three-dimensional structures of biomass, and genetic investigations have shed light on which cellular features may be essential for biofilm development. From studies of several model organisms in laboratory settings we can now present a scheme of the processes required for biofilm development on solid surfaces, which can be summarized as follows:

- Many – if not most – bacterial species will adhere to various abiotic surfaces and begin a biofilm developmental cycle (Costerton et al, 1995). In several investigated cases this adherence seems to be unspecific, although certain surface organelles such as flagella and pili have been suggested as adherence factors (O'Toole and Kolter, 1998). In contrast, adherence to tissues in connection with colonization and infection processes in the human body is often assisted by specific adhesions localized on the bacterial cell surface (Schembri et al, 2002).
- The primary necessary characteristic allowing development of a structured biofilm is that the cells stick to each other with forces that overcome the shear forces from the water flow. Water flows are important factors in the developmental cycles of biofilm development, partly because of the shear forces they induce on the biomass, partly because they may actively move bacteria from one position to another and hence influence the final distribution of biomass. If there is no cohesion between the cells the water flow may simply prevent biofilm formation, in which case single cells or single cell layers is the final outcome of surface colonization. The cell-cell sticking ensures that biomass reaching into the stream of water will sustain the shear forces, and the final out-

come is multi-layered and often rough structures of biomass developing on the surface.

- The actual biofilm structure depends on the nutritional conditions, the nature of the cell-cell sticking, the flow regime and cellular motility (including dissolution of the biofilm). In some cases extra-cellular polymeric substances (EPS) may play a specific role in addition to these other factors. The important conclusion here is that biofilm structural development is *conditional*. This means that a bacterial species, or even a specific strain, does not display a unique and reproducible biofilm development independent of the environmental conditions (Klausen et al, 2003a; Xavier et al, 2004). What you see under one set of conditions for a given strain may have absolutely no relevance for the same organism if grown on a different surface or under different conditions of nutrition, flow etc. The role of EPS is still somewhat unclear, although in several documented cases such polymer production has been found to stabilize the biofilm structure (Matsukawa and Greenberg, 2004; Friedman and Kolter, 2004a,b). In clinical contexts it is also likely that EPS plays a protective role in relation to the host defense systems (Høiby, 2002). Protection against draught and predation is also among the possible functions of EPS.
- In most cases biofilm development is a simple (mechanical) process, but some bacteria follow a more complex cycle involving some kind of population differentiation. Although a lot of research interest has been focused on gene regulation as a key parameter in biofilm development, it is clear that even quite complex biofilm structures, including micro-colonies and water channels between these, may develop as consequence of purely physico-chemical features (flow regime, diffusion rates, substrate concentrations, maximum growth rates). In some cases, however, more complex biological activities play significant roles in biofilm development, and in a few cases we can in fact describe the biofilm as the result of 'differentiation' process resembling what we know from higher multi-cellular organisms (O'Toole and Kolter, 1998).
- Different bacteria have different biofilm cycles, which reproducibly develop under identical conditions. If we study bacterial biofilm development under conditions which are fully controllable and reproducible we also observe very reproducible biofilm formation processes and kinetics for a given strain. This allows us to compare biofilm developmental cycles for a number of different bacteria, and the resulting 'pathways' then become signatures of the corresponding sessile life-styles of the different organisms. It must, however, be kept in mind that these phenotypes are not absolute such as we know it for biochemical pathways; the biofilm 'pathway' is contextual and highly influenced by the environmental conditions.
- There are important correlations between biofilm structure and properties (e.g. tolerance to drugs) (Costerton et al, 1999). Since it has been emphasized several times above that biofilm structures depend on the conditions of the environment, it is clear that biofilm properties which correlate with specific structures or structural components may also be contextual and environmentally conditional. The many claims of increased antibiotic resistance of biofilm cells relative to planktonic cells hence must be taken with a grain of salt, since an environmentally induced change of biofilm structure can give rise to significant variation in the resistance level.

The consensus developmental pathway for biofilm formation on solid surfaces derived from the above is presented in Fig 1.

The figure illustrates that single cells carried by the water flow approach the surface and some of them bind to it. Initially in a reversible way allowing them to leave the surface and continue with the flow. At some stage, surface bound cells become more permanently attached and cell proliferation results in formation of micro-colonies. A continued growth of the micro-colonies depends on 1) the nutrient composition and concentration in the water flow, 2) the strength of the water flow, 3) the cohesiveness of the cells to each other, and 4) motility of the surface associated cells. In most *in vitro* systems the initial surface coverage will be very poor, meaning that separate single cells will be found scattered on the surface after inoculation of the flow-system. If nutrients are available, and if the cells possess cohesive properties allowing them to resist the prevalent shear forces as they grow up in the water column, more or less structured biofilms will develop depending on the concentration of nutrients (the higher concentration, the thicker the biofilm; the lower the concentration, the more structured the biofilm) and on motility (if the cells can migrate on the surface the biofilm becomes flat). Eventually, the biofilm may dissolve due to starvation or stress resulting in efficient dispersal of the cells and re-colonization downstream.

