# Antibiotic Resistance Development in Bacterial Biofilms


**María D. Macià and Antonio Oliver**

**Abstract** Biofilms are organized bacterial communities embedded in an extracellular polymeric matrix attached to living or abiotic surfaces or forming aggregates. This mode of growth is characteristically associated with chronic infections, marked by the persistence of the bacteria regardless of proper antimicrobial therapies. Biofilms are up to 100–1000 times more resistant to antibiotics than planktonic cells. The basis for resistance to antimicrobials in biofilms is complex and involves both mechanisms of tolerance and classical resistance. Physical, physiological, adaptive and in vivo tolerance together with the *persister* phenomenon and the expression of specific genes lay at the origin of the recalcitrant (or tolerant) nature of the biofilm to antibiotics. On the other hand, mutational resistance and horizontal gene transfer determine the genetic changes leading to genuine resistance to antimicrobials. In this chapter, using the bacterium *Pseudomonas aeruginosa* as an archetypal microorganism, we explore the different mechanisms of antibiotic resistance development in biofilms.

## 1 Introduction

Biofilms are classically defined as organized bacterial communities embedded in an extracellular polymeric matrix attached to living or abiotic surfaces (Stoodley et al. 2002). This social bacterial structure is highly prevalent in nature (Petrova and Sauer 2012) and arises as an adaptation strategy for survival in hostile environments, including the human host (Fux et al. 2005). From a clinical point of view, the transition from a planktonic to a biofilm mode of growth frequently determines the establishment of a chronic infection (Smith et al. 2006; Oliver et al. 2007). Consequently, biofilms are representatively implicated in chronic infections, in contrast to

M. D. Macià (✉) · A. Oliver
Microbiology Service, Hospital Son Espases, Instituto de Investigación Sanitaria Illes Balears (IdISBa), Palma de Mallorca and CIBER Enfermedades Infecciosas. Instituto de Salud Carlos III, Madrid, Spain
e-mail: mariad.macia@ssib.es



K. Richter, K. Nørskov Kragh (eds.), *Antibiofilm Strategies*, Springer Series on Biofilms 11, https://doi.org/10.1007/978-3-031-10992-8_3

planktonic bacteria that are involved in acute processes (Costerton et al. 1999). Biofilm-related chronic infections are characterized by the persistence of the aetiological agent, despite (in principle) adequate antibiotic therapy and host immune responses. In general, up to 65–80% of all infections are associated with biofilm formation (Jamal et al. 2018). These infections encompass a wide range, from those related to indwelling medical devices (e.g., catheters, prosthetic joints, and surgical implants) to tissue infections, such as otitis media, osteomyelitis, rhinosinusitis, wound infections or chronic respiratory infections in cystic fibrosis (CF) patients (Høiby et al. 2015a).

In device-associated chronic infections, replacing the implant to remove biofilms is an effective intervention strategy. However, for tissue or respiratory chronic biofilm infections, the only available therapy to date is antibiotic treatment. Long-term therapies with antibiotics, despite in vitro susceptibility, frequently lead to a longstanding inflammatory response, together with a high risk of antibiotic resistance development, thus triggering an unavoidable chronic infection extremely difficult to eradicate.

One of the most important characteristics of biofilms is their increased tolerance to antimicrobial agents (Wimpenny et al. 2000). Compared to planktonic cells biofilms are up to 100–1000 times less susceptible to antibiotics and disinfectants (Costerton et al. 1987, 1999; Bjarnsholt et al. 2005).

The mechanisms of resistance and tolerance differ depending on the specific antimicrobial agent, the bacterial strain and species, the age and developmental stage of the biofilm, and the biofilm growth conditions (Ito et al. 2009; Alhede et al. 2011; Bowler et al. 2012; Haaber et al. 2012; Stewart et al. 2015).

Commonly for biofilm infections, the terms tolerance and resistance are used synonymous. Moreover, the term tolerance recurrently encompasses, or even replaces, that of resistance. However, it is necessary to distinguish their different conceptual meaning.

In general, tolerance of biofilms to antibiotics is multifactorial and includes physical, physiological, environmental, and inducible genetic factors, whereas antibiotic resistance needs the involvement of acquired or heritable processes that alter the genetic background of bacteria and do not revert in the absence of antibiotics. In other words, tolerance can be defined as the survival in the presence of the antibiotic due to the biofilm itself. In this case, single dispersed cells from biofilms are susceptible to antibiotics. The development of conventional resistance requires stable genetic changes like point mutations or horizontal gene transfer (Lewis 2008). In all likelihood, the tolerance phenomenon precedes and promotes antibiotic resistance development in biofilms. Though ultimately, both tolerance and resistance mechanisms act synergistically, thus seriously reducing the options of an effective antimicrobial treatment for chronic infections.

Understanding the mechanisms underlying biofilm-specific antibiotic resistance and tolerance will help guide the selection of efficient treatments to fight chronic infections.

In this chapter, using the bacterium *Pseudomonas aeruginosa* as a model, we examine the different mechanisms of resistance development in biofilms.

# 2 Mechanisms of Tolerance to Antibiotics in Biofilms

## *2.1 Physical Tolerance*

Traditionally, it was considered that diffusion of antibiotics was restricted by the extracellular biofilm matrix. While biofilm matrices do not inhibit the diffusion of antibiotic molecules, restricted penetration through biofilms may occur when antibiotics bind to components of the matrix or the bacterial membranes (Walters et al. 2003; Tseng et al. 2013). As an example, using fluorescently labeled antibiotics it has been demonstrated that positively charged peptides and aminoglycosides, like tobramycin, are sequestered in the negatively charged extracellular matrix (Walters et al. 2003; Chiang et al. 2013), whereas the neutral ciprofloxacin readily penetrates into the biofilms (Tseng et al. 2013). The presence of anionic extracellular DNA (eDNA) and alginate in the extracellular matrix are responsible for the capture and consequently the reduced activity observed for positively charged antibiotic classes, rather than an inability to diffuse across the biofilm mass (Cao et al. 2016; Mulcahy et al. 2008). This retardation in the entrance of the antibiotic, which ultimately exposes the biofilm to lower antibiotic concentrations, gives time and helps the bacteria to adapt to a more tolerant state (Bagge et al. 2004a; Pamp et al. 2008a; Kindrachuk et al. 2011; Ciofu and Tolker-Nielsen 2019).

## *2.2 Physiological Tolerance*

Another striking characteristic of biofilms is their compartmentalization in different metabolic states. This heterogeneity is due to and at the same time a consequence of the decreasing concentrations of oxygen and nutrients along the biofilm that established gradients of metabolic stages. Thus, the consumption of oxygen and nutrients by the metabolically active bacteria located in the periphery of the biofilm leave scarce oxygen and nutrients to the bacteria placed inside the biofilm (Stewart et al. 2016). Consequently, subpopulations at the periphery of the biofilm show an important physiological activity, whereas subpopulations positioned at the inner parts of the biofilm show a significantly reduced physiological activity or even a total lack of activity with no evidence of growth (Pamp et al. 2008a; Ciofu and Tolker-Nielsen 2019). Numerous antibiotics target processes that take place in metabolically active bacteria (e.g. replication, transcription, translation, and cell wall synthesis), thus biofilm areas with low metabolic activity may display increased antimicrobial tolerance with an impact on the general biofilm susceptibility. Likewise, it has been shown that some antibiotics that work under aerobic conditions (e.g., tobramycin, ciprofloxacin and tetracycline) are only effective on biofilm cells located at the air interface and not at other areas of the microcolony (Walters et al. 2003; Pamp et al. 2008a; Stewart et al. 2016). In addition, it has been recently shown that reactive oxygen species (ROS) are thought to play a role in antibiotic-induced

cell death, so hypoxia may additionally promote biofilm cell survival upon antibiotic exposure due to the fact that ROS production requires molecular oxygen (Van Acker et al. 2013; Jensen et al. 2014; Van Acker and Coenye 2016). Thus, other than the gradient of nutrients, low oxygen concentrations along the biofilm impair the efficacy of ROS-dependent bactericidal antibiotics and decrease bacterial metabolism (Jensen et al. 2014; Van Acker and Coenye 2016; Van Acker et al. 2016).

Related to this, it is well known that hypoxia is associated with increasing antibiotic tolerance through the upregulation of efflux pumps, which is an induced, non-specific antibiotic tolerance mechanism. Concerning biofilms, it is noteworthy that various studies have revealed differential expression of several conventional and biofilm-specific antibiotic resistance genes in biofilms (Whiteley et al. 2001). It has been shown that in oxygen-starved planktonic cells, MexEF-OprN efflux pump genes are upregulated and contribute to antibiotic tolerance (Schaible et al. 2010) like MexEF-OprN or MexCD-OprJ. Moreover, the MexCD-OprJ efflux pump locus was shown to be transcriptionally upregulated in *P. aeruginosa* biofilms grown under low oxygen conditions (Tata et al. 2016), establishing a close connection between physiological tolerance and adaptive resistance mechanisms.

