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REVIEW PAPER OPEN ACCESS

Resistance mechanisms of *Pseudomonas aeruginosa* and promising strategies to combat antibiotic resistance

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Abstract

Nowadays, antibiotic resistance constitutes one of the world-wide health crisis, food security and development. This review was designed to highlight the resistance mechanisms in *Pseudomonas aeruginosa*, a multi-resistant bacterium, and to propose strategies to combat antibiotic resistance. *P. aeruginosa* is a Gram-negative bacillus, strict aerobic and non-fermentative. It is involved in opportunistic infections, mainly in a nosocomial context. This bacterium is characterized by a natural resistance to many antibiotics, limiting the number of effective therapies. Acquisition of resistance to β-lactams is common and results from mutations leading to overproduction of chromosomal cephalosporinase (class C β-lactamase), overexpression of active efflux systems, decreased membrane permeability and/or of the acquisition of transferable genes. Resistance to aminoglycosides is common and most often the result of the acquisition of genes for modifying enzymes. Resistance to fluoroquinolones is frequently linked to mutations in genes encoding deoxyribonucleic acid (DNA) gyrase. Overexpression of efflux pumps also contributes to resistance to aminoglycosides and fluoroquinolones. A new molecule, ceftolozane-tazobactam, appears promising, particularly in the treatment of *P. aeruginosa* infections overproducing the cephalosporinase AmpC. However, multi-resistant or toto-resistant strains are being described more and more frequently throughout the world. The formation of biofilm controlled by the quorum sensing enhances the antibiotic resistance in *P. aeruginosa*. The increase of the antibiotic resistance in *P. aeruginosa* is a worrying phenomenon and biologists must be aware. New promising strategies must be developed for combating *P. aeruginosa* drug resistance.

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Introduction

Pseudomonas aeruginosa also called *Bacillus pyocyaneus* (pyocyanic bacillus) is a bacteria or agent of blue pus in superinfected wounds (Phan *et al.*, 2023). It is a Gram- bacillus which is 5 µm long and whose width is between 0.5 and 1 µm. It has a polar flagellum responsible for its motility in less viscous and aqueous environments (Bouteiller *et al.*, 2021). *P. aeruginosa* is also capable of moving on solid surfaces (Pereira *et al.*, 2023). The genome of *P. aeruginosa* strains varies between 5.2 and 7 Mbp, 10,000 to 40,000 genes with 4000 genes in common called the central genome (Jurado-mart *et al.*, 2021). This richness of the genome confers to the bacteria, metabolic flexibility and the ability to adapt to different conditions and certain hostile environments. It uses over 100 organic molecules as a carbon source (Diggle and Whiteley, 2020). It can grow at temperatures between 30°C and 42°C but 37°C is its optimal growth temperature. It is found in several biotic and abiotic places such as water, soil, plants, the digestive tract and on the skin of mammals (Balfour-lynn, 2021).

P. aeruginosa is responsible for nosocomial infections which are healthcare-associated infections acquired in a health center (Litwin *et al.*, 2021). Nosocomial infections constitute a global public health problem. They are responsible of the increase in morbidity and mortality in health establishments (Oni *et al.*, 2023). Around one hundred million patients contracted nosocomial infections causing the death of one per ten. The most common infections are urinary tract infections, respiratory tract infections and post-operation infections (Taye *et al.*, 2023). Among these infections, those caused by the bacterial species *P. aeruginosa* are difficult to treat because it develops multiple resistances to antibiotics including natural, adaptive and acquired resistance. It is classified as the pathogen presenting the greatest threat to human health (Qin *et al.*, 2022). There is between 33 to 71% mortality in patients infected with carbapenem-resistant Pseudomonas and an additional cost of care in the treatment of bacteremia (Rivera-villegas *et al.*, 2023).

Faced with this situation, it is necessary to find effective strategies for combating antibiotic resistance in *P. aeruginosa*. This review highlights the resistance mechanisms of *P. aeruginosa* and proposes strategies to combat antibiotic resistance.

Virulence factors of Pseudomonas aeruginosa

Cellular virulence factors

The flagellum: The flagellum is a unipolar filamentous multiprotein complex on the surface of the bacteria. On the one hand, it is responsible for the motility of *P. aeruginosa* in more or less viscous environments, and on the other hand it allows the pathogen to be directed and fixed on target cells (respiratory diseases) (Lovewell *et al.*, 2014). The flagellum, through its ease of attachment, initiates the start of the biofilm and allows its dispersion at the end in an appropriate timing while controlling its motility (Valentin *et al.*, 2022). Thus, the flagellum is involved in the pathogenesis of *P. aeruginosa*.

