Sapporo Medical Journal
Volume56, Issue12, December 2022

Pseudomonas aeruginosa Virulence Factors and Antivirulence mechanisms to Combat Drug Resistance; A Systematic Review

Nesrin Seder[1*], Walid Abu Rayyan[2], O'la Al-Fawares[2], Mohd Hilmi Abu Bakar[3]

[1]Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, Applied Science Private University, Amman, Jordan.
[2]Department of Medical Laboratory Analysis Faculty of Science, Al-Balqa Applied University, Al-salt, Jordan.
[3]Faculty of Health Sciences, University Sultan Zain Al Abidin, Kuala Nerus, Terengganu, 21300, Malaysia

Abstract— *P. aeruginosa*isan opportunistic microorganism that impose a versatile modifications in its genome to sustain an infection that can range from otitis media to a life threating disease. Moreover, antibiotic resistance endows *P. aeruginosa* characteristics to be one of the most resistant microorganisms to be treated. *P. aeruginosa* possess both cell associated and extracellular virulence factors. Cell associated virulence factors include flagellum, pili, adhesins, alginate, and lipopolysaccharide. While, extracellular virulence factors include hemolysins, proteases, lipases, exotoxin A, and cytotoxins.

Keywords: *P. aeruginosa,* Virulence factors, Antimicrobial resistance, Biofilm.

Pseudomonas aeruginosa

1.1 Characteristic of *P. aeruginosa*

The Gram negative bacilli *P. aeruginosa* is a pathogen normally found in humid environments such as normal flora and the soil[1]. It possesses a single whip-like flagellum located at the cell pole, together with several much shorter appendages called pili that are also localized at the cell pole. These parts are considered as one of the bacterium virulence factors. They are responsible for the bacterium motility in the aqueous environments, adhesion, and chemotaxis. In addition, they play an important role in the initiation of the inflammatory responses[2, 3]. *P. aeruginosa*is a uniquely adaptable microorganism that colonizes many ecological niches including animals, plants, and humans [4]. The pathogenicity of *P. aeruginosa*is highly dependent on the regulatory mechanisms of the intra and extracellular signals exposed during the infection state [1][5]. Antibiotic resistance of *P. aeruginosa*confers a wide range of tolerance against natural and artificial bactericidal compounds. *P. aeruginosa*infections range from nosocomial infections in immunocompromised host defenses such as in neutropenia, severe burns, or cystic fibrosis to otitis media infections[6][7-9]. Recently, the human pathogen *P. aeruginosa*is classified as the third most common hospital acquired pathogen causing approximately 10% of the hospital infections in the United States annually and leading to substantial morbidity and mortality[10][11].

*P. aeruginosa*strains possess large genomes approximately 5-7 Mbp capable of provoking life-threatening infections in several patients [12]. The adaptability encoded in its genome is

one of the versatile modifications of *P. aeruginosa* to sustain an infection that can opportunistically infect diverse organisms from insects to humans. Antibiotic resistance makes*P. aeruginosa*one of the most difficult microorganisms to be treated and to be removed from hospitals and healthcare centers. It is also the major cause of chronic lung infections in patients with cystic fibrosis and is thought to be associated with progressive deterioration of lung function and eventual death in such individuals [6][13].

1.2 Common Virulence Factors of *P. aeruginosa*

The ability of *P. aeruginosa* to cause high rates of illnesses and death is primarily related to a diverse range of virulence factors. It is possessing both cell associated and extracellular virulence factors. Cell associated virulence factors include flagellum, pili, adhesins, alginate, and lipopolysaccharide. While, extracellular virulence factors include hemolysins, proteases, lipases, exotoxin A, and cytotoxins[14]. The severity of *P. aeruginosa*infections and the limitation of antimicrobial agents are a result of the virulence factors as listed in Table Error**! No text of specified style in document.**-1.

Table **Error! No text of specified style in document.**-1: Common virulence factors of *P. aeruginosa*

Virulence factors of *P. aeruginosa*
Flagella and type 4 pili
Type 3 secretion system
Proteases
Exotoxin A
Pyocyanin and iron chelation
Quorum sensing and biofilm formation
Outer membrane proteins and efflux pumps
Antimicrobial resistance

1.2.1 Flagella and Type 4 Pili (T4P)

P. aeruginosa contains three types of appendages; polar flagellum, shorter pili and cupfimbriae. Flagellum helps the bacteria to move in rotating motion in aqueous media. While pili are essential to establish an infection by facilitating bacterial adherence to the host epithelial cells through binding to the asialyted glycolipid asialoganglio-N-tetraosylceramide (asialoGM1) [3].

However, non-flagellated mutant strains are impaired in models of acute infection [15]. Surprisingly, microorganisms isolated from models with chronic infections show a down expression of flagella or flagella mediated motility [16][6][17]. Meanwhile, type 4 pili facilitate twitching motility to help the adherence and aggregation of bacterium on target tissue which allows microcolonies of bacteria to protect themselves against the host immune system and antibiotics[18]. Pilin deficient mutants have demonstrated defective *P. aeruginosa*in twitching motility and reduce virulence ability[19][20].

1.2.2 Type 3 Secretion System (T3SS)

P. aeruginosa is a major cause of human infections and mortality in immunocompromised and cystic fibrosis patients. The host cells are infected by *P. aeruginosa* throughtheType 3 secretion system (T3SS)[21]. The pathogenic Gram negative bacterium uses T3SS which isa needle like complex to penetrate the proteinaceous toxins from the bacterial cytosol directly into host cells. These toxins are called effector proteins. Substantially, *Exo*Y*, Exo*S*, Exo*T, and *Exo*U are the only effectors that have been identified in *P. aeruginosa*[22], indicating that *P. aeruginosa*T3SS has fewer effectors compared to T3SS used by other pathogenic Gram negative bacteria. For instance, *Salmonella enterica* SPI-1 has 13 effectors and *Shigella* species have 25 effectors [21]

The majority of *P. aeruginosa*strains share both genes *Exo*Tand *Exo*Y.However, they possess only one of *Exo*S or *Exo*U genes during the infection state. *Exo*Ssecreting strains provoking directly host cell death by inducing apoptosis and inhibiting DNA repair. Whereas, *Exo*Uis a phospholipase that is 100 times more potent in accelerating host cell lysis [21]. Biogenesis and regulation of T3SS are encoded by 36 genes on five operons of the *P. aeruginosa* chromosome. While the effector proteins and their chaperones are encoded by six other genes scattered around the chromosome [21]. The T3SS is transcriptionally controlled by the regulator *Exs*A*,* a member of the *Ara*C family of transcriptional regulators (TRs) that controls multi cellular functions in the bacteria such as the virulence [17].

T3SS is considered an important factor in *P. aeruginosa*pathogenesis.Consequently, this system became a remarkable target for therapeutic intervention. Scientific researchers have focused their efforts against PcrV protein as it is a crucial component of the type III secretion system causing cytotoxicity. It is accessible at the tip of the needle like system and required for translocation of effector proteins [23]. Engineered human anti-PcrV Fab fragments have now been generated and should facilitate the clinical use of active or passive immunization. Phase I/II clinical studies are ongoing to evaluate the usefulness of PcrV specific antibodies in the settings of mechanically ventilated patients and individuals with cystic fibrosis[24].

1.2.3 Extracellular Matrix of *P. aeruginosa*Biofilm

Extracellular matrix of biofilms composed mainly of exopolysaccharides, eDNA, and proteins. ECM functions as an adhesive material, and protective barrier [25]. Until now three EPSs are identified in *P. aeruginosa*: polysaccharide synthesis locus (Psl), pellicle exopolysaccharide locus (Pel), and alginate (Alg) [26].

Psl polysaccharide is an important component of the ECM for the initiation and maintenance of *P. aeruginosa*biofilms by providing cell-surface attachment and intercellular interactions. In addition, Psl provides structural support for *P. aeruginosa*biofilms by physical interaction with eDNA to form a mesh of eDNA-Psl [27]. Meanwhile, Pel is known to be composed of cationic amino sugars, which facilitate binding with eDNA of the biofilm [28]. Pel polysaccharide is an essential component for *P. aeruginosa* to form pellicles at the air-liquid interface and solid surface associated biofilms providing protection against antibiotics such as aminoglycosides [29]. Pel can also compensate forPsl when there is a lack of Psl production in the biofilm periphery. Ultimately, the mechanisms of Psl and Pel production have a

converge point as both exopolysaccharides are under the regulation of the c-di-GMP signaling pathway owing to environmental cues [28]. Furthermore, alginate is the most studied EPS of *P. aeruginosa*biofilms and is mainly produced by *P. aeruginosa*strains isolated from cystic fibrosis patients [30]. Alginate is known as a factor used to distinguish mucoid or non-mucoid *P. aeruginosa* biofilms. Although it was found that Psl also contributes to the mucoid phenotype of the biofilms [31]. Alginate plays many important roles in biofilms. For example, alginate retains water and nutrients and provides antibiotic resistance and immune evasion [32].

