Pseudomonas aeruginosa: phenotypic flexibility and antimicrobial resistance

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Pseudomonas aeruginosa, a widespread ubiquitous opportunistic pathogen, is a leading cause of opportunistic infections in humans and is associated with an ever-widening spectrum of infections and multidrug-resistance phenomena. The evolution and spread of antibiotic resistant pathogens has become a threat to public health and strongly affects the effectiveness of most currently available antimicrobial agents, compromising the treatment of infections. This review will explore how its physiology, social behaviour and genome plasticity are shaped towards competitiveness and improved fitness. When facing a stressor, P. aeruginosa can adapt by different mechanisms, such as phenotypic switching, which might result in a worsening clinical outcome (e.g. increased antimicrobial resistances). Such phenotypic flexibility renders P. aeruginosa a feared nosocomial pathogen that continues to be problematic from a clinical perspective.

Keywords Pseudomonas aeruginosa; pathogen; virulence; multidrug resistance; phenotypic switching

1. Pathogenesis in the 21st century

Epidemics of infectious diseases are still highest because pathogens are able to overcome human defences and adapt to antimicrobial therapy. Therefore, the evolution and spread of antibiotic resistance has become a threat to public health and strongly affects the effectiveness of most currently available antibacterial agents, compromising the treatment of bacterial infections. A strong increase in the number of multi-resistant pathogens isolated from community and hospital-acquired infections has been registered in the recent years and we are now facing the possibility of a future without effective antibiotics [1]. Infections caused by multidrug-resistant bacteria are considered as the number one disease requiring priority medicines based on the potential public health impact if effective new antibiotics are not developed [2-4]. Patient health is often worsened by the coexistence of competing pathogen species or multiclonal subpopulations of one bacterial species. Co-infection results in within-host-pathogen interactions and competitiveness, which changes the predicted population dynamics and fitness, selecting for higher levels of virulence, drug resistance and pathogenicity [5-7].

Nowadays, research has been mainly focused on highly prevalent pathogens, such as Gram-positive vancomycin-resistant Enterococcus faecium, methicillin-resistant Staphylococcus aureus (MRSA) and Streptococcus pneumoniae, and the Gram-negatives Pseudomonas aeruginosa, Acinetobacter baumannii, third-generation cephalosporin-resistant Escherichia coli and Klebsiella pneumoniae [8-13]. Outbreaks of these pathogens often translate into high morbidity and mortality rates that need specific improvements in surveillance programs to surpass them with extra healthcare costs.

In this context, P. aeruginosa has become one of the best characterized opportunistic human pathogen, associated with a breach in host defences and rarely causing infection in healthy individuals [14].

2. Pseudomonas aeruginosa: an ubiquitous and opportunistic human pathogen

P. aeruginosa is a Gram-negative, rod-shaped bacterium that belongs to the group of γ-Proteobacteria [15]. It possesses a remarkable metabolic versatility and is categorised as a facultative anaerobe, achieving anaerobic growth by using nitrogen as a terminal electron acceptor in the absence of oxygen [15].

It is a ubiquitous organism present in a variety of environmental niches including soil and water and can be isolated from various living sources, including plants, animals and humans [16]. Being able to tolerate a variety of physical conditions and survive on minimal nutritional requirements, P. aeruginosa is detected in community settings in swimming pools, whirlpools, hot tubs, contact lens solution, home humidifiers, soil and rhizosphere, and vegetables [13, 14, 17]. Sinks, water fittings and tap water have also been shown to be typical reservoirs of P. aeruginosa [18, 19]. Moreover, colonisation of respiratory therapy equipment (e.g. ventilators), invasive devices and intravenous lines (e.g. catheters) has also been commonly reported as a major cause of infection outbreaks in hospital settings [20-22]. Ultimately, this pathogen can also spread through contact with medical staff or infected patients [22].

The majority of manifestations are seen in healthcare, especially in Intensive Care Units (ICU). P. aeruginosa has emerged as one of the leading causes of nosocomial infections, being responsible for urinary tract infections, respiratory system infections, skin and soft tissue infections, bone and joint infections, bacteraemia and a variety of systemic infections particularly in people with compromised immune systems including burn sufferers and cancer and AIDS
patients [14, 23]. Moreover, *P. aeruginosa* is considered the prime lung pathogen of cystic fibrosis (CF) patients, contributing to the progression and death rate of the disease [24]. Importantly, as outlined in Fig. 1, *P. aeruginosa* pathogenicity is both multifactorial, requiring the cumulative action of multiple virulence factors, and combinatorial, as pathogenicity factors may behave differently and different combinations of these determinants may result in comparable virulence phenotypes [25]. Taking into account the remarkable adaptability of these bacteria, existing both as a ubiquitous environmental organism and as an opportunistic pathogen, it is not surprising that *P. aeruginosa* genome is among the largest and most complex bacterial genomes [15].

**Fig. 1** Overview of *P. aeruginosa* features accountable for phenotypic flexibility.

### 3. Genomics of *P. aeruginosa*

The genome of strain PAO1, collected from a human wound, was the first *P. aeruginosa* genome to be completely sequenced. At the time, it was the largest fully sequenced bacterial genome, with 6.3 Mbp and 5570 predicted ORFs, contributing to its versatility, adaptability, functional diversity and virulence [15, 26]. Apparently, the genome of strain PAO1 contains many genes encoding outer membrane proteins involved in adhesion, motility, antibiotic efflux, virulence factor secretion, and quorum-sensing, genes encoding transport systems and enzymes involved in nutrient uptake and metabolism. Consequently, as expected, its content in predicted regulatory genes is one of the highest among all bacterial genomes [15, 26]. As shown in Table 1, after PAO1, several clinical *P. aeruginosa* isolates genomes have been fully sequenced.
Table 1 P. aeruginosa fully sequenced genomes [27].

