

ON THE MOLECULAR BASIS OF BIOFILM FORMATION. ORAL BIOFILMS AND SYSTEMIC INFECTIONS

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REZUMAT

Se cunoaște că bacteriile nu există sub formă de celule solitare, ci sub formă de colonii care elaborează sisteme de comunicare intercelulară, care facilitează adaptarea lor la condițiile de mediu. Microorganismele suferă modificări profunde în timpul tranziției de la faza planctonică (liberă) la faza în care se atașează unei comunități bacteriene stabile (biofilm). Parodontopatia este exemplul clasic de boală care are ca substrat formarea biofilmului. Datorită rezistenței sale în mediul bucal, biofilmul parodontal este incriminat în apariția și propagarea infecțiilor sistemice la pacienții cu imunitate deficitară. De aceea, pentru a preveni diseminarea bacteriană și apariția infecțiilor sistemice, igienizarea dentară profesională regulată și controlul cavității bucale sunt recomandate.

Cuvinte cheie: biofilm, boală parodontală, infecții sistemice

ABSTRACT

Bacteria do not exist as independent cells, but they create colonies that elaborate intercellular communication systems in order to adapt to environmental conditions. Microorganisms suffer important transformations from the planctonic (free) phase to the phase of stable bacterial community (biofilm). The periodontal disease is a classic example of disease in which biofilm formation plays an important role. Due to its resistance in the oral environment, the periodontal biofilm is responsible for the onset and evolution of systemic infections in patients with immunological disorders. Therefore, in order to prevent bacterial dissemination and occurrence of systemic infections, regular professional cleaning and control of the oral cavity are recommended.

Key Words: biofilm, periodontal disease, systemic infections

Learning Objectives: The aim of this CME article is to highlight the most significant details regarding the transformations of bacteria at the moment when they constitute the biofilm. After studying this article the readers will be able to understand the complex genetic mechanisms involved in biofilm formation, antibiotic resistance and the relationship between oral biofilms and systemic infections.

INTRODUCTION

The bacterial biofilm consists of an organized community of bacterial cells incorporated in a polymeric matrix produced by bacteria adhering to a living or inert surface.¹

1. MOLECULAR GENETIC MECHANISMS INVOLVED IN THE FORMATION OF THE BIOFILM

It was demonstrated that during the attachment of the biofilm, certain gene transcription is activated. Studies using constructor reporters in *Pseudomonas aeruginosa* showed that the gene transcription needed for extracellular polysaccharide synthesis is activated after the adherence to a solid surface.²

According to Marsh and Martin, the general characteristics of a biofilm are: protection against host defence systems and invaders, resistance to desiccation, resistance against antibacterial agents, genetic expression and new phenotypes, persistence in washing systems, spatial heterogeneity, and spatial organization which facilitates metabolic interactions.³ (Fig. 1)

In the past, biofilms were considered as simple dense aggregates of cells. An important progress in the study of biofilms was the possibility to study them

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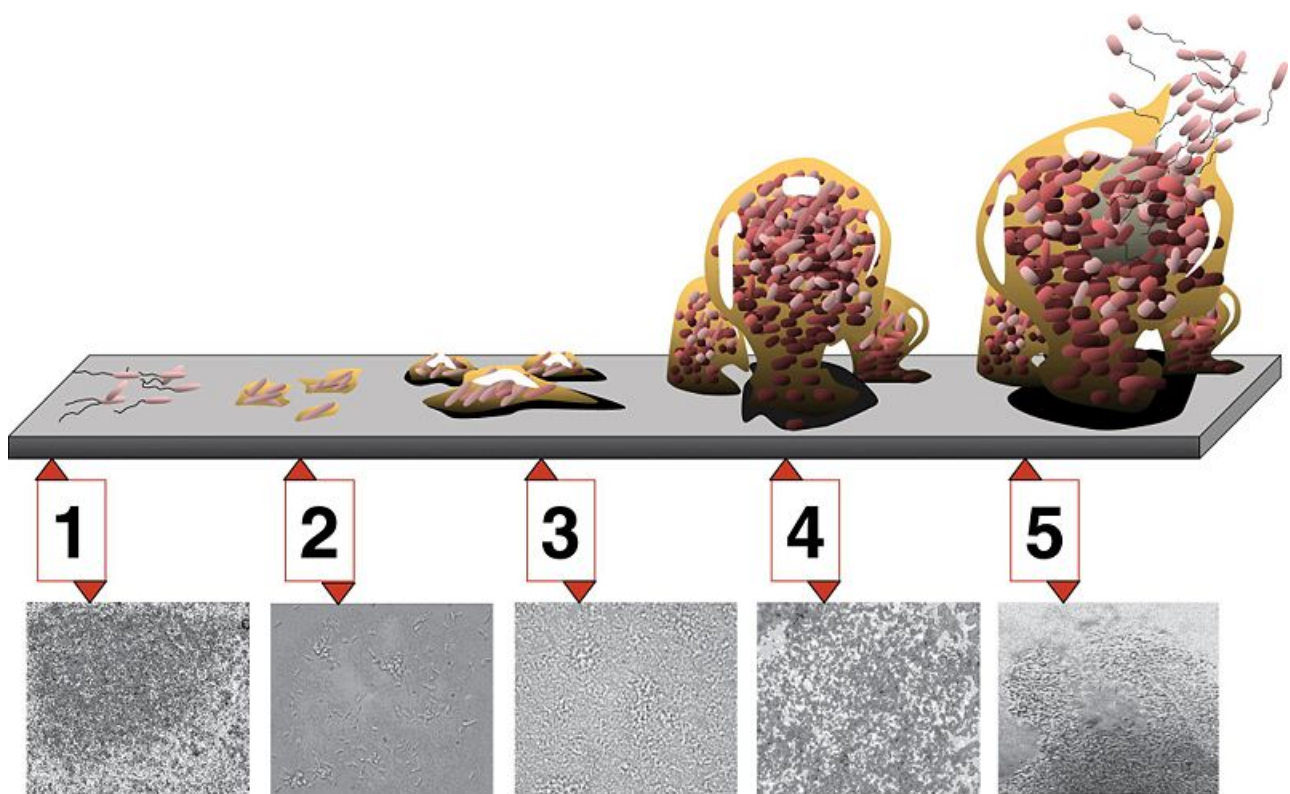


