

Mechanisms Determining Bacterial Biofilm Resistance to Antimicrobial Factors

Kamila Myszka and Katarzyna Czaczyk
Poznań University of Life Sciences
Poland

1. Introduction

In most natural environments, the process of bacterial surface association is prevailing cells lifestyle. The tendency of bacteria to colonize solid materials is advantageous from an ecological standpoint. This mechanism allows bacteria the colonization of a nutritionally favorable new niche and encouraging symbiotic relationships between the cells. Sessile mode of growth provides also some level of protection from external stresses (Costerton et al., 1995; Dunne, 2002; Russell, 2002). Anchored bacteria are being linked to common human diseases ranging from tooth decay and paradontose to nosocomial infections and both biliary tract and kidney infections (Costerton et al., 1999; Potera, 1999). According to Russell (1999) and Wood et al. (2011) 80% of bacterial chronic inflammatory and infectious human diseases involve biofilm. In industrial environments surface-bound bacteria are the potential source of contamination of processed material that in consequence may lead to spoilage or transmission of pathogens (Bower et al., 1996; Gunduz & Tuncel, 2006; Myszka & Czaczyk, 2011).

Attached bacteria to organic or inorganic surfaces form thin layer called biofilm or biological layer. Biofilms consist of a single microbial species or multiple microbial species (O'Toole et al., 2000). However mixed-species biological layers predominate in most environments, single-species biofilms occur in a variety of infections and on the abiotic surface exploited in medicine and industry practice (Adal & Farr, 1996; Donlan, 2002). Despite of difference of ecosystems in which biofilms can develop, in each case the component microbial cells reach homeostasis and are optimally organized to convert all available nutrients to usefulness products for cells (O'Toole et al., 2000; Sutherland, 2001; Myszka & Czaczyk, 2009).

Biofilm-associated bacteria perform chemically diverse biocide-resistance phenotype (Mah & O'Toole, 2001; White & McDermott, 2001). It has been estimated that biofilms can tolerate antimicrobial agents (disinfectants, antibiotics, surfactants) at concentrations of 10-1000-times that needed to inactivate genetically equivalent planktonic bacteria (Jefferson, 2004). It is worth point out that almost all clinically and industrially approved antimicrobial agents are being less active against sessile bacteria. So far selection of antimicrobial agents for industry and medical properties based on their activity against planktonic bacteria (estimation the indexes of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) for different antimicrobial agents).

The problem of high resistance of biofilm to antimicrobials has not been dissolved yet. In the United States annual cost of eradication of biofilms in hospital conditions exceeded \$ 1 billion per year (Costerton et al., 1995; Archibald & Gaynes, 1997; Potera, 1999). Recent study demonstrated that biofilm resistance has a multifactorial character (Izano et al., 2009; Simões et al., 2009). Analysis of all described data can enable control of detrimental biofilms.

2. Structure of biofilm

Tolker-Nielsen & Molin (2000) stated that biofilms communities in natural environments have unique architecture although some structural features can be considered universal. Application of scanning confocal laser microscopy performed that biofilms formed on solid surfaces and exposed to a continuous flow of nutrients, are highly hydrated layers composed of microcolonies embedded in an organic polymer matrix of microbial origins (Lawrence et al., 1991; Gilbert et al. 2002a; Czaczyk & Myszka, 2007). Microcolonies are separated by water channels that allow the fluids to flow throughout the biofilm, making the distribution of nutrients and oxygen easier (Lindsay & von Holy, 2006; Shafahi & Vafai, 2009). Moreover, the water channels between the microcolonies provide a means of removing metabolic end products (Davey et al., 2003; Lindsay & von Holy, 2006). This system of nutrients and metabolic end products distribution functions only in periphery regions of biofilms. The cells within biofilms are more tightly packed and have worse access to nutrients and oxygens. Differences in nutrients and oxygen availability within the biofilm structure affect in differences in metabolic activity among the cells. In addition, the cells within biofilms secrete signal molecules that control formation of microcolonies of complicated architecture and diverse function (Parsek & Greenberg, 2005). Structural heterogeneity of biofilm provides an effective barrier that limit penetration of antimicrobial agents throughout the biological layer (Nobile & Mitchell, 2007; Roeder et al., 2010). Kinetic diffusion of antimicrobial compound of relative molecular weight of 100kDa through mature biofilm might be reduced to 60-80% as compared with its action against planktonic cells (DeBeer et al., 1994; Stewart, 1996). Moreover, suspended cells are directly exposure to toxic compounds. Biofilm-associated bacteria are much less permeable to the biocides. DeBeer et al. (1994) observed this phenomenon investigating the rate of penetration of chlorine into *Pseudomonas aeruginosa*/*Klebsiella pneumoniae* biofilm matrix. Also Suci et al. (1994) noticed transport limitation of ciprofloxacin through *Pseudomonas aeruginosa* biofilm. In this study, during the 21-min exposure, the presence of the antibiotic in periphery region of tested biofilm reached only 20% of ciprofloxacin concentration in the bulk medium (Suci et al., 1994). Gilbert et al. (1989) used perfused biofilm fermentors, in combine with continuous culture and observed that much of resistance of Gram-positive and Gram-negative biofilms was associated with the presence of nutrient-starved microcolonies.

