ISSN 0026-2617, Microbiology, 2006, Vol. 75, No. 4, pp. 481–485. © Pleiades Publishing, Inc., 2006.
Original Russian Text © Yu.M. Romanova, T.A. Smirnova, A.L. Andreev, T.S. Il'ina, L.V. Didenko, A.L. Gintsburg, 2006, published in Mikrobiologiya, 2006, Vol. 75, No. 4, pp. 555–561.

CONFERENCE PROCEEDINGS

Formation of Biofilms as an Example of the Social Behavior of Bacteria

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Received February 27, 2006

Abstract—This paper is a brief review of data on bacterial biofilms that occur inside and outside of host organisms. Such biofilms are of great ecological and clinical importance. The role of interspecies communications in the development of bacterial biofilms and infectious diseases is particularly emphasized. Considerable attention is given to the electron microscopic study of biofilms formed by *Salmonella typhimurium* cells incubated as a broth culture in microtubes without aeration. Bacterial samples taken from the biofilm and planktonic culture grown in the same microtube were comparatively investigated by transmission electron microscopy.

DOI: 10.1134/S0026261706040199

Key words: biofilms, bacteria, *Salmonella typhimurium*, electron microscopy

For many decades, researchers have studied bacteria grown in liquid media as homogeneous cultures. It has long been believed that bacteria live in nature as free-swimming (planktonic) cells, for example, in the surface layers of sea and river waters. Most of our knowledge of the growth dynamics, metabolism, adaptation, physiology, genetics, and molecular biology of bacteria was gained from studies of planktonic cultures.

In fact, the ability of bacteria to form complex communities and the role that these communities may play in nature have been known since the time of Leeuwenhoek, although the dominance of such communities in aquatic ecosystems was recognized only in 1978 [1].

Newly developed microbiological and molecular biological methods clearly show that most bacteria live as biofilms formed on various biotic and abiotic surfaces. Biofilms were found to be dominant in all natural ecosystems except for deep ground- and seawaters.

The formation and the functioning of biofilms are an example of the social behavior of bacteria controlled by both environmental and intercellular signals. Cell-to-cell interactions are due to the functioning of a so-called quorum sensing system, which provides for a continuous exchange of information through specific chemical molecules. This mechanism allows bacteria to live collectively as a multicellular organism. The intercellular quorum sensing signals and biofilms play a key role in the symbiosis of bacteria with higher organisms, animals, and plants. These signals are also important in pathogenesis, where they may control the course of diseases [2].

The formation of biofilms is the major strategy of bacterial survival inside and outside of host organisms. Biofilms are highly organized communities of one or several species, including actively functioning and resting (nonculturable) cells. In biofilms, bacterial cells undergo complex cell-to-cell interactions which control the expression of various genes in different parts of the biofilms at different stages of their development. This is why many researchers consider biofilms to be a functional analogue of multicellular organisms.

The ability of bacteria to form films on biotic and abiotic surfaces inside and outside of host organisms gives rise to various problems in human life. In particular, biofilms can enhance metal corrosion and reduce the performance of industrial devices. They can be responsible for many chronic infectious diseases and diseases resulting from the use of contact lenses, catheters, prostheses, and the implantation of artificial cardiac valves. Bacteria in biofilms are more resistant to drugs (including antibiotics), protective immune factors, and unfavorable environmental factors (extreme temperatures, pH values, and osmolarity) than are free-living bacteria [3, 4].

Until 1990, biofilms were believed to be merely nonstructured aggregates of bacterial cells enclosed in an exopolysaccharide matrix. This belief was based on studies of biofilms by electron and light microscopy. Both techniques, however, can give artifacts because of dehydration of biofilm specimens (electron microscopy) or extrafocal effects (light microscopy). It should be noted that the confocal laser scanning microscope

(LSM) invented in the 1950s was not used to study bacteria because of the dominance of the idea of their planktonic life. With time, however, researchers came to understand the role that biofilms play in natural processes, medicine, and industry, and realized the necessity of searching for adequate methods of biofilm study.

The application of LSM allowed researchers to show that biofilms can be formed by one or several species. A combination of LSM and epifluorescence microscopy and the use of special dyes and computer programs made it possible to determine the total number of cells in a biofilm sample and to count dead, live, nonculturable, and metabolically active cells. Irrespective of their composition, biofilms developing in natural ecosystems possess a complex structure, which is rather conservative (mushroomlike or columnar entities and microcolonies enclosed in the intercellular matrix and surrounded by channels full of liquid). The channels are responsible for the supply of nutrients and oxygen and the excretion of end metabolites from bacterial cells. The channels are formed and maintained in an active state with the aid of special mechanisms involving rhamnolipids. These mechanisms keep the channels open and provide for their proper functioning, due to which the biofilms retain their ultrastructure, are supplied with necessary nutrients and oxygen, excrete end metabolic products, and are prevented from infection with foreign bacteria.

