Review Article

iMedPub Journals
www.imedpub.com

Journal of Emergency and Internal Medicine
ISSN 2576-3938

2020
Vol.4 No.2:30

DOI: 10.36648/2576-3938.100030

Resistance Mechanisms in Bacterial Biofilm Formations: A Review

Nikiforos Rodis[1], Vasiliki Kalouda Tsapadikou[1], Charalampos Potsios[2] and Panagiota Xaplanteri[3]

1 Medical School, Democritus University of Thrace, Greece
2 Department of Internal Medicine, University General Hospital of Patras, Greece
3 Department of Microbiology, University General Hospital of Patras, Greece

*Corresponding author:
Panagiota Xaplanteri

panagiota.xaplanteri@gmail.com

Department of Microbiology, University General Hospital of Patras, Greece.

Tel: + 6932914660

Abstract

The purpose of this review is to present the mechanisms that cause the emergence of resistance to antimicrobial therapy in bacterial biofilms. Biofilm-producing bacteria cause chronic and persistent infections. They develop in joint prostheses, intravenous catheters and stents, endotracheal tubes and cardiovascular devices. The problem is enormous as it results in increased hospitalization costs, multiple surgeries and prolonged antibiotic intake. The mechanisms of resistance of bacterial biofilms differ from those of planktonic cells. They include as follows. Host defense bypass mechanisms, glycocalyx and extracellular polymeric substances (EPS), enzyme-mediated resistance to antibiotics, cell heterogeneity in metabolism and growth rate, Quorum Sensing (QS, Cell to cell signaling), persister cells, genetic adaptation and mutations, efflux pumps, adverse environmental conditions, outer membrane structure, bacteriophages, interactions between different types of bacteria in polymicrobial biofilms. Which mechanism or combinations of mechanisms are used, depends on the type of microorganism. Therefore, analysis and further elucidation of their function will assist in identifying ways in which to deal with the serious infections caused by biofilms.

Keywords: Bacterial biofilm; Resistance mechanisms; Biopolymer matrix; Quorum Sensing; Persister cells; Efflux pumps

Received: April 29, 2020; **Accepted:** June 09, 2020; **Published:** June 14, 2020

Citation: Rodis N, Tsapadikou VK, Potsios C, Xaplanteri P (2020) Resistance Mechanisms in Bacterial Biofilm Formations: A Review. J Emerg Intern Med Vol.4 No.2:30

Introduction

Bacteria survive, in the environment, in two forms as the free or planktonic cells (planktonic cells) and the sessile cells attached to living or lifeless surfaces [1]. Biofilms appear in fossils, from 3.25 billion years ago and are found on surfaces both in the environment and in living tissues and medical devices [2,3]. They have a complex architectural structure formed by micro- and macro-colonies with gaps in between that allow the passage of nutrients [3]. Externally they are surrounded by a protective matrix of biopolymers. These polymeric substances (extracellular polymeric substances, EPS) are mainly polysaccharides, proteins, nucleic acids (eDNA) and lipids. They mediate cell to cell adhesion and at the same time they form the surface on which biofilm's three-dimensional architecture develops [4]. Biofilms are formed by the following bacteria: *Staphylococcus epidermidis, Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumanii, Haemophilus influenzae, Burkholderia cepacia, Enterococcus* spp., *Escherichia coli, Klebsiella* spp., Proteus spp. και *Helicobacter pylori*, *Bacteroides, Clostridium, Finegoldia, Fusobacterium,* and fungi. They may include one or more pathogens (multimicrobial biofilms) [5-7]. Biofilm-producing bacteria cause chronic infections with persistent tissue damage [8]. It is estimated that 65% of microbial infections are associated with biofilm formation, such as periodontitis, endocarditis, chronic bronchopneumonia in patients with cystic fibrosis (*P. aeruginosa*), otitis media in children (*H. influenzae*), chronic sinusitis, implants, intravenous catheters and stents (*S. aureus* and other Gram-positive *cocci*), wound infections in burn victims, and urinary tract infections *(E. coli* and other pathogens) [1-8]. On natural surfaces biofilms are formed on the teeth, cardiac valves, lungs (cystic fibrosis) and the middle ear [8]. In infections associated with prosthetic materials, biofilm development occurs on both the outer and inner surface of the foreign body. Such surfaces are joint prostheses, intravenous catheters and stents, endotracheal tubes, and cardiovascular devices [6-8]. In orthopaedic surgery; biofilm formation is associated with osteomyelitis, septic arthritis, and prosthetic joint infections (PJI). The problem that arises is huge, resulting in increased

hospitalisation cost, multiple surgeries and prolonged antibiotic intake [6].

Nosocomial infections from *A. baumanii* are due to the ability of the microorganism to form biofilms in medical devices and biological surfaces [9]. Coordinated genetic expression and bacterial communication are important factors in the development of endurance mechanisms within a bacterial biofilm [3]. Bacteria within a biofilm multiply protected from environmental pressures, the host's immune system and antimicrobial agents [10]. The higher the density of a bacterial population, the more resistant they are and the higher the dose of antimicrobials required to kill them compared to lower density populations. This phenomenon is called the "inoculum effect" [7]. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of antibiotics for biofilm bacteria are 100–1000 times higher than that of planktonic bacteria [1].

