Published in final edited form as:

Subcell Biochem. 2010 ; 53: 241–253. doi:10.1007/978-90-481-9078-2_11.

The role of *Pseudomonas* lipopolysaccharide in cystic fibrosis airway infection

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Abstract

Pseudomonas aeruginosa (PA) is a ubiquitous environmental Gram-negative bacterium found in soil and water. This opportunistic pathogen can cause infections in individuals with impaired phagocytic function, such as those with burns, exposure to chemotherapy, or cystic fibrosis (CF). PA infects the lungs of most individuals with CF, and is associated with severe progressive pulmonary disease that is the major cause of premature death in this disorder. The specific adaptations of PA to the CF airway responsible for bacterial persistence and antibiotic tolerance are not completely understood but may include increased alginate production (i.e., mucoid phenotype), biofilm formation, and specific lipid A modifications. During adaptation to the CF airway, PA synthesizes a variety of lipid A structures that alter host innate immune responses and promote bacterial persistence and chronic infection. The synthesis of specific lipid A structures is attributable to bacterial enzymes that: (1) remove the 3OH-C10:0 acyl chain from the 3-position (PagL); (2) add a C16:0 acyl chain to the 3OH-C10:0 chain at the 3'-position (PagP); (3) add C12:0 and 2OH-C12:0 acyl chains to the 3OH-C12:0 chains at the 2'- and 2-positions (HtrB and LpxO); and (4) add aminoarabinose to phosphate groups at the 1- and 4'-positions (PmrH, PmrF, PmrI, PmrJ, PmrK, and PmrE). These lipid A modifications represent an essential aspect of PA adaptation to the CF airway.

Keywords

Pseudomonas aeruginosa; cystic fibrosis; lipopolysaccharide; lipid A; chronic infection; antibiotic resistance; innate immunity; inflammation

1. Introduction

In human infection, PA is acquired from environmental reservoirs, and can cause both acute and chronic infections depending on the clinical context (Lyczak et al. 2000). In human hosts with damaged epithelial barriers (e.g., burns) or immune system defects (e.g., post-chemotherapy), acute PA infections of the skin, lungs, urinary tract, or blood can be rapidly fatal. In contrast, in specific clinical conditions such as CF, diffuse panbronchiolitis, HIV infection, and idiopathic bronchiectasis, PA can cause chronic airway infections that persist for months or years. In the setting of long-standing infection, PA isolates from CF patients are phenotypically similar to isolates from other chronic lung infections but distinct from acute infection isolates.

One such distinguishing characteristic of CF isolates is the frequent occurrence of rough or deep rough variants. Such variants represent changes in the structure of lipopolysaccharide (LPS), the major constituent of the outer leaflet of the Gram-negative outer membrane. LPS is

an important pathogenic factor of Gram-negative organisms and consists of three distinct regions: O-antigen, core, and lipid A. Both O-antigen and core consist of polysaccharide chains, whereas lipid A consists of fatty acid and phosphate moieties bonded to a central glucosamine dimer. Rough variants reflect loss of O-antigen, whereas deep rough variants reflect loss of core oligosaccharide. In PA, these changes are associated with a switch from serum resistance to serum sensitivity (Hancock et al. 1983). This switch indicates that the key structural component of PA LPS in chronic infection is its lipid A moiety, the focus of this review.

2. Cystic fibrosis is an important disease of children and young adults

CF is most common lethal autosomal recessive disorder of Caucasians, affecting about 1 in 3200 persons, and is caused by mutation of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (Moskowitz et al. 2008). The CFTR gene encodes a protein kinase A-activated epithelial cell ion channel that is selective for chloride and bicarbonate and also regulates sodium (and potentially other) channels in the plasma membrane. Thus, deficiency of CFTR results in imbalances in epithelial chloride and bicarbonate, as well as additional epithelium- and channel-specific electrolyte abnormalities. While CF affects multiple organ systems, including the gastrointestinal and reproductive tracts and the sweat glands, morbidity and mortality are primarily related to disease of the respiratory tract. In the respiratory tract, CFTR deficiency results in lack of chloride and bicarbonate secretion, and also excessive sodium absorption owing to hyperactivity of the epithelial sodium channel (ENaC) that CFTR regulates. Because the respiratory epithelium is quite water permeable, these ion imbalances result in dehydration (decreased depth) of the airway surface liquid layer that bathes cilia responsible for mucus transport across the epithelial surface (Knowles et al. 2002). These same electrolyte abnormalities also adversely affect mucous glands within the respiratory tract, leading to secretion of respiratory mucus with a higher concentration of mucins and thus greater viscosity than normal (Ballard et al. 1999; Jayaraman et al. 2001). Owing to this combination of thicker airway secretions and impaired mucociliary transport, mucus plaques tend to adhere to the surface of the CF airway and become a nidus of infection.

