Anti-PcrV Immunization for *Pseudomonas aeruginosa* Pneumonia in Cystic Fibrosis

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Abstract

Propagation of multidrug-resistant *Pseudomonas aeruginosa,* which causes endemic noso-comial infections, has become a major concern in various parts of the world. In patients with cystic fibrosis, a major cause of death is respiratory tract infections with antibiotic-resistant *P. aeruginosa*. This condition has prompted medical research aimed at develop-ing effective prophylaxis and treatments that do not rely on conventional antimicrobial agents. The pathogenesis that results in cytotoxicity and mortality in immunocompro-mised patients infected with *P. aeruginosa* is associated with the type III secretion sys-tem of this bacterium. Clinical isolates that are cytotoxic and drug-resistant are involved in acute exacerbation of chronic infectious diseases. The *P. aeruginosa* V-antigen PcrV, a *Yersinia* V-antigen LcrV homolog, is involved as an indispensable component in the translocational process of type III secretory (TTS) toxins. Vaccination against PcrV ensures survival of infection-challenged mice and decreases lung inflammation and injury. Furthermore, anti-PcrV IgG can inhibit translocation of TTS toxins. These observa-tions support the hypothesis that anti-PcrV strategies have the potential as nonantibiotic immune strategies for preventing aggravation of *P. aeruginosa* infections in patients with cystic fibrosis.

Keywords: cystic fibrosis, exoenzyme, PcrV, *Pseudomonas aeruginosa,* type III secretion system, V-antigen

1. Introduction

Propagation of multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) has become a serious concern in various parts of the world [1–4]. Recent outbreaks of extensively drug-resistant

P. aeruginosa (XDR-PA) are threatening to increase XDR-PA colonization of immunocompromised and artificially ventilated patients because efficacious antimicrobial choices against XDR-PA are limited [3–5]. In patients with cystic fibrosis (CF), a major cause of death is respiratory tract infections with *P. aeruginosa*. This condition has led to medical research on the development of effective prophylaxis and treatments that do not rely on conventional antimicrobial agents [6]. Furthermore, recent reports have suggested that XDR-PA strains appear to have a greater ability to promote bacteremia and sepsis [7]. Therefore, the pathogenic mechanisms that are responsible for this infection are important for protecting patients from the lethal consequences of these infections.

The pathogenesis responsible for mortality in *P. aeruginosa* pneumonia is associated with the development of septic shock and multiple organ failure. This is because certain *P. aeruginosa* strains have the ability to cause necrosis of the lung epithelium and disseminate into the circulation [8]. Advances in genomic analysis and cellular microbiology have shown that damage to the lung epithelium is associated with expression of toxins. These toxins are directly translocated into eukaryotic cells through the type III secretion system (TTSS) of *P. aeruginosa* [9]. Unlike classic type I and type II secretion systems, the newly identified TTSS, through which bacteria directly transfer their toxins from the bacterial cytosol to the eukaryotic cell cytosol, were discovered in most pathogenic Gram-negative bacteria (**Figure 1**) [10]. Four type III secretory (TTS) toxins, called ExoS, ExoT, ExoU, and ExoY, have been identified in *P. aeruginosa* [11, 12]. ExoS is the 49-kDa form of exoenzyme S, which is a bifunctional toxin that has ADP-ribosyltransferase and GTPase-activating protein (GAP) activities [13]. ExoS disrupts endocytosis,
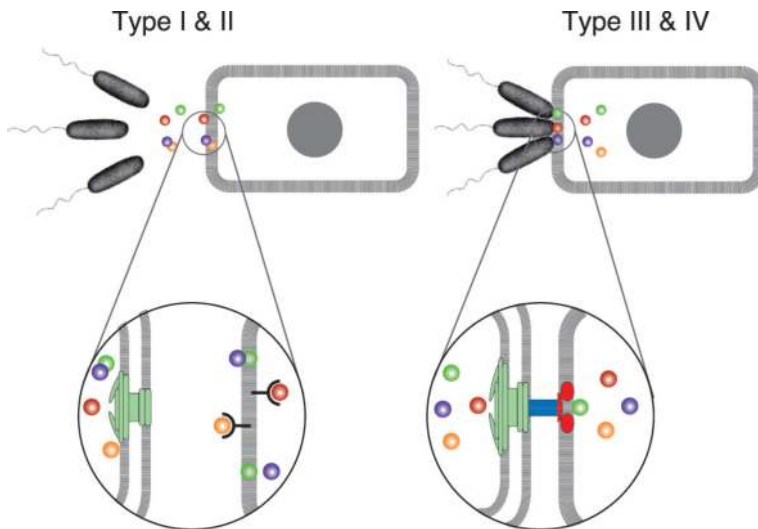

Figure 1. Toxin secretion systems in Gram-negative bacteria. In the types I and II secretion systems, bacteria secrete toxins into the extracellular space (left side of figure). As one example, secreted toxins are captured by surface receptors on the eukaryotic cell membrane and are then transferred to the cytosol. In the types III and IV secretion systems, bacteria secrete toxins directly into the cytosol of target eukaryotic cells through their secretion apparatus (right side of the figure). The mechanism whereby the secreted toxins are transferred to the eukaryotic cell cytosol is called translocation.

the actin cytoskeleton, and cellular proliferation. ExoT, which is a 53-kDa form of exoenzyme S with 75% sequence homology to ExoS, also exerts GAP activity and interferes with cell morphology and motility [13–15]. ExoY is a nucleotidyl cyclase that increases intracellular levels of cyclic adenosine and guanosine monophosphates, resulting in the formation of edema [12]. ExoU exhibits phospholipase A_2 activity activated by host cell ubiquitination after translocation. ExoU is a major pathogenic cytotoxin that causes alveolar epithelial injury and necrosis of macrophages [16–19].

