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Persister Cells Generation and their Role in Recalcitrance of Biofilms Towards Antibiotics (Part 1)

Kinga Ostrowska[1]

[1] *Laboratory of Clinical and Transplant Immunology and Genetics, Comprehensive Oncology and Traumatology Centre, Copernicus Memorial Hospital, Lodz, Poland*

Summary

In all environments nature provide evidences that two is much more than one, and that strength lies in integrity and unity. Highly complex and well organized communities formed by aggregated bacterial cells surrounded by a hydrated, self-produced matrix of extracellular polymeric substances are commonly known as the bofilms. The specific biofilm lifestyle confers on the associated bacteria a measurable decrease in susceptibility to antimicrobial agents. Once the complex structure of biofilm is established resident bacteria are able to survive after diverse types of physical and chemical aggression such as UV, heavy metals or phagocytosis. It is now clear that biofilm bacteria exhibit a characteristic ability to withstand the antibiotics killing mode of action, being directly responsible for difficulties and failures in therapeutic and clinical settings.

Key words

persister cells, bacterial biofilm, antibiotic resistance, SOS response

Introduction

Despite the passage of time, acute bacterial infections, life-threatening diseases caused by microbial pathogens such as *Yersinia pestis* or *Vibrio cholerae* are still a weak point of the mankind. Initially, due to discovery of antibiotics, vaccines and hygiene rules application, a significant reduction of lethal incidents related to bacterial infections was observed [1,2]. However, the golden age of antibiotics did not solve such challenges as occurrence of antibiotic-resistant microbes together with still rising level of chronic bacterial infections difficult to eradicate [3].

At the same time the progressive research conducted by environmental microbiologists confirmed prevalence of bacterial biofilm conglomerates in all types of natural niches and ecosystems [4]. Soon clear became the fact, that bacterial biofilms display specific biological properties in comparison to their planktonic counterparts [5]. First data pointing out the direct correlation between persistent infections and bacterial biofilm development came from J.W., Costerton and N. Hoiby during their research of *Pseudomonas areuginosa* colonization model on the lungs of CF patients [6]. Since then, a significant role of bacterial biofilms in the pathophysiology of tissue-related infections has been widely confirmed and proved.

Whilst the planktonic bacteria can be easily eradicated by diverse antimicrobial drugs, a subset of biofilm bacteria highly tolerant to antibiotics survives the treatment and becomes a cause of infection recurrence [7]. The ability of bacterial biofilms to withstand harmful bactericidal antibiotic activity, even when these bacteria are susceptible to such antimicrobial agents is called "recalcitrance of biofilm bacteria towards antibiotics" [3, 8]. Among many suggested reasons potentially able to explain the above mentioned recalcitrance phenomenon, the presence of an isogenic subpopulation of "persister cells" is now considered as the most important one [9].

Biofilm as an ancient survival strategy

In all environments nature provides evidence that two is much more than one, and that strength lies in integrity and unity. The classic view on bacterial biofilms defines those structures as highly complex and organized bacterial communities attached to a biotic or abiotic surface formed by aggregated bacterial cells surrounded by hydrated, self-produced matrix

of extracellular polymeric substances (EPS) [10, 11, 12, 13]. The first evidence of existence of those bacterial conglomerates occurred in the 17th century through simple microscopic dental plaque analysis conducted by *Anton van Leeuwenhoek* [14]. The first definition of biofilm describing it as a well-organized structure of bacterial cells arose in 1987 by Costerton et al [15]. Biofilm formation has been demonstrated for considerable number of microorganisms and is considered as an ancient prokaryotic mode of adaptation and a key factor allowing for survival in diverse, often hostile environments [16]. The formation of matrix-enclosed bacterial accretions does not only protect bacteria from the unfavorable environmental conditions but also due to various dispersal mechanism allows bacteria to efficiently colonize new niches [17].