According to the CF Foundation National Patient Registry, the median age of survival for a person with CF in 2007 was ~37 years (Cystic Fibrosis Foundation 2009). As more advances have been made in the treatment of CF airway disease, the number of adults with CF has steadily grown. As of 2007, more than 45% of the CF population in the US was age 18 and older. This increase in median survival is attributable to advances in nutritional and respiratory management. Nonetheless, pulmonary disease remains the principal cause of morbidity and mortality. Some individuals with CF still die from lung disease during their teenage years, and for those who survive into adulthood, the severity of lung disease and intensity of daily treatments needed to manage it are major determinants of functional status. These treatments include a variety of inhaled and oral medications such as hypertonic saline, mucolytics, and antibiotics. Antibiotic resistance of CF pathogens is a major problem, as is the generally unfavorable pharmacokinetics of most antibiotics with respect to the airway compartment. Clearly, improved therapies are needed that specifically target key steps in the development of CF airway infection and inflammation.

3. Persistent inflammation and chronic infection are the hallmarks of CF pulmonary disease

CF lung disease begins in infancy or early childhood, and is characterized by chronic bacterial infection and severe inflammation that leads to progressive destruction of the lungs. The airways, rather than the lung parenchyma, are the primary sites of inflammation and infection (Davis et al. 1996). CF lung disease is characterized by secretion of copious amounts of thick mucus and migration of a predominantly neutrophilic infiltrate into the airways. This chronic

neutrophilic infiltrate is accompanied by chronic lymphocytic and mononuclear cell infiltrates within adjacent submucosal tissues (Heeckeren et al. 1997). Bronchoalveolar lavage fluid obtained from teenagers and adults with CF show increased levels of the pro-inflammatory cytokines TNF- α , IL-1, and IL-8 and decreased levels of the anti-inflammatory cytokine IL-10 as compared to normal individuals (Bonfield et al. 1995). Nevertheless, these cytokine and neutrophil responses are largely ineffective in eliminating the bacterial airway infection. Most distinctive about CF lung disease is its microbiology. Although *Staphylococcus aureus* is the most common pathogen in the first five years of life, the opportunistic pathogen PA is by far the most important pathogen in severe progressive CF lung disease, infecting the majority of CF patients in later years (Rajan et al. 2002).

4. *P. aeruginosa* is an important pathogen in the CF airway

Epidemiological evidence of the importance of PA in CF lung disease comes from the National Cystic Fibrosis Patient Registry (Cystic Fibrosis Foundation 2009). Analysis of this database revealed that in 2007 PA was cultured from the airways of about 25% of toddlers and 70–80% of adults with CF. Furthermore, a combined serologic and microbiologic clinical study suggested that PA infection occurs at least intermittently in nearly all patients with CF by age 3 years (Burns et al. 2001). In addition, the bacterial load in PA infection tends to be higher than for other pathogens, up to 10^9 cfu/gm of sputum (Tummler et al. 1997). These data suggest that both in terms of numbers of patients affected and intensity of the effect, control of PA infection should be a major goal of efforts to combat CF lung disease.

5. Adaptation of *P. aeruginosa* to the airway is important for CF lung disease

Previous clinical studies have shown that following initial infection with environmental isolates, specific bacterial phenotypes are selected within the CF airway environment. These characteristics are manifested in both children and adults (mean age >9 years) and include loss of flagellar-dependent motility (Mahenthiralingam et al. 1994), loss of O-antigen and other LPS changes (Hancock et al. 1983), increased auxotrophy (Thomas et al. 2000), decreased secretion of virulence factors (Tummler et al. 1997), inability to produce pyocyanin and phage (Romling et al. 1994), antibiotic resistance (Burns et al. 1999), mucoidy (Henry et al. 1992; Boucher et al. 1997), and formation of biofilms (Singh et al. 2000). Infection with mucoid PA has been associated with worse respiratory prognosis (Li et al. 2005). Mucoidy is also observed in several other conditions including bronchiectasis (Pujana et al. 1999) and chronic urinary catheterization (Reid et al. 1992; Goto et al. 1999). Many of these adaptations are observed in PA isolates from the airways of older patients with CF; in contrast, an early adaptation is the synthesis of specific structures of lipid A, the bioactive component of LPS (Ernst et al. 1999).