The described consensus representation of biofilm development is mainly based on the laboratory based flow-chamber set-up, in which small plastic blocs with drilled channels are supplied with a continuous flow of substrate, and a glass cover slip is used to seal the channels and to function as the substratum on which bacterial colonization takes place (Christensen et al, 1999). These flow-chambers can be mounted directly under the microscope (confocal laser scanning, CSLM), and inspection can take place with intervals or continuously for time-lapse recording of biofilm development. The

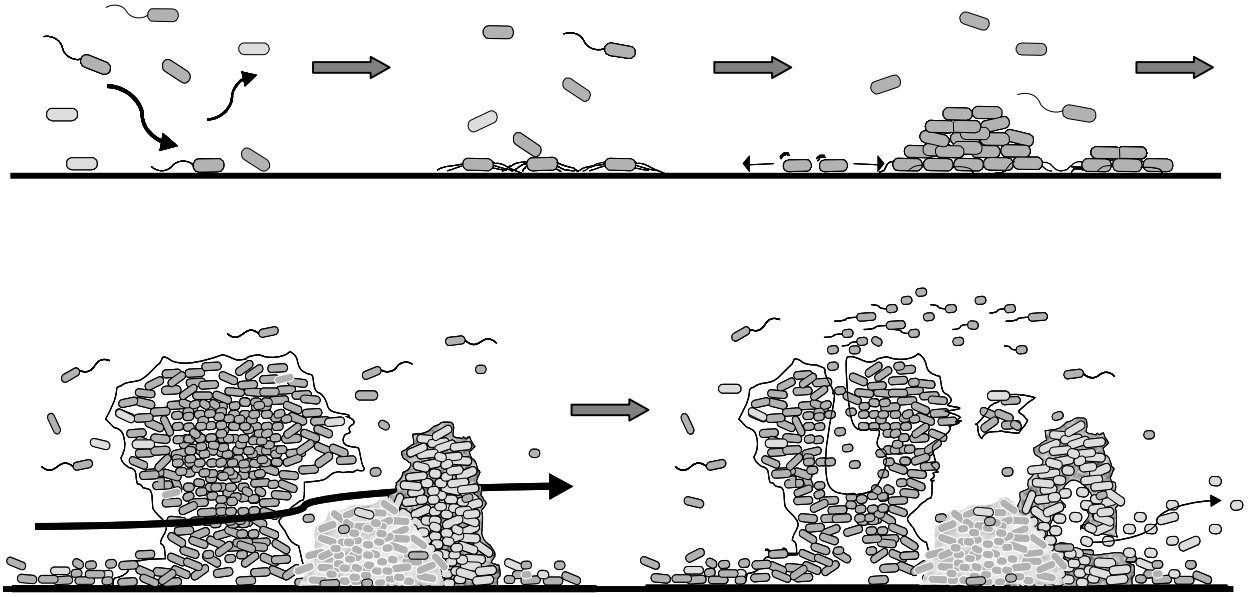

Fig 1 Schematic diagram of the biofilm 'life cycle' involving attachment, surface binding, proliferation, micro-colony development, maturation, and dissolution. Cell motility on the substratum and the flow of water through the biofilm are indicated by arrows (adapted from Hansen and Molin, 2004).

great advantage of this set-up is that it is possible to establish conditions completely controlled by the researcher, which may be reproduced not only in the same laboratory but also in other independent laboratories. The continuous flow ensures a constant environment surrounding the biofilm, but it must of course be kept in mind that within the biomass of the biofilm the conditions change constantly due to the biological processes going on here. The development of image analysis software (COMSTAT) permitting objective sampling of images and quantitative data processing has made it possible to exploit the reproducibility of biofilm development in these flow-cells for comparative investigations of strains, conditions and results obtained in different laboratories (Heydorn et al, 2000).

On the basis of a generally accepted consensus biofilm development scheme and the routine use of laboratory based set-ups including the data processing software time has now come to address the question: Is it possible to extrapolate from the laboratory systems to the human body?

In other words, can we infer from the wealth of information about *E. coli* or *P. aeruginosa* flow-chamber biofilms and from all the related genetic investigations how these organisms live in the human body in connection with the various infections they represent in patients? This important question is the focus point for the remainder of the present discussion.