Type IV pili: Like the flagellum, type IV pili are polar protein complexes. They are similar to retractable hairs. They are involved in motility, adhesion and rapid colonization of spaces (Leighton *et al.*, 2015). The minor pilin (protein) allows recognition and attachment of host cell receptors (Barnier *et al.*, 2022). The protein plays a role in biofilm formation (development, aggregation and formation of protective structures). Pili induce an inflammatory response and develop resistance to cellular phagocytosis (Rowe *et al.*, 2023).

Lipopolysaccharide (LPS): Lipopolysaccharide (LPS) is a protein structure forming part of the outer face of the bilayer of the membrane in *P. aeruginosa* . It is structured in three parts, namely lipid A, a central part and the O antigen (Caroff and Novikov, 2020). Lipopolysaccharide constitutes a barrier against large molecules and toxins, and is at the origin of pathogen-host interactions, tissue damage, and reactive oxygen species. It plays a role in antibiotic resistance and biofilm formation. It also protects the bacteria against oxidative stress (Huszczynski *et al.*, 2020).

Extracellular virulence factors

Exotoxin A: Exotoxin A is the most toxic of the virulence factors (Michalska and Wolf, 2015). It has three regions, namely the N-terminal region (host cell attachment), the intermediate region (membrane translocation) and the C-terminal region (the most toxic part) (Gholami *et al.*, 2023). Once released, it binds to host cell receptors from where it is internalized. It targets elongation factors by inhibiting the synthesis of proteins and certain substances (Il-8, Il-6) by host cells. Alkaline protein (aeruginolysin) and type IV proteases are evasion and invasion factors (Panahi *et al.*, 2024).

Exoenzymes of the type III secretion system: The type III secretion system allows the bacteria to inject toxins into the host cell (Wagner *et al.*, 2018). The injection is done through a needle-like appendage capable of puncturing the membrane of the eukaryotic cell. Its pathogenicity depends on effector exotoxins. Several types of exoenzymes are involved in the type III secretion system.

Exoenzyme S (ExoS) is a bifunctional cytotoxic protein (one ADP-ribotransferase activity and one GAP activity). Its translocation leads to a dysfunction of certain cellular functions including the production (blocking) of ROS from host cells, disruption of the cytoskeleton, internalization of the bacteria, and DNA synthesis. It induces pulmonary inflammation by production of proinflammatory cytokines and proliferation of lymphocytes (Javanmardi *et al.*, 2019).

Exoenzyme T (Exo T)

ExoT is a bifunctional exotoxin (an ADP-ribotransferase activity and a GAP activity) whose activity disrupts certain functions and the architecture of the host cell (Wood *et al.*, 2023).

Exoenzyme U (Exo U)

ExoU is a cytotoxic protein. Its phospholipase activity degrades membrane fatty acids and lung tissue, resulting in severe pneumonia and even sepsis. The expression of ExoU thus increases the virulence of the pyocyanin bacillus in a model of acute pneumonia observed in mice. ExoU has greater cytotoxicity than ExoS (Foulkes *et al.*, 2019).

Exoenzyme Y

Exoenzyme Y is an adenylate cyclase which induces an accumulation of cyclic nucleotides at the intracellular level, with a change in cell morphology and the formation of intercellular holes damaging the pulmonary endothelium (Morrow *et al.*, 2017).

Siderophores: To compensate for its iron deficiency, *P. aeruginosa* synthesizes molecules (siderophores) capable of chelating iron III necessary for its proper functioning. These are organic molecules that chelate iron III with high affinity. Their secretion contributes to the virulence of *P. aeruginosa* (Xie *et al.*, 2024).

Pyoverdine

Pyoverdine is a yellow-green pigment that chelates free iron and removes host proteins. It is a siderophore which plays an important role in the virulence of the bacteria, particularly in the regulation of other factors such as exotoxin A (Ghssein and Ezzeddine, 2022).

Pyochelin

Pyochelin is a siderophore with a lower affinity for iron. It is the major siderophore because the production of pyoverdine requires the most energy (Ghssein and Ezzeddine, 2022).