<table>
<thead>
<tr>
<th>Fully sequenced strains</th>
<th>Size (Mbp)</th>
<th>GC Content (%)</th>
<th>Year</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>P. aeruginosa PAO1</td>
<td>6.3</td>
<td>66.6</td>
<td>2000</td>
<td>26</td>
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<tr>
<td>P. aeruginosa PA14</td>
<td>6.5</td>
<td>66.3</td>
<td>2006</td>
<td>25</td>
</tr>
<tr>
<td>P. aeruginosa LESB58</td>
<td>6.6</td>
<td>66.3</td>
<td>2009</td>
<td>28</td>
</tr>
<tr>
<td>P. aeruginosa PA7</td>
<td>6.6</td>
<td>66.4</td>
<td>2010</td>
<td>29</td>
</tr>
<tr>
<td>P. aeruginosa NCGM2.S1</td>
<td>6.8</td>
<td>66.1</td>
<td>2011</td>
<td>30</td>
</tr>
<tr>
<td>P. aeruginosa M18</td>
<td>6.3</td>
<td>66.5</td>
<td>2011</td>
<td>31</td>
</tr>
<tr>
<td>P. aeruginosa DK2</td>
<td>6.4</td>
<td>66.3</td>
<td>2012</td>
<td>32</td>
</tr>
<tr>
<td>P. aeruginosa B136-33</td>
<td>6.4</td>
<td>66.4</td>
<td>2013</td>
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</tbody>
</table>

A comparative genomic analysis revealed that the P. aeruginosa genome could be considered as a mosaic consisting of two components: the core and the accessory genome. The core genome encodes a set of metabolic and pathogenic factors shared by all strains. Currently the core genome is estimated to constitute approximately 90% of the total genome [15]. However, such percentage may be over-estimated since, so far, only a limited number of isolates have been fully sequenced. Ongoing P. aeruginosa genome sequencing projects will provide valuable data on the definition of the actual core genome of this species. In contrast, the accessory genome is defined as genomic segments that vary between strains and, often, include genetic elements such as transposons, insertion sequences, plasmids, prophages and genomic islands, usually acquired via horizontal gene transfer (HGT) processes such as conjugation, transduction and transformation [15]. As seen in Fig. 2, these tend to cluster in certain loci, termed regions of genomic plasticity (RGPs), also known as genomic islands (> 10 kb) or islets (< 10 kb). The accessory genome may encode gene products that contribute to the niche-based adaptation of the bacterium, such as an increase in host range, survival in new environment and utilisation of new nutrients [15, 33].

Fig. 2 A – BLAST-based LESB58 (NC_011770) genome comparison using CGView [34]. Outermost circles depict ORFs on the positive and reverse strand, respectively. Innermost circles represent GC content and GC skew. (1) P. aeruginosa M18 (NC_017548) (2) P. aeruginosa NCGM2.S1 (NC_017549) (3) P. aeruginosa PAO1 (NC_002516) (4) P. aeruginosa B136-33 (NC_020912) (5) P. aeruginosa DK2 (NC_018080) (6) P. aeruginosa PA14 (NC_008463) (7) P. aeruginosa PA7 (NC_009656). RGPs predicted by RGP Finder [35] are indicated. B – Genomic islands predicted for LESB58 by IslandViewer [36], integrating the prediction methods IslandPick (green), IslandPath-DIMOB (blue), and SIGI-HMM (orange). Complementarity of predicted genomic islands and white gaps (corresponding to unique loci) generated by the alignment of LESB58 genome with reference strains can be confirmed.
In 2006, the conclusion of the genome sequencing of *P. aeruginosa* strain PA14 [25] revealed the presence of significant number of genes that are absent in PAO1 genome. These genes, clustered into genomic islands, were related to survival in diverse environmental conditions, including global transcriptional regulators such as GacA, genes involved in pathogenesis-related processes such as motility, quorum sensing, and phenazine biosynthesis; and genes encoding secreted cellulolytic factors and toxins such as ExoU, exotoxin A, phospholipase C, and elastase [37-39]. Nevertheless, 91.7% of total genetic background was highly similar to that of PAO1 strain. Later on, in the course of the genome sequencing project of *P. aeruginosa* strain LESB58, it was shown that both the core genome and genomic islands (including prophage clusters) were involved in *in vivo* competitiveness [28]. In 2010, comparative genomics of *P. aeruginosa* strain PA7 and PAO1 subdivided the genetic background of PA7 into a core genome and a total of 51 genomic islands, including antibiotic resistance genes, elements of transposons and prophages. Apparently, PA7 genome lacks several key virulence factor genes, notably for the type III secretion system that enables the injection of toxins into host cells (it has neither ExoS nor ExoU and lacks ToxA, ExoT, and ExoY) [29]. Nowadays there are other fully sequenced genomes, however they lack detailed comparative genomic studies.

The accessory genome component may be predicted by evaluation of different characteristics of the genomic DNA like an unusual GC content and codon usage, often associated with tRNA genes which seem to act as hotspots for the insertion of foreign DNA segments [15, 33]. Since *P. aeruginosa* is characterized by a high GC content (~66.6%), horizontally transferred genes generally have a lower GC content. There is also a close association with genetic elements facilitating mobility and a predilection for insertion at particular sites within the core genome. HGT, deletions, rearrangements, and mutations are considered the main contributors to genome evolution, conferring particular phenotypes, thus making its knowledge valuable for a more complete understanding of the pathogenic potential of *P. aeruginosa* [15].