THE *E. COLI* CASE

It was not surprising that one of the first model bacteria to investigate in relation to biofilm development was the old 'workhorse' of molecular biology, *E. coli*. The favoured microbe for genetic investigations, K-12, turned out to be less useful for biofilm studies, since it adhered quite poorly to the abiotic surfaces offered in the laboratory, glass and plastic. But it turned out that small changes of the K-12 strain, resulting in enhanced cell-cell cohesiveness, led to efficient biofilm development, which has since been studied extensively (Vidal et al, 1998; Ghigo, 2001). In a broader perspective *E. coli* is obviously of medical interest due to its role in a number of infections in the gastrointestinal (GI) tract, the urinary tract and also more severe cases of sepsis and meningitis. There is now a growing interest in the biofilm life style of *E. coli* since some of these infections are suspected of being associated with *in vivo* biofilm development (Anderson et al, 2003).

The major conclusions from the studies of *E. coli* K-12 biofilm development *in vitro* can be summarized as follows:

- Expression of a number of surface associated proteins (fimbriae, transfer pili, curli, flagella) may be essential for or have impact on biofilm development (O'Toole and Kolter, 1998; Vidal et al, 1998; Ghigo, 2001)
- Colanic acid may function as a stabilising EPS (Danese et al, 2000)

- The events leading to mature biofilm structures do not seem to involve complex differentiation steps in the population and the entire process may in fact be an example of a mechanical process mainly determined by the environmental conditions (Reisner et al, 2003).

The fact that the laboratory strain K-12 apparently had lost its biofilm properties during the many generations of growth as test tube cultures supported the view, held by many biofilm biologists, that bacteria in the 'real world' are mainly biofilm associated, but when we take them into the laboratory and investigate them we preferentially grow them in suspension, which results in loss properties related to the sessile life style. It was therefore expected that clinical isolates of *E. coli* – and especially those that were related to UTI, where the biofilm life style was assumed to dominate – would display much improved biofilm capability in comparison with K-12. In a fairly large survey of approximately 1000 clinical and commensal isolates of *E. coli* the surprising result was, however, that most of the strains were poor biofilm formers in the conventional microtitre plate or flow-cell assays, that there was a random distribution of their biofilm properties, and that there were no significant differences between the different strains obtained from very different sources (GI tract, urinary tract, bacteremia cases, or diarrhoea) (Reisner, Zechner and Molin, unpublished). In all groups the same distribution of biofilm potential was observed. The other interesting finding was that the biofilms developing in flow-cells (for the strains that were found to be positive for biofilm formation) showed a large degree of structural variation (flat, disperse colonies, fluffy, compact and others). These differences may indicate that differences in the cellular surface properties, which are expected in isolates from different environments, could be the major cause of the biofilm diversity among the isolates, and in connection with the very large genomic differences in various *E. coli* isolates the outcome of the investigation referred to above is perhaps not too surprising after all.

What the mentioned study of *E. coli* strains, however, does emphasize is that ideas about species-specific biofilm developmental pathways, which determine the structural and phenotypic features of the biofilm state of the organism, most likely should be abandoned. It does not seem likely from the above data that we can predict if, how, or when *E. coli* strains form biofilms, and consequently there is no obvious way of predicting what properties the infecting cells may have once they establish in the patient in the course of an infection.

THE *P. AERUGINOSA* CASE

The apparent complexity of *E. coli* biofilm structures could eventually be reduced to a matter of surface located adhesive proteins, which if present ensured continuous growth of micro-colonies from the solid surface despite the shear from the water flow. This relatively simple picture is not sufficient when we turn to the next very popular biofilm model organism, *P. aeruginosa*. This species has been a challenge in relation to describing the developmental processes involved in biofilm formation, and the fact that standard laboratory strains, such as PAO1, PA14 and PAK, show more or less identical developments on abiotic surfaces in the laboratory have stimulated even further the hunt for the biofilm development pathway. *P. aeruginosa* is a very versatile organism, which is found in many different environmental locations (water, soil, plants), and in addition it is a frequent opportunist infectious agent associated with humans suffering from reduced immune defence. In particular, the association of *P. aeruginosa* infections with CF patients has been a stimulatory factor for biofilm investigations since it is generally assumed that the chronic state of infection in these cases is related to biofilm developments in the lungs of CF patients.

The often referred to 'mushroom' structure of biofilms has its origin in the flow-cell induced *P. aeruginosa* biofilm structure (Costerton and Stewart, 2001; Klausen et al, 2003b). Already from the unprocessed images it is clear that biofilm development in this case may need more than just nutrition and diffusion rates to explain the mature micro-colony configuration. Moreover, when it turned out that this type of structural development was influenced by cell-cell signalling (quorum sensing) the connection to tissue development in multi-cellular organisms was tempting to make (Davies et al, 1998). There is now ample evidence showing that indeed the biofilm development for *P. aeruginosa* is complex and depends on a large number of biological properties of the organism, but before getting completely carried away it should be strongly emphasized that also in this case the particular development and the eventual mature configuration of the biofilm are conditional. What will be described in the following has so far only been documented for the standard reference strains growing in laboratory based flow-chambers and similar systems, and in fact only in connection with specific substrates. It is therefore not clear at all whether the scheme leading to 'mushroom' development has any relevance for environmental or clinical settings and conditions.