Pyocyanine: Pyocyanin (phenazine) is a toxic pigment. It has antibiotic activity and facilitates biofilm development, invasion and inhibition of phagocytosis (Chimi *et al.*, 2024). It induces neutrophil apoptosis, oxidative stress and disruption of epithelial cell function (catalase, mucosa) (Panahi *et al.*, 2024).

Rhamnolipids: Rhamnolipids are heat-stable extracellular glycolipids that solubilize pulmonary surfactant phospholipids. They are synthesized from rhamnose and 3-hydroxyalkanoate by the enzymes RhlA and RhlB (Ochsner *et al.*, 1995).

Produced in low concentrations, they improve adhesion (fixation), hydrophobicity and the release of lipopolysaccharides. In appropriate quantities, they contribute to the maintenance of channels and the structure of the biofilm. They play a role in motility; inhibit certain transports and pulmonary ciliary functions. They disrupt respiratory epithelial junctions and suppress host innate immunity by hindering immune responses (Voulgaridou *et al.*, 2021).

Elastase: Elastase is a proteolytic enzyme that catalyzes the hydrolysis of elastin. Elastases A and B are secreted by the type 2 secretion system of *P. aeruginosa* . More abundant in the body, elastase B or pseudolysin is responsible for the deterioration of epithelial junctions, affecting bacterial clearance (Cigana *et al.*, 2021). It is also capable of inactivating certain proteins such as immunoglobin A, immunoglobin G, complement compounds, fibrin, collagen. Elastase A or staphylolysin is a serine protease whose proteolytic action promotes the activity of certain proteases (elastase B) (Cigana *et al.*, 2021).

Mechanisms of antibiotic resistance in Pseudomonas aeruginosa

Reduction of the membrane permeability

The bacteria prevent the penetration of the antibiotic into the cell impeaching it interaction with its target. The “entrance door” is represented by pores, normally made up of proteins which form channels, called porins. *P. aeruginosa* reduce their number of porins and thus destabilize these channels. *P. aeruginosa* is also capable of triggering genomic mutations affecting the expression or structure of these porins (Pang *et al.*, 2019). The main mechanism of resistance to carbapenems in *P. aeruginosa* remains the membrane impermeability by an inactivating mutation of oprD, the gene encoding the D2 protein (Meletis *et al.*, 2012). Loss of this outer membrane porin confers high-level resistance to imipenem and a variable decrease in sensitivity to meropenem and doripenem (Choi and Lee, 2019).

Efflux pumps

Efflux pumps are major determinants of antibiotic resistance in *P. aeruginosa*. The bacteria are able to eliminate antibiotics by actively pumping them out of the cell, which effluxes the bacterial toxic compounds (Lorusso *et al.*, 2022). Efflux pumps enable simultaneous resistance to different classes of antibiotics. The MexEF-OprN efflux pump promotes resistance to chloramphenicol, trimethoprim, triclosan and fluoroquinolones (Scoffone *et al.*, 2021). Continuous expression of the mexEF-oprN operon is observed in mutants of mexS, a gene whose function is unknown. A mutation in mexS also affects quorum sensing (QS) mechanisms, i.e. intercellular communication (Chakraborty *et al.*, 2023). QS controls the expression of virulence factors and the formation of biofilms, observed in the respiratory tract of people with cystic fibrosis (Scoffone *et al.*, 2021).

Production of enzymes degrading antibiotics

The bacteria produce an enzyme that modifies or cleaves the antibiotic molecule, rendering it inactive (Pang *et al.*, 2019). It is the main mechanism of resistance to β-lactams (penicillin and cephalosporin family) which involves enzymes of the β-lactamase family. *P. aeruginosa* naturally expresses an inducible cephalosporinase encoded by the chromosomal ampC gene (Pachori *et al.*, 2019). Its expression can be strongly induced by certain P-lactams, notably imipenem, clavulanic acid (unlike tazobactam) and cephamycin. This induced and reversible hyperexpression confers resistance to all anti-Pseudomonas penicillin and cephalosporins, and to aztreonam. In this case, only carbapenems remain active antibiotics. However, carbapenemase-producing strains are currently spreading alarmingly in all regions of the world (Nordmann *et al.*, 2011). Although blaKPC, encoding a class A carbapenemase, has recently been described in PAMR strains, these carbapenemases most often belong to class B (Taggar *et al.*, 2020). With the exception of aztreonam, all β-lactams, including carbapenems, are hydrolyzed by these metallo-β-lactamases. Furthermore, *P. aeruginosa* is probably the pathogenic bacterial

species with the broadest diversity in terms of acquired β-lactamases. These enzymes are almost exclusively encoded by plasmids. The most common Ambler class A β-lactamases (serine-β-lactamases, susceptible to inhibitors) in *P. aeruginosa* are those of the PSE group (CARB) which inactivate all β-lactam antibiotics (Tooke *et al.*, 2019).

