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REVIEW

Biofilm—"City of Microbes" or an Analogue of Multicellular Organisms?

Yu. A. Nikolaev[1] and V. K. Plakunov

Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia

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Abstract—The definition of the term "biofilm" and the validity of the analogy between these structured microbial communities and multicellular organisms are discussed in the review. The mechanisms of biofilm formation, the types of interrelations of the components of biofilms, and the reasons for biofilm resistance to biocides and stress factors are considered in detail. The role of biofilms in microbial ecology and in biotechnology is discussed.

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INTRODUCTION

In spite of the differences in the definitions of the term "biofilm" suggested by different authors, certain specific features of these forms of microbial existence make it possible to classify them as spatially and metabolically structured microbial communities embedded in an extracellular polymer matrix and located at a phase interface.

As a rule, biofilms are formed in flow systems in the presence of the necessary growth substrates.

According to modern concepts, in natural environments 95–99% of microorganisms exist in the form of biofilms. Why are planktonic cultures (suspensions) of microorganisms the main form of cultivation under laboratory conditions? In order to answer this question, the conditions and basic stages of biofilm formation must be considered; this is the theme of the next chapter of our review.

The "pure culture" paradigm had been dominant in microbiology for a long time (since the days of R. Koch, who widely applied the method of cultivation on solid media). It enabled impressive achievements in the study of microbial diversity and in the development of methods of classification. However, this approach was not always beneficial for studies of microbial ecology and, in particular, of the contribution of microorganisms to biogeochemical processes in situ.

The paradigm shift emerged only in the middle of past century with the works of K. Zobell, who noticed the importance of the interaction between microorganisms and phase interfaces [1, 2]. J. Costerton [3] had formulated the concept of microbial life in nature mostly as structured associations, rather than as individual, freely floating ("planktonic") cells.

The most precise formulation of the new paradigm was, however, provided by G.A. Zavarzin, who instituted a change in the approaches to the study of biological objects, namely, the transition from reductionism to holism (philosophy of integrity), i.e., to the perception biological objects as integral, coevolving systems [4, 5].

As a result, the narrow view on microorganisms as unicellular forms of life is now being changed by the ever increasing realization of their ability to function in the composition of multicellular associations [6–9].

Biofilms are a kind of microbial consortia which play an important role in the biogeochemical processes of the biosphere. Molecular oxygen is one of the major factors which determine the processes of turnover of the biogenic elements. It suppresses the processes of dinitrogen fixation, denitrification, reduction of sulfates and metals, and methanogenesis. Due to the absence of the intracellular isolation of aerobic and anaerobic processes, microorganisms, and especially prokaryotes, are forced to form associations with other microorganisms and thus gain protection from the harmful consequences of the action of oxygen, especially of its active forms. This is one of the reasons for the fact that microorganisms exist in nature mostly in the form of structured communities. As we will show further, biofilms protect their microbial inhabitants not only from oxygen, but also from the consequences of other harmful environmental factors [10].

[1] Corresponding author. E-mail: nikolaevya@mail.ru

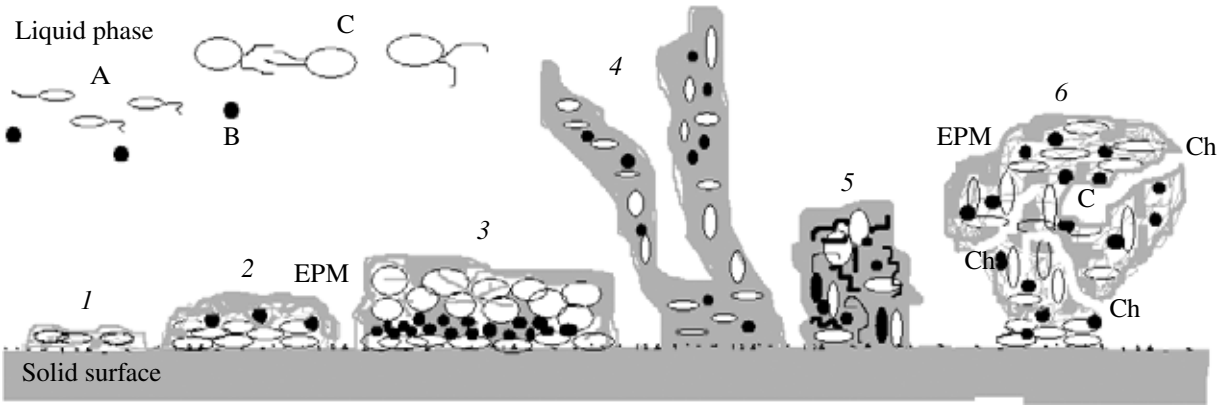

Fig. 1. The basic types of structure of bacterial biofilms: A, B, C—schematic representations of different microorganisms. (*1*) Layer of the cells of one species, submerged in the matrix of an extracellular polymeric material (EPM); (*2*) unstructured biofilm, comprised of two or several microorganisms, united under a common matrix; (*3*) mat, a laminated structure containing many species of organisms, with clearly expressed stratification, i.e., separated into morphofunctional horizontal layers (for example, the upper layer of phototrophic organisms, the middle, hydrolytic heterotrophs, and the lower one, anaerobic autotrophic or heterotrophic organisms); (*4*) biofilm with the developed surface in the form of tapes (bands) of mucous material; (*5*) dental plaque comprises many bacterial species, has a specific three-dimensional structure, and is limited in space; (*6*) mushroom-shaped body, with the narrower base, expanding upwards, comprised of many microorganisms; the structure includes channels (Ch), cavities (C), and pores (the latter not shown). Such biofilms can consist of cells of several species or of one organism (*P. aeruginosa*).