In this review, we summarize the TTSS of *P. aeruginosa* and its association with CF. We also review the development of immune therapies, including passive and active immunization against the TTSS-associated virulence of *P. aeruginosa*.

2. Cytotoxic or invasive *P. aeruginosa* strains

In most acute clinical manifestations of *P. aeruginosa* infection, severe pneumonia occurs in patients under ventilatory management and in immunocompromised patients [2, 3]. Patients with severe *P. aeruginosa* pneumonia frequently develop sepsis and subsequent multiorgan failure [7].

In the mid-1990s, researchers investigating *P. aeruginosa* reported that the strains expressing ExoS (49-kDa type exoenzyme S) had low cytotoxicity to eukaryotic cells, whereas the strains that did not express ExoS had strong cytotoxicity [20, 21]. Therefore, at that time, *P. aeruginosa* strains were classified as either a cytotoxic *exoS*⁻ type or an invasive *exoS*⁺ type, depending on the genotypes of the 49-kDa exoenzyme S [20]. The 53-kDa exoenzyme S (ExoT) was discovered as a gene product that was distinct from ExoS [14, 15]. However, because cytotoxic *exoS*⁻ type- and invasive *exoS*⁺ type-strains both possessed ExoT, the mechanism for cytotoxic virulence could not be explained solely on the basis of possessing the ExoT gene. Therefore, the mechanism whereby the cytotoxic characteristics of *exoS*⁻*exoT*⁺ type-strains develop is unknown. In 1997, a new cytotoxin, ExoU, was identified [16]. *P. aeruginosa* strains that do not have *exoU* always possess *exoS*. Therefore, the genotype of cytotoxic strains is *exoS*⁻*exoT*⁺*exoU*⁺ and the genotype of invasive strains is *exoS*⁺*exoT*⁺*exoU*⁻ [20, 21]. While searching for major cytotoxins in *P. aeruginosa*, *P. aeruginosa* exoenzymes were found to translocate directly into the eukaryotic cytosol through the TTS mechanism [22]. Among the four TTS cytotoxins, ExoS, ExoT, ExoU, and ExoY, *P. aeruginosa* clinical isolates that cause severe sepsis and mortality express TTS ExoU [16, 23–25]. After discovery of ExoU, studies showed the enzymatic action of phospholipase A_2, which is activated by a eukaryotic cell factor after translocation into the eukaryotic cell cytosol [17–19].

3. Genomic analyses of *P. aeruginosa* strains

The whole genome sequencing project by the *Pseudomonas* Genome Project was completed for the *P. aeruginosa* strain PAO1 in 2000 [26]. Since this time, comparative genomics is currently

on-going between the PAO1 reference strain and various clinical isolates of which the characteristics differ from PAO1 (**Figure 2**). In this research field, the major question is how much do the strains differ from each other at the gene level, especially between strains isolated from patients with acute infections and strains isolated from chronically infected patients with CF. The genetic mechanisms underlying multidrug resistance are also of major interest in these comparative genomic studies.

The clinical isolate UCBPP-PA14 is cytotoxic and is similar to the laboratory strain PA103 UCBPP-PA14 that has the TTS phenotype ExoS$^-$ExoT$^+$ExoU$^+$ExoY$^-$, whereas invasive PAO1 is ExoS$^+$ExoT$^+$ExoU$^-$ExoY$^+$ [16, 20]. Therefore, genomic analysis of UCBPP-PA14 was conducted to identify their phenotypic differences. As a result, two pathogenicity islands that do not exist in PAO1 were discovered in the UCBPP-PA14 genome [27]. Thereafter, researchers found that clinical strains containing *P. aeruginosa* pathogenicity island-2 (PAPI-2) and *exoU* display the cytotoxic phenotypes that are responsible for acute lung injury in animal models [16, 27]. Concurrently, strains harboring PAPI-2 and *exoU* were found to have deletional mutation of the *exoS* gene, which creates the *exoS*$^-$ genotype in PA103 and UCBPP-PA14 [27].

Figure 2. The PAO1 reference strain genome and its pathogenic gene configuration. The *P. aeruginosa* strain PAO1 possesses 5570 genes in its 6.3-Mb circular genome. The exoenzyme S regulon is a gene cluster (25.7 kb) for type III secretion. Type III secretory toxin genes, such as *exoS*, *exoT*, and *exoY*, are scattered throughout the genome. The *P. aeruginosa exoU* gene, which is located in an insertional pathogenic gene cluster in pathogenicity island-2 (PAPI-2), was discovered in the UCBPP-PA14 virulent clinical strain.

4. The type III secretion system of *P. aeruginosa*

4.1. Components of the type III secretion system and the exoenzyme S regulon

The TTSS is composed of the following: (1) secretion apparatus (injectisome), (2) translocators, (3) a set of secreted toxins, and (4) regulatory components [28]. In the *P. aeruginosa* genome, a pathogenic gene cluster called the exoenzyme S regulon encodes the genes for regulation, secretion, and translocation of the TTSS [9, 11] (**Figure 3**). In the exoenzyme S regulon, the *exsCBA* operon encodes the transcriptional activator protein ExsA, which regulates expression of exoenzyme S and its co-regulated proteins (**Figure 4**) [11]. Four TTS toxins, ExoS, ExoT, ExoU, and ExoY were identified in *P. aeruginosa* (**Table 1**) [11, 12, 16]. The genes for these TTS toxins are scattered in the genome [26]. In contrast, *exoU*, which is located in the insertional gene pathogenic cluster PAPI-2, is present with its chaperone protein gene *spcU* only in the genomes of cytotoxic strains, such as PA103 and UCBPP-PA14 [16, 27, 29].

