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Biofilms: A Survival and Resistance Mechanism of Microorganisms

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1. Introduction

Biofilms are microbial monoespecie or multispecie (consortium) communities that are the most successful colonization among microorganisms, are ubiquitous in nature and responsible for many diseases. They are considered growing communities of microorganisms embedded in a self-produced exopolysaccharide matrix and are attached to an inert surface or living tissue (Castrillón et al., 2010).

It is believed that this organization represents the mode of cell growth that allows cells to survive in hostile environments, disperse to form new niches and gives them significant advantages in protection against environmental fluctuations such as humidity, temperature, pH, the concentration of nutrients and waste removal (Costerton et al., 1987, Hall-Stoodley et al., 2004).

There is an association between the presence of biofilm-grown microorganisms with delayed wound healing and various diseases such as endocarditis, otitis media, chronic prostatitis, cystic fibrosis, periodontitis, and related infections medical devices and implants responsible for nosocomial infections (Castrillón et al., 2011, Donlan & Costerton, 2002). The latter share common features, although the causative organism and the site of infection are very different, they all evade host defenses and resist treatment with antimicrobials. In general, bacteria in biofilms tolerate high levels of antibiotics compared with planktonic cells (free). The ability of biofilm formation is not restricted to any specific group of bacteria or fungus and is now considered that under ideal conditions all microorganisms can form biofilms (Lasa et al., 2005).

2. Stages of development of biofilms

The main experimental models for studying bacterial biofilms are four: *Escherichia coli, Psedomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus* (Lopez et al., 2010) and fungi *Candida albicans* and *Aspergillus fumigatus* (Kumamoto, 2002, Müller et al. 2011). In these works describes the development of a biofilm which begins with planktonic bacteria (free) that bind irreversibly to a surface in a continuous process in accordance with various stages of development are:, b) adhesion, c) synthesis of extracellular matrix, d) maturation and e) dispersion, which leads to the formation of a uniform structure of deposits and accumulations of viscous and homogeneous material surrounding the cells by a polymer matrix with open channels for water movement (Figure 1).

Any natural or synthetic surface is covered by the constituents of the local environment, electrolytes, water and organic materials form a film before the arrival of the organism which neutralize the charge over the surface (conditioning) that prevents the aproximation between bacterial cells fungi and so begins adherence, these organic compounds can serve as nutrients for these microorganisms.

Free (or planktonic) cells form a layer that is adsorbed to the surface for short periods by electrostatic attraction forces and released from it by reversible adsorption (Bos et al., 1999). In this phase the microorganisms are still susceptible to action of antibiotics.

The microorganisms in suspension are aggregated and cell adhesion occurs with same or different cells (co-aggregation) to the surface conditioned, this process is favored by several bacterial components involved in this process by overcoming the repulsive forces such as pili or flagella, and surface polymers such as lipopolysaccharide in Gram-negative bacteria and mycolic acid in Gram-positive. The expression of these microbial structures may change depending on the environment in which they are and thus change the phase of biofilm formation. Mutants no-mobile fail to form monolayers and their union as microcolonies therefore mobility structures play an important role in the initiation of biofilm (Stickler, 1999).

The physicochemical properties of the surface can exert a strong influence on the degree and extention of adherence, the germs adhere more readily to hydrophobic surfaces, non-polarized and plastics such as Teflon, compared to hydrophilic metals such as glass or metal.

Once irreversible adhesion is achieved, the cells divide and colonize the surface and when the local concentration of chemical signals produced by microbial metabolism reaches a threshold level, suggesting that the microbial population density has reached a minimum, this determines the start of phenotypic changes in the community.

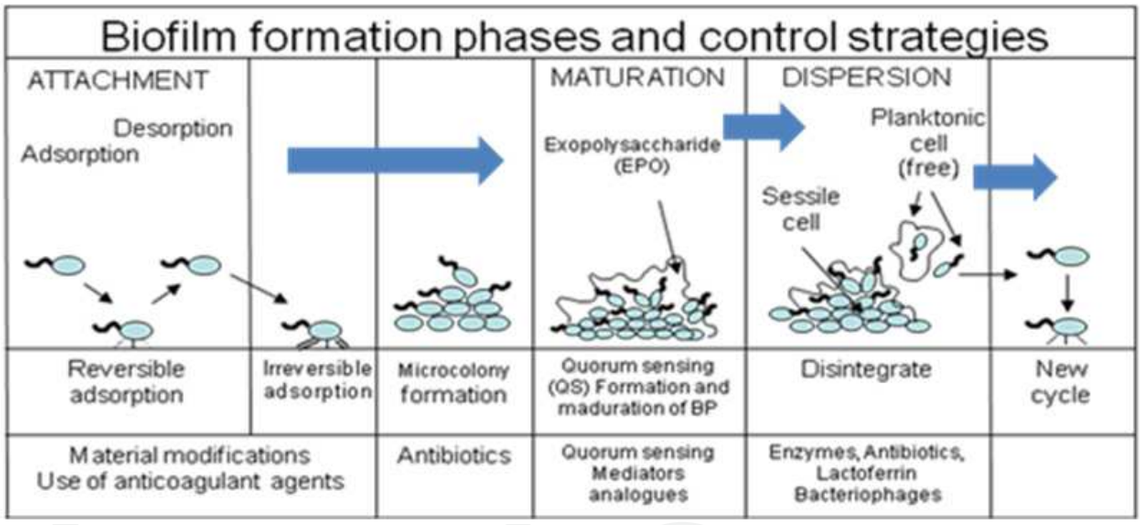
The process in which a microbial cell senses the proximity of other cells reaching a critical number in a limited space in the environment, chemical signals are generated corresponding to secondary metabolites, known as *quorum sensing*, this fact results in the autoinduction in the synthesis of the extracellular matrix or exopolysaccharide (composed of polysaccharides, proteins, nucleic acids and lipids), and thus gets to the maturation of biofilm formation with subsequent three-dimensional structure, generated by water channels that serve as the microcirculation in colonies. When the message is large enough, the organism responds like a mass and behaves as a group (Keller & Surette, 2006). The composition of the exopolysaccharide or glycocalyx is different for each bacteria and fungus, and varies depending on culture conditions, medium and substrates which are: alginate in *P. aeruginosa*, cellulose in *S. typhimurium*, rich in galactose in V. *cholerae* and poly-N-acetylglucosamine in *S. aureus* (Whitehead et al., 2001, Sutherland 1997). This matrix allows the interconnection of immobilized cells and acts as a digestive system that keeps external extracellular enzymes close to the cells and enables them to metabolize biopolymers and colloidal solids (Sauer et al., 2002, Flemming & Wingender, 2010).