### 4. Virulence factors of *P. aeruginosa*

The ability of *P. aeruginosa* to cause a wide range of infections is partly due to the potential production of a large array of virulence factors. These can be classified into cell-associated and secreted virulence factors [40]. Expression levels are dependent on a variety of determinants, particularly environmental stimuli (such as iron and nitrogen availability, temperature, osmolarity and cell density) [40].

#### 4.1. Cell-associated virulence factors

Cell-associated factors include flagella and pili that aid in motility, systems that are involved in the delivery of effector proteins into the host cells, and lipopolysaccharide (LPS) that suppresses host immune responses as well as being involved in the establishment of persistent infections [41].

Generally, in *P. aeruginosa*, there is one polar flagellum and the movement is via a screw-like motion. It is not only responsible for the primary function of bacterial motility (swimming), and thus the response to environmental signals (chemotaxis), but it also facilitates the acquisition of nutrients. In respiratory tracts, flagella of *P. aeruginosa* can also function as an adhesin to receptors of host cells, essential for an initial phase of colonisation [40, 42]. The flagellar cap protein FliD has been demonstrated to be involved in the adherence of *P. aeruginosa* to mucin on the human respiratory tract [43], whereas flagellin binds to the respiratory epithelial cell glycolipids receptors [44]. It has also been reported that the flagellum is important in an initial step of biofilm formation [45]. However, being very immunogenic, it activates a host defence response potentially leading to eradication of the bacterium. Therefore, following initial colonisation its presence can be considered a liability and in chronic infections there is often a selection for aflagellar mutants [46]. *P. aeruginosa* pili also contribute for the attachment to host surfaces. In addition, pili also seem to be required for translocation of bacterial cells along solid surfaces termed “twitching” motility [47]. This occurs via extension, tethering and retraction of type IV pili, allowing *P. aeruginosa* cells to move or glide toward environment and nutritional signals [47] and facilitates rapid colonisation of cells on surfaces as well as biofilm formation [45]. An interesting fact is that as with flagella, *P. aeruginosa* promptly “turns off” the expression of pili after binding to the host cells, to avoid clearance [48, 49]. A third type of motility regulated by the availability of nitrogen has been identified in *P. aeruginosa*. It requires both flagella and type IV pili. The ability to swarm is not present in all *P. aeruginosa* but swarmer cells tend to be elongated and hyperflagellated. It has been suggested that *P. aeruginosa* has retained these three types of motility due to the range of environments it can colonise and that swarming might play a role in colonisation of niches where nitrogen availability may be limited [50].

The outer membrane of *P. aeruginosa* is mainly composed of LPS, consisting of a hydrophobic domain, lipid A, and a hydrophilic tail formed by core polysaccharide and O-specific polysaccharide, projecting from the surface. It has been found to be critical to virulence in *P. aeruginosa* and binds a number of molecules on host cells [51-55]. These bacteria can also produce an extracellular capsule of alginate, a linear polymer of mannuronic acid and glucuronic acid, which is known to stimulate production of IgG and IgA antibodies and is thought to function as an adhesin that aids *P. aeruginosa*’s anchoring to the respiratory epithelium [40]. Mathee et al. [54] showed that mucoid bacteria produce 2-6 fold higher levels of alginate when compared to bacteria from smooth colonies. Studies on overexpressed alginate
indicate that it can protect *P. aeruginosa* from phagocytosis and antibodies [40]. It has also been found that alginate is an effective free radical scavenger which aids in clearing reactive species released from activated neutrophils [51]. Mainly in the lungs of CF patients, *P. aeruginosa* can convert from a non-mucoid to an alginate-overproducing mucoid phenotype signalling chronic infection [23].

Therefore, these virulence factors have a predominant role in colonisation, where they help bacteria to adhere and invade the host by damaging their immune responses and forming a barrier to antibiotics [23].

4.2. Secreted virulence factors

After colonisation, secreted factors can have different effects, causing extensive tissue damage, bloodstream invasion and dissemination, maintaining a persistent inflammatory state and leading to pathogenesis success [56, 57].

*P. aeruginosa* has five (type I, II, III, V and VI) of the seven secretion systems characterized in bacteria [40]. Type III secretion system (TTSS) of *P. aeruginosa* is, apparently, imperative to virulence as it delivers, at least, four exoenzymes into host cells: exoenzyme S (ExoS), exoenzyme T (ExoT), exoenzyme U (ExoU) and exoenzyme Y (ExoY). Different isolates express these cytotoxins differently [58]. Feltman *et al.* [59] investigated the presence of these genes in virulent isolates and concluded that all contained *exoT*, while the presence of other exoenzymes seemed to be mutually exclusive (particularly *ExoS* and *ExoU*). *ExoS* and *ExoT* disrupt the actin cytoskeleton of host cells, ultimately causing apoptosis. *ExoY* elevates intracellular levels of cyclic AMP (cAMP), which indirectly disrupts the actin cytoskeleton [58]. *ExoU* is the major cytotoxin of *P. aeruginosa* and it has been found to be 100 times more toxic than *ExoS* [60]. It has been proven that *ExoU* mediates killing of a variety of mammalian cells, rendering the host susceptible for secondary infections [58]. During infection, *P. aeruginosa* may also secrete exotoxin A that can cause cytotoxicity by inhibiting protein synthesis via ADP-ribosylation and inactivation of elongation factor. It is responsible for local tissue damage, including liver cell necrosis, renal necrosis and pulmonary haemorrhage and also for bacterial invasion and immunosuppression in the CF lung [61]. Its contribution to bacterial virulence in a mouse model has been shown in a study by Miyazaki *et al.* [62] where an exotoxin A deficient mutant was found to be 20 times less virulent than the wild-type strain.