In accordance with the given definition of a biofilm, they are microbial communities which are formed on interphase boundaries. There are four known types of interphase boundaries on which microbial associations develop: liquid (aqueous medium)–solid surface; liquid–air; between two immiscible liquids; and solid surface–air. Therefore, four types of biofilms should be examined. However, to date, only the biofilms developing on the boundary of liquid and solid media have been investigated in detail, and this review is dedicated mostly to these biofilms.

STRUCTURE OF BIOFILMS

The most common and best-investigated forms of biofilms formed on the boundary of solid and liquid phases have the following morphotypes (Fig. 1):

—a simple cell layer formed by one or several microbial species, without expressed morphological differentiation (only an internal and an external part can be distinguished). This is the most primitive type of biofilm. Such films are described for bacteria of the genus *Citrobacter* [11] and for the *Pseudomonas aeruginosa* strains deficient in autoregulation [12];

—mats of photosynthetic [13], methanogenic [14], and sulfate-reducing [15] bacterial communities, and also of microbial communities which develop in wastewater treatment installations [16]. The mats are multispecies associations which exhibit stratification with respect to the gradient of the factor regulating the mat structure (light for phototrophs; nutrients, oxidation potential, etc., for chemotrophs). This type of biofilm frequently reaches tens of centimeters in thickness;

—dental biofilms (plaques) formed by a complex community of many microorganisms [17, 18]. This type of biofilm is one of best-studied. The strict sequence of colonization by different microorganisms, and the types and mechanisms of their interaction have been described. Hundreds of microbial species have been isolated from dental biofilms [19, 20];

—films with bandlike outgrowths, formed by mixed bacterial populations under conditions of turbulent flow [21]. These bands can be torn off and contribute to the dissemination of the population;

—"fungi"—biofilms formed by one or several microbial species and exhibiting a specific three-dimensional structure with special functional and morphological formations (pores, channels, voids, legs) [22, 23]. The genes responsible for the synthesis of alginates, rhamnolipids, and of the signal components of the "quorum sensing" system play an important part in the formation of such differentiated biofilms by *P. aeruginosa* and *Serratia marcescens* [24]; and

—benthic and river sediments, as well as flakes suspended in the water (flocs, "river and marine snow") and different types of overgrowths, especially those formed in "extreme biocenoses" [25, 26].

For the water–air boundary, three morphotypes have been described:

—a primitive layer on the boundary of water and air, where microbes form a film; such formations have been described for vibrios [11] and bacilli [27, 28];

—massive structures of the type of "kombucha" fungus (medusomycete) [29, 30]. The community of the kombucha fungus is a symbiosis of acetic acid bacteria with yeasts of many genera. The yeasts are embedded in the polysaccharide stroma produced by bacteria,

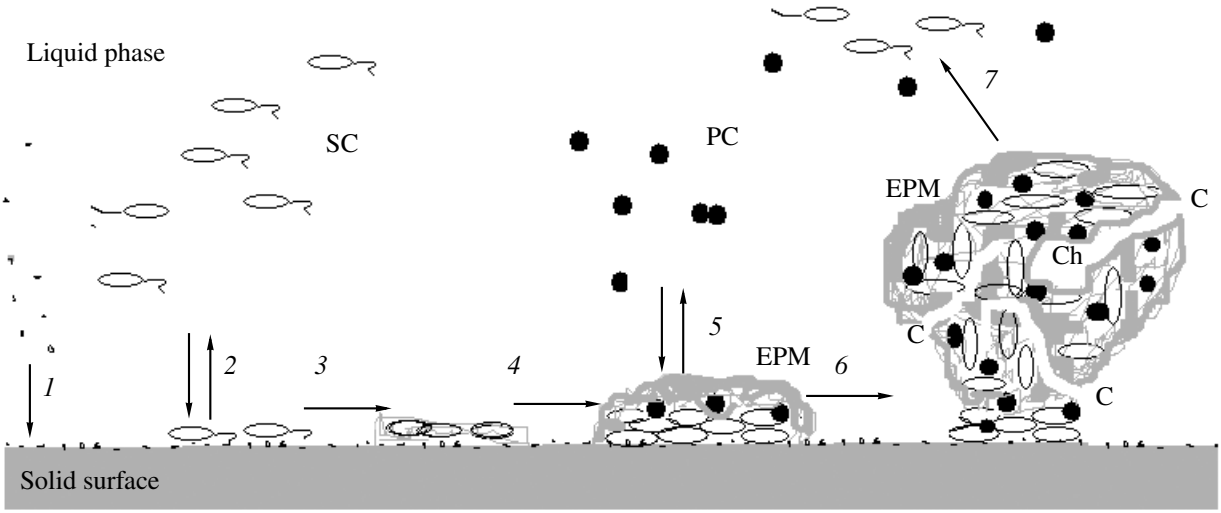

Fig. 2. Stages of formation and disintegration of biofilms at the solid–liquid interface: (*1*) Formation of layer of organic molecules on the phase interface (represented as black points); (*2*) reversible adhesion of microorganisms—primary colonizers (PC); (*3*) transition from reversible to irreversible adhesion, propagation of the primary colonizers over the surface, their growth with the formation of microcolonies and production of extracellular polymeric material, matrix (EPM); (*4*) formation of a three-dimensional structure caused by multiplication of the cells the primary colonizer; (*5*) attachment of other microorganisms—secondary colonizers (SC)—to the primary biofilm. Further development of the three-dimensional structure; (*6*) mature biofilm with an expressed macrostructure (in this case, mushroom-lke), microstructure (channels, pores, cavities), and physiological heterogeneity of microorganisms. Ch—channels, (*7*) C—cavity; some cell detach from the biofilm and become free-living.

