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Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa* biofilms

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ABSTRACT

Pseudomonas aeruginosa is a major cause of life-threatening acute infections and life-long lasting chronic infections. The characteristic biofilm mode of life in *P. aeruginosa* chronic infections severely limits the efficacy of antimicrobial therapies, as it leads to intrinsic tolerance, involving physical and physiological factors in addition to biofilm-specific genes that can confer a transient protection against antibiotics promoting the development of resistance. Indeed, a striking feature of this pathogen is the extraordinary capacity to develop resistance to nearly all available antibiotics through the selection of chromosomal mutations, evidenced by its outstanding and versatile mutational resistome. This threat is dramatically amplified in chronic infections, driven by the frequent emergence of mutator variants with enhanced spontaneous mutation rates. Thus, this mini review is focused on describing the complex interplay of antibiotic resistance mechanisms in *P. aeruginosa* biofilms, to provide potentially useful information for the design of effective therapeutic strategies.

1. Introduction

Biofilms, first described as microbial cells embedded in a self-produced extracellular polymeric matrix attached to either biotic or abiotic surfaces [1], are thought to stem from an adaptive social behaviour to survive in hostile environments [2]; including the human body during infection [3]. However, the definition of what a "biofilm" is has been adapted to advances in research to suit new findings. For instance, it has been found that some pathogenic biofilm aggregates can be formed without the need to attach to a surface, thus being able to grow within the mucus of individuals with cystic fibrosis (CF) and not in or on the lung tissue [4]. Due to the matrix protection, bacterial cells within a biofilm are significantly more recalcitrant to antibiotics and host immune defences than their planktonic counterparts, as shown by in vitro and in vivo evidence [5–8]. This fact poses a health issue, as the inability to clear the bacteria is directly related to the chronicity of the infections [9,10]. It is estimated that up to 65–80% of all infections are associated with biofilm formation [11], with chronic infections encompassing a myriad of diseases. Besides chronic respiratory infections, such as those occurring in the lungs of people with CF [12], biofilm-related chronic infections include, among others, those related to indwelling medical devices, (e.g., catheters, prosthetic joints, and surgical implants), tissue infections, such as otitis media, rhinosinusitis, osteomyelitis, and chronic wounds.

1.1. *Pseudomonas aeruginosa: a paradigmatic microorganism*

Pseudomonas aeruginosa is an opportunistic pathogen that causes severe infections, particularly in health care settings, mostly affecting immunocompromised patients [13]. There is a growing prevalence of nosocomial infections caused by *P. aeruginosa* strains, which are associated with significantly increased morbidity and mortality [14]. Likewise, *P. aeruginosa* is the main driver of chronic respiratory infections in CF and other respiratory diseases [10]. One of the most striking features of *P. aeruginosa,* which by itself is intrinsically resistant to many antibiotics, is its capacity to develop resistance to nearly all available antibiotics, through mutations in chromosomal genes, as well as acquire resistance markers through horizontal transfer, which has led to the worldwide spreading of a few specific multidrug-resistant (MDR) and extensively drug-resistant (XDR) high-risk clones [15,16]. Indeed, the outstanding capacity of *P. aeruginosa* for developing antimicrobial resistance is for seen in the extraordinary versatility of is vast mutational

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resistome, not only dependent on the exposure to a specific antibiotic but conditioned by simultaneous or even previous exposures to other [17,18]. Recent studies have estimated 4.95 million deaths associated with antimicrobial resistance (AMR) in 2019, including 1.27 million deaths attributable to bacterial AMR, *P. aeruginosa* being the sixth pathogen on the list [19].

In addition to the threat that poses this pathogen regarding AMR, *P. aeruginosa* infections are also important to be related with biofilms, being an archetypal microorganism for their study.

1.2. Tolerance to antibiotics in Pseudomonas aeruginosa biofilms

Biofilm antibiotic tolerance mechanisms involve a wide range of environmental, physical, and physiological factors that, in principle, confer a transient protection; in opposition to conventional resistance mechanisms; meaning that individual cells remain susceptible to previously exposed antibiotics despite being able to survive to lethal doses during biofilm growth mode.