6. *P. aeruginosa* lipid A structures in CF clinical isolates are distinct from those seen in acute clinical infections and isolates from the environment

The structure of PA lipid A has been elucidated using both mass spectrometry (MS) and NMR techniques (Goldman et al. 1988; Kulshin et al. 1991; Karunaratne et al. 1992). As with lipid A of other Gram-negative organisms, PA lipid A consists of a β -(1',6)-linked diglucosamine backbone with phosphates at the 1 and 4' positions, amide-linked fatty acids at the 2 and 2' positions, and ester-linked fatty acids at the 3 and 3' positions (Figure 1). The chain lengths of the fatty acids attached to the PA lipid A (C12/C10) are generally two carbons shorter than those of *S. typhimurium* and *E. coli* lipid A (C14/C12).

MS analysis of lipid A from laboratory-adapted PA strains grown in rich medium reveals a dominant ion species (m/z) of mass 1447 (Figure 2A), corresponding to a penta-acylated

molecule. This is the major structural form of lipid A isolated from laboratory-adapted PA strains (PAO1, PAK, PA14) grown in rich medium (Bhat et al. 1990; Ernst et al. 1999; Moskowitz et al. 2004). Additional lipid A species observed by MS are dependent on growth conditions (concentration of magnesium or iron, low pH and reduced oxygen levels), and the strain from which the lipid A was isolated (Goldman et al. 1988). In PA strains, penta-acylated lipid A species are usually most abundant; however, specific lipid A structures often differ between CF (m/z 1447, Figure 2A) and non-CF isolates (m/z 1419, Figure 2D).

PA adaptation to CF airways often leads to constitutive expression of lipid A modifications that are regulated in isolates from acute infection (blood, ear, eye, and urinary tract) or environmental isolates. Previously, our laboratory has shown that minimally passaged PA isolated from children with CF younger than 3 years have unique lipid A structures as compared to laboratory-adapted strains, isolates from patients with acute clinical infections (blood, ear, eye, urinary tract), or bronchiectasis, a chronic non-CF airway infection (Ernst et al. 1999). MS analysis of lipid A isolated from greater than 110 minimally-passaged clinical PA isolates, grown under conditions in which laboratory-adapted PA strains do not modify their lipid A (magnesium replete growth medium – 1 mM), demonstrated the addition of palmitate (C16:0) (m/z 1685, Figure 2B), resulting in hexa-acylated lipid A. Interestingly, growth of PA acute infection, bronchiectasis, or laboratory-adapted isolates in magnesium-limited medium resulted in the synthesis of lipid A species with palmitate, indicating that the enzymatic pathways necessary for their synthesis are intact and inducible in these non-CF clinical isolates.

More recent studies have shown that a novel hepta-acylated lipid A (m/z 1855, Figure 2C) is present in a subset of clinical isolates from patients with severe CF pulmonary disease. Formation of hepta-acylated lipid A results from loss of an enzymatic activity that ordinarily deacylates the 3-position of the diglucosamine backbone. Retention of this fatty acid at this position is associated with enhanced resistance to β -lactam antibiotics but not to aminoglycosides.

These results indicated that clinical PA isolates from various infections display marked heterogeneity with respect to the acylation state of their lipid A, reflecting differences in selective pressure that these infections impose on PA lipid A synthesis and structural modifications. Further study of the synthesis and regulation of lipid A modifications that are associated with CF lung disease is needed to understand their role in antibiotic resistance and other adaptations that are relevant to this specialized niche.