Proteases are other crucial virulence factors. To date, four types have been known to be secreted by *P. aeruginosa*: LasB elastase, LasA elastase or staphylolysin, alkaline protease and protease IV. These proteases are associated with virulence by enhancing the ability of *P. aeruginosa* to invade tissues and interfering with host defence mechanisms. For example, the elastolytic activity of these enzymes is very important in pathogenesis since a number of tissues are composed of elastin (lung, vascular and ocular tissue) [23, 56]. Protease IV causes the destruction of host proteins including fibrinogen, elastin and components of the immune system and is thought to aid in bacterial adhesion [58].

*P. aeruginosa* may produce a blue-green pigment called pyocyanin, causing a typical blue-pus wound. This pigment inhibits epidermal cell growth, cell respiration in mammalian and prokaryotic cells and plays an important role in lung infections of mice and in iron acquisition. It also represses expression of catalase [63]. Lau *et al.* [64] demonstrated that *P. aeruginosa* pyocyanin-mutant strains had substantially less pathogenicity compared to the wild-type strains as tested in mice models. Nonetheless, the mutant strains were still able to cause local inflammation, highlighting the importance of other virulence factors.

*P. aeruginosa* might also secrete two siderophores to accumulate iron: pyochelin and pyoverdine, enabling bacteria to colonise niches with limited iron availability. Iron is necessary for bacterial growth and affects *P. aeruginosa* pathogenesis, where an increase in iron concentration helps this pathogen to cause persistent infections. Biologically useful iron (Fe^{2+}) in the environment is scarce and is available mostly in the insoluble Fe^{3+} form. To help scavenge this free iron, bacteria produce siderophores, iron chelators and transporters through TonB-dependent receptors on the cell surface [40]. Even though it was shown that pyoverdine is more important for iron acquisition, both are essential for virulence and infectivity. Pyoverdine is recognised by specific receptors on the outer membrane of bacteria that act as gated porin channels allowing the entry of bound complexes [65]. Three structurally different variants of pyoverdine, with a corresponding receptor for each one, have been identified: type I, II and III. They have different peptide chains and each isolate produces only one form of pyoverdine [66]. It has also been found that pyoverdine can regulate the secretion of other virulence factors like exotoxin A as well as its own [67].

Rhamnolipid, a biosurfactant, is another virulence factor whose secretion by *P. aeruginosa* is controlled by the QS system. It is involved in development and maintenance of biofilms, and *ex vivo* studies revealed that it modulates and breaks the tight junctions between respiratory epithelial cells [68].

Other secreted virulence factors such as phospholipase C, histamine and leukocidin, even if less understood, apparently also contribute to the pathogenicity of *P. aeruginosa*.

As depicted above, the virulence of *P. aeruginosa* is multi-factorial, resulting in the use of an arsenal of virulence factors that varies from isolate to isolate and with different stages of infection, particularly from colonisation to the establishment of acute infections [23]. Moreover, *P. aeruginosa* has other features directly associated with its virulence: the Quorum-Sensing (QS) system and the ability to form a biofilm.
5. Quorum-Sensing systems

Bacteria are able to communicate with each other using cell-to-cell communication systems, known as QS. It allows bacteria to monitor their population density by sensing extracellular concentrations of self-generated signal molecules termed autoinducers. Once a threshold of autoinducer concentration is reached, they bind to specific receptors, resulting in alteration of the expression of target genes. Consequently, the expression of certain genes is induced or repressed depending on population density, synchronizing bacterial behaviors [69]. QS signal molecules control the production of numerous secreted virulence factors and it has been demonstrated that they also regulate biofilm formation [40].

In *P. aeruginosa* there are three QS systems described. Two N-acyl homoserine lactone (AHL)-dependent QS systems – *las* and *rhl* – involving two signalling molecules with different substitutions on their acyl side chains: N-(3-oxo-dodecanoyl)-L-homoserine lactone and N-butyryl-L-homoserine lactone, respectively. Briefly, a gene encoding an autoinducer synthase ("I" genes, *lasI* or *rhlI*) is activated, synthesising an autoinducer that diffuses into the environment. Once it reaches a threshold concentration, it allows binding to transcriptional activators forming a complex that activates, among others, genes coding virulence factors such as elastase, exotoxin A, TTSS apparatus proteins, alkaline protease, rhamnolipids, alginate, pyocyanin and pyoverdine [57]. They are closely connected to a third 2-alkyl-4-quinolone (AQ)-dependent QS system, involving the *Pseudomonas* quinolone signal (PQS) and its precursor HHQ. Studies have shown that PQS affects biofilm formation, regulates several virulence factors in *P. aeruginosa* and is essential for full virulence in multiple hosts. A possible important role during infection is also suggested [69]. These three QS systems are hierarchically organized, with the *las* system positively regulating both the *rhl* and *pqs* systems. Additionally, the *rhl* system negatively regulates the *pqs* system [42]. Wagner et al. [70] demonstrated that, directly or indirectly, they regulate more than 10% of the *P. aeruginosa* genome. There are also more factors that manipulate the activity of the QS systems, for instance, the two component systems GacA/GacS and PhoB/PhoR as well as the transcriptional regulators PqsR and Vfr [37, 40].

Given the implication of the QS in the pathogenesis of *P. aeruginosa* and other bacteria, strategies designed to interfere with these systems are likely to have potential as biological controls. In fact, it has been demonstrated that QS-deficient mutants display decreased virulence when compared to their QS-able counterparts [71].

6. Biofilms in *P. aeruginosa* – a sessile lifestyle

The typical *Pseudomonas* bacterium may be found in a biofilm, attached to some surface or substrate, or in a planktonic form, as a unicellular organism, actively swimming by means of its flagellum. Biofilm, by definition, is a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface [72].

