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Pathogenic factors of *Pseudomonas aeruginosa* – the role of biofilm in pathogenicity and as a target for phage therapy

Czynniki patogenności *Pseudomonas aeruginosa* – rola
biofilmu w chorobotwórczości i jako cel terapii fagowej

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Summary

Pseudomonas aeruginosa is an opportunistic pathogen that can cause several acute and chronic infections in humans, and it has become an important cause of nosocomial infections and antibiotic resistance. Biofilm represents an important virulence factor for these bacteria, plays a role in *P. aeruginosa* infections and avoidance of immune defence mechanisms, and has the ability to protect the bacteria from antibiotics. Alginate, Psl and Pel, three exopolysaccharides, are the main components in biofilm matrix, with many biological functions attributed to them, especially with respect to the protection of the bacterial cell from antibiotics and the immune system. *Pseudomonas* infections, biofilm formation and development of resistance to antibiotics all require better understanding to achieve the best results using alternative treatment with phage therapy. This review describes the *P. aeruginosa* pathogenicity and virulence factors with a special focus on the biofilm and its role in infection and resistance to antibiotics and summarizes phage therapy as an alternative approach in treatment of *P. aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa* • virulence • phage • biofilm • extracellular polysaccharide • depolymerase • therapy

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Abbreviations: **3-oxo-C12-HSL** – N-(3-oxododecanoyl)-homoserine lactone, **CF** – cystic fibrosis; **eDNA** – extracellular DNA, **EPS** – exopolysaccharide, extracellular polysaccharide, **LPS** – lipopolysaccharide, **T2SS and T3SS** – type II and III secretion systems; **Pel** – pellicle polysaccharide, **Psl** – polysaccharide synthesis locus, **PQS** – quinolone-based system, **QS** – quorum sensing.

INTRODUCTION

Pseudomonas aeruginosa is a motile, aerobic Gram-negative rod bacterium that is found in diverse biotic and abiotic habitats, including soil, water, insects, plants and animals [42]. It was first reported in 1862 by Luke, who observed rod-shaped particles in blue-green pus of human infections. Similar coloration had been previously observed by Sedillot on surgical dressings, and is now known to be caused by the pigment pyocyanin produced by *P. aeruginosa* [76]. *P. aeruginosa* is one of the opportunistic pathogens that can cause several acute and chronic infections in humans, such as ventilator-associated pneumonia, cystic fibrosis (CF), meningitis, abscess, infections of skin and soft tissues (including diabetic foot), urinary tract, bone and joint, bacteraemia corneal infections, conjunctival erythema and a variety of systemic diseases associated with genetic diseases, also in immunocompromised patients such as those with diabetes mellitus, cystic fibrosis and individuals receiving chemotherapy (Table 1) [35,42,103,106]. This bacterium is not part of human normal flora in the gastrointestinal tract, but may occur in the upper respiratory tract or skin, particularly among hospitalized patients [85]. Acute nosocomial pneumonia is typically the result of direct trauma, such as damage to the epithelium due to intubation or smoke inhalation [42], and urinary tract infection may occur by formation of bacterial biofilms on indwelling catheters [21]. Indeed, many factors play a role in development of *P. aeruginosa* infections, including immunodeficiency as in immunocompromised hosts [35] and immunosuppression connected with organ transplantation [35,115]. Genetic factors also play role in some *P. aeruginosa* infections as in individuals with cystic fibrosis (CF) [60,116] with a mutation in the cystic fibrosis transmembrane regulator (CFTR) and a cAMP-dependent chloride channel, which results in a dehydrated and thickened airway surface liquid (ASL) that hinders mucociliary clearance from the conducting airways in these conditions. The bacteria can colonize the altered ASL and cause an initial acute infection and vigorous inflammatory response. The thickened ASL severely impairs the immune response and the persistent immunological stimulation by the bacteria leads to chronic lung inflammation [42,115]. In addition to those factors, the age, the poor health status of the patients, cancer chemotherapy and medical devices are all factors also having an important role in pseudomonad infections [21,76]. On other hand, the bacterial strain and its virulence factors such as flagellum, pili, protein secretion systems, exoenzymes, lectins, quorum sensing and biofilm matrix are mainly responsible for its infection severity and multidrug resistance.

In fact, multidrug resistance and biofilm formation are frequent problems with the treatment of *P. aeruginosa* infections, which requires more investigations and a search for alternative targets for therapeutics against this infection. Phage therapy offers novel antimicrobials for eradication or suppression of *P. aeruginosa*. In this review, we attempt to summarize the recent studies of

P. aeruginosa pathogenicity and virulence factors, focusing on the biofilm matrix having a major role in infection and resistance to antibiotics, and we provide an overview of bacteriophages as alternative treatment against this pathogen.

VIRULENCE FACTORS

Pseudomonas aeruginosa exhibits a number of virulence factors (Table 2), as well as multiple antibacterial resistance mechanisms which have contributed to increasing rates of antibacterial resistance in recent years [35].

FLAGELLUM AND PILI

This bacterium possesses a single polar flagellum that plays a major role in motility, essential part of bacterial chemotaxis, initiates an inflammatory response and may mediate initial surface interactions by binding with asialylated glycolipid (aGM1) of epithelial cells of the host [35,42,76]. *P. aeruginosa* also has multiple cell surface pili (type IV) that are responsible for adherence to cell membranes and other surfaces, twitching motility [35,42], formation of biofilms and avoiding the host immune system and antibiotics by formation of microcolonies in one location on target tissues [107].

