Biofilms: a biological antimicrobial resistance system

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Biofilms are the means that bacteria use to survive in nature. They embody an area of cooperation, interchange and endurance. Cooperation attempts to achieve the same goal, i.e., an easier access to nutrients and protection from environmental damage; but, what makes this fact wondrous is that cooperating microorganisms belong not only to the same species but also to different ones, or even to different kingdoms. Interchange of information by the well-known quorum sensing alerts biofilm denizens of any event and induces a global response, which is facilitated by genetic interchange since distinct microorganisms are able to acquire enzymatic pathways from their neighbours which they could not otherwise. All the above mentioned makes biofilms a perfect setting in order to survive.

Beyond the erroneous idea that biofilms are but a bunch of microorganisms swimming in slime, biofilms present an internal structure. There we find areas filled with active microorganisms, others were cells remain quiescent or sections voided of them, were nutrients or metabolic by-products accumulate.

Presently, much is known about biofilms, but much more remains unknown. Clinical microbiology is beginning to understand the role that biofilms play in infection, but above all, clinicians are concerned about the weight of biofilms on antimicrobial resistance, from how the internal structure prevents antimicrobials from reaching their targets to the interchange of antimicrobial-resistance genes.

In this chapter we intend to review all the implications that biofilm plays on antimicrobial resistance and infection persistence.

Keywords Biofilm; antimicrobial resistance; SOS; ROS; persisters

1. Introducción

The majority of infections in the developed world are caused by biofilms. This fact sparked a renewed interest in the resistance mechanisms involved in it. A biofilm is and organized and well-structured group of microorganisms in which cells stick to each other on a surface. Biofilms contain functional characteristics and complex functions and could be considered as a super-organism.

The formation and maintenance of mature biofilms are intimately linked to the production of an extracellular matrix. Cells embedded in the biofilm matrix are well known to express phenotypes that differ from those of their planktonic counterparts, and to display specific properties including an increased resistance to biocide and antimicrobial treatments. In most cases, bacteria removed from a biofilm, isolated and recultured under laboratory conditions are generally no more resistant than the original planktonic cells of that species. However, under biofilm growth conditions, the microorganism can mutate or acquire different resistance mechanisms that allow greater resistance to biocides and antibiotics [1]. As such, biofilms have been reported as possessing susceptibilities towards biocides and antibiotics that are 100-1000 times less than equivalent populations of planktonic bacteria.

The objective of this chapter is to offer a review of the mechanisms involved in biofilm antimicrobial and biocide resistance.

2. Mechanisms involved in biofilm resistance to antibiotics

2.1. Antimicrobial Penetration failure

Biofilm are characterised by the presence of a matrix in which bacteria are embedded. This matrix is made up of an exopolysaccharide which maintains the spatial structure, but there are also a great amount of diverse types of molecules such as nucleic acids either actively secreted by bacteria or derived from cell lysis, different types of enzymes or anti-antimicrobials proteins –for example β-lactamases–, quorum sensing molecules, degradation and waste products and water [2, 3]. The composition of this matrix is not a homogeneous one and so it varies throughout its structure, creating a wide range of dissimilar environments. The effects that this extraordinary distribution exerts on antimicrobial penetration will vary greatly depending on the physicochemical characteristics of the molecule and its interactions with the matrix. Although antimicrobial penetration is blocked or slowed down, the explanation for each one is different depending on the molecule itself and the species found in the biofilm. While some molecules show sheer diffusion inhibition, others react with enzymes present within the matrix and are inactivated or fixed to structural components, thus not reaching its objective, i.e. bacteria. Different studies have shown that the thickness of a biofilm is relevant to

antimicrobial penetration; therefore the matrix itself is the initial barrier that can delay antimicrobial penetration, but other mechanisms such as inactivation are involved and act synergycally resulting in antimicrobial resistance [4].