6.1. Physiology

The physiology of biofilm cells is extremely complex and profoundly different from those grown planktonically. The physiological status of cells is heterogeneous and determined by the location of each individual cell within the multiple layers of cells that form the biofilm. Cells located in the surface of the biofilm may have easy access to nutrients, including oxygen, and have fewer problems with the discharge of metabolic waste products, hence they are metabolically active. These cells are likely to have identical properties to their planktonic counterparts. On the other hand, cells embedded within the thick glycocalyx matrix are likely to be less metabolically active because of poor access to essential nutrients. These cells are at the stage of dormancy and are likely to be smaller since they are not actively engaged in cell division. However, the development of biofilms generates a sheltered community of cells in which environmental stresses are greatly reduced, promoting bacterial survival in a hostile environment [73].

6.1.1. Stages of biofilm development

Sauer et al. [74] proposed that the maturation of biofilms of *P. aeruginosa* involves five distinct stages:

1. **Reversible attachment**: Planktonic bacteria are attached to substratum via the flagellated pole. *P. aeruginosa* is transiently fixed to the substratum and is able to detach freely, independently of the QS signal.

2. **Irreversible attachment**: Reorientation of bacteria axis possibly causes the loss of flagella thus ceasing the swimming motility. *P. aeruginosa* then aggregates and forms microcolonies. Type IV pili, responsible for the twitching motility, has an important role in this stage [45]. After attachment, genes including *algC*, *algD* and *algU*, are activated leading to the synthesis of extracellular matrix composed of alginate [47].

3. **Early Maturation**: At this stage, approximately 540 genes were upregulated [47]. Proteins involved in anaerobic processes have been suggested to contribute during this maturation period, implying an oxygen limitation.

4. **Maturation**: Biofilms reach maximum thickness. They have a mushroom-shaped multicellular structure separated by water-filled channels, but the formation of such structure might depend on nutrient sources. Rhamnolipids, secreted by cells within the biofilms, have been proposed as a maintenance factor for the open channels surrounding microcolonies.
Furthermore, bacterial population within the biofilm during this stage exhibits different physiological activities, which may correlate to the oxygen distribution within the mature biofilms [74].

(5) Detachment: As the biofilm develops, single cells or groups of cells can detach and actively leave to form new biofilms. This detachment can be caused by external perturbations, however, Sauer et al. [74] suggested that this could be an active process that the biofilm is programmed to do, allowing colonisation of new niches. Bacteria within microcolonies actively swim away from the interior part of the cluster and enzymes degrading the matrix might also play roles in the dispersion.

6.2. Antibiotic resistance in biofilms

Bacteria organized in biofilms predominate, numerically and metabolically, in virtually all nutrient-sufficient ecosystems and their low susceptibility to antibiotics and disinfectants is recognized. The exopolysaccharide (EPS) matrix encasing the *P. aeruginosa* biofilms also protects cells from the host immune response [75].

The reduced susceptibility of biofilms to antibiotics is probably closely related to their structural and chemical heterogeneity. The structural heterogeneity is given by the non-uniform distribution of cells and polymers within the biofilm matrix and variable biofilm thickness. The chemical heterogeneity is indicated by the local variation in the concentrations of metabolic substrates, products, and microbial species. In addition, biofilm bacteria undergo a number of physiological, metabolic and phenotypic changes leading to the emergence of population diversity [76]. This seems to guarantee the survival of persisting bacteria even under harsh conditions, which would be able to re-establish a biofilm afterwards.

7. Antibiotic resistance

*P. aeruginosa* is also associated to antibiotic resistance phenomena, as a significant number of strains show innate and acquired resistance against a wide range of antimicrobial agents (e.g. β-lactams, aminoglycosides, fluoroquinolones, cationic peptides), leading to difficulty in treating infections [77]. Multi-drug resistance often results from a combination of mechanisms to circumvent antibiotic stress. The strategy varies between isolates and a cross-talk between mechanisms may occur, usually conferring resistance to several classes of antimicrobials. Common mechanisms involve i) reduction of cell wall permeability to the antibiotic, ii) expression of modifying enzymes that selectively target and destroy the activity of antibiotics, iii) overexpression of membrane-associated efflux pumping proteins that extrude the toxic compound to the extracellular environment and iv) modification of the antibiotic target (e.g. through mutations in chromosomal genes or regulators of resistance genes, or even by reprogramming of biosynthetic pathways) [13, 78]. Additionally, resistance might be acquired through addition of resistance genes from other organisms via plasmids, transposons, integrons and bacteriophages [78]. As summarized in Table 2, multidrug-resistance is a complex phenomenon since a cross-talk between resistance mechanisms employed by bacterial cells as response to a specific antibiotic might confer additional resistance to other classes of drugs.

*P. aeruginosa* is naturally resistant to some β-lactams (e.g. some penicillins such as ampicillin) as it encodes chromosomal β-lactamases AmpC [79] and PoxB [80], and up to 7 penicillin-binding proteins (PBPs) [81, 82]. AmpC is a well-characterized class C cephalosporinase, frequently related to β-lactam resistance in clinical strains whereas class D oxacillinase PoxB expression was only detected in ampC laboratory mutants. PBPs are involved in the cross-link of peptidoglycan and are inhibited by β-lactams. However, the redundancy of the PBPs repertoire in *P. aeruginosa* and their different affinities to bind β-lactams, allow these bacteria to overcome some of these first-line antimicrobials [83]. Moya *et al.* [84] described that mutations on dacB (PBP-4) resulted in an AmpR-dependent overexpression of ampC in 10 ceftazidime-resistant clinical isolates and PAO1 strain. Moreover, overexpression of ampC by mutations of ampR (positive regulator) and ampD (negative regulator) have also been described to promote resistance to penicillins, cephalosporins and monobactams [83, 85, 86]. Mutations and silencing of regulators, such as *nalB* (MexR [85, 87]), *nfsB* [88] and *nfsC* (MexT) [86] may also lead to downregulation or alteration of porin OprD in the outer membrane [89, 90], the main channel responsible for the uptake of β-lactams (e.g. carbapenems) and overexpression of efflux systems MexAB-OprM [83, 91], MexCD-OprJ [83, 91, 92] or MexEF-OprN [83, 93], which also play important role on the reduction of fluoroquinolones activity. Pan-resistance to β-lactams usually appears when isolates express extended spectrum β-lactamases (ESBL) or metallo-β-lactamases (MBL), acquired by HGT of mobile elements [94-97], that are able to hydrolyse broad-spectrum monobactams, cephalosporins and carbapenems.