The extracellular polymeric material (or matrix) is an important structural component of biofilms. The matrix is composed of exopolysaccharides (the best-studied component), proteins, nucleic acids, and other substances. One of the most important functions of the matrix is to protect the biofilm bacteria from various stressful factors, such as UV radiation, extreme pH values, osmotic shock, dehydration, antibiotics, and the defense mechanisms of the host organism. The complex structure of biofilms provides for the metabolic cooperation of bacterial cells within spatially well-organized systems by establishing either symbiotic or antagonistic interactions between different bacterial species.

Most of our knowledge of the properties and the genetic control of biofilm formation has been obtained from the study of the clinically important bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* [5, 6]. The electron microscopic study of implanted materials showed that they were covered by bacterial biofilms [7]. It is beyond doubt that the bacterial biofilms formed on implanted artificial cardiac valves are the main causative agents of endocarditis in patients bearing such valves. Millions of cardiac, intravenous, and urinary catheters used annually in clinics are potent substrates for the formation of biofilms. The biofilms produced on contact lenses often cause keratitis. On average, about 60% of nosocomial infections in patients with implanted materials are caused by bacterial films.

The role of biofilms in the infection of patients without implanted materials is not yet understood. As far as we know, only one pulmonary disease, cystic fibrosis, is undoubtedly caused by *P. aeruginosa* and *Burkholderia cepacia* biofilms. Those who suffer from this hereditary disease are very susceptible to chronic pulmonary infections caused by these pathogenic bacteria. The mechanism of this susceptibility is unknown. Cystic fibrosis is associated with an acute inflammation of pulmonary tissue, dyspnea, and a high rate of patient mortality. Electron microscopic analysis of pulmonary specimens taken from patients who died of cystic fibrosis showed the presence of *P. aeruginosa* biofilms [8]. As a rule, the *P. aeruginosa* strains isolated from individuals afflicted with cystic fibrosis are characterized by enhanced synthesis of alginate or exopolysaccharide, which is a key component protecting biofilm bacteria from antimicrobial agents.

It is known that changes in the range and degree of expression of the genes of the causative organism in response to the signal molecules produced by the host organism is an attribute of the infectious process. Infectious diseases are characterized by complex interactions between pathogens and their host organisms. As a rule, the phlegm of cystic fibrosis patients shows the presence of not only pathogenic *B. cepacia* and *P. aeruginosa* but also nonpathogenic indigenous bacteria. The study of the interaction between pathogenic *P. aeruginosa* strains and nonpathogenic oropharyngeal microflora in laboratory rats showed that the microflora itself does not induce damage to the pulmonary tissue but may contribute to the damage induced by the pathogenic *P. aeruginosa* strains, regardless of the degree of their invasion [8]. The promoter library of *P. aeruginosa* constructed by Duan et al. contained a luminescent reporter gene. Experiments with the use of this gene allowed the authors to show that the nonpathogenic microflora induces the expression of some genes in *P. aeruginosa*, in particular, the genes of elastase and exotoxin, which serve as the pathogenicity factors of this bacterium.

There is evidence that autoinducer AI-2, a type of quorum sensing signal molecule of bacteria, provides for interspecies signaling [2] and induces the expression of virulence genes. Unlike *P. aeruginosa*, oropharyngeal bacteria are able to produce AI-2 in large amounts. Thus, there are sufficient grounds to believe that the oropharyngeal bacteria contribute to the pathogenesis of cystic fibrosis through their interspecies regulatory signals [8]. The addition of the *P. aeruginosa* autoinducers to *B. cepacia* cells enhances the expression of virulence factors in the latter bacterium [9]. Taken together, all these data show that interspecies communications help mixed populations of pathogenic *P. aeruginosa* and *B. cepacia* cells to coordinate the production of virulence factors and thereby enhance the development of infection regardless of its type (either mono- or heteroinfection). Cystic fibrosis patients are usually treated with macrolid antibiotics, which allow