Literature Review

Antibiotic resistance of bacteria in biofilms is not related to host factors and has significant economic consequences and environmental impacts [3-11]. These include not only the contamination of medical implants and the distribution of drinking water, but also the paper industry, metallurgy and food processing [3]. 60-70% of nosocomial infections are due to the formation of biofilm in implants [2]. The resistance mechanisms (**Figure 1**) developed in biofilms differ from conventional antimicrobial resistance [11].

Resistance Mechanisms

Mechanisms of escape from Host defence

Biofilm bacterial cells can bypass or escape the host's defences in a number of ways. White blood cells and the various enzymes they produce cannot enter the biofilms. At the same time reduced phagocytic capacity is observed. This phenomenon is called frustrated phagocytosis and macrophages and neutrophils cannot engulf the biofilm bacteria. In addition, reduction of respiratory burst is detected and thus, suppression of leukocyte functions. In the biofilm formed by *P. aeruginosa, neutrophils* produce less peroxide than planktonic cells. The same applies for lysozyme and lactoferrin as well [12].

Extracellular polymeric substances (EPS): Glycocalyx and matrix

Glycocalyx is present in all biofilms. It has a thickness of 0.2-1.0

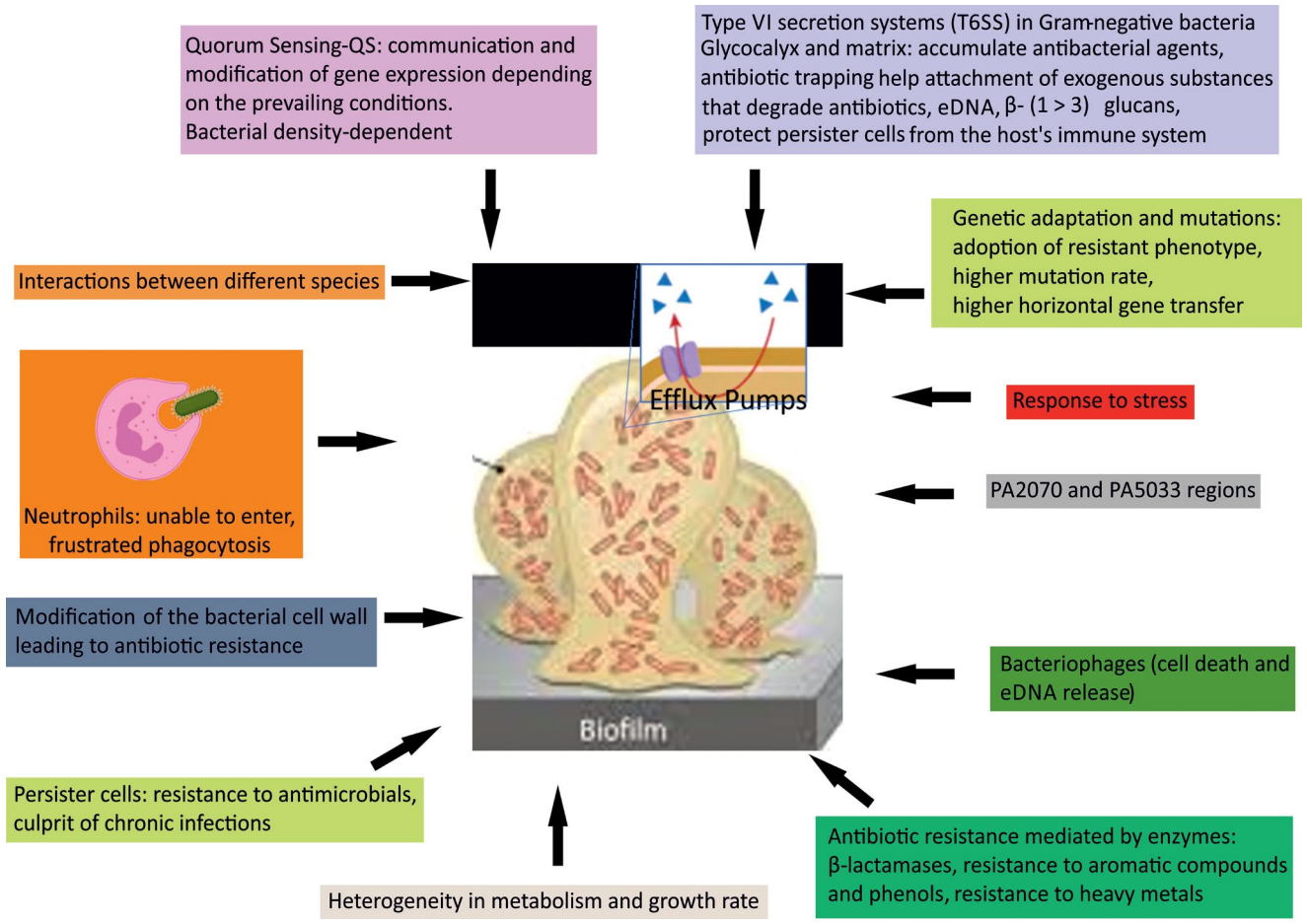

Figure 1 Schematic presentation of the main resistance mechanisms in biofilm formations.

