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Characteristics and significance of microbial biofilm formation

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Abstract

Biofilm is a microbially derived sessile community characterized by cells that are attached to an abiotic or living surface and embedded in a matrix of extracellular polymeric substances that they have produced. This polymicrobic community has an altered phenotype and it is physiologically different from planktonic microorganisms. Epidemiological studies have shown that biofilm formation is associated with more than 60% of all human infections. Since microorganisms growing in a biofilm are highly resistant to antimicrobial agents and host's immune system, it is necessary to employ effective methods for the prevention or control of biofilm formation. Formation and persistance of a biofilm is a complex and dynamic process that needs to be studied as better understanding of biofilm characteristics will enable the development of new therapeutic strategies.

INTRODUCTION

Biofilm is a community of microorganisms attached to substrate surface and submerged into extracellular slimy matrix. Genetic diversity of organisms that form the biofilm *(1, 2)* and variety of environmental conditions where it emerges *(3, 4, 5)* prove that biofilm is an ancient ubiquitous life form of microorganisms.

Bacterial biofilm, as a sessile life form, ensures existence of bacterial life forms and it is a dominant phenotype in the nature over the free floating, planktonic form (6). Biofilm bacteria are protected from negative environmental influence *(7),* they can disperse *(8)* and are highly resistant to antibiotics *(9)*.

Biofilm has positive effects in biotechnology *(10),* but it is extremely harmful in industry *(11)* and in medicine *(12)*.

Biofilm causes numerous chronic infections, such as chronic osteomyelitis *(13),* chronic cystitis *(14),* chronic prostatitis *(15),* chronic otitis media *(16),* chronic pneumonia in patients with cystic fibrosis *(17, 18)*. In addition, biofilm also causes various infections of biomaterial used in medicine, such as infections associated with the use of intravascular *(19)* and urethral catheters *(20),* infections of orthopedic devices *(21),* contact lenses *(22),* prosthetic heart valves *(23),* vocal cord prosthesis *(24)*.

BIOFILM FORMATION

Biofilm formation (Figure 1) is regulated by different genetic and environmental factors. Genetic studies have shown that bacterial mobility, cell membrane proteins, extracellular polysaccharides and signal-

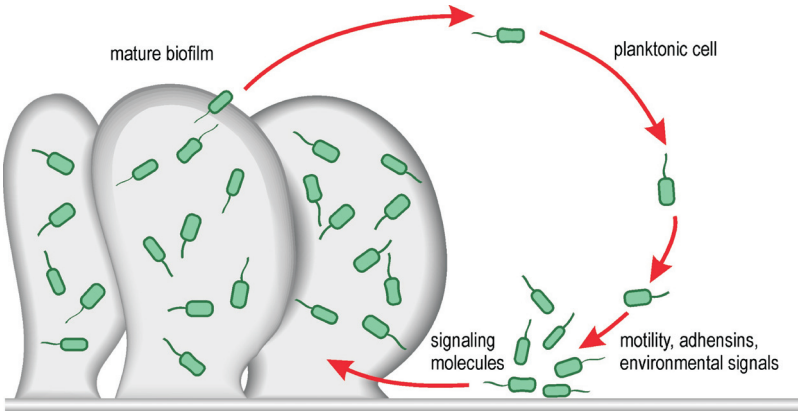

Figure 1. *Biofilm formation.*

ling molecules play significant roles in biofilm formation.

Bacterial mobility is enabled by two types of protein growths on the cell surface, flagella and fimbriae. Flagella are long, spiral growths that enable bacteria to float in liquid medium, and fimbriae are short, straight growths that enable limited, twitching movements of bacteria on substrate surface. Microscopic studies of wild-type strains and immobile mutant bacteria

Escherichia coli (25) and *Pseudomonas aeruginosa (26)* showed that both kinds of bacterial mobility are necessary for biofilm formation. Bacterial mobility enabled by flagella is necessary for establishing the connection between the bacteria and the surface, while the mobility enabled by fimbriae is necessary for the formation of microcolonies.

Initial interaction being established, stable connection between bacteria and substrate surface is maintained by specific cell membrane proteins, adhesins. If adhesin activity is inhibited, there is no biofilm formation, which was proved by studies carried out on *E. coli (25)* and *Vibrio cholerae (27)*.