4.2. The *pcrGVHpopBD* translocation operon

One operon in the exoenzyme S regulon, called *pcrGVHpopBD*, encodes five proteins associated with translocational processes for TTS toxins [11]. These proteins are PcrV, PopB, and PopD, and their chaperones are PcrG and PcrH. PcrV, which is the *P. aeruginosa* V-antigen, is a cap structure component on the tip of the injection needle formed by PscF in the secretion apparatus [30] (**Figure 5**). The genetic organization for the exoenzyme S regulon shares
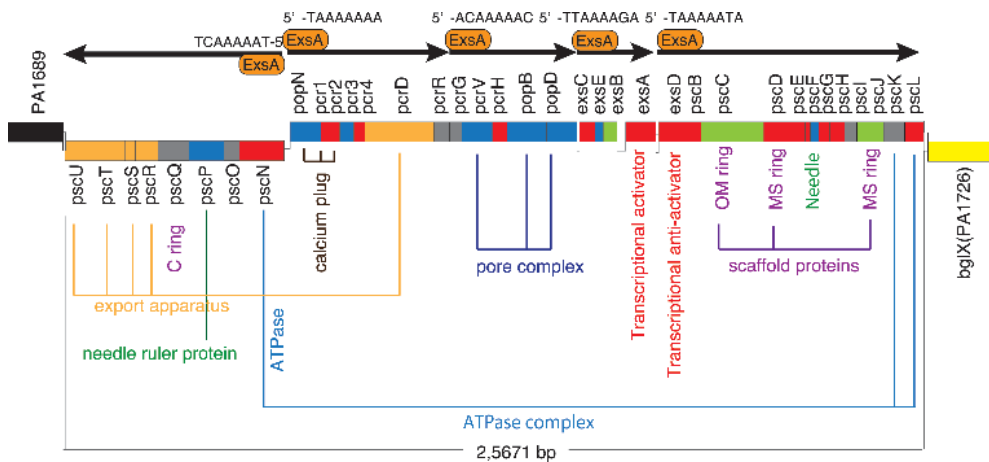

Figure 3. Genomic structure of the exoenzyme S regulon. The type III secretion regulatory region (25.5 kb), found as a gene cluster, was named the exoenzyme S regulon. The exoenzyme S regulon comprises five operons, including 36 genes for transcription (*exsA-exsD*), genes encoding the secretion apparatus (*pscB-pscU*), and others encoding translocation-related proteins (*pcrGVHpopBD*). The *exsCBA* operon encodes the transcriptional activator ExsA protein, which regulates expression of exoenzyme S and co-regulated proteins.
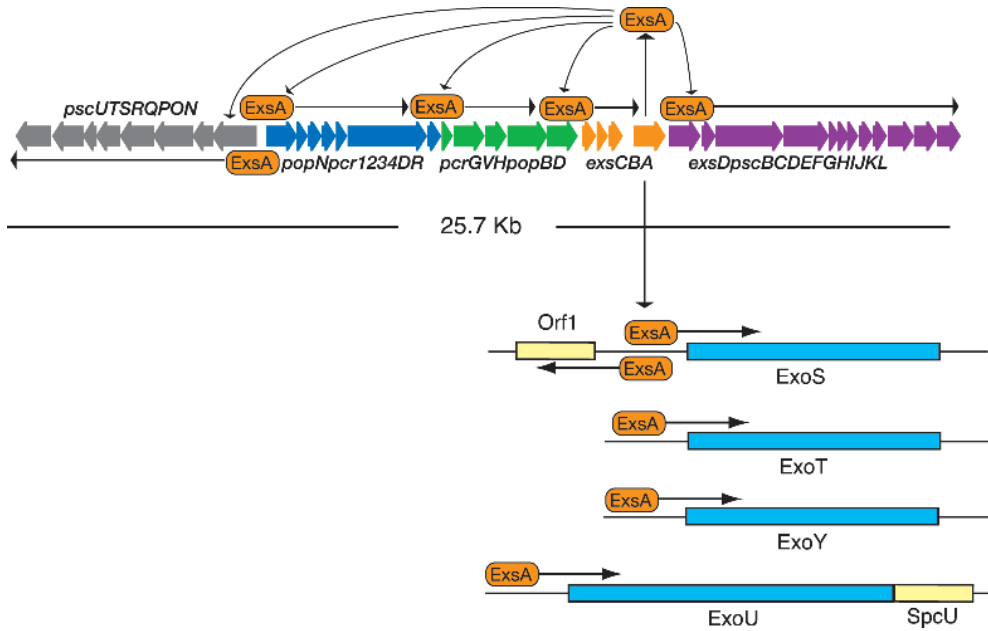
Figure 4. Type III secretion regulation in *P. aeruginosa* by the ExsA transcriptional activator. The ExsA transcriptional activator protein activates five operons in the exoenzyme S regulon via five ExsA binding motifs "TxAAAAxA". ExoT, ExoU, and ExoY were identified in *P. aeruginosa*. Genes for ExoS, ExoT, and ExoY are scattered in the genome. The *orf1* gene, which encodes a chaperone protein for ExoS, is next to *exoS*. The *exoU* gene, which is located in the insertional gene pathogenic cluster PAPI-2, is present with its chaperone protein gene *spcU* only in the genomes of cytotoxic strains, such as PA103 and UCBPP-PA14.