Another component of the ECM is eDNA. It is a central component of the biofilm matrix that is necessary for both initial attachment and early biofilm formation. It also plays an important role in the formation of cation gradients and antibiotic resistance [33]. Moreover, eDNA is a major pro-inflammatory factor for *P. aeruginosa* biofilms [34].

Proteins also contribute to the formation of the biofilm matrix [33]. For example, flagella act as adhesion to help initial bacterial attachment to the surface, type IV pili contribute to the formation of mushroom like biofilm cap structures [35]. The outer membrane transporter cyclic diguanylate regulater partner A (*Cdr*A) interacts with Psl and increases the stability of biofilm. Cup fimbriae are also one of the proteinaceous components of the ECM that plays important roles in cell tocell interaction during the initial stages of biofilm formation [25].

1.2.4 Proteases

The opportunistic human pathogen *P. aeruginosa*can elaborate a large number of secretory products and proteins including proteases. Proteases are considered an important virulence factor that is capable of damaging host tissues and interfering with host antibacterial defense mechanisms [36]. Proteases are enzymes that hydrolyze peptide bonds and can, therefore, degrade proteins and peptides like various plasma proteins such as complement and coagulation factors [37]. Proteases of*P. aeruginosa*help to establish and maintain infections and thereby controlling and modifying the environment according to the needs of the bacterium within the host tissue[38]

Several potent proteases have been isolated and characterized in *P. aeruginosa*infectionssuch as *Pseudomonas* elastase (PE), alkaline protease (AprA), and protease IV [39]. *P. aeruginosa* produces two elastases; *Las*A which known as staphylolysin and *Las*B. Both enzymes are regulated by the *las*I quorum sensing system and secreted via type 2 secretion systems [40, 41]. *P. aeruginosa* proteases are detected in ocular infections and the lungs of cystic fibrosis patients such as elastase B, alkaline protease, and protease IV [42]. *Las*A, is a serine protease has high staphylolytic activity as it can hydrolyze the pentaglycine bridge required for peptidoglycan stabilization in the cell wall of staphylococci. Whereas, it has a low elastolytic ability compared with that of *Las*B and rather it is thought to enhance the proteolytic activity of *Las*B [43]. *Las*B has been observed to degrade the opsonizing lung surfactant proteins A and D [44]. As a result, *las*B mutants are more susceptible to phagocytosis and are attenuated for virulence [45].

Alkaline protease is a zinc metalloprotease that has been shown to degrade host complement proteins and host fibronectin [46]. It has a direct function in invasion and hemorrhagic tissue

necrosis in infections caused by *P. aeruginosa*. Moreover, it can interfere with flagellin signaling through host Toll like receptor 5 (TLR5) by degrading free flagellin monomers and thereby helping *P. aeruginosa* to avoid immune detection [47].

Furthermore, leukocytes are components of the host immune system that is affected by proteases of *P. aeruginosa*. It was shown that the secretion of alkaline protease and *Las*B proteins hinder the phagocytic activity against *P. aeruginosa*. This effect of *P. aeruginosa* is based on the cleavage of the cell receptors on the cell surface which are necessary for phagocytosis [48].Protease IV has been shown to inhibit the phagocytosis of *P. aeruginosa*by alveolar macrophages. The degradation exerted on host surfactant proteins A and D demonstrates a crucial role for proteases in *P. aeruginosa* survival during infection [49]. In the immune response, both protease and elastase B can impede the function of neutrophils, especially by interfering with their chemotaxis [50]. This promotes escaping of the bacterium from phagocytes of the host defense system[51].

1.2.5 Lipopolysaccharides

Lipopolysaccharides (LPS) which known as endotoxin is an important virulence factor for *P. aeruginosa*. LPS is a complex glycolipid that is the major component of the outer membrane in Gram negative bacteria [52][53]. LPS constitutes a physical barrier protecting the bacterium from host defenses, mediates direct interactions with host cell receptors, and functions as endotoxin leading to host tissue damage [54]. LPS composed of three parts: lipid A which is embedded in the outer membrane, the core oligosaccharide, and the O antigen polysaccharide [6]. Lipid A can induce fatal reactions of the human immune system at very low concentrations. Core oligosaccharide is bound to lipid A and relatively well conserved among closely related bacteria. The third component is the O antigenpolysaccharide which represents the outermost region of the LPS and is involved in many of the interactions between the bacterium and the environment or host [52, 54 2006][55].

During the chronic infections of *P. aeruginosa,* LPS interferes with the immune system through mediating an essential modification at the level of Lipid A structure. This leads to a loss of O antigen which promotes immune evasion. Furthermore, LPS and O-antigen are hotspots of genetic variation during bacterial infections namely during chronic CF infections[52]. The modifications of Lipid A are performed through an acylation or addition of positively charged components to render the outer membrane resistance to the host antimicrobial peptides. Finally, cyclic di-GMP modulate O antigen biosynthesis which facilitates the switch from motile to biofilm lifestyle during *P. aeruginosa* infections[6].

1.2.6 Exotoxin A

*P. aeruginosa*produce an extracellular enzymeknown as exotoxin A. Thisenzymebelongs to the mono-ADP-ribosyltransferase (ADPRT) family. It is considered one of the most important virulence factors of *P. aeruginosa.* It enables this pathogenic bacterium to survive and initiate infectious responses in host cells[56]. Exotoxin A plays an important role in host pathogenicity by inhibiting host elongation factor 2 (EF2) which inhibits proteins synthesis leading ultimately to the death of the host cells [57]. Additionally, it has direct cytopathic effects and can interfere with cellular immune functions of the host [58].Several studies

conducted on strainsproducing exotoxin A showed increased virulence compared with deficient mutantstrains of exotoxin A [56].

1.2.7 Pyocyanin and Iron Chelation

The source of blue green color for *P. aeruginosa* colonies is derived from pyocyanin pigment which confers the distinct color. The ability of *P. aeruginosa* to produce pyocyanin is considered a marker of virulence. It leads to oxidative stress in the host by inactivating host catalases, and disrupting of mitochondrial electron transport chain [59]. Thus, itexerts cytotoxic effect in the host cells through secretion of cellular catalases to confer a protection against reactive oxygen species produced by host tissues. Additionally, pyocyanin facilitates biofilm formation by intercalating of eDNA to the biofilm matrix [60]. During cystic fibrosis infections, pyocyanin secretion causes damage to lung epithelium and plays a vital role in the pathogenesis of pneumonia [61]. Recent studies on pyocyanin have approved the potential role in *vitro* to induce apoptosis of leukocytes especially in neutrophils, as well as promoting the phagocytosis process in the innate immune response of apoptotic bodies by macrophages [59, 62]. Moreover, it inhibits the mucociliary activity of the pseudostratified columnar epithelial cells [63, 64].

Another virulence factor is the ability of the *P. aeruginosa*to chelate iron which is essential to establish an infection and to progress into a chronic infection [13]. In the host environment, there are a few free iron molecules in the bloodstream due to the presence of iron binding proteins such as lactoferrin and transferrin which function as sequester molecules. *P. aeruginosa* produce two siderophores; pyoverdine and pyochelin to sequester iron from host depots. Iron bound pyoverdine interacts with the*Pseudomonas* cell receptor FpvA. This complex, in turn, interacts with the anti sigma factor FpvR, causing the upregulation of exotoxin A, endoprotease, and pyoverdine itself [65]. Eventually, several other iron siderophore transport systems exist enabling the uptake of iron complexed with endogenous siderophores such as host heme, or the siderophores of other microorganisms [66].

1.2.8 Quorum Sensing

Quorum sensing (QS) is a mechanism implemented by different bacteria such as *E. coli, P. aeruginosa, S. aureus,* and *S. epidermidis* toexecute spatial and temporal coordination of the bacterial population to the environmental changes. This adaptation is mediated by small membrane diffusible molecules called autoinducers (AIs)[67]. QS allows a coordinated responses to a variety of environmental signals by inducing alterations in gene expression [68, 69]. AIs molecules are constitutively produced as cofactors to act as specific transcriptional regulators when the threshold is reached. The threshold is proportional to two conditions; firstly, the concentration of autoinducer molecules in the medium, and secondly, the number of bacteria. For instance, when the bacterial population increases to a certain level the autoinducers activate the expression of specific downstream genes resulting in a coordinated response across the entire bacterial population[70]. It is estimated that around 10% of the genome and more than 20% of the expressed bacterial proteome are regulated by QS [68]. QSregulates the expression of several virulence factors in *P. aeruginosa*such as pyocyanin to destruct host cells. Additionally, biofilm formation in *P. aeruginosa* is directly regulated by QS. The pathogenicity of *P. aeruginosa*is mediated through a communication

between bacterial cells under the regulation of QS mechanism. Recently, QS inhibitors are implemented as antimicrobial agents to hinder the bacterial pathogenicity in chronic infections [70][71].

