“Weapons of a pathogen: Proteases and their role in virulence of *Pseudomonas aeruginosa*”

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Pathogenic bacteria need to interact with their host to establish an infection and to maintain it successfully afterwards. This requires to “understand” signals of the immune-system and to “respond” appropriately when the host tries to defend himself. Part of the response of successful pathogens is secretion of so called virulence factors which manipulate or even destroy defense lines of the host. An important part of the arsenal of bacterial virulence factors are proteases. Proteases are enzymes that hydrolyze peptide bonds and can therefore degrade proteins and peptides. As proteins are one of the basic building blocks in nature, proteases can influence a broad range of biological functions including the infection process which is not just a simple and rapid multiplication of bacterial cells in the human body. The opportunistic human pathogen *Pseudomonas aeruginosa* has an arsenal of impressively efficient proteases that helps establishing and maintaining an infection and thereby controlling and modifying the environment according to the needs of the bacterium within the host tissue.

**Keywords** pathogen, protease, immune system, secretion

### 1. Introduction

*Pseudomonas aeruginosa* is an aerobic Gram-negative bacterium that is an important cause of both community-acquired and hospital-acquired infections [1]. Community-acquired infections include among others ulcerative keratitis (usually associated with contact lens use), otitis externa, skin and soft tissue infections (including diabetic foot infections). Hospitalized patients may be colonized with *P. aeruginosa* on admission or during hospital stay. Nosocomial infections include pneumonia, urinary tract infections, bloodstream infections, surgical site infections and infection of the skin after burn injuries. *P. aeruginosa* infections also occur in immunocompromised patients e. g. AIDS especially in patients who have a compromised phagocytic system [2]. *P. aeruginosa* is the leading cause of respiratory tract infections with patients which are intubated during a hospital stay and has a high mortality of 40% to 50% [3]. Chronic sinopulmonary colonization and recurrent infections of *P. aeruginosa* are seen in patients with Cystic Fibrosis. In a survey in 2004 57.3% of all reported respiratory cultures from CF patients contained *P. aeruginosa* and in another survey 97.3% of CF children had a *P. aeruginosa* infection by the age of three [1]. In Europe *P. aeruginosa* is the most prevailing bacterium that caused chronic lung infection in CF patients [4]. CF lung infections are characterized by a vigorous inflammatory response with increased cytokine production including interleukin-8 (IL-8) and interleukin-6 (IL-6) and a neutrophil-dominant inflammatory response in the airways [5]. *P. aeruginosa* proteases are detected in the lungs of CF patients such as elastase B, alkaline protease, protease IV and PasP [6-7]. Wound infections due to *P. aeruginosa* are especially difficult in burn patients [3]. A high percentage of the wound infections will lead to sepsis with significant mortality rates. Protease-deficient strains are generally less virulent than protease producers in burned mouse models [8]. *P. aeruginosa* infections of the eye usually follows minor trauma to the cornea [3]. These infections are frequently associated with contact lens use. Proteases play an important role during infection with *P. aeruginosa* and are a characteristic for invasiveness as determined in clinical strains [9].

Proteases or peptidases are enzymes that can hydrolyze peptide bonds within peptides and proteins [10]. For a long time the function of proteases were solely dedicated to protein turnover or digestion of proteins as a food source. More and more it becomes clear that the hydrolysis of a peptide bond of proteins can have a wide range of biological functions which can be very subtle and specific.

Proteases can be divided into 6 different catalytic types: aspartic-proteases, metallo-proteases, serine-proteases, cysteine-proteases, threonine-proteases and glumatic-proteases depending on their active residues that are involved in the catalysis. The aspartic, metallo and serine-proteases are definitely the most abundant protease groups [10]. Genomes of different *P. aeruginosa* strains are available and several of them can be found in the MEROPS peptidase database. The well known PAO1 laboratory strain of *P. aeruginosa* contains about 5568 predicted genes [11]. Of these genes 155 are predicted proteases and deposited in the MEROPS database [12]. This is about 2.8% of the genome. The proteases are classified in the different catalytic types: 84 serine-proteases (54% of total proteases), 45 (29%) metallo-proteases, 11 (7%) cysteine-proteases, 5 (3%) threonine-proteases, 3 (2%) aspartic-proteases and 7 (5%) unassigned proteases.