PROTEIN SECRETION SYSTEMS AND EXOENZYMES

The severity of *P. aeruginosa* infections is due to its secretions of exoenzymes that cause host tissue damage by disrupting normal cytoskeletal structure, depolymerization of actin filaments and cleavage of immunoglobulin G (IgG) and A (IgA), and all of these processes lead to invasion, dissemination and development of chronic infections [21]. As Gram-negative bacteria, *P. aeruginosa* has protein secretion systems that play a role in several physiological processes such as adhesion, pathogenicity, adaptation and survival [26,97]. Type II and III secretion systems (T2SS and T3SS) secrete toxins that may play independent roles in death due to *Pseudomonas* lung infection [59]. T2SS is composed of multiprotein secretions encoded by the *xcp* and *hxc* operons [59]. These proteins secrete from the periplasm into the extracellular environment, and they include various hydrolysing enzymes such as pseudolysin [26], LasA and LasB elastases, type IV protease, alkaline protease, protease IV, phospholipase H, lipolytic enzymes as well as exotoxin A [21,59]. LasA and LasB elastases are regulated by the *lasI* quorum-sensing system [42]. LasB elastase degrades collagen and noncollagen host proteins, facilitates spread of infection by destroying host physical barriers and inhibits monocyte chemotaxis to prevent early clearance of *P. aeruginosa* from wound sites by phagocytosis and then stopping bacterial antigens' presentation to the host immune system [76]. Type IV protease protects *P. aeruginosa* during infection by degradation of host surfactant proteins A and D to inhibit the association of *P. aeruginosa* with alveolar macrophages [42], and also it is capable of degrading multiple immunoregulatory pro-

Table 1. Description of *Pseudomonas aeruginosa* infections

Infection	Route of infection and characteristics
Cystic fibrosis (CF)	Chronic, in individuals with genetic disease
Pneumonia: healthcare-associated pneumonia (HCAP), hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP)	Acute and nosocomial infection, the poor health status of the patients, the high carriage rate of often multidrug-resistant strains in hospital wards, and prior use of broad spectrum antibiotics
Chronic lung infection	In an immunocompromised patients, cystic fibrosis patients, individuals receiving chemotherapy
Urinary tract infections	Acute and nosocomial infections, urinary catheterization
Skin and soft tissue infections	Acute and nosocomial infections, burn, wounds and surgical site infections
Diabetic foot infections	Community-acquired infections
Burn infection	Acute and nosocomial infection
Bacteraemia and septicaemia	Acute, in immunocompromised patients
Central nervous system infections	Meningitis and brain abscess by contiguous spread, bacteraemia or direct infection as in trauma and surgery
Otitis externa	Community-acquired infection, in immunocompromised patient such as those with diabetes mellitus and swimmer's ear
Corneal infections	Acute ulcerative keratitis, community-acquired infection usually associated with users of extended-wear soft contact lenses

teins, including complement, immunoglobulins, fibrinogen, plasminogen and antibacterial peptides [21,35]. Alkaline protease degrades host complement proteins and host fibronectin [21,42], and it effectively degrades the host Toll-like receptor 5 (TLR5) ligand monomeric flagellin, while polymeric flagellin (involved in bacterial motility) and TLR5 itself resist degradation, thereby helping *P. aeruginosa* to avoid immune detection [11]. Phospholipase H and lipolytic enzymes break down surfactant lipids and the phospholipids of host cell membranes, leading to an increase in surface tension [42], and it can lyse human and sheep erythrocytes [87]. Exotoxin A plays an important role as a virulence factor in host cell death by inhibiting eukaryotic elongation factor 2 (EF2), thereby affecting protein synthesis [21,35,42].

T3SS is double-membrane embedded nanomachine in *Pseudomonas* and promotes the transfer of bacterial effector proteins to the cytoplasm or the plasma membrane of target eukaryotic cells, thereby promoting bacterial invasion and colonization [26]. Also it is a major determinant of virulence that is frequently associated with acute invasive infections and increased mortality in infected patients. Four effector proteins (ExoY, ExoS, ExoT and ExoU) of *P. aeruginosa* T3SS are expressed variably in different strains and isolates and have different activities [35,42,49]. Their role in pathogenicity is not clear exactly, but it is thought that T3SS may allow *Pseudomonas* to exploit breaches in the epithelial barrier by antagonizing wound healing during colonization and to promote cell injury leading to the symptoms of bacterial pneumonia [42]. ExoS is a bifunctional toxin with both GTPase activating protein (GAP) activity and adenosine

diphosphate ribosyl transferase (ADPRT) activity. The ADPRT activity of ExoS has a number of adverse effects on the host cell, including cell death (e.g. apoptosis and necrosis), actin cytoskeletal disruption to facilitate *P. aeruginosa* penetration through epithelial barriers, and inhibition of DNA synthesis, vesicular trafficking and endocytosis [49]. ExoT is also a bifunctional toxin with amino terminal GAP activity and carboxy-terminal ADPRT activity. This toxin is one of the reasons for delays in wound healing, and it causes apoptosis-like cell death predominantly through its ADPRT activity at later time points (10 hours, compared to 2-5 hours for ExoS-mediated killing). The GAP and ADPRT activities of ExoT work together to alter the actin cytoskeleton, block the cell division at the stage of cytokinesis, inhibit cell migration, adhesion and proliferation. These activities lead to blocking phagocytosis and disrupting epithelial barriers to facilitate bacterial dissemination [49]. ExoU is a potent phospholipase causing rapid necrotic death (within 1-2 hours) of eukaryotic cells due to irreversible damage of cellular membranes [21,49]. ExoY is an adenylate cyclase with two domains that act together to bind ATP. Although the significance of ExoY in infection remains unclear, its injection into mammalian cells results in a high concentration of intracellular cAMP, leading to disruption of the actin cytoskeleton, inhibition of bacterial uptake by host cells, and increased endothelial permeability [49].

The type V secretion system (T5SS), also known as the autotransporter system [26], and it is a macromolecular machine secreting mainly virulence factors that target eukaryotic cells and also plays roles in biofilm formation and cellular adherence [26,29]. The T5SS includes auto-