Toxins	Size (kDa)	Enzymatic activity	Action
ExoS	49	FAS-dependent ADP-ribosyltransferase	Antiphagocytosis, inhibition of endocytosis
ExoT	53	Small GTPase activating protein activity	Inhibition of tissue repair
ExoU	74	Phospholipase A2	Cytotoxin, lipid degradation
ExoY	42	Adenylate cyclase	Edema formation, anti-inflammatory

Table 1. Type III secretory toxins in P. aeruginosa.

the most homology with the *Yersinia* Yop virulon [31]. *Yersinia* LcrV has been reported to be a molecular target competing with TTSS virulence. Similarly, *P. aeruginosa* PcrV also is a molecular target that can compete with TTSS virulence in *P. aeruginosa* [32, 33]. This competition with TTSS virulence will be discussed later in this review.
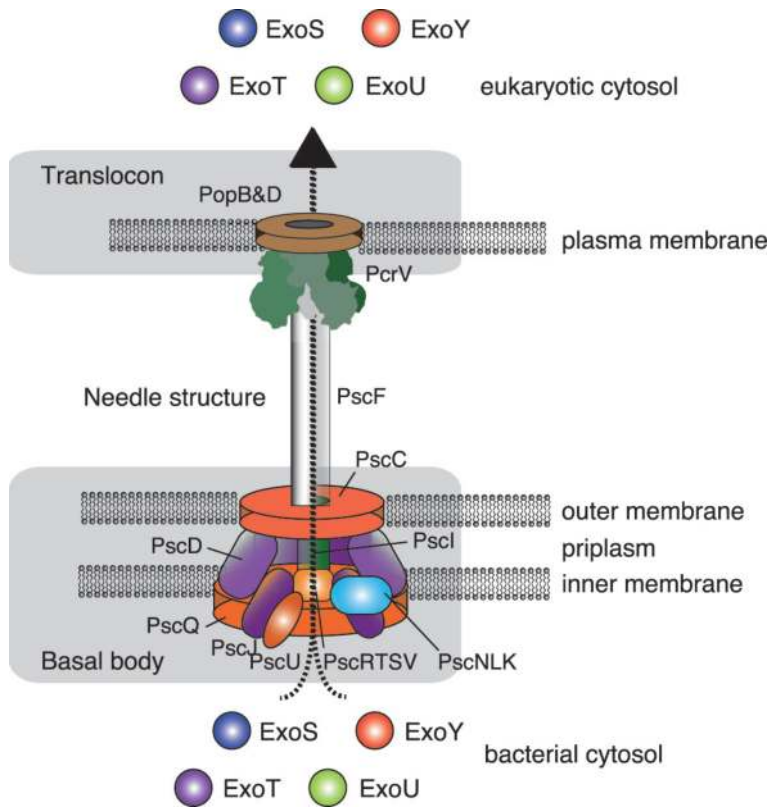
Figure 5. The type III secretory apparatus of *P. aeruginosa*. The *P. aeruginosa* type III secretory apparatus comprises many protein components as follows: a cap component, PcrV; a needle component, PscF; an outer ring component, PscC; and basal components, including PscJ, ATPase PscN, and others. Four type III secretory toxins, ExoS, ExoT, ExoU, and ExoY, are injected directly into the cytosol of target eukaryotic cells through the type III secretory apparatus. Translocated toxins are activated by specific eukaryotic cell cofactors. Following activation, ADP-ribosyltransferase activity is shown by ExoS, whereas ADP-ribosyltransferase and GTPase-activating protein activity is shown by ExoT. Activated ExoU has phospholipase A$_2$ activity, and ExoY exhibits adenylate cyclase activity.

4.3. *Yersinia* V-antigen LcrV and *P. aeruginosa* PcrV

Historically, *Yersinia* LcrV has been referred to as the *Yersinia* V-antigen. Approximately 50 years ago in the UK, Burrows et al. reported that *Yersinia* V-antigen was an antigen substance associated with the pathogenic toxicity [34–38] of this bacterium. They found that only *Y. pestis*, with the antigenic factor they called the V-antigen, induced immunity in a mouse model of infection [34–38]. In 1986, the gene encoding the LcrV V-antigen, *lcrV*, was cloned from the Low-calcium response (LCR) operon of the *Y. pestis* pCD1 plasmid [39]. A genetic mutation experiment then showed that LcrV is essential for translocation of the toxin [32]. Additionally, antibodies against LcrV were reported to be capable of blocking transfer of the toxin [32]. As well as *Yersinia* LcrV in the TTSS, *P. aeruginosa* PcrV is essential for transition of the TTSS toxin, and the antibody against PcrV can block transition of the TTS toxin [33]. PcrV might play a role

in connecting the needle rod (composed of PscF) to the pore (formed with PopB/PopD) on the eukaryotic cell membrane. Indeed, electron microscopy has successfully visualized V-antigens as cap structures on the mushroom needle tip portion of the protein [30]. Specific blocking antibodies against the V-antigen also block translocated toxin from binding to the top part of the cap structure [32, 40].

5. *P. aeruginosa* type III secretory toxins

5.1. ExoS and ExoT

In the late 1970s, *P. aeruginosa* exoenzyme S was discovered as an adenosine diphosphate ribosyltransferase that was distinct from exotoxin A [41, 42]. However, in the mid-1990s, exoenzyme S activity was determined to be the result of two highly homologous toxins, termed ExoS (49-kDa exoenzyme S) and ExoT (53-kDa exoenzyme S), which are encoded in two separate portions of the *P. aeruginosa* genome [14, 15]. ExoS and ExoT were also found to be secreted by the TTS mechanism [15, 22].

5.1.1. ADP-ribosyltransferase activity

ExoS and ExoT are two immunologically indistinguishable proteins that co-fractionate with exoenzyme S activity [14]. ExoS and ExoT encode proteins of 457 and 453 amino acids, respectively, and share 75% amino acid identity. ExoT possesses approximately 0.2% of the ADP-ribosyltransferase activity of ExoS [14, 15]. ExoT diminishes motility of macrophages and phagocytosis, at least in part through disrupting the eukaryotic cellular actin cytoskeleton, and also blocks wound healing [43, 44]. The ExoS carboxyl terminal catalyzes transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide to a number of different proteins, including the intermediate filament protein, vimentin [45–47].

5.1.2. GTPase-activating protein activity

The amino terminal domains of ExoS and ExoT have been characterized as GAPs of Rho GTPases [48]. The Rho GAP activity of ExoS stimulates reorganization of the actin cytoskeleton by inhibiting Rac and Cdc42, and induces formation of actin stress fibers by inhibiting Rho [49]. These domains, which include catalytic arginines, share sequence homology with not only *Yersinia* YopE and *Salmonella* SptP, but also with mammalian Rho GAP proteins, such as hsp-120GAP, hsNF1, dmGAP1, and sclRA1. Biochemical studies have shown that ExoT possesses GAP activity for RhoA, Rac1, and Cdc42, and interferes with Rho signal transduction pathways, which regulate actin organization, exocytosis, cell cycle progression, and phagocytosis [50, 51].