2.2. Outer Membrane Vesicles

Outer membrane vesicles (OMV) are small spherical structures release by bacteria that disseminate far from the cell and have different compositions and functions. They play a role in nutrient acquisition, quorum sensing, pathogenesis or horizontal gene transfer depending on its content. OMV are not randomly produced, instead they are the final step of a directed biological mechanisms which has an energy cost that is controlled by many a gene among which the Tol/Pal-envelope-spanning complex is principal. They can be found within the biofilm matrix [5] and are thought of being responsible of spreading resistance genes to other members of the biofilm community, fixing or inactivating antimicrobials and transmitting quorum sensing molecules [5, 6], all of which have a noticeable connotation in biofilm antimicrobial resistance.

2.3. Stress response

Bacteria have evolved a number of mechanisms that help them continue to live whenever they suffer an untoward situation. Many different biochemical pathways are activated in response to critical circumstances. Whether it is lack of nutrients or antimicrobial insult, bacteria switch on these means in order to survive.

2.3.1. General Stress Response

Bacteria living under environmental stress present a slow growth rate that is regulated by the alternate sigma factor RpoS, which is essential to biofilm formation and induction of bacterial transformation into persister cells, other sigma factors might be involved such as AlgT for Pseudomonas aeruginosa. The chief aim of this activation is to preserve bacteria from the detrimental effects of environmental stress, including starvation, heat, cold, changes in pH and chemical or antimicrobial agents [7]. Production of catalase or trehalose (an osmoprotectant) is enhance by RpoS, but also, DNA repair proteins like MutS are down-regulated, allowing mutations to occur, which can lead to antimicrobial resistance development.

There exist other genes involved in general stress response such as sigma factor RpoH which was once considered responsible of heat-shock response, but that now it has been demonstrated that it also regulates stress response to other types of insults like oxidative or antimicrobial stress. [8]

The final result of this group of genes is slow growing cells adapted to an adverse environment and with a high mutation-rate capacity, all of which have been linked to chronic infections and antimicrobial treatment failure [9].

2.3.2. Oxidative Stress

Bacteria exposed to oxygen need a strong protection against reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide or hydroxyl radicals, in order to maintain within safe limits ROS derived of bacterial insult or metabolism, or coming from external sources like macrophages. Antimicrobial lethality is due in part to increase production of ROS; therefore, ROS protective enzymes have a role in antimicrobial resistance [10-12]. ROS also have some implications in persister cells formation and antimicrobial tolerance through SoxRS activation, a promoter of AcrAB-TolC multi-drug pump [13]. There are several genes involved in ROS regulation such as OxyR, PerR or OhrR, and interestingly, OxyR is also involved in biofilm formation [14].

2.3.3. SOS

The SOS response system is an operon regulated by two proteins –LexA a replication inhibitor, and RecA a replication promoter– associated with DNA repair that reacts to damaged DNA. The operon encodes type II, IV and V DNA polymerases which do not have proof-reading activity; therefore, errors may occur that will lead to genetic mutations. This adaptive system has evolved in order to provide a number of mutants at a high rate so that they be more apt to new environments or challenging stresses [8, 15].

Starvation and the SOS response have been linked to antimicrobial tolerance in biofilms [16], and although antibiotics like fluoroquinolones may induce a SOS response in Escherichia coli, others like novobiocin seem to inhibit it in Staphylococcus aureus [17]. As expected with biological systems, SOS response to antimicrobials depends greatly on the microorganism and the antibiotic. Moreover, SOS response also enhances acquisition of transposons and integrons with resistance genes and regulates its rearrangement within the bacterial chromosome; therefore, a bacterium susceptible to an antimicrobial can become resistant to it after SOS response-induced rearrangement [15].

2.3.4. Toxin-Antitoxin Systems

Toxin-Antitoxin systems (TAS) are composed of two genes in an operon that encodes a stable toxin that interrupts essential bacterial processes and a labile antitoxin –RNA or a protein– that prevents toxicity. TAS functions range from programmed cell arrest, persister cells formation or programmed bacterial death, to growth control, gene regulation and stabilization of genomic parasites. However, TAS have recently been associated to biofilm formation, general stress response regulation and SOS response, especially the TAS pair MqsR/MqsA which regulates RpoS production [18]. Although many TAS can be found in bacteria, they are not redundant since they seem to respond to different types of stress.

Thus, antimicrobial resistance obtained by different pathways are ultimately regulated by TAS.