1.2.9 Outer Membrane Porins and Efflux Pumps

Porins are beta barrelouter membrane proteins that cross the cellular membrane of the bacterium. This is a general feature shared among Gram negative bacteria. Porins function to form a water filled pore through the membrane from the exterior to the periplasm. This region is located between the outer and inner membranes of the bacterium results in asemi permeable membrane [72].The outer membrane proteins of *P. aeruginosa* form a significant barrier to the penetration of antibiotics, restricting the rate of penetration of small molecules and excluding larger molecules [73]. Small hydrophilic antibiotics such as the β-lactams and quinolones can only cross the outer membrane by passing through the aqueous channels provided by porin proteins [74 2003].

Porins are of great importance since they allow the transportation of small hydrophilic molecules through the periplasm such as a variety of nutrients required by the bacterium for growth and survival.The size of the diffusing molecule depends on the size of the channel.Moreover, porins have relevance in antibiotic resistance by mutating the gene that encodes the porins.The outer membrane of *P. aeruginosa*has four types of porins: 1) General/nonspecific porins, 2) Substrate specific porins, 3) Gated porins and 4) Efflux porins (also called channel-tunnels) [72][75]. The sequencing of genomic DNA of *P. aeruginosa*wild type PAO1 has revealed three large families of porins: The OprD family of specific porins which consists of 19 members, the TonB interacting family of gated porins consists 35 members and the OprM family of efflux porins that consists of 18 members [76].

1.3 Development Stages of *P. aeruginosa* Biofilm

1.3.1 Attachment of *P. aeruginosa*

There are several structures in *P. aeruginosa* involved in the attachment process such as adhesins, type IV pili, and lipopolysaccharide.These structures are regulated by environmental cues, through an increase in levels of cyclic diguanylate monophosphate (c-di-GMP), an intracellular second messenger [77, 78].

Many types of environmental cues can cause an increase in cyclic di-GMP level, which activates the production of adhesions, Psl, Pel, alginates, and various extracellular matrix products[26][78]. Biofilm formation is also regulated by small RNA (sRNA)produced in many bacterial species [79]. The shift between motile and sessile states is influenced by several regulatory systems that appear to intersect at various nodes. The *Gac*A/*Gac*S two component system has been implicatedfor many years in both biofilm formation and virulence. An activated *Gac*A response regulator (RR) promotes the transcription of the two small regulatory RNAs, *Rsm*Y and *Rsm*Z, which then bind and inactivate the translational repressor *Rsm*A [80]. Sequestered *Rsm*A causes the formation of biofilm through increase the production of Pel and PsI polysaccharides and increase the second messenger cyclicdi-GMP level[81][82]. The hybrid sensor *Ret*S influences this system by repressing *Gac*A and by

affecting cyclic di-GMP production via the diguanyl cyclase and *Wsp*R regulator[82]. Many other regulators contribute in regulating biofilm formation such as diguanylate cyclases by controlling the production of cyclicdi-GMP[83].

1.3.2 Maturation of *P. aeruginosa*Biofilm

After the attachment of *P. aeruginosa* to surfaces, they start to grow, produce ECMs, synthesize water channels, and form microcolonies. Biofilm maturation requires the production of the EPS components of the matrix. The EPS genes are upregulated by small RNAs linked to cyclic di-GMP when *P. aeruginosa*attaches to a host surface [84][85]. The biofilm maturation is mediated through a quorum sensing signaling system. Additionally, the bacteria became more adapted and resistant to environmental conditions and antibiotics due to the protective rigid polymeric barrier that supports the cells and retards the diffusion of molecules such as antibiotics [67][86].

1.3.3 Detachment of *P. aeruginosa*Biofilm

A detachment process is required to create new biofilms in new niches. There are several types of biofilm detachment mechanisms including sloughing, erosion, and dispersal [87].Sloughing is the detachment of a large portion of a biofilm from the original mass. While, erosion is a washout of a small portion of biomass or bacteria from the outer surface. Finally, dispersal is a single planktonic cell or microcolonies released from the center of the biofilm, leaving an empty cavity [87]. The dispersal mechanism involves ECM degradation and autolysis of a biofilm subpopulation. This process is induced by environmental cues, such as nutrients, oxygen availability, nitric oxide (NO), pH, and various chemicals[88][77].Metal chelators, cis-2- decanoic acid, and anthranilate are examples of chemicals contribute to the dispersal of *P. aeruginosa* biofilms[89]. Additionally, in the conditions of a sudden increase in glucose supply, a decrease of intracellular cyclic di-GMP happens which increases flagella production and induces dispersal [90]. Moreover, nitric oxide stimulates phosphodiesterase activity, which decreases the intracellular c-di-GMP level in *P. aeruginosa* and leads to the dispersal of the biofilm [91].

1.4 Infections of *P. aeruginosa* Biofilm

*P. aeruginosa*is considered the major cause of hospital acquired infections (HAI) owing to its ability to express several mechanisms of resistance including biofilm formation. The conversion from the planktonic to the biofilm stage, and change the gene expression pattern contributes to the cause severe infections [88].*P. aeruginosa* biofilm causes several infections including skin and soft tissue infections, osteomyelitis, endophthalmitis, endocarditis, meningitis, and septicemia. Also, it may develop on devitalized tissue and medically implanted devices[88].*P. aeruginosa* biofilm is frequent in the lungs of cystic fibrosis (CF) and diffuse panbronchiolitis (DPB) patients. These biofilm infections cause death in more than 90% of these patients. The clinical course of both CF and DPB can be divided into two pathogenic modes originating from the chronic biofilm colonization. The first mode, being an acute exacerbation caused by planktonic bacteria released from the biofilm, and the second mode, being a slowly progressive insult induced by harmful immune reactions mediated by matrix components of the biofilm [92][93].

Biofilms are a major medical problem since biofilm infections cause high resistance to antibiotics, disinfectants, and the host immune response. Chronic infections are caused mainly by biofilm of microbial pathogens[94]. The microbes in biofilms can be hundreds of times more resistant than their planktonic counterparts [95][96]. Biofilm associated infections are either due to direct infections in host tissues, such as chronic pneumonia in CF patients, chronic otitis media, and palindromic urinary tract infection [97], or due to indwelling medical devices such as urinary catheters, peritoneal dialysis catheters, contact lenses and venous catheters [92]. Biofilms have been isolated from almost all medical devices, and their related infections are very difficult to be eradicated[98]. These infections are at a high risk of progression to systemic infections. The only treatment of biofilm infections on medical devices is the removal of the device [99].

P. aeruginosa like other microbes in biofilms use several biofilm specific mechanisms that are different than those commonly used by planktonic counterparts. These mechanisms include the physical barrier provided by the ECM that prevents the distribution of antibiotics into the biofilm [100] andresistance against strong external stress such as antibiotics. This resistance provided by metabolic growth arrest of persister cells which are biofilmsub population of*P. aeruginosa* that proliferate extremely slowly or stop growth altogether when exposed to antibiotics[101][102].Moreover, glucan production and efflux pumps are important antibiotic resistance mechanisms in biofilm[103][104]. Furthermore, the biofilm under starvation conditions is restricted concerning oxygen and nutrient supply, leading to the inhibition of growth and increase of amino acid synthesis for survival. This starvation induced response plays an important role in enhancing biofilm resistance [105]. Ethanol oxidation, eDNA, and iron acquisition are also thought to contribute to antibiotic resistance with mechanisms that are still unknown[106].

1.5 Antibiotic Resistance in *P. aeruginosa*

P. aeruginosa presents a serious therapeutic challenge for treatment of both, community acquired and nosocomial infections. The selection of the most appropriate antibiotic is complicated by the ability of *P. aeruginosa* to develop resistance to multiple classes of antibacterial agents[107]. This resistance is attributable to the acquisition of resistance genes on mobile genetic elements such as plasmids or through mutations in chromosomal genes which regulate resistance genes [108]. In addition to this acquired resistance, *P. aeruginosa* is intrinsically resistant to antimicrobial agents due to outer membrane impermeability, low permeability of its cell wall, drug efflux mechanisms, and antibiotic degrading enzymes [74]. Although resistance of *P. aeruginosa* to individual drug or drug classes is a concern, the prevalence of multidrug resistant strains is an even more serious therapeutic challenge[95].

