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## Pseudomonas aeruginosa and Its 4 Arsenal of Proteases: Weapons 5 to Battle the Host

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# *Pseudomonas aeruginosa* and Its Arsenal of Proteases: Weapons to Battle the Host

Anna Clara M. Galdino, Marta H. Branquinha, André L.S. Santos and Lívia Viganor

## Abstract

*Pseudomonas aeruginosa* is a ubiquitous and opportunistic human pathogen that represents a critical problem to the clinician due to the increased number of resistant strains isolated from hospital settings. In addition, there is a great variety of pathologies associated with this versatile Gram-negative bacterium. *P. aeruginosa* cells are able to produce an incredible arsenal of virulence factors, especially secreted molecules that act singly or together to ensure the establishment, maintenance, and persistence of a successful infection in susceptible hosts. In this context, pseudomonal proteases roles are highlighted due to their ability to cleave key host proteinaceous substrates as well as to modulate several biological processes, for example, escaping and modulating the host immune responses in the bacterial own favor. Proteases secreted by *P. aeruginosa* include elastase A (LasA), elastase B (LasB), alkaline protease (AP), protease IV (PIV), *Pseudomonas* small protease (PASP), large protease A (LepA), MucD, and *P. aeruginosa* aminopeptidase (PAAP). In the present review, we discuss the role of each of these relevant proteases produced by

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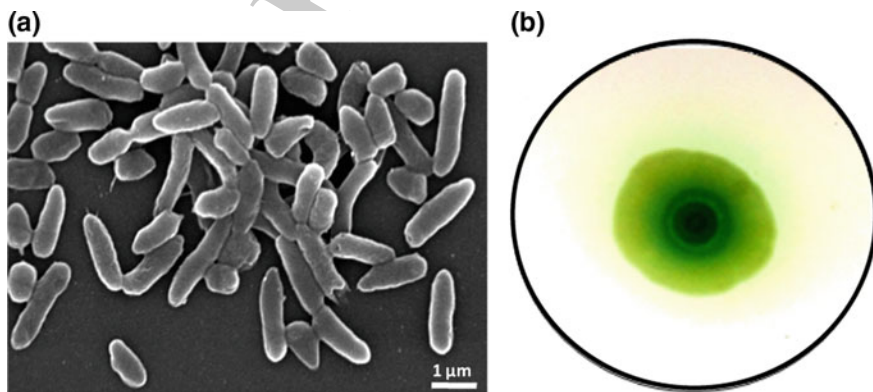
26 *P. aeruginosa* taking into consideration their main biological functions in the  
 27 bacterium–host interaction that favors the establishment of the infectious  
 28 process.

### 30 **Keywords**

32 *Pseudomonas aeruginosa* • Proteases • Virulence factors

## 33 **1 Introduction**

35 Pseudomonads are bacteria well known for their metabolic versatility and wide-  
 36 spread spatiotemporal distribution [1]. One of the most important species of  
 37 pseudomonads is, with no doubt, *Pseudomonas aeruginosa*, which is a fascinating  
 38 ubiquitous Gram-negative bacterium with rod shape measuring  $0.5\text{--}0.8\ \mu\text{m} \times 1.5\text{--}$   
 39  $3.0\ \mu\text{m}$  (Fig. 1a) [1, 2]. *P. aeruginosa* presents the following metabolic features:  
 40 non-fermentative, catalase positive, oxidase positive, ammonia producer, and  
 41 usually aerobic, but it also can grow in an anaerobic environment if nitrate, citrate,  
 42 and arginine are available [3]. The production of 2-aminoacetophenone by the  
 43 bacterial cells generates the fruity grape-like odor that is characteristic of this  
 44 pseudomonad species. On blood agar plates, colonies of *P. aeruginosa* often dis-  
 45 play beta-hemolysis and a greenish metallic sheen due to the production of pig-  
 46 ments [2]. The characteristic that most distinguishes *P. aeruginosa* from the other  
 47 pseudomonads, and from the other species of Gram-negative non-fermenting bac-  
 48 teria, is its ability to produce pyocyanin, a blue-green phenazine pigment that gives  
 49 the green color to the bacterial colony (Fig. 1b) and also to the pus. This pigment  
 50 and several others, such as pyochelin (purple-cyan), pyoverdinin (yellow, green and



**Fig. 1** Scanning electron microscopy (a), showing the characteristic bacterial rod shape, and colony morphology (b), evidencing the pyocyanin pigment, of *Pseudomonas aeruginosa*