7. Synthesis of cystic fibrosis-specific lipid A modifications in *P. aeruginosa*

Based on analyses of lipid A structures synthesized by various PA strains, the following biosynthetic scheme for the generation of CF-associated lipid A molecules from lipid IV_A is proposed (Figure 3) (Ernst et al. 2003). Starting from PA lipid IV_A (Figure 3A), a lauryltransferase, **HtrB2** catalyzes the acyl-oxy-acyl addition of a laurate group (C12:0) to the amide-linked 3OH-C12:0 at the 2'-position (Figure 3B) and a second lauryltransferase, **HtrB1**, combined with the lipid A-specific hydroxylase, **LpxO**, catalyzes the addition of a 2-hydroxylaurate group (2OH-C12:0) to the 3OH-C12:0 at the 2-position (Figure 3C) to generate the hexa-acylated lipid A species, m/z 1617 (Figure 3D). Both of these chromosomally-encoded lauryltransferases are required for the synthesis of the hexa-acylated lipid A species shown in Figure 3D. Hydroxylation of the laurate group (C12:0) that is attached to the amide-linked 3OH-C12:0 at the 2'-position, resulting in lipid A species with two 2-hydroxylaurate groups, may also be observed.

A deacylase, **PagL**, the gene for which has recently been identified (Trent et al. 2001; Geurtsen et al. 2005), catalyzes the removal of 3-hydroxydecanoate (3OH-C10:0) at the 3-position of the hexa-acylated structure to generate a penta-acylated lipid A species, m/z 1447 (Figure 3E)

that is often the predominant lipid A structure in CF isolates. A palmitoyltransferase, **PagP**, can modify this penta-acylated species through the acyl-oxy-acyl addition of palmitate (C16:0) at the 3' position to generate a second hexa-acylated species, m/z 1685 (Figure 3F). Finally, a hepta-acylated lipid A species, m/z 1855 (Figure 3G) is observed in a subset of PA isolates from patients with severe CF pulmonary disease, presumably the result of loss of deacylase activity (**PagL**) in these isolates. The key steps in the synthesis of CF-specific lipid A include the addition of 2-hydroxylaurate (2OH-C12) and palmitate (C16:0), as well as deacylation of the 3-position fatty acid, leading to the synthesis of hexa-acylated lipid A species shown in Figure 3D and 3F, and the penta-acylated lipid A species shown in Figure 3E, respectively.

These enzymatic activities include three acyltransferases (HtrB1, HtrB2, PagP) and a deacylase (PagL). Depending on the specific PA isolate background, these activities can be classified as inducible (lipid A structures only observed under specific growth conditions that induce modification in these isolates), constitutive (lipid A structures always observed under any growth condition in these isolates), or deficient (lipid A structures never observed under any growth condition in these isolates). In CF isolates, lipid A modifications that are constitutively expressed are typically stable after repeated passage under non-inducing growth conditions.

8. *P. aeruginosa* lipid A modifications promote CAP resistance

PA has the genetic capacity to modify the structure of its lipid A as a mechanism for resisting the effects of elements of host innate immunity, such as cationic antimicrobial peptides (CAPs). PA accomplishes this in part through the addition of aminoarabinose to the terminal phosphates of its lipid A (Bhat et al. 1990; Ernst et al. 1999; Moskowitz et al. 2004). PA possesses the two-component regulatory system, PhoPQ (sensor-kinase, PhoQ and phosphorylated transcriptional activator, PhoP). PhoP regulates structural lipid A alterations (addition of palmitate and aminoarabinose) essential for CAP resistance (Ernst et al. 1999; Macfarlane et al. 1999; Macfarlane et al. 2000). The addition of aminoarabinose to PA lipid A is also regulated by a second two-component regulatory system, PmrAB, that contributes to resistance to polymyxins, a family of acylated cyclic CAMPs (McPhee et al. 2003; Moskowitz et al. 2004; Pamp et al. 2008).

Both the PhoPQ and PmrAB systems regulate induction of the *pmrH* operon (McPhee et al. 2003), which encodes enzymes necessary for the synthesis of aminoarabinose as well as its transfer to the 1- and 4'-phosphate groups of lipid A (Figure 4). In PA laboratory-adapted strains, addition of aminoarabinose ($\Delta m/z$ +131) to the penta- and hexa-acylated species (m/z 1447 or m/z 1685) can be observed under appropriate inducing conditions, resulting in lipid A species of m/z 1578 or m/z 1816, respectively.

In polymyxin-resistant clinical isolates, in addition to constitutive aminoarabinosylation, loss of the lipid A modifying enzyme LpxO, an oxygenase required for the synthesis of 2-OH fatty acids (Gibbons et al. 2000), appears to contribute to CAP resistance, as many such isolates exhibit relative or absolute deficiency of 2-hydroxylaurate (Moskowitz et al. 2000). Thus, addition of aminoarabinose to a hexa-acylated species, m/z 1601, a penta-acylated species, m/z 1431, and a hexa-acylated species, m/z 1669 (corresponding to loss of a hydroxyl group from the structures shown in Figure 3D, 3E, and 3F, respectively), is commonly seen in polymyxin-resistant clinical isolates.