Extracellular polysaccharide matrix (EPS) has a significant role in biofilm formation. Molecular genetic studies on *P. aeruginosa* showed that activation of genes necessary for extracellular polysaccharide synthesis took place after establishing stable connection between bacteria and substrate surface *(28)*. Studies conducted on *Staphylococcus epidermidis (29)* showed that the bacteria lose ability to form biofilm if the genes responsible for synthesis of EPS matrix are inactivated. Interactive communication via signalling molecules enables bacteria to organize into a community so that the biofilm functions as a multicellular organism.

Different signals from environment, such as availability of certain nutrients, presence of oxygen, temperature and pH, take part in regulation of a biofilm formation. Studies on *Listeria monocytogenes* biofilm formation showed that a too low or too high level of phosphates in the environment reduces biofilm formation, while the presence of carbohydrates mannose and trehalose stimulates biofilm formation *(30)*. Biofilm formation in *E. coli* is regulated by the presence of oxygen. In case of insufficient oxygen supply biofilm does not form, since bacteria cannot adhere to substrate surface *(31)*. Studies on the influence of temperature on *L. monocytogenes* showed that biofilm did not form if temperature was high, because the process of connecting bacteria to substrate surface was inhibited *(32)*. Environmental pH is also important for biofilm formation, which was shown by studies carried out on *V. choleare*. Optimal pH for multiplication of *V. cholerae* is 8.2, and if pH value is less than 7, that is if the solution is acid, the ability of this bacteria to form a biofilm is reduced due to the fact that bacterial cells lose their mobility *(33)*. Unlike *V. cholerae*, bacteria *S. epidermidis* and *E. coli* do not need alkaline environment to multiply so that they can form a biofilm on urethral catheters where urine pH is acidic.

BIOFILM STRUCTURE

Basic structural units of a biofilm are microcolonies, separate communities of bacterial cells embedded into EPS matrix. These microcolonies are in most cases mushroom-shaped or rodlike and they can consist of one or more types of bacteria *(34)*. Depending on bacteria type, microcolonies consist of 10–25% of cells and 79–90% of EPS matrix *(35)*. EPS matrix protects biofilm cells from various negative environmental conditions, such as UV radiation, abrupt changes in pH values, draining *(36)*.

Between microcolonies, there are channels through which water flows *(37)*. These water channels function in a biofilm as a simple circulatory system distributing nutrients to microcolonies and receiving harmful metabolites *(38)*.

Biofilm is also affected by environmental factors, such as nutrient availability and hydrodynamics.

Biofilm is polymorphic and it can adjust its structure to changes in the amount of nutrients, which was demonstrated by experiments with different glucose concentrations. When glucose concentration is high, microcolonies grow fast and consequently biofilm thickness increases significantly. When glucose concentration is

decreased, biofilm biomass is reduced and the former structure is restored.

Studies of biofilm in different hydrodynamic conditions, such as laminar and turbulent flow, have shown that biofilm structure changes depending on the flow type. In laminar flow bacterial microcolonies become round, and in turbulent flow they extend in downstream direction *(39)*.

BIOFILM MATURATION

Microscopic analysis and gene expression analysis during development of *P. aeruginosa* biofilm identified several developmental phases, each phase having different phenotype *(40)*. In every phase of biofilm development, bacteria cells were physiologically different from cells in the other phase. In a mature biofilm, all phases can exist simultaneously.

In *P. aeruginosa* biofilm maturation, five phases can be distinguished: reversible adsorption, irreversible attachment, maturation I, maturation II and dispersion *(40)*.

Initial event in biofilm development is interaction between planktonic bacteria and substrate surface. This phase is called reversible adsorption because some bacteria attach to the substrate surface only for a brief period and then detach from it. This phase lasts a few minutes.

In the second phase, irreversible attachment, bacteria adhere firmly to substrate surface and lose their mobility. Bacterial cells attach to each other and to the substrate surface and thus formation of bacterial microcolonies begins. This phase lasts two hours.

Protein analysis of a first two phases in biofilm formation determined that there were significant differences in regulation of the large number of proteins, which showed that there is physiological difference between reversibly and irreversibly attached cells.

Maturation I is the third phase in biofilm formation. In this phase, a matrix of extracellular polysaccharide substances (EPS) is produced. Microcolonies increase and become multi-layered, and their thickness is up to 10 μm. This phase lasts three days.

