Organelles as virulence factors of an opportunistic pathogen *Pseudomonas aeruginosa*

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Type IV pili (T4P) and flagellum are well known virulence factors of an opportunistic pathogen *Pseudomonas aeruginosa*. T4P pili provide twitching motility, adherence to biotic/abiotic surfaces, intercellular association that is essential to biophilm formation, and are implicated in chemotaxis, bacteriophage adsorption and DNA uptake. Flagellum provides swimming/swarming motilities and chemotaxis, adhesion and biofilm formation. Both T4P and flagellum play a central role in virulence and host invasion, and induction of innate and adaptive immune response. Structure, assembly, functions of T4P and flagellum and their role in host defense are the the subjects of the review.

Keywords: *Pseudomonas aeruginosa*, type IV pili; flagella, immune response

1. *Pseudomonas aeruginosa* as an opportunistic pathogen

Members of the genus *Pseudomonas* are found in soil, decaying organic matter, vegetation and water. The broad environmental distribution of pseudomonades is made possible by their simple growth requirements and nutritional versatility. They are capable of using many organic compounds as sources of carbon and nitrogen, and some strains can even grown in distilled water [1] by using trace nutrients. Generally, pseudomonades utilize carbohydrates through aerobic respiration using the oxygen as the terminal electron acceptor. In addition, they can grow an aerobically using nitrate or arginine as an alternative electron acceptor.

Pseudomonades are uncommon in humans as part of the normal microbial flora [2]; however healthy people can carry these bacteria without having any problems. At the same, pseudomonades are recognized as serious opportunistic pathogens in immunocompromised patients, *i.e.* persons having an immune system that has been impaired by evoked diseases (*e.g.* AIDS [3]), inherited diseases that affect the immune system [4] or medical treatment (*e.g.*, patients with cancer after chemotherapy [5] and transplant patients who are taking certain immunosuppressive drugs [6; 7] are at high risk group). *Pseudomonas aeruginosa* is the most common pathogen among the genus members. The bacterium is the most common cause of respiratory failure in cystic fibrosis patients (CF), genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene [8], which results in thick, sticky mucus.

P. aeruginosa is responsible for an increasing proportion of infections acquired in the modern hospital setting (nosocomial infections), especially in intensive care units (ICU) and in urology patients [9], and has held a nearly unchanged position in the rank order of pathogens causing ICU infections during the last 4 decades [10]. The major risk factor associated with an increased mortality rate in patients with *P. aeruginosa* is antimicrobial resistance [11; 12].

It is surprising, that pseudomonades are not more common pathogens, considering their ubiquitous presence, ability to grow in virtually any environment, resistance to many antibiotics and virulence factors. The latter means specific substances that enable a microorganism to establish itself on or within a host and enhance its potential to cause disease via adhesion, colonization, invasion, inhibition of immune response and ability to cause tissue damage. List of *P. aeruginosa* virulence factors from data base 'Virulence factors of pathogenic bacteria' (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Pseudomonas) includes type IV pili (T4P) and flagellum that are the subjects of the review.

2. Pili

2.1 Structure and assembly

T4P are very strong flexible filaments up to 4 μm in length and 50-80Å in diameter, composed of thousands of copies of a single protein, PilA, that are assembled into long polar surface appendages [13]. Two main T4P subtypes are recognized: type IVa (T4aP) and type IVb (T4bP) that differ in length, leader sequence and N-terminal modification of major and minor pilin subunits, and in assembly system components [14; 15].

P. aeruginosa pilins belong to the type IVa group, with major pilin subunit PilA. *P. aeruginosa* has five types of *pilA* alleles, with differences in post-translational modifications of PilA and different associations with CF; group I pilins is the most prevalent type in CF patients [16]. Of the five pilA alleles, all but one (group II) are strictly associated with its cognate accessory gene; *tfpO* with $pilA_{I}$, *tfpY* with $pilA_{III}$, *tfpX* and *tfpW* with $pilA_{IV}$, and *tfpZ* with $pilA_{V}$ [17]. An accessory gene *tfpO* (*pilO*) from the group I encodes a pilin glycosyltransferase that posttranslationally modifies each

pilin subunit on a C-terminal Ser residue with an O-antigen unit prior to assembly into the pilus [18], which confers resistance to opsonization during host-mediated phagocytosis [19]. Pilins from the group IV are also posttranslationally glycosylated; the glycan is a homopolymer of alpha-1,5-linked D-arabinofuranose [20]. TfpW was identified as arabinosyltransferase [21]; *tfpX* encodes a putative pilin accessory protein with unknown function [16]. The accessory proteins TfpY and TfpZ from the group III and V, respectively, modulate pilus retraction dynamics [17].

The pilin subunits of T4aP share a common architecture: the N-terminal ~53 residues form an extended α-helix, α1; the N-terminal half of this helix, α1-N, protrudes from the protein and the C-terminal half of α-helix, α1-C, is embedded in a globular domain and interacts with an anti-parallel four- to five-stranded β-sheet; and the D-region encompassed by the conserved cysteines that links β-sheet to the C-terminal segment of pilin [22]. Functional diversity of T4P comes from two hypervariable loop regions: the αβ-loop, which is situated between α1 and the β-sheet; and the disulfide-bonded loop (DSL) formed by the D-region [22]. The D region can function as the adhesive component of the pilus and an important immunodominant epitope, which leads to development of anti-*P. aeruginosa* vaccines containing peptides corresponding to the region.