and the entire association is located at the water–air boundary; its thickness can be up to several centimeters; and

—a film of bacilli at the liquid–air boundary, with loose main part consisting of chains of cells and with spore-forming fruiting bodies turned upwards (to the air phase) [27, 28].

STAGES AND MECHANISMS OF BIOFILM FORMATION AND REGULATION

Under natural conditions, a solid surface immersed in water is immediately covered by a so-called primary (conditioning) film, which changes the properties of this surface [31]. The formation of this layer of molecules is the first stage, preceding the formation of a bacterial film in the strict sense (Fig. 2). For example, the full-fledged formation of dental plaques on the teeth occurs only in the presence of saliva. The proteins of saliva form the surface layer to which the first bacterial colonizers become attached [32, 33].

The next stage is that of microbial adhesionproper, reversible adhesion, when microorganisms become reversibly fastened to the solid surface. At this stage, nonspecific physicochemical forces of interaction act between the molecules and structures on the surfaces of the microorganism and the solid substrate (van der Waals', hydrophobic, electrostatic, and London dispersion forces) [34, 35]. Both living and killed microbial cells are capable of this type of adhesion. For example, *P. fluorescens* cells killed by UV irradiation, γ-rays, or heating, do not lose their adhesive capacity [36].

The following stage, that of irreversible adhesion, begins when the cell becomes irreversibly attached to the surface. This phase, in turn, consists of several independent stages of biofilm formation. For a certain period after attaching to the surface, the cells can move along the surface by means of flagella and IV type pili [37–39]. Afterwards, the cells lose motility and some of them adhere to each other; excretion of extracellular polymers begins (polysaccharides, lipopolysaccharides, glycoproteins) [40, 41], which form the extracellular polymeric matrix (EPM). As a result of cell division, compact microcolonies emerge, bound with this matrix.

Afterwards, the secondary colonizers arrive, i.e., microorganisms which become attached to the cells localized on the surface. For example, in the course of the formation of dental plaque, streptococci are the primary colonizers; to their cells fusobacteria are attached, and subsequently other bacteria as well, up to several hundreds of species in total [17, 19, 20]. Simultaneously with an increase in the thickness of the biofilm, its specific structures are formed: cavities, channels, outgrowths, and pores. Under favorable conditions, this stage of the build-up of a mature biofilm continues for a sufficiently long time; under unfavorable conditions, the biofilm enters its last stage, disintegration, degradation, and loss of some cells and liberation of the others in the form of free-floating (planktonic) cells, as has been demonstrated for *P. aeruginosa, S. marcescens, Vibrio cholerae*, and *P. tunicata* [42].

Environmental factors and the properties of microbial cells affect the process of biofilm formation and its

characteristics. The most important environmental factors are pH, salinity, osmolarity, oxygen partial pressure, accessibility of nutrient sources, and also the hydrophobicity of the phase interface, the force and type of liquid motion relative to this surface.

The flow of liquid is the main factor affecting the displacement of both motile microorganisms and those incapable of motion to the phase interface. Bacterial adhesion to solid surfaces occurs both under conditions of turbulent fluid flow and when the aqueous phase is relatively immobile [35]. A zone of low-mobility water ("viscous layer") exists near the solid surface. The capillary (drainage) forces, which are caused by the pressure of the liquid flowing between the surface of the solid phase and the bacterial surface, also affect the movement of microorganisms. Sedimentation can play a significant role only in systems with slow flow and only for particles of significant size, such as large bacteria or bacterial aggregates. The inherent motility of microorganisms also plays a certain role, since the motile cells actively move towards the surface and are retained there by the forces of attraction, which act close to the surface [43].

The hydrophobicity of microbial cells plays an important role in their adhesion. Thus, in mutants of *Burkholderia cepacia, Shewanella alga,* and *Enterococcus fecalis* whose surface is considerably less hydrophobic compared to the wild type, the capacity for adhesion is reduced considerably [44, 45]. The surface charge also influences adhesion of microorganisms. Negative charge inhibits adhesion of *P. fluorescens* [46], whereas the presence on the surface of Fe^{3+} cations considerably increases the number of adhered anaerobic bacteria *Desulfomonile tiedjei*, *Syntrophomonas wolfei*, *Syntrophobacter wolinii*, and *Desulfovibrio* sp. (the cells of these bacteria are negatively charged) [47].

The effect of pH on bacterial adhesion on pH medium is to a large degree species-specific. The number of adhered cells of the gliding bacterium *Flexibacter* sp. increases with pH decrease. In the case of *Enterobacter cloacae* and *Chromobacterium* sp., the pH optimum for adhesion lies in the range of 5.5–7. Beyond this range, the number of attached cells is minimal. For *P. fluorescens*, the maximum adhesion occurs at pH 7 [48]. On the contrary, *Archaeoglobus fulgidus* forms a biofilm in the case of a sharp increase pH to extreme limits [49].

