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REVIEW

Pseudomonas aeruginosa; Virulence Factors and Host Defense Mechanisms

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Abstract

As an opportunistic pathogen, Pseudomonas aeruginosa (P. aeruginosa) can cause both acute and chronic infections. Variable virulence components and antibiotic resistance markers in the bacterium's genome constitute the bacterium's pathogenic profile and provide the bacterium with outstanding metabolic adaptability to many conditions. The interactions of P. aeruginosa with the host are poorly understood, complicating the treatment of its infections and the development of vaccines against them. Despite decades of scientific research focusing specifically on this challenge, vaccines to prevent these dangerous infections still do not exist. The major virulence factors of P. aeruginosa and host immune responses against the bacteria are discussed in this review.

Keywords: *Pseudomonas aeruginosa, virulence factors, host immune response*

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Introduction

Pseudomonas aeruginosa is a Gram-negative non-fermentative bacteria that causes hospital infections and it is resistant to many molecules[1]. In addition to being naturally resistant to many antibiotics, it is a serious problem with an increasing incidence in recent decades, gaining multiple antibiotic resistance owing to its ability to acquire resistance rapidly even during antibiotic use[2–4]. *P. aeruginosa* is on the World Health Organization (WHO) list of "critical" bacterial pathogens demanding new antibiotic development and research[5,6].

There are many incomprehensible parts of *P. aeruginosa*-host interactions. Therefore, the treatment difficulties of infections continue, and preventive vaccines have not yet been developed despite several decades of research[7]. Many extracellular components are concerned with the pathogenesis of *P. aeruginosa.* These systems can increase the movement of bacteria (twitch motility) and enable them to reach nutrients more easily. In addition, they allow bacteria to penetrate tissues more easily or cause more damage to the tissue they colonize through enzymes (elastase, protease, and DNase) that can break down various substances. However, the most important virulence factor for bacteria is biofilm production[8]. The virulence factors of *P. aeruginosa* act on molecules and enzymes in the host cell cytoplasm and activate multimolecular signaling stages in immune cells known as inflammatory cells[9].

Virulence factors of *P. aeruginosa*

P. aeruginosa carries a wide range of virulence factors that contribute to its pathogenicity. Several virulence factors may induce pathogenicity while targeting the extracellular matrix, facilitating adhesion, and/or busting host cell signaling pathways. *P.aeruginosa* can cause a variety of diseases that occupy the host and its immune system, causing infections that are almost impossible to heal[10-12].

The lipopolysaccharide (LPS) is the major component of the outer membrane of *P. aeruginosa*; it involves a distal polysaccharide (O-antigen), a hydrophobic endotoxic element (lipid A), and a single-core oligosaccharide[13]. The LPS activates the host's both innate and adaptive immune responses and ultimately dysregulates inflammation responses that result in increased morbidity and mortality[14].

Flagel, which has a strong immunogenic structure, plays a critical role in the pathogenesis by binding to membrane components of epithelial cells such as AsiolaGM1 and providing adherence[15]. *P. aeruginosa* easily reaches nutrients through flagella, evades the harmful effects of immune system elements and antibacterial agents, translocates into the host cell, and can move freely in the

biofilm[16]. The flagellum stimulates the NFχB-dependent inflammatory response by interacting with TLR5 and TLR2, so leads to the initiation of IL-8 synthesis by activation of the calcium-dependent kinase pathway. It is found in most of the strains isolated from the environment and hospital infections, whereas the 10 strains isolated from patients with cystic fibrosis and chronic infections do not have flagella to evade the host immune system response[17].

Type-IV pili have the ability to adhere to cells, thereby providing tissue tropism and attachment to certain tissues. These adhesions mediate non-opsonic phagocytosis and biofilm formation[18-20].