The detachment may be seen as another stage of the life cycle of the biofilm, which can be reached or not depending on environmental conditions such as nutrient availability, oxygenation, pH and specific compounds because at some point the high density cell can result in severe, dynamic gradients of nutrients and toxic metabolic sub-products, then some

cells are released from the matrix to colonize other surfaces closing the process of formation and development of biofilms, this process may be the result of several factors such as are: mechanical forces as the flow of blood vessel, cessation of production of exopolysaccharides and detachment factors such as enzymes that destroy the matrix or surfactants.

Fragments of biofilm with viable cells can be dispersed in liquids or aerosols. The scattering process is of interest for their potential to promote the spread of bacteria or fungi in the ambient or their ability to exploit these processes to combat infections (Hall-Stoodley & Stoodley, 2005).

For the development cycle of *Candida albicans* biofilms has shown that scattered cells show a distinct phenotype associated with increased virulence (Uppuluri et al., 2010). When the extracellular medium accumulates enough of these molecules activate specific receptors that alter gene expression and affect different phenotypes that produce virulence factors such as enzymes and toxins or rhamnolipid of *P. aeruginosa* cell that are protective of fagocytosis, the *quorum sensing* determines tolerance to antibiotics and innate inflammatory response dependent on polymorphonuclear cells.



Biofilm formation occurs as a series of sequential events that depend on the interaction of microorganisms on inert surfaces or living, by overcoming the repulsive forces to achieve irreversible adsorption followed by the formation of a microcolony. Upon reaching a certain population density, induce the synthesis of secondary metabolites (*quorum sensing*) that produces an exopolysaccharide formation until maturation of the biofilm. Disintegration allows the formation of a new colony or elimination. It shows the treatment options for different stages of biofilm development.

Fig. 1. Phases of biofilm formation and dispersal strategies.

The main characteristic that best distinguishes chronic infections associated with biofilm to acute infections is their response to treatment with antibiotics, in general biofilm microorganisms tolerate high levels of antibiotics compared to planktonic cells and cause recurrent episodes. In the case of acute infections these are eliminated after a short treatment. In addition, acute infections are more aggressive than those associated with chronic infections or implants as the latter persist for months or years and progress through periods of rest alternating with exacerbations.

3. Host resistance to biofilm

Biofilms cause chronic infections characterized by persistent inflammation and tissue damage despite treatment with antibiotics and innate and adaptive immune responses of the host.

Planktonic cells that are released directly from the biofilm was removed by the action of antibiotics and phagocytic cells activated, but the organization as a biofilm is considered as a very efficient defensive strategy adopted since these microorganisms grow slowly and are protected mechanisms of host resistance through various strategies among which are a) inability of antibodies, complement and lysozyme to penetrate these organizations multicellular b) production of catalase bacteria that prevents the action of hydrogen peroxide produced by oxidative mechanisms of phagocytic cells c) inhibition of host immune function such as chemotaxis, opsonization and bactericidal potential exopolysaccharide (Lasa et al., 2005).

A study has demonstrated inability of the immune system clearance sessile cells that persist for weeks and months was observed when the peritoneal cavity of rabbits were inoculated mature biofilms of *P. aeruginosa* in immunocompetent animals, the penetration of phagocytic cells in the biofilm was detected, however, these cells were unable to phagocytose the bacteria (Ward et al., 1992). A similar response was described with the inoculation of fragments of the same biofilm bacteria trapped in agar beads and introduced into the lung (Woods et al., 1980).

4. Identification tests and antibiotic susceptibility in biofilms

In clinical samples, a biofilm is difficult to detect in routine diagnosis but may be recognized by light microscopy and accurate identification of bacteria in a biofilm can only be done by techniques of hybridization, fluorescein staining, the molecular probe 16SRNA domain eubacteria (EUB 338), determining live cell / dead BacLight staining or by identifying the matrix components by specialized staining techniques (Veeh et al., 2003).

Routine microbial cultures provide misleading results because they do not reflect the increasing resistance of bacteria growing in biofilms. The minimum inhibitory concentration (MIC) of bacteria grown as biofilm is 100 to 1000 times higher compared to planktonic cells despite antibiotic susceptibility in the laboratory (Costerton et al., 1999).

There are no standardized methods to date used routinely to determine the antibiotic sensitivity of bacteria grown as biofilms. When sampling swab and plating growth obtained in cultures performed standardized susceptibility testing, these same antibiotics fail to solve conventional bacterial infections This is because bacteria grow attached and the surface as a biofilm. However, in many cases it is not possible to recover the bacteria by traditional culture methods. This has been reported in infections where *Staphyococcus* biofilms emerging vascular grafts stimulate the production of antibodies against biofilms initiated within 10 days of colonization, however, cells were never recovered by conventional techniques of microbial culture (Costerton al ., 2003), another case is related to infections in medical devices where antibiograms shown susceptibility against some microorganisms but the infection fails to be eliminated by these antibiotics (Fux et al., 2005).