Although surface-associated bacterial biofilm communities are widespread in all natural habitats, where they play an important positive role, mostly they are known for their negative and harmful activity, in particular including biofilm-related infections. Unlike natural biofilms, which usually are multispecies structures, pathological biofilms are mostly created by a single species of bacteria [13]. Based on the phenomenon of *Pseudomonas aeruginosa* lungs tissue colonization observed in cystic fibrosis patients, J.W. Costerton with N. Høiby and their collaborators were the very first researchers to point out a direct correlation between persistent infections and biofilm development [3, 18]. The next decades of research were about to confirm the crucial role of biofilms in the pathophysiology of tissue-related infections [19]. The direct link between microorganism living on the surfaces and human infections development became clear and rose to final estimation that 65% to 80% of infectious diseases were correlated with bacterial biofilms [3]. The specific properties of bacterial biofilms are highly problematic and posing an ever increasing problem mainly because of their enhanced tolerance to multiply in unfavorable conditions including high concentrations of antibacterial agents such as antibiotics.

The architecture of bacterial biofilms: three-dimensional structures formation step by step

The unique form of existence presented by bacterial populations living on surfaces was pointed out in the 20th century by pioneering studies of Henrici and further by Costerton and collaborators. Virulent biofilms for-

med on multiple types of surfaces are biological phenomenon associated with diverse infections and ilnesses such as native valve endocarditis, oral diseases or nosocomial infections [20].

Biofilm formation is a compex process arising as a combination of physiological and molecular events. It can be classified into five stages including: (a) surface film development, (b) cells attachment to the surface, (c) microcolonies formation, (d) differentiation and maturation of the biofilm along with expression of matrix polymers and (e) finally dispersal of bacterial cells from biofilm structure [21].

The first and crucial stage in the biofilm formation process involves attachment/adhesion. It occurs in two steps: initial, weak and fully reversible attachment and strong permanent irreversible adhesion of bacterial cells to a surface. Reversible attachment to a surface might be a response to the absence of nutrient availability, also due to the impact of hydrodynamic forces and usage of different diverse appendages and structures, such as extracellular organelles including outer membrane proteins, curli fibers, flagella, intimins, invasions, fimbriae and pili. Microbial cells adsorb irreversibly due to specific binding of their adhesins to the ligands present on the solid surface, then replicate and form microcolonies with physical dimensions equal to tens or hundreds of microns in diameter [12, 22]. When bacterial biofilm is formed inside macroorganism (*in vivo*) a significant role in this process play the extracellular matrix proteins (ECM). Many of bacteria like Gram-positive *Staphylococcus aureus* posses on their surface adhesins for EMC, included in the microbial surface components recognizing adhesive matrix (MSCRAMMs). Attached irreversibly to the solid surface, bacterial cells secrete an extracellular polymeric substance (EPS) which forms a physical barrier (hydrogel layer) between bacterial community and extracellular environment. Highly hydrated (98% water) EPS consisting of lipids, proteins, lipopolysaccharides and DNA provide the structure and the matrix that define a biofilm as a structure extremely heterogeneous, both over time and in space [22, 23]. The composition of the EPS is different and depends on growth conditions, species and, moreover, chemical interaction between bacterial cells within the biofilm that stimulate its secretion and formation [24]. One of the widely described type of chemical communication is Quorum sensing (QS). This specific type of chemical interaction is crucial phenomenon in biofilm formation and a central mechanism used by bacteria to query extracellular environ-

ment [12]. Quorum sensing modulates various cellular functions such as motility, pathogenesis, conjugation or nutrient exposition [25]. As bacterial cells replicate and the EPS accumulate, the microbial community arises into a three-dimensional structure and matures into a biofilm. Biofilm resident bacterial cells are bonded together by the EPS, which provides structural stability, protection against antimicrobials, immune effectors, phagocytosis and other clearance mechanisms [26]. Moreover EPS "glues" bacterial cells together, thereby protecting bacterial community against detachment from the surface [27]. The last but not least step for biofilm propagation and self-renewal of the microbial community is bacterial cells detaching and dispersing into the bulk fluid. Microbial cells detached from some regions of biofilm structure may attach to the solid surface within new environmental niches and give rise to new biofilms [15].