The presence of an extracellular matrix is probably one of the most characteristic factors involved in biofilm antibiotic tolerance. As it is well known, the matrix consists of exopolysaccharides, proteins, extracellular DNA (eDNA) and lipids [20,21], yet its exact composition will vary depending on the bacterial strain, the growth conditions, and the biofilm maturation stage [22]. Interactions with the matrix components, as anionic eDNA and alginate [23–25] can capture positively charged classes of antibiotics, such as colistin or tobramycin, and consequently reduce their activity [23,26–28], whereas neutral antibiotics, like ciprofloxacin, penetrate more easily into the biofilms [24]. In general, the transition through the matrix entails a delay in the antibiotic-bacteria contact and the exposition to lower concentrations, giving bacteria time to become tolerant [29,30]. In fact, regarding the pharmacokinetic (PK) and pharmacodynamic (PD) parameters of antibiotics, biofilms have been proposed as a third pharmacological compartment, in addition to the blood and the target tissue of the infection [31,32]. Accordingly, the existence of nutrients and oxygen gradients results in differential subcompartments [33] differentiating two main subpopulations; one metabolically active, frequently in the outer layers of the biofilm, and one less active (or even inactive), in the deeper ones [34,35]. This should be considered, since several antibiotics only target processes of growing bacteria (like replication, transcription, translation, or cell wall synthesis), while others are effective on metabolically inactive cells, to establish the basis for combination therapies [33,36,37].

The most extreme case of decreased metabolism, also found in the biofilm, is represented by the *persisters*. This is a special growth state that means less than 0.1% of the biofilm population, refractory to antibiotics, a kind of spore-like cell state activity that can become active after finishing the treatment [38,39].

The lack of oxygen inside the biofilm also plays an important role in tolerance to some antibiotics that work under aerobic conditions (e.g., beta-lactams, aminoglycosides, fluoroquinolones and tetracyclines) [40], being only effective against bacteria on the periphery [23,41]. Fortunately, polymyxins and other membrane-targeting compounds such as SDS, EDTA, and chlorhexidine, maintain their anti-biofilm activity [34,42–44] despite hypoxia.

Loss of antibiotic activity could also be partly explained by the deficiency of reactive oxygen species (ROS) production under anaerobic conditions. Induction of ROS by some bactericidal antibiotics is thought to contribute to their killing effect, as evidenced by the emergence of cytotoxic hydroxyl radicals (•OH) in *P. aeruginosa* biofilms treated with ciprofloxacin [45]. The antioxidant systems are also upregulated due to the stringent response in biofilms [46,47], participating in their tolerance to antibiotics. On the contrary, in response to different forms of stress (oxidative stress, nitrosative stress and membrane-damaging agents) the up-regulation of the efflux pumps, like MexXY-OprM, MexEF-OprN and MexCD-OprJ, can be triggered in *P. aeruginosa* as non-specific antibiotic tolerance mechanism [48,49].

Other than biofilm environment intrinsic factors leading to physiological tolerance, there is also an in vivo contribution of the host immune system mediated, for example, by the polymorphonuclear (PMNs) leukocytes action as it is demonstrated in the endotracheal mucus from people with CF infected by *P. aeruginosa* [50,51] where PMNs are known to consume oxygen and release eDNA that traps cationic antibiotics [51–53].

The differences between planktonic and biofilm modes of life are also related with differential gene expression even in the absence of antibiotics [54–58]. This is the case, for instance, of *brlR*, which is a Mer-like transcriptional activator [59] that stimulates the expression of the MexAB-oprM and MexEF-oprN efflux pumps [60], the production of the ABC (ATP-Binding Cassette) transporters, (like the ABC transporter PA1874-1877, 10 times more expressed in *P. aeruginosa* biofilms [59,61]), or alters the expression of genes encoding modification of lipopolysaccharide (LPS), membrane protein composition, or metabolism and energy generation [62]. Consequently, increased expression of *brlR* in biofilms lowered the susceptibility to hydrogen peroxide and five different classes of antibiotics by increasing the minimum inhibitory concentrations (MICs) up to 6-fold [63]. On the contrary, *brlR* represses *phoPQ* expression increasing susceptibility to colistin so; the reciprocal role of *brlR* enhancing colistin susceptibility while increasing resistance to other antibiotics, like tobramycin, provides the genetic basis for their use in combination [64]. The expression of other genes, like *ndvB*, coding a glycosyltransferase involved in the formation of cyclic glucans [65] that can sequester aminoglycoside antibiotics is also augmented in biofilms [64,66,67].