In addition to the reduction of metabolic activity, impairment of the efficacy of ROS-dependent antibiotics, and upregulation of efflux pumps; the compartmentalization of the biofilm microenvironment due to the declining gradients of oxygen and nutrients could lead to areas of starved cells. It has been shown that responses to nutrient and iron starvation, like the stringent response, also play an important role in the tolerance of biofilms to antibiotics (Nguyen et al. 2011; Bernier et al. 2013).

## 2.3 *Persisters*

The most extreme stage regarding metabolic inactivity could be represented by the characteristic dormant or persistent cells of biofilms; popularly called *persisters*. This is a spore-like cell state, dormant and incapable of growth, that protects bacteria from the action of antibiotics (Lewis 2008, 2010). However, as a tolerance mechanism, the refractoriness to antibiotics is not heritable and when *persister* cells are segregated from the biofilm and grown individually, they display the same pattern of antibiotic susceptibility as the original population (Brauner et al. 2016). Importantly, after treatment they can revert to the vegetative state, repopulating the biofilm, and thus reconstituting the infection, leading to prolonged treatment duration (Lewis 2008, 2010; Barraud et al. 2013). *Persisters* represent less than 0.1% of the biofilm population and have been documented in a number of bacterial species, including *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Escherichia coli*, *Salmonella entérica* subsp. *enterica* serovar Typhimurium and *P. aeruginosa*. Several mechanisms, such as reduced levels of cellular energy (low intracellular ATP levels), and adaptive responses have been associated in *persister* cell formation (Wilmaerts et al. 2019).

## 2.4 Expression of Specific Genes

A number of genes have been identified as being specifically involved in the tolerance of biofilms to antibiotics. In general, these genes are characteristically overexpressed in biofilm growth and this increase in their expression is related to tolerance to antibiotics. This is a different concept from adaptive resistance which requires the presence of the antibiotic to induce the resistance mechanism.

For instance, the biofilm resistance locus regulator, BrlR, was found to be a Mer-like transcriptional activator of biofilm-specific antibiotic tolerance in *P. aeruginosa* by its interaction with a variety of different potential targets (Poudyal and Sauer 2018). For example; activating the expression of the MexAB-oprM and MexEF-oprN efflux pumps (Liao et al. 2013), inducing the production of the ABC transporters, like the ABC transporter PA1874–1877, which has been found to be 10 times more expressed in *P. aeruginosa* biofilms compared to planktonic cells (Poudyal and Sauer 2018; Zhang and Mah 2008), or altering the expression of genes encoding modification of LPS, membrane protein composition, or metabolism and energy generation.

As mentioned before, multidrug efflux pumps efficiently eject antibiotics out of the cell and their induction through different ways is an important factor for antibiotic tolerance in biofilms. Different forms of stresses can induce efflux pumps in paradigmatic biofilms of *P. aeruginosa*, such as oxidative stress, nitrosative stress and membrane-damaging agents can activate MexXY-OprM, MexEF-OprN and MexCD-OprJ, respectively (Poole 2014).

## 2.5 Adaptive Tolerance

We refer to adaptive tolerance as an inducible mechanism that depends on the antibiotic presence and does not require genetic changes, consequently; it is considered a tolerance mechanism. Adaptive tolerance is mediated by regulatory networks that lead to its switch off or reversion when the antibiotic molecules disappear. Thus, similarly to *persisters*, an on/off model with long-term consequences can be evidenced; if cells are not completely eradicated, once the treatment is stopped resettlement of susceptible biofilms can occur.

Examples of adaptive tolerance in *P. aeruginosa* are the induction of AmpC β-lactamase, LPS alteration and the upregulation of efflux pumps.

The induction of AmpC β-lactamase through the exposure to β-lactam antibiotics, as imipenem or ceftazidime, is likely the main adaptive tolerance mechanisms in *P. aeruginosa* biofilms (Giwercman et al. 1991, 1992). Bagge et al. demonstrated that the expression of this enzyme followed a special structural distribution being characteristically concentrated at the periphery of the biofilms (Bagge et al. 2004b).

Similarly, exposure of *P. aeruginosa* biofilms to colistin was shown to upregulate the *pmr* and *arn* genes in the metabolically active peripheral subpopulations (Pamp

et al. 2008a). The *arn* gene products alter the negative charge of LPS that leads to a decrease in the uptake of polymyxins and consequently, an increase in the biofilm tolerance to the cationic peptides (Lewenza 2013). Upregulation of MexAB-OprM efflux pump is also involved in colistin biofilm tolerance (Pamp et al. 2008a). Interestingly, tolerance to colistin is linked to metabolically active cells, thus establishing a pattern for combination of antibiotics with different mechanisms of action (Pamp et al. 2008a).

### *2.6 In Vivo Tolerance*

What has been exposed so far refers to intrinsic factors of the biofilm special atmosphere that leads to the physiological tolerance but, as well as biofilm micro-environment inherent conditions, there is also the in vivo contribution of the host immune system by, for example, the involvement of polymorphonuclear leukocytes (PMNs). These cells are known to consume oxygen, thus, further impacting the mechanisms of tolerance described previously, and to release eDNA which, also contributes to the antibiotic tolerance through the sequestration of cationic antibiotics (Kolpen et al. 2010; Kragh et al. 2014; Worlitzsch et al. 2002).

Apart from the PMNs action, several circumstances are implicated in the in vivo tolerance of biofilms, for example, the different in vitro/in vivo sizes of biofilms (Høiby et al. 2015b), their placement in clusters (Bjarnsholt et al. 2013) or the polymicrobial nature of in vivo biofilms (Burmolle et al. 2010). Regarding the accessibility of antibiotics, biofilm has been considered as a third compartment after tissue (second) and blood (first) (Cao et al. 2015).

## 3 Antibiotic Resistance Development in Biofilms

### *3.1 Mutational Resistance*

The described tolerance mechanisms have been extensively studied and clearly related to the recalcitrance of biofilms to antibiotics; however, there is still an important gap of knowledge regarding the effect and relevance of conventional resistance mutational mechanisms in the reduced antibiotic susceptibility of biofilms.

According to current knowledge, it would be reasonable to summarize that tolerance mechanisms provide a fertile ground for the emergence of antibiotic-resistant mutants (Levin-Reisman et al. 2017).

Traditionally, the balance between the evolution, driven by mutation and selection and determining the adaptability to new environments or to adverse conditions, and stability of genetic information, related with the population fitness and the perpetuation of species, shapes the mutation rate (Levin-Reisman et al. 2017).

A priori, exceeding limits of the normal mutation rate could lead to an evolutionary death by no adaptability (hypomutation) or to short-time death by the accumulation of deleterious mutations (hypermutation), (Radman et al. 1999). However, in some situations, e.g., when confronting new, fluctuating or stressful environments, mutators can be amplified by being co-selected with adaptive mutations (Sniegowski et al. 1997; Taddei et al. 1997; Mao et al. 1997) and thus hypermutation could be advantageous.

Hypermutators have a spontaneous mutation rate increased from 100- to 1000-fold due to defects in DNA repair or errors in the avoidance systems, frequently affecting the key genes of the methyl-directed mismatch repair system (MMSR) *mutS*, *mutL* or *uvrD* (*mutU*), (Miller 1996). Moreover, mutator phenotype can arise from mutations affecting the 8-oxo-dG (or GO) repair system genes (*mutM*, *mutY* and *mutT*), as well as genes involved in the prevention of oxidative damage produced by reactive oxygen species (ROS) (*oxyR*, *sodA* and *pfpI*), (Miller 1996; Rodríguez-Rojas and Blázquez 2009).

It has been reported that hypermutator strains were extremely high prevalent in the context of chronic respiratory infections in CF patients (Oliver et al. 2000). Mutators, mainly due to deficiencies in the DNA mismatch repair (MMR) system (frequently in the *mutS* gene), represented 20% of the total number of *P. aeruginosa* isolates and were found in 37% of patients (Oliver et al. 2000, 2002), which is in contrast to the low prevalence (<1%) of this feature in acute infections (Gutiérrez et al. 2004). Likewise, in other respiratory infections such as bronchiectasis, or chronic obstructive pulmonary disease (COPD) (Maciá et al. 2005), a very high prevalence of hypermutable strains was also documented and again, hypermutation was mainly due to errors in MMRS, especially in *mutS (*Oliver et al. 2002*;* Maciá et al. 2005*)*. Similarly, an association between hypermutation and chronic infections was also established in other microorganisms (Watson et al. 2004; Labat et al. 2005; Prunier et al. 2003).

The proportion of hypermutable isolates can vary from less than 1% at acute infections, going through 6% at environmental settings, or 10% at the onset of chronic infection, to up to 65% at chronical phases (Oliver et al. 2000; Gutiérrez et al. 2004; Maciá et al. 2005; Martínez-Solano et al. 2008; Montanari et al. 2007; Kenna et al. 2007; Mena et al. 2008).

Nevertheless, one of the characteristics of hypermutator strains with the greatest clinical impact is their association with high antibiotic resistance rates (Oliver et al. 2000; Maciá et al. 2005). Indeed, it was shown by in vitro and in vivo experiments that hypermutation dramatically favors resistance development during antibiotic exposure (Oliver et al. 2000; Maciá et al. 2005; Woodford and Ellington 2007; Blázquez et al. 2002).