1.5.1 Intrinsic Resistance of *P. aeruginosa*

Generally, intrinsic resistance is encoded in the microorganism's chromosome. For instance,the antibiotics used to treat *P. aeruginosa* infections have to cross the cell wall to reach their targets.*P. aeruginosa*is characterized by low permeability of its outer membrane and the size of its pores is too small to allow efficient penetration of most antibiotics having molecular weights generally higher than 400 daltons [109].Another contributing factor is the

low permeability of water filled channel porins within the outer membrane depending on their size which restricts the diffusion of small hydrophilic agents such as b-lactam antibiotics [110]. For instance, alterations in the porins lead to a rising number of *P. aeruginosa*clinical isolates that share mutations either in the small size channel porins OprC, OprB, and OprD or the large channel porins OprF which are the most abundant porins. The mutations cause the cells to become more resistant to antibiotics. For example, the antibiotic imipenem (carbapenem) diffuses specifically into the bacterium through the porin OprD. Imipenem resistance results from exclusion of its diffusion which is associated with an alteration in the three dimensional structure of OprD [111]. Therefore, identifying the variability and alterations of porins on the molecular level will facilitate the drug industry to synthesize antimicrobial agentscapable to penetrate the mutated channels.

In the majority of Gram negative bacteria, multidrug efflux pumps are characterized by a common three component organization: a membrane fusion protein that is associated with the cytoplasmic membrane, a transporter protein that exports the substrate across the inner membrane, and outer membrane proteins that facilitate the passage of the substrate across the outer membrane [72][112]. They are responsible for extruding drugs, toxic metal ions, organic solvents, and other substances from inside the cell. The largest number of predicted pumps belongs to the resistance nodulation cell division (RND) family [112]. The multidrug efflux systems expressed in *P. aeruginosa* outer membrane participate in intrinsic resistance. The RND family membrane efflux (Mex) pumps which are mexAB-oprM, mexXY-oprM, mexCD-oprJ, and mexEF-oprN are capable of extruding a wide range of antibiotic classes and disinfectants by one or more of the efflux systems [113]. For example, MexAB-oprM is responsible for extrusion of b-lactams, quinolones, MexXY-oprM extrudes aminoglycosides and mexEF-oprN extrudes carbapenems and quinolones. On the other hand, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim are other antibiotics that can be collectively extruded by these efflux pumps [114, 115].All *P. aeruginosa* strains encode the previous mentioned systems in their genomes, but the expression level is not adequate between different strains. However, increased expression of efflux system genes can result from a mutation in regulatory genes such as *mexR*, which controls the expression of the mexAB-oprM genes [116].

Another participant in intrinsic resistance is the natural inducible chromosomal gene b-lactamase (*Amp*C*)* which is possessed by all strains of *P. aeruginosa*. The enzyme is naturally located in the bacterium periplasm [117]. It plays its role through the ability to hydrolyze various b-lactam antibiotics such as penicillins and cephalosporins since these antibiotics can strongly induce the production of *AmpC* even in sub inhibitory concentrations [118]. Restricted outer membrane permeability is related to the resistance acquired by efflux pumps and *Amp*C. The concentration of b-lactams in the periplasm is dependent on the efficiency and rate by which they are transported through the porins of the outer membrane [119]. Substantially, resistance conferred by overproduction of *Amp*C provides a variability in antibiotics resistance [120].

1.5.2 Acquired Resistance of *P. aeruginosa*

Thistype of resistance acquired by mutations that occur in the intrinsic genes of the human pathogen. These mutations confer acquiring resistance to different antimicrobial agents. The acquisition of resistance happens through carrying genetic alleles or through transferring of resistance encoding genes in plasmids. Alleles encode for resistance cassette in the genome of*P. aeruginosa*which leads to emerging an irreversible resistant population [121-123].

- **Mutation in Resistance Gene of *P. aeruginosa***

The high rate of antibiotic resistance via intrinsic resistance genes confers a lower susceptibility of *P. aeruginosa* to narrow spectrum antibiotics. More specifically, a mutation in the regulatory pathway of intrinsic resistance genes could increase the promoter activities. The over expression of regulatory genes leads to overproduction of *Amp*C and multi drug efflux pump systems causing a higher level of resistance to antibiotics [124]. For instance, a shared feature was found in *P. aeruginosa* resistant clinical isolates, they display a constitutively high level of *Amp*C production even in the absence of antibiotic inducers. The point mutations of *Amp*R; expression regulator of *Amp*C and inactivation of *Amp*D; repressor of *Amp*C, is conferring induction of the *Amp*C gene [125]. Additionally, the expression of the mexAB-oprM operon in *P. aeruginosa* is under the negative control of some regulatory genes such as *mex*R*, nal*D*, nal*B*, and nal*C. In case of loss of function for the previous genes,an overexpression of the mexAB-oprM operon will lead to confer greater resistance to antibiotics such as carbapenem [76, 126]. Similarly, overexpression of other multidrug efflux pumps such as MexXY and MexCD-OprJ occurs in cases of mutations in regulatory genes leading to high resistance to different antimicrobial agents [127]. On the other hand, mutation of the carbapenem specific porin OprD which is localized in the outer membrane of *P. aeruginosa* results in a loss or decrease of OprD activity which can reduce the permeability of the outer membrane to carbapenems [76, 128]. In addition, the occurrence of *Opr*D mutations has led to the emergence of imipenem resistant strains and reduced susceptibility to meropenem[76]. Furthermore, a downregulation of *Opr*D expression can be mediated by other regulatory factors such as MexT which itself concurrently upregulates mexEF-oprN expression [129, 130]. Furthermore, *P. aeruginosa*possesses resistance to fluoroquinolone by mutational changes within the DNA gyrase such as gyrA and gyrBor overproduction of efflux pumps[131, 132].

- **Plasmid Mediated Resistance of *P. aeruginosa***

Bacterial plasmids serve as a potent vehicle for acquiring resistance genes and subsequent delivery to the recipient host. This is called horizontal gene transfer whereby genetic elements can be transferred between bacterial cells particularly via conjugation. Some resistance plasmids are broad host range which can be transferred among various species via bacterial conjugation. While narrow host range plasmids are transferred among a small number of cells from similar bacterial species. For example, plasmid RP1 can transfer resistance genes to most Gramnegative bacteria [133]. Plasmid encoded antibiotic resistance confers resistance against different classes of antibiotics that are currently applied in the frontline of clinical treatments such as β-lactams, fluoroquinolones, and aminoglycosides [134]. However, *P. aeruginosa*resistance via horizontal gene transfer has been reported for

the genes encoding β-lactam hydrolyzing enzymes known as the extended spectrum β-lactamases [135]. The genes encoding extended spectrum β-lactamases and carbapenemase are clinically important due to their hydrolyzing activity on a wide range of β-lactams such as carbapenems and extended spectrum cephalosporins[124, 136, 137]. The global epidemiology of carbapenem resistant *P. aeruginosa* was recently analyzed by Park *et al*[138]. They reported the geographical prevalence of these genes differs from country to country. The genes encoding carbapenemases such as *IMP, VIM,* and *NDM*type Metallo-β-lactamases have been found in all continents [138-140]. Almost all types of transferable carbapenemases, except *SIM*-1, have been detected in *P. aeruginosa*[140, 141]. It is of concern that transferable plasmids carrying some of the resistance genes are mobile among a wide range of unrelated Gram negative bacteria. This can increase the antimicrobial resistance transfer rate between different microorganisms causing an increase in treatment complications [141].

Liu *et al*., reported the first evidence of plasmid mediated colistin resistance from China [142]. Colistin belongs to the family of polymyxins. The members of this class of antibiotics such as polymyxin B and colistin have been the last shelter for antibiotic treatment of carbapenemresistant bacteria such as *P. aeruginosa* isolates and *Enterobacteriaceae*[143]. Resistance to polymyxins was previously reported to occur via chromosomal mutations [144, 145]. However, new evidence suggests plasmid mediated resistance through the mobilization of the *MCR*-1gene which consequently confers resistance to colistin. This gene was discovered in *E. coli* strain SHP45 collected from agricultural products. It is more concerning that the plasmid carrying *MCR*-1was mobilized into *Klebsiellapneumoniae* and*P. aeruginosa*via conjugation [142].

1.5.3 Adaptive Resistance of *P. aeruginosa*

The reduction in antimicrobial killing capability in originally susceptible bacterial populations after initial exposure to an antibiotic is defined as adaptive resistance [141]. *P. aeruginosa* is a highly adaptable microorganism due to the additional genetic capacity compared with other microorganisms. It can resist several antibiotics and disinfectants by altering its properties in response to changes in the environmental conditions. Additionally, modifications can occur under various stress conditions such as extensive use of antibiotics or exposure to sub inhibitory antibiotic concentrations, biofilm formation, and crowded surfing motility[146]. This type of resistance refers to adaptive resistance which differs from other types in that it is unstable transient and reversed upon removal of external stimuli leading to regain susceptibility [147]. The adaptive mechanism is still not well understood but it mainly depends on altering antibiotic targets, changing gene expression, and protein production resulting in up regulation of the genes that confer resistance [110].