An increase in the ionic force of the solution increases adhesion when the initial ionic force is low (in solutions with NaCl concentration less than 0.1 M) [50]. At the higher values of this index, there is a spectrum of variants [36, 46]. Thus, for instance, NaCl concentrations up to 0.1 M increased adhesion of *Vibrio alginolyticus* and *P. fluorescence* above this value, inhibition of attachment was observed. Increased osmolarity of the medium enhances adhesion of *Staphylococcus epidermidis* [51].

The effect of temperature on microbial adhesion is also highly species-specific. Two types of temperature dependence are known:

—the maximum number of attached cells correlates with the temperature profile for the growth rate, i.e., the microorganisms are attached under conditions normal for their growth. This dependence is known for some strains of *P. fluorescens*, *Enterobacter cloacae*, and for *Chromobacter* sp. [48] and

—the maximum of adhesion is observed under non-optimal temperatures and even under temperatures in no way compatible with growth of these microorganisms. Elevated temperature causes adhesion of *S. epidermidis* [51]. In response to temperature stress, *A. fulgidus* attached to the surface of the cultivator and within 2–3 h produced significant quantities of a well-differentiated biofilm [49].

Oxygen concentration can also affect bacterial adhesion, as can certain toxic compounds [47] and ultraviolet radiation [49].

It seems likely that the factors which a microbe considers as stressors under given conditions are usually precisely those that have positive effect on adhesion (and subsequent biofilm formation).

The effect of biotic factors on adhesion has also been investigated.

The growth phase of a bacterial culture determines the adhesive properties of its cells to a considerable degree. For the cultures of anaerobic bacteria *Syntrophomonas wolfei* and *Desulfovibrio*, the adhesive capacity is most pronounced in the early logarithmic phase, decreases with age, and becomes negligibly low with the exhaustion of the nutrient sources in the medium. The enhanced adhesive properties of actively growing cells can be related to their mobility. The process of adhesion is characterized by a saturation curve; the maximum is reached after 2 h. The density of the cell suspension does not have a great effect [47]. The mode of nutrient supply, their qualitative composition, and limitation can be the regulatory factors for bacterial adhesion. Copiotrophic bacteria incubated in a medium with an extremely low content of carbon sources, i.e., under starvation conditions, react with an increase in the adhesive properties of cells [52].

A number of biochemical and genetic mechanisms are involved in the process of biofilm formation. The presence of genes that react to attachment and are active only (or mostly) in biofilms can be considered a specific genetic mechanism [9, 53]. Several genes react to reversible attachment, while irreversible attachment causes a change in the activity of dozens of genes. In *Bacillus subtilis*, biofilm formation is regulated by glucose according to the type of catabolic repression [54]. The presence of specific regulator systems of biofilm formation has been shown for *E. coli* [55, 56], staphylococci [57], and streptococci [58].

A number of authors stress the importance of the surface structures, flagella and IV type pili [37–39], and

of specific "sticky molecules," lectins and adhesins [59]. We agree with the last author and believe that adhesins are specific molecules responsible for the recognition of a surface to which attachment occurs, and also for the attachment itself due to hydrophobic, hydrogen, ionic, and covalent bonds. These molecules, naturally, are localized on the surface structures of microorganisms.

The process of adhesion is also regulated by the formations of antiadhesins, specific extracellular metabolites which prevent reversible adhesion. Antiadhesins are used both for the regulation of adhesion of the antiadhesin-producing organism and for dealing with competitors, hampering their introduction into the already formed biofilm. Thus, the cells of one of the film-forming marine strains of gliding bacteria *Cytophaga* do not suppress the viability of another strain; they do, however, inhibit its adhesion by excretion of an inhibitor, a glycoprotein consisting of five neutral sugars and 18 amino acids [60].

For *P. fluorescens*, gaseous regulators of adhesion have been described [61], as well as antiadhesins, represented by a mixture of *n*-alkanes [62], protease [63], and other hydrolases [40, 64–67].

Bacillary antiadhesins, such as lipocyclopeptide of *B. licheniformis* [68] and surfactin, a well-known biosurfactant produced by *B. subtilis*, have a similar antiadhesive effect. Surfactin inhibited adhesion and biofilm formation by *Salmonella enterica* [69]. Microbial biosurfactant production in biofilms was originally described as a protective mechanism against surface colonization by other strains on the example of probiotic bacteria in the human urogenital system, bowels, and larynx. For example, *Streptococcus thermophilus* and species of the genus *Lactobacillus* are capable of biosurfactant production in the course of growth [70–72].

Intercellular communication by means of specific autoregulators is extremely important for the development and functioning of biofilms. The mutants of *P. aeruginosa* incapable of autoregulation by homoserine lactones formed atypical, degenerate flat biofilms [12]. Regulators of another type, halogenated furanones, produced by marine algae, prevent the formation of bacterial biofilms [73]. A number of cases are known when the bacteria of a biofilm produce hydrolytic enzymes in response to the exhaustion of nutrients in the medium; this reaction enables them to use the polymers of the matrix as nutrient sources and simultaneously liberates them in the form of planktonic cells, able to find more favorable growth conditions [40, 63–67]. It was demonstrated that production of the rhamnolipid surfactant by *P. aeruginosa* is necessary to prevent dense attachment of newly formed bacteria inside the biofilm; it is also necessary for their removal from the biofilm, and therefore for the formation and maintenance of cavities within it [74].