In recent years, studies focussing on the effects of hypermutation on biofilms development showed that mutagenesis is intrinsically increased in biofilms and that hypermutation plays an important role in development, adaptation and diversification processes (Conibear et al. 2009; Webb et al. 2003; Boles and Singh 2008; Driffield et al. 2008).

In fact, it was demonstrated that MMRS deficient mutants were associated with enhanced micro-colony development and increased rates of phenotypic diversification, which conferred improved competitiveness (Luján et al. 2011).

Recently, studies have been conducted to assess the effect of hypermutation on the resistance to antibiotics in biofilms. For example, the antibiotic azithromycin showed promising results in the treatment of chronic respiratory infections by *P. aeruginosa*. Although macrolides are not active against *P. aeruginosa* in standard in vitro susceptibility tests, these antibiotics showed bactericidal activity on stationary phase cells (Imamura et al. 2005; Saiman et al. 2003). In a biofilm model using the Calgary device it was shown that azithromycin was active against *P. aeruginosa* biofilms, however, resistant mutants were readily selected, especially in hypermutable strains (MMRS deficient mutants) (Mulet et al. 2009). The main resistance mechanism was found to be the hyperexpression of MexCD-OprJ, due to *nfxB* mutations, which collaterally showed cross-resistance to other unrelated antipseudomonal agents, such as ciprofloxacin or cefepime, and hypersusceptibility to other drugs, such as imipenem or tobramycin (Mulet et al. 2009). This work demonstrated the impact of hypermutation on antibiotic resistance in biofilms and provides guidance in the selection of appropriate antipseudomonal therapies in CF patients under azithromycin maintenance treatment (Mulet et al. 2009).

Similarly, in a model of theoretically optimized pharmacokinetic/pharmacodynamic (PK/PD) parameters of ciprofloxacin to treat flow cell biofilms formed by hypermutable strains, deficient in *mutS*, this antibiotic, which was one of the most active antibiotics on biofilms according to previous works (Moskowitz et al. 2004), led to the selection and amplification of resistant mutants (Macià et al. 2011). Particularly relevant was the demonstration that mutational mechanisms played a major role in biofilm antibiotic resistance and that theoretically optimized PK/PD parameters failed to suppress resistance development. The results from this work supported the hypothesis that the increased antibiotic tolerance (physical, physiological and adaptive), driven by the special biofilm microenvironment, may raise the effective mutant preventive concentration (MPC) and favors gradual mutational resistance development, especially in mutator strains. Accordingly, overexpression of MexCD-OprJ or MexEF-OprN was the primary cause of ciprofloxacin resistance development in biofilms. Importantly, it was also demonstrated for the first time that mutator populations were dramatically amplified under antibiotic treatment by co-selection with resistance mutations in *P. aeruginosa* biofilms. In addition, the results highlighted the need to adapt the classical PK/PD target values to the biofilm mode of growth, in order to optimize the therapeutic management of chronic infections (Macià et al. 2011).

More recently, with the same aim of optimizing PK/PD strategies, the impact of ciprofloxacin and meropenem as monotherapy and in combination was evaluated on hypermutable strains using the dynamic in vitro CDC biofilm reactor (CBR) (Bilal et al. 2020). Concentration-time profiles attainable in epithelial lining fluid (ELF) following FDA-approved doses were simulated in the CBR. Overall, all monotherapies failed and combination treatments demonstrated synergistic bacterial killing and resistance suppression in hypermutable strains (Bilal et al. 2020).

In an analogous study, different dosage regimens of meropenem (standard and in continuous infusion) and tobramycin, as monotherapies and in combination against hypermutable carbapenem-resistant *P. aeruginosa* were studied (Bilal et al. 2019). In accordance with the work by (Bilal et al. 2020), monotherapy of meropenem and tobramycin were ineffective against planktonic and biofilm bacteria, while the combination treatment in continuous infusion exhibited enhanced bacterial killing and resistance suppression of carbapenem-resistant hypermutable *P. aeruginosa*. The results from these works emphasize the importance of using PK/PD optimized combinations of antibiotics when treating hypermutable *P. aeruginosa* biofilm-related infections (Bilal et al. 2020, 2019).

The role of mutational resistance has been revealed as a key mechanism for antibiotic resistance in biofilms. In a recent study, *P. aeruginosa* mutants showing diverse resistance mechanisms, such as β-lactamase or efflux pumps hyperexpression, were studied in biofilms (Rojo-Molinero et al. 2019). As expected, biofilms formed by AmpC (*dacB*) and MexAB-OprM (*mexR*) hyperproducing mutants showed enhanced β-lactam (cefepime) resistance (Rojo-Molinero et al. 2019). Resistant mutants (*dacB*) were selected and amplified in the competition with the wild type under cefepime treatment (Rojo-Molinero et al. 2019). Under the pressure of antibiotics, resistant mutants were also selected and amplified in biofilms. Interestingly, in mixed biofilms, resistant mutants shielded susceptible populations from antibiotic treatment (Rojo-Molinero et al. 2019). This finding could help to decipher the role of the frequent phenomenon of multi-strain/species in biofilms (Ciofu et al. 2012) and to determine why susceptible variants are not effortlessly eradicated from CF patients despite long-term intensive antimicrobial treatments (Rojo-Molinero et al. 2019).

The impact of the resistance mechanisms on biofilm community was also studied in preceding studies. In a work by Mulet et al. (2011) it was demonstrated that protective resistance mechanisms are different in biofilm vs planktonic growth. As it is well known, the most relevant *P. aeruginosa* resistance mechanisms include the overexpression of the chromosomal β-lactamase AmpC, the inactivation of the carbapenem porin OprD or the overexpression of different efflux pumps encoded in its genome (Livermore 2002; Poole 2004). Unfortunately, the combinations of these resistance pathways are often cumulative or synergistic, frequently driving to multidrug resistance. However, one of these mechanisms, the overexpression of the MexCD-OprJ efflux pump (caused by inactivation of its negative regulator NfxB), collaterally yields a notable hypersusceptibility to other relevant antipseudomonal agents, including most β-lactams and aminoglycosides (Gotoh et al. 1998). The key to this is that overexpression of the MexCD-OprJ efflux pump causes major changes in the cell envelope physiology, impairing the backbone of *P. aeruginosa* intrinsic resistance, including the major constitutive (MexAB-OprM) and inducible (MexXY-OprM) efflux pumps and the inducible AmpC β-lactamase. In addition, it also impaired the constitutive AmpC overexpression through a dramatic decrease in periplasmic-lactamase activity, produced by an abnormal permeation of AmpC out of the cell. Interestingly, although non-protective on planktonic cells, this permeation of AmpC into the matrix resulted in a protective mechanism for biofilm

communities against β-lactams. Therefore, in accordance with other works previously mentioned, hyperexpression of MexCD-OprJ efflux pump by *nfxB* mutants is a resistance mechanism that has been found to be beneficial and protective in biofilms (Mulet et al. 2009, 2011; Macià et al. 2011).

Similar findings have been confirmed in other bacterial species like *Acinetobacter baumanii* where, by using experimental evolution experiments, it was demonstrated that antibiotic resistance mechanisms were dependent upon environmental structure and bacterial lifestyle (Santos-Lopez et al. 2019). Similarly, in evolution experiments with *A. baumanii* and *P. aeruginosa* exposed to increasing concentrations of tobramycin in planktonic and biofilm environments, both species acquired antibiotic resistance-associated mutations that were more prevalent in the biofilm lifestyle than in planktonic cells (Scribner et al. 2020).

The development of mutational resistance has been demonstrated for almost all antibiotic classes in the chronic respiratory infection context. For example, in addition to the selection of mutants hyperexpressing MexXY-OprM, high-level aminoglycoside resistance driven by mutations in *fusA1*/*fusA2* has been documented in the CF setting (López-Causapé et al. 2017, 2018). Similarly, the PhoPQ regulatory system, PmrB, and ParS/ParR have been identified as hot spots of mutation that promote polymyxin resistance of *P. aeruginosa* isolated from colistin-treated CF patients (Gutu et al. 2013; Moskowitz et al. 2012; Greipel et al. 2016).

In summary, mutation-driven resistance has been shown to be a key factor in the development of resistance to antibiotics in biofilms. As a whole, the tolerance processes, which precede and support the development of classical resistance, as well as the biofilm microenvironment, which enhances mutagenesis and genomic and phenotypic diversification, are convergent forces that promote the development of mutational resistance. Figure 1 schematically illustrates this sequential process.

## 3.2 Horizontal Gene Transfer

In general, horizontal gene transfer, either by conjugation, transformation, or phage transduction mechanisms, is a principal source of evolution.

Within a biofilm, horizontal transfer of resistance-conferring genes between bacterial cells has been reported as being 700 times more efficient than among free-living, planktonic bacterial cells (Flemming et al. 2016).

Biofilms are ideally suited to the exchange of genetic material of various origins since conditions, such as high cell density and proximity, as well as increased genetic competence and accumulation of genetic elements are provided in their microenvironment (Flemming et al. 2016; Molin and Tolker-Nielsen 2003). Indeed, the conjugation mechanism itself may stimulate biofilm development (Madsen et al. 2012). Likewise, transformation seems to be part of a biofilm-related life cycle and both of these gene-transfer mechanisms may be auto-catalytically promoted in biofilms (Madsen et al. 2012).