A defining functional characteristic of T4P proteins is their ability to reversibly assemble into polymeric fibers in a helical manner. Assembly requires cleavage and methylation of a hydrophilic leader peptide of PilA by a type IV prepilin peptidase PilD during pilin secretion [23]. Minor pilins FimU, PilV, PilW, PilX, and PilE (see below) also are processed by PilD before incorporation in pilus filament [24].

The first T4P model was proposed based on a single pilin structure, that of *N. gonorrhoeae* GC pilin [25]. In this model, the conserved hydrophobic α1 helices are twisted in a helical array in the core of the filament, anchoring the structurally variable globular head domains, which form the outer surface. Each head domain generally consists of a 4- to 7-stranded antiparallel β-sheet; structurally variable parts of head domain are αβ loop and DSL. The current model [26] predicts that the heads are separated by deep grooves. The αβ loop and the C-terminal domain are located at the base of the grooves and polar interactions are observed between the αβ loop of one subunit and the C-terminal region of the adjacent subunit. Inside the assembled pilus, hydrophobic α1 helices make multisubunit contacts to provide mechanical strength and flexibility. Outside, adaptable heads contribute surface variation for specificity of pilus function in antigenicity, motility, adhesion, and colony formation [27].

X-ray diffraction analysis of the *P. aeruginosa* strain PAK fibres reveals that they have a 52 Å outer diameter and a 12 Å inner diameter, with the subunits arranged in either a right-handed one-start helix with four subunits per turn or a left-handed three-start helix with four subunits per turn [14].

T4P are assembled at the cytoplasmic membrane by complex nanomachines whose molecular mechanisms and regulation are not yet fully understood. There are over 40 genes involved in the assembly and regulation of the T4P system in *P. aeruginosa* [15; 28]. These genes can be divided into two groups; structural components that form the assembly machinery and the pilus itself, and regulatory components.

2.1.1 The pilus fiber

Besides PilA, key structural component of T4P, other proteins are incorporated into the mature T4P fiber and required for T4P biogenesis [24]. There are five pilin-like proteins (core minor subunits FimU, PilV, PilW, PilX and noncore minor subunit PilE) and non-pilin protein PilY1 known as pilus-associated adhesin and anti-retraction protein [29]; all are encoded by an operon fimU-pilVWXY1Y2E [30].

In accordance with the model proposed by Nguyen et al. [31], PilVWXY1 (a priming complex) and either FimU or PilE comprise the minimal set of components required for pilus assembly. PilV, PilW, and PilX require PilY1 for inclusion in surface pili and *vice versa*; PilE requires PilVWXY1 for inclusion. FimU is incorporated independently of the others and is proposed to couple the putative minor pilin-PilY1 complex to the major subunit, PilA.

Minor pilins FimU-PilVWX are homologs of the type II secretory system (T2SS) minor pseudopilins (called XcpUVWX in *P. aeruginosa*); a mutant lacking the minor pilin operon produces small amounts of T4P since minor pseudopilins can prime T4P assembly, although there are no equivalents of PilE and PilY1 in the T2SS [31].

Among proteins encoded by an operon fimU-pilVWXY1Y2E, PilY1 is the most enigmatic. Minor pilins promote surface display of the PilY1 adhesin; trafficking of PilY1 to the cell surface allows for production of pili of sufficient length to support adherence and motility [31]. Indeed, PilY1 has additional roles in antagonizing pilus retraction and mediating attachment to host epithelial cells, since *pilY1* mutant of *P. aeruginosa* is defective in early biofilm formation [32].

A crystal structure of the C-terminal domain of PilY1 revealed a beta propeller fold with a novel calcium-binding motif [330]. A second calcium-binding site was identified in the N-terminal domain of PilY1, and pull down experiments showed that both sites regulate integrin binding [34]. It was supposed that binding of PilY1 to integrins expressed on the surface of the target cell could allow *P. aeruginosa* to sense attachment and pull itself towards the host.

A recent study [35] shows that association with rigid surfaces induces *P. aeruginosa* virulence toward multiple hosts. Virulence induction depends on the mechanical, but not chemical, properties of the surfaces and requires the surface-exposed protein PilY1 containing N-terminal region homologous to the mechanosensitive von Willebrand factor type A domain. The data are consistent with the mechanochemical model of Persat et al. [36]. According to this model, a

chemosensory system measures the mechanically induced conformational changes in stretched T4P, which leads to a signaling cascade that results in the expression of genes associated with pathogenicity and surface-specific twitching motility.

Luo et al. [32] recently proposed a model that includes pilY1 as a key component in regulation of surface behavior (*viz.*, repressing of swarming motility and induction of biophilm formation), and integrates the protein with chemotaxis Chp system and two low molecular regulators, cAMP and c-di-GMP. According to the model, signaling through the Chp system leads to activation of the adenylate cyclase CyaB, an increase in cAMP and Vfr (virulence factor regulator)-dependent transcription of the minor pilin operon, which includes *pilY1*. The resulting increase in PilY1 surface expression leads to activation of diguanylate cyclase SadC; signal is transduced through the PilMNOP complex. Increased SadC activity leads to increased c-di-GMP level, which downregulates motility and promotes sessility. Interestingly, PilW and PilX also are possible modulators of intracellular c-di-GMP level, which is important for biophilm formation [37].