Extracellular polymeric substances (EPS)

Biofilms are well organized multicellular conglomerates encased in an extracellular polymeric substances (EPS). The EPS are extremely important for the biofilm communities since their unique biochemistry promotes recalcitrance to antimicrobial agents such as antibiotics and plays a crucial role in the resistance phenotype of the biofilm conglomerates [28]. Moreover, as the EPS encase the bacterial cells, they become the first line of interaction with the human immune system [29, 30, 31]. Mutants unable to produce EPS are not capable to create a stable and sustainable biofilm [32, 33, 34]. The EPS production depends on multiple important factors, which include growth phase, nitrogen and carbon sources together with their ratio, role of such nutrients as phosphorus, micronutrients/trace elements or vitamins, also impact of pH, temperature, metals, growth conditions (anaerobic versus aerobic) or type of bacterial culture (pure versus mixed culture) [35]. The EPS may influence the physicochemical properties of the cells, such as polymeric features or hydrophobicity [36]. Depending on the bacteria that initiate formation of the EPS, their composition may be very diverse, but most are comprised of polysaccharides, extracellular DNA (eDNA), bacterial proteins and phospholipids.

Polysaccharides constitute the largest part of the EPS matrix and mediate adhesion and cohesion, play crucial role in stabilization of biofilm structure. The Gram-negative bacteria like *Providencia* typically produce

negatively charged polysaccharides due to the presence of such compounds in their sugar backbone as uronic acids, phosphate and sulfate groups or pyruvate. Negative charge of the surface permits binding of calcium and magnesium cations, which by the cross-linking binding with the polymer chains stabilize the structure of the biofilm [12]. Polysaccharide complexes are composed of long and thin chains connected together and with the bacterial cells by means of electrostatic and hydrogen bonds (an alternative way of binding to bacterial cells may also occur by lipids). There are diverse classifications of polysaccharides produced within the matrix according to which we can distinguish homopolysaccharides, heteropolysaccharides and their linear, branched or cyclic counterparts. An important group of polysaccharides presents the capsular (cell-associated) and slime (not cell-linked) form [37].

Most Gram-negative bacteria produce the outer membrane vesicles (OMVs) containing diverse groups of molecules, which oversee courses of different biological processes. For instance the bacterial OMVs can enable transport of molecules to other microbial cells within their environment. Experimental studies revealed that OMVs are components of the nontypeable *Haemophilus influenzae* (NTHI) and *Pseudomonas aeruginosa* biofilm EPS [35, 38, 39]. Although the OMVs role in modulating EPS either biofilm structure has not been fully evaluated, mutant organisms able to modulate both *NTHI* and *Escherichia coli* OMV biogenesis have been identified [40].

Despite the fact that the number of proteins in the matrix is approximately 5 times lower than the polysaccharides, those compounds are recognized for their increasing importance in bacterial biofilm function and structure. Large-scale proteomic analyses performed on *NTHI* and *Pseudomonas aeruginosa* EPS showed abundance of both outer membrane proteins and type IV pili. Matrix proteins include motility organelles, secreted proteins as well as components of adhesins [41,42]. The participation of two classes of proteins, which are lectins and lyases, is discussed in the formation of the matrix. Lectins are named to be a carbohydrate-binding proteins, occurring mostly on outer membrane of Gram-negative bacteria, also peptidoglycan and fimbrias [37]. This molecule abundance plays a mediating role in adhesion process between macroorganism cells and bacterial cells. Lectin may be also responsible for binding cells with EPS and cell co-aggregation, thereby enables the biofilm formation

[43]. One of the best and most widely studied proteins are lectins LecA and LecB produced by *Pseudomonas aeruginosa*. Both lectins are carbohydrate-binding proteins committed in biofilm formation process [24, 44]. Moreover, among others well known proteins we can name large surface protein BapA, associated with robust biofilm formation in Gram-negative *Salmonella* species [45]. The biofilm EPS promotes the recalcitrance toward antibiotics and limited killing by innate immune components. Negatively affected by the EPS are also such processes as opsonization by immunoglobulins and complementation. It is widely accepted that resident biofilm bacteria respond by producing components and factors which significantly limit the non-oxidative and oxidative powers of phagocytic cells succor survival abilities of bacteria [46].