Table 2. *Pseudomonas aeruginosa* virulence factors and their roles

Virulence factor	Role
	Motility, chemotaxis, mediate initial surface interactions [34,41,75]
Pilli	Adhesion on cell surface, twitching motility, formation of biofilms and avoid host immune system and antibiotics [34,41,107]
T2SS	
Exotoxin A	Inhibits eukaryotic protein synthesis [21,34,41]
LasA	Staphylolysin, zinc metalloprotease with elastase and staphylolytic activity [41]
LasB	Elastase, degrades collagen and noncollagen host proteins, host physical barriers, degrade the opsonizing lung surfactant proteins A and D and inhibits monocyte chemotaxis [75]
Type IV protease	Degrades immunoglobulins, complement, surfactant proteins A and D, fibrinogen, plasminogen and antibacterial peptides [21,34]
Alkaline protease	Inhibits complement activation, neutrophil phagocytosis and fibronectin [11,41]
Protease IV	Cleaves transferrin, elastin, lactoferrin, decorin and casein [21]
Phospholipase H	Destroys surfactant lipids and phospholipids, hemolyse erythrocytes [75,87]
T3SS	
ExoS	Apoptosis, necrosis, killing of host immune cells, cytoskeletal disruption and inhibition of DNA synthesis [48]
ExoT	Apoptosis, blockage of cell division, alter the actin cytoskeleton, blocks phagocytosis, inhibits cell migration, adhesion, and proliferation [48]
ExoU	Necrosis and killing of phagocytes as well as epithelial barriers [48]
ExoY	Disruption of the actin cytoskeleton and increase endothelial permeability [48]
T5SS	Target eukaryotic cells, biofilm formation, cellular adherence and antibacterial functions [78,97]
Lectins LecA and LecB	Adhesion on host cells and induce lung injury [22], biofilm formation [32]
Siderophores	Iron acquisition from iron limited environments [115]
Pyocyanin	Ciliary dysfunction in the respiratory tract, exerts pro-inflammatory and oxidative effects that damage host cells [31,106]
Quorum sensing	Biofilm formation, regulation expression of several virulence factors, response to changing microbial population density, adaptation to the metabolic demands of living in community [31,36,98]
Lipopolysaccharide	
lipid A	Endotoxic shock [90]
O-specific polysaccharide	Responsible for conferring serogroup specificity and antibodies production [18]
Biofilm	
Alginate	Protective factor from environments in CF lungs [114] and immune defenses [105], structural stability and protection of biofilms [114] and antibiotics resistance [104]
Psl	Attachment [17,54,100] resistance of immunity [80], antibiotics resistance [12], structural scaffold in mature biofilms [24,120] and formation of eDNA–Psl fibres to give bacteria structural support [113]
Pel	Primary structure scaffold for biofilms formation [24,25], antibiotic resistance [25] and adhesins [110]
eDNA	antibiotic resistance [112]

transporters (ATs) and two partner secretion (TPS) systems that are secreted through T5aSS and T5bSS, respectively. Recently, *P. aeruginosa* was shown to secrete PlpD protein, (a lipase of the patatin-like family protein) through the type T5dSS, which causes lipid destruction by inserting into the outer lipid bilayer of target cells, leading to establishment of infection [29]. The T5SS also has antibacterial functions to target other bacteria by a contact-dependent growth inhibition (CDI) mechanism [29].

LECTINS

Two soluble lectins, LecA and LecB, are present in the outer membrane of *P. aeruginosa* that may participate in adhesion on host cells [22] and play a major role in the severity of *P. aeruginosa*-induced lung bacterial load and injury, and dissemination of the pathogen, influencing its survival [22] and biofilm formation [33].

IRON CHELATION

Iron chelation is a vital part of establishing infections and the progression to a chronic status. When free iron becomes unavailable for bacteria as a result of acquisition by the host cell of lactoferrin and transferrin from its environments, the bacteria are able to sequester iron from these environments by their iron chelation with siderophores (pyoverdine and pyochelin) that act as signalling molecules and transport system for iron [21,42]. Pyocyanin (blue-green pigment) is one of the *P. aeruginosa* virulence factor secreted metabolites causing ciliary dysfunction in the respiratory tract, exerting pro-inflammatory and oxidative effects that damage host cells by disrupting host catalase and mitochondrial electron transport, and playing a protective role against the reactive oxygen and nitrogen species produced by phagocytic cells during infection [67].

QUORUM SENSING

Quorum sensing (QS) is a generic regulatory mechanism used by many bacteria to perceive and respond to such varied factors as changing microbial population density and the expression of specific genes [32]. It comprises intercellular, small, membrane diffusible signalling molecules, called autoinducers, released into the environment and playing an important role in regulating expression of several virulence factors and biofilm formation [32,37]. Pathogenic bacteria use this mechanism not only to modulate virulence factor production but also to adapt to the metabolic demands of living in a community. The bacterial genome (in 4-10%) and the expressed bacterial proteome (in $\geq 20\%$) can be influenced by QS [32]. Many genes in *P. aeruginosa* are regulated and expressed by the QS system including pathogenesis genes such as those for alkaline protease, pyocyanin, pyoverdine, cyanide, lipase, twitching movement, alginate, azurin, chitinase, catalase, superoxide dismutase, lasA, lasB, XCP transport machine, etc [81]. *P. aeruginosa* produces three autoinducers; two are acyl homoserine lactones, the lactone-based systems (AHLs) Las and Rhl, and one is a quinolone-based system (PQS) [32,98]. The Las system consists of a *lasI*-encoded acyl-HSL synthase and the *lasR*-encoded transcriptional activator. The Rhl system consists of an *rhlI*-encoded acyl-HSL synthase and an *rhlR*-encoded transcriptional activator. Each system produces and responds to a specific acyl-HSL; LasI directs the synthesis of N-(3-oxodecanoyl)-homoserine lactone (3-oxo-C12-HSL) and RhlI directs the synthesis of N-butyryl-homoserine lactone (C4-HSL) [37,81,104].

C12-HSL autoinducer causes IL-8 production in human lung structural cells such as fibroblasts and bronchial epithelial cells. The autoinducer also stimulates the production of cyclooxygenase-2 and prostaglandin E2 in lung fibroblasts, thereby playing a role in inflammation. Moreover, the molecule can induce apoptosis of neutrophils and macrophages [81]. Both 3-oxo-C12-HSL and C4-HSL have also been detected in biofilms [37].

Moreover, the Rhl QS regulates production of rhamnolipid, which is important for biofilm formation *in vitro* and persistence *in vivo*, whereas PQS regulates the generation of the eDNA matrix component [98] and acts as a link between Las and Rhl systems [81]. Due to the role of QS in the regulation, control and formation of biofilm and many virulence factors, QS inhibition has been suggested as a potential target for new preventive and/or therapeutic strategies of *P. aeruginosa* infections [37,81]. Indeed, knowledge on biofilm formation and quorum sensing results in identification of new targets for therapeutics against *P. aeruginosa* infection [104].