The activity of bacteriophages leads to the lysis of some of the infected bacteria in a biofilm and to the formation of cavities and channels. In old films of *P. aeruginosa, S. marcescens, V. cholerae*, and *P. tunicata*, induced cell death was observed; it provided nutrients for the remaining cells and contributed to the structurization of the biofilm—to the formation of cavities, channels, and pores [42, 75].

In the beginning of this review, we raised the question of why microorganisms, as a rule, grow as planktonic cultures under laboratory conditions, while in nature they grow in the form of biofilms. Let us attempt to formulate the answer which we believe to be the most well-founded.

It is possible to assume that several such reasons exist. First of all, the inability to form biofilms may be the result of selection conducted by researchers and directed towards rapid growth of a microorganism in the form of a homogeneous culture; such cultures facilitate analytical procedures in the course of growth, obtaining the standard biomass, and isolation of cellular components. However, in the institutions where the processes performed by attached or aggregated cultures are studied (for example, for water purification), efforts are applied to maintain them in their native, aggregated state.

Moreover, under laboratory conditions, rich media and optimal growth conditions are used for the cultivation of microorganisms; this is possibly the main reason for microbial growth in the form of planktonic cultures. This assumption is in accord with the position of the majority of authors, who consider biofilms as a method of protection of microorganisms from stress factors.

DIVERSE INTERRELATIONS BETWEEN THE COMPONENTS OF BIOFILMS

The metabolic heterogeneity of microorganisms in the biofilms is caused by many reasons; the spatial localization of microbial cells in different sections of the three-dimensional matrix is the most obvious. Biofilms do not fundamentally differ in this respect from stratified marine and freshwater sediments. However, since the thickness of biofilms is, as a rule, less, the stratification processes are usually less pronounced.

Nevertheless, microsensor research has demonstrated that the preferable sequence of electron acceptors, inherent in sediments, is retained in freshwater biofilms [76]. The electron acceptors form a series, according to the value of the free energy change in the reaction of their reduction: $O_2 > NO_3^- > MnO_2 > Fe^{3+} > SO_4^{2-} > CO_2$. Therefore, first of all, O_2 is used (in the processes of respiration, nitrification and sulfide oxidation), and then NO_2^- and NO_3^-, formed in the process of denitrification or received from the environment. Deni-

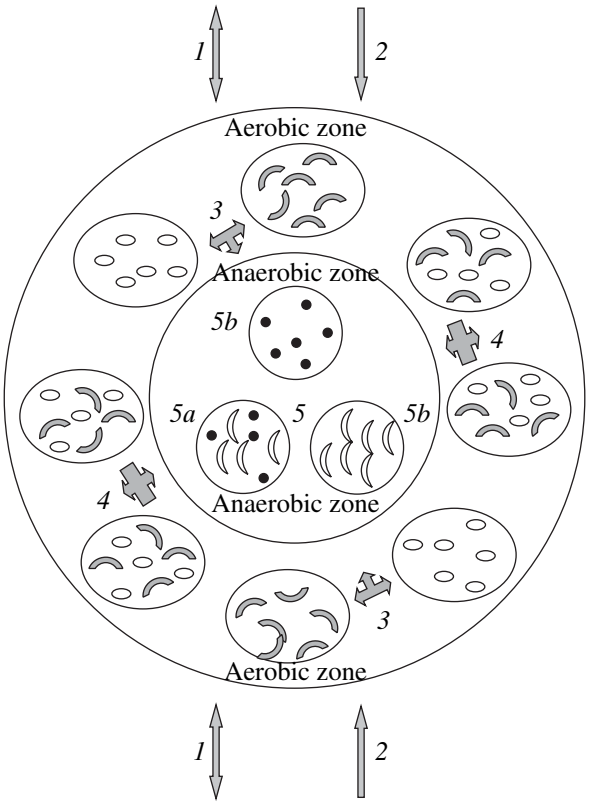

Fig. 3. Schematic diagram of microzonality in a microbial biofilm which consists of four microorganisms, a horizontal section (according to [33], with changes): (*1*) inflow of organic substrates and/or CO_2 and outflow of the products; (*2*) influx of oxygen and/or of light energy; (*3*) trophic and signal interactions of primary consumers/producers with formation of substrates for anaerobic processes (with the possible formation of syntrophic associations); (*4, 5*) anaerobic processes with (*5a*) or without formation (*5b*) of syntrophic associations.

trification can be coupled with sulfide oxidation. Sulfate reduction occurs below the zone of denitrification. Finally, methanogenesis is spatially isolated from sulfate reduction and occurs in the deepest zones of the biofilm.

On the whole, in a simplified form, the distribution of functions in the biofilm can be represented as follows (Fig. 3).

However, this orderly and logically substantiated model is by no means realized in all cases. The microzonality of biofilms results, for example, in sulfate-reducing bacteria being present not only on the boundary of the aerobic and anaerobic zones, but even in the aerobic sections; and in the latter case, they actively metabolize propionate [15].

Since biofilms, as a rule, are heterogeneous associations which consist of microorganisms of different physiological groups, it is necessary to discuss the basic types of interrelations (first of all the trophic relationships) between their components.