There are several well known bacterial proteases that interact with their hosts during a pathogenic infection. The highly lethal Anthrax Toxin of the pathogenic bacterium *Bacillus anthracis* consists for example of a complex of three...
different proteins of which one is called the lethal factor. The lethal factor of the antrax toxin is a metallo-protease that’s able to specifically cleave and inactive MAP kinase kinases [13]. Another example is the botulinum neurotoxin (BoNT) from Clostridium botulinum. BoNT is regarded as one of nature’s most lethal toxins’s known to man with a LD₅₀ of roughly 0.005–0.05 µg/kg [14]. BoNT posses a metallo-protease domain which is able to block acetylcholine release at peripheral nerve ending by the cleavage the SNAP-25 protein that plays a role in the storage and depletion of acetylcholine [15]. P. aeruginosa also secretes several proteases that play a role in the pathogenic interaction between bacterium and host. This review gives an insight in the most important proteases in the weapons armoury of Pseudomonas aeruginosa.

2. Elastase B

2.1 Protease classification and structure

Elastase B is a metalloprotease belonging to the M₄ thermolysin peptidase family as classified in the MEROPS database [16]. The lasB gene codes for a preproelastase of 498 amino acids and consists of 3 different domains [17]. The first two domains represent the propeptide. The third domain harbours the catalytic thermolysin metalloprotease centre of the M₄.005 subfamily [17].

The first 23 amino acids from the N-terminal code for a signal peptide that directs the protein for translocation over the inner membrane to the periplasm by the secretory system (Sec) during which the signal is cleaved of [18]. During the translocation across the inner membrane the preproelastase is in an unfolded state. Subsequently, before the proelastase is secreted to the extra cellular environment, the protein is folded to its active and secretion competent form by the propeptide. The two domains of the propeptide act as a chaperone in which they support a correct refolding along with other periplasmatic chaperones and folding catalysts [19]. The propeptide domain is processed after correct folding by autoproteolytic cleavage and will remain non-covalently attached to the mature elastase [20]. After correct folding and autoproteolytic cleavage, the propeptide-elastase complex is subsequently translocated across the outer membrane. The translocation is mediated by the Xcp machinery (T2SS) of the general secretory pathway (Fig. 1). A correct conformation of the protein is essential for secretion. The attached propeptide acts as an inhibitor of the mature elastase and dissociates and is degraded in the extracellular environment [21].

2.2 Regulation

Elastase is one of the prototype virulence factors of P. aeruginosa regulated by the quorum sensing cascade [22]. Quorum sensing is an important communication system involved in the pathogenicity of many bacteria. Many virulence factors of P. aeruginosa are regulated by quorum sensing such as proteins as exotoxin A and the non-protein virulence factors e. g. pyocyanin and rhamnolipids. The production and subsequent secretion of elastase B is regulated by quorum sensing as it is part of the las and rhl regulons [22]. Quorum sensing is a kind of bacterial cell-to-cell communication via small molecular chemical signals. At a certain threshold these messengers such as the group of N-acylhomoserin lactones (AHL) will bind to the transcriptional regulator LasR which subsequently activates transcription of its las regulon. Another transcriptional regulator called RhlR which is part of the las regulon also binds to certain members of the AHL signal molecules. Upon AHL binding RhlR will start transcription of its rhl regulon. The transcription is initiated by binding of the regulator to the las-rhl box within the promoter region of the respective target genes. A las-rhl box can function as binding sites for either or both regulators [22]. Upstream of the lasB gene lays a LasR transcriptional binding site. Elastase B depends on both las and rhl systems as deletion mutants strains of both lasB and RhlR results in reduced and or - in the case of double mutants - in completely abolished elastolytic activity [23]. Also another system within quorum sensing network is called the PQS system which is hierarchically in-between the las-rhl systems but all intercalate with each other and plays a role in the regulation of elastase B. The Pseudomonas quinolone signal (PQS) is a small mw molecule that can bind to the transcriptional regulator PqsR (MvfR). Activation of the PQS system by addition of PQS leads to induction of lasB transcription [24], as inactivation of the PQS signal results in decreased elastolytic activity [25].