It is very important to point out that the systems sensitivity to antibiotics were traditionally performed on cells in suspension, which is equivalent to the population of planktonic cells, for this reason, is necessary to design new laboratory techniques that reveal the sensitivity of these substances directly on biofilms, this idea has been reported that antibiotics active against stationary phase bacteria *in vitro* are successful in removing biofilms *in vitro* infections (Zimmerli et al., 1998).

This information is important to consider that when it is mentioned that biofilm infection has hematogenous dissemination must specify if they are planktonic cells or biofilm fragments because there are differences in their ability to resist antibiotics, adherence and host response resistance.

5. Horizontal gene transfer

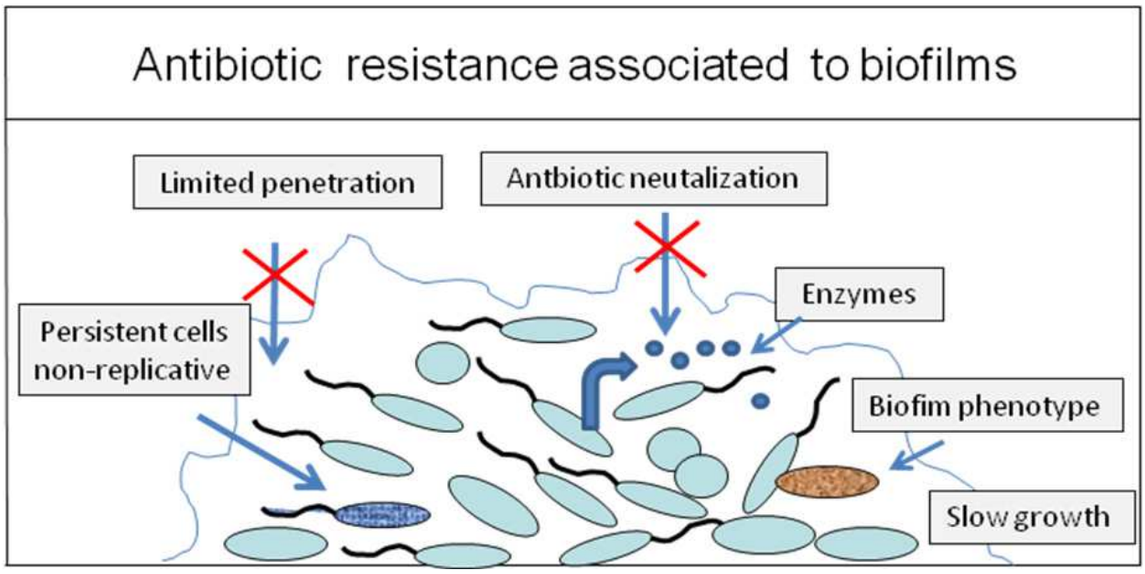
Mobile elements such as plasmids and transposons, have proven important in the transfer of antibiotic resistance is enhanced when the cell density increases and competition genetic, hence that biofilms are an ideal state to promote the horizontal transfer of genes (Ghigo, 2001). However, there is evidence that when bacteria of a biofilm is dispersed is rapidly becoming susceptible so their resistance is not the result of mutations and mobile elements (Stewart & Costerton, 2001, Stewart, 2002).

Increasing resistance to beta-lactams, aminoglycosides and fluoroquinolones has been correlated to the frequency of mutations in bacteria that grow as biofilms (Hoiby et al., 2010). These facts lead to rapid and global spread of genes in natural environments and in hospitals favoring nosocomial infections associated with biofilms.

6. Mechanisms of resistance associated with biofilms

The conventional mechanisms of resistance to antibiotics and biocides fall into four categories: direct inactivation of the active molecule, altering the body's sensitivity by changing its target of action, reducing the concentration of the drug reaches its target unchanged its chemical composition and efflux systems (Hogan & Kolter, 2002, Poole 2002). However, most information comes from studies that were performed in suspension cultures and in general, bacteria in biofilms tolerate high levels of antibiotics compared to what their planktonic cells. In different settings, the level of antibiotic resistance may vary and the factors causing this increase may differ.

The primary evidence indicates that conventional mechanisms do not explain the high resistance to antimicrobial agents associated with biofilms, although this evidence does not exclude the possibility of resistance in the growth of adherent cells. This suggests that the development of resistance in bacteria that are aggregated on surfaces or biofilm has its own intrinsic mechanisms are different and are responsible for those conventional antibiotic resistance, and although currently no single accepted mechanism, we have explored several potential candidates as responsible for this high resistance characteristic of biofilms among which are: Diffusion limited, neutralizing enzymatic, functional heterogeneity, slow growth, persistent cells and biofilm phenotype corresponding to adaptive mechanisms to stress such as efflux pumps and alterations in membrane. (Figure 2).



The antibiotic may be retained by interactions with the extracellular matrix or be neutralized by the production of enzymes that modify it. The metabolic heterogeneity may alter the growth preventing antibiotic action if its molecular target requires active metabolic pathways, or the oxygenation or pH gradients inhibit the action of the antimicrobial. The appearance of persistent or phenotype within biofilm makes it insensitive to the antibiotic

Fig. 2. Antibiotic resistance associate to biofilms.

6.1 Low penetration

Antibiotics can diffuse through the biofilm matrix, to inactivate the cells trapped, but this exopolysaccharide behaves as a physical barrier affecting its spread to deeper layers by direct interaction of these molecules to modify their transport to the interior, causes resistance to these antimicrobials, as well as high molecular weight molecules with cytotoxic properties as lysozyme and complement. So, while planktonic cells are quickly exposed to high concentrations of antibiotics, the microorganisms in deep layers are gradually exposed to increasing the concentration of antibiotics.