In the next phase, maturation II, bacterial microcolonies grow to their maximum size and their thickness is about 100 μm. This phase lasts six days.

Studies of protein expression have shown a significant difference between maturation I and maturation II phases. It is assumed that changes in protein structure are directly correlated to phenotype adaptations of bacterial cells.

Comparison of cells in maturation II phase and planktonic cells has shown significant difference in protein structure, which proves that there is great physiological difference between biofilm bacteria and planktonic bacteria.

The last phase in biofilm development is dispersion. In this phase, microcolony structure changes since the bacteria cells situated in their central part regain their mobility and detach from the previously formed structure. Microcolonies are therefore not mushroom-shaped or rodlike any longer, but adopt shell-like structure having an inner empty cavity and the wall consisting of immobile bacteria. The process dispersion probably takes place to allow bacterial cells better access to nutrients. During this phase, water channels form between microcolonies. It lasts nine to twelve days.

Protein expression in the dispersion phase is similar to protein expression in planktonic cells, which proves that some bacteria return into planktonic phenotype.

QUORUM SENSING

Intercellular communication of bacterial cells is provided by extracellular signalling molecules, autoinductors.

Accumulation of signalling molecules in the medium enables every single bacterial cell to estimate the total number of bacteria, that is cell density. This phenomenon is known as *quorum sensing*. At exactly determined critical cell density, concentration of autoinductors in the medium reaches the level required for activation of specific target genes *(41)*.

Signalling molecules in gram-negative bacteria are non-essential amino acids named acyl-homoserine lactones (acyl-HSL) *(42)* (Figure 2). Synthetized acyl-HSL

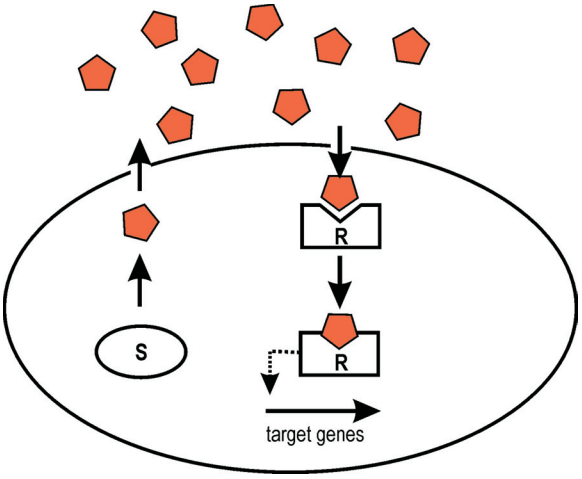

Figure 2. *Mechanism of quorum sensing in Gram-negative bacteria. pentagons = acylated homoserine lactone (acyl-HSL), S = acyl-HSL synthases, R= acyl-HSL binding protein*

produce acyl-HSL molecules, which diffund through the cell membrane and gradually accumulate in the medium. When the concentration of signalling molecules in the medium becomes high enough, they enter the cell and bind to the HSL receptor. A complex consisting of a signalling molecule and a receptor binds to suitable target genes and activates their transcription. Gram-positive bacteria use oligopeptides *(43)* as signalling molecules

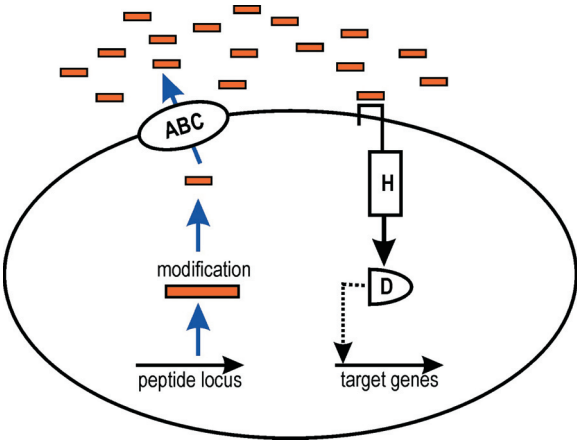

Figure 3. *Mechanism of quorum sensing in Gram-positive bacteria ABC = transporter protein complex, H = histidin kinase, D = regulator protein*

(Figure 3). Protein complex ABC transports oligopeptides out of the cell into intercellular space. At sufficiently high concentration of autoinductors in the medium, the signal is sensed by a protein system consisting of protein kinase and a regulatory protein. After binding signalling molecules protein kinase phosphorylates and thus becomes activated. The activated protein kinase activates the regulatory protein which then binds to specific target genes and activates their transcription.