Despite the emerging of aminoglycosides as an alternative antibiotic for β-lactams-resistance, clinical isolates able to counteract their activity (e.g. kanamycin [98], gentamicin [99], amikacin, tobramycin [100]) quickly appeared. Activity of aminoglycoside-modifying enzymes (AME) has been extensively reported as the major mechanisms responsible for resistance to this class of antimicrobials [101-104]. AME inactivate the target through phosphorylation by aminoglycoside phosphotransferases (APhS), adenylation by aminoglycoside nucleotidyltransferases/ adenylyltransferases (ANTS/AADs) and acetylation by aminoglycoside acetyltransferases (AACs). AME are frequently found in mobile genetic elements, harbouring other resistance-conferring genes (e.g. ESBL, MBLs and chloramphenicol acetyltransferases) and thus, aminoglycoside-resistant isolates usually display a multi-resistant phenotype [101, 102,
On the other hand, AME-independent mechanisms involving overexpression of efflux pumps (e.g. MexAB-OprM [106], MexXY-OprM [107]) and reduced accumulation/uptake of antibiotic molecules [108] have also been described in some strains, particularly in CF clinical isolates showing resistance to amikacin [109], gentamicin and tobramycin [110]. Moreover, the appearance of multiple aminoglycoside resistant clinical isolates has also been reported due to the combination of transport-related mechanisms with AME expression [110, 111]. Regulatory mutations play a minor role in aminoglycoside resistance but are also reported in some CF isolates. Although they seem to be mutually exclusive, mutations in mexZ [112], a regulator of MexXY-OprM pump, and mutations in the two-component regulatory system parRS [113], which influence OprD levels, are responsible for deregulation of non-AME mechanisms. In the last 10 years, a new mechanism accountable for high level of aminoglycoside-resistance has been studied, consisting in the methylation of the binding site of aminoglycosides molecules to the 16S ribosomal RNA of 30S ribosomal subunit. This mechanism catalysed by 16S rRNA methylases has emerged among Gram-negative bacteria. These methylases are similar to those expressed by aminoglycoside-producing actinomycetes and are typically located on transposons and plasmids, disseminated by HGT events [114]. In P. aeruginosa, several 16S rRNA methylases were identified in some clinical isolates: RmtA [115], RmtB [116], RmtD [117], ArmA [116].

Table 2 Overview of the major resistance mechanisms to antipseudomonal antibiotics.

<table>
<thead>
<tr>
<th>Resistance mechanism</th>
<th>Class of antibiotic</th>
<th>Genes envolved</th>
<th>References</th>
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<tr>
<td></td>
<td>Cationic peptides</td>
<td>phoPQ, pmrAB, parRS</td>
<td>108,128,129</td>
</tr>
<tr>
<td>Antibiotic inactivation</td>
<td>β-lactams</td>
<td>Chromosomal β-lactamas</td>
<td>79,80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ampC, poxB</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasmid-mediated β-lactamas</td>
<td>94-97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ESBL (TEM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>metallo-β-lactamas (IMP, VIM, SPM, GIM, SIM, AIM)</td>
<td></td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>ME</td>
<td>aac(6 ′)-Ib’, ant(2 ′′)-Ia, ant(4 ′)-IIa, ant(4 ′)-IIb, aph(3 ′)-Ib, ant(4 ′)-Iib</td>
<td>101-104,110</td>
</tr>
<tr>
<td>Increased efflux</td>
<td>β-lactams</td>
<td>mexAB-oprM, mexCD-oprJ, mexEF-oprN</td>
<td>93,95-97</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>mexAB-oprM, mexXY-oprM</td>
<td>106,107</td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>mexAB-oprM, mexCD-oprJ, mexEF-oprN, mexXY-oprM</td>
<td>91,106,121</td>
<td></td>
</tr>
<tr>
<td>Target modification</td>
<td>β-lactams</td>
<td>redundancy of PBPs</td>
<td>81-83</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>16S rRNA methylases (rmtA, rmtB, rmtD, armA)</td>
<td>115-117</td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>gyrA, gyrB, parC, parE</td>
<td>119,120</td>
<td></td>
</tr>
<tr>
<td>Mutation of regulators</td>
<td>β-lactams</td>
<td>ampR, ampD, nfxB, nfxC (mexT), nalB (mexR)</td>
<td>83,85,86,88</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>mexZ, parRS</td>
<td>112,113</td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>nalB (mexR), nalC, nalD, mexZ, nfxB, nfxC (mexT)</td>
<td>119,122-126</td>
<td></td>
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</table>