In the initial stages of *P. aeruginosa* biofilm development in flow-chambers, single cells attach to the glass surface, and soon after some of them begin to move on the surface, whereas others stay fixed to the original posi-

tion of attachment. On the surface the cells move with the aid of type IV pili in a motility form called twitching motility (Semmler et al, 1999). Subsequently, the motile cells both proliferate and disperse across the glass surface, whereas the non-motile cells divide and begin micro-colony development. At some point the entire surface is covered by the motile sub-population seen as a flat layer of cells on the substratum, whereas the micro-colonies rise from the substratum into the water column. In a final developmental process the migrating cells begin to climb the micro-colonies leading eventually to the formation of a 'cap' on top of the micro-colony 'stalks', and after some further time most of the surface attached cell layer has either disappeared or climbed to the mushroom tops (Klausen et al, 2003b).

The described sequence of events is indicative of a population differentiation process initiated soon after attachment of single cells to the glass surface. The regulation of this differentiation is presently unknown but the actual process depends on the presence of a fully functional type IV pilus motility apparatus. It has also been shown that the climbing process is influenced by EPS and surfactant production from the micro-colony cells (Lequette and Greenberg, 2005). In absence of rhamnolipid surfactant the 'mushrooms' will not mature completely leaving behind considerable biomass between the colonies (Davey et al, 2003), which suggests that the climbing process is less efficient in this case. Rhamnolipid production is partly controlled by cell-cell signalling (Pearson et al, 1997). An interesting component in the biofilm EPS of *P. aeruginosa* is extra-cellular DNA excreted in the micro-colonies and deposited on the outside of these (Whitchurch et al, 2002). Climbing may be enhanced by the DNA matrix (Aas et al, 2002), and removal of DNA in the early stages of biofilm development leads to disintegration of the biofilm (Whitchurch et al, 2003). Moreover, excretion of extracellular DNA is partly regulated by quorum sensing (Allesen-Holm et al, submitted). These findings suggest that *P. aeruginosa* biofilm development in flow-chambers is a complex process involving at least two rapidly emerging, phenotypically different sub-populations, which come together in the formation of the 'mushroom' structures. The combined activities of the two sub-populations ensure the development of stable structures able to resist the water flow shear forces and chemical treatments with detergents. Weakening of these structures occurs if the motile population is prevented in climbing the micro-colonies (type IV mutants), or partly inhibited in the climbing process (quorum sensing mutants, EPS reduction, surfactant reduction).

The efficiency of biofilm development observed for all the most used reference strains of *P. aeruginosa* (PAO1, PA14, PAK), and the suggestion that they probably all share the described processes leading to 'mushroom' formation, has made it a generally accepted conclusion that at least for this organism there is a specific and reproducible pathway of biofilm development, which may be investigated in flow chambers in the laboratory and extrapolated to *in vivo* scenarios in patients with *P. aeruginosa* infections. On this background it was therefore a surprising discovery that isolates of this organism from CF patients with chronic infections showed great variation in their capacities to develop *in vitro* biofilms (Li et al, 2005), and that even if they eventually did develop a biofilm the structural features of these often showed no resemblance with the 'mushrooms' described so far. It is of course not easy to interpret such findings, and they certainly do not exclude that formation of biofilms in the CF lungs is directly correlated to chronic infections, but they do caution against any simple extrapolations from the *in vitro* data. If flow-chamber experiments have indicated that for example antibiotic resistance is associated with the specific features of 'mushroom' development (Bjarnsholt et al, 2005), this cannot be taken as information with predictive value concerning the state of bacterial sensitivity or tolerance to the drugs in the lung of the patients.

CONCLUSIONS

In the comparisons made between laboratory reference strains and clinical isolates with respect to biofilm development the following conclusions can be suggested:

- Biofilm development is clonally very diverse. This suggests that many different factors have impacts on the process and the final outcome of surface attachment and growth, and in fact the cell surface is a variable of bacteria which traditionally has been used to distinguish specific strains in groups of serotypes, and which also may be highly relevant in connection with biofilm properties.
- There is no obvious correlation between *in vitro* biofilm development and presumed *in vivo* behaviour. This means that we must develop realistic analytical assays for the relevant organisms if biofilm development is suspected of being involved in the pathogenicity (or other properties) of the organism.
- It is not clear if the *in vitro* set-ups are irrelevant, if we use the wrong model strains, if biofilms as we know them from *in vitro* work do not exist *in vivo*, or if in general all our assumptions are totally wrong. There is only one way to find out: Let us start all over with much better approaches addressing directly the conditions in the patient or in the environment, whichever our research target.

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