3. Heterogeneity

When a biofilm is formed, different environments are also created within its matrix which will have a great impact on bacterial growth and behaviour. This will render a great heterogeneity among bacteria living in the biofilm; even though they belong to the same species they are exposed to varying types of environmental stress. Different gradients of nutrients, waste products, oxygen or quorum sensing molecules organise this complexity where we can find very active, fast growing cells near the biofilm-environment interface, but slow growers or persister cells towards the centre. The differences found allow us to assert that each single bacterium has its own specific environment. Thus, the response to antimicrobial agents will vary depending on the location of specific cells within the biofilm community. [3, 4]

4. Persister cells

Persister cells neither grow nor die in the presence of bactericidal agents, and thus exhibit multidrug tolerance (MDT). They are a small subpopulation of bacteria that survive lethal concentrations of antimicrobials without specific resistance mechanisms. Persister cells present a transient phenotypic switch so that if they are recultured, they revert to a wild type. Generally this subpopulation is about 0.1 to 10%, even under prolonged antibiotic exposure. All resistance mechanisms do essentially the same thing: prevent the antibiotic from hitting a target; by contrast, tolerance apparently works by shutting down targets. Bactericidal antibiotics kill bacteria by corrupting their targets, rather than merely inhibiting them. Shutting down targets then protects from killing [19-21].

The mechanism of persister cells formation is still unknown and redundant, which makes its eradication a hard task. What we do know is that TAS and general stress response are involved but are independent of quorum sensing [19, 21]. Inhibition of translation by TAS leads to a shutdown of other cellular functions as well, preventing antibiotics from corrupting their targets [19].

An example in E. coli is overproduction of the TAS HipA, RelE or MazF. For instance, HipA inhibits translation by phosphorylating an Ef-Tu kinase causing a sharp increase in persisters. Deletion of the hipBA module produces a sharp decrease in persisters in both stationary and biofilm cells. There are also studies that include strains deletion in individual TA (toxin/antitoxin) loci or with transposon insertions, which overall suggest that there is more than one mechanism of persiter cell formation [19, 22].

Studies show that in the presence of DNA damage, an SOS response activates up regulation of DNA repair functions. This may lead us into another possible mechanism of persister cell formation. This response triggers TA genes to express tisB toxin gene that encodes a small membrane acting peptide. This creates an anion channel in the membrane which causes a decrease in ATP, followed by cell arrest. Thus induction of TisB by SOS response also controls production of persisters and multidrug-tolerant cells [19].

Some existing antibiotics can kill persister cells and could be of great use in treating chronic infectious disease. An example is mitomycin, a prodrug that when converted into a reactive compound once it enters the cell, forms adducts with DNA. When amynoglicosides are used in long-term treatments, it causes mistranslation and therefore misfolded peptides that have proven to sterilize stationary cultures of P. aeruginosa. Rifampicin, and inhibitor of RNA polymerase, is a bactericidal agent that kills by preventing persister resuscitation [19].

The ability of a biofilm to limit the access of the immune system components, and the ability of persister to sustain an antibiotic attack could then account for the recalcitrance of such infections in vivo and for their relapsing nature. Perhaps, combination of a conventional antibiotic with a compound inhibiting persister formation or maintenance may produce an effective therapeutic which could become an important turning point in long-term antimicrobial therapeutics.

5. Resistance of bacterial biofilms to antiseptics and disinfectants

While antimicrobials kill bacteria by acting upon specific bacteria and specific targets, antiseptics are nonspecific biocides with multiple targets that produce effects such as alterations in bacterial permeability, in structural proteins, in

nucleic acids and in microbial enzymes. Resistance to these substances is achieved by the aforementioned mechanisms but with some peculiarities. Moreover, resistance to disinfection is frequently associated with the presence of biofilms on surfaces. The definition of resistance needs to be clarified as it changes depending on whether planktonic or biofilm cells are considered. In the first case, a bacterial strain is defined as being resistant to a biocide if it is not inactivated by a specific concentration or period of exposure that usually inactivates the majority of other strains. Biofilm cells, on the contrary, are generally said to be resistant by comparison with their planktonic counterparts. Their insusceptibility is sometimes considered to be a tolerance rather than a real resistance since it is mainly induced by a physiological adaptation to the biofilm mode of life and can be lost or markedly reduced when biofilm cells revert to the planktonic state. Nevertheless, stable resistant variants can appear in biofilms.