2.3 Role of elastase B in host-pathogen interaction

Elastase B was first identified as an elastolytic protease and this activity is believed to play a key role in the CF lung infection [5]. Elastase B can degrade human and bovine elastin [26-27]. Elastin is a protein forming a biopolymer in organs and tissues of vertebrates that gives them elastic properties [28]. It is known for Cystic Fibrosis patients that the lung tissue shows decreasing levels of elastin and increasing levels of collagen resulting in lung fibrosis [5]. Histologic studies have detected abnormal elastin fibers in lung alveoli of CF patients on autopsy [29]. Elastin can also be found around vascular tissue in the external elastic lamina and its disintegration is associated with vasculitis during P. aeruginosa infection [30].
Elastase B is not only able to degrade elastin but also collagen - another important human biopolymer. Collagen can be found throughout a whole range of different human tissues and can be subdivided in different types. Elastase B was shown to degrade collagen type III and IV [20, 31]. Collagen type III can be found in interstitial extracellular matrixes and type IV collagen in basement membranes. Basement membranes (BM) are specialized extracellular matrices which form thin acellular layers that underlie cells and separate the cells from and connect them to their interstitial matrix [32]. Bejarano and co-workers were able to show that elastase B was responsible for the degradation of intact basement membranes obtained from bovine lenses and lungs [33]. The formation of BMs is a prerequisite for normal tissue development and function and regulates different biological activities during cell development. The degradation of BMs was also demonstrated in a mouse model after subcutaneous injections of purified elastase B and caused severe hemorrhage and muscle damage [34]. Pathological studies showed a loss of endothelial integrity along with changes in the structure of the vascular wall. These findings were confirmed within cultured endothelial cells. The pathological effects were described to be the result to the fibrinogenolytic and fibrinolytic activity of elastase B [34].

In another study elastase B producing P. aeruginosa strains were shown to degrade proteins from human wound fluids and human skin biops ex vivo. Elastase B was also shown to inhibit fibroblast growth [35]. All of these features are characteristics of chronic ulcer infections with P. aeruginosa [35].

Elastase B can also interact with proteins of the human immune defense system and degrade them. Heck and co-workers were able to show that elastase B could degrade immunoglobuline A (IgA) [36]. IgA is a major serum immunoglobulin and the predominant antibody class in the external secretions that bathe mucosal surfaces such as the respiratory and gastrointestinal tract and the eye which plays a key role in the defense of micro-organisms [37]. Elastase B is also able to degrade immunoglobuline G (IgG), the most predominant and important antibody [38]. It was shown that elastase B is not only processed but that the degradation products of IgG obtained from CF patients inhibit bacterial uptake by human neutrophils and therefore prevent opsonophagocytosis [38]. So the action of elastase B is ambiguous, first inactivation of IgG and secondly inhibition of bacterial uptake by phagocytosis by the degradation products. Phagocytosis is probably inhibited because of the binding of the degradation productions to the IgG receptors on the human neutrophils that prevent uptake of bacterial invaders bound by IgG as an opsonin [38].

As part of the innate immune system the complement system eliminates invading pathogens [39]. Elastase B is able to inactivate key components of the complement system such as fluid-phase and cell-bound C1 and C3 and fluid-phase C5, C8 and C9 [30].

Another part of the innate immune system is inactivated by elastase B in its ability to degrade Surfactant Protein A and D (SP-A and D) [40]. Surfactant Proteins, members of the collectin family, are synthesized by alveolar type II epithelial cells and consists of a N-terminal collagen region and a C-terminal lectin domain. Complexes of the SPs or collectins are pattern recognition molecules that bind oligo saccharides presented on the surface of many bacteria and by doing so indentify them as alien intruders. SP5D binds among others P. aeruginosa as was protease IV. Deficient SP5D mice were more susceptible to cornea infection after inoculation with P. aeruginosa compared to wild-type animals, only the wild-type mice recovered completely of the infection [41]. Besides the respiratory tract, SP-D can also be found in tear fluid and cornea. Elastase B was suggested to be responsible for the SP-D degradation in the eye, as was protease IV.

P. aeruginosa is a significant cause of corneal infections. Elastase B was shown to cause damage to the stromal proteoglycan extracellular matrix after topical application or injection of wounded cornea with purified protease in different studies [43-45]. Elastase B deficient strains of P. aeruginosa were shown to be less virulent during keratitis after injection to guinea pig cornea. Co-infection of the cornea of elastase B producing strains with elastase B antibodies or the protease inhibitor ovomacroglobulin showed a significant infection reduction [46]. Kessler and co-workers were also able to show that the elastase B inhibitor phosphoramidon could protect the corneas of rabbits for elastase B damage [47].

The elastase B corneal virulence was also shown in an assay by expressing recombinant elastase B in the non-pathogen Pseudomonas putida and subsequent infection of rabbit cornea. Corneal damage was significantly increased in the P. putida expression the elastase B in comparison to the strain without elastase B [48].