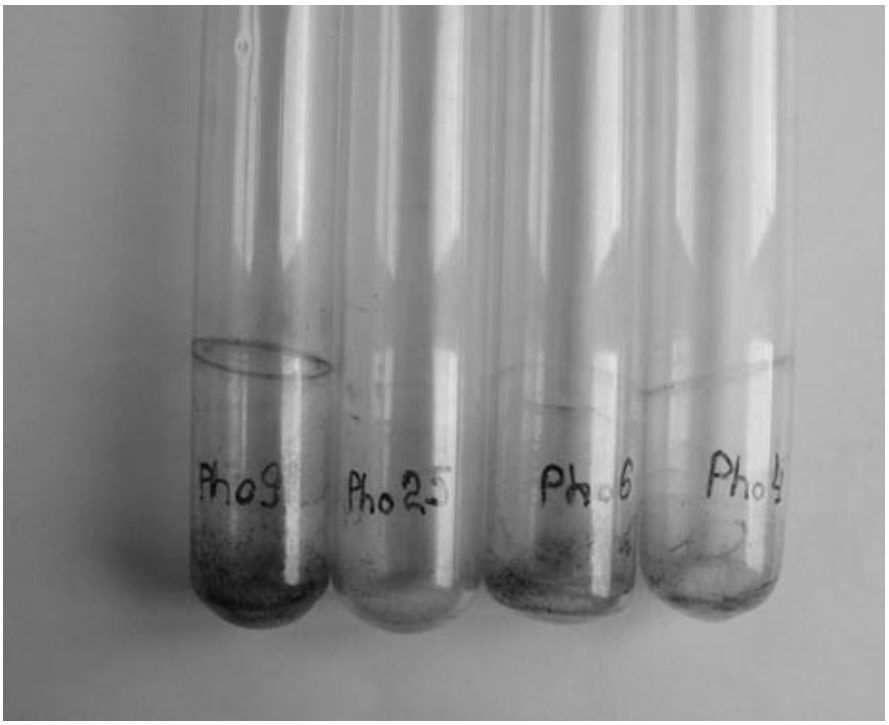

Fig. 1. Biofilm formation by salmonella cells incubated in glass tubes.

the patients to cope with the disease, although *P. aeruginosa* are resistant to these antibiotics. According to the aforementioned publication [8], the therapeutic effect of the macrolid antibiotics can be explained by their ability to affect the nonpathogenic oropharyngeal microflora of lungs, to reduce the synthesis of AI-2 by this microflora, and, consequently, to eliminate the transcriptional signal necessary for the synthesis of pathogenicity factors in *P. aeruginosa.*

To facilitate the study of biofilms, researchers have invented special devices for manufacturing biofilms under laboratory conditions on various plastic, teflon, and metal surfaces, and developed approaches to evaluate the produced biofilm. Relevant studies showed that biofilm formation proceeds via several stages (for example, as many as five in the case of *P. aeruginosa*). The growth of the biofilm of this bacterium begins with transition from planktonic life to life on a solid surface, when bacterial cells attach first reversibly and then irreversibly to the surface (substrate). The optimal attachment to the substrate requires the presence of flagella or IV- type pili. The attachment is associated with the immigration and division of bacterial cells, the formation of small cell clusters (first maturation stage), followed by their development into microcolonies (second maturation stage) and the formation of an exopolysaccharide matrix. The development of biofilms is completed by the stage of their degradation (dispersion) [10]. This stage depends on the availability of organic nutrients, iron, and oxygen in the environment.

The ever-increasing range of bacteria used in biofilm studies enriches our knowledge with new ideas of the mechanisms responsible for bacterial interactions in natural communities. Relevant research projects conducted at the Laboratory of Genetic Engineering of Pathogenic Microorganisms at the Gamaleya Institute of Epidemiology and Microbiology include the quantification of biofilms, the derivation of mutants with impaired ability to form biofilms, the testing of virulence of parent and mutant strains in model systems, and the electron-microscopic study of biofilms. It should be noted that biofilm formation can be simulated using 96-well plates, plastic or glass tubes, plastic petri dishes, and cover slips. In our laboratory, biofilms were studied quantitatively by the method of bound dye (crystal violet) using an iEMS Reader MF photometer and qualitatively using a digital camera or visual observations. The best formation of biofilms by the bacterium *Salmonella typhimurium* was observed in LB broth without NaCl at an initial cell density of 10^6–10^7, incubation temperature of 28°C, and incubation time of 24 h. In water, the bacterium formed biofilms very poorly.