Figure 1. Five stages of biofilm development. Stage 1- initial attachment; stage 2 - irreversible attachment; stage 3 - maturation I; stage 4 - maturation II; stage 5 - dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. All photomicrographs are shown to same scale.⁵³

in situ, without any processing of the samples that could deteriorate their structures. The use of confocal microscopy to study the biofilm architecture without chemical fixation was also a remarkable achievement. Very thin optical sections can be obtained out of the entire biofilm structure and these can be processed by mean of image software to obtain three-dimensional images. Moreover, specific organism location can be detected by using immunological or nucleic acid probes. Confocal microscopy can be combined with the reporter gene technique in order to identify the genes expressed only in one biofilm. The method consists in insertion of a marker in the bacterial chromosome, downstream of a promoter, so that a signal is produced when the gene is activated. The application of these modern techniques to the bacterial ecosystem showed that biofilms developing in poor nutritional environments, especially those in aqueous habitats, have a more open structure than previously estimated. This structure allows bacteria to survive in apparently hostile environments.³

An interesting property of biofilms is the “quorum-sensing” through acyl homoserine lactone (AHL). It has become obvious that numerous microorganisms have the ability to recognize the presence of another bacterial population and to communicate together. A large number of signal molecules and response

pathways have been defined lately and many of them belong to this regulation process. Quorum-sensing (QS) serves as an intra- and interspecies bacterial communication system.⁴

QS bacteria regulate gene expression in response to increasing cell population density by producing and releasing auto-inducers. The mechanism is as follows: when a threshold stimulatory concentration of cells is achieved, the autoinducer binds a regulatory protein which is activated as a transcription factor. The regulatory protein is a member of the LuxR family of transcription factors and activates the transcription of genes that encode the LuxR protein and LuxI homologous. A feedback positive mechanism is involved. The autoinducer (AI-1) is a homoserine lactone (HSL) produced by LuxI proteins. The LuxI proteins are recognized by the receptor proteins LuxR. The activated receptor (R) induces transcription of several virulent genes. The QS LuxR/LuxI system is involved in intraspecies communications. AI-2, another autoinducer, is produced by LuxS and is involved in interspecies communications.

Several chemical classes of bacterial-derived signaling molecules have been recently identified. These can be divided into two categories: amino acids and short peptides, frequently utilized by Gram-positive bacteria, and fatty acids derivatives, commonly

utilized by Gram-negative bacteria.^{5,6} The first phenotype in which regulation via such a QS system was identified, was the control of bioluminescence in a marine bacterium, called *Vibrio fischeri*. When living independently in seawater, at low cell densities, *V. fischeri* is non-luminescent. When grown to high cell densities in the laboratory, a *V. fischeri* culture exhibits a blue-green light. This organism usually forms symbiotic relationships with some marine species. The most studied example of such a symbiosis is that between *V. fischeri* and *Euprymna scolopes*. The latter, a small squid, appears bioluminescent in dark environments due to the maintenance of high density of *V. fischeri* in a specialized light organ. Thus, the squid becomes camouflaged against predators and, in return, provides the *V. fischeri* with nutrients. In recent years, similar systems have been found in different species of Gram-negative bacteria. It came out that these systems monitor the cell density by producing AHL. Their structure depends on the bacteria that produce them.^{7,8} In *Vibrio harveyi* the first system has been described as system 1, hence the auto-inducer which controls it is described as AI-1. In this case, the hydroxybutyryl homoserine lactone is the auto-inducer.