P. aeruginosa resistance against β-lactams, aminoglycosides, polymyxins, and fluoroquinolones is mediated by adaptive resistance [148].For example, when the cells are exposed to stress conditions such as depletion ofmagnesium ion (Mg^{2+}), exposure to amino peptides, and epithelial cell interaction. The sensor kinases (SKs) such as *Pho*Q, *Pmr*B, *Par*S, *Cpr*S, and *Cbr*A are simultaneously expressed inducing the overexpression of the *arnBCADTEF-udg* operon. The overexpression of the operon causes the synthesis and

addition of amino arabinose to lipid A. In turn,the interactions of cationic peptides with the outer membrane will be decreased by reducing the negative charge of lipopolysaccharide[149]. Furthermore, multiple mutations in known resistance genes including the *gyr*A, *gyr*B, *nfx*B, and *orf*N have conferred resistance of *P. aeruginosa*to fluoroquinolones [150]. In addition, the resistance to polycationic antimicrobials such as aminoglycosides, polymyxins, and cationic antimicrobial peptides is mediated by altering the lipid A structure in LPS. This modification is mediated by multiple mutations in cognate regulatory proteins such as the two component systems *Pho*P-*Pho*Q, *Pmr*A-*Pmr*B, *Cpr*R, *Cpr*S, and *Par*R-*Par*S[151, 152]. Moreover, polymyxin resistance is acquired through alterations affecting regulatory pathways such as *Pho*Q and *Pmr*Bcausing amino acid substitutions. This explains the mechanism of *P. aeruginosa*resistance to colistin found among various isolates [99]. In addition, resistance tothe biocide chlorhexidine was mediated through an expression of*mexCD-oprJ* genes. The resistance is specifically dependent on the sigma factor *Alg*U.*Alg*U is a major stress response sigma factor that positively regulates overproduction of alginate in mucoid isolates increasing the biofilm formation [153].

1.6 Clinical Significance of *P. aeruginosa*

The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed for this organism to persist in both community and hospital settings. In the hospital, it can be isolated from a variety of sources, including respiratory therapy equipment, antiseptics, soap, sinks, mops, medicines, and physiotherapyand hydrotherapy pools. Community reservoirs of this organism include swimming pools, whirlpools, hot tubs, contact lens solution, home humidifiers, soil, and vegetables [98][92].*P. aeruginosa* is especially problematic for immunocompromised patients such as patients who have experienced trauma or a breach in cutaneous or mucosal barriers by mechanical ventilation, tracheostomy, catheters, surgery, or burns. Also patients with leukemia, lymphoma, cystic fibrosis, and AIDS are vulnerable to *P. aeruginosa* biofilm infections [127 2004]. A review of surveillance data collected by the CDC National Nosocomial Infection Surveillance System from 1986 to 1998 shows that *P. aeruginosa* was identified as the fifth most frequently isolated nosocomial pathogen, accounting for 9% of all hospital acquired infections in the United States [31].

1.7 Conclusion:

Biofilms are considered the notorious form of bacterial infections, the biofilm forming bacteria as P. aeruginosa and S. pyogenes commonly colonize various surfaces such as skin, teeth, and medical implants, which result in severe chronic infections. Malaysian Trigona honey has successfully degraded the biofilm of the opportunistic bacteria S. pyogenes and P. aeruginosa. Hence, biofilm associated infections are more difficult to be eradicated and financially more expensive to be treated compared with the infections caused by planktonic bacteria. Thus, urgent new antibiofilm agents with low cost and high margin of safety are required. Therefore, Malaysian Trigona stingless bee honey can be the resort for treating such infections, as it has been used as a traditional remedy for several decades, due to its effectiveness in treating human ailments, and remarkably there are no reports about resistant strains to Trigona honey.

1.8 Conflict of interest

Authors declare no conflict of interest

Reference:

[1] Pachori, P., R. Gothalwal, and P. Gandhi, *Emergence of antibiotic resistance Pseudomonas aeruginosa in intensive care unit; a critical review.* Genes & diseases, 2019. **6**(2): p. 109-119.

[2] Wong, G., *Surface Sensing, Motility Appendages, and Extracellular Electron Transport in P. Aeruginosa and S. Oneidensis.* Biophysical Journal, 2018. **114**(3): p. 28a.

[3] Miao, E.A., et al., *TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system.* Semin Immunopathol, 2007. **29**(3): p. 275-88.

[4] Spangenberg, C., T.C. Montie, and B. Tummler, *Structural and functional implications of sequence diversity of Pseudomonas aeruginosa genes oriC, ampC and fliC.* Electrophoresis, 1998. **19**(4): p. 545-50.

[5] Balasubramanian, D., et al., *A dynamic and intricate regulatory network determines Pseudomonas aeruginosa virulence.* Nucleic Acids Res, 2013. **41**(1): p. 1-20.

[6] Faure, E., K. Kwong, and D. Nguyen, *Pseudomonas aeruginosa in chronic lung infections: how to adapt within the host?* Frontiers in immunology, 2018. **9**: p. 2416.

[7] Lyczak, J.B., C.L. Cannon, and G.B. Pier, *Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist.* Microbes Infect, 2000. **2**(9): p. 1051-60.

[8] Seder, N., M.H. Abu Bakar, and W.S. Abu Rayyan, *Transcriptome Analysis of Pseudomonas aeruginosa Biofilm Following the Exposure to Malaysian Stingless Bee Honey.* Adv Appl Bioinform Chem, 2021. **14**: p. 1-11.

[9] Abdelmalek, S., et al., *Changes in public knowledge and perceptions about antibiotic use and resistance in Jordan: a cross-sectional eight-year comparative study.* BMC Public Health, 2021. **21**(1): p. 750.

[10] Azam, M.W. and A.U. Khan, *Updates on the pathogenicity status of Pseudomonas aeruginosa.* Drug discovery today, 2019. **24**(1): p. 350-359.

[11] Hancock, R.E. and D.P. Speert, *Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment.* Drug Resist Updat, 2000. **3**(4): p. 247-255.

[12] Stover, C.K., et al., *Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen.* Nature, 2000. **406**(6799): p. 959-64.

[13] Cohen, T.S. and A. Prince, *Cystic fibrosis: a mucosal immunodeficiency syndrome.* Nature medicine, 2012. **18**(4): p. 509-519.

[14] Haghi, F., et al., *Diversity of virulence genes in multidrug resistant Pseudomonas aeruginosa isolated from burn wound infections.* Microbial pathogenesis, 2018. **115**: p. 251-256.

[15] Brimer, C.D. and T.C. Montie, *Cloning and comparison of fliC genes and identification of glycosylation in the flagellin of Pseudomonas aeruginosa a-type strains.* J Bacteriol, 1998. **180**(12): p. 3209-17.

[16] Kilmury, S.L. and L.L. Burrows, *The Pseudomonas aeruginosa PilSR two-component system regulates both twitching and swimming motilities.* Mbio, 2018. **9**(4): p. e01310-18.

[17] Wolfgang, M.C., et al., *Pseudomonas aeruginosa regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients.* Proc Natl Acad Sci U S A, 2004. **101**(17): p. 6664-8.

[18] Craig, L., M.E. Pique, and J.A. Tainer, *Type IV pilus structure and bacterial pathogenicity.* Nat Rev Microbiol, 2004. **2**(5): p. 363-78.

[19] Laventie, B.-J., et al., *A surface-induced asymmetric program promotes tissue colonization by Pseudomonas aeruginosa.* Cell host & microbe, 2019. **25**(1): p. 140-152. e6.

[20] Kipnis, E., T. Sawa, and J. Wiener-Kronish, *Targeting mechanisms of Pseudomonas aeruginosa pathogenesis.* Med Mal Infect, 2006. **36**(2): p. 78-91.

[21] Hauser, A.R., *The type III secretion system of Pseudomonas aeruginosa: infection by injection.* Nat Rev Microbiol, 2009. **7**(9): p. 654-65.

[22] Feltman, H., et al., *Prevalence of type III secretion genes in clinical and environmental isolates of Pseudomonas aeruginosa.* Microbiology, 2001. **147**(Pt 10): p. 2659-69.

[23] Mueller, C.A., et al., *The V-antigen of Yersinia forms a distinct structure at the tip of injectisome needles.* Science, 2005. **310**(5748): p. 674-6.