Bacteria that are deficient in polysaccharide synthesis and therefore of produce biofilm, escape from the biofilm and are susceptible to attack by immunocompetent cells. An antibiotic may be inactivated or sequestered by binding to the extracellular matrix as in the case of the alginate exopolysaccharide of *P. aeruginosa* which is anionic nature. Which explains why the fluoroquinolones and aminoglycosides penetrate slowly rapidly since the latter positively charged bind to the matrix has a negative charge, but this mechanism can be saturated if repeated doses are administrated (Lewis, 2001, Gordon et al. , 1988, Mah & O'Toole, 2001).

The penetration of chlorine does not reach concentrations greater than 20% in mixed cultures of *K. pneumoniae* and *P. aeruginosa* biofilms. In case of biofilms of *S. epidermidis* vancomycin reaches deep layers but not rifampin (Mah & O'Toole, 2001).

Has also been observed that the thickness of the biofilm is important for the penetration of hydrogen peroxide was allowed in layers with 3.5 log CFU *P. aeuroginosa* and not diffusion

when the layer was 7.6 log CFU, however, the absence of catalase gene (kata) makes it easy access even if the biofilm is thick (Stewart et al., 2000).

The dissemination and death from alkaline hypochlorite (pH 11) and chlorosulphamate (pH 5.5) was evaluated on biofilms of *P. aeruginosa.* The chlorosulfamate transport was not affected unlike hypochlorite delaying their penetration, however both biocides enter to the biofilm and fail to kill cells suggesting an alternative mechanism to explain the resistance to these substances (Stewart et al., 2001).

Other explanations for the failure to altering the penetration of antimicrobial agents in biofilms *K. pneumoniae* are that the cells are stacked or is the result of problems of bioavailability of the drug (Smith, 2005).

Reduced mobility of an antibiotic is not an impenetrable barrier and is not sufficient to explain the resistance, it is assumed that other mechanisms must be involved. Recently it has been suggested that the delay in permeability through the biofilm allows the bacteria have enough time to implement adaptive responses to stress.

6.2 Neutralization

If an antibiotic penetrates the biofilm enzyme production by microorganisms can degrade or modify are synthesized by enzymes that selectively destroy the activity of antibiotics. These enzymes are a series of proteins that use multiple adaptive strategies to confer resistance such as hydrolysis (β-lactams, macrolide esterases epoxidase) and modification of antibiotics by acyltransferases, phosphorylation, glycosylation, nucleotidilación, ribosylation and transfer of thiol groups (Wright, 2005, Castrillón et al., 2003, Gallant et al., 2005, Martinez-Suarez et al., 1985). These enzymes accumulate in the glycocalyx as a result of its secretion or cell lysis (by action of the antibiotic on the microorganisms from the biofilm surface or planktonic).

Neutralization acts synergistically with delayed diffusion and degradation of the antimicrobial into the biofilm. An important mechanism of resistance in cystic fibrosis by *P. aeruginosa* is due to the overproduction of cephalosporinase AmpC enzymes which is its main mechanism of resistance to beta lactam in the presence of high levels of carbapenems such as imipenem which is a strong inducer in contrast with ceftazidime is weak probably due to its inactivation in the biofilm (Del Valle, 2009, Giwercman, 1991).

The filters impregnated with antibiotics and its direct action on biofilms *K. pneumoniae* has shown that the antibiotic diffuses only in the presence of mutant cells β-lactamases but growth is observed, suggesting that another mechanism of resistance must be considered (Anderl et al., 2000).

6.3 Heterogeneity

To determine the rate of microbial growth within a biofilm microelectrodes were used with probes for direct measurement of oxygen in different areas of the biofilm, and the use of acridine orange to identify fast-growing cells (stained orange) or slow (stained yellow/ green) according to their relative concentration of RNA / DNA. (Mah & O¨Toole 2001). These studies demonstrate that biofilms are structurally and metabolically heterogeneous in which aerobic and anaerobic processes occur simultaneously and display areas so that

metabolically inactive antimicrobial response may vary depending on the location of an individual cell within the community and that the high level of activity on the surface and limited or absent growth inside reduces the susceptibility to antibiotics.

These studies have shown that biofilms are heterogeneous structures with three chemical patterns that correspond to differences in concentration gradients from outside to inside the biofilm. The pattern of metabolic substrate induces a higher concentration on the outside and less inside, the metabolic product pattern is reversed to the previous and the pattern of metabolic intermediates shows a greater concentration between the boundary of the biofilm in the aqueous phase (Stewart & Franklin , 2008). These patterns bring the result that within these structures are established differences in pH gradients and oxygenation as it has been shown that the penetration of oxygen as high as 25% in the depth of the biofilm (Borriello et al., 2004). These facts are installed microbial populations aerobic or facultative anaerobes within the different layers of the biofilm, allowing us to understand the differences in susceptibility to treatment with antibiotics, which is different from the response to the free forms (plankton) that the attached (sessile).

Deprivation of oxygen and anaerobic growth of microorganisms affects the action of aminoglycosides which is modulated by the availability of oxygen and pH gradients (Wimpenny, 2000).

6.4 Slow growth

When an organism is limited nutrients, slow growing and may cause resistance to antibiotics. Cells within biofilms are under a gradient of nutrients resulting in metabolically active cells with access to these nutrients in the surface layer or on the periphery of the biofilm, in contrast, metabolically inactive cells are found within its interior. These different areas of metabolic activity correspond to different areas of antimicrobial susceptibility

The decrease in growth rate and low metabolic activities decrease the cell permeability and therefore the access of antimicrobial substances, metabolic inactivity can also reach a level where the bacteria are viable but have lost their ability to be cultivated this state of non-culturable viable cells is the main reason for the low detection of biofilm infections by standardized culture methods.