Besides the increased expression of specific genes on biofilms compared to planktonic**,** adaptive tolerance mechanisms induced by the presence of the antibiotic (especially favoured by sub-inhibitory concentrations [68–70]) results in an emerging transient tolerant phenotype that reverts to susceptibility once the molecules have disappeared. For example, the presence of colistin upregulates the two-component regulatory system *pmr* which, in turn, regulates *arn* genes, leading to a reduction of the negative charge of the LPS thus, protecting the biofilm surface against the cationic peptide colistin [34].

The induction of AmpC β-lactamase by the exposure to β-lactam antibiotics, as imipenem or ceftazidime, is probably the main adaptive tolerance mechanism in *P. aeruginosa* biofilms [71,72]. Bagge et al. demonstrated that the expression of the enzyme showed a special structural distribution characteristically concentrated at the periphery of the biofilms [29]. In addition, as seen in *P. aeruginosa* biofilms from people with CF, these β-lactamases are partially excreted by membrane vesicles [73], consistent with their extracellular location [71].

Hence, adaptive tolerance mechanisms operate in a similar way than *persisters* and an on/off model with long term consequences can be evidenced; if survivor cells are present once the treatment has stopped, the re-emergence of biofilms might happen.

In summary, concerning recalcitrance of biofilms, there are different pathways that act synergistically with intrinsic tolerance providing a fertile ground that helps traditional antibiotic resistance mechanisms to develop [74]. Fig. 1 gives a general view of all the mechanisms of both, tolerance, and resistance, that will be discussed in the next section.

2. Mechanisms of conventional antibiotic resistance in biofilms

Whereas the previously reported tolerance mechanisms show clear impact on biofilm recalcitrance to antibiotics and have been broadly studied, the role that traditional resistance mechanisms play in the decreased susceptibility of biofilms towards antibiotics has been less examined.

Nonetheless, recent studies seem to point towards tolerance as a preceding stage that favours the establishment of antibiotic resistance mutations that could otherwise occur, but might not be selected for [74,75].

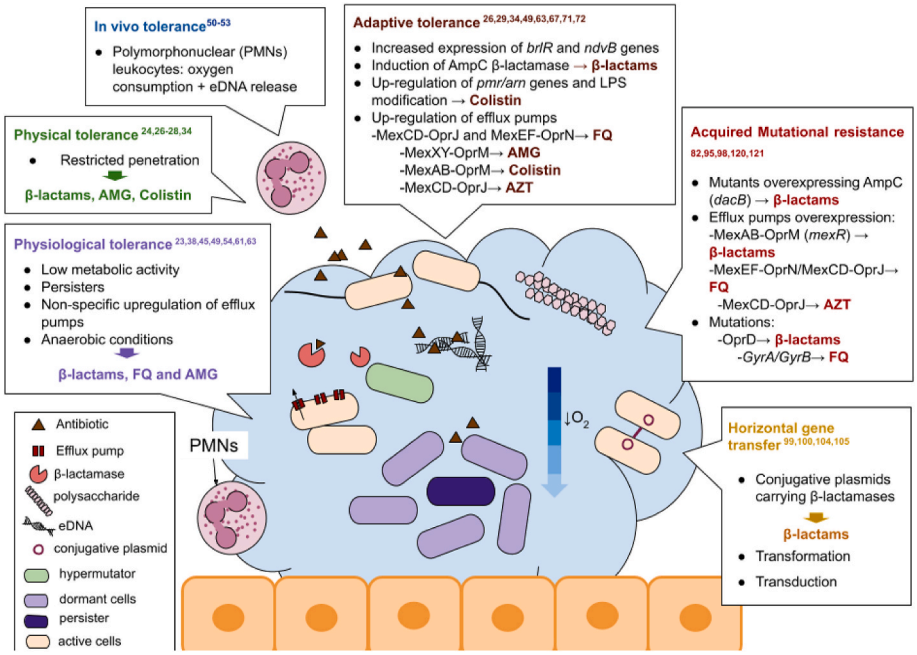

Fig. 1. Mechanisms of antimicrobial tolerance and resistance found in biofilms and antibiotics affected by them. FQ: Fluoroquinolones; AMG: Aminoglycosides; AZT: Azitromycin; LPS: lipopolysaccharide. Figure adapted from Ciofu O et al., Nat rev, 2022 [62].

Furthermore, the tolerance conditions described above, particularly, the high density and proximity of cells, together with an accumulation of available genetic elements, make the biofilm a great environment for horizontal gene transfer [76,77].