Quorum sensing is a signalling mechanism that regulates specialized processes in bacteria, such as bioluminiscence, expression of virulence factors, beginning of the resting phase, production of antibiotics.

In studies on *Vibrio fischeri* culture, has been observed that luminiscence appears only in case of high density of bacterial population. It was found that bacteria produce acyl-HSL molecules into the medium which accumulates and thus causes luminiscence *(44)*.

Virulence of many human pathogenes, among which are bacteria *P. aeruginosa (45)* and *Staphylococcus aureus (46)* is regulated by quorum sensing. To avoid activation of the host's immune system, pathogenic bacteria coordinate their virulence by postponing the production of virulence factors until the bacteria population becomes large enough to cause infection.

In bacteria *Bacillus subtilis* (47) and *E. coli (48),* quorum sensing is a signalling system that regulates the genes included in transition of bacteria into the resting phase if environmental conditions are unfavorable.

Quorum sensing system regulates biosynthesis of the antibiotic karbapenem in bacteria *Erwinia carotovora*. Acyl-HSL signalling molecules activate proteins that act as transcription activators and induce expression of genes responsible for synthesis of this karbapenem antibiotic *(49)*. In mutant bacteria that cannot synthesize acil-HSL molecules, karbapenem is not synthetized *(50)*.

Intercellular communication is also possible between bacterial cells of different types, which has been demonstrated by studies on mixed biofilm consisting of bacteria *P. aeruginosa* and *Burkholderia cepacia*. Both types of bacteria use the same kind of signalling molecules, acyl-HSL, which enables communication between them and coordinates expression of virulence factors. The signalling between these two types is one-way, from *P. aeruginosa* towards *Burkholderia cepacia*. Actually, acyl-HSL signalling molecules produced by *P. aeruginosa* stimulate expression of target genes in *Burkholderia cepacia,* but the process cannot function reversely *(51)*.

GENERAL STRESS RESPONSE

It is assumed that slow growth of certain subpopulations of cells within a biofilm is a consequence of general stress response, a regulatory mechanism that enables bacteria to survive in unfavorable environmental conditions.

General stress response includes numerous physiological changes in bacterial cells and their passing into stationary phase. This results in bacterial cell resistence to various unfavorable environmental conditions, such as lack of nutrients, unfavorable temperature, pH changes, action of various chemical agents *(52)*.

At molecular level, general stress response is regulated by RpoS protein which acts as a RNA polymerase sigma subunit. RpoS controls a complex network of genes responsible for the passing of bacterial cells into stationary phase *(53)*.

It is considered that general stress response is initiated by cell density. At high density of bacterial population, RpoS quantity abruptly increases, which leads to expression of genes regulated by RpoS *(54)*.

The fact that RpoS is significant for the life of bacteria in a community was confirmed by studies on *E. coli*. Actually, deletion mutants of RpoS gene rendered *E. coli* incapable of forming a normal biofilm, while rpoS deletion did not significantly affect planktonic cells *(55)*.

Interaction of regulatory factors is complex, which is confirmed by the fact that RpoS also acts as a regulator of genes included in quorum sensing system *(56)*.

PERSISTERS

Persisters are a fraction of bacterial cells resistant to the concentration of antibiotics that destroys most of the population of a certain bacterial type.

Existence of such bacterial cells was discovered while studying effects of penicillin on a population of streptococci. It was discovered that a culture did not become sterile after penicillin treatment, but there was a small fraction of cells left (10^{-6}) that survived. Those cells were named persisters *(57)*.

Effects of different concentrations of antibiotics on *P. aeruginosa* biofilm were studied and the results showed that most biofilm cells were successfully destroyed by relatively low, clinically acceptable antibiotic concentra-

tions (?5μg/ml), almost not different from concentrations required to destroy planktonic cells *(58)*.

It is therefore assumed that biofilm survival can be explained by effects of persisters. When bacterial cells are treated by antibiotics, most planktonic cells are destroyed as well as most biofilm cells. A small population of planktonic persisters that is left after the treatment is destroyed by host's immune system, so that they do not represent clinical problem. However, unlike planktonic persisters, biofilm persisters are protected from immune system by polysaccharide matrix *(59),* so that a small fraction of persisters is responsible for high biofilm resistance to destruction. Actually, when the concentration of antibiotics decreases, the persisters restore the biofilm, which then starts to release new planktonic cells. This dynamics explains relapsing nature of biofilm infections *(60)*.