The cytotoxic action of many antibiotics is dependent on the growth of microorganisms such as penicillins that are active are active only in growing cells, many antibiotics are targeting some kind of molecular synthesis and have no effect on bacteria where this synthesis has stopped, and cells in the interior might be protected from the cytotoxic action of these substances (Brown and Allison, 1988). Penicillin and ampicillin do not attack cells that are not growing and its action is proportional to its activity, other antibiotics such as β-lactams, cephalosporins, aminoglycosides and fluoroquinolones attack stationary phase cells, but are more active in dividing cells (Costerton et al., 1999). It has been determined resistance to cetrimide on *E. coli*, ciprofloxacin on *S. epidermidis*, tobramycin and piperacillin in *P. aeruginosa*, this effect is associated with decrease in growth rate (Donlan & Costerton, 2002).

Antimicrobial peptides are natural products produced as part of the arsenal of protection in the host innate responses and target microbial membrane (Castrillón et al., 2007). Colestine peptide (polymyxin E) has been used in the treatment of multidrug-resistant cancer patients

and cystic fibrosis by *P. aeruginosa* (Hachem et al., 2007), this antibiotic is the only antimicrobial activity against the central part of biofilms *in vitro*, while the metabolically active at the surface become tolerant due to the regulation system *pmr* operon genes and the MexAB-OprM. Ciprofloxacin and tetracycline are able to clear metabolically active cells so it is suggested that combination therapy with these antibiotics colistin for early eradication of *P. aeruginosa* in patients with cystic fibrosis (Pamp et al., 2008).

6.5 Persistent cells

A small percentage of the cell population remains viable after prolonged exposure (or overdoses) to antibiotics known as persistent, and gives (or not) their resistance to progeny once the selective pressure is removed. This susceptibility to the threshold of growth varies depending on the mode of action of antibiotic used.

Persistent cells are cells that temporarily quit the replication for the survival of the community and their strategy is different from the stress-related adaptive responses in which the population expresses resistance proteins in response to potential environmental damage. Persistent cells survive doses of antibiotics that kill normal cells and increase in number when there is a high cell density reached the highest number in the stationary phase suggesting that their main role is to ensure the survival of cells that are not growing (Lewis 2008).

These cells are different from the antibiotic-resistant mutants do not produce offspring resistant to the antibiotic in his absence and can grow in the presence of the antibiotic while maintaining the same minimum inhibitory concentration (MIC) in contrast to the mutants.

The main evidence of the existence of persistent cells in biofilms are: a) there is a biphasic dimension in biofilms wich means that much of the population is attacked fast and another is not affected even with a prolonged course of antibiotics, b) description of gene of persistence (*hip*) that act as regulatory circuits that allow them to enter and leave this state as a protective response, c) bacteriostatic antibiotics inhibit the growth of sensitive cells are those that contribute to persistent cell growth and preservation of biofilm d) when therapy is withdrawn biofilm again reshape (Herrera 2004).

The production of persistent cells in biofilms in bacteria is highest during the stationary phase in planktonic culture of the biofilm, however, in the case of *Candida albicans* their formation occurs only when growth occurs as a biofilm (Spoering & Lewis, 2001).

Although the date is unknown the basics of the physiology of these persistent cells, several genes involved has been described for their generation, including locus have identified three hip (high-level-persistence): A, B and AB control the frequency of this phenotype. The identification of genes and their products may be targets for developing new therapies (Keren et al., 2004).

All *hip* mutant cells produce a thousand times more cells persistent than the wild variant (Moyed & Broderick 1986). The importance of the appearance of these cells determines the success of treatment with antimicrobial use as the minimum bactericidal concentration would kill 99.9% of cells in biofilms, and the remaining would be eliminated by the immune system, without however, the presence of persistent cells limits the removal of the population of microbial cells or in the case of a dysfunction in the patient's immune response may be the cause of recurrent infections.

6.6 Biofilm phenotype

Nutritional starvation and high cell density in a limited space are important features in in the physiology of planktonic cultures reaching stationary phase. Hence the formation of a biofilm represents this natural phase of bacterial growth by increasing production of secondary metabolites such as antibiotics, pigments and other molecules, which act as signaling molecules to form (or inhibition of growth of other microorganisms) of biofilms (Lopez et al., 2010).

The response to environmental stresses such as heat shock, pH changes, oxygen and chemicals among others, cause physiological changes that act as protective antagonizing the harmful effects by inducing protective mechanisms such as efflux pumps of antibiotics, changes membrane level or phase variation.

In biofilms in response to treatment with antibiotics, appear subpopulations with different phenotypes that vary in their gene expression but not in their genetic material (Fux et al, 2005). This was confirmed when performing subcultures in fresh medium in which not only provide nutrients but also dilutes the cell-cell signaling, the cells regain susceptibility to the antibiotic, demonstrating the absence of mutations.

The gene expression patterns in biofilms of *P. aeruginosa* produce different phenotypes that differ from their planktonic counterparts (Sauer et al., 2002) and a small proportion of cells develop a protective phenotype that coexists with the cells sensitive to antibiotics and has been suggested by some authors that corresponds to that expressed by a spore (Stewart & Costerton, 2001).

A biofilm community that shows resistance to treatment by antibiotics and develops a characteristic phenotype such as biofilm growth has been called "biofilm phenotype" and have come to propose the existence of specific genes and reference to their therapeutic targets, however , DNA microarrays and gene expression in *Bacillus subtilis* biofilms differ only 6% compared to their planktonic cells and only 1% in *Pseudomonas aeruginosa*. At present, the differential expression of these genes has not proven useful for this purpose (Fux et al., 2005).