Darouiche et al. (1994) noticed that although the presence of vancomycin in a *Staphylococcus epidermidis* biofilm exceeded bactericidal concentration, it was not sufficient to kill surface-bound bacteria. These authors support the notion that vancomycin resistance of *Staphylococcus epidermidis* biofilm, was not due to limited diffusion of the compound through biological layer, but rather to a reduction in the antimicrobial effect of the drug (Darouiche et al., 1994). Anderl et al. (2000) observed similar effect during investigation of rate of penetration of ampicillin and ciprofloxacin through *Klebsiella pneumoniae* biofilm. In this work, the inability of transport of ampicillin through biofilm was affected by the production

of the drug degrading enzyme β -lactamase. Ampicillin was able to penetrate biological layer formed by a β -lactamase-deficient mutant without difficulty. In contrast, ciprofloxacin diffused through *Klebsiella pneumoniae* biofilm without delay. Differences in the effect of penetration of both ciprofloxacin and ampicillin through *Klebsiella pneumoniae* wild-type and β -lactamase-deficient mutant biofilms, suggesting that biofilm resistance is multifactorial (Anderl et al., 2000).

3. Glycocalyx

Costerton et al. (1978) termed glycocalyx as the integral part of the biofilms of Gram-positive and Gram-negative bacteria. Glycocalyx known as either as slime or capsule may provide the forces responsible for cohesion and adhesion to the solid surfaces (Flemming, 1995; Mayer et al., 1999). This is performed by the weak interaction such as electrostatic interactions, hydrogen bonds and van der Waals forces (Flemming, 1995; Dunne, 2002). During biofilm maturation process, slime cementing and immobilizing the cells (Sutherland, 2001). Glycocalyx in biofilm structure varies in its thickness from 0.2 to 1.0 μ m (Flemming et al., 1992; Flemming & Wingender, 2001; Branda et al., 2005). Its composition is remarkably flexible and is control by the nature of the biofilm growth environment (Brown & Williams, 1985; Costerton, 1988; Anwar et al., 1990). The fibrous polysaccharides and globular glycoproteins components of the capsule are influenced by the condition applied upon cultivation. Brown & Williams (1985) and Costerton (1988) demonstrated that for the bacterial biofilm it is pivotal importance to maintain plasticity in the composition of its envelope to respond to changes in the growth environment. Such mechanisms enable the pathogenic bacteria surviving an extremely hostile environment when they enter the host (Anwar et al., 1990).

Recent reports suggest that slimes are responsible for the microbial biofilm resistance (Drenkard, 2003; Leid et al., 2005). Glycocalyx may cause alterations in the gaining access of antibacterial molecules to its targets located inside the cells (Anwar et al., 1990; Beech et al., 2005). According to Lewis (2001) glycocalyx matrix provides effective resistance for biofilm bacteria against large molecules such as antimicrobial proteins and their components. This physiological barrier is also effective against smaller peptides-defensins and their analogs (Lewis, 2001). Studies from a number of laboratories have concluded that the glycocalyx acting as a barrier, trapping antibacterial molecules from external environment and isolating the enclose cells from fluctuations in the surrounding environments (Gilbert et al., 1990; Flemming, 1995). The slime changes the charge and the free energy on bacterial surfaces, thereby limiting biocides transport (Hogt et al., 1986). Molecules binding capacity based on estimated number of available carboxyl and hydroxyl groups. The diffusion barrier's role of glycocalyx may also vary according to its soluble state (Siegrist & Gujer, 1985; Hoyle et al., 1992). Glycocalyx have been shown to accumulate antibacterial molecules up to 25% of their weight (Jang et al., 1990; Drenkard, 2003). Extracellular alginate, a slime produced by *Pseudomonas aeruginosa* has been studied for its ability to trap antimicrobial agents. This ability appears to be related to anionic nature of the exopolymer. Cationic substances can thus be retained within the matrix and prevented from acting upon the biofilm bacteria. Alginate has also been shown to bind positively-charged biocides and inhibit their activity (Suci et al., 1994). Also Hentzer et al. (2001) observed that alginate overproduction affects *Pseudomonas aeruginosa* biofilms resistance to antibiotic tobramycin treatment. On the other

hand, Dunne et al. (1993) and Yasuda et al. (1994) noticed that rifampicin, vancomycin, cefotiam and ofloxacin penetrated *Staphylococcus epidermidis* biofilms that formed on the dialysis membrane upon long-term exposure to antibiotics. These results support the notion that limitation of diffusion by glycocalyx matrix cannot always define resistance to antibacterial compounds. Transport limitations of biocides by glycocalyx depends on the present of the adsorption sites in the matrix (Carlson & Silverstein, 1998). After long-term exposure to antibiotics, saturating all possible binding sites in the glycocalyx matrix by the drugs enabled delivering and killing *Staphylococcus epidermidis* and *Staphylococcus aureus* biofilm (Dunne et al., 1993; Boles & Horswill, 2011).