A second QS was described in *V. harveyi*.^{9,10} The structure of this autoinducer (named AI-2) is still uncertain, although it has been reported that its synthesis depends on the LuxS genes.^{9,11} This system seems to be more widespread among the microbial world than the first, and homologues for LuxS have been identified in a large number of Gram-positive and Gram-negative bacteria.⁹

The achievements of medical care in industrialized societies are currently impaired due to chronic opportunistic infections that are increasingly apparent in immunocompromised patients and in the aging population. Chronic infections remain a major challenge for the medical profession and are of great economic relevance because traditional antibiotic therapy is usually not sufficient to eradicate these infections. One major reason for persistence seems to be the capability of the bacteria to grow within biofilms that protect them from adverse environmental factors. *Pseudomonas aeruginosa* is not only an important opportunistic pathogen and causative agent of emerging nosocomial infections but can also be considered a model organism for the study of diverse bacterial mechanisms that contribute to bacterial persistence. Thus, the elucidation of the molecular mechanisms responsible for the switch from planktonic growth to a biofilm phenotype and the role of inter-bacterial communication (QS) in

persistent disease should provide new insights in *P. aeruginosa* pathogenicity, contribute to a better clinical management of chronically infected patients and should lead to the identification of new drug targets for the development of alternative anti-infective treatment strategies.^{4,5}

Over the recent few years, *P. aeruginosa* was the microorganism on which the most QS-related studies have been done. It is well known that this bacterium may cause pneumonia, septicemia, and chronic lung infections in cystic fibrosis patients. Virulence of *P. aeruginosa* depends on cellular and extracellular factors. This microorganism is recognized for its ability of producing virulent extracellular factors such as proteases, exotoxins, the exoenzyme S and the exoenzyme A. These factors are capable of being destructive both in humans and other mammals.^{12,13}

Two QS systems have been described for *P. aeruginosa*. The first system consists of *lasI* and *lasR* (encode an AHL synthase and a transcriptional factor) and the AHL signal molecule N-3-oxo-dodecanoyl homoserine lactone.¹⁴⁻¹⁶ The second QS system consists of *rhlI* and *rhlR* (encode an AHL synthase and a transcriptional factor) and the AHL signal molecule N-butyryl homoserine lactone.¹⁷

There is now overwhelming evidence that QS system is required for *P. aeruginosa* to cause the disease.¹⁸⁻²¹ A *P. aeruginosa* *lasR* mutant has been found to be avirulent in a neonatal murine model of acute pulmonary infection.¹⁸ Disruption of QS genes or elimination of virulence factors result in mild and treatable infections.

2. ANTIBIOTIC RESISTANCE

Bacteria growing in biofilms are antibiotic resistant, as opposed to planktonic ones. In order to elucidate this aspect, two hypotheses were taken so far into consideration: either antibiotics cannot penetrate biofilms, or the altered chemical microenvironment within the biofilm influences the effect of antibiotics.²²⁻²⁴ Reduced penetration into the biofilm may result in antibiotic inactivation because of secretion of certain enzymes, such as β -lactamases, or binding of the agent by the exopolysaccharide matrix. The exopolysaccharides could inhibit antimicrobial penetration by either binding the antimicrobial or forming a protective coating that prevents or delays diffusion through the biofilm.^{22,25,26} The complex heterogeneity within biofilms is demonstrated by studies analyzing different microenvironments throughout the biofilm that differ in metabolic activity,

pH, and oxygen distribution.^{27,28} A third hypothesis, more speculative, is that a subpopulation of microorganisms forms a highly protected state, similar to spore formation, thus being less susceptible to be killed. This phenotype is not a response to nutrient limitation; it is a biological response programmed for a future association of bacteria with a surface.¹ Thus, the “biofilm phenotype” is a collective term used to describe a biologically programmed response to growth on a surface that involves specific physiologies and patterns of protein and gene expression that are quite different from those of planktonic cells.²⁹ Increased resistance to antimicrobial agents is most probably a combination of all of the above mentioned mechanisms and may involve many of these factors working together as the biofilm matures.³⁰

3. ORAL BIOFILMS AND THEIR RELATIONSHIP WITH SYSTEMIC INFECTIONS

Biofilms have been found to be involved in a wide variety of microbial infections, by one estimate 80% of all infections.³¹ Infectious processes in which biofilms have been involved include common problems such as urinary tract infections, catheter infections, middle-ear infections, formation of dental plaque and gingivitis, coating contact lenses, and less common but more lethal processes such as endocarditis, infections in cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses and heart valves.³²⁻³⁵ (Fig. 2)