5.2. ExoU

A specific isogenic mutant of the cytotoxic *P. aeruginosa* strain PA103, which does not have ExoS and is genetically modified to lack ExoT, is still cytotoxic *in vitro.* This mutant causes epithelial injury *in vivo*, indicating that another cytotoxin is responsible for the observed pathology [16, 17]. In 1997, ExsA-activated ExoU was discovered to be a major virulence factor causing lung injury and the *exoU* gene was cloned from the cytotoxic strain PA103 [16]. A region downstream

of *exoU* encodes a small 15-kDa protein named SpcU, which functions as a chaperone for ExoU [29]. ExoU is a TTS protein of *P. aeruginosa* that is necessary for epithelial cell cytotoxicity *in vitro* and virulence in a mouse model of pneumonia [16].

5.2.1. Patatin-like phospholipase A_2 activity

ExoU contains a potato patatin-like phospholipase A (PLA) domain [17]. Patatin is a member of a multigene family of vacuolar storage glycoproteins with lipid acyl hydrolase and acyl transferase activities. Alignment of ExoU, potato patatin, and human PLA2 shows three highly conserved regions in the ExoU amino acid sequence as follows [17]: (1) a glycine-rich nucleotide binding motif, GXGXXG/A (position 111–116 in ExoU); (2) a serine-hydrolase motif, which includes a serine active site for cPLA2, GXSXG/S (position 140–144 in ExoU); and (3) an active site motif containing aspartate for cPLA2, DGG/A (position 344–347 in ExoU) (**Figure 6**).
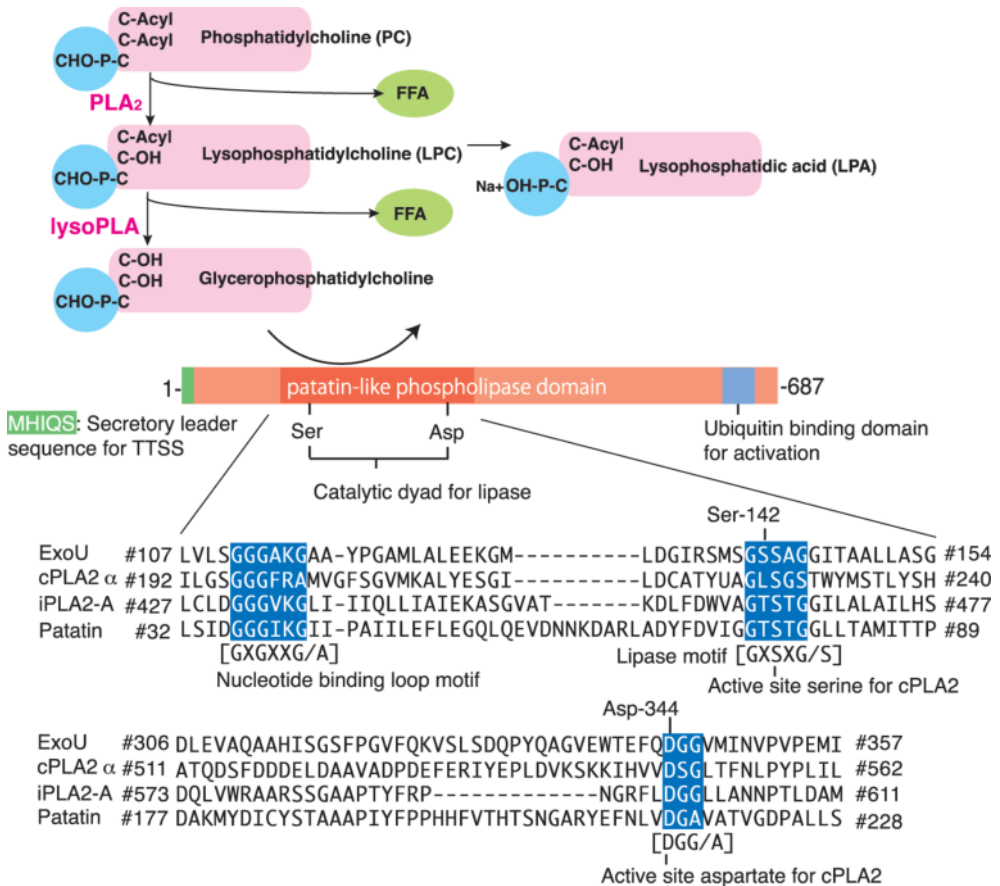
Figure 6. Enzymatic activity and consensus motifs in ExoU. *P. aeruginosa* ExoU, a major factor causing cytotoxicity and epithelial injury in the lungs, contains a patatin domain that catalyzes membrane phospholipids through its phospholipase A_2 activity. Homology in the amino acid sequence, with a catalytic dyad in the primary structure, is found among patatin, mammalian phospholipase A_2 (cPLA$_2$-α and iPLA$_2$), and ExoU. FFA: free fatty acids.

5.2.2. Phospholipase A$_2$ activity and acute lung injury

Site-directional mutations in the predicted catalytic site of ExoU cause a loss of lysophospholipase A activity [52]. Airspace instillation of virulent *P. aeruginosa* expressing ExoU causes acute lung injury and death in infected mice [53]. However, airspace instillation of isogenic mutants secreting catalytically inactive ExoU is non-cytotoxic and this does not cause acute lung injury or death in these mice [53]. Therefore, virulent *P. aeruginosa* causes acute lung injury, with concomitant sepsis and mortality, via cytotoxic activity derived from the patatin-like phospholipase domain of ExoU. Cells targeted by ExoU through the TTSS are not only epithelial cells, but also macrophages [54]. Through the TTSS, ExoU is activated after its translocation into the cytosol of eukaryotic cells [55–57]. Ubiquitin and ubiquitin-modified proteins are associated with the activation of ExoU [18, 19].

