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REVIEW PAPER

The Mechanism of Formation of *Pseudomonas aeruginosa* Biofilm, a Type of Structured Population

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Abstract—The paper is an attempt to analyze and generalize molecular and cell biology data on the formation of polysaccharide matrix–based biofilms. The conception of biofilms as structured populations sharing the characteristics of uni- and multicellular organisms and population is proposed.

Key words: structured population, bacteria, biofilms, *Pseudomonas.*

INTRODUCTION

The conception of structured bacterial populations that I have recently proposed [1] defines this type of microbial population with specific properties to distinguish them from all other types of microbial populations. Formally, any microbial population can be considered to be structured in one or another sense, so that at first sight it may seem illogical or unnecessary to introduce a new term. However, this conception was conceived to distinguish specific populations (such as bacterial colonies, foulings, methanogenic granules, dental plaques, films of polysaccharide matrix–enclosed pseudomonads, and other stable consortia and communities) from conventional homogeneous submerged cultures. In other words, I shall consider structured populations in a restricted sense, that is, as stable systems that are regulated and reproduce as a whole and share the properties of uni- and multicellular organisms and population.

In a simple case, the members of structured populations are bound physically, for instance, by a common solid substrate or polysaccharide matrix, the latter being produced by one or several members of the population. In more sophisticated cases, the members of structured populations are bound by coordinated metabiotic bonds. Structured populations, particularly heterogeneous ones, may contain subpopulations that are regulated, grow, and even evolve more or less independently. There is evidence that the interactions (physiological, biochemical, and genetic) of organisms in structured populations between each other and with the environment considerably differ from those observed in canonical aqueous submerged cultures. My conception of structured populations is free of the popular (but, in my opinion, straightforward and one-sided) analogy of bacterial populations with macroorganisms [2].

Because of a great body of recent experimental data concerning structured populations, the problems discussed in the present paper will be restricted to the biofilms of alginate-enclosed pseudomonads. Other biofilms of this type (as the polysaccharide matrix–enclosed *Escherichia coli* and some staphylococci), dental plaques, and foulings will be invoked only for comparison.

It should be noted that biofilms have attracted the great interest of researchers in the last decade. The comprehensive review of Costerton *et al.* [3] on this subject was primarily devoted to the general properties of biofilms, their structure, and research methods. The present review primarily concentrates on other subjects, namely, the cell biology and regulation mechanisms functioning in biofilm-grown bacteria.

Let us first consider some relevant problems. The definition given by Costerton *et al.* [3], which defines biofilms as the populations of bacteria enclosed in a matrix and attached to one another or to solid surface–medium interfaces, holds also for microbial aggregates, globules, and populations in porous media. Comprehensive as it is, this definition, however, leaves outside itself some types of structured populations, such as dental plaques, in which bacteria belonging to different species and genera are tightly bound by specific intercellular bonds in a three-dimensional network, while the microcolonies of streptococci enclosed in a dextran matrix play an insignificant part in the functioning of the dental plaque. The history of biofilm research goes back to the work of Zobell [4], who studied the effect of solid surfaces on the biology of marine bacteria. Since then, this problem has been given great attention from researchers. Bearing this in mind, I will concentrate on another type of structured populations, i.e., polysaccharide matrix–based biofilms.

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SOME PROBLEMS OF THE GENERAL BIOLOGY OF *PSEUDOMONAS* BIOFILMS

Distribution and habitats. Pseudomonads form biofilms on solid surfaces, both biotic and abiotic, in aqueous or sufficiently humid aerial environments. In bodies of water, pseudomonads are found primarily on the surface of aquatic plants and bottom sediments [5]. In lakes and ponds in the temperate zone, they can be detected in lacustrine plankton over a short period of late spring–early summer [6]. In wet soils, pseudomonads inhabit mineral surfaces. In anthropogenic environments, they are found on the glass, metal, plastic and other surfaces [3, 7, 8].

Great attention was given by researchers to the *Pseudomonas aeruginosa* films formed on the human internal organs affected by cystic fibrosis. The cystic fibrosis of the human airway tree [9], which is associated with the oversynthesis of the extracellular polysaccharide alginate, is one of the most dangerous diseases of this type.