LIPOLYSACCHARIDE

Lipopolysaccharide (LPS) is a complex glycolipid and forms part of the outer membrane of the cell wall of *Pseudomonas aeruginosa* and other Gram-negative bacteria. This molecule plays a role in antigenicity, the inflammatory response, exclusion of external molecules and in mediating interactions with antibiotics [63], and also acts as structural integrity of *P. aeruginosa* biofilms [117]. Lipopolysaccharide contributes to biofilm function and architecture by influencing bacterial adhesion, cell-to-cell adherence and viscoelastic properties of biofilms [68], and it is able to form a stable monolayer at the air-water interface by the amphiphilic LPS molecules involved in initial steps of pellicle formation [2]. *P. aeruginosa* produces a three-domain, typical LPS, consisting of a membrane-anchored lipid A, oligosaccharide core region, and a highly variable O-specific polysaccharide (O-antigen or O-polysaccharide) [63]. Although LPS is a prominent factor in mediating both bacterial pathogenicity and the host immunity response, this impact varies depending on the susceptibility of the patient to infection, the isoform of the LPS, particularly the lipid A component responsible for endotoxic activity of LPS, and structural variation in the O-antigen side chain that impacts host immunity [90].

Lipid A is composed of an N- and O-acylated diglucosamine bisphosphate backbone [4-P- β -D-GlcpN¹-(1 \rightarrow 6)- α -D-GlcpN¹-(1 \rightarrow P)] with chemical variation in the number of primary acyl groups and the types of fatty acids substituting the primary and secondary acyl groups [66,91]. Structurally, the number, position, and nature of the linked acyl groups and the type of substituent to the phosphate groups can vary according to isolation source, strain and growth condition [66,91]. Modifications to lipid A can alter some bacterial pathogenicity properties such as sensitivity to polymyxins and cationic antimicrobial peptides as well as change its inflammatory properties and then severity of infection [36]. Recognition of lipid A by TLR-4 mediates both effective host resistance to infection as well as some of the pathology associated with LPS-induced shock. Lipid A bound to CD14 can interact with the extracellular or intraluminal domains of TLR4 in the presence of the co-factor MD2, leading to activation of transcription factors, notably NF- κ B, which enters the nucleus and promotes production of inflammatory cytokines such as inter-

leukin (IL)-1, IL-6, IL-8, tumour necrosis factor α and other host factors, eventually causing endotoxic shock [90]. TLR4-mediated responses are highly dependent on the level of acylation of lipid A, which can be controlled by the bacterial strain and the growth conditions as mentioned previously. In general, production of a fully hexa-acylated lipid A is associated with a more vigorous inflammatory response induced by *P. aeruginosa*, whereas production of lipid A is associated with lower levels of acylation resulting in reduced cellular responses and production of inflammatory cytokines [90]. In fact, the penta-acylated form of the *P. aeruginosa* LPS is predominant in laboratory strains and in isolates from acute infections [63]. Conversely, hexa- and sometimes hepta-acylated species were isolated from chronically infected lungs of CF patients [36].

There are two different glycoforms of the oligosaccharide core region which contains an *N*-alanylated galactosamine residue, 3 D-glucose residues, and one L-rhamnose residue the positions of which differ in the two glycoforms. This oligosaccharide core is linked with lipid A on one side, the reducing end of the core, and the O-polysaccharide domain on another side [18,19].

The O-polysaccharide or O-antigen domain of LPS is responsible for conferring serogroup specificity, which is defined by antibodies specific to the different variants of this antigen, where the O-antigens can be diversified into at least 11 structural variants. The sugars within the O-antigenic structure include *N*-acyl derivatives of different amino sugars along with rhamnose. The sugars are arranged in repeating units containing 3 to 4 individual monosaccharides, except for serogroup O7, which is a disaccharide repeating unit [18]. Generally, two types of O-antigen exist simultaneously within the *P. aeruginosa* cell, but with different structural and serological properties. A-band ('common') polysaccharide is a homopolymer of D-rhamnose, approximately 70 sugars long, and leads to a weak antibody response. In contrast, B-band ('O-specific') polysaccharide is a strain-variable heteropolymer both in chain length and in the nature of the sugars, and it stimulates a strong antibody response which is the chemical basis for serotyping [63].

BIOFILM MATRIX

Biofilm is one of the most important virulence factors that arise on the surface of bacteria which are embedded within the extracellular matrix (Fig. 2) [27,114], and it can be formed on a variety of surfaces such as natural, industrial and hospital niches [114]. Biofilm acts as a protective mode of bacteria that allows them to survive in hostile environments and to colonize under desirable conditions [114,120], as a protective barrier to antimicrobials and the host immune system [12,25,120]. *P. aeruginosa* is an avid biofilm former that is implicated in both chronic and acute infections. It can cause particularly devastating chronic infections or enable life-threatening nosocomial infections in short time courses [12]. Clin-

ically, biofilm formation within the cystic fibrosis (CF) airways is believed to facilitate the infection as well as protect the bacteria from antimicrobial treatment and host defences [24]. Moreover, biofilm has been shown to be formed readily on catheters and ventilator tubes, in urinary tract infection and ventilator-associated pneumonia (VAP), respectively, and it is present in chronic leg wounds, where a higher prevalence of biofilm-like formations was found in this infection [83].

The biofilm extracellular matrix is composed of secreted extracellular polymeric substances which consist of exopolysaccharides (EPS), proteins, nucleic acids and lipids (Fig. 2) [12,25,27,114], which function as a matrix holding bacterial cells together [114].