[24] Baer, M., et al., *An engineered human antibody fab fragment specific for Pseudomonas aeruginosa PcrV antigen has potent antibacterial activity.* Infect Immun, 2009. **77**(3): p. 1083-90.

[25] Wei, Q. and L.Z. Ma, *Biofilm matrix and its regulation in Pseudomonas aeruginosa.* Int J Mol Sci, 2013. **14**(10): p. 20983-1005.

[26] Marmont, L.S., et al., *PelA and PelB proteins form a modification and secretion complex essential for Pel polysaccharide-dependent biofilm formation in Pseudomonas aeruginosa.* Journal of Biological Chemistry, 2017. **292**(47): p. 19411-19422.

[27] Wang, S., et al., *The exopolysaccharide Psl-eDNA interaction enables the formation of a biofilm skeleton in Pseudomonas aeruginosa.* Environ Microbiol Rep, 2015. **7**(2): p. 330-40.

[28] Jennings, L.K., et al., *Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the Pseudomonas aeruginosa biofilm matrix.* Proc Natl Acad Sci U S A, 2015. **112**(36): p. 11353-8.

[29] Colvin, K.M., et al., *The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa.* PLoS Pathog, 2011. **7**(1): p. e1001264.

[30] Ryder, C., M. Byrd, and D.J. Wozniak, *Role of polysaccharides in Pseudomonas aeruginosa biofilm development.* Curr Opin Microbiol, 2007. **10**(6): p. 644-8.

[31] Wozniak, D.J., et al., *Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 Pseudomonas aeruginosa biofilms.* Proc Natl Acad Sci U S A, 2003. **100**(13): p. 7907-12.

[32] Leid, J.G., et al., *The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-gamma-mediated macrophage killing.* J Immunol, 2005. **175**(11): p. 7512-8.

[33] Yang, L., et al., *Distinct roles of extracellular polymeric substances in Pseudomonas aeruginosa biofilm development.* Environ Microbiol, 2011. **13**(7): p. 1705-17.

[34] Fuxman Bass, J.I., et al., *Extracellular DNA: a major proinflammatory component of Pseudomonas aeruginosa biofilms.* J Immunol, 2010. **184**(11): p. 6386-95.

[35] O'Toole, G.A. and R. Kolter, *Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development.* Mol Microbiol, 1998. **30**(2): p. 295-304.

[36] Oldak, E. and E.A. Trafny, *Secretion of proteases by Pseudomonas aeruginosa biofilms exposed to ciprofloxacin.* Antimicrob Agents Chemother, 2005. **49**(8): p. 3281-8.

[37] Wretlind, B. and O.R. Pavlovskis, *The role of proteases and exotoxin A in the pathogenicity of Pseudomonas aeruginosa infections.* Scand J Infect Dis Suppl, 1981. **29**: p. 13-9.

[38] Vidaillac, C. and S.H. Chotirmall, *Pseudomonas aeruginosa in bronchiectasis: infection, inflammation, and therapies.* Expert Review of Respiratory Medicine, 2021. **15**(5): p. 649-662.

[39] Caballero, A.R., et al., *Pseudomonas aeruginosa protease IV enzyme assays and comparison to other Pseudomonas proteases.* Analytical biochemistry, 2001. **290**(2): p. 330-337.

[40] Toder, D.S., et al., *lasA and lasB genes of Pseudomonas aeruginosa: analysis of transcription and gene product activity.* Infect Immun, 1994. **62**(4): p. 1320-7.

[41] de Kievit, T.R. and B.H. Iglewski, *Bacterial quorum sensing in pathogenic relationships.* Infect Immun, 2000. **68**(9): p. 4839-49.

[42] Upritchard, H.G., S.J. Cordwell, and I.L. Lamont, *Immunoproteomics to examine cystic fibrosis host interactions with extracellular Pseudomonas aeruginosa proteins.* Infect Immun, 2008. **76**(10): p. 4624-32.

[43] Li, X.-H. and J.-H. Lee, *Quorum sensing-dependent post-secretional activation of extracellular proteases in Pseudomonas aeruginosa.* Journal of Biological Chemistry, 2019. **294**(51): p. 19635-19644.

[44] Mariencheck, W.I., et al., *Pseudomonas aeruginosa elastase degrades surfactant proteins A and D.* Am J Respir Cell Mol Biol, 2003. **28**(4): p. 528-37.

[45] Kuang, Z., et al., *Pseudomonas aeruginosa elastase provides an escape from phagocytosis by degrading the pulmonary surfactant protein-A.* PLoS One, 2011. **6**(11): p. e27091.

[46] Laarman, A.J., et al., *Pseudomonas aeruginosa alkaline protease blocks complement activation via the classical and lectin pathways.* J Immunol, 2012. **188**(1): p. 386-93.

[47] Bardoel, B.W., et al., *Pseudomonas evades immune recognition of flagellin in both mammals and plants.* PLoS Pathog, 2011. **7**(8): p. e1002206.

[48] Kharazmi, A., et al., *Effect of Pseudomonas aeruginosa proteases on human leukocyte phagocytosis and bactericidal activity.* APMIS, 1986. **94**(1-6): p. 175-179.

[49] Malloy, J.L., et al., *Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions.* Am J Physiol Lung Cell Mol Physiol, 2005. **288**(2): p. L409-18.

[50] Kharazmi, A., et al., *Pseudomonas aeruginosa exoproteases inhibit human neutrophil chemiluminescence.* Infection and immunity, 1984. **44**(3): p. 587-591.

[51] Skopelja-Gardner, S., et al., *Regulation of Pseudomonas aeruginosa-mediated neutrophil extracellular traps.* Frontiers in immunology, 2019. **10**: p. 1670.

[52] Huszczynski, S.M., J.S. Lam, and C.M. Khursigara, *The role of Pseudomonas aeruginosa lipopolysaccharide in bacterial pathogenesis and physiology.* Pathogens, 2020. **9**(1): p. 6.

[53] Rietschel, E.T. and H. Brade, *Bacterial endotoxins.* Scientific american, 1992. **267**(2): p. 54-61.

[54] King, J.D., et al., *Lipopolysaccharide biosynthesis in Pseudomonas aeruginosa.* Innate immunity, 2009. **15**(5): p. 261-312.

[55] Bystrova, O., et al., *Full structure of the lipopolysaccharide of Pseudomonas aeruginosa immunotype 5.* Biochemistry (Moscow), 2004. **69**(2): p. 170-175.

[56] Zahran, D., et al., *Production of Polyclonal Antibodies against Exotoxin A of Pseudomonas aeruginosa.* NVEO-NATURAL VOLATILES & ESSENTIAL OILS Journal| NVEO, 2021: p. 10075-10091.

[57] Michalska, M. and P. Wolf, *Pseudomonas Exotoxin A: optimized by evolution for effective killing.* Front Microbiol, 2015. **6**: p. 963.

[58] Dieffenbach, M. and I. Pastan, *Mechanisms of Resistance to Immunotoxins Containing Pseudomonas Exotoxin A in Cancer Therapy.* Biomolecules, 2020. **10**(7): p. 979.

[59] Lau, G.W., et al., *The role of pyocyanin in Pseudomonas aeruginosa infection.* Trends in molecular medicine, 2004. **10**(12): p. 599-606.

[60] Van Laar, T.A., et al., *Pseudomonas aeruginosa gshA mutant is defective in biofilm formation, swarming, and pyocyanin production.* Msphere, 2018. **3**(2): p. e00155-18.

[61] Alonso, B., et al., *Characterization of the virulence of Pseudomonas aeruginosa strains causing ventilator-associated pneumonia.* BMC infectious diseases, 2020. **20**(1): p. 1-8.

[62] Bianchi, S.M., et al., *Impairment of apoptotic cell engulfment by pyocyanin, a toxic metabolite of Pseudomonas aeruginosa.* American journal of respiratory and critical care medicine, 2008. **177**(1): p. 35-43.

[63] Lau, G.W., B.E. Britigan, and D.J. Hassett, *Pseudomonas aeruginosa OxyR is required for full virulence in rodent and insect models of infection and for resistance to human neutrophils.* Infection and immunity, 2005. **73**(4): p. 2550-2553.

[64] Vinckx, T., et al., *The Pseudomonas aeruginosa oxidative stress regulator OxyR influences production of pyocyanin and rhamnolipids: protective role of pyocyanin.* Microbiology, 2010. **156**(3): p. 678-686.

[65] Jimenez, P.N., et al., *The multiple signaling systems regulating virulence in Pseudomonas aeruginosa.* Microbiology and Molecular Biology Reviews, 2012. **76**(1): p. 46-65.

[66] Cornelis, P., *Iron uptake and metabolism in pseudomonads.* Applied microbiology and biotechnology, 2010. **86**(6): p. 1637-1645.