Initiation of biofilm formation. It will be reasonable to begin this section with the review of the article *Initiation of Biofilm Formation in Pseudomonas fluorescens WCS365 Proceeds via Multiple, Convergent Signalling Pathways* [8]. The article's authors, O'Toole and Kolter, showed that the formation of biofilm by *P. fluorescens* WCS365 cells upon their contact with a polyvinyl chloride surface began with the synthesis of extracellular proteins, presumably cell adhesins. This synthesis was stimulated by the high osmolarity of the medium, which served as an additional external signal to biofilm formation. The ClpP subunit of the cytoplasmic Clp protease was also somehow involved in initiating biofilm formation. The negative mutants that were unable to attach to the surface under normal conditions acquired this ability after the addition of iron ions to the growth medium with citrate, glutamate, or glucose, but not malate or mannitol, as the sources of carbon and energy. Along with the growth substrates, unfavorable environmental factors (such as nitrogen deficiency, low water activity, high osmolarity, etc.) could also serve as signals to biofilm formation. The genetic pathways involved in this process may evidently converge, which makes the initiation of biofilm formation possible under varying environmental conditions [8].

In another work, O'Toole and Kolter showed that both flagellar and twitching type IV pili–mediated motilities are required for *P. aeruginosa* biofilm formation and its further growth [10]. Flagellar motility is necessary to bring bacterial cells in contact with a surface. Type IV pili are involved in the attachment of the cells to the surface; however, the major part in this process is played by some unknown factor. In the case of wild-type cells, they first form a monolayer film on the surface and then aggregate into separate colonies in a way dependent on both flagellar and type IV pili–mediated twitching motilities. This follows from the fact that mutants with the defective synthesis of pilus proteins were able to form a monolayer film on the surface but were unable to produce colonies and then a mature multilayer biofilm [10].

Biofilm formation in *E. coli* requires flagella and type I pili (the latter are necessary for the primary adherence of cells to a surface), while chemotaxis is dispensable for normal biofilm formation [11]. This finding of Pratt and Kolter contradicts the accepted hypothesis that the biological significance of biofilms lies in the localization of bacterial cells at the surface–medium interface, where nutrient concentrations seem to be high [3, 4]. It should be noted that the process of biofilm formation is also influenced by many other factors, which are not so important as cell motility. For instance, biofilm formation depends on the concentration of some ions, oxygen, aliphatic compounds, and other substances in the medium [3, 4, 12].

O'Toole and Kolter found that the formation of multilayer colonies in *P. aeruginosa* resembles the formation of fruiting bodies in *Myxococcus*, in which type IV pili are also involved [10]. There is evidence that the quorum sensing system also plays a role in the formation of biofilms by *P. aeruginosa* [13, 14]. Furthermore, this system may control some defense mechanisms in bacterial biofilms, in particular, it regulates the susceptibility of film-grown bacteria to oxidative stress [15].

P. aeruginosa* and cystic fibrosis.** ***P. aeruginosa cells specifically bind to the epithelium of fibrotic animal tissues with the involvement of sensory sphingolipids of at least three kinds: gangliosphingolipids, sialic acid-containing glycosphingolipids, and lactosylceramide-containing glycosphingolipids [16]. The interaction of bacterial cells with these receptors is strain-specific and is mediated by alginate capsule and pili. Solid surfaces are colonized by cells capable of synthesizing alginate in small amounts (this is a typical phenotype of *P. aeruginosa*, which is isolated from diverse habitats). At the same time, fibrotic tissues are colonized by cells of the mucoid phenotype, which is characterized by the oversynthesis of alginate.

The mechanism of conversion of ordinary cells into mucoid cells (M forms) will be considered in the following section.

REGULATION OF ALGINATE SYNTHESIS

Alginate synthesis and its regulation in M forms. Alginate is a linear copolymer of D-mannuronic acid and the C-atom 5 epimer of L-glucuronic acid linked by β-1,4-bonds. The glucuronic acid is partially acetylated at oxygen atoms. The pathway of alginate biosynthesis is not clearly understood. The *alg* genes of alginate synthesis, whose number is about ten, are presumably organized in an operon located close to the 35th min in the chromosome map [17]. The key enzyme of alginate synthesis is likely GDP-mannose dehydrogenase encoded by the *algD* gene.