5.3. ExoY

ExoY has adenylate cyclase activity and is secreted by the TTS mechanism [12]. The primary ExoY sequence shares homology with sequences of the extracellular adenylate cyclases of *Bordetella pertussis* (CyaA), *Bacillus anthracis* (EF), and *Y. pestis* insecticidal toxin [12]. An unknown eukaryotic cell factor, distinct from calmodulin, enhances recombinant ExoY catalysis. Infection of eukaryotic cells with *P. aeruginosa* that produce catalytically active ExoY results in an elevation of intracellular cAMP and morphological changes in cells. ExoY increases the permeability of lung endothelial cells and alters Chinese hamster ovary cell morphology but does not result in acute cytotoxic responses. Ninety percent of clinical isolates that are tested show the presence of the *exoY* gene in DNA hybridization experiments [12]. ExoY production may play a role in protecting the bacterium from local phagocytic cells [58].

6. Cystic fibrosis and *P. aeruginosa* type III secretion

6.1. *P. aeruginosa* pneumonia and cystic fibrosis

Respiratory infections with *P. aeruginosa* are the major causes of morbidity and mortality in individuals with CF. *P. aeruginosa* isolates from newly infected patients with CF resemble those from acutely infected non-CF patients, and have a number of virulence factors including flagella, pili, pyocin, pyoverdin, and the TTSS [59, 60]. Expression of these virulence factors is considered to be essential for successful development of infection at an early stage of infection in patients with CF. However, at the chronic stage of infection, triggered by high selective pressure in CF lungs and by antibiotic treatments, *P. aeruginosa* gradually generates genotypes and phenotypes that are specially adapted to the lungs in CF. These include overproduction of alginate (mucoid phenotype), loss of lipopolysaccharide O-antigen components, loss of motility, resistance to antibiotics, virulence factor loss, and adapted metabolism [61]. These changes might be essential for *P. aeruginosa* to facilitate evasion of the host defense mechanisms and immune surveillance [62].

6.2. Epidemiological studies of isolates from patients with cystic fibrosis

In our epidemiological study that analyzed clinical isolates, there was a subset of isolates that displayed the TTS phenotype ExoS$^-$ExoU$^-$ with extensive drug-resistant characteristics [63]. Most of these isolates were from chronic infections in patients with CF. Therefore, clinical isolates of *P. aeruginosa* are classified into three subgroups depending on their ExoS and ExoU phenotypes. ExoS$^+$ExoU$^-$ strains are invasive and cause infections in burns tissues, whereas ExoS$^-$ExoU$^+$ strains are cytotoxic and cause acute pneumonia and sepsis. Most strains isolated from chronic infections in CF patients are ExoS$^-$ExoU$^-$ (**Figure 7**). *P. aeruginosa* strains that are isolated from acutely infected patients show positive phenotypes for TTS proteins (ExoS, ExoU, and PcrV) and the positive O-antigen phenotype. However, strains that are isolated from chronic infections of patients with CF are frequently the O-antigen phenotype (−), TTS protein phenotypes (−), and the mucoid phenotype (+) with increased antibiotic resistance (**Figure 8**). Recent studies have shown that TTSS production, as well as other virulence factors, such as flagella, pili, pyocin, and pyoverdine, are attenuated in many isolates from chronically infected patients with CF [64–66]. The results of several studies that investigated the relationship between CF clinical isolates and the TTSS in *P. aeruginosa* are shown in **Table 2** [61, 67–71]. Two of the six studies were longitudinal and followed the same patients with CF. Additionally, four studies performed genotype analysis on strains, and five others performed immunoblot analysis of TTS proteins. Findings from these epidemiological studies suggest that CF isolates from children are more virulent with a positive TTSS phenotype than isolates that are recovered from adults. These studies also suggest that isolates from initial infections are more virulent than isolates from subsequent infections. The ratio between ExoS$^+$ExoU$^-$ and ExoS$^-$ExoU$^+$ differed in each study. However, a more recent report from Hu et al. showed that 7 isolates among 40 in total from subsequently occurring infections were ExoU$^+$ [71]. These findings suggest the potential pathogenic involvement of ExoU-associated virulence, even in patients with CF.
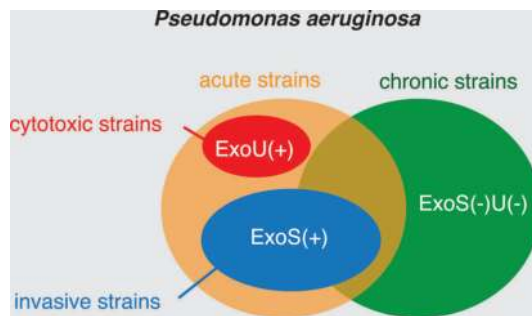