P. aeruginosa has six secretory systems (Type I to VI) that release hydrolytic enzymes and various toxins to invade the host[21,22]. Type I (T1SS) and Type V (T5SS) secretion systems are simple secretory mechanisms that secrete substances into the extracellular environment. T1SS is a one-step secretory system without the need for a periplasmic intermediate. It consists of an outer membrane protein (OMP), an ATP-binding cassette (ABC) transporter, and an adapter protein binding the two together. Alkaline protease (AprA) and heme acquisition protein (HasAp) are known substrates of T1SS; they are directed into the T1SS secretion tool by a C-terminal secretory signal. The type II secretion system (T2SS) is the most multipurpose secretion system of *P. aeruginosa*[21]. The type III secretion system (T3SS) is the most important secretory system used to disable and destroy the host's immune system[23]. The type VI secretion system (T6SS) is one of the most recently described bacterial secretion systems. T6SS consists of a hemolysin-coregulated protein (Hcp-TssD) tube containing double-spike proteins. It is important for bacterial competition as it produces bacterial toxins (Tse) that destroy the host's microbial flora but also play a minor role in host defense[24]. Unlike the other three systems with a one-step mechanism, T2SS and T5SS use a two-step secretion mechanism that involves the passage of secreted proteins into the periplasm. On the other hand, T3SS and T6SS inject proteins directly into the cytoplasm of the host cell[21,22].

The exotoxin A is secreted through T2SS, which uses a pilus-like tool to secrete proteins into the extracellular environment, including lipase, phospholipase, alkaline phosphatase, and protease. The contribution of these factors to the virulence of bacteria has been demonstrated in animal experiments[25]. Besides, exotoxin A has been demonstrated to be involved in regional tissue invasion and injury [26]. Elastase LasB type metalloproteases also cause tissue damage in the host, especially in burned wound infections and pulmonary infections[27].

Alginate, another substance secreted by *P. aeruginosa*, is an anionic mucoid exopolysaccharide. It consists of repetitive polymers of D-mannuronic acid and L-glucuronic acid and protects bacteria against adverse environmental conditions such as oxidative stress, host defense systems and especially the harmful effects of aminoglycoside antibiotics[28,29].

Quorum Sensing (QS) is a "cell-to-cell" bacterial communication mechanism via diffusible chemical compounds. The quorum is required to produce a sufficient amount of a secreted signal molecule (auto-inducer) to prompt the expression of a large regulon[30]. The most common class of autoinducers are the acyl-homoserine lactones (AHL) used by Gram-negative bacteria. The autoinducer diffuses freely throughout the bacterial membranes. Butanoyl-homoserine lactone and oxohexanoyl-homoserine lactone are the AHL signals of the bacteriae[31]. These signals generated by AHL synthase (LasI/RhlI) are emitted into the microenvironment. The increase in bacterial density during infection also increases the autoinducer concentration. When a certain bacterial population density is reached, AHL molecules accumulated in the medium activate genes that induce biofilm formation and encode virulence factors[32].

A recently discovered QS System (IQS) uses "2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde" as a new signaling molecule. The cognate receptor is not yet known[33]. IQS inhibits host cell growth, impairs host DNA damage repair, and induces apoptosis dose-dependently[34].

Biofilms play an important role in the development of resistant to chronic infections caused by *P. aeruginosa.* Biofilms are microbial communities adhering to a surface and surrounded by an exopolysaccharide (EPS) matrix. The function of the biofilm is to ensure that the microorganisms contained in them are protected from attack by the internal and external environment. Thus, it gains resistance to antibiotics, disinfectants, and host defenses and impairs bacterial clearance[35,36]. Compared to the planktonic form, biofilm formation of *P. aeruginosa* is generally associated with higher drug resistance and leads to evading the host immune response. Microorganisms in the biofilm also exhibit altered phenotypes concerning growth rate and gene transcription[37–42].