There are several mechanisms of regulation of biofilm formation. Bis-(3 \rightarrow 5 \rightarrow)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a general key regulator of the bacterial biofilm lifecycle [38]. C-di-GMP is synthesized and degraded via activities of diguanylate cyclases (DGCs) with a GGDEF domain and phosphodiesterases (PDEs) with EAL or HD-GYP domains, which contain sensory domains that sense and respond to environmental cues [96]. *P. aeruginosa* encodes several DGCs and PDEs that increase or decrease the intracellular c-di-GMP levels, causing stimulation or prevention of biofilm formation respectively [64,114]. At least four c-di-GMP effectors, including Alg44, FimX, PelD and FleQ, were identified in *P. aeruginosa* [38]. Alg44 is a c-di-GMP-binding protein with a putative PilZ domain [79], involved in the synthesis of the matrix exopolysaccharide alginate in *P. aeruginosa* [79,95]. PelD, a protein encoded in the *pel* operon, acts as c-di-GMP receptor that mediates c-di-GMP regulation of *pel* polysaccharide biosynthesis [70]. FleQ protein is a transcriptional regulator for several genes, as in flagella and outer membrane adhesin *CdrA* biosynthesis genes [9,50,99]. FleQ is a type of c-di-GMP binding protein that controls the transcriptional regulation of *pel* biosynthesis genes in *P. aeruginosa* [50], where it works as a repressor and an activator of *pel* transcription [10]. The FimX effector has been proposed to regulate twitching motility in response to alterations in the c-di-GMP level [56].

Quorum sensing, as mentioned above, plays an important role in regulating virulence and biofilm formation genes in *P. aeruginosa* both in its natural environment and in infection sites. In biofilm formation, LasI as one of the QS systems is expressed during the initial stage of biofilm formation [30], while the RhlR/RhlI system is activated during the maturation stage of *P. aeruginosa* biofilm development [101]. Although Psl itself can act as a signalling molecule to stimulate its own expression via two diguanylate cyclases [53], the QS regulator LasR can bind to the promoter region of the *psl* operon, perhaps to regulate *psl* expression [44]. On the other hand, AHL- and *Pseudomonas* quinolone signal (PQS)-mediated quorum sensing systems function as regulators for extracellular DNA (eDNA) generation [4]. Furthermore,

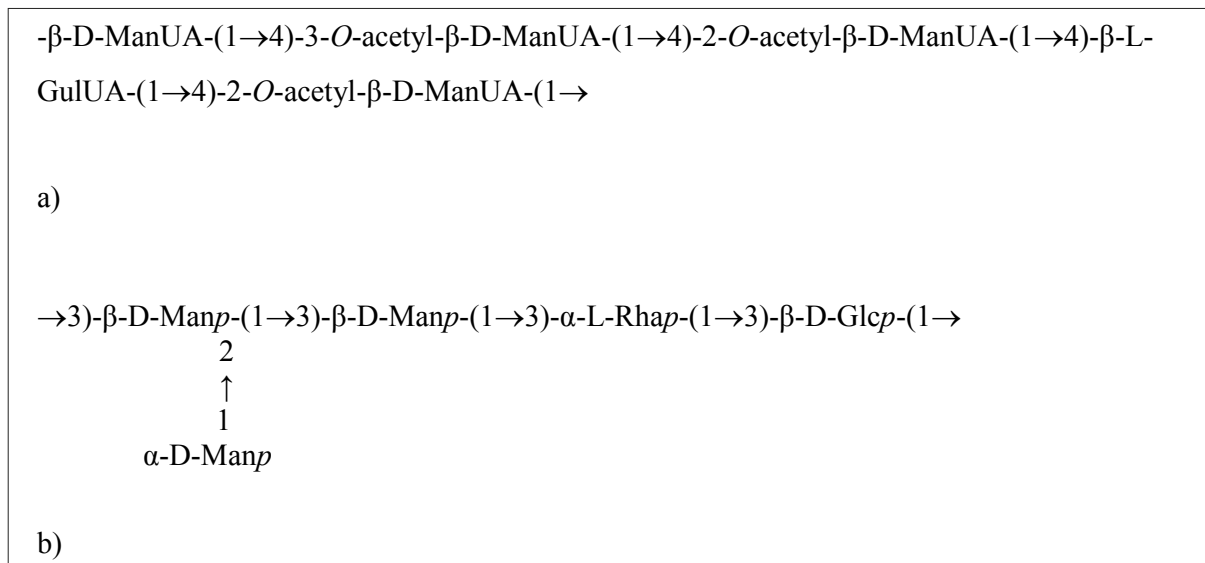


Fig. 1. Structures of *P. aeruginosa* (a) alginate, composed of β -D-mannuronate and α -L-gulonate with O-acetyls at C-2 and/or C-3 position of the mannuronate residues and of (b) Psl polysaccharide, composed of repeating pentasaccharide units [39]

QS signalling controls the production of the biosurfactant rhamnolipid, which was shown to be important for biofilm formation by *P. aeruginosa* and to play a role in the tolerance of *P. aeruginosa* biofilms towards immune cells [38]. QS also regulates the production of LecA and LecB lectins [33] and siderophores [8], which play a role in *P. aeruginosa* biofilm formation [8,33].

Besides the aforementioned mechanisms, the GacA/GacS two-component system also has a role in regulation of *pel* and *psl* gene expression for exopolysaccharide production in *P. aeruginosa*, via the control of transcription of two small regulatory RNAs (sRNAs), *rsmY* and *rsmZ*, leading to the decrease or increase in the translation of the *pel* or *psl* operon. This mechanism involves two histidine kinases, RetS and LadS, that act in opposing ways on the GacA/GacS two-component system [111]. To produce the RNAs *rsmZ* and *rsmY*, sensor kinase GacS can phosphorylate response regulator GacA, and this activity is stimulated by LadS and antagonized by RetS [46,111].

In addition to the typical regulation of biofilm development, biofilm formation also involves other types of regulation, such as fatty acid-mediated signalling, which may play a role in regulating *P. aeruginosa* biofilm dispersal. The signal, cis-2-decenoic acid, appears to be involved in dispersal of mature biofilms [6]. Also Psl was found to be regulated by RpoS transcriptionally, and post-transcriptionally by RsmA and RNA binding protein [54] as well as the transcriptional regulator AmrZ, which directly binds to the promoter region of the *psl* operon to repress its transcription [78]. Finally, the total amount of exopolysaccharides in *P. aeruginosa* is under control of the sugar precursor pool for exopolysaccharide synthesis, where the overexpression of one exopolysaccharide could reduce the production of the

other via metabolic regulation mediated by AlgC [114].

Extracellular polysaccharides (EPS) as biofilm components play a role as a primary biofilm scaffold, initial attachment and adhesion to surfaces and other cells, as well as protecting the bacterial cell from antimicrobials and host defences [12,24,25]. In *P. aeruginosa* at least three extracellular polysaccharides occur that can contribute to biofilm formation [100,114].