5.1. Diffusion/reaction limitations of disinfectants in biofilms

Since disinfectants are often highly chemically reactive molecules, the presence of organic matter such as proteins, nucleic acids or carbohydrates can profoundly impair their efficacy as these molecules are themselves key targets, which reduces the concentration available for action against the microorganisms. Thus, potential interactions between biocides and biofilm components seem more likely to explain the limitations of penetration into the biofilm matrix.

Extracellular polymeric substances of the matrix are electrically charged and may be responsible for binding the biocide agents before they have the opportunity to reach specifics targets in the cell, hindering their diffusion [23]. It has been shown that in the absence of any electrostatic interactions, the majority of particles tested could penetrate and diffuse into a biofilm. Also, bacterial cell wall hydrophobicity could alters the diffusion of nanoparticles within a biofilm, suggesting that cell wall interfacial components such as peptidoglycan, fimbriae, capsules and the surface-layer affects diffusion of compounds within the biofilm [24].

Transport limitations due to physicochemical interactions between the biocide and matrix components or bacterial cells rather than steric hindrance contribute to biofilms resistance to disinfectants.

On the other hand, other components such as enzymes are present in the extracellular matrix and play a role in neutralizing toxic compounds. An example is the presence of peroxidases and catalases that reduce the attacking concentration of biocides such as hydrogen peroxide [1].

5.2. Phenotypic adaptations of biofilm cells to sublethal concentrations of biocides

During a disinfection process, the reaction-diffusion limited penetration of biocides into a biofilm results in low levels of exposure to the antimicrobial agent in specific regions of the biofilm. These cells will therefore develop adaptive responses to sublethal concentrations of the disinfectant. Adaptation depends on the microorganism and the disinfectant. For instance, Salmonella biofilm cells display better adaptation to benzalkonium chloride than their planktonic counterparts after continuous exposure. The up regulation of specific proteins involved in energy metabolism, protein biosynthesis, adaptation and detoxification, together with a shift in the fatty acid composition explain survival [25].

Additionally, the conditions prevailing during initial adhesion to a substratum are also relevant for biofilm resistance to a biocide: cell morphology, spatial distribution and the relative amounts of exopolymer matrix in Pseudomonas biofilms differ in the presence of sublethal doses of chlorhexidine, benzalkonium chloride or triclosan, while chlorine dioxide at sublethal doses stimulates biofilm formation in Bacillus subtilis [26].

5.3. Gene transfers and mutations

Horizontal gene transfer participates in microbial adaptation to the environment through the exchange of genetic sequences including plasmids, transposons or integrons that confer specific phenotypic traits on cells including metabolic capabilities, virulence expression and antimicrobial resistance. Biofilms constitute an optimum environment for the exchange of genetic material, leading to the dissemination of biocide resistance cassettes within the population as conjugation and transformation processes are promoted [27].

As said before, biofilm growth leads to the emergence of extensive genetic diversity within a bacterial population [28] and the production of variants creates more resistant subpopulations that will enhance the fitness of the bacterial community under stressful conditions, i.e. biocides. Endogenous or exogenous oxidative stress promotes mutants through the ROS and SOS responses [29].

5.4. Pathogen protection in multispecies biofilms

In their natural environments, biofilms are complex mixtures of different species rather than the laboratory single species structures. In these complex consortia, species interactions can lead to the emergence of specific biofilm phenotypes. Multi-species biofilms are generally more resistant to disinfection than mono-species biofilms but the mechanisms involved are numerous and remain unclear. An explanation can be found in the chemical interactions between the polymers produced by each species which may lead to a more viscous matrix with a lower permeation to biocides [30, 31].

Similarly, because a biocide can be inactivated in a biofilm matrix by enzymes, as the catalase-mediated inactivation of hydrogen peroxide in a P. aeruginosa biofilm, the enzymes produced by the different species may act synergistically against toxic compounds so that non-productive species will benefit from the association through enzyme complementation.

Specific spatial arrangement of certain bacterial species within a biofilm could be another explanation, some strains may be protected from a biocide by their aggregation with others within the three-dimensional structure.