μm, consists of glycoprotein's and polysaccharides and with the help of Van Der Waals forces and hydrogen bonds, it favours the attachment of the biofilm to solid surfaces. In addition, it helps in the maturation of the biofilm and the survival of the microorganism in the unfavourable environment of the host. Glycocalyx can also accumulate antibacterial agents up to 25% of its weight. Its absorbent surfaces limit the transport of microbicides and are also used for the attachment of exogenous substances that degrade antibiotics [3-13]. B-lactamases accumulate in the extracellular polymeric substance produced by *P. aeruginosa,* giving resistance to β-lactams. *P. aeruginosa* PAO1 strains produce Psl polysaccharide, which consists of repeating D-glucose, D-mannose and L-ramnose units and plays an important role in the formation of biofilm. Such strains are resistant to colistin, polymyxin B, tobramycin and ciprofloxacin. The structural ingredient Pel of *P. aeruginosa* biofilm encoded by pellocus provides protection against tobramycin and gentamicin. The Pel molecule is a linear cationic polysaccharide consisting of 1 → 4 glycosidic bonds of N-acetylgalactosamine and N-acetyl glucosamine. It is positively charged and reacts with eDNA in biofilm microcolonies. Pel's role in antibiotic resistance to biofilms, however, needs further investigation [14].

Matrix is the first component of the biofilm with which the antibiotic agent comes in contact. Matrix acts as a diffusion barrier. This means that the antibiotic must penetrate a thick layer of exopolysaccharide (EPS), DNA and proteins to penetrate the biofilm. As a result, the antibiotic reaches the bacteria in a reduced amount and therapeutic dose and favours the development of resistance [15]. In addition, the presence of the antibiotic itself induces an increase in the composition of the biofilm matrix. This phenomenon has been confirmed for *P. aeruginosa* and coagulase negative *Staphylococci* that produce slime biofilms. In these cases sub-therapeutic concentrations of β-lactam antibiotics induce increased matrix synthesis [8]. Another mechanism for the development of resistance through the biofilm matrix is the binding of the antibiotic to the matrix (antibiotic trapping). Thus, the matrix acts as a natural and chemical barrier for specific antibiotics [14-16]. The resistance of biofilms to positively charged amino glycosides cationic peptides is because they are bound to the negatively charged extracellular substance which leads to the saturation of the binding points [2-8]. In addition, exposure to extracellular DNA (eDNA), which is negatively charged, results in the accumulation of spermidine on the cell surface, which protects against polymyxins [2-15]. Spermidine on the cell surface binds lipopolysaccharide (LPS) and stabilizes the outer membrane against antibiotic damage and oxidative stress [17,18]. The above mechanisms and properties of the extracellular polymer are attributed to the fact that *P. aeruginosa* mucosal strains that live in biofilms are 1000 times more resistant to tobramycin than biofilms formed by non-mucosal strains. The biofilm of the wild-type strain of Klebsiella pneumoniae may be penetrated by ciprofloxacin, but not ampicillin, which is inactivated by the extracellular substance [16]. Oxacillin, cefotaxime, and vancomycin cross the biofilm of *S. aureus* and *S. epidermidis* to a limited extent [14].

eDNA in the extracellular polymer is derived endogenously not only as a result of cell lysis, but also active secretion by neutrophilic polymorph nuclear cells in sites of inflammation. Its presence increases the resistance of the biofilm to specific antibiotics with the following mechanism: eDNA is negatively charged and forms chemical bonds with cautions such as magnesium ions. As a result, the concentration of magnesium ions in the environment is reduced (*P. aeruginosa* and *Salmonella enterica* serovar Typhimurium biofilms). It triggers the activation of the systems PhoPQ and PmrAB and induces resistance via up regulation of the operon PA3552-3559 (arnBCADTEFugd or pmrHFIJKLM-ugd). The enzymatic action of encoded proteins induces resistance to amino glycosides and cationic antimicrobial peptides by adding aminoarabinose to LPS lipid A. The addition of eDNA to wild-type strains of *P. aeruginosa* has been shown to increase resistance to cationic peptides such as polymyxin B and colistin by 4 and 8 times, respectively [14]. In addition, the eDNA in the *P. aeruginosa* biofilm is involved in the synthesis of spermidine. Spermidine is a polyamine found in the outer membrane of bacterial cells and protects against aminoglycosides and cationic antimicrobial peptides, reducing its permeability to positively charged molecules [14-18]. In addition, eDNA is involved in the horizontal transfer of antibiotic-resistant genes between the biofilm bacterial cells. This has been proven for *Campylobacter jejuni* and its resistance to kanamycin (*aphA3* gene) and chloramphenicol (*cat gene*) [14].