The interaction between the components begins in the process of biofilm formation. It has been repeatedly shown that biofilms consisting of microorganisms of different taxa are denser and thicker than biofilms which consist of microorganisms of one species [77]. Interaction apparently occurs at the stage of formation of the extracellular matrix.

Unlike planktonic cultures, interspecies competition is seldom observed in "mature" biofilms. Even when one of its species dominates due to a higher growth rate, the second retains viability and high abundance. Such interrelations have been discovered, for example, in binary biofilms (consisting of the cultures of two species) comprised of populations of rapidly growing cultures of *Klebsiella pneumoniae* and *P. aeruginosa* [78].

A less widespread variety of competition, ammensalism, can be caused by the production by one of the microorganisms of agents which inhibit other members of the community, or by the creation of unfavorable physicochemical conditions (for example, pH shifts). This situation was revealed in the binary biofilm formed by two *Ruminococcus* species, one of which forms a bacteriocin active against the other [79].

Commensalism and protocooperation are the most common types of interrelations between the microbial components of biofilms.

The term "commensalism" designates a one-way influence of one of the biofilm components on the activity of another component. Oxygen consumption by an aerobic microorganism, facilitating the growth of microaerophilic or anaerobic "room-mates," is a common example [25]. This type of interaction plays an important role in microbial corrosion performed by sulfate reducers located in anaerobic micro-niches [80].

Protocooperation leads to the mutual positive influence of the biofilm components on each other. Such interrelations exist, for example, in biofilms that contain phototrophic and heterotrophic microorganisms. The interaction between fermentative cellulolytic bacteria and methanogenic archaea is another characteristic example of protocooperation. Methanogens utilize molecular hydrogen and formate, which are formed in the course of fermentation, and thus shift the thermodynamic equilibrium, preventing the accumulation of reduced coenzymes in the cells of fermentative bacteria and stimulating ATP synthesis in their cells [81].

The previous case is an example when protocooperation borders on synergism, since both components of the biofilm benefit from this collaboration, and the formation or consumption of any product in the biofilm is higher than the values characteristic of the individual populations. A typical example is cellulose hydrolysis by biofilms containing both cellulolytic microorganisms and those incapable of cellulose decomposition. The latter stimulate cellulose hydrolysis and growth of cellulolytics by consumption of the low-molecular

products of hydrolysis, which repress the biosynthesis of cellulases [82].

Other examples of interactions between the microbial components of biofilms are given in the reviews [19, 53].

Exchange of genetic information plays a notable role in intercellular interactions in biofilms. This is to some degree the result of the high densities of the microbial populations. Massive evidence exists for the higher intensity of horizontal gene transfer in biofilms compared to planktonic cultures [83, 84, 53]. For example, the mechanism of the "toxin–antitoxin" type for protection of plasmids from elimination exists in biofilms (the role of this model for the existence of "persister" cells will be considered further) [85]. In principle, this protection consists of the encoding of a stable protein (toxin) and a labile protein (antitoxin) by the plasmid. If the daughter cells after division do not contain plasmids and are incapable of antitoxin resynthesis, then the stable toxin kills such cells when the residual antitoxin is decomposed. The effectiveness of horizontal gene transfer in situ has been convincingly proven by the application of scanning confocal laser microscopy (SCLM) with the aid of reporter genes encoding the fluorescent proteins: green (Gfp), red (Rfp), blue (Cfp), yellow (Yfp), and dark-blue (Bfp). Moreover, it is possible not only to determine the spatial localization of migrating plasmids in the population, but also to isolate the relevant subpopulation by the methods of cell sorting [86].

Let us consider one of the specific examples, when the combination of SCLM with the method of reporter genes made it possible to reveal the spatial and metabolic reconstruction of the biofilm community consisting of two bacterial components: *Burkholderia* sp., capable of degrading chlorobiphenyl, and *Pseudomonas* sp., "catching" the chlorobenzoate which is formed during the first stage. The green fluorescent protein (Gfp) discovered in the jellyfish *Aequorea victoria* was used as the reporter system [87]. It was found that both organisms grow as isolated colonies in the presence of citrate, which both of them can use as a carbon source. Mixed colonies which actively metabolize chlorobiphenyl are formed when this xenobiotic is used as a substrate.

Besides the metabolic heterogeneity inherent in the biofilm as an integral system and determined by the biofilm zonality, many authors also note essential differences in the physiological and biochemical properties of the cells integrated in biofilms, in comparison with their planktonic analogues. As a result, the concept has emerged of the special "biofilm" phenotype, which expresses, for example, decreased sensitivity to antibiotics and toxic agents [88].

The analysis of the activity of individual genes, performed by the methods of proteomics (by means of two-dimensional electrophoresis of proteins), has made it possible to establish the differences in gene expression in the course of biofilm formation and in the course of their maturation [89]. With the use of the GFP system, it has been possible to localize the regions of synthesis of individual proteins in the biofilm [90]. Thus, for instance, it was shown that *P. aeruginosa* genes *lasI* and *rhlI*, responsible for the synthesis of quorum sensing factors, are predominantly expressed in the cells located at the interface of solid and liquid phases; moreover, the expression of the first gene decreased with time, whereas the second gene was expressed at a constant rate [91].