2.1.2 The pilus assembly machinery

The assembly machinery consists of three subcomplexes implicated in T4aP biogenesis and function activity: (*i*) the cytoplasmic/inner membrane motor subcomplex, (*ii*) the inner membrane/periplasmic space alignment subcomplex and (*iii*) outer membrane secretin subcomplex.

The cytoplasmic/inner membrane motor subcomplex is composed of an integral membrane protein PilC that recruits from the cytoplasm three ATPases, PilBTU [38; 39]. Assembly ATPase PilB powers pilus assembly, retraction ATPases PilT and PilU drive depolymerization of the pilus filament, which results in extension and retraction of the fiber, respectively. PilU in *P. aeruginosa* is unipolar, unlike PilB and PilT that are found at both poles [38]. Without PilU, *P. aeruginosa* may extend and retract pili at both poles simultaneously, resulting in the net-zero motility phenotype characteristic of a *pilU* mutant [40]. Thus, PilU control the direction of motility.

The outer membrane secretin subcomplex is composed of protein PilQ known as secretin, a multimer of 12-14 PilQ subunits, forming a pore through which the pilus is extruded [41], and its pilotin PilF [42; 43. PilF is lipoprotein required for the localization and multimerization of PilQ. PilF interacts directly or indirectly with the PilQ monomer after translocation of both proteins through the inner membrane and acts as a co-chaperone with the Lol system, a lipoprotein sorting mechanism, to facilitate transit of PilQ across the periplasm to the outer membrane [44]. In a *pilF* mutant of *P. aeruginosa*, cell surface piliation and twitching motility were abolished, and the mutant was resistant to infection by T4P retraction-dependent bacteriophage [42].

The secretin and motor subcomplexes are linked by the inner membrane/periplasmic space alignment subcomplex PilMNOP encoded by the highly conserved *pilMNOPQ* gene cluster [45] and potentially, FimV. In the absence of any of PilMNOP proteins, T4P are not expressed on the surface of the bacteria except in a retraction-deficient background [45; 39]. The formation of the complex includes several stages. The type II membrane proteins, PilN and PilO, dimerize via their periplasmic domains and anchor the alignment subcomplex in the inner membrane. PilNO binds PilP, a periplasmic lipoprotein. PilP interacts via its C-terminal domain with the N0 domain of the outer membrane secretin PilQ, and N-terminal cytoplasmic tail of PilN binds to the actin-like protein PilM (nominally cytoplasmic protein, colocalized to the inner membrane) [45]. In accordance with the model proposed by Tammam et al. [46], the PilNOP complex could provide a stable anchor in the inner membrane, while the PilMNOPQ transenvelope complex facilitates transit of the pilus through the periplasm and clamps the pilus in the cell envelope. The PilMN interaction is responsible for communicating signals from the cytoplasmic to periplasmic components of this macromolecular machine. Indeed, transenvelope complex increases local concentrations of PilA monomers (re-deposited into the inner membrane upon disassembly of the pilus fiber [48; 47] for recycling [49]) to promote the efficiency of fiber assembly [46]. Role of FimV in pilus assembly is to promote PilQ secretin multimer formation.

In the absence of the pilin subunit, the levels of membrane-bound components of the inner membrane complex are negatively regulated by the PilR/S two-component transcriptional regulatory system [45]. The system consists of PilS, a cytoplasmic sensor protein, and PilR located in inner membrane. The PilS stimulated by the appropriate environmental signals activates PilR through kinase activity. Under wild-type conditions, PilR/S functions to promote PilA expression and has no impact on the inner membrane complex PilMNOP. When pilA is not expressed, the PilR/S pathway negatively affects the stability of the inner membrane complex by altering the equilibrium between the two dimerization states of PilO, favoring PilO homodimerization and resulting in decreased PilN stability [45].

2.2 Functions of pili: motility, chemotaxis, intercellular (tip – to - tip) association, adherence to biotic/abiotic surfaces and others

T4P are implicated in "twitching motility", a unique form of surface-associated movement whereby the bacteria pull themselves rapidly towards or along a surface by retracting their T4P. Pilus-mediated twitching motility arises from extension and retraction of pili from their site of assembly in the inner membrane. Molecular basis of extension/retraction is assembly/disassembly of fiber. The rate of disassembly is estimated to be between 1,000 and 1,500 subunits per second [47; 49]. Retraction behavior of bacteria was explained by T4P deformation which was

modeled as a compound elastic body consisting of multiple helical strands under axial load [50]. Pilus retraction is capable of generating forces >100pN [48], making the pilus one of the strongest nanomachines currently characterized.

The "twitching" motility mode employed for surface exploration uses T4P as linear actuators to enable directional crawling. T4P-mediated crawling in *P. aeruginosa* consistently alternates between two distinct actions: a translation with constant velocity and a combined translation-rotation that is approximately 20-fold faster in instantaneous velocity; the former is due to multiple T4P, whereas the latter is due to single T4P [51]. The translation-rotation action leads to a fast "slingshot" motion that can turn the cell body efficiently and enables bacteria to move through extracellular polymeric substances that bacteria secrete on surfaces during biofilm formation. In *P. aeruginosa*, vertically oriented walking, by which the bacterium moves with low directional persistence and high instantaneous velocity, is an alternative to horizontally oriented crawling, by which the bacterium moves lengthwise with high directional persistence [52]. The vertical orientation facilitates surface detachment and could influence biofilm morphology [53].

T4P are required for chemotaxis, i.e., preferential movement of bacteria toward or away from specific signals, such as oxygen tension, pH, and specific chemicals that are either attractants or repellents. The two known chemotaxis pathways, a flagella-mediated pathway and a putative pili-mediated system, are reviewed by Sampedro et al. [54].