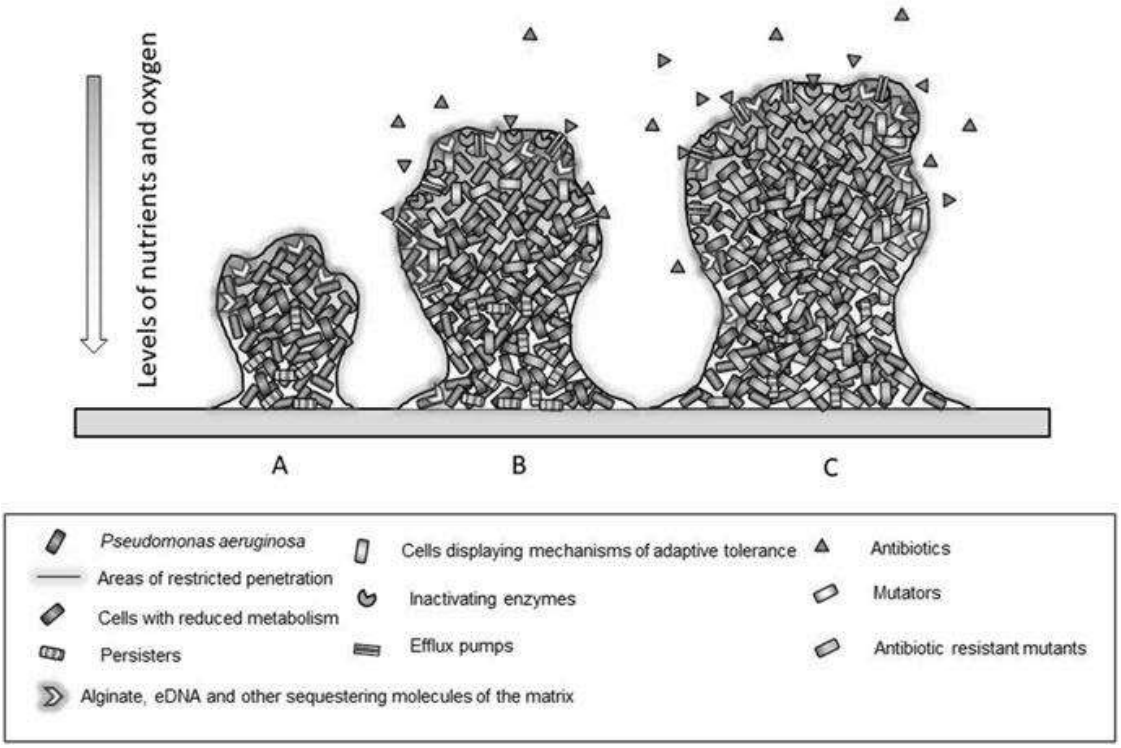


**Fig. 1** Graphic representation of the sequential process of antibiotic resistance development in biofilm. (**a**) Innate tolerance of biofilms represented by physical (restricted penetration, sequestering molecules), physiological (gradients of oxygen and nutrients, reduced metabolism) and *persisters* phenomena. (**b**) Addition of adaptive tolerance represented by cells displaying mechanisms such as hyperexpression of inactivating enzymes and/or efflux pumps. Presence of mutators. (**c**) Addition of antibiotic conventional resistance mechanisms represented by the selection and amplification of antibiotic-resistant mutants

Moreover, it has been shown that biofilms facilitate horizontal gene transfer and plasmids induce biofilm formation (Ghigo 2001). Plasmid transfer has been proven to occur at a higher frequency in biofilm cultures of different bacterial species (Kouzel et al. 2015; Savage et al. 2013; Hennequin et al. 2012; Tanner et al. 2017).

Table 1 summarizes the mechanisms of antibiotic resistance described in biofilms.

## *3.3 Conclusion and Future Perspectives*

Biofilms are hotspots for the development of antibiotic resistance due to the intrinsic phenotypic tolerance linked to the mutagenic microenvironment that helps emergence, selection and amplification of mutational resistance, together with the proficiency in transferring resistance genes.

In recent years, many studies have investigated the different mechanisms underlying antibiotic resistance in biofilms; however, there are still important knowledge

**Table 1** Mechanisms of biofilm antibiotic resistance

| | Tolerance | | | Acquired resistance | |
|---|---|---|---|---|---|
| Antibiotics | Physical tolerance | Physiological tolerance | Adaptive tolerance | Mutational resistance | Horizontal gene transfer |
| β-lactams | • Restricted penetration | • Low metabolic activity (Walters et al. 2003)<br>• *Persisters* (Lewis 2008; Lewis 2010)<br>• Non-specific upregulation of efflux pumps (MexAB-oprM) (Liao et al. 2013; Poole 2014) | • Induction of AmpC β-lactamase (Giwercman et al. 1991; Giwercman et al. 1992; Bagge et al. 2004b)<br>• Repression of OprD (Livermore 2002; Poole 2004) | • Mutants overexpressing AmpC (*dacB*) (Rojo-Molinero et al. 2019)<br>• Mutants overexpressing MexAB-OprM (*mexR*) (Rojo-Molinero et al. 2019)<br>• Mutations in OprD (Bilal et al. 2019, 2020) | • $bla_{NDM-1}$ plasmids (Tanner et al. 2017)<br>• conjugative plasmid carring bla(CTX-M-15), bla(OXA-1) and aac (6′)-Ib-cr genes (Hennequin et al. 2012) |
| Fluoroquinolones | • No effect | • Low metabolic activity (Walters et al. 2003; Bernier et al. 2013)<br>• Anaerobic conditions (Jensen et al. 2014)<br>• Non-specific upregulation of efflux pumps (MexCD-OprJ, MexEF-OprN) (Whiteley et al. 2001; Liao et al. 2013; Poole 2014)<br>• *Persisters* (Lewis 2008; Lewis 2010) | • Upregulation of efflux pumps (MexCD-OprJ, MexEF-OprN) (Poole 2014) | • Mutants overexpressing MexEF-OprN/MexCD-OprJ (Macià et al. 2011)<br>• Mutations in *GyrA/GyrB (*Macià et al. 2011*)* | |
| Aminoglycosides | • Restricted penetration (binding alginate, eDNA) (Tseng et al. 2013; Chiang et al. 2013; Cao et al. 2016; Mulcahy et al. 2008) | • Low metabolic activity (Walters et al. 2003)<br>• Anaerobic conditions (Walters et al. 2003)<br>• *Persisters* (Lewis 2008, 2010) | • Upregulation of efflux pumps (MexXY-OprM) (Poole 2014) | • Mutants overexpressing<br>• MexXY-OprM, *fusA1/fusA2* mutants (López-Causapé et al. 2017, 2018) | |

| | | | | | |
|---|---|---|---|---|---|
| | | • Non-specific upregulation of efflux pumps (MexXY-OprM) (Liao et al. 2013; Zhang and Mah 2008; Poole 2014) | | | |
| Colistin | • Restricted penetration (binding alginate, eDNA) (Pamp et al. 2008a) | | • Upregulation of efflux pumps (MexAB-OprM) (Pamp et al. 2008a)<br>• Upregulation of *pmr/arn* genes and LPS modification (Mulcahy et al. 2008) | • PhoPQ (Gutu et al. 2013), PmrAB (Moskowitz et al. 2012), and ParRS mutants (Greipel et al. 2016) | |
| Azithromcycin | | | • Upregulation of efflux pumps (MexCD-OprJ) | • Mutants overexpressing MexCD-OprJ (Mulet et al. 2009) | |

gaps. A better understanding of these molecular mechanisms could lead to improvements in the effective treatment of biofilm-related infections.

Considering the multifactorial nature of antibiotic resistance in biofilms, strategies adopted to overcome resistance should take into account the different systems acting together and efficiently combine different strategies to treat biofilm-related infections. For instance, the use of quorum sensing inhibitors to prevent and/or disrupt biofilms (Jakobsen et al. 2013), the use of SDS, EDTA, chlorhexidine or antibiotics like colistin to activate metabolically inactive cells (Pamp et al. 2008b), or restocking lacking metabolites and electron acceptors to revert tolerance to susceptibility (Crabbé et al. 2019), hyperbaric oxygen treatment (HBOT) to enhance antibiotic activity (Gade et al. 2018; Kolpen et al. 2016; Lerche et al. 2017), treatment with eDNAses (Yu et al. 2015) and/or oligo G to attack the matrix (Hengzhuang et al. 2016), use of efflux pumps inhibitors (EPIs) directed to the adaptive resistance (Kvist et al. 2008), use of antioxidants like N-Acetylcysteine to target hypermutation, SOS, and oxidative stress (Skov et al. 2015), or improving therapeutic antibiotic strategies, as well as PK/PD parameters, to overwhelm mutation selection like combination of antibiotics (Bilal et al. 2019, 2020; Jakobsen et al. 2013; Maciá et al. 2006; Plasencia et al. 2007), innovative sequential regimens (Rojo-Molinero et al. 2016), or topical administration (Krajewski et al. 2014; Heijerman et al. 2009). Progress in new alternative therapeutic approaches like formulation of antibiotics in nanoparticles (Pelgrift and Friedman 2013), novel anti-biofilm compounds, bacteriophage therapy (Pires et al. 2017), CRISPR gene editing technologies and photodynamic therapies is necessary. In addition, an important effort on transferring all these treatment options into real and practical therapeutic guidelines for the treatment of biofilm-related infections is urgently needed.