Elastase B is also able to inactivate the Proteinase-activated receptor-2 (PAR2). PAR2 belongs to a family of G-coupled transmembranc receptors which can be activated after cleavage at an activation site within the N-terminus of their own exodomain by a variety of proteases [49]. After cleavage, this small peptide functions as a tethered ligand which binds intramolecularly to the receptor and activates it. PAR2 is functionally expressed in different tissues among them the respiratory epithelium. Activation of PAR2 leads to a variety of responses such as triggering the secretion of prostanoids, cytokines and metalloproteases. In vivo activation leads to modulation of bronchomotor activity as shown in guinea pigs, neutrophil extravasation and oedema in rats and eosinophil infiltration in mice [49]. It was postulated that secreted bacterial proteases are the activators of the PAR2 receptor as thereby initiating a defense response. In vitro experiments with cell cultures show that elastase B is able to disarm the PAR2 receptor by cleaving within the
exodomain without activating the receptor and therefore prevent a response shown by a lower secretion of interleukin-8 (IL-8) and prostaglandin E₂ (PGE₂) [49].

Elastase B can also cleave the urokinase-type plasminogen activation receptor (uPAR) purified after recombinant expression or expressed in human monocytic and bronchial epithelial cell lines [50]. The uPAR (also designated CD87 or Mo3 antigen) is a highly glycosylated glycosylphosphatidylinositol (GPI)-anchored cell membrane protein which is composed of three domains D1, D2 and D3. Its expression is increased upon the exposure of cells to a wide range of inflammatory mediators. uPAR has a high affinity for different types of proteins that are involved in cell adherence and migration. uPAR can e.g. bind the serine protease uPA (urokinase-type Plasminogen Activator) which on binding converts plasminogen into plasmin. uPAR-deficient mice are impaired in recruitment and activation of leukocytes at sites of infection, resulting in impaired bacterial clearance and an increased host mortality. Besides that uPAR-deficient animal models may indicate its role in tissue repair and healing after an inflammatory event [50]. Elastase B can inactivate the binding of uPAR to substrates by cleavage at several different parts of the protein.

Research done on guinea pigs showed an increase of lung epithelial permeability after treatment with elastase B aerosol respiration. This epithelial permeability was the result of the disruption of the tight junctions between epithelial cells [51]. The same researcher showed that elastase B could enhance IL-8 production in rabbit alveolar epithelial cells by activation of the mitogen-activatedprotein kinase (MAPK) pathway via extracellular signal-regulated (ERK1/2) proteins [52]. This activation was abolished in the presence of the ERK activation inhibitor U0126.

Human α₁-proteinase inhibitor (α₁-PI) is responsible for the tight control of elastase activity secreted from neutrophils by inhibiting it. Down regulation of α₁-PI may cause excessive tissue degradation. Elastase B from P. aeruginosa is able to inactivate α₁-PI by cleavage [53].

In rats elastase B can induce neutrophil accumulation after injection in the air-pouch cavity of the respiratory tract. After injection of elastase B the volume of exudate and neutrophil amounts were increased significantly and showed a peak 8 hours after infection. IL-8 levels were highest 4 hours after infection which production was also enhanced by elastase B [54].
Fig. 1  Schematic gene structure and secretion of the major virulence proteases of *P. aeruginosa*, elastase B (LasB), elastase A (LasA), protease IV (PIV), alkaline protease (AprA). a) Schematic gene structure with domains. Abbreviations are signal peptide (SP) and amino acids (AA). b) Schematic representation of secretion. Abbreviations are cytoplasmic membrane (CP), inner membrane (IM), periplasm (PP), outer membrane (OM), extra cellular (EC), ferric uptake regulator (Fur), alternative sigma factor (PvdS), signal peptide (SP), Secretory pathway (Sec), Xcp machinery (Xcp), alkaline secretion proteins (AprD, AprE and AprF), propeptide domains (p) and catalytic domains or mature proteases (c). Scissors represent (auto)protolytic activity and arrows indicate the direction of secretion.
3. Elastase A

3.1 Protease classification and structure

Elastase A (LasA), also known as staphylolysin, is one of the most abundant of at least four secreted endopeptidases of *P. aeruginosa*. It was originally identified by a mutation (*lasA1*) in strain PA01 that results in reduced elastolytic activity [55]. Elastase A is a zinc metalloprotease [56] and belongs to the subgroup A of M23 family of staphylolytic or β-lytic zinc metallo-endopeptidases [12, 57]. The *lasA* gene encodes a 40 kDa protein [58] which represents the elastase A preproprotein. The mature LasA that can be found in the extracellular environment has a molecular weight of 20 kDa [58-59]. After its synthesis LasA is secreted together with its propeptide via the Xcp (type II) machinery [18, 60] and is subsequently activated when it has reached the extracellular space (Fig. 1) [60]. In contrast to LasB the cleavage of the LasA propeptide is not autocatalytic [59]. Instead, the LasA propeptide is processed by different endopeptidases secreted by *P. aeruginosa* named LasB, LysC and protease IV [61-62]. The propeptide sequence of LasA shows in principle little identity to those of other β-lytic proteases. Therefore it is difficult to predict functional domains within the propeptide.