T4P interactions promote intercellular (tip – to - tip) association and moderate swarming of *P. aeruginosa* [55] that is important for biofilm formation. Though flagellar-mediated swarming of *P. aeruginosa* does not require T4P extension or retraction, a *pilR* and a *pilA* mutant, both deficient in T4P, were unable to swarm [56].

A binding of the bacterium to a cellular or an abiotic substratum is the initial stage of colonization for both initiation of an infection [57] and biofilm formation [58]; at that biofilm formation is a major factor contributing to the chronic infection characteristic of CF patients [59].

The T4P is the major virulence-associated adhesin of *P. aeruginosa.* It accounts for about 90% of the adherence capability of *P. aeruginosa* to human lung pneumocyte A549 cells and is responsible for more than 90% of the virulence in AB.Y/SnJ mice [60]. T4P make an attractive target for the design of novel antimicrobials as bacteria lacking T4P cannot adhere to surfaces and are therefore avirulent.

The binding of T4P to the carbohydrate moiety of the glycosphingolipids (GSL), gangliotriaosylceramide (Gg_3) and gangliotetraosylceramide (Gg_4) [61; 62; 63], also commonly termed asialo-GM2 and asialo-GM1, respectively, has implicated these molecules as receptors for adhesion of *P. aeruginosa* to epithelial cells [64; 65]. A role for pili in attachment to mucins has been excluded by use of nonpiliated mutants [66].

The disaccharide sequence GalNAc-β-D-(1→4)-Gal, common in both asialo-GM1 and asialo-GM2, likely represents the minimal structural receptor motif recognized by T4P of *P. aeruginosa* [67; 62]. The pilus-associated epithelial cell-binding domain is located at the tip of the pilus [61], within residues 128–144 of the C-terminal region of the end molecule PilA [68]. Interestingly, Gg_4 has been reported to be increased in cells containing non-functional CFTR [69; 65] due to reduced sialylation [70], providing an attractive hypothesis for the increased *P. aeruginosa* colonization of respiratory epithelium of CF patients.

More recent studies have provided evidence that allow calling into question the role that Gg_4 might play as a receptor for *P. aeruginosa* [71] and the role of T4P as molecule responsible for bacterial adhesion to human cells [72]. In particular, the investigation of glycolipid-binding specificity of 15 laboratory and clinical *P. aeruginosa* strains [72] showed that none of the tested strains specifically bound GSLs, and that selective depletion of GSLs from target respiratory epithelial cells had no effect on *P. aeruginosa* binding. In contrast to whole piliated *P. aeruginosa*, T4P sheared from such bacteria showed significant Gg_3 and Gg_4 binding, which provide a potential explanation for the results of previous binding studies. Possible explanation is the disassembly of the pilus subunits detached from the bacterium, and differential binding of the numerous small fragments, each of which contains a receptor-binding-tip domain.

The possibility that other molecules, e.g. human corneal epithelial extracellular matrix perlecan [73], human corneal epithelial proteins [74], annexin II [75] and CFTR [76] serve as receptors for T4P has been suggested. New cell receptors for T4P were identified recently [77; 78; 79]. T4P-mediated binding to host N-glycans located at the apical surface of polarized epithelium results in activation of PI3K/Akt pathway (phosphatidylinositol 3-kinase/protein kinase B pathway, a signal transduction pathway that promotes survival and growth in response to extracellular signals and serves pleiotropic functions, such as cell survival, motility and phagocytosis; in partilular, the parthays modulates expression of proinflammatory genes) and bacterial entry [77; 78]. In other study was shown that T4P associated protein PilY1 specifically recognizes still none identified host receptor localized or enriched on basolateral epithelial cell surfaces [79].

In addition to attachment of bacteria to human cells, T4P mediate attachment to artificial surfaces including stainless steel and two widely used plastics, polyvinylchloride and polystyrene [80], which contributes to morbidity of patients with medical implants including catheters [81], prosthetics [82] and stainless steel implants [83]. The same pilin domain is implicated in T4P adhesion to biotic and abiotic surfaces though the affinities are differing by several orders of magnitude [80].

It was shown that T4P are not specifically required for the type III secretion system (T3SS) of *P. aeruginosa* to deliver exoenzymes directly into the eukaryotic cell [84; 85].

Other known functions of T4P are bacteriophage adsorption [86; 87] and DNA uptake [88; 89]. The latter is important for *P. aeruginosa* clearance via neutrophil extracellular traps (NETs). NET formation was for the first time identified in 2004 by Volker B. et al. [90]. In this study, NETs were released by neutrophils upon *in vitro* activation with the pharmacological agent phorbol myristate acetate, interleukin 8 (IL-8) or lipopolysaccharide. NET formation was identified as a third way to kill invading pathogens, complementing the other two neutrophil strategies, namely engulfment of microbes and secretion of anti-microbials. NETs consist of stretches of DNA (which bind pathogens) and globular protein domains (which contain antimicrobial proteins such as neutrophil elastase and cathepsin G) [90]. I*n vivo*, NETs are released during NETosis, a form of pathogen-induced cell death. Although NET formation is an important innate immune mechanism, in certain pathological conditions NETs release damages host tissues [91] and has been linked to several diseases including CF.