Alginate is secreted by mucoid strains isolated from cystic fibrosis patients [100]. On the other hand, the non-mucoid strains isolated from environments other than CF lung produce primarily the Psl and Pel polysaccharides for biofilm formation [25,27,100].

Alginate is a high molecular mass unbranched and anionic copolymer containing β -D-mannuronate and α -L-gulonate with O-acetyl groups at the C-2 and/or C-3 position of the mannuronate residues (Fig. 1a) [39,100]. Under atomic force microscopy, *P. aeruginosa* FRD1 alginate appeared as a soft loosely adhered polymer that surrounds the cells [39]. Alginate protects *P. aeruginosa* from harsh environments in CF lungs by providing an extracellular matrix in biofilms [114] and from immune defences via inhibition of bacterial uptake by macrophages during phagocytosis [105] and prevents activation of the complement alternative pathway, protecting cells from antibody-independent phagocytosis [91]. It plays important roles in structural stability and protection of biofilms, and it is necessary for water and nutrient retention in biofilms [114]. Despite information about its role in antibiotic resistance, however, a recent study indicated that it does not contribute to resistance to gentamicin [43]. Moreover, another study reported that alginate production did not play a significant role in bacterial attachment, biofilm formation, or biofilm development [71].

Psl polysaccharide (polysaccharide synthesis locus), identified as a repeating pentasaccharide, contains D-mannose, L-rhamnose and D-glucose residues (Fig. 1-b) [17,39]. Fluorescent staining and confocal laser scanning microscopy of *P. aeruginosa* PAO1 biofilm suggest that Psl forms a fabric-like matrix connecting the biofilm cells [39]. The *polysaccharide synthesis locus* operon was shown to be essential for biofilm formation in strain PAO1 and is composed of 15 genes (*pslA-O*, PA2231-2245), only 11 genes of those encoding the Psl biosynthesis [17,55,100]. Psl polysaccharide plays an important role in cell-surface and cell-cell attachment [25,77,100,120] and resistance to immune attacks by inhibition of opsonization, resulting in reduced neutrophil reactive oxygen species (ROS) production and decreased killing by phagocytes [80]. On the other hand, Psl in the *P. aeruginosa* biofilm matrix represents the first line of defence toward antibiotics, with diverse biochemical properties during the initial stages of biofilm development [12]. Also it has a major role in biofilm formation via acting with Pel as a structural scaffold in mature biofilms. Both Pel and Psl polysaccharides are required for type IV pilus-independent microcolony formation in the initial stages and for macrocolony formation in the later stages of *P. aeruginosa* PAO1 biofilm formation [25,120]. Furthermore, it can react with eDNA to form a web of eDNA-Psl fibres, which resembles a biofilm skeleton in the centre of pellicles to give bacteria the structural support and capability against agents targeted at one matrix component [113].

Pel polysaccharide (pellicle) composition was suggested by Friedman and Kolter as a glucose-rich polysaccharide-like cellulose [40], but the exact structure remains unknown [30,39,53]. Indeed, using specialized carbohydrate chemical analyses, a recent study could define Pel as a positively charged exopolysaccharide composed of partially acetylated 1→4 glycosidic linkages of *N*-acetylgalactosamine and *N*-acetylglucosamine. By this cationic charge, Pel cross-links eDNA in the biofilm matrix [58]. Scanning electron microscopy of a *P. aeruginosa* PA14 pellicle shows a fabric-like matrix that surrounds and connects the cells to form a microbial mat (a multi-layered sheet of microorganisms) at the air-water interface [39]. A seven-gene operon (*pelA-F*) is responsible for Pel synthesis and was identified in a mutagenesis screen for the loss of pellicle formation in PA14 [40]. Pel polysaccharide was shown as a primary structure scaffold for the bacterial community by maintaining the cell-to-cell interactions in PA14 biofilms and plays a protective role by enhancing resistance to aminoglycoside antibiotics in biofilms [24,25]. As mentioned above, both Pel and Psl polysaccharides are essential for subpopulation interactions and macrocolony formation in the later stages of *P. aeruginosa* PAO1 biofilm formation, but Psl polysaccharide is more important than Pel in PAO1 biofilm formation and antibiotic resistance [120]. In addition, Pel polysaccharide in the *P. aeruginosa* PAK strain can compensate as an attachment factor in the absence of other adhesins such as type IV pili [110].

ANTIMICROBIAL RESISTANCE

Antibiotic resistance of *P. aeruginosa* is a serious medical problem which is responsible for chronic and ~10% of all hospital-acquired infections worldwide, leading to a high rate of morbidity and mortality among patients [21,85]. *P. aeruginosa* exhibits multiple mechanisms of resistance, including antibiotic-modifying enzymes such as aminoglycoside-modifying enzymes and β -lactamases, acquisition of chromosomally or plasmid encoded antibiotic resistance genes, mutations, limited membrane permeability for the antibiotics [74,93,109] and antibiotic efflux pumps such as MexAB-OprM, MexEF-OprN and MexCD-OprJ, which provide resistance to β -lactam antibiotics, MexEF-OprN and MexCD-OprJ, which confer resistance to fluoroquinolones, and MexXY-OprM, affecting aminoglycoside resistance [72,75,93]. Moreover, *P. aeruginosa* is able to adapt to various stresses of the environment as in antibiotic concentrations or other effectors by different ways such as biofilm formation, swarming or surfing motility, or association with epithelial surfaces leading to increased resistance [13], or by modifications of the lipopolysaccharide (LPS) by the addition of a 4-amino-4-deoxy-L-arabinose moiety in the lipid A structure as in resistance to polymyxin B involving a large array of chromosomal genes [69,75,84]. Sometimes the bacteria enter into the latent state to protect themselves from antibiotics such as penicillin, which act by inhibiting cell wall synthesis during the growth period [104].