General regulation of the *algD* gene. The expression of this gene is mainly regulated by the AlgU protein, which is probably the sigma factor of RNA polymerase analogous to the σ^E factor of *E. coli*. The *algD* gene is regulated with the involvement of the gene clusters *algU–mucA–mucB* and *algP–algQ–algR*, located at the 68th and 10th min of the chromosome, and the individual genes encoding the AlgR, AlgB, and AlgZ transactivators [17]. Let us consider all these components of the regulatory system of alginate synthesis.

AlgR protein. This protein has long been considered the major regulator of alginate synthesis, which modulates the transcription of the *algD* gene with the involvement of three upstream enhancer-like elements. The AlgR protein is involved in the sensing of the concentration and uptake of nitrogen in the medium independently of the main mechanism, which includes the sigma-54 subunit of RNA polymerase encoded by the *rpoN* (*ntrA*) gene [17, 18]. The AlgR protein is similar to the NtrC regulatory proteins of binary regulatory systems. The AlgR protein, together with its sensor FimS (formerly AlgQ), regulates not only alginate synthesis but also the twitching motility of bacterial cells by a mechanism which is hitherto unknown [19]. Twitching motility is also controlled by the AlgU (AlgT) protein of another regulatory system.

AlgB regulatory protein. Like AlgR, the AlgB regulatory protein is similar to the members of the NtrC subclass of prokaryotic regulators and has an unidentified sensor protein [20]. The AlgR and AlgB regulator proteins bind to different domains of the regulatory region of the *algD* gene between the three enhancer-like elements, which are close to each other because of the rigid DNA loop formed with the involvement of the histone-like AlgP protein and integration host factor (IHF) [21]. These two proteins play an important part in the regulation of the *algB* gene transcription; however, the main regulatory function in this process belongs to the alternative subunit of RNA polymerase, AlgU (AlgT), which provides for the active synthesis of alginate in mucoid strains and falls into the class of the *E. coli* sigma factors (such as σ^{22}) [20, 21]. The aforementioned regulatory region of the *algB* gene also contains binding motifs for another σ^{54} factor, RpoN. However, genetic analysis showed that RpoN is not involved in the transcription of the *algB* gene. Mutant analysis also showed that the transcriptional AlgB activator provides for the maximal synthesis of alginate in mucoid strains [20]. Therefore, this activator, together with its sensor, may respond to variation in the environmental conditions.

AlgZ. There is evidence [22] that this protein depends on AlgU and is involved in the formation of the transcriptional DNA loop described above.

It should be noted that the molecular mechanism of the *algD* gene expression is still the subject of debate. For instance, it remains unclear whether the phosphorylation of the AlgR protein stimulates alginate synthesis [23] or not [24]. Furthermore, there is some uncertainty as to the binding site of the AlgR protein. As mentioned above, some authors consider that AlgR binds to the regulatory region of the *algD* gene containing three enhancer-like elements, RB1, RB2, and RB3. The affinity of AlgR for RB1 and RB2 is higher than for RB3, so that AlgR actually binds to only RB1 and RB2. Conversely, other authors believe that AlgR efficiently binds to all three enhancer-like elements [17, 25].

CONTROL OF AlgU AND MUTUAL CONVERSION OF THE Alg^- AND Alg^+ PHENOTYPES

In 1980, Fife and Govan obtained muc mutation in the late region of the chromosome of the wild-type *P. aeruginosa* strain, which converted the original Alg^- (nonmucoid) phenotype into the Alg^+ (mucoid) phenotype [26]. The *mucA* (*algS*) gene identified in this region was found to be responsible for the spontaneous mutual conversion of the mucoid and nonmucoid phenotypes through the control of the *algU* (*algT*) gene expression [27]. The adjoining *algN* (*mucB*) gene is also involved in the control of alginate synthesis [28, 29].