2.3 Pili and immune response

T4P pili are found in a variety of different bacteria with conservation of the pilin molecule and are important virulence factors [14]; they have all features that are typical of microbial determinants recognized by innate immune receptors. It was shown [92] that purified pilin of *P. aeruginosa* strain PA103 introduced into mouse macrophages by liposomal delivery activated caspase-1 (that proves inflammasome assembly, see below) and led to secretion of mature IL-1β. The process was not dependent on the host inflammasome NLRC4 and NLRP3 (NLR family, CARD domain containing 4 and 3, respectively) proteins or melanoma 2 (AIM2)-like receptors (ASC), since pilin was introduced into cells by liposomal-mediated transfection. Mutants of strain PA103 lacking pilin did not activate the inflammasome following infection of macrophages with live bacteria. TTSS remained intact in the absence of pili, showing that it was not due to a lack of effector delivery. These observations suggest that pilin is a novel activator of the inflammasome in addition to flagellin [93] and PrgJ protein family (*e.g*., PscI protein of *P. aeruginosa*, the basal body rod component of the T3SS) [94]. It was hypothesized [92] that in patients infected by pseudomonades pilin can activate the inflammasome when it introduced into the cytoplasm of host cells via 'accidental'passage through the TTSS. Indeed, pilin may be delivered into the host cell cytoplasm as result of detachment from bacteria within the phagocytic vacuole.

T4P induce adaptive immune response and have been used for a generation of vaccines against *P. aeruginosa*. As was shown above, an accessory gene *pilO* from *P. aeruginosa* strain 1244 (T4P from group I) encodes a pilin glycosyltransferase that covalently links an O-antigen subunit (of serotype O7) and pilin [18]. The O antigen is the dominant surface polysaccharide of the lipopolysaccharide molecule of most gram-negative bacteria, and consequently, antibodies directed against this cellular component have been shown to protect against infection. The PilO has low glycan specificity. Expression of plasmid-borne pilAO 1244 in nonserotype O7 *P. aeruginosa* strains resulted in pilin glycosylation, in which the glycan consisted of the host's O subunit. This system allows producing glycoconjugate vaccines to provide O-antigen-specific protection, as it was confirmed in two separate infection models [95].

3. Flagella

3.1 Structure, swimming/swarming motilities and chemotaxis

The typical bacterial flagellum of gram negative bacteria (*e.g. P. aeruginosa*) consists of six major elements [96]. The passive (non-motor) structural elements are (*i*) the rod with three rings, MS, L and P, where L ring is in outer membrane, and P ring and MS ring are in peptidoglycan layer and inner membrane, respectively; all elements collectively called the basal body, (*ii*) the hook connecting the filament to the basal body and associated hook–filament junction zones, and (*iii*) the hollow filament composed of about 20,000 identical flagellin subunits, FliC, and the pentameric FliD cap at the distal end of the filament. Elements (*ii*) and (*iii*) are outside of bacterial cell.

The motor consists of (*iv*) a rotor/switch element or C ring, a peripheral membrane structure mounted on the cytoplasmic face of the MS ring, and (*v*) stator elements or Mot complexes that are embedded in the cell membrane and surround the MS and C rings. Rod acts as a driving shaft and L and P rings act as a bushing; the hook acts like a joint between motor and propeller (filament) when the flagella is rotating. Finally, there is (*vi*) the type III flagellar protein export apparatus, which consists in part of integral membrane components located in the center of the MS ring, and soluble or peripheral components such as the ATPase that drives the export process. Bacterial flagellum structure is well presented in two review studies [97; 98].

In the best studied systems, those of *Escherichia coli* and *Salmonella enterica* sv. *Typhimurium*, >50 genes are involved in flagellar biosynthesis and function [96]. The flagellar transcriptional hierarchies are reviewed by Tsang J. and Hoover T.R. [98]. The aflagellate phenotype of *P. aeruginosa* is the result of a single amino acid change (G240V) in the master flagellar regulator, FleQ [99].

The flagellum is assembled in a stepwise fashion [96]. First the basal body is inserted into the membrane, next the hook, finally the filament. Units of flagellin reach the tip through its hollow centre. Proteins needed to allow the flagella to rotate are added late in organelle assembly. The flagellar cap acts as the promoter of flagellin self-assembly [100].

The motor proteins form the stator, which is the ion-conducting channel complex that generates the torque. Until recently it was accepted that the stator is comprised of MotA and MotB proteins and anchored to the cell wall via MotB that possesses a peptidoglycan-binding domain. Tethering is dynamic and there is rapid turnover of MotB in the motor. To generate torque, MotA4B2 complex undergoes a conformational change triggered by the ion flux and interacts directly with FliG, a component of the rotor, via Mot A. Thus, the protons energizing the motor induce conformational changes in the stator that drive movement of the rotor [101].

Current opinion is that *P. aeruginosa* flagellum made of one rotor and two sets of H^+-driven stators [102], PA1460/1461 (MotCD) and PA4954/4953 (MotAB), in accordance with nomenclature of Kuchma et al. [103]. Either MotAB or MotCD rotors can generate torque for swimming (see below). The number and identity of rotors in a motor change dynamically. MotCD, along with the additional motor protein MotY (PA3526), is required for swarming (see below). Under the conditions of elevated c-di-GMP, the MotCD rotors are excluded from the motor since high c-di-GMP levels interfere with proper localization the MotD protein, whereupon swarming motility is greatly impeded by the MotAB stators [104]. The ability of *P. aeruginosa* to control stator composition by c-di-GMP is unique in comparison with other bacteria that modulate a motile-to-sessile lifestyle switch via c-di-GMP, e.g. *Escherichia coli* and *Salmonella enterica*. These bacteria employ a specific c-di-GMP receptor/effector protein, YcgR. Some studies suggest that at elevated c-di-GMP levels YcgR–c-di-GMP interferes with flagellar rotation via binding to the FliG and FliM subunits of the rotor [105], while others suggest interactions with the stator subunit MotA [106].