In addition, adsorption sites within glycocalyx may also serve to anchor exoenzymes from external environments. Such immobilized enzymes are capable to impede the penetration and action of susceptible drugs (Hoyle et al., 1990). Giwerzman et al. (1991) found that β -lactamases may accumulate in the glycocalyx of *Pseudomonas aeruginosa* giving the whole biofilm population the potential for decreased β -lactam susceptibility. In addition, in mixed-species biological layers, the synthesis of neutralizing enzymes by one member of the community may confer protection for whole tested sessile populations (Stewart et al., 2000). Exoenzymes trapped within the biofilm matrix, may not only protect the sessile population from the antimicrobial activity of particular agents but also serve as a source of substrates scavenging the metabolites of biocides degradation and elimination (Morton et al., 1998).

Another form of biocides quenching by glycocalyx matrix has been demonstrated by Characklis (1989). The author found that chlorine react with extracellular polysaccharides in the mature biofilms and that this results in disruption of the structure of biological layer. The effect of this process may cause problems especially in industry practice by release of biofilm fragments of pathogenic microorganisms into water phase (Characklis, 1989). On the other hand, under particular circumstances, released biofilm fragments are more sensitive to biocides treatment. Gaylarde and Videla (1994) reported that eradication of biofilm from The North Sea pipelines by biocides caused initially increasing of the sulphate reducing bacterial count in the liquid from 2×10^2 CFU/ml to 3.1×10^3 CFU/ml. Interestingly, 2 hours later, the amount of the sulphate reducing bacterial amount fell to the value of 5.0×10^1 CFU/ml. The study of Gaylarde and Videla (1994) indicated that liberated sessile bacteria are susceptible to antimicrobials agents.

4. Metabolic and growth rate heterogeneity

Differences in nutrients and oxygen availability within biofilm affect in differences in growth rate and metabolic activity of bacteria. Wentland et al. (1996) and Xu et al. (1998) used fluorescent probes and reporter genes to visualized patterns of bacterial growth and cells metabolic activities in biofilm. Different concentrations of the key metabolic substrates and products within biofilm proved that surface-bound communities contain cells at all phases of bacterial growth and cells at the different activity levels (Stewart, 2002). This leads to microbial population heterogeneity. The problem occurs both in single-species and mixed-species bacterial biofilms (Xu et al., 2000). Better access to nutrients and oxygen in the periphery region of biofilm promotes metabolic activity of cells. In this part of biological layer the bacteria are able to proliferate. In contrast, in the deeper part of biofilm the metabolic potential of bacteria is limited by the worse diffusion process of nutrients (Senior, 2004). Chapman et al. (1993), Wentland et al. (1996) and Xu et al. (1998) identified slow-

growing or stationary-phase cells inside biofilm matrix. It was characterized by the decreased level of RNA (tRNA and rRNA) synthesis and accumulation of a guanine nucleotide-guanosine 3',5'-bis-pyro-phosphate (ppGpp). The authors demonstrated these effects in *in vitro* experiments by changing a conditions of biofilm maturation process from a nutrient-rich to a minimal ones (Chapman et al., 1993; Wentland et al., 1996; Xu et al. 1998).

Similar information concerning the metabolic and growth rate heterogeneity of cells within biofilms has come from studies of cellular enzyme synthesis (Poulsen et al., 1993; Wimpenny et al., 2000). Mitchison (1969) performed that level of enzyme synthesis is influenced by a series of sequenced changes in the particular stage of the bacteria growth cycle. For instant in periphery sphere of bacterial communities where cells are able to proliferate, part of the cellular enzymes are continuously active, and part of them only double at specific point to allow equality in the daughter cells (Mitchison, 1969). Mitchison (1969) also demonstrated that during division stage cellular enzymes may be proportional to cell mass. In slow-growing or stationary-phase bacteria, cellular enzymes synthesis is arrested (Sternberg et al., 1999).

Because most of biocides killing metabolically active bacteria, it has been proposed that bacteria at the dormant growth phase in the deeper region of biofilm are less susceptible to antimicrobial agents (Evans, et al., 1989; Toumanen, et al., 1989; Lewis, 2001; Stewart, 2002; Gilbert et al., 2002b; Bulter et al., 2010). These effects were observed in amino acid-starved communities where the cells were able to produce ppGpp (Pissbaro et al., 1990). Evans et al. (1989), Toumanen et al. (1989) and Duguid et al. (1992) investigated growth-rate-related effects upon laboratory conditions for biofilms of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus epidermidis*. The authors stated that the sensitivities of biofilms cells to penicillin, tobramycin and ciprofloxacin increased with the increasing growth rate of examined bacteria. These results suggest that the dormant phase of bacteria biofilm protects the cells from antimicrobial action of antibiotics (Evans et al., 1989; Toumanen et al., 1989; Duguid et al., 1992). The slow growth rate plays also an important role in mediating resistance of *Pseudomonas aeruginosa* biofilm to β -lactams (Tanaka et al., 1999; Alvarez-Ortega et al., 2010). According to Betzner et al. (1990) *Escherichia coli* at the dormant growth phase, activates the RelA-dependent synthesis of ppGpp that limits anabolic processes in cells. The presence of ppGpp suppressed the activity of a major *Escherichia coli* autolysin, SLT that makes the bacteria in non-growing zones of biofilm more tolerant to antibiotic treatment (Betzner et al., 1990). In addition, a mutation in *relA*, a gene coding ppGpp synthase, did not effect the growth rate. The population of *relA* mutants was more sensitive to killing by antibiotics. Rodionov and Ishiguro (1995) stated that ppGpp inhibits peptidoglycans production, that would explain the reduced levels of activity of the bacteria cell wall inhibitors. From a practical standpoint, it would be interesting to examine whether *relA* mutants become also eliminating by other antimicrobial agents that do not target the cell wall.