**Acknowledgments** María D. Macià and Antonio Oliver are supported by the Spanish Network for Research in Infectious Diseases (REIPI RD16/0016).

## References

Alhede M, Kragh KN, Qvortrup K, Allesen-Holm M, van Gennip M, Christensen LD, Jensen PØ, Nielsen AK, Parsek M, Wozniak D, Molin S, Tolker-Nielsen T, Høiby N, Givskov M, Bjarnsholt T (2011) Phenotypes of non-attached *Pseudomonas aeruginosa* aggregates resemble surface attached biofilm. PLoS One 6(11):e27943. https://doi.org/10.1371/journal.pone.0027943. Epub 2011 Nov 21. PMID: 22132176; PMCID: PMC3221681

Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP et al (2004a) *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. Antimicrob Agents Chemother 48:1175–1187. https://doi.org/10.1128/AAC.48.4.1175-1187.2004

Bagge N, Hentzer M, Andersen JB, Ciofu O, Givskov M, Høiby N (2004b) Dynamics and spatial distribution of beta-lactamase expression in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemother 48:1168–1174. https://doi.org/10.1128/AAC.48.4.1168-1174

Barraud N, Buson A, Jarolimek W, Rice SA (2013) Mannitol enhances antibiotic sensitivity of persister bacteria in *Pseudomonas aeruginosa* biofilms. PLoS One 8:e84220. https://doi.org/10.1371/journal.pone.0084220

Bernier SP, Lebeaux D, DeFrancesco AS, Valomon A, Soubigou G, Coppee JY et al (2013) Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. PLoS Genet 9:e1003144. https://doi.org/10.1371/journal.pgen.1003144

Bilal H, Bergen PJ, Kim TH, Chung SE, Peleg AY, Oliver A, Nation RL, Landersdorfer CB (2019 Oct 22) Synergistic meropenem-tobramycin combination dosage regimens against clinical hypermutable *Pseudomonas aeruginosa* at simulated epithelial lining fluid concentrations in a dynamic biofilm model. Antimicrob Agents Chemother 63(11):e01293–19. https://doi.org/10.1128/AAC.01293-19. PMID: 31427301; PMCID: PMC6811397

Bilal H, Bergen PJ, Tait JR, Wallis SC, Peleg AY, Roberts JA, Oliver A, Nation RL, Landersdorfer CB (2020 Jun 23) Clinically relevant epithelial lining fluid concentrations of meropenem with ciprofloxacin provide synergistic killing and resistance suppression of hypermutable *pseudomonas aeruginosa* in a dynamic biofilm model. Antimicrob Agents Chemother 64(7): e00469–20. https://doi.org/10.1128/AAC.00469-20. PMID: 32366710; PMCID: PMC7318014

Bjarnsholt T, Jensen PO, Burmolle M et al (2005) *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology 151:373–383

Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sorensen SR, Moser C, Kuhl M et al (2013) The in vivo biofilm. Trends Microbiol 21:466–474. https://doi.org/10.1016/j.tim.2013.06.002

Blázquez J, Oliver A, Gómez-Gómez JM (2002 Aug) Mutation and evolution of antibiotic resistance: antibiotics as promoters of antibiotic resistance? Curr Drug Targets 3(4):345–349. https://doi.org/10.2174/1389450023347579

Boles BR, Singh PK (2008 Aug 26) Endogenous oxidative stress produces diversity and adaptability in biofilm communities. Proc Natl Acad Sci U S A 105(34):12503–8. https://doi.org/10.1073/pnas.0801499105. Epub 2008 Aug 21. PMID: 18719125; PMCID: PMC2527941

Bowler PG, Welsby S, Towers V, Booth R, Hogarth A, Rowlands V, Joseph A, Jones SA (2012 Aug) Multidrug-resistant organisms, wounds and topical antimicrobial protection. Int Wound J 9(4):387–96. https://doi.org/10.1111/j.1742-481X.2012.00991.x. Epub 2012 May 29. PMID: 22640181; PMCID: PMC7950580

Brauner A, Fridman O, Gefen O, Balaban NQ (2016) Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 14:320–330

Burmolle M, Thomsen TR, Fazli M, Dige I, Christensen L, Homoe P et al (2010) Biofilms in chronic infections–a matter of opportunity– monospecies biofilms in multispecies infections. FEMS Immunol Med Microbiol 59:324–336. https://doi.org/10.1111/j.1574-695X.2010.00714.x

Cao B, Christophersen L, Thomsen K, Sønderholm M, Bjarnsholt T, Jensen PØ, Høiby N, Moser C (2015 Jul) Antibiotic penetration and bacterial killing in a *Pseudomonas aeruginosa* biofilm model. J Antimicrob Chemother 70(7):2057–2063. https://doi.org/10.1093/jac/dkv058. Epub 2015 Mar 18

Cao B, Christophersen L, Kolpen M, Jensen PØ, Sneppen K, Høiby N, Moser C, Sams T (2016 Apr 21) Diffusion retardation by binding of tobramycin in an alginate biofilm model. PLoS One 11(4):e0153616. https://doi.org/10.1371/journal.pone.0153616. PMID: 27100887; PMCID: PMC4839563

Chiang WC, Nilsson M, Jensen PO, Hoiby N, Nielsen TE, Givskov M et al (2013) Extracellular DNA shields against aminoglycosides in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemother 57:2352–2361. https://doi.org/10.1128/AAC.00001-13

Ciofu O, Tolker-Nielsen T (2019 May 3) Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents-how *P. aeruginosa* can escape antibiotics. Front Microbiol 10: 913. https://doi.org/10.3389/fmicb.2019.00913. PMID: 31130925; PMCID: PMC6509751

Ciofu O, Mandsberg LF, Wang H, Høiby N (2012 Jul) Phenotypes selected during chronic lung infection in cystic fibrosis patients: implications for the treatment of *Pseudomonas aeruginosa* biofilm infections. FEMS Immunol Med Microbiol 65(2):215–25. https://doi.org/10.1111/j.1574-695X.2012.00983.x. Epub 2012 Jun 1. Erratum in: FEMS Immunol Med Microbiol. 2012 Oct;66(1):120

Conibear TC, Collins SL, Webb JS (2009) Role of mutation in *Pseudomonas aeruginosa* biofilm development. PLoS One 4:e6289

Costerton JW, Cheng KJ, Geesey GG et al (1987) Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Crabbé A, Jensen PØ, Bjarnsholt T, Coenye T (2019 Oct) Antimicrobial tolerance and metabolic adaptations in microbial biofilms. Trends Microbiol. 27(10):850–863. https://doi.org/10.1016/j.tim.2019.05.003. Epub 2019 Jun 6

Driffield K, Miller K, Bostock JM, O'Neill AJ, Chopra I (2008 May) Increased mutability of *Pseudomonas aeruginosa* in biofilms. J Antimicrob Chemother 61(5):1053–1056. https://doi.org/10.1093/jac/dkn044. Epub 2008 Feb 6

Flemming H-C, Wingender J, Szewwzyk U, Steinberg P, Rice SA, Kjelleberg S (2016) Biofilms: an emergent form of bacterial life. Nat Rev Microbiol 14(9):563–575

Fux CA, Costerton JW, Stewart PS, Stoodley P (2005 Jan) Survival strategies of infectious biofilms. Trends Microbiol 13(1):34–40. https://doi.org/10.1016/j.tim.2004.11.010

Gade PAV, Olsen TB, Jensen PØ, Kolpen M, Høiby N, Henneberg KÅ, Sams T (2018 Jun 14) Modelling of ciprofloxacin killing enhanced by hyperbaric oxygen treatment in *Pseudomonas aeruginosa* PAO1 biofilms. PLoS One 13(6):e0198909. https://doi.org/10.1371/journal.pone.0198909. PMID: 29902223; PMCID: PMC6002088

Ghigo JM (2001 Jul 26) Natural conjugative plasmids induce bacterial biofilm development. Nature 412(6845):442–445. https://doi.org/10.1038/35086581

Giwercman B, Jensen ET, Høiby N, Kharazmi A, Costerton JW (1991 May) Induction of beta-lactamase production in *Pseudomonas aeruginosa* biofilm. Antimicrob Agents Chemother 35(5):1008–10. https://doi.org/10.1128/AAC.35.5.1008. PMID: 1906694; PMCID: PMC245148

Giwercman B, Meyer C, Lambert PA, Reinert C, Høiby N (1992 Jan) High-level beta-lactamase activity in sputum samples from cystic fibrosis patients during antipseudomonal treatment. Antimicrob Agents Chemother 36(1):71–6. https://doi.org/10.1128/AAC.36.1.71. PMID: 1590704; PMCID: PMC189229