6.6.1 Efflux pumps

Accumulation of antibiotics in the periplasmic space inside the bacteria is antagonized by efflux pumps that are resistant to several classes of antibiotics including tetracyclines, macrolides, fluoroquinolones, β-lactam and reducing their concentration at sub-toxic level (Van Bambeke et al ., 2003).

Efflux pumps are protein structures that are able to expel from the bacterial cytoplasm and periplasm for bacteria toxic compounds such as antibiotics. The expression of these pumps can be permanent (constitutive expression) or intermittent (expression can be induced). These pumps may be specific to a substrate or similar compounds can be transported and may be associated with multidrug resistance (MDR). (Sánchez-Suarez et al., 2006, Grkovic et al., 2002).

In prokaryotes there are five families of efflux transporters: MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), SMR (small multidrug resistance) and ABC (ATP binding cassette). All of them require proton motive power and power supply.

The main systems reported in bacteria of interest in the clinic are: *Campylobacter jejuni* (CmeABC), *E. coli* (AcrAB-TolC, TolC-AcrEF, EmrB, EmrD), *Pseudomonas aeruginosa* (MexXY-OprM, MexCD-OprJ, and OprN MexEF Mex-XY-OprM), *Streptococcus pneumoniae* (PmrA), *Salmonella typhimurium* (AcrB) and *Staphylococcus aureus* (NorA) and *Candida albicans* (MRD1, CDR1 and CDR2) (Webber & Piddock, 2003).

It has been speculated the possibility of antibiotic resistance in biofilms of *P. aeruginosa* by the expression (or overexpression) of these pumps, however, none of the four efflux pumps in the genome of this bacterium contributes to the resistance (De Kievit et al., 2001). In contrast to these results, resistance to azithromycin is associated pumps MexAB-OprM and MexCD-OprJ to biofilm resistance mechanisms in *P. aeruginosa* (Gillis et al., 2005) and PA1874-1887 pump that is expressed at high level in both biofilms and planktonic cells (Zhang & Mah, 2008). Although the results are still inconclusive, have proposed the use of anti-inhibitor drugs efflux pumps (EPI) as potential anti-biofilm treatment have been well tolerated in humans (Kvist et al., 2008).

When cells bind to a surface, expressed a different phenotype to the planktonic cells and may be expressed as a resistance mechanism multidrug efflux pump as reported in *Escherichia coli* (AcrAB operon *mar)*. When *mar* expression was evaluated in a bioreactor and as growth in biofilm, the results support the idea that *mar* operon was expressed in biofilms where the lowest level was detected compared with the equivalent in stationary phase fermenter cultures (Maira Litrán et al., 2000a). The loss of acrAB *mar* did not affect the growth as biofilms of *E. coli* and resistance to ciprofloxacin is not dependent on the regulation of *mar* operons or acrAB (Maira Litrán et al., 2000b).

In the case of *Candida albicans* pumps for azoles was noted that in mutants *cdr* planktonic cells and *mdr* were hypersusceptible to fluconazole in contrast to cells that were resistant biofilm showing that resistance is a complex phenomenon that can not be explained by a single mechanism (Ramage et al., 2002).

6.6.2 Alterations in membrane proteins

The diffusion of any antibiotic depends on the permeability of its outer membrane that allows its diffusion of different routes to the periplasmic space. Porins are channel proteins of the outer membrane of Gram-negative bacteria involved in the transport of hydrophilic molecules from the external environment to the periplasmic space.

The genes encoding porins can mutate and produce nonfunctional or altered proteins can decrease their expression. Both processes give rise to mutant bacteria deficient in porins, which have low permeability to hydrophobic molecules pass (Hancock, 1997).

A quick change of balance in the expression of porins in response to antibiotic therapy confers an advantage to the pathogen compared with the commensal microflora that is susceptible to β–lactams (Pagés et al., 2008). In the case of *P. aeruginosa porin* OprD is used for the dissemination of imipenem and resistance is associated with its three-dimensional disturbance.

Porins in *E. coli* are OmpF and OmpC operated in response to changes in osmolarity. Mutations in ompB (regulator of OmpF and OmpC) increase resistance to β-lactam antibiotics, the mutants lacking OmpF are resistant to chloramphenicol and tetracycline.

The genes encoding porins are differentially expressed in biofilms and may contribute to antibiotic resistance. The expression of ompC and three other osmotically regulated genes are increased when the bacteria grow as biofilms in the environment a protective mechanism (Mah & O'Toole 2001).

6.6.3 Phase variation

In biofilm there is capacity development of subpopulations of bacteria or fungi to switch to the dormant metabolic state as small-colony known variants (SCVs) in which they are less susceptible to growth-dependent antibiotic killing, have a defective catalase activity interfere with oxidative metabolism and uptake of aminoglycoside modifying its minimum inhibitory concentration of 8 to 16 times compared with large colonies and normal as in the case of *Enterobacter aerogenes* (Neut et al., 2007, Rusthoven al., 1979).

The phase variation plays an integral role in the formation of diverse phenotypes within biofilms and is largely responsible for the recalcitrance of infections caused by biofilms, the increase in the reversal phase coincides with the antibiotic treatment. This phenomenon has been reported for several genera and species, including *Staphylococcus* and *Pseudomonas* genus, and certain species of Enterobacteriaceae and fungi. (Costerton et al., 1999).

The phase variation causes detectable changes in colonial morphology, the small colony variants of phase variant (SCVs) in biofilms develop properties hyperadherence, autoaggregation, increased hydrophobicity and reduced motility, it has been suggested that tolerate a wide variety of aggressive environmental conditions so that this process is considered a survival mechanism.

It was considered that the phase variation is a process of cellular internal rearrangement, however recently it has been considered to occur by interactions with genetic elements outside the cell as an internal bacteriophage genetic rearrangement by suggesting a model where mobile genetic elements generate the phase variation through a collective mechanism (Chia et al., 2008).