Fluoroquinolones (FQs) inhibit DNA transcription and replication, by targeting DNA gyrase and topoisomerase IV [118]. Due to their broad-spectrum, FQs were extensively used against β-lactam- and aminoglycoside-resistant isolates. However, this massive application resulted in the appearance of FQs-resistant clinical isolates which showed modifications of FQs targets. FQ-resistant isolates usually carry mutations in genes encoding subunits of DNA gyrase (gyrA and to a lesser extent, gyrB), and topoisomerase subunits (parC and to a lesser extent, parE) [119,120]. In combination to target point mutations, expression of multidrug efflux pumps also play an important role in FQ resistance. The four efflux systems MexAB-OprM [91], MexXY-OprM [91, 106], MexCD-OprJ [91], and MexEF-
OprN [121] have been reported to be overexpressed in some FQ-resistant clinical isolates by inactivation of their respective regulators. MexAB-OprM may be deregulated by mutations on NalB (MexR [122]), NalC [123] and NalD [124]. The other common hyperexpressed pump MexXY-OprM is repressed by MexZ and mutations in this regulator are frequent in FQ-resistant strains [125]. MexCD-OprM and MexEF-OprN overexpression are less common in FQ-resistant clinical isolates. The former is related to mutations in the repressor NfxB [119] while the latter pump is related to an activating mutation in NfxC (MexT) [126], which is coded in an inactive form and is converted to an active regulator.

Taking into account the versatility displayed by many clinical isolates of *P. aeruginosa* to overcome the action of antibiotics and emerge as multidrug resistant strains, new powerful and efficacious antipseudomonalas were introduced in therapeutic strategies: the cationic peptides polymixin B and colistin [127]. However, there are already some reports of resistance to both agents in clinical isolates, involving changes in LPS composition (e.g. Lipid A substitution by aminoarabinose) regulated by PhoPQ [108,128], PmrAB [128] and ParRS [129] two-component regulatory systems.

## 8. Phenotypic flexibility of *P. aeruginosa* as response to external stimuli

External factors are continuously challenging bacteria that must adapt in order to survive. Bacteria can face several stressful conditions like starvation, temperature and pH shocks, oxygen limitation, antimicrobial agents and immune host defences [131]. The adaptive response involves physiological, behavioural and genetic changes and can be achieved by different mechanisms, such as phenotypic switching or phase variation, described for both pathogenic and non-pathogenic species [132].

Phenotypic switching refers to a reversible adjustment between two phenotypic states, leading to subpopulations with different gene expression: one with altered expression of one or more phase variable genes and other expressing the full genes profile [133]. If beneficial, the microorganisms retain the possibility to switch again when new stimuli occur, or return to the previous phenotype state when the external stressor is eliminated [132, 134]. Phenotypic diversity can be mediated by specific genetic alterations or by epigenetic mechanisms [134]. The best approach to bacteria achieve adaptation by genetic alterations is through mutations and HGT. Spontaneous mutations occur at very low frequency but increased mutation rates, named hypermutability, could be an efficient approach to acquire genetic diversity [132].

Another striking feature of bacteria is their inherent phenotype heterogeneity, since it is possible to have cell-to-cell phenotypic variability within an isogenic population. This heterogeneity, not involving genome modifications, may be owed to, for instance, fluctuation in the rates of protein synthesis and degradation [132]. Hence, it is fundamental to bacterial fitness and development since strain variability allows colonisation of different ecosystems and enhances their resistance against environmental fluctuations [135].

### 8.1. Morphological variants

The most visible evidence of phenotypic switching demonstrated in some strains of *P. aeruginosa* is colony morphology variation, termed as “dissociative behaviour” [136, 137]. Modifications in size, opacity, pigmentation and texture may be a sign of different expression of one or more bacterial traits and might be attributed to phase variation of several surface-exposed proteins, capsule and cell wall composition [134]. The correlation between bacterial characteristics and colony morphologies is remarkably important [132]. Macroscopic visualization may help predict which traits were altered. Equally, colonies inspection will help associate morphology changes with virulence, persistence and antimicrobial resistance.

The best studied colony morphotypes are the small colony variants (SCV) [137-140] and the mucoid morphotype [48], that may be associated with antimicrobial resistance, altered metabolism and reduced immunogenicity, contributing to increased bacterial pathogenicity and persistence. SCV have been implicated in persistent and recurrent human infections like CF [139, 141] and device-related infections. These variants have been characterized as hyperpiliated with enhanced ability to form biofilms [137-140], increased twitching motility, increased fitness under human infections like CF [139, 141] and device-related infections. These variants have been characterized as contributing to increased bacterial pathogenicity and persistence. SCV have been implicated in persistent and recurrent infections [48], that may be associated with antimicrobial resistance, altered metabolism and reduced immunogenicity, ultimately resulting in the arising of autoaggregative SCV with defects in swimming, twitching and swarming abilities [143, 144]. These variants are hyperadherent and putatively negatively controlled by the phenotype variant regulator (PrvR) [144]. Interestingly, von Gotz et al. [140] described a SCV with increased TTSS expression, that was observed to be linked to an increased *in vitro* cytotoxicity and enhanced virulence in murine respiratory tract infection. Thus, the assumption that *P. aeruginosa* is subject to selection for reduced cytotoxicity and attenuated virulence during chronic CF lung infection might not apply to all variants. SCV may comprise a large number of variants with diverse virulent and resistance behaviour [132]. Also, Drenkard and Ausubel [142] described the emergence of a SCV subgroup, the RSCV, when *P. aeruginosa* PA14 was treated with kanamycin. Mutations in *wspF* ultimately result in elevated intracellular cyclic di-GMP (c-di-GMP) levels and Hickman et al. [145] demonstrated that this mutation is
capable of converting a wild-type strain into an RSCV, and subsequent depletion of c-di-GMP in a wspF mutant strain can convert the morphology back to a wild-type morphology, proving the implication of c-di-GMP signalling in the RSCV phenotype.

The mucoid phenotype has been associated by several authors [48, 141, 146] with the overproduction of EPS (alginate in P. aeruginosa) with many functions in the context of the pathogenesis of respiratory infections, as in CF. During respiratory infection, P. aeruginosa converts from non-mucoid to mucoid form, usually due to a mutation in mucA [48]. As mentioned before, the overproduction of alginate protects bacteria from host defences and antibiotic therapy, prolonging the infection persistence [48, 147].