Gotoh N et al (1998) Characterization of the MexC-MexD-OprJ multidrug efflux system in ΔmexA-mexB-oprM mutants of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 42:1938–1943

Greipel L, Fischer S, Klockgether J, et al. (2016) Molecular epidemiology of mutations in antimicrobial resistance loci of *pseudomonas aeruginosa* isolates from airways of cystic fibrosis patients. Antimicrob Agents Chemother 60(11):6726–6734. Published 2016 Oct 21. https://doi.org/10.1128/AAC.00724-16

Gutiérrez O, Juan C, Pe'rez JL, Oliver A (2004) Lack of association between hypermutation and antibiotic resistance development in *Pseudomonas aeruginosa* isolates from intensive care unit patients. Antimicrob Agents Chemother 48:3573–3575

Gutu AD, Sgambati N, Strasbourger P, Brannon MK, Jacobs MA, Haugen E, Kaul RK, Johansen HK, Høiby N, Moskowitz SM (2013 May) Polymyxin resistance of *Pseudomonas aeruginosa* phoQ mutants is dependent on additional two-component regulatory systems. Antimicrob Agents Chemother 57(5):2204–15. https://doi.org/10.1128/AAC.02353-12. Epub 2013 Mar 4. PMID: 23459479; PMCID: PMC3632916

Haaber J, Cohn MT, Frees D, Andersen TJ, Ingmer H (2012) Planktonic aggregates of *Staphylococcus aureus* protect against common antibiotics. PLoS One 7(7):e41075. https://doi.org/10.1371/journal.pone.0041075. Epub 2012 Jul 18. Erratum in: PLoS One. 2012;7(10). https://doi.

org/10.1371/annotation/08d0f2a8-0c40-4a0c-b546-0025648e73f0. PMID: 22815921; PMCID: PMC3399816

Heijerman H, Westerman E, Conway S, Touw D, Döring G (2009) Inhaled medication and inhalation devices for lung disease in patients with cystic fibrosis: a European consensus. J Cyst Fibros 8:295–315

Hengzhuang W, Song Z, Ciofu O, Onsøyen E, Rye PD, Høiby N (2016 Apr 22) OligoG CF-5/20 disruption of mucoid *pseudomonas aeruginosa* biofilm in a murine lung infection model. Antimicrob Agents Chemother 60(5):2620–6. https://doi.org/10.1128/AAC.01721-15. PMID: 26833153; PMCID: PMC4862494

Hennequin C, Aumeran C, Robin F, Traore O, Forestier C (2012 Sep) Antibiotic resistance and plasmid transfer capacity in biofilm formed with a CTX-M-15-producing Klebsiella pneumoniae isolate. J Antimicrob Chemother 67(9):2123–2130. https://doi.org/10.1093/jac/dks169. Epub 2012 May 10

Høiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G et al (2015a) ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infect 21(Suppl. 1): S1–S25. https://doi.org/10.1016/j.cmi.2014.10.024)

Høiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G et al (2015b) ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infect 21(Suppl. 1): S1–S25. https://doi.org/10.1016/j.cmi.2014.10.024

Imamura Y, Higashiyama Y, Tomono K, Izumikawa K, Yanagihara K, Ohno H, Miyazaki Y, Hirakata Y, Mizuta Y, Kadota J, Iglewski BH, Kohno S (2005 Apr) Azithromycin exhibits bactericidal effects on *Pseudomonas aeruginosa* through interaction with the outer membrane. Antimicrob Agents Chemother 49(4):1377–80. https://doi.org/10.1128/AAC.49.4.1377-1380.2005. PMID: 15793115; PMCID: PMC1068619

Ito A, Taniuchi A, May T, Kawata K, Okabe S (2009 Jun) Increased antibiotic resistance of *Escherichia coli* in mature biofilms. Appl Environ Microbiol 75(12):4093–100. https://doi.org/10.1128/AEM.02949-08. Epub 2009 Apr 17. PMID: 19376922; PMCID: PMC2698376

Jakobsen TH, Bjarnsholt T, Jensen PØ, Givskov M, Høiby N (2013 Jul) Targeting quorum sensing in *Pseudomonas aeruginosa* biofilms: current and emerging inhibitors. Future Microbiol 8(7): 901–921. https://doi.org/10.2217/fmb.13.57

Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz MA, Hussain T, Ali M, Rafiq M, Kamil MA (2018) Bacterial biofilm and associated infections. J Chin Med Assoc 81(1):7–11. https://doi.org/10.1016/j.jcma.2017.07.012

Jensen PO, Briales A, Brochmann RP, Wang H, Kragh KN, Kolpen M et al (2014) Formation of hydroxyl radicals contributes to the bactericidal activity of ciprofloxacin against *Pseudomonas aeruginosa* biofilms. Pathog Dis 70:440–443. https://doi.org/10.1111/2049-632X.12120

Kenna DT, Doherty CJ, Fowerake J, Macaskill L, Barcus VA, Govan JRW (2007 Jun) Hypermutability in environmental *Pseudomonas aeruginosa* and in populations causing pulmonary infection in individuals with cystic fibrosis. Microbiology (Reading). 153(Pt 6): 1852–1859. https://doi.org/10.1099/mic.0.2006/005082-0

Kindrachuk KN, Fernandez L, Bains M, Hancock RE (2011) Involvement of an ATP-dependent protease, PA0779/AsrA, in inducing heat shock in response to tobramycin in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 55:1874–1882. https://doi.org/10.1128/AAC.00935-10

Kolpen M, Hansen CR, Bjarnsholt T, Moser C, Christensen LD, van Gennip M et al (2010) Polymorphonuclear leucocytes consume oxygen in sputum from chronic *Pseudomonas aeruginosa* pneumonia in cystic fibrosis. Thorax 65:57–62. https://doi.org/10.1136/thx.2009.114512

Kolpen M, Appeldorff CF, Brandt S, Mousavi N, Kragh KN, Aydogan S, Uppal HA, Bjarnsholt T, Ciofu O, Høiby N, Jensen PØ (2016 Feb) Increased bactericidal activity of colistin on *Pseudomonas aeruginosa* biofilms in anaerobic conditions. Pathog Dis 74(1):ftv086. https://doi.org/10.1093/femspd/ftv086. Epub 2015 Oct 12. PMID: 26458402; PMCID: PMC4655427

Kouzel N, Oldewurtel ER, Maier B (2015 Jul) Gene transfer efficiency in gonococcal biofilms: role of biofilm age, architecture, and pilin antigenic variation. J Bacteriol 197(14):2422–31. https://doi.org/10.1128/JB.00171-15. Epub 2015 May 11. PMID: 25962915; PMCID: PMC4524189

Kragh KN, Alhede M, Jensen PØ, Moser C, Scheike T, Jacobsen CS, Seier Poulsen S, Eickhardt-Sørensen SR, Trøstrup H, Christoffersen L, Hougen HP, Rickelt LF, Kühl M, Høiby N, Bjarnsholt T (2014 Nov) Polymorphonuclear leukocytes restrict growth of *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients. Infect Immun 82(11):4477–86. https://doi.org/10.1128/IAI.01969-14. Epub 2014 Aug 11. PMID: 25114118; PMCID: PMC4249348

Krajewski J, Bode-Böger SM, Tröger U, Martens-Lobenhoffer J, Mulrooney T, Mittelstädt H, Russlies M, Kirchner R, Knobloch JK (2014 Oct) Successful treatment of extensively drug-resistant *Pseudomonas aeruginosa* osteomyelitis using a colistin- and tobramycin-impregnated PMMA spacer. Int J Antimicrob Agents 44(4):363–366. https://doi.org/10.1016/j.ijantimicag.2014.05.023. Epub 2014 Aug 13

Kvist M, Hancock V, Klemm P (2008 Dec) Inactivation of efflux pumps abolishes bacterial biofilm formation. Appl Environ Microbiol 74(23):7376–82. 10.1128/AEM.01310-08. Epub 2008 Oct 3. PMID: 18836028; PMCID: PMC2592912

Labat F, Pradillon O, Garry L, Peuchmaur M, Fantin B, Denamur E (2005 Jun 1) Mutator phenotype confers advantage in *Escherichia coli* chronic urinary tract infection pathogenesis. FEMS Immunol Med Microbiol 44(3):317–321. https://doi.org/10.1016/j.femsim.2005.01.003

Lerche CJ, Christophersen LJ, Kolpen M, Nielsen PR, Trøstrup H, Thomsen K, Hyldegaard O, Bundgaard H, Jensen PØ, Høiby N, Moser C (2017 Sep) Hyperbaric oxygen therapy augments tobramycin efficacy in experimental *Staphylococcus aureus* endocarditis. Int J Antimicrob Agents 50(3):406–412. https://doi.org/10.1016/j.ijantimicag.2017.04.025. Epub 2017 Jun 29

Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ (2017) Antibiotic tolerance facilitates the evolution of resistance. Science 355:826–830. https://doi.org/10.1126/science.aaj2191

Lewenza S (2013) Extracellular DNA-induced antimicrobial peptide resistance mechanisms in *Pseudomonas aeruginosa*. Front Microbiol 4:21. https://doi.org/10.3389/fmicb.2013.00021