Previous studies offered several hypotheses to explain the role of biofilms in resistance to antibiotics. The penetration limitation hypothesis assumes that the biofilm represents a barrier to prevent penetration of antibiotic into bacterial cells [108], where the lethal dose of the antibiotic may be adsorbed by components of the biofilm extracellular matrix or consumed and deactivated in biofilm by inactivating enzymes and efflux pumps [104]. Indeed the biofilm capacity for antibiotic exposure is not similar for the same antibiotic; for example, the penetration of ampicillin was inhibited by the production of a β -lactamase. However, ampicillin was fully able to penetrate the biofilms of a β -lactamase deficient mutant, which indicates that the β -lactamase can accumulate in the biofilm matrix, deactivating β -lactam antibiotics on the surface layers of the biofilm before they can diffuse into the substratum [7]. Another way to prevent antibiotic penetration occurs with positively charged aminoglycosides which bind to the negatively charged alginate component of biofilms, leading to slow diffusion through the biofilm, providing additional time for bacteria to mount a stress response [112]. In the same way, extracellular DNA can delay penetration of positively charged aminoglycosides across *P. aeruginosa* biofilms through electrostatic interactions, but for a short time before saturation of eDNA with antibiotic [23].

Another hypothesis was defined by alteration of biofilm microenvironment. Consumption of oxygen in surface layers and formation of anaerobic conditions in deep

layers of the biofilm can lead to hindering of antibiotic action as observed with aminoglycoside antibiotics which become less effective in an oxygen-limited environment [61]. Additionally, alteration of the osmotic environment within a biofilm may cause an osmotic stress response which results in antibiotic resistance [94].

TREATMENTS AND PHAGE THERAPY

P. aeruginosa has many virulence factors which are the main reason for the high incidence of infections and the emergence of resistant strains to antibiotics constantly leading to an increase of morbidity and mortality among patients. Therefore it is critical to develop therapeutic interventions to treat pseudomonas infections. Currently, multidrug resistance is the hardest problem associated with the treatment of *P. aeruginosa* infections, which made it imperative to search for alternative treatment strategies. This alternative strategy must target one or more virulence factors to eradicate *P. aeruginosa* successfully. Here we will discuss phage therapy as a novel treatment method of treating *P. aeruginosa* infections briefly.

In fact, despite the problems associated with this non-conventional therapy, numerous bacteriophages were isolated and used clinically in the Middle East, Asia and Eastern European countries such as Russia, Poland and Georgia to treat infections in humans [1,3]. The ability of lytic bacteriophage to target and kill bacteria suggests some of the potential advantages of phage antibacterial therapy, where they can multiply at the infection site and target only specific bacteria with no effect on commensal flora, and when their host disappears they also gradually vanish, as well as having an effect on biofilms in antibiotic resistant strains [65,89]. Despite these advantages, phages are still not used as antimicrobial agents because of their ever-changing, dynamic nature. Some of them can acquire undesirable genes such as toxins or transfer bacterial genes between bacteria, as occurs in transduction, and bacteriophages potentially may interact with the human immune system [65,86].

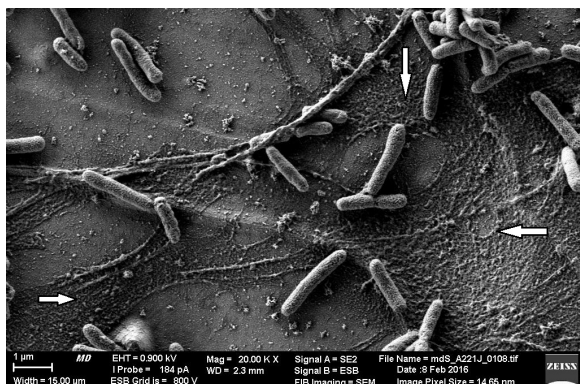


Fig. 2. *Pseudomonas aeruginosa* PAR5 surrounded by extracellular polymeric substances; the image was taken by scanning electron microscopy at low voltage (LV-SEM)

The effect of phage on bacterial cells occurs by several mechanisms. The most common is bacteriolysis by disruption of the cell wall with the virolysin-holin system or the single lytic factor (Fig. 3) [89]. In another mode of action by genetically modified phages, *P. aeruginosa* filamentous phage can be genetically modified by replacing the transportation gene with a restriction enzyme gene, so that the phages lose the ability to extrude from bacterial cells and lyse them, but acquire the ability to digest the bacterial nucleic acid. This modification reduces the release of cell wall components, thereby avoiding a Jarisch–Herxheimer reaction, which occurs when lytic phages induce bacterial lysis, releasing bacterial endotoxins, which simulate the general pathological aspects of septicaemia. Such modification leads to therapeutic efficiency better than therapies using lytic phages [86]. With regard to the *P. aeruginosa* biofilms formed on medical implants such as catheters and on airways of the CF patients, by strains extremely resistant to antibiotics, bacteriophages can infect the bacteria and disrupt their growth in the biofilm matrix and then replicate themselves near to the site of the infection [73,82,92]. As also noted, using a bacteriophage cocktail [5,41] and bacteriophage in combination with an antibiotic [28] reduces and disperses *P. aeruginosa* biofilm. Further, bacteriophages produce alginate, an enzyme that depolymerizes the alginic acid capsule of *P. aeruginosa* in the biofilm matrix. Also other enzymes can degrade the bacterial exopolysaccharides in biofilms [34,45,47,51,62]. In other words, both bacteriophage and their encoded proteins can act as an anti-biofilm matrix.

Using bacteriophage components as antimicrobial agents seems to be more common in the fermentation industries, such as the addition of lysozyme to yogurt and other milk fermented products to prevent bacterial contamination. Phage-encoded lysozymes are of two kinds: endolysin, which is produced by lytic phage at the end of its replication cycle to degrade the peptidoglycan of the bacterial host from inside, resulting in cell lysis and release of progeny virions, and phage tail-associated murein lytic enzymes (TAME), which can hydrolyse cell wall bonds

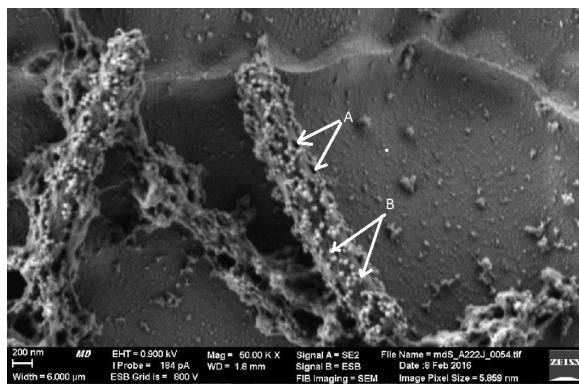


Fig. 3. *Pseudomonas aeruginosa* PAR5 after phage treatment; the image was taken by scanning electron microscopy at low voltage (LV-SEM). Signs with (A) arrows indicate the holes on the bacterial surface and with (B) heads of phages

from outside after phage adsorption to the host [3]. Actually, using bacteriophage components for therapy is safer and more beneficial in avoiding the undesired advantages which appear with whole phage therapy.