Resistance and tolerance: dangerous composition leading to biofilm recalcitrance phenomenon

The biofilm lifestyle confers on the associated bacteria a measurable decrease in susceptibility to antimicrobial agents. Once the complex structure of biofilm is established, the resident bacteria are capable of surviving the diverse types of physical and chemical aggression, such as heavy metals, modulation in salinity, phagocytosis, UV light or acidity [3, 47, 48]. It is now clear that biofilm bacteria reveal characteristic ability to withstand killing mode of action mediated by antibiotic, which is directly responsible for difficulties and failures in the manner of therapeutic and clinical settings.

Nowadays it is obvious that wide-studied mechanisms involved in antibiotic resistance, like for instance modifying enzymes or efflux, play no more than marginal role in the abilities of bacterial biofilms to survive conventional antibiotics [3, 49]. Bacterial cells embedded in a biofilm are capable to partially withstand high dosage of antibiotics, even when these microorganisms are completely susceptible to those antibiotic under planktonic life conditions. This complex phenomenon is called "recalcitrance of biofilm bacteria towards antibiotics" and is due to many mechanisms including resistance and tolerance [10]. *In vitro* studies of how planktonic bacterial cells can escape and survive antibiotic treatment brought scientist to the two concepts of tolerance and resistance.

Resistance is simply defined as the ability of bacterial cells to survive (multiply) in the presence of both bacteriostatic and bactericidal an-

timicrobial agent [50, 51, 52]. Typically resistance phenomenon is tested by evaluation of minimal inhibitory concentration (MIC) of antimicrobial compound. Many mechanisms stand behind resistance, including reduced permeability to antimicrobial agents (antibiotics), efflux pomp or enzymes destroying antibiotics. Moreover, it is important to realize that resistance is very often genetically inherited and hence transmitted between bacterial generation, or it also can be acquired through horizontal gene transfer [3].

Unlike resistance, tolerance is only associated with usage of bactericidal antimicrobial agents, that is to say an antimicrobial agent kills at least 99.9% of bacterial cells within 12 hours [51]. According to Clinical and Laboratory Standards Institute (CLSI), the lowest concentration of antimicrobial agent capable to reach this killing threshold is hereafter named minimal bactericidal concentration (MBC) [51]. In other words, the definition of tolerance can be expressed as the absence of growth, but the presence of microorganisms survival in the presence of a bactericidal antimicrobial agent. Two types of tolerance have been distinguished. First the genotypic one in which presence of a genetic alterations and modifications lead to ability of the antimicrobial agent to kill bacterial cells to be reduced, moreover can be transferred to the next generation cells. Diverse examples have been described e.g. in small colony variants of Gram-positive *Staphylococcus aureus* or *Streptococcus pneumoniae* [3, 53]. In the case of phenotypic tolerance the environment leads to decreased capacity of antibiotics to kill. However, noteworthy is the fact that this type of tolerance is fully reversible once returned to a growth-promoting growth media [54]

Study of bacterial biofilms has offered at least several physiological explanations of the recalcitrant nature of those bacterial conglomerates. Despite the fact that recalcitrance can be defined as a combination of tolerance and resistance, biofilm phenotype is more prone to tolerance rather than resistance [55, 56]. Biofilm recalcitrance phenomenon in most of the cases is non-inherited and can be reversed when biofilm structure is disrupted and microorganisms return to a planktonic form [57].

Antibiotic penetration and drug indifference

Bacterial biofilm recalcitrance is a multifactorial and thus extremely complex phenomenon. Moreover, depending on the type of antibiotics used it

involves diverse mechanisms like drug indifference or impaired antibiotic diffusion [3].