In contrast, the Tanaka et al. (1999) researchers also demonstrated that growth rate heterogeneity in *Pseudomonas aeruginosa* biofilm did not limited bactericidal action of fluoroquinolones (Tanaka et al., 1999). In addition, Brooun et al. (2000) observed that *Pseudomonas aeruginosa* in non-growing zones of biofilms are resistant only to part of commercially available antibiotics. For instant, slow growth rate increased resistance of *Pseudomonas aeruginosa* to tetracycline, but did not influence on the resistance of examined

bacteria to tobramycin. In this experiment the susceptibility of majority of *Pseudomonas aeruginosa* cells within biofilms were not much different from what is stated for planktonic bacteria. The greater parts of *Pseudomonas aeruginosa* biofilm were killed by clinically achievable range of antibiotics concentrations (about 5 μ g/mg) (Brooun et al., 2000). Brooun et al. (2000) also reported that after biofilm maturation, further increase in the antibiotic concentration had no effect on killing of *Pseudomonas aeruginosa* biofilm. The results of Tanaka et al. (1999) and Brooun et al. (2000) reinforced the idea that under the particular circumstances metabolic and growth rate heterogeneity may only contribute to increasing tolerant of bacterial biofilms to antimicrobials agents. Brooun et al. (2000) also stated that only a small fractions of bacteria are responsible for the very high level of resistance of *Pseudomonas aeruginosa* biofilms. According to Lewis (2000) the greater number of bacteria in biofilms are usually not more resistance to killing than free-floating cells and die more rapidly after treatment with a lethal dose of antibiotics. Under particular circumstances bacteria in non-growing zones of biofilms are preserved by the presence of biocides that only inhibits their growth (Lewis, 2000; Singh et al., 2006).

In biofilms metabolic activities of bacteria are controlled by oxygen availability. Biofilms of *Pseudomonas aeruginosa* grow in a gaseous environment of pure oxygen were killed by ciprofloxacin and tobramycin antibiotics (Walters et al., 2003). In contrast, Tresse et al. (1995) reported that reduction of oxygen availability enhanced of antibiotic resistance of agar-entrapped *Escherichia coli*. Also Hill et al. (2005) observed that anaerobically biofilm-grown isolates of *Pseudomonas aeruginosa* were significantly less susceptible for meropenem, tobramycin and ciprofloxacin treatments. According to Yoon et al. (2002) under strict anaerobic conditions, bacteria form robust biofilm, and that specific gene products were essential to develop such anaerobic biofilms. Metabolic and phenotypic changes under anaerobic conditions lead to increased levels of biocide resistance of bacterial biofilms. Sauer et al. (2002) based on analysis of protein patterns of *Pseudomonas aeruginosa* mature biofilm, demonstrated that a large part of biological layer is exposure to oxygen limitation.

5. Persister phenomena

Bacterial biofilms include persisters, cells that neither grow nor die during exposure to bactericidal agents, thus exhibit multidrug tolerance (MDT) (Lewis, 2005; Cheng & Hardwick, 2007; Lewis, 2008). While measuring a dose-response of a *Pseudomonas aeruginosa* biofilm to ofloxacin, Brooun et al. (2000) observed that a fraction of persister cells was not killed even by very high doses of the antibiotics. These cells appeared invulnerable in contrast to fairly sensitive *Pseudomonas aeruginosa* biofilm (Brooun, et al., 2000). Also in *Escherichia coli*, increasing concentration of ciprofloxacin or imipenem led to an initial 100- to 1000-fold reduce of live cells of a biofilm, remaining small population insensitive persisters to further increases in drug concentration (Ashby et al., 1994). These data suggest that most of the cells in the biofilm are as susceptible to bactericidal agents as planktonic bacteria. Only the persister fraction is responsible for survival of the whole sessile population (Ashby et al., 1994; Brooun et al., 2000). Also Spoering & Lewis (2001) noticed that stationary phase planktonic and sessile bacteria were tolerant to antimicrobials at similar level and that resistance of stationary phase and biofilm bacteria was dependent on the persister fraction. In addition, the increased resistance to killing of biofilm is due to high level of persisters produced by stationary phase bacteria inside biofilm (Spoering & Lewis,

2001). It is also important to emphasize that persisters are not simply non-growing cells in stationary culture. Keren et al. (2004b) noticed that fluoroquinolones and mitomycin C eliminated the bulk of *Escherichia coli* biofilm and left 1-10% intact persisters. From a medical perspective, the presence of persisters in biofilm is problematic. In planktonic population, a fraction of persisters that survive antibiotic action, is eliminated by the immune system (Hoyle et al., 1990; del Pozo & Patel, 2007). Biofilm persisters are protected from the immune system by glycocalyx matrix. In sessile bacterial population persisters are responsible for biofilm regrowth when the antibiotics concentration decrease or when the treatment is discontinued (Hoyle et al., 1990; Lewis, 2000).