3.2 Role of elastase A in Host-pathogen interaction

LasA has both low elastolytic and high staphylolytic activities [63]. LasA cleaves a wider range of glycine-containing proteins, including tropoelastin-derived pentapeptides [56], glycine-rich synthetic peptides and specific sequences present in elastin [56]. Such sequences are rare in elastin resulting in the limited elastolytic power of LasA protease [64]. Besides the own intrinsic elastolytic activity, LasA increases significantly the elastolytic activity of other proteases, including that of LasB [56, 65-66]. Therefore LasA enhances the virulence activity of LasB in the establishment of a *P. aeruginosa* infection. Moreover, LasA enhances also the activity of several other host elastolytic proteases, including human leukocyte elastase, human neutrophil elastase [67] and other proteases [56, 65]. The concerted action of several enzymes in elastin degradation and the relationship of the two *Pseudomonas* elastases LasA and LasB appear to be involved in generating the invasive phenotype of some *P. aeruginosa* strains [56, 65, 68-69].

Elastase activity of *P. aeruginosa* has effects on tight junctions of epithelial cells [70]. The loss of either LasB or LasA decreases *in vitro* invasion of epithelial cells about 70 % and the loss of both proteases leads to a further significant decrease of the ability to invade host cells [69]. Inactivation of the *aprA* gene encoding for the alkaline protease in addition to *lasA* and *lasB* mutation, however, did not further decrease invasion of epithelial cells. These results indicated that both LasA and LasB might play critical roles in the regulation of tissue invasion [69]. One opportunity by which LasA and LasB may induce invasion could be the degradation of inhibitors of invasion or of degradation of proteins involved in biosynthesis, processing and delivery of invasion inhibitors. The secreted effector proteins ExoS and ExoT are able to inhibit the invasion of epithelial cells by cytotoxic strains of *P. aeruginosa*. There are indications that LasA and LasB directly or indirectly decrease the levels of the toxins ExoS and ExoT that leads to a reduction of invasion inhibition [69].

In addition to the protease activity of LasA against substrates like elastin the protease possesses staphylolytic activity. This staphylolytic activity causes rapid lysis of *S. aureus* cells by cleaving the pentaglycine bridges of their cell wall peptidoglycan. The overall staphylolytic activity of *P. aeruginosa* is mainly LasA dependent. LasA-induced lysis of *Staphylococci* is stimulated and enhanced by other proteases like LasB or the alkaline protease [59]. Physiologically the staphylolytic activity of *P. aeruginosa* may represent a defense strategy against a competing organism and may give *P. aeruginosa* an important advantage to outcompete *S. aureus* during colonization of the CF lung [71-72].

Beside the already mentioned interactions of LasA with host proteins like elastin the influence of LasA on the so called shedding process is of particular importance to enhance pathogenesis of *P. aeruginosa*. The shedding process constitutes the cleavage of cell surface proteins by proteases and the release of ectodomains from the surface as soluble effectors [73-74]. Shedding is an elemental biological mechanism of protein secretion. Surface molecules like growth factors and growth factor receptors, adhesion molecules and cytokines are shed as soluble ectodomains. Shed ectodomains are involved in several pathophysiological events like tissue repair, host defense, septic shock, Alzheimer's disease and wound healing processes [74-77]. *P. aeruginosa* seems to use the host cell’s shedding mechanism to increase its virulence.