Quorum sensing

Quorum Sensing (QS) is a cellular communication mechanism. It allows different bacterial groups to coordinate gene expression in various environments and also control bacterial metabolism (Goo *et al.*, 2015). Metabolites of the QS system of *P. aeruginosa* can induce immune cell death by dissolution of the lipid domain on the cell surface (Song *et al.*, 2019). The transcription of many *P. aeruginosa* virulence genes is under the control of bacterial density-dependent QS. Two QS systems, named las and rhl, have been identified in *P. aeruginosa* . Each system is defined by a pair composed of a regulatory protein and an autoinducing enzyme: LasR/LasI for the las system and RhlR/RhlI for rhl (Ruimy and Andremont, 2004).

The las system, the first to have been described, comprises the lasR gene encoding the regulatory protein LasR and the lasI gene encoding an auto-inducer synthase enzyme (LasI), necessary for the synthesis of a type of AHL: N -(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (Alfiniyah *et al.*, 2019). As in V. ficheri, 3-oxo-C12-HSL has the property of easily crossing bacterial membranes and thus constitutes a real means of communication between bacteria. When the concentration of 3-oxo-C12- HSL reaches a critical threshold, indicating a high bacterial concentration, an AHL molecule binds to two LasR proteins to form a complex that activates the transcription of several genes (Kumar *et al.*, 2022). This activation is triggered synchronously throughout the bacterial population at the junction between the exponential growth phase and the beginning of the stationary phase. The genes activated by this system include: (i) lasB, lasA, aprA coding respectively for two elastases, and for an alkaline protease each contributing to the destruction of lung tissues, (ii) toxA coding for an ADP-ribosylating exotoxin, (iii) xcpR and xcpP, encoding proteins of the type II secretion machinery necessary for the export of these factors out of the bacteria, (iv) lasI, allowing a rapid increase in the synthesis of 3-oxo-C12-HSL and therefore an amplification of the signal by self-induction (Kiratisin *et al.*, 2002; Aceves-Soto *et al.*, 2022).

The second rhl system functions according to the same scheme and includes the rhlR gene, coding for the regulatory protein RhlR and the rhlI gene, coding for an auto-inducing synthase enzyme, RhlI necessary for the synthesis of a second type of AHL: N -butyryl-L-homoserine lactone (C4-HSL) (Elnegery *et al.*, 2021). The RhlR-C4-HSl complex controls the expression of the rhlAB operon necessary for rhamnolipid production, and the expression of a series of genes including lasB, lasA, aprA, and rhlI (Chadha *et al.*, 2022).

Biofilm formation

A biofilm is a population of microorganisms organized into a community and attached to a surface which can be biotic or abiotic (Salinas *et al.*, 2022). Bacterial biofilms have intrinsic characteristics that make them more resistant to harsh environmental conditions (pH, oxygen, UV, flux) (Salinas *et al.*, 2022). In the context of an infection, the establishment of the biofilm also results in a bacterial population that is difficult to eliminate by the immune system but is also resistant to antibiotics. Major molecular determinants which are involved in the formation of the biofilm are the flagellum, the type IV pili and the Cup fimbriae for attachment, or even the exopolysaccharides which are, with DNA, essential components of the extracellular matrix of the biofilm that encompasses the bacterial population (Nadar *et al.*, 2022). In *P. aeruginosa* , if alginate is the major polysaccharide, it has been shown that non-mucoid strains also form biofilms and that in this context, the synthesis and secretion of the major polysaccharide of the matrix are dependent on pel genes (Nadar *et al.*, 2022). Bacteria located within

biofilms become resistant to phagocytosis, antibodies, disinfectants and antibiotics. The nature of the mechanisms of this resistance has not yet been completely elucidated. These biofilms represent the main cause of persistence of pneumonia in cystic fibrosis patients and of ocular infection in contact lens wearers (Tuon *et al.*, 2022).