Another resistance mechanism is related to the binding of the cell membrane-bound to ndvB glucosyltransferase, which is responsible for the production of cyclic β- (1 → 3) glucans. The β- (1 → 3) glucans are located in the periplasmic space and matrix of the biofilm and present anionic subunits that react with the cationically charged antibiotics kanamycin and tobramycin and prevent them from reaching biofilm cells [14]. The ndv B gene of *P. aeruginosa* encodes the synthesis of tobramycin-binding glucans and is associated with resistance to tobramycin [15]. In Streptococcus mutans biofilm, the dltABCD operon offers resistance to gentamicin. This is important because the microorganism causes infectious endocarditis. dltABCD adds D-alanine to the wall acids of many Gram-positive bacteria and gives a more negative charge than to platonic bacteria in *S. mutans*. As a result, the positively charged gentamicin is bound and resistance is induced [14].

Antibiotic resistance mediated by enzymes

Enzymes present in the biofilms via ion reduction convert bactericidal agents into a non-toxic form, thus inducing resistance [3]. The enzymatic inactivation of antibiotics in the outer areas of the biofilm prevents the antibiotic from reaching the deeper layers, allowing the susceptible bacteria that reside there to survive. In contrast, the resistant bacteria cells that express these enzymes occupy external positions, where the concentration of the antibiotic is higher [7].

The diffusion barrier created by the biofilm matrix, due to the presence of β-lactamase, plays a key role in the resistance of *P. aeruginosa* and hydrolyzes β-lactam antibiotics before they

reach the bacterial cells [8]. In addition, the use of imipenem and piperacillin induces β-lactamase production in *P. aeruginosa* biofilms. The source of β-lactamase is thought to be derived from a layer of bacteria that have been dissolved and to secrete defensive enzymes in the extracellular space or membrane vesicles that contain β-lactamase and are secreted by resistant strains of *P. aeruginosa* [8]. With this mechanism the mature biofilm of *P. aeruginosa* shows greater resistance to ceftazidime and meropenem compared to the newly formed biofilm which does not have an increased amount of β-lactamases in the matrix [14].

Further, the resistance to aromatic compounds and phenols used as bactericides are decomposed by the biofilm of specific bacteria. Resistance to heavy metals such as mercury, antimony, nickel, cadmium, arsenic, cobalt, zinc, lead, copper, chromium and silver is due to their enzymatic reduction encoded by heavy metal resistance genes, which are of plasmid or chromosomal origin. *P. aeruginosa* and *Pseudomonas putida* reduce formaldehyde through a NAD + -glutathione-dependent dehydrogenase, to non-toxic formaldehyde NAD + oxidoreductase. This enzyme activity is encoded by a gene that is based on a plasmid and is constantly expressed [19].

Heterogeneity in metabolism and growth rate

Heterogeneity in a population increases the chances of survival. The rate of growth and metabolic activity in a biofilm is affected by the different availability of nutrients and oxygen. As a result, in the peripheral region of the biofilm the metabolic activities of the cells are enhanced, while in the interior - due to the poor diffusion of nutrients and oxygen - the metabolic activity and the development of the cells are reduced [3].

Biofilm bacteria in anaerobic conditions show resistance to a variety of antibiotics, with the exception of *P. aeruginosa,* where in anaerobic conditions there is an increased sensitivity to colistin. Hypoxia reduces the potency of *P. aeruginosa's* outer membrane, contributing to antibiotic resistance to amino glycosides, which cannot be transported within the bacterial cell. The responsible mechanism is the activation under conditions of hypoxia of the *mexEF-oprN* and *mexCDoprJ* genes encoding efflux pumps [14].

Another mechanism by which the hypoxia environment contributes to the resistance and survival of microorganisms in the biofilms is related to the production of reactive oxygen species (ROS). ROS, which play an important role in killing microorganisms, require the presence of molecular oxygen. What exactly is happening with the production of ROS in the deeper layers where there is hypoxia, is a point that still remains to be clarified. In addition, *P. aeruginosa* biofilms have been shown to neutralize ROS by KatA catalase [14].

It is known that microbial agents affect the metabolically active bacteria, while metabolically inactive ones are protected [3-8]. This applies to antibiotics such as penicillin, ampicillin, cephalosporins, amino glycosides, and fluoroquinolones, which are more active against dividing bacteria [1-11] Colistin, which disrupts the cell membrane, may be more effective in slow-growing biofilm subpopulations, as it is thought that the integrity of the membrane has been affected [14].

Differences in biofilm morphology also affect resistance. After exposure to biofilm at inhibitory bactericidal concentrations or in difficult environmental conditions such as increased temperature or nutrient poverty, the resistant population leads to phenotypic adaptation. This phenomenon is reversed after the removals of adverse conditions [3]. *P. aeruginosa* mutated strains with reduced antibiotic susceptibility have been described. These strains synthesize biofilm thicker than the corresponding wild-type strains, either due to overproduction of extracellular alginic polymer or due to changes in stationary phase. As a result resistance to tobramycin occurs. A mutation in the gacA gene gives resistance to various antibiotics [11].