Lewis K (2008) Multidrug tolerance of biofilms and persister cells. Curr Top Microbiol Immunol 322:107–131. https://doi.org/10.1007/978-3-540-75418-3_6

Lewis K (2010) Persister cells. Annu Rev Microbiol 64:357–372. https://doi.org/10.1146/annurev.micro.112408.134306

Liao J, Schurr MJ, Sauer K (2013) The MerR-like regulator BrlR confers biofilm tolerance by activating multidrug efflux pumps in biofilms. J Bacteriol 195:3352–3363. https://doi.org/10.1128/JB.00318-13

Livermore DM (2002) Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin Infect Dis 34:634–640

López-Causapé C, Sommer LM, Cabot G, Rubio R, Ocampo-Sosa AA, Johansen HK, Figuerola J, Cantón R, Kidd TJ, Molin S, Oliver A (2017 Jul 17) Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international Cystic Fibrosis clone. Sci Rep 7(1):5555. https://doi.org/10.1038/s41598-017-05621-5. PMID: 28717172; PMCID: PMC5514035

López-Causapé C, Rubio R, Cabot G, Oliver A (2018) Evolution of the *pseudomonas aeruginosa* aminoglycoside mutational resistome in vitro and in the cystic fibrosis setting. Antimicrob Agents Chemother 62(4):e02583–17. Published 2018 Mar 27. https://doi.org/10.1128/AAC.02583-17

Luján AM, Maciá MD, Yang L, Molin S, Oliver A, Smania AM (2011) Evolution and adaptation in *Pseudomonas aeruginosa* biofilms driven by mismatch repair system-deficient mutators. PLoS One 6(11):e27842. https://doi.org/10.1371/journal.pone.0027842. Epub 2011 Nov 17. PMID: 22114708; PMCID: PMC3219696

Maciá MD, Blanquer D, Togores B, Sauleda J, Pérez JL, Oliver A (2005) Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. Antimicrob Agents Chemother 49(8):3382–3386. https://doi.org/10.1128/AAC.49.8.3382-3386.2005

Maciá MD, Borrell N, Segura M, Gómez C, Pérez JL, Oliver A (2006 Mar) Efficacy and potential for resistance selection of antipseudomonal treatments in a mouse model of lung infection by hypermutable *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 50(3):975–83. https://doi.org/10.1128/AAC.50.3.975-983.2006. PMID: 16495260; PMCID: PMC1426455

Macià MD, Pérez JL, Molin S, Oliver A (2011 Nov) Dynamics of mutator and antibiotic-resistant populations in a pharmacokinetic/pharmacodynamic model of *Pseudomonas aeruginosa* biofilm treatment. Antimicrob Agents Chemother 55(11):5230–7. https://doi.org/10.1128/AAC.00617-11. Epub 2011 Aug 22. PMID: 21859941; PMCID: PMC3195006

Madsen JS, Burmølle M, Hansen LH, Sørensen SJ (2012 Jul) The interconnection between biofilm formation and horizontal gene transfer. FEMS Immunol Med Microbiol 65(2):183–195. https://doi.org/10.1111/j.1574-695X.2012.00960.x. Epub 2012 Apr 23

Mao EF, Lane L, Lee J, Miller JH (1997 Jan) Proliferation of mutators in A cell population. J Bacteriol 179(2):417–22. https://doi.org/10.1128/jb.179.2.417-422.1997. PMID: 8990293; PMCID: PMC178711

Martínez-Solano L, Macia MD, Fajardo A, Oliver A, Martinez JL (2008 Dec 15) Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. Clin Infect Dis 47(12):1526–1533. https://doi.org/10.1086/593186

Mena A, Smith EE, Burns JL, Speert DP, Moskowitz SM, Perez JL, Oliver A (2008 Dec) Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. J Bacteriol 190(24):7910–7. https://doi.org/10.1128/JB.01147-08. Epub 2008 Oct 10. PMID: 18849421; PMCID: PMC2593214

Miller JH (1996) Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. Annu Rev Microbiol 50:625–643. https://doi.org/10.1146/annurev.micro.50.1.625

Molin S, Tolker-Nielsen T (2003 Jun) Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Curr Opin Biotechnol 14(3):255–261. https://doi.org/10.1016/s0958-1669(03)00036-3

Montanari S, Oliver A, Salerno P, Mena A, Bertoni G, Tümmler B, Cariani L, Conese M, Döring G, Bragonzi A (2007 May) Biological cost of hypermutation in *Pseudomonas aeruginosa* strains from patients with cystic fibrosis. Microbiology (Reading). 153(Pt 5):1445–1454. https://doi.org/10.1099/mic.0.2006/003400-0

Moskowitz SM, Foster JM, Emerson J, Burns JL (2004 May) Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. J Clin Microbiol 42(5):1915–22. https://doi.org/10.1128/JCM.42.5.1915-1922.2004. PMID: 15131149; PMCID: PMC404629

Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, Johansen HK, Høiby N (2012 Feb) PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother 56(2):1019–30. https://doi.org/10.1128/AAC.05829-11. Epub 2011 Nov 21. PMID: 22106224; PMCID: PMC3264203

Mulcahy H, Charron-Mazenod L, Lewenza S (2008) Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. PLoS Pathog 4:e1000213. https://doi.org/10.1371/journal.ppat.1000213

Mulet X, Maciá MD, Mena A, Juan C, Pérez JL, Oliver A (2009 Apr) Azithromycin in *Pseudomonas aeruginosa* biofilms: bactericidal activity and selection of nfxB mutants. Antimicrob Agents Chemother 53(4):1552–60. https://doi.org/10.1128/AAC.01264-08. Epub 2009 Feb 2. PMID: 19188376; PMCID: PMC2663112

Mulet X, Moyá B, Juan C, Macià MD, Pérez JL, Blázquez J, Oliver A (2011 Oct) Antagonistic interactions of *Pseudomonas aeruginosa* antibiotic resistance mechanisms in planktonic but not biofilm growth. Antimicrob Agents Chemother 55(10):4560–8. https://doi.org/10.1128/AAC.00519-11. Epub 2011 Aug 1. PMID: 21807976; PMCID: PMC3186965

Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K et al (2011) Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334:982–986. https://doi.org/10.1126/science.1211037

Oliver A, Cantón R, Campo P, Baquero F, Blázquez J (2000) High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science 288:1251–1253

Oliver A, Baquero F, Blázquez J (2002) The mismatch repair system (mutS, mutL, and uvrD genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. Mol Microbiol 43:1641–1650

Oliver A, Mena A, Macià MD (2007) Evolution of *Pseudomonas aeruginosa* pathogenicity: from acute to chronic infections. In: Baquero F et al (eds) Evolutionary biology of bacterial and fungal pathogens. American Society for Microbiology, Washington, DC, pp 433–434

Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T (2008a) Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Mol Microbiol 68:223–240. https://doi.org/10.1111/j.1365-2958.2008.06152.x

Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T (2008b Apr) Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Mol Microbiol 68(1):223–240. https://doi.org/10.1111/j.1365-2958.2008.06152.x. Epub 2008 Feb 28

Pelgrift RY, Friedman AJ (2013) Nanotechnology as a therapeutic tool to combat microbial resistance. Adv Drug Deliv Rev 65:1803–1815

Petrova OE, Sauer K (2012 May) Sticky situations: key components that control bacterial surface attachment. J Bacteriol 194(10):2413–25. https://doi.org/10.1128/JB.00003-12. Epub 2012 Mar 2. PMID: 22389478; PMCID: PMC3347170

Pires DP, Melo L, Vilas Boas D, Sillankorva S, Azeredo J. Phage therapy as an alternative or complementary strategy to prevent and control biofilm-related infections. Curr Opin Microbiol 2017 Oct;39:48–56. https://doi.org/10.1016/j.mib.2017.09.004. Epub 2017 Sep 28

Plasencia V, Borrell N, Maciá MD, Moya B, Pérez JL, Oliver A (2007 Jul) Influence of high mutation rates on the mechanisms and dynamics of in vitro and in vivo resistance development to single or combined antipseudomonal agents. Antimicrob Agents Chemother 51(7):2574–81. https://doi.org/10.1128/AAC.00174-07. Epub 2007 Apr 30. PMID: 17470655; PMCID: PMC1913281

Poole K (2004) Efflux mediated multiresistance in gram-negative bacteria. Clin Microbiol Infect 10:12–26

Poole K (2014) Stress responses as determinants of antimicrobial resistance in *Pseudomonas aeruginosa*: multidrug efflux and more. Can J Microbiol 60:783–791. https://doi.org/10.1139/cjm-2014-0666

Poudyal B, Sauer K (2018) The ABC of biofilm drug tolerance: the MerR-like regulator BrlR is an activator of ABC transport systems, with PA1874-77 contributing to the tolerance of *Pseudomonas aeruginosa* biofilms to tobramycin. Antimicrob Agents Chemother 62:e01981–e01917. https://doi.org/10.1128/AAC.01981-17