Other morphologies are underestimated and poorly studied, being their role and impact on diversity and dynamic of bacterial populations unknown.

8.1.1. Biofilm as niches of phenotypic variants

Throughout biofilm development, bacteria adopt a specific phenotype, usually different from the planktonic one. This switch involves physiological, metabolic and phenotypic variations. For example, it has been described that bacteria regulate the expression of proteins implicated in resistance to oxidative damage, EPS production, phospholipids synthesis and membrane transport [74]. Hereafter, it can prompt mechanisms accountable for antimicrobial resistance, enhanced virulence and persistence [132].

Microniches within biofilms translate their heterogeneity imposed by chemical gradients. The morphotypes that may result from P. aeruginosa biofilms emphasize the diversity of colonymorphologies presented by biofilm-growing cells [132]. Stewart and Franklin [148] stated that in a mature biofilm there are at least three physiological states: cells in the superficial layer similar to planktonic cells, cells in the middle zone and cells in the deeper zone. In a study by Sousa et al. [132], biofilms of one P. aeruginosa clinical isolate originated morphotype diversity almost 4-fold higher than the observed for the wild-type strain.

Colony diversity of biofilms is also influenced by the development stage of these structures [148]. Different proportions of each variant during biofilm formation and after exposure to stressors, suggests that they have specialized functions in establishment and survival [149, 150] explaining why phenotypic heterogeneity within biofilms is one of the key motives of their survival in harsh environments [149].

8.2. Virulent variants

Phenotypic switching assists bacteria to evade immune mechanisms during colonisation and infection, by affecting the host-pathogen interactions, since the immune system reacts against recognized antigens and the generated subpopulations exhibit different sets of antigens [132]. One of the crucial stages for certain chronic infections, such as CF, is this phenotype switch. As mentioned before, in CF lungs P. aeruginosa might convert to a mucoid form by overproduction of alginate. This EPS has a protective role in harsh environments in which bacteria are under attack by the immune system and oxidative stress, turning bacteria more difficultly eradicated [132, 151]. This conversion and the possible hyperbiofilm ability of colony variants are important virulence aspects in pathogenesis. It has been proven that biofilms have reduced activation of the complement system and biofilm-growing bacteria are more resistant to phagocytosis and, in some cases, to antibody-mediated immune response [132, 152].

Mutations leading to loss of virulence-associated phenotypes are common among CF isolates of P. aeruginosa, where the loss of known virulence factors may represent an adaptive response to chronic infection, with such factors playing a crucial role in acute infections but are not required for persistence in the CF lung. On the other hand, a Liverpool Epidemic Strain (LES) hypervirulent subtype, more adapted to acute infections and associated with transmission to the non-CF parents of a CF patient, has also been identified [133]. This population diversification regarding pathogenic abilities leads to subtypes better adapted to causing either acute or chronic infections, or exhibiting transmissibility.

8.3. Antibiotic-resistant variants

Antibiotic resistance is generally associated with costs for bacteria, however, it was demonstrated a mechanism that contradicts those bacterial costs, the arising of SCV. This phenomenon was observed in S. aureus [153], where exposure to gentamicin led to the emergence of SCV, whereas in the absence of the antibiotic, the SCV reverted to normal phenotype, avoiding unfavourable costs related to irreversible resistance mutation. Similarly to other bacterial species, in P. aeruginosa the typical characteristics of SCV, such as small size, slow growth rate, pigmentaton and higher resistance to antibiotics and cell-wall inhibitors, are commonly attributed to defects in electron transport chain [138] caused by genetic mutations in menD, hemB and ctaA [132].

Another study combining data from phenotypic, gene expression and proteome analysis, demonstrated that resistance to aminoglycosides in one SCV mutant is multifactorial, resulting from the combination of several mechanisms including overexpression of efflux mechanisms and LPS modification, and is accompanied by a drastic down-regulation of the pqs system [154].
8.4. Persisters cells

Persisters cells correspond to the small fraction of a bacterial isogenic population, highly tolerant to antibiotics since they survive to exposure to lethal conditions in the absence of drug resistance mechanisms [155-158]. They are nor resistant nor have genetically acquired antimicrobial resistance. Instead, they are dormant cells remaining in a metabolic inactive state [155, 159]. After reinstatement of normal conditions, they might return to normal growth rates and generate a new population, equally susceptible. This reversion ability is suggested to be a sign of phenotypic switching [132]. The small amount of such cells and their only temporary expression justifies the little knowledge about the mechanism and traits of persisters colonies. There are however various theories in literature explaining their formation, however, the most relevant assumes that persistence is a phenotypic state [155].

Studies in *P. aeruginosa* have proven that there is also an association between persisters and antibiotic resistance since they can prevent clearance of bacteria, especially within biofilms [132, 155, 158]. A study performed by Möker *et al.* [159] has established that this phenotype can be affected by QS-linked molecules, since pyocyanin and AHL significantly amplified the persister quantities.

9. Concluding remarks

In order to colonise new niches, to adapt or to survive in a changing environment, bacteria, like *P. aeruginosa*, usually resort to evolution and diversification, and the great range of phenotypic variants reported is evidence of the significance of this phenomenon. This phenotypic flexibility has been shown to influence *P. aeruginosa* virulence and antimicrobial resistance, being crucial to bacterial fitness and competitiveness.

It is still largely unclear how different factors such as environmental regulation and gene products contribute to the success of the bacterial population. Nevertheless, the continued efforts to identify environmental signals and regulatory networks that influence phenotypic switching will contribute to the knowledge of the fluctuations that allow bacteria to adapt and survive to continuously changing external factors.

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