The flagellum mediates two motility mechanisms: near-surface swimming and surface-anchored spinning, which often precedes detachment from a surface [52]. Indeed, flagellum is implicated in swarming motility [107]. The common characteristics of swarming cells navigating the surface of semisolid media are: (*i*) elongation accompanied by multiflagellation (*P. aeruginosa* have two polar flagella); (*ii*) movement in a coordinated fashion, either en masse at the colony edge or as rafts of migrating cells temporarily leaving the colony behind; and (*iii*) production of extracellular slime consisting of polysaccharide and/or biosurfactant. Cells with mutations in *fliC* and the *las* quorum-sensing system showed diminished swarming behavior, while mutants in genes of *rhl* quorum-sensing system were completely unable to swarm [56]. The *pilR* and *pilA* mutants deficient in T4P were unable to swarm, though swarming of *P. aeruginosa* does not require T4P extension or retraction, and T4P-T4P interactions between cells slow swarm expansion [55].

The bacterial flagellar motor drives rotary motion at speeds reaching 100000 rpm [108].The motor rotates in both counter-clockwise (CCW) and clockwise (CW) directions giving rise to a 'run-and-reverse' trajectory. Additionally, the flagellar motor exhibits multiple speeds in the CCW but not in the CW direction. In the presence of a chemoattractant (serine), the cells alter their run-length, switching frequency and motor speeds in order to move toward favorable environments. Therefore, in chemotaxis, apart from varying the switch frequency, the *P. aeruginosa* flagellar motor has an additional mechanism that allows it to favor the higher rotation speed state [109]. For a review on role of flagellum in chemotaxis, the reader is referred to two reviews [54; 110].

3.2 Flagella in virulence and host invasion, adhesion and biofilm formation

In addition to providing swimming/swarming motilities and chemotaxis, flagella play a central role in virulence and host invasion, adhesion and biofilm formation, and induction of host immune response [111], the points that will be discussed below in details.

P. aeruginosa colonizes surfaces *in vitro* by either biofilm formation or swarming motility. The choice of behaviour is influenced by the physical properties of the surface and specific nutrient availability. In isolates from 237 hospitalized patients without CF, biofilm formation and swarming were negatively associated, while swarming was positively associated with the secretion of both proteases and type 3 exoenzymes [112]. By other words, *P. aeruginosa* exists in host either as invasing or colonizing bacteria, and flagella play a critical role in the acute infection and in the initial stages of chronic infection [113].

The motility-to-biofilm transition, if necessary, likely involves two steps. In the short term, flagella are functionally regulated to either inhibit rotation or modulate the basal flagellar reversal frequency. Over the long term, flagellar gene transcription is inhibited and in the absence of *de novo* synthesis, flagella are diluted to extinction through growth. Both short-term and long-term motility inhibition is likely important to stabilize cell aggregates [114].

It was established long ago that flagella *per se* and flagellar motility are required for full virulence [115], as it was later confirmed in numerous studies with bacteria carrying mutations in flagellar genes. A nonmotile *flgK* mutant (with mutation in gene encoding hook-associated protein 1; HAP1) is defective in surface attachment [116]; mutants in *fliM* (gene encoding one of the flagellar switch proteins) and *cheY* (gene encoding chemotaxis cytoplasmic protein, that interacts directly with the flagellar motor switch to control direction of rotation) are also nonmotile and are unable to form biofilm cap structures on initial biofilm colonies [117]. Additionally, *flgE* and *fliC* mutants (with mutations in genes encoding protein of the hook and flagellin, respectively) make less pyocyanin, a redox-active toxic secondary metabolite that is crucial for lung infection [118; 119.].

Flagella facilitate the bacterial adhesion to mucus and epithelial cells, which confer the ability to colonize and invade throughout the early phases of infection [120]. Two mechanisms of adhesion (specific binding *versus* nonspecific adsorption) find experimental support and can be taken into account.

In numerous studies flagellin (FliC) and flagella cap protein (FliD) of *P. aeruginosa* were detected as interacting with non-identified epithopes of mucin and MUC1 [121; 122; 123]. Later it was shown that flagellum recognizes carbohydrate chains of respiratory mucins. Among these are neutral oligosaccharides and several forms of acidic oligosaccharides derived from the Lewis antigens [124], *viz.*, sialyl-Lewis^X and its derivative sulfo-sialyl-Lewis^X [125]. Sialyl Lewis^X, also known as sialyl Le^X, is a tetrasaccharide carbohydrate NeuAc(alpha2-3)Gal(beta1-4)[Fuc(alpha1-3)]GlcNAc-R that is usually attached to O-glycans on the surface of cells. Sialyl-Lewis^X and sulfo-sialyl- Lewis^X are overexpressed in mucins from CF patients and could be responsible in part for the persistence of lung infection in CF patients.