In *Pseudomonas aeruginosa* has shown that under different environmental pressures will favor the appearance of morphological variants that relate to the phenotype of biofilm among which are the small-colony variants (SCVs), rough small-colony variant (RSCVs), wrinkled variants, and rugose colonies autoaggregating cells. The phenotypes RSCVs and SCVs play a critical role in the colonization in cystic fibrosis and mutations in the *psl* locus in variants RSCVs lose their hyperadherence and autoaggregation abilities (β Häubler et al, 2003, Kirisits et al., 2005).

The SCVs of *S. aureus* differ from normal phenotype in size as they are ten times smaller than the wild colonies and are deficient in electron transport by auxotrophism to hemin / menadione, thiamine or thymidine. Their colonies are non-pigmented on agar plates and reduced coagulase production increases resistance to aminoglycosides and cell-wall active antibiotics. The specific role of the SCVs of *S. aureus* resistance to antibiotics in biofilms is still unknown (Proctor & Peters, 1998) although its presence in mixed biofilms with *Pseudomonas* has proven to be a survival mechanism against the attack of the exotoxins of *Pseudomonas* for which its wild form is sensitive (Biswas et al., 2009).

In staphylococcal biofilm formation requires intercellular adhesin (PIA) is a polymer whose main component is N-acetylglucosamine and is synthesized by several enzymes encoded by

intercellular adhesion cluster (*ica*), the presence of these genes correlated with the morphology colonial and the ability to form biofilms, so the net growth in Congo red agar form black colonies when the adhesin is present and red in its absence (Ziebuhr et al., 1997). The *ica* operon is constituted by a group of four structural genes icaA, icaB, icaC and a regulatory component icaR (Diemond & Miranda, 2007). Adhesin negative mutants do not produce biofilms due to an IS256 transposon in the gene icaC (Cho et al., 2002).

It has recently been reported in strains of methicillin resistant *Staphylococcus aureus* (MRSA) that the presence of *ica* locus does not guarantee that its expression and does not directly reflect the ability of biofilm formation. Has been evaluated the participation of three regulatory genes *agr* and *sar*A and as well as the alternative transcription sigma factor *sig*B latter being responsible for the variation of biofilm (Jong-Hyun et al., 2008, Eftekhar & Dadaei T, 2011).

In *S. pneumoniae* have described two variants of colonial morphology between colonies spontaneously switched between transparent and opaque, the latter capable of forming two to six times the capsule, with limited bonding capacity and the possibility of evasion of host immune system, this variation observed both in planktonic growth conditions and in biofilm. Other variants described in aged biofilms are small and not mucoid without capsule (SCVs) with capacity to form hyperadherent biofilms, in contrast to the large and mucoid variants that appear late in the biofilm adhere poorly to surfaces forming flat structures unable to form biofilms. The SCVs of *S. pneumoniae* correlated with reduced capsule production and an increase in initial attachment instead to the opaque and transparent colonies, the SCV non capsule cells are not reversible due to a deletion in the capsule operon cps3DSU (Allegrucci & Sauer, 2007).

In *Candida parapsilosis* was previously thought that it was not able to form true filaments and biofilms, we now know they are not as large as those of *Candida albicans* and concentric phenotype forms quantitatively more biofilm in contrast to the smooth phenotype as it does in lesser extent and does not invade the agar (Laffey & Butler, 2005).

The coexistence of microorganisms in biofilms may lead to the emergence of phenotypic variants as in the case of *Pseudomonas putida* and *Acinetobacter* strain C6 where the excretion of benzoate by *Acinetobacter* as a result of the metabolism of benzyl alcohol, induces phase variation in *Pseudomonas* as rough colony (Kirkelund et al., 2007).

The importance of knowledge and isolation of these slow-growing variants (SCVs) are often misdiagnosed by routine microbiological analysis due to its unusual morphology and biochemical reactions which complicates eradication by failures in the antibiotic treatment

7. Biofilm control

Biofilms can be reformed if: a) there is growth of fragments, followed by debridement and cleaning, b) planktonic bacteria is spread, released from the biofilm residual, c) there is new growth of microorganisms in the biofilm (Cooper & Okhiria, 2010) .

Antibiofilms actions can be divided in two: 1) Prevention of formation. and 2) removal or destruction of biofilms. Among the prevention strategies for catheter-related infections that have developed protocols aqre aseptic filtered air in operating rooms which has reduced the incidence of these infections and are based on the correct implementation of the measures of asepsis during insertion and maintenance of vascular pathways. The formation and training

of staff on the recommendations of the indication, insertion and maintenance of intravascular devices are the backbone of the prevention of catheter-associated infections.

The methods for controlling biofilms are basically: prevent adhesion (material handling, use of antibiotics or anticoagulants) to prevent bacterial differentiation and congregation (quorum sensing antagonists or use of lactoferrin), matrix elimination (enzymes) and recently the administration of specific bacteriophages (Figure 1).

Many of catheter-related infections due to microorganisms present in the skin are acquired when the catheters are inserted so that alternative strategies anti-colonization are being explored. Other alternatives would be to coat catheters or medical devices with antimicrobial agents (antibiotics, antiseptics and silver) incorporated into the implant material, with limited success. This is due to several reasons among which are the fact that biofilm infections are chronic and the half-life of these substances is shorter on the other hand the incorporation of these drugs can damage the implanted material or incompatibility with the host.

The coating of catheters with antibiotics or biocides such as rifampin and minocycline or cefazolin, chlorex, silver sulfadiazine and silver impregnation decreases the possibility of colonization, has also proved successful when the catheter is used for short periods and as a prophylactic measure, but counterproductive in the long term the huge problem of resistance (Lewis, 2001, Raad & Hanna, 1999).