The cluster of several genes at the 68th min of the chromosome involved in the transcriptional control of alginate synthesis may be presented as *algU*(*algT*)–*mucA–mucB*(*algN*)–*mucC*(*algM*)–*mucD*(*algY*) [30]. As mentioned above, the product of the *algU* gene is the alternative σ^{22}-type factor of RNA polymerase, which controls the *algR*, *algB*, *algD* and *P. aeruginosa* promoters and its native promoter and is required for the defense of *P. aeruginosa* against oxidative and temperature stresses. All other genes are regulatory. Some authors believe that MucA is a negative regulator of AlgU, which blocks transcription as the antisigma factor [31, 32]. However, MucA is localized in the cytoplasm, although it has cytoplasmic and periplasmic domains, which implies the presence of a mediator between AlgU and MucA. Another negative regulator of AlgU, MucB, is localized in the periplasmic space. The suggestion that MucC may serve as the mediator has not received strong experimental underpinning. All this shows that the regulatory system of AlgU is more complex than it seems at first sight [32].

There is another putative mechanism of the regulation of AlgU in terms of protein stability. With this mechanism, MucB serves as a signal for the degradation of σ^{22} through the membrane-mediated interaction of MucB with MucA and with the involvement of MucD, whose function is analogous to the periplasmic protease HtrA (DegP) of the heat shock response system of *E. coli* [30]. This mechanism remains poorly understood.

The suggestion that the *mucC* gene is a positive regulator of AlgU contradicts the observation that the inactivation of this gene in the *mucA* and *mucB* mutants is accompanied by the active synthesis of alginate in high-

osmolarity media. This observation suggests that the *mucC* gene is a negative regulator of alginate synthesis and shows that this synthesis may be activated in response to a combined action of several signals [9]. Mutants defective in the *algU* gene exhibited enhanced resistance to high temperature and oxidative stress factors, such as superoxide radical and hypochlorite. Therefore, AlgU may perform two functions: (1) to control alginate synthesis and (2) to protect cells from unfavorable environmental conditions [33].

THE ROLE OF OTHER GENES IN THE CONTROL OF ALGINATE SYNTHESIS

The *algC* gene. This gene codes for the second key enzyme of alginate synthesis, the bifunctional enzyme converting mannose-6-phosphate into mannose-1-phosphate and glucose-6-phosphate into glucose-1-phosphate [34]. The AlgC protein is involved in the synthesis of alginate as phosphomannomutase and in the synthesis of lipopolysaccharides as phosphoglucomutase. In biofilms, the expression of the *algC* gene is about 20 times more active than in planktonic pseudomonads and correlates with the glass-clinging ability. However, the activation of this gene is not sufficient for the attachment of bacterial cells to solid surfaces. It is possible that the signal to such an attachment is generated in response to the contact of cells with a surface possessing certain properties [34]. The *algC* promoter is modulated by the phosphorylated AlgR regulatory protein in the same way as in binary systems, and its transcription requires the sigma factor RpoN. This promoter, like that of the *algD* gene, is regulated by the osmolarity of the medium. It should be noted that the *algC* gene is located beyond the gene cluster of alginate synthesis [35].

The *algL* gene. This gene codes for alginate lyase. The inactivation of this gene, as well as of the upstream *algX* gene, suppresses alginate biosynthesis [36]. The mechanism of this phenomenon is not clearly understood, although it is suggested that the partial hydrolysis of alginate by the lyase is a prerequisite for active alginate synthesis.

The conversion of some nonmucoid strains of *P. aeruginosa* into mucoid ones was found to be associated with the insertion elements IS-PA-4, IS-PA-5, and IS-PA-6 of the gene locus located upstream of the *toxA* gene of exotoxin A [37]. However, further investigations are needed to gain a better understanding of this phenomenon.

Mucoidy and the antagonism of sigma factors. As mentioned above, the enhanced expression of the key *algD* gene of alginate synthesis is believed to be primarily due to the alternative σ^{22} subunit of AlgU. At the same time, the *algD* promoter contains a motif which is similar to the binding site of another sigma factor, RpoN (σ^{54}). This motif overlaps the binding site of AlgU. Under certain conditions, RpoN suppresses AlgU, both in vivo and in vitro [38]. The σ^{54} factor is unable to form the open transcriptional complex. In other words, when bound to the promoter, this factor acts as a nonspecific repressor. It is known that transcription is initiated only if a regulator, such as NtrC, binds to a closed complex and makes it open. In much the same way, the regulatory AlgB protein initiates the transcription of the regulatory *algD* gene with the aid of RpoN [38]. A similar regulator may be responsible for the nitrogen-dependent signal transduction.