Beyond interactions with other bacterial species, bacteria in a biofilm can also be protected by eukaryotic microorganisms. Many bacterial species survive within various amoebal species [32]. Trophozoites are the actively dividing forms of amoebae; increased resistance to disinfection has been reported for bacteria internalized within trophozoites. Various bacterial species, including Legionella pneumophila, L. micdadei, more than 15 mycobacterial species, Francisella tularensis and Vibrio cholerae have been reported to survive within amoebal cysts, thus benefiting from the extremely efficient protection they afford.

6. The biofilm phenotype

The acquisition of the biofilm phenotype is the global answer to antimicrobial resistance. There is not one single mechanism by which bacteria living within a biofilm environment are resistant or tolerant to antimicrobial insults, instead a "matrix" of interconnected systems makes it possible.

From the attachment of cells to the development of a three-dimensional structure, the growth of a biofilm is associated with physiological adaptations of cells that may lead to an increase in resistance to antimicrobials. These phenotypic adaptations result from the expression of specific genes in response to their direct microenvironmental conditions and heterogeneity. Right after a bacterium contacts a surface, genes coding flagellar proteins are repressed and other genes coding for exopolysaccharides and adhesin proteins are induced, MqsA is degraded and MqsR and RpoS are activated [18]. These changes induced by cell adhesion lead to the appearance of more resistant phenotypes, as suggested by studies reporting the greater resistance of cells that are merely adhered to a surface when compared with their planktonic counterparts [33].

Following adhesion, bacteria start to develop into a biofilm with a three-dimensional structure. A direct consequence of the growth of this structure is the emergence of chemical gradients within the biofilm. Cells located at the periphery of the cluster have access to nutrients and oxygen, while bacteria in internal biofilm layers experience nutrient-poor microenvironments where the concentrations of metabolic waste products are higher. This chemical heterogeneity governs the onset of physiological heterogeneity. Protein synthesis and active cell growth are restricted to the zone where oxygen is available and represent a narrow band in the biofilm-environment interface. Cells with distinctive metabolic rates are present throughout the matrix, thus constituting a physiologically heterogeneous population. Alterations to growth and activity rates induce modifications to membrane composition and the expression of defence mechanisms that lead to an increased resistance of bacteria to antimicrobials. Bacteria show different degrees of resistance according to their growth state: slowly growing bacteria are known to be less sensitive to antimicrobials than more actively metabolizing cells [34].

The ROS response affords protection against the activity of oxidizing agents [35], and is associated to up regulation of genes coding to multidrug efflux pumps in biofilms is another mechanism to explain antimicrobial resistance. Efflux pumps are systems that enable cells to rid themselves of toxic molecules and allow bacteria to survive in the presence of such substances [34].

The appearance of a biofilm-specific phenotype is partly induced by quorum sensing. Indeed, cell-to-cell communication has been identified as controlling biofilm development in a number of bacterial species, and the expression of catalase and superoxide dismutase genes coding protective enzymes against oxidizing stress has been shown to be under the control of quorum sensing [36].

Mutant bacteria with antimicrobial resistance can arise after antimicrobial exposure through the SOS response, and although this system must be controlled under ordinary circumstances, under stress the fittest are selected. [37, 38]

A final adaptation that contributes to bacterial resistance in biofilms is that a fraction of the population enters a highly protected state displaying dramatic resistance and referred to as persister cells. These bacteria are phenotypic variants but not genetic mutants. One assumption is that persisters develop more frequently in a biofilm than in a planktonic culture, perhaps induced by the specific environmental conditions prevailing within the structure, and may therefore contribute to better antimicrobial protection in the biofilm [39].

7. Conclusion

As we have seen, biofilm mediated antimicrobial resistance is a complex issue. A great amount of known mechanisms act together in order to block, fix, suppress or ignore antimicrobial stress, but the number of unidentified responses remains unknown and yet to be discovered. Moreover, we still have to understand the interactions between different stress responses.

Nevertheless, we do know the final result of this intricate system: antimicrobial treatment failure. Knowing the way these systems work collectively, and finding means to hinder them is the next obvious step in clinical microbiology.

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