The formation of persisters is dependent on the bacteria growth state (Lewis, 2007). Keren et al. (2004b) performed a test for measuring a rate of persisters after adding spent stationary medium to early log cells of *Escherichia coli* and *Pseudomonas aeruginosa*. Authors noticed that spent medium did not increase persisters of examined bacteria. In addition, persisters are rapidly lost if a stationary population is diluted (Keren et al., 2004b). The work of Keren et al. (2004b) demonstrated that formation of persisters dependent on the level of bacterial metabolic activity.

Falla & Chopra (1998) suggested that persisters are not mutant, but rather dormant variant of the wild type cells. Keren et al (2004a) observed that repeated reinoculation maintaining the cells in an log phase affects to a complete loss of persisters in *Escherichia coli* population. The work of Keren et al. (2004a) suggest that persisters are not formed in response to bactericidal agents exposure. According to Lewis (2005) persisters representing specialized survival cells whose formation is controlled by the growth stage of the bacterial culture. Moreover persisters are the cells with forfeiting rapid propagation system which ensures survival of cells in presence of lethal doses of antimicrobial factors (Lewis, 2005).

The tolerance of persisters to antibiotics works, not by preventing bactericidal binding, but by interfering with the lethal action of the compounds. Lewis (2007) postulated that persisters produce multidrug resistance protein (MDR protein) that shut down the antibiotic targets. It is worth point out that bactericidal properties of antibiotics occur by corrupting the target function of cells, rather than by inhibiting it. For instant, erythromycin blocks protein synthesis (Menninger & Otto, 1982). Streptomycin leads translational misreading, that produces truncated toxic peptides, causing the cell death. Shutting down the ribosome in a persister cells would produce tolerance to bactericidal aminoglycosides (Kornder, 2002; Lewis, 2005). According to Lewis (2005) persister protein can shut down most of antibiotics targets, formatting the resistant, dormant persister cells.

The phenomenon of tolerance of persisters to antimicrobial agents has also been linked with programmed cell death (PCD) system (Webb et al., 2003; Lewis, 2005; Lewis, 2007). Lewis (2000) suggests that actions of antimicrobial compounds are not responsible for cell death, but that they lead to cell damage that indirectly trigger PCD. The most common observation of PCD in bacterial biofilm is autolysis of cells. Autolysis is a self-digestion of the cell wall by peptidoglycan hydrolases termed autolysin (Shockman et al., 1996). Both production and hydrolysis of peptidoglycan are essential for creating the cell wall, therefore some autolysins are the part of normal bacteria growth activity in biofilm (Lewis, 2000). Because a bactericidal compound that diffuses throughout biofilm would not able to eliminate whole sessile population, Lewis (2005) proposed that persisters have a defective PCD mechanism.

The work of Moyed & Bertrand (1983) supported this statement. Moyed & Bertrand (1983) discovered in *Escherichia coli* a toxin-antitoxin system (*hipAB* locus) that has a potential of both killing the cells and improving survival after exposure to lethal doses of antimicrobial factors. The inactivation of the toxin-antitoxin systems by insertional elements or by mutation, induced defects in PCD system in *Escherichia coli* and made the bacteria more susceptible to antimicrobial agents (Han et al., 2011).

6. Quorum sensing

A mechanism which cannot be overlooked when discussing bacterial biofilm resistance to antimicrobial factors is *quorum sensing*. Within biofilm, bacteria are able to sense an increase of the cell population density and respond to it by the induction of particular set of genes (Whitehead et al., 2001; Shirtliff, et al., 2002; González & Keshavan, 2006; Turovskiy et al., 2007). *Quorum sensing* termed also cell-to-cell signaling system, includes in gram-negative bacteria the production and secretion of an acyl homoserine lactones (AHL), which diffuse through the cell wall, from the cell to the medium (Eberl, 1999; Williams et al., 2007). *Quorum sensing* mechanism in gram-positive bacteria typically use secreted peptides as signal compounds and a two-component regulatory system (composed of a membrane-bound histidine kinase receptor and an intracellular response regulator) to detect the peptide and trigger the required changes in gene expression (Kleerebezem et al., 1997; Suntharalingam & Cvitkovitch, 2005). A third examined form of *quorum sensing* mechanism employes a family of related molecules termed autoinducers-2. This system was found in both gram-negative and gram-positive bacteria (Platt & Fuqua, 2010).

According to Whitehead et al. (2001) and González & Keshavan (2006) several important biofilm features are likely to affect signal molecules production. The number of active cells in the biological layer, which is influenced by the bacteria growth and the synthesis of both of glycocalyx matrix and degradative enzymes, may affects signal molecules production (Chopp, 2003; Mentag et al., 2003; Newton & Fray, 2004; Sakuragi & Kolter, 2007). Moré et al. (1996), Schaefer et al. (1996) and Parsek et al. (1999) observed that metabolic activity of gram-negative bacteria will likely affect the availability of cellular substrates pools for signal molecules production, *S*-adenosylmethionine and acyl-carrier protein, thereby increasing signal molecules production. For gram-negative bacteria, *S*-adenosylmethionine is the amino acid substrate necessary for the synthesis of *quorum sensing* signal compounds, whereas acyl carrier protein is the donor of fatty acid chain in the biosynthesis of signal molecules of *Vibrio fischerii* (Eberhard et al., 1991).