Figure 7. Type III secretory toxin phenotypes in *P. aeruginosa* clinical isolates. *P. aeruginosa* clinical isolates can be classified into three subgroups depending on their ExoS and ExoU phenotypes. ExoS(+)ExoU(−) stains are invasive and cause infections in burnt tissue, whereas ExoS(−)ExoU(+) strains are cytotoxic and cause acute pneumonia and sepsis. Most strains that are isolated from chronic infections in patients with cystic fibrosis are ExoS(−)ExoU(−).
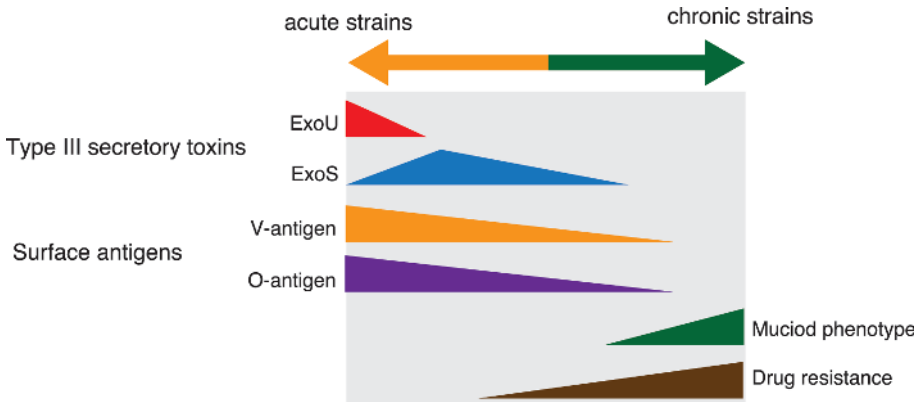
Figure 8. Phenotypic variation in *P. aeruginosa* clinical isolates. *P. aeruginosa* strains that are isolated from acutely infected patients are positive for type III secretory proteins, such as ExoS, ExoU, and PcrV, and are O-antigen positive. In contrast, strains that are isolated from chronic infections in patients with cystic fibrosis are frequently O-antigen-negative and type III secretory protein-negative, but are mucoid with increased antibiotic resistance.

6.3. Type III secretion and cystic fibrosis isolates

Most studies have reported that the proportion of *P. aeruginosa* strains secreting TTS proteins decreases over the duration of *P. aeruginosa* infection. Jain et al. showed a significant inverse correlation between the percentage of TTS proteins and the duration of *P. aeruginosa* infection [67]. They also reported an association between the proportion of TTS protein-secreting isolates and a decline in the rate of forced expiratory volume in 1 s in patients who still harbor at least some TTS-positive isolates. Other reports that investigated the genotype and phenotype of the TTSS showed that all *P. aeruginosa* strains harbor at least some TTSS genes (*exoS, exoT, exoU, exoY*), regardless of the expression of TTS proteins (ExoS, ExoT, ExoU) [66, 69–71]. This suggests that the TTSS regulon may remain intact and the expression of TTSS can be reversible. There are other variants called rough small-colony variants in *P. aeruginosa*, and these have been isolated from chronically infected patients with CF [72, 73]. These variants are hyperpiliated and hyperadherent, and differ from the mucoid phenotype in their secretion of TTS proteins. Their remarkably high resistance to several antibiotic classes enables their persistence in the lungs in CF.

6.4. Comparative genome studies on recent *P. aeruginosa* isolates

Comparative genomics on the reference PAO1 strain and isolates from patients with CF are on-going. In 2003, two comparative studies between CF isolates and PAO1 were reported. These studies demonstrated that clinical strains do not express TTSS, whereas most of them that are isolated from chronic infections possess this gene cluster [74, 75]. Additionally, these studies show that 10% of genes in CF isolates do not exist in the PAO1 genome, and half of them are newly identified genes.

Year	Author	Reference	CF clinical isolates, n	TTSS secretion (+), n (%)	Exoenzyme genotype, n				Exoenzyme phenotype, n (% positive by genotype)			
					S	T	Y	U	S	T	Y	U
2000	Dacheux et al.	[69]	29	8 (27.5%)	28	28	28	3	8 (28.6)	8 (28.6)	NA	0 (0)
2004	Jain et al.	[61]	235 (CI, children)	40 (18%)	NA	NA	NA	NA	35 (88)	40 (100)	NA	2 (5)
			200 (CI, adults)	8 (4%)					8 (100)	8 (100)	NA	0 (0)
			35 (NI)	17(49%)					17 (100)	17 (100)	NA	0 (0)
2005	Lee et al.	[68]	7	5 (71.4%) (initial)	7	NA	NA	0	5 (71.4)	NA	NA	0 (0)
				3 (42.8%) (subsequent)	6	NA	NA	1	2 (28.6)	NA	NA	1 (14.3)
2007	Wareham et al.	[70]	75	39 (52%)	66	75	74	12	NA	NA	NA	9 (75)
2008	Jain et al.	[67]	1299 (CI, children)	29.1%	NA	NA	NA	NA	NA	NA	NA	NA
			1217 (CI, adult)	11.5%								
			135 (NI)	45.2%								
2013	Hu et al.	[71]	52 (initial)	43 (82.7%)	44	NA	NA	8	36 (82)	NA	NA	7 (88)
			40 (subsequent)	26 (65%)	33	NA	NA	7	19 (58)	NA	NA	7 (100)

Table 2. Studies on the relationship between cystic fibrosis and type III secretion in *P. aeruginosa*.

Recent reports have indicated that the combination of carbapenem and fluoroquinolone resistance and the presence of the gene encoding the TTSS ExoU effector in *P. aeruginosa* are the strongest predictors of development of pneumonia [76–78]. Further investigations have suggested that the fluoroquinolone-resistant phenotype and the *exoU+* genotype of *P. aeruginosa* cause poor clinical outcomes in patients with *P. aeruginosa* pneumonia [76–79]. Several genome sequence analyses of small colony variants of *P. aeruginosa* have been reported recently [80–85]. These studies showed multifactorial antibiotic-resistance mechanisms, such as overexpression of efflux mechanisms, LPS modification, and a drastic downregulation of the *Pseudomonas* quinolone signal quorum-sensing system. These reports suggest that, over the last 15 years, wide-spread global carbapenem and fluoroquinolone use has rapidly enhanced propagation of virulent and drug-resistant *P. aeruginosa* strains.

7. Anti-PcrV strategies in *P. aeruginosa* infections

Recent outbreaks of XDR-PA are threatening to increase colonization by MDR-PA in immunocompromised patients because efficacious antimicrobial choices are extremely limited. Therefore, this situation requires development of new prophylactic or therapeutic strategies that do not rely on conventional antimicrobial agents [86, 87].