2.1. *Mutational resistance*

As mentioned before, in biofilms under treatment, matrix restrictive penetration results in areas of lower antibiotic concentration that, together with nutrient limitation, can prompt stress responses and lead to mutation emergence [49,78]. In addition, heterogeneity of nutrient and oxygen availability, results in different ecological niches that ensure the formation of distinct subpopulations and, consequently, fixation of beneficial mutations is more easily enabled [78]. Typically, the balance between evolution and genetic change shapes the rate of mutation [74]. If that rate is augmented more than usual, a priori, premature death could happen due to the additive effect of deleterious mutations [79]. Even so, in certain situations, especially those involving stressful environments, increased mutation rate might be beneficial for the bacteria [80,81].

One characteristic of *P. aeruginosa* biofilms, particularly described in CF individuals with chronic respiratory infections, is the elevated prevalence of mutator (or hypermutable) strains, present in over one-third of the studied patients [82]. The high prevalence of mutators in this setting strongly contrasts with what has been documented for onset of chronic infections (10%) [83], environment (6%) [84], or acute infections (<1%) [85]. Mutators, bacteria with an increased spontaneous mutation rate, frequently emerge due to defects in DNA repair mechanisms, such as the mismatch repair system (MMR), or stress responses [86–88]. These mutators are especially relevant in the clinical setting as they are frequently associated with high antibiotic resistance rates [82,89]. Moreover, it has been demonstrated that mutagenesis is naturally increased in biofilms and that this condition favours development, adaptation, and diversification processes that could, ultimately, lead to the occurrence of resistance under antibiotic exposure [90–94].

Mutation-driven resistance has been shown to be relevant also for antibiotics showing potent activity against *P. aeruginosa* biofilms, but not against planktonically growing cells, such as azithromycin (AZM). Indeed, marked selection of resistant mutants was demonstrated to be linked to hyperproduction of the multidrug efflux pump MexCD-OprJ, associated with inactivation mutations in its negative regulator *nfxB* [95]. The emergence of such resistant mutants was dramatically enhanced in biofilms formed by hypermutable strains [95]. Hyperexpression of MexCD-OprJ, showed cross-resistance to other unrelated antipseudomonal agents as ciprofloxacin or cefepime but hypersusceptibility to others such as imipenem or tobramycin [95]. Therefore, this work was helpful in guiding the selection of appropriate antipseudomonal therapies in CF individuals under AZM maintenance treatment.

Selection and amplification of resistant mutants and, even the hypermutator strains themselves, has been demonstrated in *P. aeruginosa* biofilms treated with ciprofloxacin [70]. Results showed that mutational mechanisms were playing a major role in biofilm antibiotic resistance and that theoretically optimized PK/PD parameters failed to suppress resistance development, suggesting that the increased antibiotic tolerance driven by the special biofilm physiology and architecture probably raises the effective mutant prevention concentration (MPC), favouring gradual mutational resistance development, especially in mutator strains [70].

Differential acquisition of resistance mutations in *P. aeruginosa* planktonic and biofilm growth have been documented in some cases, such as aminoglycoside (tobramycin) resistance development seemingly linked to LPS biosynthesis genes (*orfKHLN*) and electron transport chain components (*cyoAB*) [96,97].

Other relevant mutations involved in biofilm antibiotic resistance are those connected to β-lactamase hyperexpression. In a study looking into mixed biofilm communities of wild-type PAO1 and mutants with hyperproduction of either, the AmpC β-lactamase (*dacB* knockout), or the MexAB-OprM efflux pump (*mexR* knockout), it was shown that, under treatment with cefepime PAO1Δ*dacB* resistant mutants were selected for and amplified [98]. Furthermore, both, PAO1Δ*mexR* and PAO1Δ*dacB* mutants, seemed to locate themselves in the outer biofilm layers surrounding the sensitive PAO1 subpopulation and exert a shielding effect, which did not happen during planktonic growth [98]. Thus, this study demonstrated that, in biofilms, mutants showing diverse resistance mechanisms such as β-lactamase hyperproduction protect the

whole community, preserving wild-type susceptible populations from the effect of the antibiotics. Therefore, these findings represented a step forward to figure out antibiotic resistance dynamics in biofilms, as well as to understand the population biology of bacterial pathogens in chronic infections, where the coexistence of susceptible and resistant variants in dense communities is a hallmark.

Summing up, the biofilm microenvironment and population relationships and structure, together with its associated tolerance phenomenon, potentiate adaptive mutagenesis; all of which creates a breeding ground for the emergence of antibiotic resistance.