We still know rather little about the nature of persisters. However, it was determined that persisters are neither a separate phase in the cell-cycle, nor mutants, but a variant of a wild strain of a certain bacterial type *(61, 62)*.

Although survival mechanism of persisters is still unknown, several genes related to resistance have been described based on the studies on *E. coli (63)* and *Streptococcus pneumoniae (64)*. It is assumed that persisters might be cells with damaged apoptosis mechanism. In normal bacterial cells, antibiotics cause damage that activates apoptosis which initiates cell self-destruction. High tolerance of persisters to effects of antibiotics might be a consequence of inefficient apoptosis in these cells, which enables their survival *(65)*.

BIOFILM RESISTANCE TO ANTIMICROBIAL AGENTS

It is difficult to eradicate bacterial biofilm which is therefore the cause of numerous chronic infections. The bacteria within the biofilm are 10–1000 times more resistant to antibiotics than planktonic cells *(52),* but their resistance mechanism is still unexplained. So far three hypothesis have been formulated in attempt to explain biofilm resistance to antibiotics.

The first hypothesis is based on slow or incomplete diffusion of antibiotics into biofilm inner layers. EPS matrix containing embedded biofilm bacteria represents a diffuse barrier for a great number of bacteria *(66)*. Studies on *P. aeruginosa* biofilm showed that polymeric substances in a matrix with negative charge bind to antibiotics with positive charge, thus reducing their diffusion *(67, 68)*. Penetration of antibiotics into *Klebsiella pneumoniae* biofilm is restricted due to deactivation of antibiotics that occurs in outer biofilm layers. This process takes place at higher speed than diffusion *(69)*.

The second hypothesis is based on changes that occur in biofilm microenvironment. According to this hypothesis some biofilm bacteria fall into a state of slow growth due to lack of nutrients or accumulation of harmful metabolites, and therefore they survive *(9)*. Experiments conducted on planktonic cells and biofilm cells of *P. aeruginosa, E. coli* (70) and *Staphylococcus epidermidis (71)* confirmed the assumption that slow growth protects biofilm cells from effects of antibiotics.

According to the third hypothesis, up to now only a theoretical one, there is a subpopulation of cells within the biofilm whose differentiation resembles the process of spore formation. This subpopulation has a unique, highly resistant phenotype that protects them from effects of antibiotics. This phenotype does not develop as a result of insufficient nutrient provision, it is a biologically programmed response to the sessile life form of bacteria *(36)*.

Application of various molecular-biological and microscopic techniques proved that bacteria within a biofilm are physiologically heterogenous *(72, 73, 74),* which is highly significant for resistance to antibiotics. Actually, thanks to the great diversity of metabolic stages coexisting within a biofilm, the survival of certain number of cells is ensured in case of any metabolic threat to the biofilm.

CONCLUSION

Biofilm represents a specific life form of microorganisms which provides not only efficient protection from negative outside influence, but also physically and chemically suitable micro-environment necessary for growth and survival.

The fact that biofilm is the cause of many chronic diseases *(13, 14, 15, 16, 17, 18),* infections of catheters *(19, 20)* and other biomaterials *(21, 22, 23, 24)* used in medicine, makes the research on biofilm extremely important for medicine. It is estimated that 65% of all bacterial infections are caused by biofilm *(58)*.

Contemporary interdisciplinary research, based on genetic analyses, microscopic observations and studies of gene expression, has resulted in advanced knowledge of molecular and genetic basis of biofilm development and survival.

It has also contributed to an increasing number of strategies for biofilm prevention and control. Biofilm formation can be prevented by signalling molecules that block the attachment of bacterial cells to substrate surface *(75),* and by chemical reactions that prevent synthesis of polymers in extracellular matrix *(76)*. Substances that block communication between bacteria can prevent biofilm formation or stimulate its dispersion *(77, 78)*. Biofilm dispersion can be induced by enzymes that break down polymers in extracellular matrix *(79)*. To develop new treatments for biofilm destruction, it is extremely important to carry on research on mechanisms that lead to increased biofilm resistance to antimicrobial agents *(80, 81, 82)*.

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