The relatively new method of DNA microarrays is highly promising; it is similar to the dot-blotting method, but provides considerably more possibilities [92]. It enables determination of the differences in the transcription activity of each section of the genome via the comparison of the level of appropriate mRNA. For example, the application of this method to the study of gene expression in planktonic *E. coli* cells and in those embedded in biofilms revealed differences in the activity of 22 genes, including the genes of stress response (*hslS, hslT, hha*, and *soxS*), the gene responsible for fimbria formation (*fimG*), the metabolic (synthesis of amino acids) gene (*metK*), and of 11 other genes whose functions have not yet been determined [93].

Many examples of the differential expression of genes in biofilms are considered in detail in reviews [9, 53, 94].

RESISTANCE TO CHEMICAL AGENTS AND STRESS FACTORS

It should be noted that until recently primary attention was devoted not so much to the metabolic differences between planktonic cultures and the cultures included in biofilms, but rather to the differences in their sensitivity to stress factors. One of the concepts concerning the reason for biofilm formation is based on the assumption that these structured associations are a means of protecting microorganisms from stress conditions [53]. Actually, microorganisms in the biofilms are more resistant to various stress factors: substrate limitation, pH changes, and oxidation by reactive oxygen species. However, researchers pay the greatest attention to the resistance of biofilms to antibiotics and different biocides. This is caused primarily by the importance of such studies for medicine, since many pathogenic microorganisms form biofilms in the infected macroorganism or on the surface of various medical devices (catheters, contact lenses, artificial heart valves, etc.). According to the data of various authors, at least 60% of infections are caused by agents which are localized in biofilms. It has been found that, compared to planktonic cultures, microorganisms embedded in biofilms are 100–1000 times less sensitive to most antibiotics and other biocidal substances [95, 96]. Many works are devoted to the nature of this stability. However, due to the differences in the methods used and the experimental details of the very process of obtaining the biofilms,

the views of the authors on the mechanisms of this stability vary significantly. One highly popular concept states that the ability of the exopolysaccharide matrix to bind antibiotics plays a substantial role in this resistance [97]. There is also no doubt concerning the significant role of the special features of the "biofilm" cell phenotype, which are expressed in selective expression of the genes of resistance. These genes, for example, include the *P. aeruginosa* gene, regulating the response to amino glycosides (*arr*), which encodes the membrane phosphodiesterase. The "secondary messenger," cyclic diguanosine monophosphate, which regulates the degree of the adhesiveness of bacterial cell surfaces, is the substrate for this enzyme [98]. A number of authors have attempted to explain antibiotic resistance in biofilms by the increased production of so-called "systems of multiple resistance" (of CDR and MDR types), which are based on the active removal (excretion) of antibiotics from microbial cells. However, thorough analysis by dot-blotting and DNA microarrays did not confirm this assumption. Both in the case of *P. aeruginosa* [99] and in the case of *C. albicans* [100], intensive formation of such systems was not observed. Moreover, the *C. albicans* double mutant in these systems, which lost fluconazole resistance in planktonic culture, completely retained it in the biofilm. Hence follows the completely logical conclusion that resistance to antibiotics (and, probably, to other toxic agents) in biofilms is a complex phenomenon, which can not be completely explained by any single mechanism.

Many authors have stressed the fact that the rate of cell growth is usually considerably lower in biofilms than in planktonic cultures. Since it is known that rapidly growing cells are more sensitive to antibiotics than slowly growing ones, the low growth rate was considered as one of the reasons for resistance to antibiotics (and to a number of other unfavorable factors) in biofilms [101]. A mathematical model was constructed, based on the concept of the local substrate limitation, which leads to impeded growth; it predicted the existence of a direct dependence between the growth rate and the rate of cell loss caused by an antibiotic [102].

Nevertheless, the concept of existence of special persisting cells in biofilms is nowadays, perhaps, the most prevalent [103].

The idea that within a bacterial population there exist cells which are less sensitive to the action of inhibitors emerged sufficiently early, at the dawn of the era of antibiotics [104]; these cells were termed persisters. The presence of such cells in the composition of bacterial populations has been repeatedly confirmed [105]. The more detailed works of the recent years have demonstrated that the properties of the *P. aeruginosa* population in a biofilm were similar to the properties of the stationary stage culture; increased resistance of these populations to the toxic agents is determined by their low growth rate and by the presence of the fraction of persister cells [106]. The number of such cells in the stationary culture is 1–10%.

Detailed studies of the nature of persister cells have been carried out by K. Lewis and coworkers [107, 108] and summarized in his review [97].

The nature of persister cells remains mysterious in many respects. It has been suggested that persisters are specialized surviving cells, which appear at a specific phase of growth. During the logarithmic growth phase, these cells are present in insignificant amounts; they are incapable of division, and repeated passages of the culture through this stage remove persisters from the population [104, 109]. The authors assume that specific genes are expressed in the persister cells (in particular, *hipA*), which act according to the "toxin–antitoxin" model and block the targets of antibiotics; cell growth is therefore limited (the cells are converted to the quiescent state), but the cells acquire resistance to the given inhibitors [97].

The formation of persister cells can be considered as a method of adaptation to changing environmental conditions, when the cell population has to choose between two strategies: either to continue growth in spite of the risk of extinction caused by stress conditions, or to suppress growth and gain "insurance" against extinction by transferring into the quiescent state. This suggestion has been confirmed by the development of mathematical models [110, 111]. These models are supported by data confirming the participation of persister cells in the resistance of the biofilm population not only to antibiotics [112], but also to other bactericidal agents: the cations of heavy metals [113] and hydroxy anions of metals and metalloids (CrO_4^{2-}, AsO_4^{2-}, SeO_3^{2-}, etc.) [114].