Three stages can be distinguished in the formation of biofilms: an initial stage of attachment to the surface of a mucous membrane or foreign material, followed by a stage of proliferation leading to the formation of microcolonies and finally a stage structuring of the biofilm (Zubair *et al.*, 2017). QS has been shown to play a role in the latter stage; which is not surprising since the QS is dependent on a high bacterial density. The biofilm obtained from a strain mutated in the lasI gene is thinner, poorly structured, and sensitive to the action of a 0.2% SDS detergent (Sharma *et al.*, 2023). On the other hand, a mutant strain in the rhlI gene gives a minimally modified biofilm comparable to that obtained with the parental strain. The addition of 3-oxo-C12-HSL restores a structured biofilm and confirms the essential role of QS via lasI (Sharma *et al.*, 2023). The production of 3-oxo-C12-HSL within the biofilms was measured on an artificial bladder model and on urinary catheters taken from patients. The role of QS in the constitution of biofilms opens therapeutic perspectives for the use of QS.

Therapeutic targets against antibiotic resistance in P. aeruginosa

Inhibition of positive QS regulators or inhibition of lasR–lasI or rhlR–rhlI expression

A therapeutic target could be the inhibition of positive QS regulators such as GacA or Vfr. Indeed, bacteria mutated on these genes have a reduction in the transcription of lasR and/or rhlR as well as virulence factors dependent on QS (Elnegery *et al.*, 2021). Another theoretical approach is the use of antisense oligonucleotides which would bind specifically to lasR–lasI or rhlR–rhlI transcripts and thus inhibit the expression of genes regulated by these transcriptional activators.

Degradation or inhibition of the AHL synthesis

The synthesis of AHL requires in particular the presence of a molecule, S-adenosyl methionine as an amino acid donor. Analogues of this substance (such as S-adenosylhomocysteine, S-adenosylcysteine, sinefungin) can block the synthesis of AHL produced by *P. aeruginosa*. However, these compounds are still not specific enough and interfere with bacterial growth. The administration of 2 µg of azithromycin/ml to a culture of *P. aeruginosa* interfered with the QS: this antibiotic reduces the transcription of lasI by 80% and of rhlI by 50%, reduces the production of 3-oxo-C12- HSL and C4-HSL, and decreases the amount of elastase and rhamnolipid (Kumar *et al.*, 2022). Azithromycin acts by inhibiting the synthesis of autoinducer molecules (Kumar *et al.*, 2022). The first enzyme inactivating AHL (named AiiA) was discovered in Bacillus sp. The introduction of this gene into *P. aeruginosa* leads to a reduction in the synthesis of AHL and certain virulence factors such as pyocyanin, elastase and rhamnolipids (Ruimy and Andremont, 2004). Other virulence factors such as motility and adhesion to surfaces are not affected. Much research focuses on other molecules inhibiting AHLs. In humans, the respiratory epithelium produces molecules that inactivate QS autoinducers. These would be AHL-cleaving enzymes (acylases or lactonases) (Pang *et al.*, 2019). When AHLs diffuse out of the bacteria, they may be accessible to anti-AHL antibodies. Thus, in *P. aeruginosa* , antibodies directed against 3-oxo-C12-HSL are capable of preventing the activation of the lasB transcript as well as the production of IL-8 by epithelial cells.

The most promising therapeutic strategy appears to be blocking AHL at its protein receptor. This blocking can be done either by competitive inhibitors, having a structure similar to that of AHL, or by non-competitive inhibitors which would have a structure different from AHL and which would bind to different sites on the protein receptor. Natural QS inhibitors with antimicrobial properties, furanones, have been discovered. The mechanism of action of these compounds is not fully understood but it appears to inhibit the interaction of AHL with its protein receptor.

Conclusion

P. aeruginosa possesses numerous virulence factors. The main interbacterial communication systems in this bacterium are the las and rhl systems. Among the genes regulated by QS are genes encoding virulence factors such as elastase, exotoxin A, pyocyanin, rhamnolipids. Models of *P. aeruginosa* infections have highlighted the crucial role of QS in pathogenicity. Inhibiting this interbacterial communication could reduce the production of virulence factors. Given the complexity of QS signaling, many blocking pathways are theoretically possible. Among the compounds currently studied, furanones prevent the expression of numerous virulence genes dependent on quorum sensing and experimental data in animals are already encouraging.

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