Quorum Sensing (QS)

The formation of the biofilm is a result of the inextricable cooperation of the microorganisms involved in it10. In recent years, it has been proved that bacteria communicate with each other through various communication systems (Quorum Sensing -QS) and modify gene expression depending on the prevailing conditions. The function of these systems depends solely on the bacterial density (density-dependent) and through them the bacteria regulate the expression of various genes and the production of infectious agents such as extracellular enzymes and lysines. These are necessary for the pathogenicity of infections, but also affect antibiotic resistance, inflammatory response and biofilm development [1-10].

Activation of these intercellular signaling systems appears to contribute to the resistance of *P. aeruginosa* by the following mechanism: increased ROS production and aggregation induced by simultaneous reduction of membrane potential leading to cell proliferation and eDNA release to extracellular matrix [20]. Biofilms formed by DlasR DrhlR strains of *P. aeruginosa,* which do not activate communication systems, were much more susceptible to tobramycin than wild-type strains [14]. In *S. aureus*, when the agr system is not activated, a decrease in rifampicin resistance was observed in relation to the wild-type strains [14-21]. Also in *S. aureus* the activation of the agr system has been associated with resistance to cephalosporins, vancomycin, daptomycin, linezolid, rifampicin and fusidic acid. In Enterococcus faecalis it has been shown that resistance to gentamicin, daptomycin and linezolid requires the activation of the fsr system and the production of gelE protease, the production of which is controlled by this gene [14].

Persister cells

Persister cells are the population of bacterial cells within a biofilm that are resistant to antimicrobials and cause chronic infections. Those cells are multidrug-resistant can survive in the presence of microbicides in lethal concentrations at a higher rate than planktonic cells [3-11]. The resulting resistance is not related to genetic or inherited changes, but to a phenotypic switch from the susceptible wild type strain to a resistant one [22]. After end of antibiotic treatment these cells begin to multiply and re-form the biofilm. They are dormant variants of the wild type and not mutated. In addition, the presence of extracellular polysaccharide protects persister cells from the host's immune

system [3]. Pyocyanin molecules, acyl-homo serine lactone 3-OC12-HSL and 2-amino acetophenone (whose production is controlled by quorum-sensing systems) have been shown to increase the number of persisters in *P. aeruginosa* [22]. In the *S. epidermidis* biofilm, the large population of persister cells is the most important resistance mechanism [2]. The presence of persisters explains the resistance to low-thickness biofilms better than other theories [11]. Although persister cells are a variety of phenotype, studies have shown that modified genetic activation of glpD, glpABC, and plsB regulators of glycerol-3-phosphate is a mechanism through which persisters show resistance to ampicillin [15]. Chromosomal toxin/antitoxin (TA) systems, which are associated with programmed bacterial death, are a major factor in the formation of persistent cells in Gram-negative bacteria. TA systems appear to inhibit the activity of specific cellular mechanisms such as ribosomes (translation), resulting in stasis of the bacterial cell. The TA molecules dinJ/yafQ, relBE, and mazEF are activated in *E. coli.* The over expression of the relE toxin gene leads to resistance to ofloxacin, cefotaxime and tobramycin. Also, the hipBA TA locus is associated with the formation and maintenance of persister cells, while mutation of the hipA toxin gene leads to an increase in persisters in *E. coli.* [14-15]. In Burkholderia cenocepacia, over expression of specific toxins in biofilm cells resulted in the formation of persister cells after treatment with tobramycin or ciprofloxacin. *S. aureus* biofilms do not use TA systems, but the formation of persister cells is due to a decrease in adenosine triphosphate (ATP) [14]. *S. aureus* cells, which cause a drastic reduction in ATP levels, are 325 times more likely to produce persister cells [23].

Genetic adaptation and mutations

Genetic adaptation within the biofilm is necessary to reduce susceptibility and to adopt a resistant phenotype [8]. The rate at which biofilm cells mutate is significantly higher than that of planktonic cells, and a higher horizontal gene transfer is also observed. In addition, due to the proximity of the cells, the transport of plasmids is accelerated [11]. Another factor that appears to enhance mutations is oxidative stress. This is due to the increased production of endogenous ROS by activated neutrophil polymorphonuclear cells plus a defective antioxidant system. As a result, genetic adaptation and evolutionary changes increase. For fermenters, the cells adapt to stress conditions within a few hours of exposure to the harmful agent [8]. Oxidative stress is thought to enhance mutations in biofilm microenvironment and to express defenses against oxidative factors [1]. Such genes encode various catalysts, such as superoxide dismutases, hydroperoxide reductases, alkyl glutathione reductases, the production of DNA repair enzymes and regulatory genes that determine intracellular oxidative-reductive potential such as *asoxyR* and *soxR3*. In *P. aeruginosa,* overproduction of a chromosome-encoding AmpC is considered the major mechanism of resistance to β-lactams and is further induced by the presence of β-lactam antibiotics. In the biofilm of the same microorganism, colistin does not act on metabolically active surface cells due to activation of the PmrA-PmrB regulatory system involved in acquired resistance to cationic peptides by adding aminoarabinose to LPS lipid A. In addition, resistance to tobramycin is due to the low metabolic activity of cells [8]. Horizontal transfer of genetic material is favored in the biofilm environment. This is achieved through:

a) eDNA of the extracellular polymer [14]

b) **Plasmids:** In *E. coli* biofilm transfer of multiresistance genes take place in areas with hypoxia, which means that heterogeneity of biofilms also contributes to this process. In *E. faecalis*, biofilm cells had an average of 1.6-2 times the number of plasmid copies compared to the planktonic.

c) With the increased expression of the integrase in the biofilm cells compared to the planktonic cells and the mobilization of antimicrobial resistance genes that is, genetic elements that through recombination incorporate gene cassettes of broader regions that encode antibiotic resistance.