Quorum sensing mechanism controls also the biofilm maturation process (Davies et al., 1998; Costerton, 1999; Watnick & Kolter, 2000). The work of Kjelleberg & Molin (2002) and Williams et al. (2007) demonstrated that the diffusion process of signal molecules within biofilm is unlimited. Inside biological layers there are shorter-distance migration of signal molecules and therefore the contact between the cells and reaction to signal molecules by the cells is more probable (Whitehead et al., 2001). The role of signal molecules-mediated *quorum sensing* in biofilm formation has been examined for *Brukholderia cenocepacia*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Serratia marcescens* (Davies et al., 1998; Huber et al., 2001; Lynch et al., 2002; Steidla et al., 2002; Labbate et al., 2007). Davies et al. (1998) demonstrated that cell-to-cell signal *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) is needed for the development of *Pseudomonas*

aeruginosa biofilm with a wild-type structure: loosely packed biomass with a mushroom appearance with notable amount of extracellular polysaccharides and water channel traversing the entire the biological layer. Whereas, signal molecules-negative mutants of *Pseudomonas aeruginosa*, *Brukholderia cenocepacia* and *Aeromonas hydrophila* showed defects in the late stages of biofilm maturation and thus were unable to form biofilms with the wild-type architecture (Huber et al., 2001; Lynch et al., 2002; Steidla et al., 2002; Labbate et al., 2007).

Because heterogonous architecture of biofilms and the synthesis of degradative enzymes deactivate biocides, it seems reasonable to speculate that biofilm antimicrobial agents resistance could also be influenced by *quorum sensing* system. Moreover, coordinated expression of *quorum sensing*-mediated phenotypes is crucial in cells migration to a more suitable environment/better nutrient supply and in adaptation to a new modes of growth, which may afford protection from deleterious environment (Whitehead et al., 2001; Abee et al., 2011). However, to date *quorum sensing* system as factor decreasing the biofilm susceptibility to antimicrobial agents has been studied in a limited number of strains. Davies et al. (1998) and Hassett et al. (1999) reported that exposure of *quorum sensing*-negative mutant biofilms to the antimicrobial agents SDS and hydrogen peroxide caused detachment and dispersion of surface-anchored bacteria. In addition, Hassett et al. (1999) have reported that cell-to-cell signaling mechanism in *Pseudomonas aeruginosa* controls the expression of the catalase and superoxide dismutase genes and mediates biofilms resistance to hydrogen peroxide. According Shih & Hoang (2002) *quorum sensing*-deficient mutant biofilms susceptibility to kanamycin correlated with thinner biofilm formation and lower EPS production. Above results provide evidences that biofilm respond directly or indirectly to environmental stress via a *quorum sensing* system.

Interestingly, resent reports have also demonstrated chelating properties of cell-to-cell signals (Schertzer, et al. 2009). Such non-signaling features were stated for *Pseudomonas aeruginosa quorum sensing* molecules. Weinberg (2008) examined multiple meaning of *quorum sensing* system in mixed-species bacterial population. The author performed that *Pseudomonas aeruginosa* may kills competing bacteria in the growth environment by hijacking the bacteria's iron stores using 2-heptyl-3-hydroxy-4-quinolone signal. According to Weinberg (2008) *Pseudomonas* quinolone signal is a high affinity iron chelator. The ability of signal molecules to trap external positive-charged compounds is similar to antimicrobial action of glycocalyx matrix (Schertzer, et al, 2009). However, this role of cell-to-cell signal molecules to biofilm resistance properties needs to be examined in more detail.

7. General stress response

A general stress response is characterized by numerous changes in bacteria physiology and morphology that increasing cellular stress resistance (Hengge-Aronis, 1999; Lee et al., 2009). The formation of cell envelope and synthesis of thin aggregative fimbriae in *Escherichia coli* and *Salmonella enteritis* serovar *Typhimurium* are both under control of general stress response. These features affect cell to cell contact (Atlung & Brøndsted, 1994; Römling et al., 1998). Moreover, the study of Hengge-Aronis et al. (1993) performed that under extreme conditions, the general stress response functions as a factor preventing cellular damage rather than repaired it. This mechanism induced by many different stresses including nutrients deprivation (which results in stationary phase of bacteria growth cycle), high or

low temperature, high osmolarity and acidic pH (Lange & Hengge-Aronis, 1991; Lee et al., 1995; Xu et al., 2001). Some evidences suggest also that biofilm development process leads to an early general stress response (Brown & Barker, 1999).