Table 1. Virulence factors and pathogenicity mechanisms of *P. aeruginosa*

Virulence factors	Mechanism
Lipopolysaccharide (LPS)	Activates the host's innate and adaptive immune system by Toll-like receptor 4 (TLR4), NOD-like receptor pyrin domain containing 1 (NLRP1), NLRP2, and NLRP3), and dysregulates inflammation responses.
Flagellum	Strongly immunogenic; interacts with TLR2 and TLR5; binds to membrane components of epithelial cells (Asialo GM1). Gives motility to access nutrients, escape from immune system elements and antibacterial agents, migrate in the host cell, and move freely within the biofilm.
Type-IV Pili	Type-IV pili have the ability to adhere to cells, thereby providing tissue tropism and attachment to certain tissues.
T1SS (Type 1 Secretory System)	One-step secretory machinery consists of an outer membrane protein (OMP), an ABC transporter, and an adapter protein. The substrates are alkaline protease (AprA) and Heme acquisition protein (HasAp).
T2SS (Type 2 Secretory System)	This molecular nano-machine consists of three parts: General secretory pathway (Gsp) proteins, a large channel embedded in the OM, and the pseudo-pilus functioning as a piston that secretes exotoxin A.
T3SS (Type 3 Secretory System)	The most important secretory system used to disable and destroy the host's immune system. Supports the transfer of virulence proteins called effectors from the bacterial cytoplasm to the eukaryotic cell in a single step.
T4SS (Type 4 Secretory System)	The most cosmopolitan secretory system. It differs from other secretion systems by having the ability to transfer DNA in addition to proteins.
T5SS (Type 5 Secretory System)	Consists of a two-stage secretory mechanism involving the passage of secreted proteins into the periplasm.
T6SS (Type 6 Secretory System)	An integrated secretory system within the cell membrane. Transfers toxic substrates to eukaryotic and prokaryotic cells. It plays a crucial role in pathogenesis and competition among bacteria.
Exotoxin A	It is secreted via T2SS. It plays a role in local tissue damage and invasion.
Proteases	Several proteases are "elastase LasB-type" metalloproteases. They destroy the host tissue and play a crucial role in both acute lung and burn wound infections.
Alginate	A mucoid anionic exopolysaccharide. Protects the bacteria against adverse environmental conditions, oxidative stress, the host defense system, and especially the harmful effects of aminoglycoside antibiotics.
Quorum Sensing	A bacterial "cell-to-cell" communication mechanism via diffusible chemical compounds. Quorum must produce a sufficient amount of a secreted signaling molecule (auto-inducer) to activate the expression of a large regulon. Acyl-homoserine lactones (AHL) are the most common class of autoinducers used by Gram-negative bacteria.
Biofilm Formation	Biofilms are communities of microorganisms that adhere to a biotic or abiotic surface surrounded by an exopolysaccharide (EPS) matrix. They protect the microorganisms in their contents from the microbial attack of the external or internal environment.

Host immune response against *P. Aeruginosa*

P. aeruginosa is an opportunistic pathogen and causes secondary infections in immunocompromised humans, so the main factor determining the occurrence of infections is the immune status of the host[43,44]. The immune system is suppressed in cases where barrier integrity is impaired as a result of the use of broad-spectrum antibiotics, catheter applications, trauma, wound, ulcer, or burn infections, etc. Bacteria, which find the opportunity for colonization, produce alginate from extracellular virulence factors in such cases, causing tissue damage and spreading in the bloodstream[45].