In patients with cystic fibrosis, *P. aeruginosa* strains with defective methyl mismatch repair or DNA oxidative repair systems have been isolated. These strains are more resistant to antibiotics than cells that have no damage to DNA repair systems. This mechanism has been found to be associated with resistance to ciprofloxacin in strains of *P. aeruginosa* and *Campylobacter jejuni*, resistance to mupirocin and rifampicin in strains of *S. aureus,* and resistance to clarithromycin in strains of *H. Pylori*. In addition, the cells in the biofilm are likely to undergo automatic mutations due to the increased endogenous oxidative stress that causes DNA damage. This hypothesis has been proved for of *S. aureus, Streptococcus pneumoniae* and *P. aeruginosa* biofilms. The cells that form small colony variants (SCVs) have been associated with persistent and recurrent osteomyelitis and infections associated with implants due to *S. aureus* and also associated with *S. pneumoniae* and *P. aeruginosa* infections. These cells have a variety of phenotypes and differences compared to the wild-type such as smaller colony size, low growth rate, and increased antibiotic resistance [14]. Resistance to β-lactams results from mutations in the regulatory genes of β-lactamase production that lead to the formation of strains with stable or partially stable AmpC [8]. Resistance to ciprofloxacin in cystic fibrosis *P. aeruginosa* strains is due to mutations in the gyrA gene and to two efflux systems, MexCD-OprJ and MexEF-OprN. Accordingly, biofilm cells of *P. aeruginosa* exposed to azithromycin induce the expression of the MexCD-OprJ efflux pump, which is not observed in planktonic cells [2]. Colistin resistance results from mutations in the pmr system associated with the structure of LPS8. The succession of the IS1669 sequence that inactivates the ampD gene is blamed for resistance to clinically isolated *P. aeruginosa* strains that produce β-lactamase [8]. Biofilm strains of *P. aeruginosa* isolated from cystic fibrosis patients have alterations in genes related to DNA repair (the mismatch repair system, MMR and DNA oxidative lesions repair system GO). Mutations in both systems express efflux pumps and determine the appearance of multi-resistance8. In addition, resistance of *P. aeruginosa* strains to aminoglycosides and fluoroquinolones in cystic fibrosis biofilms has been shown to be due to mutations that lead to increased pumping activity [8]. A further resistance mechanism involves

transcription regulators, such as the PA0756-0757 system in the *P. aeruginosa* biofilm and the rapA protein in the urethral biofilms of *E. coli* (UPEC). More specifically, the expression of the PA0756-0757 system in the biofilm of *P. aeruginosa* is increased compared to planktonic cultures and is associated with 4-8 times increase in resistance to tobramycin and 2-4 times increase in resistance to gentamicin. It is possible that the resistance is due to the suppression of the expression of the *opdH* gene encoding the OpdH porin, which allows the passive diffusion of specific antibiotics into the bacterial cell. *PA0756-0757* locus is analogous to the TctDE system (tricarboxylate transport) in *S. enterica serovar typhimurium* [14]. rapA protein also contributes to the resistance of biofilm bacteria to planktonic uropathogenic *E. coli* (UPEC) cells, in which it is not detected at all. It gives antibiotic resistance to biofilm with two mechanisms. According to the first mechanism, in the absence of rapA protein, the transcription of 22 genes is reduced, including the *yhcQ* gene, which encodes a multidrug efflux pump. The second mechanism is related to the synthesis of less extra polysaccharide compared to wild-type UPEC biofilms, which allows faster penetration of the antibiotic into the inner layers [14].

Efflux pumps

Bacteria use specialized proteins to excrete various substances from the cytoplasm called efflux pumps [24]. They are associated with both endogenous and induced antibiotic resistance through the movement of antimicrobial agents away from their intracellular targets [14]. Their overproduction leads to multi-resistance. There are several studies showing that genes controlling their production are located on plasmids [3]. Five classes of active efflux pumps are described:

1) The major family facilitator superfamily (MF)
2) The resistance-nodulation-division family (RND)
3) The small multidrug resistance family (SMR)
4) The ATP binding cassette family (ABC)
5) The multidrug and toxic compound extrusion family (MATE) [3].