One family of cell surface transmembrane glycoproteins is termed syndecans which has four members [78-79]. Syndecans or heparin sulphate proteoglycans (HSPGs) possess a heparin sulfate chain which enables binding and modulation of the activity of a several soluble and insoluble ligands. The extracellular part of syndecan can be shed and after the release as soluble ectodomains they can function as soluble effectors. The constitutive shedding process of syndecan is a normal host-regulated mechanism. Besides there are hints that syndecan shedding is induced by tissue injury. This host-regulated response leads to enhanced shedding and the released ectodomains function as regulators of inflammation [80]. During infection secreted virulence factors of bacteria enhance host ectodomain shedding that leads to epithelial barrier disruption, tissue penetration and endothelial damage [81-82]. One of these secreted virulence factors is LasA of *P. aeruginosa*. LasA enhances shedding of syndecan-1 *in vitro* [81] and also *in vivo* [82].
released soluble syndecan-1 ectodomains subsequently enhance bacterial virulence through their heparan sulfate chains in newborn mice [81-82]. Keeping in mind that *P. aeruginosa* is a dominant pathogen in CF and burn wounds it is not surprising that syndecan-1 is the major syndecan of the lung epithelia and epidermal keratinocytes cells. The exact reason for enhancement of *P. aeruginosa* virulence because of the heparan sulfate chains remains to be determined. It is clear so far that the ectodomains do not interact directly with *P. aeruginosa* [82]. It was suggested that shed ectodomains may promote bacterial pathogenicity by interfering with host defense system [82]. There are several hints that soluble, distinct host defense factors like neutrophil elastase and cathepsin G [83] are inhibited by shed syndecan-1 in turn facilitating the attack of *P. aeruginosa*.

4. Protease IV

4.1 Protease classification and structure

Protease IV was first indentified and characterized as a 26 kDa serine protease present in the culture supernatant of *P. aeruginosa* [84]. Protease IV is also known as lysyl endopeptidase or iron-regulated protein PrpL and belongs to the chymotrypsin family S1 according to the MEROPS database [85]. The catalytic domain contains three active residues His-72, Asp-122 and Ser-198 that form a triad in the catalytic cavity predicted based on homology with other proteases of this family and proven by mutagenesis studies [86]. The serine (Ser-197) adjacent to Ser-198 was also shown to be essential for catalytic activity. The *piv* gene, however, encodes a protein of 463 amino acids with a calculated size of 48.2 kDa. The *piv* gene consists of three domains; a short N-terminal secretion signal followed by a propeptide and a C-terminal catalytic domain. The protease is synthesized intracellular as a pre-proenzyme of 48 kDa. Bioinformatic analysis predicts a signal peptide consisting of 24 amino acids. Expression studies with the whole open reading frame of protease IV in *P. putida* confirms the presence of a proenzyme of about 45 kDa [87]. In comparison to other proteases such as elastase B it is likely that the pre-proenzyme is translocated across the inner membrane to the periplasm after which the signal peptide is cleaved of. In the periplasm the propeptide is probably removed by autoproteolysis thereby liberating the mature protein. Evidence for autoproteolysis was shown by processing of the inactive mutated proenzyme His-72-Ala after addition of purified protease IV [86]. It was suggested that autoprocessing involving the cleavage of the 45 kDa proenzyme takes place at the lysine residue at the junction between the propeptide and the mature protease domains (Fig. 1). The function of the propeptide is unknown but probably it acts as a intermolecular chaperone in analogy to elastase B and assists in folding of the mature protease, secretion of the enzyme and it may have an inhibitory function. Traidej and coworkers hypothesized that protease IV could be secreted by the type II secretion apparatus for translocation across the outer membrane (Fig. 1) [87].

4.2 Regulation

The expression of protease IV has been shown to be regulated by the alternative sigma factor PvdS because it was absent in culture supernatants of a *P. aeruginosa* PvdS deletion mutant [62]. PvdS belongs to the extracytoplasmic factor class of regulatory proteins and regulates virulence genes such as exotoxin A [62]. The PvdS itself is regulated by the ferric uptake regulator (Fur) which contributes to the expression of many virulence factors. The human host iron-binding proteins lactoferrin and transferrin are normal constituents of airway secretion which is a large biopolymer and part of the blood clotting system [91]. Fibrinogen is conferred to a fibrin cloth after vascular damage. Dysfunction of fibrinogen will lead to hemorrhage which is a characteristic of *P. aeruginosa* infection [84]. Moreover protease IV can degrade a whole range of biological important host proteins such as fibrinogen and plasminogen and immunoglobulin G (IgG) and the complement components 3 and C1q all belonging to the immune defense system [61].