Exposure of *Escherichia coli* to adverse environments can induce RpoS, a sigma subunit of RNA polymerase, that acts as a central regulator. In *Escherichia coli* above 50 sigma factor-controlled genes determine stress tolerance of cells, whereas others mediate the physiological rearrangement or redirect the metabolism of bacteria upon stress condition (Hengge-Aronis, 1999; Whiteley et al., 2000). Analysis of the molecular reactions in dense population of *Escherichia coli* revealed the influence of sigma factor-controlled genes on production of trehalose (Liu et al., 2000). Trehalose is the stress protectant in bacteria. In *Escherichia coli*, this molecule acts as osmoprotectant and is essential for bacteria desiccation tolerance (Strøm & Kaasen, 1993; Welsh & Herbert, 1999). Trehalose also plays an important role in thermotolerance of *Escherichia coli* (Hengge-Aronis et al., 1991). *rpoS* mutants that devoided of the typical features associated with the general stress response were unable to accumulate trehalose and they died off rapidly in stationary phase (Hengge-Aronis et al., 1991; Lange & Hengge-Aronis, 1991; McCann et al., 1991).

In bacterial populations, RpoS-controlled promoter regions include multiple binding sites for additional regulators such as cAMP-CRP or the histone-like proteins H-NS, leucine-responsive regulatory protein (Lrp), integration host factor (IHF) and FIS (Barth et al., 1995; Marschall et al., 1998). These regulators determining RpoS specificity (Marschall, et al. 1998).

As focused in literature, the general stress response acts both as a rapid emergency response and as a long-term mechanism, that enables the cell adaptation to nutrient deprivation and other environmental stresses that cause changes in cellular metabolism (Gentry et al., 1993; Hengge-Aronis, 1999). Activation of the general stress response in the cells, immobilized in biofilm matrix, may results in increasing resistance to biocides action (Drenkard, 2003). However, this mechanism needs to be examined in more detail. Drenkard (2003) demonstrated that the general stress response maintain cell viability upon stationary phase when nutrients availability is limited. It is highly probable that environments within biofilm would promote the expression of the RpoS. This process affecting the physiological changes that mediate protection of biofilms to environmental stresses (Drenkard, 2003). Adams & McLean (1999) observed that *Escherichia coli* that lack *rpoS* are unable to form biofilm of wild type architecture. The study of Cochran et al. (2000) demonstrate that thin biofilms formed by *Pseudomonas aeruginosa* mutants of *rpoS* are susceptible to hydrogen peroxide.

8. Efflux pumps

Efflux pumps can affect both intrinsic and acquired resistance to antimicrobial agents by applying the energy to limit the cytoplasmic compound concentration to subtoxic level (Nikaido, 1992; Hogan & Kolter, 2002; Liaw, et al., 2010). Efflux system was first described as a mechanism of negatively impact to tetracycline susceptibility in *Escherichia coli* population. It was the plasmid-encoded single component Tet protein export of tetracycline throughout the cytoplasmic membrane (Ball et al., 1980).

A set of efflux systems facilitates bacteria to survive in extreme environments. Bacterial efflux pumps are involved in the multidrug resistance (MDR) phenotype combined with other more specific resistance systems including target mutation and enzymatic

modification of antimicrobial agents (Zgurskaya & Nikaido, 2000; Davin-Regli et al., 2008; Bolla et al., 2011). The mechanism of efflux pumps in *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* may also serve down regulation of porin production that slow down the penetration of hydrophilic solutes, and decrease the transmembrane diffusion of lipophilic solutes (Nikaido & Vaara, 1985; Plésiat & Nikaido, 1992; Li & Nikaido, 2004; Pagés et al., 2008). However, under particular circumstances, the outer membrane barrier cannot be the whole explanation of the bacteria resistance to inhibitors (Nikaido 1996). In fact, the equilibration across the outer membrane is reached very quickly, in the part of the surface-to-volume ratio that is very large to compare with bacterial cell size. Thus, the periplasmic concentration of many antibiotics may achieve 50% of their external value (Nikaido, 1989).

In the literature, numerous plasmid and chromosome-encoded efflux systems, both agent- or class-specific and multidrug have been performed in a various of microorganisms where they are the major determinant in the intrinsic resistance of the bacteria to action of dyes, detergents and different classes of antibiotic including β -lactams (Nikaido, 1989; Nikaido, 1994; Markham & Neyfakh, 2001; Butaye et al., 2003). Bacterial efflux pumps compose of five classes of systems including: the major facilitator superfamily (MF), the ATP-binding cassette family (ABC), the resistance-nodulation-division family (RND), the small multidrug resistance family (SMR), and the multidrug and toxic compound extrusion family (MATE) (Putman et al., 2000; Kumar & Schweizer, 2005; Poole & Lomovskaya, 2006). To drive antimicrobial agents efflux, the ABC family system hydrolyses ATP, whereas the MF family, the RND family and the MATE family function as secondary transporters, catalysing drug-ion antiport (H^+ or Na^+) (Poole, 2005).

The RND family transporters are most commonly found in bacteria cells (Poole, 2001). In gram-negative bacteria this system operates as a part of a tripartite mechanism that includes: a membrane fusion protein that is associated with the cytoplasmic membrane, a transporter protein that export substrates throughout the inner membrane, and an outer membrane factor (OMF) that enables the passage of the substrate throughout the outer membrane (Poole, 2005). The RND family transporters are the first line of bacterial defense that can promote the acquisition of additional resistance mechanisms such as target mutations or drug modification (Davin-Regli et al., 2008; Li & Nikaido, 2009). Pagés et al. (2008) and Pagés et al. (2010) performed that the expression of RND efflux pumps is an important prerequisite for the selection of fluoroquinolone resistant strains carrying the target mutation. According to Stover et al. (2000), *Pseudomonas aeruginosa* encode 12 efflux systems of the class of the RND family. However, to date only MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OmpD, MexJK and MexXY have been detailed characterized (Poole & Srikumar, 2001; Chuanchuen et al., 2002; Blair & Piddock, 2009; Breidenstein, et al., 2011).