[67] Rajkumari, J., et al., *Attenuation of quorum sensing controlled virulence factors and biofilm formation in Pseudomonas aeruginosa by pentacyclic triterpenes, betulin and betulinic acid.* Microbial pathogenesis, 2018. **118**: p. 48-60.

[68] Deep, A., U. Chaudhary, and V. Gupta, *Quorum sensing and bacterial pathogenicity: from molecules to disease.* Journal of laboratory physicians, 2011. **3**(1): p. 4.

[69] Heeb, S., et al., *Quinolones: from antibiotics to autoinducers.* FEMS microbiology reviews, 2011. **35**(2): p. 247-274.

[70] Haque, S., et al., *Developments in strategies for Quorum Sensing virulence factor inhibition to combat bacterial drug resistance.* Microbial pathogenesis, 2018. **121**: p. 293-302.

[71] Cocotl-Yañez, M., et al., *Virulence factors regulation by the quorum-sensing and Rsm systems in the marine strain Pseudomonas aeruginosa ID4365, a natural mutant in lasR.* FEMS Microbiology Letters, 2020. **367**(12): p. fnaa092.

[72] Fluit, A.C., et al., *Fatal Carbapenem Resistance Development in Pseudomonas Aeruginosa Under Meropenem Monotherapy, Caused by Mutations in the OprD Outer Membrane Porin.* The Pediatric infectious disease journal, 2019. **38**(4): p. 398-399.

[73] Ude, J., et al., *Outer membrane permeability: Antimicrobials and diverse nutrients bypass porins in Pseudomonas aeruginosa.* Proceedings of the National Academy of Sciences, 2021. **118**(31).

[74] Lambert, P., *Mechanisms of antibiotic resistance in Pseudomonas aeruginosa.* Journal of the royal society of medicine, 2002. **95**(Suppl 41): p. 22.

[75] Hancock, R.E. and F.S. Brinkman, *Function of pseudomonas porins in uptake and efflux.* Annu Rev Microbiol, 2002. **56**: p. 17-38.

[76] Kao, C.-Y., et al., *Overproduction of active efflux pump and variations of OprD dominate in imipenem-resistant Pseudomonas aeruginosa isolated from patients with bloodstream infections in Taiwan.* BMC microbiology, 2016. **16**(1): p. 107.

[77] Tolker-Nielsen, T., *Biofilm Development.* Microbiol Spectr, 2015. **3**(2): p. MB-0001-2014.

[78] Gjermansen, M., et al., *Characterization of starvation-induced dispersion in Pseudomonas putida biofilms: genetic elements and molecular mechanisms.* Mol Microbiol, 2010. **75**(4): p. 815-26.

[79] Chambers, J.R. and K. Sauer, *Small RNAs and their role in biofilm formation.* Trends in microbiology, 2013. **21**(1): p. 39-49.

[80] Mikkelsen, H., M. Sivaneson, and A. Filloux, *Key two-component regulatory systems that control biofilm formation in Pseudomonas aeruginosa.* Environmental microbiology, 2011. **13**(7): p. 1666-1681.

[81] Robitaille, S., et al., *An experimentally evolved variant of RsmA confirms its central role in the control of Pseudomonas aeruginosa social motility.* bioRxiv, 2020.

[82] Moscoso, J.A., et al., *The Pseudomonas aeruginosa sensor RetS switches Type III and Type VI secretion via c-di-GMP signalling.* Environmental microbiology, 2011. **13**(12): p. 3128-3138.

[83] Yeung, A.T., et al., *Swarming of Pseudomonas aeruginosa is controlled by a broad spectrum of transcriptional regulators, including MetR.* Journal of bacteriology, 2009. **191**(18): p. 5592-5602.

[84] Moradali, M.F. and B.H. Rehm, *The Regulation of Alginate Biosynthesis via Cyclic di-GMP Signaling.* Microbial Cyclic Di-Nucleotide Signaling, 2020: p. 223.

[85] Petrova, O.E. and K. Sauer, *A novel signaling network essential for regulating Pseudomonas aeruginosa biofilm development.* PLoS Pathog, 2009. **5**(11): p. e1000668.

[86] Donlan, R.M., *Biofilms: microbial life on surfaces.* Emerg Infect Dis, 2002. **8**(9): p. 881-90.

[87] Kim, S.K. and J.H. Lee, *Biofilm dispersion in Pseudomonas aeruginosa.* J Microbiol, 2016. **54**(2): p. 71-85.

[88] Thi, M.T.T., D. Wibowo, and B.H. Rehm, *Pseudomonas aeruginosa biofilms.* International Journal of Molecular Sciences, 2020. **21**(22): p. 8671.

[89] Fullagar, J.L., et al., *Antagonism of a zinc metalloprotease using a unique metal-chelating scaffold: tropolones as inhibitors of P. aeruginosa elastase.* Chem Commun (Camb), 2013. **49**(31): p. 3197-9.

[90] Borlee, B.R., et al., *Pseudomonas aeruginosa uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix.* Mol Microbiol, 2010. **75**(4): p. 827-42.

[91] Harmsen, M., et al., *An update on Pseudomonas aeruginosa biofilm formation, tolerance, and dispersal.* FEMS Immunol Med Microbiol, 2010. **59**(3): p. 253-68.

[92] Maurice, N.M., B. Bedi, and R.T. Sadikot, *Pseudomonas aeruginosa biofilms: host response and clinical implications in lung infections.* American journal of respiratory cell and molecular biology, 2018. **58**(4): p. 428-439.

[93] Kobayashi, H., O. Kobayashi, and S. Kawai, *Pathogenesis and clinical manifestations of chronic colonization by Pseudomonas aeruginosa and its biofilms in the airway tract.* Journal of infection and chemotherapy, 2009. **15**(3): p. 125-142.

[94] Gellatly, S.L. and R.E. Hancock, *Pseudomonas aeruginosa: new insights into pathogenesis and host defenses.* Pathog Dis, 2013. **67**(3): p. 159-73.

[95] Gebreyohannes, G., et al., *Challenges of intervention, treatment, and antibiotic resistance of biofilm-forming microorganisms.* Heliyon, 2019. **5**(8): p. e02192.

[96] Hoiby, N., et al., *Antibiotic resistance of bacterial biofilms.* Int J Antimicrob Agents, 2010. **35**(4): p. 322-32.

[97] Donlan, R.M., *Biofilms and device-associated infections.* Emerg Infect Dis, 2001. **7**(2): p. 277-81.

[98] Pelling, H., et al., *Bacterial biofilm formation on indwelling urethral catheters.* Letters in applied microbiology, 2019. **68**(4): p. 277-293.

[99] Lee, K. and S.S. Yoon, *Pseudomonas aeruginosa Biofilm, a Programmed Bacterial Life for Fitness.* J Microbiol Biotechnol, 2017. **27**(6): p. 1053-1064.

[100] Stewart, P.S., *Theoretical aspects of antibiotic diffusion into microbial biofilms.* Antimicrob Agents Chemother, 1996. **40**(11): p. 2517-22.

[101] Karami, P., et al., *The correlation between biofilm formation capability and antibiotic resistance pattern in Pseudomonas aeruginosa.* Gene Reports, 2020. **18**: p. 100561.

[102] Mulcahy, L.R., et al., *Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis.* J Bacteriol, 2010. **192**(23): p. 6191-9.

[103] Pragasam, A.K., et al., *An update on antimicrobial resistance and the role of newer antimicrobial agents for Pseudomonas aeruginosa.* Indian journal of medical microbiology, 2018. **36**(3): p. 303-316.

[104] Sadovskaya, I., et al., *High-level antibiotic resistance in Pseudomonas aeruginosa biofilm: the ndvB gene is involved in the production of highly glycerol-phosphorylated beta-(1->3)-glucans, which bind aminoglycosides.* Glycobiology, 2010. **20**(7): p. 895-904.

[105] Nguyen, D., et al., *Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria.* Science, 2011. **334**(6058): p. 982-6.

[106] Chen, M., Q. Yu, and H. Sun, *Novel strategies for the prevention and treatment of biofilm related infections.* Int J Mol Sci, 2013. **14**(9): p. 18488-501.

[107] Driscoll, J.A., S.L. Brody, and M.H. Kollef, *The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections.* Drugs, 2007. **67**(3): p. 351-368.

[108] Botelho, J., F. Grosso, and L. Peixe, *Antibiotic resistance in Pseudomonas aeruginosa–Mechanisms, epidemiology and evolution.* Drug Resistance Updates, 2019. **44**: p. 100640.

[109] Nakae, T., *Role of membrane permeability in determining antibiotic resistance in Pseudomonas aeruginosa.* Microbiol Immunol, 1995. **39**(4): p. 221-9.