7.1. Active and passive immunization against *P. aeruginosa* PcrV

The first experimental trial on immunotherapy against the TTSS of *P. aeruginosa* was performed using *E. coli*-derived recombinant PcrV protein to actively immunize mice [33]. In this experiment, the immunized mice survived lethal challenge infections with *P. aeruginosa* pneumonia. Together with the active immunization trial, passive immunization was carried out in mice with a purified protein A binding γ-globulin fraction, which was separated from the sera of rabbits that were actively immunized with recombinant PcrV [30]. In this series, the immunized mice survived pulmonary administration of a lethal dose of *P. aeruginosa*. A correlation between the survival rate of the mice and the dose of the polyclonal anti-PcrV antibody was found. The effects of this polyclonal anti-PcrV antibody were later tested in various animal models of burns and chronic bacterial pneumonia [88, 89].

The mechanism responsible for the positive effect of the polyclonal anti-PcrV antibodies, in terms of whether the effect depends on the Fc-portion of the antibody, was investigated. The anti-PcrV polyclonal antibody F(ab)′$_2$ was tested in a rabbit model, and the same effect as whole IgG was confirmed [90]. This finding strongly suggests that the prophylactic and therapeutic effects of anti-PcrV polyclonal antibodies are derived by blocking the action involved in the pathogenicity of the antigen. Monoclonal antibody screening on normal mouse hybridomas was then performed and the clone mAb166 was discovered as the strongest TTSS blocker [40]. The clone mAb166 displayed equivalent therapeutic and prophylactic effects to those of the anti-PcrV polyclonal antibody [40, 91, 92]. The mAb166 Fab fragment also conferred the same therapeutic effect as the original whole IgG in *P. aeruginosa* pneumonia [85]. In particular, mAb166 exerted a strong therapeutic effect following airway administration of *P. aeruginosa* in a pneumonia model in rats [93]. By using this mAb166 antibody as a template with the

bacteriophage gene shuffling recombination technology, the US venture company KaloBios Inc. started a project to create a humanized anti-PcrV antibody. Consequently, KB001-A was developed as a humanized monoclonal antibody [94]. This antibody underwent phase I and phase II clinical trials in the USA and France [95, 96].

7.2. Immunization against PcrV in immunocompromised models

Active immunization with PcrV was examined in immunocompromised mice that were pre-treated with cyclophosphamide [97]. Cyclophosphamide treatment induced immunosup-pression in the mice, decreased immunity against *P. aeruginosa*, and decreased the lethal dose of *P. aeruginosa*. In this study, five truncated PcrV fragments and full-length-PcrV were tested as vaccine candidates in a mouse model of *P. aeruginosa* pneumonia. Acute systemic infec-tion was introduced by intraperitoneal injection of a lethal dose of *P. aeruginosa* in this mouse model [97]. This study showed that active immunization with either full-length $PcrV_{1-294}$ or $PcrV_{139-294}$, both of which contain the $_{PcrV144-257}$ blocking epitope region of monoclonal anti-PcrV IgG mAb166, successfully protected the immunocompromised mice from lethal *P. aeruginosa* infection. This finding suggested that the anti-PcrV strategy might be effective in neutropenic conditions in which human patients frequently develop *P. aeruginosa* infections.

The intravenous immunoglobulin (IVIG) was recently shown to confer significant protection against lethal infection with virulent *P. aeruginosa* [98, 99]. The effect of administrating 2.5 mg of IVIG was comparable with that of administrating 10 μg of specific anti-PcrV polyclonal IgG. The mechanism of protection is likely to involve the synergic action of anti-PcrV titers and some surface antigen to block the TTSS-associated virulence of *P. aeruginosa* [98]. There is considerable variation in anti-PcrV titers in adult subjects without any obvious history of infection with *P. aeruginosa* [100]. IVIG extracted from high anti-PcrV titer human sera confers protective effects in a mouse model of lethal *P. aeruginosa* pneumonia [101]. These results sug-gest that, not only monoclonal strategies against PcrV, but serum-derived immunoglobulin therapy with specific titers against PcrV also has great potential as effective immunotherapeu-tic tool against lethal *P. aeruginosa* infections.

8. Conclusions

In this review, we summarize the current status of research on the pathogenesis and treatment of *P. aeruginosa* infections from the viewpoint of acute and chronic infections. First, there are two phenotypes of *P. aeruginosa* strains: one causes acute types of infection, whereas the other causes chronic types of infection. Genomic level differences exist between these two pheno-types. In the course of evolution, acquisition of virulence gene cassettes, especially PAPI-2, created subtypes with increasing toxicity. Second, exposure to antibiotics enhances their drug resistance together with a loss of cytotoxicity and antigenicity that can be targeted by host immunity. Third, some mutant cytotoxic and drug-resistant *P. aeruginosa* strains may be involved in acute exacerbation of chronic infectious diseases. The lifespan of patients with CF has improved via various medical advances. Rather than focusing on eradication of infectious pathogens, prophylaxis against lethal pathogenic factors to avoid acute exacerbation during

the chronic infection state should probably be given more priority at present. Currently, the monoclonal antibody strategies that are used against bacterial infections have not yet reached the level of practical application that is found in cancer therapy. The on-going challenge for anti-PcrV immunotherapy is realizing its potential to improve the clinical outcome of *P. aeruginosa* infections occurring in patients with CF.

Acknowledgements

Abbreviations

CF	cystic fibrosis
GAP	GTPase-activating protein
IVIG	intravenous immunoglobulin
MDR-PA	multidrug-resistant *Pseudomonas aeruginosa*
PAPI-2	*P. aeruginosa* pathogenicity island-2
PLA	Phospholipase A
TTS	Type III secretory
TTSS	Type III secretion system
XDR-PA	extensively drug-resistant *P. aeruginosa*

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