Prunier AL, Malbruny B, Laurans M, Brouard J, Duhamel JF, Leclercq R (2003 Jun 1) High rate of macrolide resistance in *Staphylococcus aureus* strains from patients with cystic fibrosis reveals high proportions of hypermutable strains. J Infect Dis 187(11):1709–1716. https://doi.org/10.1086/374937. Epub 2003 May 15

Radman M, Matic I, Taddei F (1999 May 18) Evolution of evolvability. Ann N Y Acad Sci 870:146–155. https://doi.org/10.1111/j.1749-6632.1999.tb08874.x

Rodríguez-Rojas A, Blázquez J (2009 Feb) The *Pseudomonas aeruginosa* pfpI gene plays an antimutator role and provides general stress protection. J Bacteriol 191(3):844–50. https://doi.org/10.1128/JB.01081-08. Epub 2008 Nov 21. PMID: 19028889; PMCID: PMC2632063

Rojo-Molinero E, Macià MD, Rubio R, Moyà B, Cabot G, López-Causapé C, Pérez JL, Cantón R, Oliver A (2016 Apr 22) Sequential treatment of biofilms with aztreonam and tobramycin is a novel strategy for combating *Pseudomonas aeruginosa* chronic respiratory infections. Antimicrob Agents Chemother 60(5):2912–22. https://doi.org/10.1128/AAC.00196-16. PMID: 26926631; PMCID: PMC4862541

Rojo-Molinero E, Macià MD, Oliver A (2019 Mar 22) Social behavior of antibiotic resistant mutants within *Pseudomonas aeruginosa* biofilm communities. Front Microbiol 10:570. https://doi.org/10.3389/fmicb.2019.00570. PMID: 30967851; PMCID: PMC6438888

Saiman L, Marshall BC, Mayer-Hamblett N, Burns JL, Quittner AL, Cibene DA, Coquillette S, Fieberg AY, Accurso FJ, Campbell PW (2003 Oct 1) 3rd; macrolide study group. Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*: a randomized controlled trial. JAMA 290(13):1749–1756. https://doi.org/10.1001/jama.290.13.1749

Santos-Lopez A, Marshall CW, Scribner MR, Snyder DJ, Cooper VS (2019 Sep 13) Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. Elife 8:e47612. https://doi.org/10.7554/eLife.47612. PMID: 31516122; PMCID: PMC6814407

Savage VJ, Chopra I, O'Neill AJ (2013 Apr) *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. Antimicrob Agents Chemother 57(4):1968–70. https://doi.org/10.1128/AAC.02008-12. Epub 2013 Jan 28. PMID: 23357771; PMCID: PMC3623343

Schaible B, Schaffer K, Taylor CT (2010 Dec 31) Hypoxia, innate immunity and infection in the lung. Respir Physiol Neurobiol 174(3):235–243. https://doi.org/10.1016/j.resp.2010.08.006. Epub 2010 Aug 13

Scribner MR, Santos-Lopez A, Marshall CW, Deitrick C, Cooper VS (2020) Parallel evolution of tobramycin resistance across species and environments. mBio 11(3):e00932–20. Published 2020 May 26. https://doi.org/10.1128/mBio.00932-20

Skov M, Pressler T, Lykkesfeldt J, Poulsen HE, Jensen PØ, Johansen HK, Qvist T, Kræmer D, Høiby N, Ciofu O (2015 Mar) The effect of short-term, high-dose oral N-acetylcysteine treatment on oxidative stress markers in cystic fibrosis patients with chronic *P. aeruginosa* infection -- a pilot study. J Cyst Fibros 14(2):211–218. https://doi.org/10.1016/j.jcf.2014.09.015. Epub 2014 Oct 23

Smith EE et al (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103:8487–8492

Sniegowski PD, Gerrish PJ, Lenski RE (1997 Jun 12) Evolution of high mutation rates in experimental populations of E. coli. Nature 387(6634):703–705. https://doi.org/10.1038/42701

Stewart EJ, Ganesan M, Younger JG, Solomon MJ (2015 Aug 14) Artificial biofilms establish the role of matrix interactions in staphylococcal biofilm assembly and disassembly. Sci Rep 5:13081. https://doi.org/10.1038/srep13081. PMID: 26272750; PMCID: PMC4536489

Stewart PS, Zhang T, Xu R, Pitts B, Walters MC, Roe F et al (2016) Reaction-diffusion theory explains hypoxia and heterogeneous growth within microbial biofilms associated with chronic infections. NPJ Biofilms Microbiomes 2:16012. https://doi.org/10.1038/npjbiofilms.2016.12

Stoodley P, Sauer K, Davies DG, Costerton JW (2002) Biofilms as complex differentiated communities. Annu Rev Microbiol 56:187–209. https://doi.org/10.1146/annurev.micro.56.012302.160705. Epub 2002 Jan 30

Taddei F, Radman M, Maynard-Smith J, Toupance B, Gouyon PH, Godelle B (1997 Jun 12) Role of mutator alleles in adaptive evolution. Nature 387(6634):700–702. https://doi.org/10.1038/42696

Tanner WD, Atkinson RM, Goel RK, Toleman MA, Benson LS, Porucznik CA, VanDerslice JA (2017 Apr 1) Horizontal transfer of the blaNDM-1 gene to *Pseudomonas aeruginosa* and Acinetobacter baumannii in biofilms. FEMS Microbiol Lett 364(8). https://doi.org/10.1093/femsle/fnx048

Tata M, Wolfinger MT, Amman F, Roschanski N, Dötsch A et al (2016) RNASeq based transcriptional profiling of *Pseudomonas aeruginosa* PA14 after short- and long-term anoxic cultivation in synthetic cystic fibrosis sputum medium. PLoS One 11(1):e0147811. https://doi.org/10.1371/journal.pone.0147811

Tseng BS, Zhang W, Harrison JJ, Quach TP, Song JL, Penterman J, Singh PK, Chopp DL, Packman AI, Parsek MR (2013 Oct) The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. Environ Microbiol 15(10):2865–78. https://

doi.org/10.1111/1462-2920.12155. Epub 2013 Jun 10. PMID: 23751003; PMCID: PMC4045617

Van Acker H, Coenye T (2016 Jun 10) The role of efflux and physiological adaptation in biofilm tolerance and resistance. J Biol Chem 291(24):12565–12572. https://doi.org/10.1074/jbc.R115.707257. Epub 2016 Apr 21. PMID: 27129224; PMCID: PMC4933474

Van Acker H, Sass A, Bazzini S, De RK, Udine C, Messiaen T et al (2013) Biofilm-grown Burkholderia cepacia complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. PLoS One 8:e58943. https://doi.org/10.1371/journal.pone.0058943

Van Acker H, Gielis J, Acke M, Cools F, Cos P, Coenye T (2016 Jul 20) The role of reactive oxygen species in antibiotic-induced cell death in Burkholderia cepacia Complex Bacteria. PLoS One 11(7):e0159837. https://doi.org/10.1371/journal.pone.0159837. PMID: 27438061; PMCID: PMC4954720

Walters MC III, Roe F, Bugnicourt A, Franklin MJ, Stewart PS (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 47:317–323. https://doi.org/10.1128/AAC.47.1.317-323.2003

Watson ME, Burns JL, Smith AL (2004 Sep) Hypermutable Haemophilus influenzae with mutations in mutS are found in cystic fibrosis sputum. Microbiology (Reading) 150(Pt 9):2947–2958. https://doi.org/10.1099/mic.0.27230-0

Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, Koch B, Givskov M, Kjelleberg S (2003 Aug) Cell death in *Pseudomonas aeruginosa* biofilm development. J Bacteriol 185(15):4585–92. https://doi.org/10.1128/JB.185.15.4585-4592.2003. PMID: 12867469; PMCID: PMC165772

Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, Greenberg EP (2001 Oct 25) Gene expression in *Pseudomonas aeruginosa* biofilms. Nature 413(6858):860–864. https://doi.org/10.1038/35101627

Wilmaerts D, Windels EM, Verstraeten N, Michiels J (2019) General mechanisms leading to persister formation and awakening. Trends Genet 35:401–411

Wimpenny J, Manz W, Szewzyk U (2000) Heterogeneity in biofilms. FEMS Microbiol Rev 24:661–671

Woodford N, Ellington MJ (2007 Jan) The emergence of antibiotic resistance by mutation. Clin Microbiol Infect 13(1):5–18. https://doi.org/10.1111/j.1469-0691.2006.01492.x

Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC et al (2002) Effects of reduced mucus oxygen concentration in airway pseudomonas infections of cystic fibrosis patients. J Clin Invest 109:317–325. https://doi.org/10.1172/JCI0213870

Yu S, Su T, Wu H, Liu S, Wang D, Zhao T, Jin Z, Du W, Zhu MJ, Chua SL, Yang L, Zhu D, Gu L, Ma LZ (2015 Dec) PslG, a self-produced glycosyl hydrolase, triggers biofilm disassembly by disrupting exopolysaccharide matrix. Cell Res 25(12):1352–67. https://doi.org/10.1038/cr.2015.129. Epub 2015 Nov 27. PMID: 26611635; PMCID: PMC4670989

Zhang L, Mah TF (2008) Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. J Bacteriol 190:4447–4452. https://doi.org/10.1128/JB.01655-07