[110] Breidenstein, E.B., C. de la Fuente-Nunez, and R.E. Hancock, *Pseudomonas aeruginosa: all roads lead to resistance.* Trends Microbiol, 2011. **19**(8): p. 419-26.

[111] Xu, Y., et al., *Mechanisms of Heteroresistance and Resistance to Imipenem in Pseudomonas aeruginosa.* Infection and Drug Resistance, 2020. **13**: p. 1419.

[112] Drenkard, E., *Antimicrobial resistance of Pseudomonas aeruginosa biofilms.* Microbes and infection, 2003. **5**(13): p. 1213-1219.

[113] Poole, K., *Multidrug resistance in Gram-negative bacteria.* Curr Opin Microbiol, 2001. **4**(5): p. 500-8.

[114] Livermore, D.M., *Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare?* Clin Infect Dis, 2002. **34**(5): p. 634-40.

[115] Schweizer, H.P., *Efflux as a mechanism of resistance to antimicrobials in Pseudomonas aeruginosa and related bacteria: unanswered questions.* Genet Mol Res, 2003. **2**(1): p. 48-62.

[116] Rocha, A.J., et al., *Pseudomonas aeruginosa: virulence factors and antibiotic resistance genes.* Brazilian Archives of Biology and Technology, 2019. **62**.

[117] Strateva, T. and D. Yordanov, *Pseudomonas aeruginosa - a phenomenon of bacterial resistance.* J Med Microbiol, 2009. **58**(Pt 9): p. 1133-48.

[118] Oliver, A., et al., *The increasing threat of Pseudomonas aeruginosa high-risk clones.* Drug Resist Updat, 2015. **21-22**: p. 41-59.

[119] Jacoby, G.A., *AmpC beta-lactamases.* Clin Microbiol Rev, 2009. **22**(1): p. 161-82, Table of Contents.

[120] Umadevi, S., et al., *Detection of extended spectrum beta lactamases, ampc beta lactamases and metallobetalactamases in clinical isolates of ceftazidime resistant Pseudomonas Aeruginosa.* Braz J Microbiol, 2011. **42**(4): p. 1284-8.

[121] Davies, J.E., *Origins, acquisition and dissemination.* Antibiotic resistance: origins, evolution, selection and spread, 2008. **787**: p. 15.

[122] Davies, J. and D. Davies, *Origins and evolution of antibiotic resistance.* Microbiology and molecular biology reviews, 2010. **74**(3): p. 417-433.

[123] Li, M., et al., *HigB of Pseudomonas aeruginosa enhances killing of phagocytes by up-regulating the type III secretion system in ciprofloxacin induced persister cells.* Frontiers in cellular and infection microbiology, 2016. **6**.

[124] Blair, J.M., et al., *Molecular mechanisms of antibiotic resistance.* Nature Reviews Microbiology, 2015. **13**(1): p. 42-51.

[125] Juan, C., et al., *Molecular mechanisms of β-lactam resistance mediated by AmpC hyperproduction in Pseudomonas aeruginosa clinical strains.* Antimicrobial agents and chemotherapy, 2005. **49**(11): p. 4733-4738.

[126] Quale, J., et al., *Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of Pseudomonas aeruginosa clinical isolates.* Antimicrobial agents and chemotherapy, 2006. **50**(5): p. 1633-1641.

[127] Lister, P.D., D.J. Wolter, and N.D. Hanson, *Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms.* Clinical microbiology reviews, 2009. **22**(4): p. 582-610.

[128] Epp, S.F., et al., *C-terminal region of Pseudomonas aeruginosa outer membrane porin OprD modulates susceptibility to meropenem.* Antimicrobial agents and chemotherapy, 2001. **45**(6): p. 1780-1787.

[129] Michéa-Hamzehpour, M., et al., *Characterization of MexE–MexF–OprN, a positively regulated multidrug efflux system of Pseudomonas aeruginosa.* Molecular microbiology, 1997. **23**(2): p. 345-354.

[130] Ochs, M.M., et al., *Negative regulation of the Pseudomonas aeruginosa outer membrane porin OprD selective for imipenem and basic amino acids.* Antimicrobial agents and chemotherapy, 1999. **43**(5): p. 1085-1090.

[131] Lee, J.K., et al., *Alterations in the GyrA and GyrB subunits of topoisomerase II and the ParC and ParE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of Pseudomonas aeruginosa.* International journal of antimicrobial agents, 2005. **25**(4): p. 290-295.

[132] Sun, J., Z. Deng, and A. Yan, *Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations.* Biochemical and biophysical research communications, 2014. **453**(2): p. 254-267.

[133] Yang, B., et al., *Paclitaxel and its derivative facilitate the transmission of plasmid-mediated antibiotic resistance genes through conjugative transfer.* Science of The Total Environment, 2021: p. 152245.

[134] Bennett, P., *Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria.* British journal of pharmacology, 2008. **153**(S1).

[135] Poole, K., *Pseudomonas aeruginosa: resistance to the max.* Frontiers in microbiology, 2011. **2**.

[136] Paterson, D.L. and R.A. Bonomo, *Extended-spectrum β-lactamases: a clinical update.* Clinical microbiology reviews, 2005. **18**(4): p. 657-686.

[137] Sullivan, R., et al., *Extended spectrum beta-lactamases: a minireview of clinical relevant groups.* J Med Microbiol Diagn, 2015. **4**(203): p. 2161-0703.1000203.

[138] Park, J.-W., et al., *Pathophysiological changes induced by Pseudomonas aeruginosa infection are involved in MMP-12 and MMP-13 upregulation in human carcinoma epithelial cells and a pneumonia mouse model.* Infection and immunity, 2015. **83**(12): p. 4791-4799.

[139] Johnson, A.P. and N. Woodford, *Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance.* Journal of medical microbiology, 2013. **62**(4): p. 499-513.

[140] Meletis, G. and M. Bagkeri, *Pseudomonas aeruginosa: multi-drug-resistance development and treatment options*, in *Infection Control*. 2013, InTech.

[141] Hong, D.J., et al., *Epidemiology and characteristics of metallo-β-lactamase-producing Pseudomonas aeruginosa.* Infection & chemotherapy, 2015. **47**(2): p. 81-97.

[142] Liu, Y.-Y., et al., *Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study.* The Lancet infectious diseases, 2016. **16**(2): p. 161-168.

[143] Falagas, M.E., S.K. Kasiakou, and L.D. Saravolatz, *Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections.* Clinical infectious diseases, 2005. **40**(9): p. 1333-1341.

[144] Moskowitz, S.M., et al., *PmrB mutations promote polymyxin resistance of Pseudomonas aeruginosa isolated from colistin-treated cystic fibrosis patients.* Antimicrobial agents and chemotherapy, 2012. **56**(2): p. 1019-1030.

[145] Gutu, A.D., et al., *Polymyxin resistance of Pseudomonas aeruginosa phoQ mutants is dependent on additional two-component regulatory systems.* Antimicrobial agents and chemotherapy, 2013. **57**(5): p. 2204-2215.

[146] Pang, Z., et al., *Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies.* Biotechnology advances, 2019. **37**(1): p. 177-192.

[147] Fernandez, L., E.B. Breidenstein, and R.E. Hancock, *Creeping baselines and adaptive resistance to antibiotics.* Drug Resist Updat, 2011. **14**(1): p. 1-21.

[148] Khaledi, A., et al., *Transcriptome Profiling of Antimicrobial Resistance in Pseudomonas aeruginosa.* Antimicrob Agents Chemother, 2016. **60**(8): p. 4722-33.

[149] McPhee, J.B., et al., *Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg2+-induced gene regulation in Pseudomonas aeruginosa.* J Bacteriol, 2006. **188**(11): p. 3995-4006.

[150] Wong, A., N. Rodrigue, and R. Kassen, *Genomics of adaptation during experimental evolution of the opportunistic pathogen Pseudomonas aeruginosa.* PLoS Genet, 2012. **8**(9): p. e1002928.

[151] Barrow, K. and D.H. Kwon, *Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of*

Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy, 2009. **53**(12): p. 5150-5154.

[152] Fernández, L., et al., *The two-component system CprRS senses cationic peptides and triggers adaptive resistance in Pseudomonas aeruginosa independently of ParRS.* Antimicrobial agents and chemotherapy, 2012. **56**(12): p. 6212-6222.

[153] Fraud, S., et al., *MexCD-OprJ multidrug efflux system of Pseudomonas aeruginosa: involvement in chlorhexidine resistance and induction by membrane-damaging agents dependent upon the AlgU stress response sigma factor.* Antimicrobial agents and chemotherapy, 2008. **52**(12): p. 4478-4482.