The *P. aeruginosa* genome contains at least 12 RND operons encoding active efflux pumps [24]. MaxAB-OprM pump overexpression simultaneously with the presence of β-lactamases plays an important role in resistance to piperacillin in *P. Aeruginosa* [8]. Biofilm exposure to antibiotics and disinfectants induces expression of efflux pumps [3]. For *P. aeruginosa* it has been shown that under the pressure of tobramycin, transcription of efflux pumps genes can be induced [11]. In addition, the *P. aeruginosa* MexCD-OprJ pump is a special defense mechanism against azithromycin [14-24]. The MexAB-OprM pump provides defense against colistin in *P. aeruginosa* [8-24]. An important efflux pump in *P. aeruginosa* biofilms is PA1874-1877, which is expressed 10 times more in biofilm than in planktonic cells. The genes *PA1874-1877* encode the proteins PA1874 or opmL (outer membrane), PA1876 (transporter protein) and PA1877 (membrane fusion protein), the deletion of which leads to an increase of up to 4 times the susceptibility to tobramycin, gentamicin and ciprofloxacin in relation to planktonic cells [14]. *P. aeruginosa* planktonic cells in conditions of hypoxia have an increased expression of the MexEF-OprN pump. This could mean that in the lungs of patients with cystic fibrosis where hypoxia is observed, the bacteria of *P. aeruginosa* with this mechanism develop resistance [24]. In Burkholderia cepacia biofilm, also pathogenic in patients with cystic fibrosis, RND, BCAM0925-0927 (RND-8) and BCAM1945-1947 (RND-9) pumps give resistance to tobramycin, while BCAL1672-1DNN R pump gives resistance to ciprofloxacin. The expression of four RND pumps in the *H. pylori* biofilm explains their increased resistance to clarithromycin. Burkholderia pseudomallei biofilms via efflux pumps show resistance to ceftazidime and doxycycline [14-25].

Response to stress

Stress caused by lack of nutrients, exposure to extreme temperatures, hyperosmolarity and acidic pH leads to changes in the morphology and physiology of biofilm cells with the ultimate goal of increasing resistance and preventing cell damage. In *E. coli* the sigma subunit of RNA polymerase RpoS is induced. The same sigma factor controls 50 stress resistance genes, while other factors regulate cell metabolism to respond to stress, including resistance to bactericidal agents [3]. In *P. aeruginosa* and *E. coli* the lack of nutrients leads to resistance to fluoroquinolones. Also in *P. aeruginosa* the response to heat shock leads to resistance to aminoglycosides through the AsrA intracellular protein [2-25].

Structure of the outer membrane

To act, antibacterial agents must penetrate the cell wall to reach their target. As a result, modification of the bacterial cell wall like degeneration or over expression of external membrane proteins is associated with resistance to antibiotics [3].

Bacteriophages

P. aeruginosa biofilms show an increased concentration of chromosomal Pf phage genes. Bacteriophages contribute to cell death and eDNA release, and thus contribute to the development of resistance. In addition, their presence forces *P. aeruginosa* to organize itself into a biofilm of a specific architecture (liquid crystalline biofilm) that gives resistance to tobramycin [14].

Interactions between different species

Given that many infections are multimicrobial, research into antibiotic resistance in biofilms consisting of more than one microbial species demonstrates a greater range of resistance compared to biofilms consisting of only one. Such models have shown greater resistance to gentamicin in *P. aeruginosa* involved in polymicrobial biofilms with *S. aureus, E. faecalis* and *Finegoldia magna*, compared to the biofilm consisting only of *P. aeruginosa*. The mechanism is unknown. Similarly, in a mixed Moraxella catarrhalis and *S. pneumoniae* biofilm in otitis media, *Moraxella catarrhalis* secretes β-lactamase and gives *S. pneumoniae* resistance to ampicillin. *S. pneumoniae* also gives *M. catarrhalis* resistance to azithromycin through an unknown mechanism. In a mixed *P. aeruginosa* and *Stenotrophomonas maltophilia* biofilm, an intracellular signaling molecule produced by *S. maltophilia* leads to activation of the genes *PA3552-3559* and *PA4773-4775* in *P. aeruginosa*, which leads to resistance to polymyxins [14].

Biofilm reactions consisting of bacteria and fungi have also been studied. In *S. epidermidis* biofilm with *Candida albicans*, an increase in *S. epidermidis* resistance to vancomycin was observed [14]. Mixed biofilm by *C. albicans* and *S. aureus* increased resistance to vancomycin due to the β-1, 3-glucan of the fungus, which acts as a barrier to the diffusion of vancomycin into the biofilm. In *E. coli* and *C. albicans* polymicrobial biofilm, β-1, 3-glucan gives resistance to ofloxacin. In multimicrobial biofilms, horizontal gene transfer is favoured. The transfer of the *vanA* (vancomycin resistance gene) gene to *S. aureus* by *E. faecium* strain involved in the same multimicrobial biofilm has been proved [14].

Various other mechanisms

tssC1: Type VI secretion systems (T6SS) in Gram-negative bacteria, which serve as secretory pathways that facilitate protein transport are involved in the antibiotic resistance in biofilms. The *P. aeruginosa* genome contains three different T6SS, the so-called Hcp secretion islands (HSI-I, -II and -III) and the *tssC1* (*hsiC1*) gene in the *HSI-I* locus that give resistance to antibiotics. The mechanism needs further elucidation [14].

PA2070 and PA5033 regions: PA2070 and PA5033 regions are associated with resistance to tobramycin and gentamicin in the biofilms of *P. aeruginosa* by unknown mechanism [14-25].