The relationship between alginate synthesis and cell metabolism. Alginate synthesis requires GTP, which is consumed during the synthesis of GDP-mannose from mannose-1-phosphate. The increased consumption of GTP may shift the proportion between nucleoside triphosphates in cells and alter the entire synthesis of nucleic acids [39]. In *P. aeruginosa*, the positive regulator of alginate synthesis, AlgR, is also an activator of nucleoside-diphosphate kinase and succinyl-CoA synthase, the key enzymes of nucleotide synthesis in cells. As a result, alginate synthesis is most active when the activities of these biosynthetic enzymes are maximal, i.e., in the early and late stationary growth phases [40]. This may be accounted for by the fact that alginate synthesis is stimulated under conditions of nitrogen and/or phosphorus deficiency, which is observed just in the early and late stationary growth phases [39]. On the other hand, this period of bacterial growth is characterized by the accumulation of inorganic polyphosphates and synthesis of guanosine tetraphosphate (ppGpp). The latter compound is a global regulator capable of mobilizing all intracellular reserves for the synthesis of nucleic acids and proteins. The activation of energy metabolism in mucoid cells within this growth period is associated with their enhanced respiration [41, 42].

Cell-to-cell contacts in biofilms. *P. aeruginosa* produces at least two types of extracellular signal molecules involved in cell-to-cell contacts and quorum sensing and related to the functioning of the *lasR–lasI* and *rhlR–rhlI* gene systems [3, 14]. The *lasI* gene controls the synthesis of excretory *N*-(3-oxododecanoyl)-L-homoserine lactone. The transcriptional regulator LasR requires this lactone in sufficient amounts to activate virulence genes and the *rhlR-rhlI* gene system. RhlI controls the synthesis of another extracellular signal molecule, *N*-butyryl-L-homoserine lactone, which is necessary for the expression of the *rhlR* gene encoding the stationary-phase sigma factor RpoS. Both signal lactones were found in *P. aeruginosa* biofilms, where they obviously play an important part at the stage of maturation, as judged from their increased concentrations in mature biofilms. The double *lasI–rhlI* mutant produces thin, even, and flat films on glass surfaces, which considerably differ from those produced by the wild-type strain in the appearance and architectonics [14]. The wild-type biofilm is thick (up to several hundred μm in thickness) and represents a system of mushroom-shaped, columnar, and conical alginate structures

with enclosed bacterial microcolonies. These structures are separated by channels (gaps) with a circulating liquid, which provided bacterial cells with nutrients and oxygen. The channels have a diameter of up to 0.3 μm [3]. The addition of acyl-homoserine lactone to the medium with a growing mutant biofilm led to the formation of a normal biofilm typical of wild-type bacteria. Therefore, cell-to-cell contacts are necessary for the formation of mature biofilms [14].

CONCLUSION

As follows from the foregoing, biofilms are formed with the involvement of ordinary enzymes of microbial metabolism and their regulatory systems. Researchers failed to find enzymes and metabolic pathways which would be specific to only biofilm-grown cells. Peculiar properties of biofilms, such as their extreme resistance to toxic compounds, are due to a great number of cells enclosed in a matrix, which is produced by the film-grown cells and possesses specific permeability properties. It is the matrix that allows the respective toxin-inactivating enzyme to be accumulated in sufficient amounts [43]. Biofilms are products of the complex synevolutionary process. This type of structured population allows bacteria to survive unfavorable environmental conditions and to occupy new ecological niches. The fact that the evolution of bacteria in biofilms is rapid was confirmed by the measurements of the horizontal gene transfer rates in biofilms, which appear to be almost the same as in planktons [44]. It should be noted that we are as yet far from a complete understanding of the phenomenon of structured bacterial populations.

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