Da Silva and coworkers showed that *P. aeruginosa* is able to bind human plasminogen (Plg) to his extra cellular surface [92]. Plasminogen plays an important role in the delicate equilibrium of blood clotting. Plasminogen can be
activated by other proteases such as the urokinase-type (uPA) proteolytic activator by conversion into the active plasmin. Plasmin is a serine protease that degrades a fibrin clot. It is thought that plasminogen can also have other physiological functions that may be misused by pathogenic bacteria. Several human cells bind Plg that assists and directs migration throughout the body [92]. Pathogenic bacteria, among them *P. aeruginosa* can bind Plg which helps them to invade the whole host and the attached protease can degrade extracellular matrix (ECM) proteins or helps in escaping from fibrin networks that serve as a focus of infection [92]. In order to be effective the bound Plg has to be converted into the active plasmin (Pm). Beaufort and coworkers showed that Plg could not be activated by elastase B nor protease IV directly but indirectly by activation of pro-uPA into uPA [92]. uPA on its turn will convert Plg in Pm and therefore activates it [92].

Like elastase B protease IV is also able to degrade several different surfactant proteins (SP) such as SP-A, SP-D and SP-B. Malloy and coworkers showed that protease IV is able to degrade SP-A, SP-D and SP-B in a time- and dose depended fashion in cell-free bronchoalveolar lavage fluid (BAL) [93]. In the course of the original observation that *P. aeruginosa* was able to cleave SP-A and SP-D it was suggested that two different proteases were responsible [40]. During this study only one protease, elastase B, was identified as a responsible protease. Later on protease IV could be identified in a follow-up study as the second protease [93]. The degradation of SP-A and SP-D resulted in the inhibition of their ability to aggregate bacteria and enhance bacterial uptake by alveolar macrophages. Furthermore it was shown that the surface tension lowering effect of large surfactant aggregates (LA) was abolished by protease IV [93]. This effect on LA could be inhibited by TLCK. Protease IV thus alters both functions of the surfactant proteins; surface tension reduction and innate immune system. In addition to elastase B, protease IV can also degrade SP-B in BAL. As mentioned before SP-D was shown to play a role in *P. aeruginosa* keratitis [41].

Most research on the virulence of protease IV has been done in elucidating its role in keratitis of the cornea [94-95]. Protease IV was identified in attenuated and deficient *P. aeruginosa* strains to contribute to the virulence of corneal infection in rabbits and mice [96-97]. These results could be confirmed by restoring the virulence in a keratitis rabbit infection model by co-applying purified protease IV together with a protease IV deficient *P. aeruginosa* strain [98]. Thibodeaux and coworkers investigated the protective value of immunization against protease IV in preventing keratitis in rabbits [99]. For this rabbits were immunized with purified protease IV and urea-soluble recombinant protease IV. Antibody titers were raised against recombinant protease IV and purified protease IV. Nevertheless the antibodies could not neutralize enzyme activity in vitro neither protecting infected rabbits in developing keratitis.

### 5. Alkaline protease

#### 5.1 Protease classification and structure

The alkaline protease (AprA), also known as aeruginolycin, is one of the secreted zinc-dependent metalloendopeptidase of *P. aeruginosa*. AprA belongs to the subfamily B of the M10 peptidase family and is a member of the so called metzincin superfamily [12].

The *apr* locus of *P. aeruginosa* contains five open reading frames and encodes for proteins that are involved in either the synthesis or secretion of the alkaline protease [100] among them the gene *aprA*, encoding for the alkaline protease itself and the gene *aprC*, which encodes the protease inhibitor APRin. The three proteins AprD, AprE and AprF are membrane proteins that are necessary for AprA secretion. The genes encoding these membrane proteins are clustered adjacent to the 5' end of the alkaline protease structural gene *aprD* [101-102]. The AprD, AprE and AprF proteins show a high homology to proteins necessary for secretion of proteases from *Erwinia chrysanthemi* and to the α-haemolysin of *Escherichia coli* [102] and form the type I secretion machinery for translocation of alkaline protease (Fig. 1) [101]. AprF is localized within the outer membrane, AprE functions as a membrane fusion protein and AprD represents the ATP-binding cassette. Like all proteins secreted by this type of mechanism, AprA possesses a C-terminal secretion signal located within the last 50 amino acids residues (Fig. 1) [103].

#### 5.2 Role of alkaline protease in host-pathogen interaction

It was shown that AprA cleaves a large number of physiological substrates in vitro [95]. One of these substrates is laminin which is an important and biologically active part of the basal lamina [104]. Soluble laminin is rapidly cleaved by purified *P. aeruginosa* alkaline protease. Laminins are big trimeric proteins that contain an α-chain, a β-chain and a γ-chain. AprA rapidly cleaves the α-chain but slowly cleaves the β-chain [104] while degrading of both α-chain and β-chain by LasB occurs rapidly. Because of laminin degradation, alkaline protease could probably have a direct function in invasion and hemorrhagic tissue necrosis in infections caused by *P. aeruginosa*.