The electron microscopic study of *S. typhimurium* cultures incubated in microtubes without aeration showed the formation of a dense film at the air–liquid interface (Fig. 1). Cell samples taken from this film and planktonic culture were comparatively analyzed in a JEM-100B transmission electron microscope (Japan) using specimens fixed according to Ito and Karnovsky [11], dehydrated in a series of alcohol solutions of

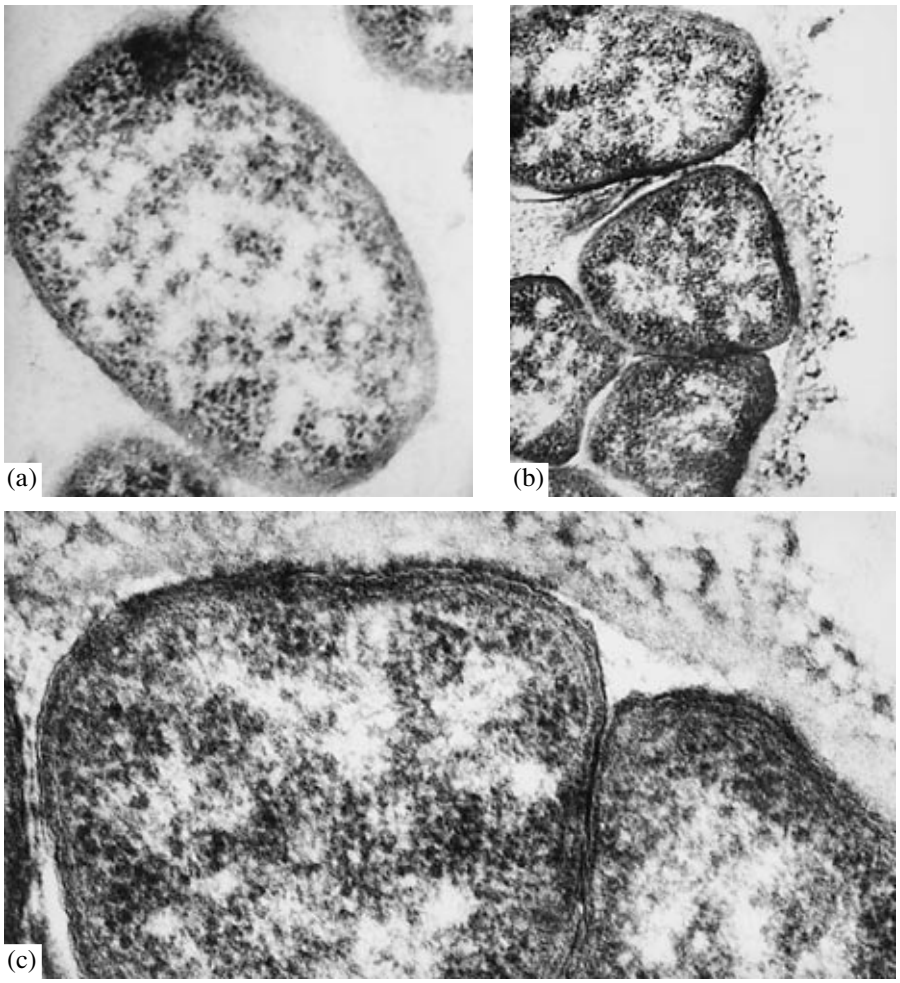

Fig. 2. Electron micrographs of *S. typhimurium* cells taken from (a) planktonic culture (magnification 70500×), (b) biofilm (magnification 50000×), and (c) fragment of this biofilm (magnification 110000×).

increasing concentration, embedded into LR-White methacrylate resin, and cut into thin sections with an LKB-3 microtome.

Figure 2 shows the micrographs of cells taken from the planktonic culture (Fig. 2a) and the biofilm (Fig. 2b, 2c). It can be seen that planktonic cells have a typical shape and ultrastructure; in particular, the cell wall is not damaged and the cytoplasm contains ribosomes, polyribosomes, and thin DNA threads. In contrast, the ultrastructure of the salmonella cells taken from the biofilm was characterized by a 3-fold thickened cell wall and a regular geometric shape of close-packed cells (Fig. 2b) enclosed in a honey- combed fibrillar envelope (Figs. 2b, 2c). The close contact of cells in the biofilm must facilitate intercellular communication, signaling, and gene exchange (for example, conjugal transfer of plasmids carrying multiple drug resistance). The intercellular space of biofilm cells is filled with a matrix necessary to maintain the biofilm integrity. These preliminary observations show that transmission microscopy may be useful for detecting differences in the structure of biofilms formed by the parent cells and those carrying mutations in the genes involved in biofilm formation.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, grant no. 04-05-49379.

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