Conclusion

During their evolutionary course, bacteria develop strategies that ensure their survival. One such strategy is the creation of a biofilm. The increased resistance to antimicrobial agents observed in biofilms is due to a combination of mechanisms. These are their reduced diffusion, the binding of the antibiotic to the extracellular polymer, resistance mediated by enzymes, the level of metabolic activity, and the presence of efflux pumps, persister cells and extra polysaccharide structure. Changes in the level of expression of various genes in response to stress protect superficially bound bacteria. Which mechanism will be used depends on the type of microorganism. In addition, the genetic and biochemical properties of biofilms should be further investigated *in vivo* in order to create antimicrobial agents capable of providing effective protection. The is fundamentally important in infections of prosthetic materials and catheters, as biofilm bacteria express multidrug resistance and this affects the treatment of the patient, the destruction of persisters and the reduction of recurrences.

Acknowledgments:

Final English edit: Charmaine McAneny

Image preparation: penofcreation by Xristos Mosxos

References

1 Paraje MG (2011) Antimicrobial resistance in biofilms, Science against Microbial Pathogens. Communicating Current Research and Technological Advances 9: 736–744.

2 César de la FN, Reffuveille F, Fernández L, Hancock REW (2013) Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Current Opinion in Microbiology 16(5): 580-589.

3 Singh S, Singh SK, Chowdhury I, Singh R (2017) Understanding the Mechanism of Bacterial Biofilms Resistance to Antimicrobial Agents. The Open Microbiology Journal 11: 53-62.

4 Abee T, Kovacs AT, Kuipers OP, van der VS (2011) Biofilm formation and dispersal in Gram-positive bacteria. Current Opinion in Biotechnology 22: 172–179.

5 Rafii F, Hart ME (2015) Antimicrobial resistance in clinically important biofilms. World J Pharmacol 4(1): 31-46.

6 Dibartola AC, Swearingen MC, Granger JF, Stoodley P, Dusane DH (2017) Biofilms in orthopedic infections: a review of laboratory methods. APMIS 125: 418–428.

7 Vega NM, Gore J (2014) Collective Antibiotic Resistance: Mechanisms and Implications. Current Opinion in Microbiology 21: 28-34.

8 Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents 35: 322–332.

9 Emami S, Eftekhar F (2015) The Correlation between Biofilm Formation and Drug Resistance in Nosocomial Isolates of *Acinetobacter baumanii*. Avicenna J Clin Microb Infec 2(2): e23954.

10 Solano C, Echeverz M, Lasa I (2014) Biofilm dispersion and quorum sensing. Current Opinion in Microbiology 18: 96–104.

11 Stewart P (2002) Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol 292: 107-113.

12 Leid JG (2009) Bacterial biofilms resist key host defences. Microbe 4(2): 66-70.

13 Myszka K, Czaczyk K (2012) Mechanisms Determining Bacterial Biofilm Resistance to Antimicrobial Factors. Antimicrobial Agents 26: 215-216.

14 Hall CW, Mah TF (2017) Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS Microbiol Rev 41(3): 276-301.

15 Fletcher M (1992) Bacterial Metabolism in Biofilms. Biofilms Science and Technology 1: 113-124.

16 Taraszkiewicz A, Fila G, Grinholc M, Nakonieczna J (2013) Innovative strategies to overcome biofilm resistance. Bio Med Research International 150653: 13.

17 Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S (2012) Surface-localized spermidine protects the *Pseudomonas aeruginosa* outer membrane from antibiotic treatment and oxidative stress. J Bacteriol 194(4): 813-826.

18 Petropoulos AD, Xaplanteri MA, Dinos GP, Wilson DN, Kalpaxis DL (2004) Polyamines affect diversely the antibiotic potency: insight gained from kinetic studies of the blasticidin S AND spiramycin interactions with functional ribosomes. J Biol Chem 279(25): 26518-26525.

19 Cloete TE (2003) Resistance mechanisms of bacteria to antimicrobial compounds, International Biodeterioration & Biodegradation 51(4): 277-282.

20 Hazan R, Que YA, Maura D, Benjamin S, Paul AM, et al. (2016) Auto

poisoning of the respiratory chain by a quorum-sensing-regulated molecule favours biofilm formation and antibiotic tolerance. Curr Biol 26: 195–206.

21 Liang TJ (2017) Drug resistance mechanism of *Staphylococcus aureus* bacterial biofilm. Journal of Clinical and Nursing Research 1(1): 62-65.

22 Admassie M (2018) Current Review on Molecular and Phenotypic Mechanism of Bacterial Resistance to Antibiotic. Science Journal of Clinical Medicine 7(2): 13-19.

23 Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, et al. (2016) Persister formation in *Staphylococcus aureus* is associated with ATP depletion. Nat Microbiol 1: 51.

24 Van Acker H, Coenye T (2016) The Role of Efflux and Physiological Adaptation in Biofilm Tolerance and Resistance. The Journal of Biological Chemistry 291(24): 12565–12572.

25 Balcázar JL, Subirats J, Borrego CM (2015) The role of biofilms as environmental reservoirs of antibiotic resistance. Front Microbiol 6: 1216.