Alkaline protease and elastase B of *P. aeruginosa* inactivate human γ-interferon and human tumor necrosis factor-α [105-106]. Both cytokines are critical for effective host immune responses. γ-interferon is the key factor for innate and adaptive immunity against viral and bacterial infections and for control of tumor formation. A lack of γ-interferon results in autoimmune-inflammatory and autoimmune diseases [107-108]. Tumor necrosis factor-α is an important factor for the
regulation of immune cells. It is involved in systemic inflammation and is able to stimulate the acute phase reaction by induction of apoptotic cell death. Apoptosis is induced to initiate inflammation and to inhibit tumorigenesis and viral replication [109-110].

Both alkaline protease and Elastase B are able to inhibit the function of neutrophils, especially interfering with their chemotaxis [111] which gives the bacterium an advantage in escaping from phagocytes of the host defense system. Another component of the host immune system that is affected by proteases of P. aeruginosa are leucocytes. It was shown that alkaline protease and LasB reduce their phagocytic activity against P. aeruginosa [112]. It was assumed that this effect of P. aeruginosa is based on cleavage of the cell receptors on the cell surface which are necessary for phagocytosis [112]. A further factor which is influenced by both proteases in vitro is the Natural Killer (NK) cell function. Alkaline protease and LasB are able to inhibit the effector/target cell conjugate formation. The inhibition of NK cell binding to the target cell is probably caused by the protease activity that enables the cleavage of surface receptors associated in the binding [113]. Beside the already mentioned substrates it was demonstrated that AprA can also cleave a variety of physiological substrates in vitro. These substrates are fibrin, fibrinogen and different complement factors, especially C3 [114]. Moreover AprA is able to inactivate different human protease inhibitors such as serpins, the C1-inhibitor and α-1-antichymotrypsin, IgG from rat [115], the human RANTES, epithelial neutrophil-activating protein-78 (ENA-78) and monocyte chemotactic protein 1 (MCP-1) [116]. Furthermore it was demonstrated that elastase and alkaline protease were capable to interfere with human lymphocyte function, assumedly based on degradation of IL-2 [117].

Furthermore AprA is capable to degrade interleukin-6 very efficiently [118]. Cytokine IL-6 is an important factor in the host immune response during infection. Leu-Lys-AprA is more effective compared to the shorter variant in the degradation of IL-6. Another difference between both AprA forms is the efficiency of IL-6 intermediate degradation. The shorter form of AprA degrades IL-6 without any intermediates. It was suggested that the cytokine IL-6 takes part in a mechanism to regulate the inflammatory signaling activated by airway epithelial cells [119]. AprA may hinder these IL-6 dependent regulatory systems. Potentially both types of AprA interfere with the cytokine circuits in lung [118]. However, the presence of Leu-Lys-AprA in patients sputum needs to be verified.

The role of P. aeruginosa alkaline protease in keratitis is still controversial. First it was reported that strains of P. aeruginosa PA103 deficient in alkaline protease production were not able to establish corneal infections and could not colonize traumatized cornea [120]. Afterwards an in vivo infection study in mice showed that active alkaline protease was present in corneal tissues [121]. In contrast Pillar and coworkers constructed alkaline protease-deficient mutants of P. aeruginosa to analyze the role of this protease in corneal infection and they were able to show that the alkaline protease has no influence on the ocular virulence of P. aeruginosa [122]. Because of these results it was concluded that alkaline protease is not essential for P. aeruginosa keratitis contrary to the former opinion. The role of secreted proteases in the pathogenesis of corneal disease was further examined by using protease mutant strains of P. aeruginosa [123]. This study showed that the three proteases alkaline protease, LasA, and LasB are not essential for the establishment of the ocular virulence of P. aeruginosa. However, it was demonstrated that the alkaline protease seems to be a crucial mediator of virulence. This function of the alkaline protease depends on the location of P. aeruginosa within the cornea and on the presence of simultaneous elastolytic activity [123].

In a current study the two proteases LasB and AprA were expressed separately by the non-pathogenic organism P. putida using a rabbit model to determine the specific virulence capability of P. aeruginosa proteases on bacterial keratitis [48]. Recombinant Elastase B expression in P. putida caused significant enhancement of corneal damage. The expression of alkaline protease caused limited corneal erosions. These results indicated that the potential of alkaline protease to cause corneal erosions during P. aeruginosa keratitis is limited.

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