During *P. aeruginosa* infections, potent agonists of Toll-like receptors (TLR) -TLR2, TLR4, TLR5, and TLR9 - which recognize bacterial lipopolysaccharide (LPS), lipopeptides, unmethylated bacterial CpG DNA, and flagellin, are expressed in the host[46–48]. Of these, the most crucial for infection clearance is the TLR4-dependent inflammatory response to LPS[47]. TLR4 sensing of LPS leads to activation of important inflammatory cytokines such as TNF-α, IL-6, and IL-8[49]. The absence of TLR4 increases sensitivity to two distinct signaling pathways: the primary response pathway of myeloid differentiation 88 (MyD88) and the adaptive pathway involving the beta interferon-inducing Toll/IL-1R domain (TRIF pathway). The MyD88 pathway activates the nuclear factor kappa light chain enhancer of activated B cells (NF-κB), thereby releasing numerous proinflammatory cytokines and chemokines – TNF-α, IL-6, and macrophage inflammatory protein (MIP). The TRIF pathway regulates the transcription of chemokines such as IFN-α and IFN-β, IP-10 (Interferon γ-inducible protein 10) and RANTES (regulated in the activation of normally expressed and secreted T cell)[50]. UT12 - the TLR4/MD2 agonistic monoclonal antibody - was shown to support host defense against chronic *P. aeruginosa* lung infection, increase neutrophil levels and inflammatory MIP-2 concentrations in the lungs, and improve bacterial clearance in mice[51]. Animal experiments have shown that TLR4 and TLR5 are required for the appropriate host immune response[52-55]. It has been shown that lung cells infected with *P. aeruginosa* in mice were unable to induce invasive lung infection when TLR5 was blocked with anti-TLR5 antibodies[56]. Inflammatory responses dependent on TLR2 and TLR9 are shown to be important in bacterial clearance. The deficiencies of TLR-2 and TLR-9 increased bacterial clearance and protected mice against *P. aeruginosa* pulmonary infection[57, 58].

Studies have shown that the MyD88 pathway is essential for the rapid migration of neutrophils to the infection site[59]. Substances released by *P. aeruginosa* target the host cell cytoplasm and activate the assembly of multimolecular signaling stages in inflammatory cells. NOD-like receptors (NLRs) are a group of patern recognition receptors (PRRs) that can recognize

various endogenous and exogenous ligands and thus play a critical role in innate immunity. NLR inflammatories - NLRC4 and NLRP3- are involved in the detection and reaction of *P. aeruginosa* infections[60]. NLRP3 is involved in the recognition of *P. aeruginosa* infection, followed by macrophage secretion of caspase-1 and IL-1β. As a secondary signal, the human cathelicidin LL-37/h-CAP18 promotes the death of epithelial cells infected with *P. aeruginosa* and acts as a "fire alarm" to stimulate inflammatory responses that will counteract uncontrolled infection. Then IL-1β and IL-18 are released from infected epithelial cells in order to promote neutrophil efflux[61].

P. aeruginosa also activates NLRs by the release of outer membrane vesicles (OMV). Thus, TLR-dependent reactions occur in epithelial cells via proteins and LPS. These membrane vesicles also activate NF-κB signaling and mitogen-activated protein kinase (MAPK) within epithelial cells[48,62]. This may suggest the use of NLRs as therapeutic adjuvant targets during *P. aeruginosa* infection, thereby reducing inflammatory responses in bacteria-infected cells[48].

P.aeruginosa and innate immunity

Recognition of *P. aeruginosa* pathogen-associated molecular patterns (PAMPs) is the first step of a robust inflammatory response that facilitates bacterial clearance and the migration of macrophages and neutrophils to the site. A weak immune response results in a poor response of phagocytic cells and failure of bacteria-killing and clearance, while an excessive immune response causes host tissue damage. Therefore, the host response should be optimal[48,63–67]. The chemokine receptors - CXCR1 and CXCR2- are the chemokine receptors on the neutrophils that regulate host defense and neutrophil migration, especially in pulmonary infections caused by *P. aeruginosa*. CXCR1 regulates anti-*Pseudomonas* neutrophil responses by modulating reactive oxygen species and interacting with TLR5[48,67–69]. CXCR1, also binds to IL-8 and to GCP-2 specifically leading to a proinflammatory effect[48,68]. Both CXCR1 and CXCR2 are essential in the response to *P. aeruginosa* because they recruit neutrophils that provide bacterial clearance and have other functions in the immune system[70,71]. While killing *P. aeruginosa*, neutrophils may also contribute to host lung injury due to the synthesis of reactive oxygen compounds (ROS) and proteins released from their acidophilic granules[72]. Therefore, a neutrophil recruitment level that will provide bacterial clearance but not cause excessive tissue damage is crucial during infection control.