Occasional polymyxin-resistant clinical isolates appear to have lost the second lauryltransferase, HtrB1. Lipid A from these isolates does not contain either of the corresponding hexa-acylated lipid A species, m/z 1601 or 1617 (Figure 3D), indicating the presence of only a single laurate (C12:0), presumably in acyl-oxy-acyl linkage to the 2'-position 3OH-C12:0. *Rarely, polymyxin-resistant clinical isolates may lose the deacylase, PagL.* However, as compared to constitutive aminoarabinosylation and deficient 2-hydroxylation of

laurate, these additional changes do not seem to be strongly associated with CAP resistance, as they may also be observed in polymyxin-susceptible CF isolates. Interestingly, in polymyxin-resistant clinical isolates that have lost HtrB1, deacylation of the penta-acylated lipid A species shown in Figure 3B (m/z 1419) can still occur, as well as palmitoylation of both the resulting tetra-acylated species (m/z 1249) and the preceding penta-acylated species, resulting in MS peaks at m/z 1487 and m/z 1657 respectively. This indicates that the later enzymatic steps in CF-specific lipid A synthesis (Figure 3) are not strictly dependent on the earlier steps.

9. *P. aeruginosa* lipid A modifications modulate host inflammatory responses

Persistent lung infections in CF cause the airways to produce a heightened proinflammatory response. Over time, this repetitive cycle progressively damages the lungs. Lipid A is a major stimulator of inflammatory responses through a receptor complex that includes TLR4, the GPI anchored protein CD14, and the secreted protein component MD-2 (Palsson-McDermott et al. 2004; Miller et al. 2005). PA can synthesize a variety of lipid A structures and these structures have varying inflammatory stimulatory properties in human and mouse cells through a specific domain of TLR4 (Hajjar et al. 2002; Ernst et al. 2003). PA synthesizes a more highly-acylated lipid A structure containing palmitate during adaptation to the cystic fibrosis airway that stimulates increased NF- κ B-mediated responses suggesting that the acylation state may affect LPS-mediated responses (Ernst et al. 1999; Hajjar et al. 2002; Ernst et al. 2003). The fact that this adaptation significantly increases the inflammatory properties of the PA lipid A for humans leads to the hypothesis that an initial lack of recognition of non-palmitoylated lipid A may promote colonization in humans. Therefore, understanding the synthesis of lipid A that occurs in CF clinical isolates should provide important information about the complex host-bacterial interactions that take place within the lungs of CF patients infected with PA.

10. Conclusions

PA isolates from CF patients constitutively synthesize lipid A with diverse structural modifications. The synthesis of these structures may make a critical contribution to the pathogenesis of CF lung disease. PA with unique lipid A could contribute to CF lung disease in two ways: by increasing host inflammatory responses, and by increasing bacterial resistance to antibiotics or to elements of host innate immunity such as CAPs. Future investigation of the relevance and regulation of these lipid A structural modifications to CF pulmonary disease will require the construction and testing of PA mutant strains unable to synthesize specific lipid A structures. The components of the bacterial machinery responsible for lipid A structural modifications represent potential drug targets that may enable the development of specific therapies to reduce inflammation and render PA more susceptible to host cell killing.

Acknowledgments

The review was supported by grants from the U.S. National Institutes of Health (NIH) to SMM (R01AI067653) and RKE (R01AI047938).

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Figure 1.
Chemical structure of *P. aeruginosa* lipid IV_A.

Figure 2.

Chemical structure of PA penta-, hexa, and hepta-acylated lipid A from (A and B) CF isolates, (C) a subset of isolates from patients with severe CF pulmonary disease, and (D) acute clinical infection or non-CF bronchiectasis isolates.

Figure 3. Biosynthesis of *P. aeruginosa* lipid A in cystic fibrosis patient isolates
 For all structures, the mass / charge ratio of the singly charged lipid A species is indicated.

A. Operon Structure

B. Biosynthetic Pathway

Figure 4.
Biosynthesis of aminoarabinose-modified lipid A in *P. aeruginosa*.