Same epithelial membrane components were identified as interacting with flagella, *viz*., GM1, GD1a, asialo-GM1 [113], the latter was mentioned above as an adhesin for T4P, and heparan sulfate proteoglycans (HSPGs) [78]. Importantly, HSPGs are inaccessible to bacteria as long as the epithelium is not damaged, since they are located at basolateral membrane of polarized cells. Thus, cell polarity contributes to defense against *P. aeruginosa* [126], and injury predisposes tissue to infection [77]. Flagella-mediated binding to heparan sulfate itself and HSPGs activates the epidermal growth factor receptor (EGFR), adaptor protein Shc, and PI3K/Akt, and induces bacterial entry at the basolateral surface of polarized epithelial cells [78]. It was shown in *in vitro* study [127] that coating the polystyrol wells with collagen and/or hyaluronan significantly increased the biofilm biomass of attached *P. aeruginosa* cells compared to an uncoated control. The data explain the known biofilm forming abilities of *P. aeruginosa* in wounds that easily become chronically infected.

In accordance with the model proposed by Rossez Y et. al. [128], biophysical properties of flagella lend themselves towards nonspecific adhesion and turn flagella into a tool for pathogenic colonisation of a diverse range of niches. Firstly, flagella organelles are long filaments that can reach up to 20 μm from the bacterial cell surface. It can act as probes of uneven surfaces during biofilm formation, attaching within crevices. Individual cells of bacteria effectively become tethered to artificial surfaces that resemble microvilli; a scenario that is very likely to hold true for interactions with biotic surfaces [129]. Secondly, flagella motor can spin filament at speeds in excess of 15,000 rpm, which not only increases the chances of the filament coming into contact with surfaces, but also ensures it does so with force. Thirdly, the flagellum filament is a polymeric structure, comprised of repeating epitopes of one or more flagellin types which give high avidity by definition, since low affinity ionic interactions can be consolidated, amplified, and relevant if the binding substrate is also repetitive. With very few exceptions (innate immune receptors being the most notable), published examples of "specific" flagella binding interactions are with factors that are repetitive, such as polymeric proteins, proteoglycans, glycolipids, and phospholipids. Thus, flagella are a tool for pathogenic colonisation of a diverse range of niches.

3.3 Flagella in induction of host defense

Innate immunity, primarily through inflammatory cytokine production, cellular recruitment, and phagocytic clearance by neutrophils and macrophages, is the key to endogenous control of *P. aeruginosa* infection [130]. Nevertheless, flagellum triggers both adaptive and innate immune response, mainly via variable and conservative parts of flagellin, respectively.

The filament of flagellum consists of 11 protofilaments composed of several thousand flagellin monomers. Flagellin molecule consists of four linearly connected domains labeled D0, D1, D2, and D3; the N-terminal chain starts from D0, going through D1, D2, and reaches D3, and then comes back through D2 and D1, and the C-terminal chain ends in D0. While all three domain connections are formed by pairs of short antiparallel chains, the one that connects domains D0 and D1 is significantly longer than the other two, and therefore it is called the "spoke" region. [131]. The central region of flagellin is variable in sequence, forms two globular domains and is exposed in native flagellin [133; 132]. Sequence variation explains the observed differences in antigenic variation and the adhesive functions of flagellins from different bacterial strains or species. The conserved N- and C-termini of monomeric flagellin are involved in flagella assembly via polymerization of flagellin. Besides, they contain sites recognized as pathogen-associated molecular patterns (PAMP) that trigger innate immune response. These sites are only accessible to immune system in flagellin monomers released during bacterial growth, because the long flagellum tail is easily disrupted [134].

The outermost regions of the polypeptide chain of flagellin are involved in binding to Toll-lile receptor 5 (TLR5) expressed mostly at the basolateral surface of intestinal epithelial cells, monocytes and fibroblasts [135; 136; 137]. Activation of TLR5 signaling leads to expression of proinflammatory cytokines and chemokines in airway epithelial cells [138], which is important for host immune defense. The biophysical and structure-based modeling study on *P. aeruginosa* FliC, the FliC-TLR5 complex, and the filament suggests that D1 domain of FliC is folded into a rod-shaped structure and provides major TLR5-binding sites, while the D2 domain would be exposed to solution and could play an important role in immunogenicity [139].

Sites located closer to the center of the polypeptide chain compared to sites responsible for binding with TLR5 interact with cytoplasmic NOD-like receptors in human cells and induce NLRC4 inflammasome formation [140; 141]. NLRC4 inflammasome (where NLRC4 is subset of the NLR family, previously known as Ice Protease-Activating Factor, IPAF) [142]) is multiprotein oligomer responsible for the activation of the inflammatory response, *viz.,* maturation of cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) [143]. Thus, when N- and C-termini of

monomeric flagellin are exposed, they independently signal the production of cytokines (via TLR5) and drive their maturation via caspase-1 (via NLRC4 inflammasome).

TLR5 cooperates with asialoGM1 and Toll-like receptor 2 (TLR2) in response to flagellin. In study of Adamo et al. [144] *P. aeruginosa* flagella signaling in polarized airway epithelial cells was initiated by interactions with asialoGM1 and TLR2 at the apical surface. By 4 h of exposure to flagella, TLR5 expression was induced, mobilized to the apical surface of the cells, and colocalized with superficial flagella. The IL-8 expression was activated by flagella through induction of Ca^{2+} flux, proto-oncogene tyrosine-protein kinase Src, small GTPases Ras, extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase and nuclear factor-kappaB (NF-kB) activation, a pathway previously associated with asialoGM1-mediated stimuli. Mutations in genes of TLR2 or TLR5 inhibited IL-8 induction by 78% and 35%, respectively, and increased asialoGM1 correlated directly with increased signaling. In other study [145], flagellin can interact with both TLR5 and the cell surface glycolipid, asialoGM1, to activate an innate immune response; interdependence between the asialoGM1 parthway (asialoGM1 ligation, ATP receptor signaling, Ca^{2+} mobilization, and Erk1/2 activation) and TLR5 pathway (that leads to activation of NF-kB) was shown.