Molecular analysis of efflux pumps assesses the role of this mechanism in biofilm resistance to antimicrobial agents. Exposure the bacterial biofilms to insufficient dose of antibiotics, such as tetracycline and chloramphenicol, and to xenobiotics, such as salicylate and chlorinated phenols, induces the expression of multi-drug resistance operons and efflux pumps (Levy, 1992; Ma et al., 1993). Also DNA microarray analysis of mature *Pseudomonas aeruginosa* PA01 biofilm demonstrated that none of genes encoding the RND efflux system were induced in sessile bacterial population grown in antibiotic-free environments (Whiteley et al., 2001).

Numerous of works have focused on the identification of genes that could contribute efflux system-mediate resistance of bacterial biofilms. Maira-Litran et al. (2000) examined the systems of *mar* and *acrAB* that confer on *Escherichia coli* biofilm the multidrug resistance phenotype. The *mar* operon is a regulator controlling the expression of various genes in *Escherichia coli* cells constituting the *mar* regulon. Upregulation of *mar* in planktonic bacteria effects a resistance phenotype to antimicrobial agents such as antibiotics (penicillins, cephalosporins, rifampicin, nalidixic acid and fluoroquinolones), oxidative stress agents and organic solvents (Alekhshun & Levy, 1997). *mar* can be induced by sub-lethal doses of commonly used therapeutics such as tetracycline, chloramphenicol, salicylate and paracetamol (Cohen et al., 1993; Seoane & Levy, 1995). The *acrAB* efflux pump is upregulated in *mar* mutants and determined the multidrug resistant phenotype of *mar* mutant isolates (Ma et al., 1995; Ma et al., 1996). According to Maira-Litran et al. (2000) the constitutive expression of *acrAB* efflux pump effects lower susceptibility of *Escherichia coli* biofilm to sub-lethal doses of ciprofloxacin. In addition, the expression of *mar* and its target genes is related to stationary phase of bacteria growth. Authors observed the highest level of *mar* expression within the depth of *Escherichia coli* biofilm, where the metabolic activity of examined bacteria were the most suppressed (Maira-Litran et al., 2000).

Brooun et al. (2000) and De Kievit et al. (2001) examined the expression of the genes associated with efflux pumps (MexAB-OprM and MexCD-OprJ) in developing biofilms of *Pseudomonas aeruginosa*. Brooun et al. (2000) underscored the importance of these pumps in the resistance to ofloxacin. Authors demonstrated that at low concentration of ofloxacin *Pseudomonas aeruginosa* mature biofilm with lacking MexAB-OprM was less resistant to antibiotic than mature biofilm that overexpressed the pump (Brooun et al. 2000). De Kievit et al. (2001) found that expression of the genes that encode MexAB-OprM and MexCD-OprJ, are decreased over time during biofilm maturation. In addition, authors, using the overexpressing and efflux pumps mutants of *Pseudomonas aeruginosa* revealed that none of efflux pumps analyzed plays a significant role at decreasing susceptibility of *Pseudomonas aeruginosa* biofilm to antibiotics (De Kievit, et al., 2001). Therefore to assess the true function of efflux pump in bacterial biofilm resistance to antimicrobial agents, further experiments of additional not yet characterized loci with homology to efflux system are needed.

9. Conclusion

Survival of bacterial after disinfection and antibiotic treatment represents a problem for the modern medicine and industry practice. Commonly applied antibiotics and disinfectants are able to eliminate planktonic bacteria released from the biofilm but often are unable to treat biofilm-embedded cells. This may cause difficult to eradicate infectious.

Biofilm resistance to bactericidal agents is usually multifactorial and may vary from one microorganism to another. Environmental heterogeneity that exists inside the biofilm might promote the formation heterogeneous communities of bacteria, such that different levels of resistance can be employed throughout the entire population. For instant, the bacteria at periphery region of biofilm might be protected by the glycocalyx matrix, by the efflux systems and by the enzymes that inactivate certain antimicrobial compounds. The cells in the intermediate position of biofilm became starved for a particular nutrient, and they slow their growth. Transition from exponential to slow or no growth/persisters phenomena is also be accompanied by the increased in resistance of bacteria biofilm to bactericidal agents.

Upon the extreme conditions setting the general stress response mechanism by surface-bound bacteria may prevent cellular damage.

Whatever new biocides/antibiotics are developed, the high number of bacteria within biofilms will combine to overcome their action and lead to resistance formation. The only way to avoid or to slow the speed of excess resistance formation is systematic and in-depth investigation of resistant bacteria isolated from naturally occurred biofilms. The information derived from lab investigations can provide insight strategies to subvert both biocide and antibiotic resistance of surface-bound bacteria.

10. References

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