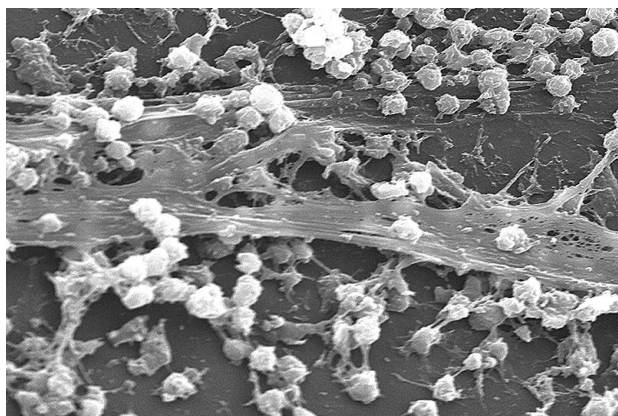


Figure 2. *Staphylococcus aureus* biofilm of the surface of a catheter (from CDC Public Health Image Library³⁴)

It has also recently been shown that biofilms are present on the removed tissue of 80% of patients undergoing surgery for chronic sinusitis. The patients with biofilms were shown to have been denuded areas of cilia and goblet cells, unlike the controls

without biofilms who had normal cilia and goblet cell morphology.³⁶ Biofilms were also found on samples from two of ten healthy controls as mentioned above. The species of bacteria from interoperative cultures did not correspond to the bacteria species in the biofilm on the respective patient’s intraoperative tissue sample. In other words, the cultures were negative though the bacteria were present.³⁷

With respect to the dental plaque, most of oral bacteria exhibit the property of intergeneric coaggregation.³⁸ Partnerships are specific and, sometimes, the interactions are site-specific. For example, *Veillonellae* isolated from the surface of the tongue co-aggregate with streptococci isolated from the same site, while *Veillonellae* isolated from subgingival plaque coaggregate with streptococci colonizing the same site.³⁹ Coaggregation may be intra-, inter- or multigeneric. The secondary colonizers synthesize adhesins that recognize receptors situated on primary colonizers such as streptococci.⁴⁰

Fusobacterium is considered to be a bridge between early and late colonizers. Early colonizers coaggregate among each other and together with *F. nucleatum*. Late colonizers do not coaggregate with early colonizers, but they coaggregate exclusively with *F. nucleatum*.⁴¹ George and Falkler demonstrated that five species of *Eubacterium* coaggregate with six strains of *F. nucleatum* in a group of 33 isolates representing 10 species that include *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia*.⁴² The great majority of early colonizers consist of streptococci, representing between 47% and 85% of cultivable species found during the first four hours after professional teeth cleaning.⁴³ Most of early colonizers recognize the components of the acquired pellicle, a thin layer that coats the freshly cleaned dental surface and consists mainly of glycoproteins, mucins and salivary enzymes. Within 12 hours after teeth are cleaned, the microbial population diversifies and includes *Actinomyces*, *Capnocytophagae*, *Haemophili*, *Prevotellae*, *Propionibacteria* and *Veillonellae*.⁴⁴

Gingival biofilms represent a permanent reservoir of Gram-negative bacteria, having direct access to periodontal tissues and circulation. Systemic reactions to Gram-negative bacteria or lipopolysaccharides induce major vascular responses, such as inflammatory response in the vessel walls, smooth muscle proliferation, or intravascular coagulation.^{45,46}

Regarding the periodontal pathogens, it is demonstrated that *P. intermedia*, *F. nucleatum*, and *P. gingivalis* exhibit signals similar to AI-2, capable to stimulate the production of light in *V. harveyi*.⁴⁷ In

addition, a genetic sequence (open reading frame) was found in *P. gingivalis*, which has 30% identity and 50% homology with luxS from *V. harveyi*.

Periodontal disease and chronic pulmonary infection in cystic fibrosis patients are classical examples of diseases that are associated with biofilms.^{48,49} Oral bacteria, poor oral hygiene, and periodontitis seem to influence the incidence of pulmonary infections, especially nosocomial pneumonia episodes in susceptible individuals.^{50,51}

The concept of biofilm is important in understanding the mechanisms of periodontal disease and its relationship with systemic diseases. The complete removal of subgingival dental plaque is difficult and, although the dental plaque is in permanent contact with the defense systems of the subgingival fluid, bacteria are not seriously affected. Very often, the antimicrobial therapy, administered either locally or systemically, is inefficient in killing the bacterial population that colonizes the dental plaque. Therefore, in order to prevent bacterial dissemination and the occurrence of systemic infections, especially in patients with depressed immunity, regular teeth cleaning, systematic removal of the dental plaque and biofilm and professional control of the oral cavity are recommended.

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