The possibility of microbial survival in biofilms due to the formation of other resting forms (for example, classical endospores), and the formation of ultramicrobacteria (nanobacteria) should not be underestimated. However, the scale and role of these processes is as yet insufficiently understood [115, 116].

THE ROLE OF BIOFILMS IN MICROBIAL ECOLOGY AND BIOTECHNOLOGY

The materials presented in the previous sections provide a basis for a satisfactory understanding of the role of biofilms in natural ecosystems. The participation of biofilms in processes related to anthropogenic interference with these systems, as well as the possibility of using biofilms in biotechnological processes, have recently attracted much attention. Let us briefly review some aspects of this problem.

The formation of biofilms with low sensitivity to antibiotics in the course of chronic infections, in particular, cystic fibrosis, is presently a matter of great concern [9, 117]. Furthermore, under natural conditions, biofilms may harbor pathogenic microorganisms, which, under these conditions, are hardly susceptible to

disinfection processes and can rapidly acquire antibiotic resistance due to the horizontal transfer of the genes of resistance.

The corrosion of metalware (including pipelines), which is caused or intensified by microorganisms, including sulfate-reducing bacteria [118], is a source of many complications. However, recently acquired data indicate the possibility of decreasing corrosion by the formation of biofilms of genetically modified microorganisms on the surface of steel articles. These organisms are capable of producing both inhibitors of corrosion and antibiotics, which suppress the growth of corrosion-causing bacteria. For example, biofilms which include gramicidin-producing *B. brevis* have been demonstrated to protect metals from corrosion in the presence of sulfate-reducing bacteria [119].

Biofilms can act as biocontrol agents in the rhizosphere of plants, especially against fungal and bacterial infections [120]. The commercial *B. subtilis* strain, which constitutes a part of such biofilms, produces antifungal and antibacterial substances and thus protects plants from phytopathogenic microorganisms [121].

The artificial construction of biofilms acting as "bioreactors" causes increasing interest. In such systems, it is possible to obtain new physiologically active products [122, 123].

Finally, some bacteria have been found to apply a curious method of protection from grazing by protozoa. In the process of biofilm formation, they arrange in large colonies (or aggregates), which are inaccessible to endocytosis [124]. This property can be useful for microbiological bioremediation of soils, as well as for the formation of associations (bioaugmentation) in the process of wastewater purification [125].

It should be noted that biofilm formation is not always properly taken into account in evaluating the role of microbial communities in the environment, especially in places subjected to anthropogenic action. For example, a recently published monograph noted that biofilms with the participation of oil-oxidizing microorganisms were formed in ca. 40 sections, associated with a typical petroleum deposit, including different forms of overgrowths [126]. Meanwhile, unlike methods for the analysis of planktonic cultures, methods for the analysis of the microflora of biofilms in such complex systems do not always adequately record the microorganisms which are localized there. Precise estimation of the specific contribution of these structured communities is necessary both to counteract the harmful effect of biofilms and to utilize their useful properties.

CONCLUSIONS

The complexity of the organization of biofilms and the variety of interrelations between their components has allowed some authors to consider these structured associations as analogous to multicellular organisms, and the microorganisms in biofilms as specialized cells, which possess the capacity for cooperative "altruistic" behavior [127, 128], which violates the principles of "Darwinian" evolution [129]. The discovery of the phenomenon of apoptosis, programmed cell death, in bacteria provides evidence in favor of this concept [130, 131]. Apart from the already mentioned "toxin–antitoxin" mechanism, apoptosis includes mechanisms involving caspases, which are characteristic of multicellular organisms [132].

The recent hypothesis about the possible origin of the first multicellular animals (sponges) from biofilms which contain bacteria and flagellate protozoa, is a further development of this concept [133].

However, this concept meets serious objections, primarily because of the fundamental differences between the cells of the tissues of a multicellular organism and bacterial cells. Although bacteria in the biofilm can adapt to environmental conditions, they do not undergo any permanent differentiation which would distinguish them from planktonic cultures; upon their decay, the biofilms return to the original planktonic form. The cells of the tissues of multicellular organisms behave differently; they preserve the properties inherent to the cells of a particular tissue even in cell culture. In this case, differentiation is irreversible, since it does not respond to environmental signals. The basic concept of the development of multicellular organisms is the vector model, which assumes the presence of certain checkpoints; after passing these points, cells cannot "turn back" the process of differentiation. In contrast to the differentiation of tissues, the processes of formation (colonization) and disintegration of biofilms are easily reversed and depend completely on the signals from the environment. Thus, there is a "biofilm phenotype," but no "biofilm genotype".

Nevertheless, it was shown in the previous sections of this review that biofilms cannot be considered as a simple sum of their constituent cells. They are rather a qualitatively new type of association, which can be considered as a "city of microbes" [8]; they are formed for the purpose of supplying various conveniences for their inhabitants. If existence in such a city becomes less comfortable, its inhabitants leave. The inhabitants of this "city" select the neighbors convenient for themselves; inter-district communications exist, as well as means of communication and protection from unfavorable environmental conditions. It can be hoped that the development and improvement of ever more precise methods of the analysis of the interrelations between the components of these unusual associations will make it possible to decipher the details of their interactions and on this basis to obtain tools to regulate their activity under natural and artificial conditions.

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