Although the endolysins can lyse peptidoglycan of Gram-positive bacteria due to the absence of an outer membrane in their cell wall, recent studies demonstrated that these proteins can also be active against Gram-negative bacteria, but with peptidoglycan different structure, reflecting the differences in cell wall architecture between these major bacterial groups [16]. Endolysins from Gram-positive bacteria consist of two functional domains. One is termed cell wall binding domain (CBD), which targets the protein to its substrate and keeps it tightly bound to cell wall debris after cell lysis, thereby likely preventing diffusion and subsequent destruction of surrounding intact cells that have not yet been infected by the phage. The second one is enzymatically active domain (EAD), cleaving specific bonds within the bacterial peptidoglycan. By contrast, the outer membrane (OM) of Gram-negative bacteria acts as a protective wall for peptidoglycan from the outside. This might explain why endolysins from phages infecting Gram-negative hosts are mostly small single-domain globular proteins (molecular mass between 15 and 20 kDa), usually without specific CBD domains [102]. *P. aeruginosa* bacteriophage endolysins KZ144 (phage ϕ KZ) and EL188 (phage EL) are exceptions, where their lysins are highly lytic peptidoglycan hydrolases and contain an N-terminal CBD and a C-terminal EAD [15]. Both domains are required for the antibacterial activity of the endolysin; the C-terminal enhances the permeabilization of the *P. aeruginosa* outer membrane and N-terminal for enzymatic activity [14,16]. In the case of KZ144 endolysin, the N-terminal domain binds to the *P. aeruginosa* cell wall with high affinity and is also active against peptidoglycan of a broad range of Gram-negative species [82]. Recently, endolysins have been used successfully in various medical applications *in vivo*, including decolonization of mucous membranes, treatment of systemic infections and controlling pathogenic bacteria. Additionally, various endolysins have been demonstrated to reduce or eradicate bacterial biofilms, which are a problem in human infections, food production and other industries as well as antibiotic resistance [3,102].

The biofilm matrix is a barrier preventing phage infection, where bacterial microcolonies are surrounded by a matrix that may pose a problem for phages in reaching their receptors on the cell surface target. However, it has been observed that some phages are able to overcome this obstacle and penetrate the extracellular matrix due to their “accompanying” enzymes which hydrolyse the EPS matrix [88]. Bacteriophages encode two kinds of these enzymes that are capable of depolymerizing components of the EPS matrix; one of these depolymerases is important for release of bacteriophages from the host cell (these also include endopeptidases), and the second are tail spike proteins that serve infection. Although

these proteins are restricted in activity within the virus particle, they can be released from lysing cells in a more generally active form, which can affect the biofilm matrix [48]. EPS depolymerases therefore can be used as anti-biofilm agents because of their ability to structurally degrade this biofilm matrix, where they dissolve a biofilm faster than phages may infect and lyse bacteria. This degradation can be accomplished either with or without phage association, while the use of whole phages in addition to EPS depolymerases can allow for bacterial killing and lysis as well [20]. EPS depolymerases have been classified as endorhamnosidases, alginate lyases, endosialidases and hyaluronidases (glycoside hydrolases) [119]. Overall, EPS depolymerase activities include alginate lyases, amylases, cellulases, dextranases, endohexosaminidases, exopolysaccharide lyases, galactosidases, glucosidases, guluronan lyases, hyaluronate lyases, and pullulanases, where they are not just enzymes that degrade the biofilm matrix but also degrade the glycocalyx more generally, such as that making up bacterial capsules or slime layers [20], and they degrade their substrate by acting as endo-glycanohydrolases [52]. Hanlon et al. in 2001 demonstrated EPS depolymerase as anti-*P. aeruginosa* biofilm with the ability to reduce the viscosity of the alginate and EPS in *P. aeruginosa* [47], while Glonti et al. in 2010 identified haloes in cultures of a bacteriophage infecting cystic fibrosis strains of *P. aeruginosa* and purified a depolymerase protein from the bacteriophage using electrophoresis. This protein has the ability to degrade extracellular alginate acids [45].

CONCLUSIONS AND PERSPECTIVES

Pseudomonas aeruginosa is a pathogen having many virulence factors which are the cause of nosocomial infections and cases of antibiotic resistance (table 2). Biofilm represents an important factor in *P. aeruginosa* infections, avoids immune defence and protects the bacteria from antibiotics. Here, we describe the composition, formation and role of biofilms in an attempt to understand their function and the mechanism of antibiotic resistance. Also, further understanding of *Pseudomonas* regulatory systems and the factors which contribute to biofilm formation may help to find successful resolutions for decreasing nosocomial infection. There are several suggestions on the role of the biofilm matrix in antibiotic resistance, but they need further investigations, particularly with respect to its components and their properties. Increased spread of antibiotic resistant strains and the role of biofilms in this phenomenon require research on alternative methods of treatment. Phage therapy is an alternative method having much promise for the future. Although many *in vitro* and pre-clinical studies [31,51,102] as well as a few human clinical trials [57,118] gave a clear picture of the importance of phage therapy for controlling *P. aeruginosa* infections, there are still many difficulties and challenges to widespread clinical use. Indeed, many precautions must be taken into consideration when applying phage therapy,

such as phage selection regarding their specificity for individual bacterial strains, purification, storage, dose, route of uptake, sterility control, bacterial strain and ability to acquire new virulence from the phage as well as the immune system of the host, where some studies report that the phage treatment may be neutralized by anti-phage antibody production and subsequently reduce the therapeutic effects. Using two or a cocktail of phages will be a better choice for treatment. Also, suffi-

cient biofilm removal by phage components such as EPS depolymerases is perhaps better than the use of whole phages.

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