Bacterial biofilms are sessile communities of cells resided in a matrix consisting of extracellular polymeric substances (EPS). Historically, it was postulated that the intrinsic antimicrobial resistance in biofilms is caused by self-produced and adhesive matrix, which was proposed to be the main culprit responsible for biofilm recalcitrance phenomenon [48]. Many data suggest that physicochemical properties of the EPS can retard or delay penetration of multiple diverse compounds, including antibiotics and antiseptics. The activity of an antibiotic can be reduced by adsorbtion on the matrix due to electrical interactions with polymers that surround bacteria within the biofilm [58]. Moreover, penetration of positively charged aminoglycosides is also slowed by polymers of the biofilm matrix which are negatively charged [59]. Observation of antibiotic diffusion through cardiac vegetation in endocarditis indicated that diffusion gradient is possible in the case of penicillin and glycopeptides – teicoplanin [60]. Conversely, Hoyle et al. revealed that EPS of *Pseudomonas aeruginosa* were capable of binding tobramycin, thereby exhibit delayed and reduced diffusion of this antibiotic *in vitro*. Moreover, it was indicated that planktonic cells were 15-fold more susceptible to this antibiotic than their biofilm counterparts. Other studies showed that diffusion of chlorine, a commonly used antiseptic was also reduced in the case of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [61, 62]. *Staphylococcus aureus [63, 64]* and *Staphylococcus epidermidis* [65] slime remarkably decrease the activity of the vancomycin. Moreover, the efficacy of such antimicrobial agents as: cloxacillin, imipenem, cefpirome, erythromycin, roxithromycin, clindamycin, fusidic acid, trimethoprim, doxycycline, gentamicin, netilmicin, isepamicin or ofloxacin, also has been negatively affected by the EPS of *Staphylococcus epidermidis*. Moreover, the penetration profile was suggestive of a substrate being consumed within the matrix of the biofilm. Other studies by Suci et al. [66] revealed a reduced rate of ciprofloxacin transport to colonized surface compared with a transport to a sterile surface, suggesting that ciprofloxacin was captured and bonded to the components of biofilm matrix. In the light of foregoing studies, the chemical structure of the biofilm matrix is clearly significant and has been proved that, depending from the type of single pathogen, different types of exopolysaccharides can

be involved, due to the environment immediately surrounding the cells within a biofilm [3, 67].

Nevertheless, the biofilm recalcitrance towards antimicrobial agents cannot be fully explained by reduction or handicap of antibiotic penetration. Mathematical models suggest that for many groups of antimicrobial agents there should be no barrier preventing their diffusion inside a biofilm structure. Such antibiotics as for example ampicillin or gatifloxacin are capable of strong penetration through the biofilm matrix, even though they miss to kill the whole population of biofilm bacteria [68, 69]. Even in the case of antibiotics that slowly diffuse through the biofilm matrix, a significant percentage of them finally reach all biofilm resident bacteria [8, 69]. Furthermore, delayed and retarded antibiotic diffusion through biofilm may have significant consequences in the physiology of bacterial cell, which can adapt to the presence of antimicrobial compounds. Moreover, a very dangerous trend associated with slow or limited diffusion is the transient exposition of biofilm bacteria to subinhibitory concentrations of antibiotics. It is also very likely that limited diffusion protects the biofilm structure from destructive activity of antimicrobials.

Drug indifference together with alteration with microenvironment is another serious problem in the biofilm recalcitrance phenomenon. Most antimicrobial agents is known to be more active against metabolically active microorganism. Microenvironment within deep layers of the biofilm due to a lack of nutrients, pH, or just anoxia can simply have antagonistic influence on antibiotic activity [70]. This problem is particularly conspicuous in the case of β-lactam antibiotics, which are active and fully effective only in combating metabolically active and dividing bacteria. Analogous other physico-chemical characteristic like low oxygen concentration prevailing within the deep layers of the biofilm reduce the bactericidal effect of such antibiotics like tobramycin or ciprofloxacin of *Pseudomonas areuginosa* biofilm [71].

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Address for correspondence / Adres do korespondencji

Kinga Ostrowska
Pracownia Immunologii Klinicznej, Transplantacyjnej i Genetyki
Wojewódzkie Wielospecjalistyczne Centrum Onkologii i Traumatologii im. M. Kopernika
ul. Pabianicka 62, 93-513 Łódź
tel. (42) 689 52 80
e-mail: ostrowska.uni.lodz@wp.pl