Interestingly, in order to prevent immune activation and thus favor survival in the host, bacteria secrete many proteins, e.g. alkaline protease (AprA). The enzyme was identified as a TLR5 signaling inhibitor as evidenced by a marked reduction in IL-8 production and NF-kB activation [146]. Since the cleavage site of AprA is within the conserved domain of flagellin, ArpA does not affect the structure of flagellin in the flagellum but disrupts pathogen-derived monomeric flagellin that results in a more persistent infection.

In was shown that NAIP5 (NLR family, apoptosis inhibitory protein 5) is a universal component of the flagellin-NLRC4 pathway [147]. Direct interaction of flagellin with a single NAIP5 promotes a physical NAIP5-NLRC4 association and leads to formation of a hetero-oligomeric inflammasome [141]. The data of Matusiak et al. [148 Matusiak et al., 2015] prove a biphasic activation mechanism for the NLRC4 inflammasome in which Ser533 phosphorylation prepares NLRC4 for subsequent activation by the flagellin sensor NAIP5; the flagellin D0 domain was required and sufficient for NLRC phosphorylation; NAIP5 engagement by flagellin promoted a physical NAIP5-NLRC4 association.

Flagellin must occur in cytoplasm of host cells to induce inflammasome activation. FliC was identified as a secretion substrate of both the injectisome- and flagellum-associated T3SSs; the first 20 amino-terminal residues of FliC are sufficient for secretion by the injectisome-T3SS and the first 100 residues are sufficient for translocation of FliC into host cells. Indeed, the flagellar cap (FliD), hook-associated (FlgK and FlgL), hook (FlgE), and rod (FlgE) proteins are secretion substrates of the injectisome-T3SS. While a role in inflammasome activation for these proteins has been excluded, these data raise the possibility that the flagellar components conserved between different bacterial species trigger other specific host responses contributing to the pathogenesis of *P. aeruginosa* [149]. Interestingly, in the absence of flagellin, NLRC4 inflammasome may be activated by T3SS itself [150] and NAIP2 protein functioning analogously to NAIP5, serves as a specific inflammasome receptor for T3SS rod proteins [147; 151].

Importantly, both flagella *per se* and its motility are important in induction of an immune response. The flagellar motility is a key PAMP recognized by phagocytes; susceptibility to phagocytosis in swimming bacteria is proportional to *mot* gene function; decreases in flagellar motility result in proportional phagocytic evasion [152]. The mechanism underlying the preferential phagocytic response to motile bacteria was identified by Lovewell et al. [153]. It was shown that PI3K/Akt signaling parthway is activated in response to *P. aeruginosa* flagellar motility and required for bacterial uptake. Moreover, exclusively motile strains of *P. aeruginosa* induce activation of the NLRC4 inflammasome, and loss of bacterial motility leads to reduced inflammasome activation and antibacterial IL-1β host response independently of flagellar expression [142]. It was shown that loss of bacterial motility, consistently observed in clinical isolates from chronic *P. aeruginosa* infections, confers a dramatic resistance to phagocytosis that is independent of both flagellar expression and TLR signaling [154]. In sum, nonmotile strains of *P. aeruginosa* may have an advantage during chronic infection and inhibition of flagellar gene transcription is part of strategy to avoid the host immune response. For example, *P. aeruginosa* growing in mucopurulent human respiratory mucus from CF patients represses the expression of its flagellin to escape bactericidal effect of elastase, an innate immunity effector molecule, produced and secreted into mucus by neutrophils [155].

Interestingly, the host is unable to eradicate flagellated *P. aeruginosa* efficiently, since flagellin induces myeloid-derived suppressor cells [156]. The cells are induced by both purified flagellin and flagellated *P. aeruginosa* and are capable of suppressing polyclonal T cell proliferation and modulating Th17 responses. This mechanism is an alternative to another known method of evading the immune response, namely inhibition of flagellar gene transcription.

On the other hand, *P. aeruginosa fliC* mutants are unable to upregulate transcription of *lasI* and *rhlI* genes which encode enzymes that synthesize quorum sensing homoserine lactones [157]. These quorum sensing molecules regulate production of exoproteases, which degrade human surfactant protein-A (SP-A), that opsonizes and permeabilizes membranes of lung pathogens. In the absence of these exoproteases, the *P. aeruginosa fliC* mutants are cleared from the lung [157]. *P. aeruginosa fliE* and *fliD* mutants (with mutations in genes encoding basal body protein and cap protein, respectively) as well as *flgE* and *fliC* mutants are also attenuated in biosynthesis of lipopolysaccharides, which compromises the integrity of their outer membranes making them more susceptible to SP-A [158]. Thus, a role of flagellum/flagellar motility in induction of an immune response is contradictory.

Since flagellin plays a well-documented role in innate immunity and known as a dominant antigen that induces adaptive immunity, it has been used for a generation of vaccines against *P. aeruginosa* [159], specifically, as a vaccine adjuvant together with poorly immunogenic antigens [160].

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