2.2. Horizontal gene transfer

Another traditional source of antimicrobial resistance is horizontal gene transfer, which can occur via conjugation, transformation or transduction, as it represents a major source of genetic variability.

For instance, it is known that conjugation in biofilms occurs at higher frequencies than in planktonic cultures [99–101]. Actually, biofilms could act as plasmid reserves maintaining and preserving MDR plasmids [102,103].

The biofilm architecture and the elevated bacterial density together facilitate the encounter of donor and recipient cells [77]. However, individual cells from within the diverse biofilm microenvironments were found to differ in their capacity to maintain incoming plasmids [100].

Thus, a high number of conjugation events does not guarantee effective gene transfer in all cases and could partially explain why some and not all cells acquire resistance mechanisms.

Similarly, transformation also seems to experience a boost within the biofilm domain [99]. The main hypothesis behind this is that the presence of eDNA in the matrix triggers a natural state of competence which in turn activates DNA release systems further promoting said state, that also contribute to the stabilisation of the biofilm matrix [77,99,104]. Whilst the state of competence might play an important part in biofilm matrix formation, it might also result in resistance gene acquisition as an after effect.

Lastly, along the same line as the case of transformation, given the role bacteriophages might play in biofilm matrix development and maintenance through the release of bacterial cytoplasmic components, the contribution of transduction to antibiotic resistance acquisition should not be ignored. On this note, phage mediated conversion of *P. aeruginosa* into a mucoid phenotype has already been reported and associated with poor outcomes for people with CF [105].

3. Final thoughts and future perspectives

Altogether, *P. aeruginosa* biofilms encompass an intricate environment conducive to antibiotic therapy failure; either through its natural tolerance and adaptive mechanisms or via an increased rate of conventional resistance events favourably selected.

So, improving knowledge regarding *P. aeruginosa* biofilm, research should be guided to find new strategies to overcome resistance, considering the multitude of factors that make the eradication of *P. aeruginosa* infections difficult. From a clinical perspective, the severity of chronic *P. aeruginosa* infections is having public health consequences, particularly affecting the most severely infected patients in our hospitals and people with CF.

After all the nuances about biofilms explained in this mini-review, it is obvious that, apart from antimicrobials, we need other compounds that help the action of our selected antibiotic, specifically targeting the biofilm mode of growth [106]. Starting with the numerous quorum *sensing* inhibitors that plays a key role in the regulation of *P. aeruginosa* biofilm formation but does not affect the viability of the bacteria. Other strategies to stand out would be the ones activating metabolic inactive cells by using SDS, EDTA and chlorhexidine, antibiotics like colistin or other compounds as organic acids or carbon sources [34,42,46,107–109]. More recently, the use of hyperbaric oxygen therapy (HBOT) metabolism-stimulating has been shown to redirect bacterial metabolism towards an antibiotic-susceptible phenotype [44,110,111]. Similarly, Oligo-G (alginate oligosaccharide), has been demonstrated to be able to inhibit biofilm formation and disrupt established biofilm matrix in vitro, as well as c-di-GMP modulators do [112,113]. Treatment with eDNAases [114]; use of efflux pumps inhibitors (EPIs), directed to the adaptive resistance [115]; use of antioxidants like N-acetylcysteine to target hypermutation, SOS, and oxidative stress [116]; bacteriophages [117]; photodynamic inactivation [118]; or even revert acidic environment to neutral conditions to prevent chronic *P. aeruginosa* infection and colonization [119], should be added to the list of possible therapies.

Beyond looking for new compounds or non-antibiotics strategies, it would be sensible to strive to improve therapeutic antibiotic strategies already available, as well as PK/PD parameters, to overwhelm mutation selection like combination of antibiotics [120–124], innovative sequential regimens [125,126] or improved drug-delivered for topical administration [127,128] recently demonstrated by ultrasound patches [129]. Progress in new alternative therapeutic approaches like formulation of antibiotics on nanoparticles [130,131], novel anti-biofilm compounds, CRISPR gene editing technologies and photodynamic therapies [132–134] is necessary. In addition, a determined attempt on transferring all these options into clinical practice for the treatment of biofilm-related infections will need to be done.

CRediT authorship contribution statement

María Fernández-Billón: Writing – original draft. **Aina E. Llambías-Cabot:** Writing – original draft. **Elena Jordana-Lluch:** Writing – review & editing. **Antonio Oliver:** Conceptualization, Writing – review & editing. **María D. Macià:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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