The coating material with enzymes may be another option to prevent infections resulting from medical devices, recently reported peroxidase titanium coating which can generate antimicrobial hypothiocyanite hypoiodite or to form hydrogen peroxide or thiocyanate. This coated material and a liquid environment with substrates of the enzyme has been shown to limit the formation of biofilms of *Candida albicans* (Ahariz & Courtois, 2010).

Recently have proposed new alternatives for delivery of antibiotics into the biofilm with the use of liposomes or biodegradable complexes that allow the drug concentration at the interfaces of the biofilm (Smith, 2005).

The discovery of bacterial communication systems (*quorum sensing*) as a temporary facility during the infectious process has given an opportunity to decrease the bacterial infection by means other than growth inhibition. Because many bacteria use this communication system and control of virulence, *quorum sensing* mediators are the new targets for drug design (Hentzer & Givskov, 2003). These substances are known as quorum sensing inhibitors (QSI), which have been identified in nature and analogs have been synthesized by modifying its structure and assessed its activity in experimental systems *in vivo* and *in vitro*. QSI resistance occurs only in bacterial mutations.

In the case of gram-negative bacteria depends on the communication mechanism of the synthesis of N-acyl homoserinlactones (AHL), so they have developed analogs of this substance that are aimed at inhibiting biofilm formation by several mechanisms: a) inhibition of AHL signal generation, b) inhibiting the spread of the intracellular signal and c) inhibiting the reception of AHL. In Gram-positive bacteria that use peptides as signaling molecules of *quorum sensing*. A synthetic peptide called RIP interferes with the reception of these signals in *Staphylococcus aureus*, is active in its ability to inhibit biofilm formation in animal models (Balaban et al., 2007).

Substances that interfere in the formation of exopolysaccharide as xylitol and gallium have been used in formulations of oral biofilms management and iron chelating agents such as

lactoferrin, deferoxamine and EDTA are candidates for use in controlling biofilms. Recently it was shown that lactoferrin, a ubiquitous and abundant substance in secretions, stimulates the disintegration of biofilms depends on its ability chelator of iron, essential for bacterial growth, and stability of the links necessary for the extracellular matrix biofilms. Their use encourages the release of planktonic cells rather than their aggregation and biofilm (Castrillón 2010, Rodríguez-Franco et al., 2005).

Endogenous production of enzymes allows degradation of exopolysaccharides of the biofilm to achieve dispersion of microorganisms for the generation of a new colony once the biofilm is mature and begin a new cycle of development, this allows us to propose the use of different enzymes for removal, however, due to the heterogeneity of extracellular polysaccharide, it is necessary to use a mixture enzymes for degradation. Among the most commonly used are dispersin D alginase, phage depolimerase, proteases, glycosidases: pectinase arabanase, cellulase, hemicellulase, beta-glucanase, xylanase, glucose oxidase and lactoperoxidase (Johansen et al., 1997, White, 2006, Kaplan et al., 2004).

A different approach for the treatment of biofilms is the use of bacteriophages, viruses that are specific for the bacteria to replicate inside and kill them. It has been demonstrating its effectiveness with the use of bacteriophage T4, which can infect and replicate in *Escherichia coli* breaking up the morphology of the biofilm and killing the bacteria, or in the case of phage 456 on *S. epidermidis*. (Curtin & Donlan, 2006). A bacteriophage expressing enzymes that degrade the biofilm matrix has been designed and simultaneously attack the bacterial cells of *Escherichia coli*. This design eliminates the need to express, purify and deliver large doses of enzymes to specific sites of infection that impede access by the presence of the extracellular matrix (Lu & Collins, 2007).

Pretreatment of catheters with hydrogel with a hydrolyzate of bacteriophage *P. aeruginosa* M4 reached lower cell density in biofilms after bacterial inoculation suggesting its potential use to prevent biofilm formation (Fu et al., 2010).

8. Conclusions and perspectives

The organization of the microorganisms to grow as a biofilm has been shown to have their own intrinsic mechanisms of resistance differ from those described stop the growth of microorganisms in free form. Therefore, these strategies should be considered resistance to explain therapeutic failure in the treatment of patients for whom laboratory results provide suitable sensitivity patterns.

Growth as a biofilm is a risk factor for the spread of resistance to antibiotics and biocides as a long-term treatment with a microorganism determines their survival by developing a biofilm phenotype.

Therapy in the future against biofilm-related infections should be considered as a priority to have standardized methods of diagnosis (still non-existent at the routine level) to determine differential management strategies of these infections.

As biofilms are heterogeneous in nature, antibiotics are useful to control those who are active in cells with low metabolic activity or non-actively growing cells so requires the search for new antibiotics that fit this profile.

The main strategy for controlling these infections is the use of agents that prevent biofilm formation as (*quorum sensing* inhibitors, inhibitors of synthesis of exopolysaccharides or

material handling to prevent sticking) and its growth has been kept as planktonic cells to be susceptible to the action of antibiotics and host immune system. Other possible control strategies for mature biofilm consisting of dispersal of the organism by specific enzymes responsible or bacteriophage that allow differential lysis.

In conclusion, biofilm growth as a major advantage for microorganisms because of the variety of strategies developed by them not only to ensure their survival in hostile environments but to evade the antibiotics, so knowledge of the process and the mediators involved will allow us to direct them to our benefit.

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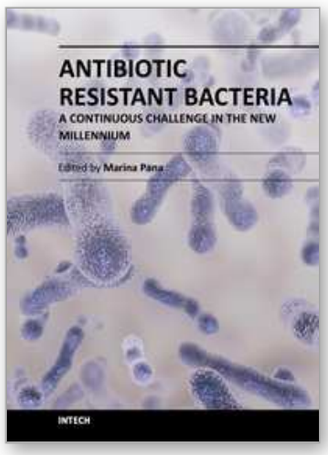

Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium

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Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

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