There is increasing evidence that *P. aeruginosa* can survive inside mammalian cells. In the study of Garai et al., it was shown that *P. aeruginosa* can be detected first in phagosomal vacuoles and then in the cytoplasm of macrophages. This indicates phagosomal escape of the bacteria.

Among the *P. aeruginosa* virulence factors, T3SS and ExoS play an important role in the intramacrophage survival of bacteria and can induce host macrophage lysis[73].

Complement activation also plays a role in the host response to *P. aeruginosa*. The OprF porin located in the outer membrane of the bacterium acts as a binding acceptor molecule for C3b, enabling the formation of the membrane attack complex (MAC). A study by Mishra et al. showed that C3b binding was significantly reduced in an OprF-deficient *P. aeruginosa* strain[74]. The innate immune system is important in the control of *P. aeruginosa* infections, but further studies are needed on the details of these pathways and how they are integrated in vivo.

P. aeruginosa and adaptive immunity

If proinflammatory pathways are weakened during the acute phase of *P. aeruginosa* infections, the inflammatory response may resolve. T helper cells (Regulatory T cells; Treg) secrete anti-inflammatory cytokines, inhibit the secretion of pro-inflammatory cytokines, and dendritic cells initiate adaptive responses. *P. aeruginosa* infection, which cannot be eradicated during the acute phase, progresses to a chronic infection characterized by mucoid biofilm formation[75]. As neutrophil inflammation prolongs, high expression of IFN-γ, IL-6, IL-1β and IL-17 and a decrease in IL-10 and Treg are observed, followed by an effector T cell response[48]. This response inhibits bacterial antigen presentation and an effective host immune response against *P. aeruginosa*[75]. A Th1-like response may improve lung function by releasing IFN-γ[76-78]. With the induction of IFN-γ by alveolar macrophages, apoptotic neutrophils are removed, progression to necrosis occurs and thus further inflammation is prevented[75]. Increasing the Th1 response reduces IL-8 - a chemoattractant for neutrophils- thus reducing inflammation in the lung due to decreased neutrophil recruitment. A low Th2 response reduces both tissue damage and the formation of immune complexes. Furthermore, decreased IL-13 levels may result in decreased mucus production[79].

Another acquired immune response in *P. aeruginosa* infections is antibody production and subsequent immune complex (IC) formation. Although IgG antibody production in chronic *Pseudomonas* infections is associated with high NF-KB expression, particularly in cystic fibrosis (CF) patients, it should be kept in mind that the response to specific antigens may vary depending on the stage of infection. While some antigens are more expressed in the acute phase, causing an intense immune response, some antigens are recognized in the chronic phase. The presence of specific antibodies against antigenic structures of the mucoid phenotype of *P. aeruginosa* has been associated with a poor prognosis[75].

Immunoglobulin A (IgA), the dominant antibody isotype of the mucosal immune system, is also of great importance in the humoral immune response against respiratory tract infections caused by *P. aeruginosa*[80]. Based on the concentration of secretory IgA against *P. aeruginosa* in nasal secretions and saliva, it can be predicted whether patients are colonized, infected, or chronically infected with *P. aeruginosa*[81,82].

Conclusion

The numerous virulence factors of *P aeruginosa* and their expression at different levels according to the environment and conditions cause infections and treatment approaches to be quite complex. Despite more than 50 years of research to develop a vaccine against *Pseudomonas*, no marketable product has yet been produced. *P. aeruginosa* remains one of the most resistant organisms to antibiotics in the pharmaceutical industry. Researchers are still looking for new drugs or new treatment options that could stop infections caused by this multi-drug-resistant, problematic bacterium.

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