51 fluorescent), pyomelanin (light-brown), and pyorubin (red-brown), are secondary  
52 metabolites of *P. aeruginosa*, which play an important role in bacterial nutrition,  
53 such as iron acquisition and pathogenesis [2, 3]. Almost all *P. aeruginosa* strains  
54 are motile due to the presence of a single polar flagellum that facilitates the loco-  
55 motion and colonization of a wide range of environmental niches [2]. This  
56 microorganism can grow within the temperature range from 4 to 42 °C in terrestrial  
57 (soil) and aquatic habitats (polluted, salt, and freshwater) as well as on the surface  
58 of animate hosts (insects, plants, animals, and humans) and inanimate surroundings,  
59 mainly in the hospital environment (distilled water, disinfectants, sinks, medical  
60 devices, and equipment), being an important causative agent of nosocomial  
61 infections, particularly in intensive care units (ICUs) [1–4]. One of the interesting  
62 characteristics of *P. aeruginosa* is its pan-genome, which presents a larger genetic  
63 repertoire than the human genome. This intriguing feature explains the broad  
64 metabolic capabilities of *P. aeruginosa* and its distribution and adaptability in  
65 diverse environments [5].

66 *P. aeruginosa* is one of the most important bacterial species for public health  
67 considerations due to its high resistance to different classes of antibiotics and its  
68 capability to cause serious health care-associated as well as nosocomial infections  
69 [6, 7]. Results reported from an International Nosocomial Infection Control Con-  
70 sortium (INICC) surveillance study, performed between 2007 and 2012, in Latin  
71 America, Asia, Africa, and Europe, in which prospective data were collected from  
72 605,310 patients hospitalized in 503 ICUs, displayed frequencies of 42.8% of  
73 *Pseudomonas* isolates resistant to amikacin and 42.4% to imipenem [8]. In the  
74 USA, an estimated 51,000 health care-associated *P. aeruginosa* infections occur  
75 each year, in which more than 6,000 (13%) of these are multidrug-resistant and 400  
76 deaths per year are attributed to these infections [9]. The analyses based on data  
77 extracted from the Public Health England (PHE) voluntary surveillance database in  
78 the period 2008–2012 showed that 92% of *Pseudomonas* spp. isolates identified  
79 from bacteremia in 3,457 reports were *P. aeruginosa* [10]. In Brazil, the National  
80 Health Surveillance Agency (ANVISA), through the National Monitoring Micro-  
81 bial Resistance Network Health Services (RM Network), published a report that  
82 shows the main etiologic agents and the resistance phenotypes responsible for  
83 causing primary bloodstream infections associated with the use of central venous  
84 catheter in adult patients interned at ICUs from Brazilian hospitals between January  
85 and December 2013. According to that study, 18,233 notifications were reported,  
86 of which 1,850 (10.1%) were caused by *P. aeruginosa*, being the fifth pathogen most  
87 often reported as the etiologic agent. The resistance rate to the carbapenems reached  
88 37.4% (692 *P. aeruginosa* isolates) [11]. Additionally, the Infectious Diseases  
89 Society of America has highlighted *P. aeruginosa* as part of a faction of  
90 antibiotic-resistant bacteria, called ‘the ESKAPE pathogens’—*Enterococcus fae-*  
91 *cium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*,  
92 *Pseudomonas aeruginosa*, and *Enterobacter* spp., capable of ‘escaping’ the bac-  
93 tericidal action of antibiotics and mutually representing new paradigms in patho-  
94 genesis, transmission, and resistance [12].

95 *P. aeruginosa* is extensively resistant to multiple drugs and is increasingly  
96 resistant to most available antibiotics, being a great emergency problem in the  
97 hospital settings worldwide [13]. Interestingly, *P. aeruginosa* has evolved over time  
98 in its ability to find new ways to be resistant to different classes of chemical  
99 compounds as well as to build strategies to exchange genetic materials, allowing  
100 that other bacteria also become drug-resistant [5]. Generally, resistance usually  
101 occurs due to a combination of factors acting synergistically: (i) *P. aeruginosa* is  
102 intrinsically resistant to antimicrobial agents due to its outer membrane/cell  
103 envelope composition that reduces the permeability of several drugs; and  
104 (ii) *P. aeruginosa* expresses a powerful repertoire of resistance mechanisms that can  
105 be developed through mutations in the genomic content that regulates resistance  
106 genes, and also acquired from other organisms via plasmids, transposons, or bac-  
107 teriophages [14].

108 As a major opportunistic pathogen for humans, *P. aeruginosa* causes a plenty  
109 variety of acute and chronic infections and presents significant levels of morbidity  
110 and mortality [15, 16]. *P. aeruginosa* typically infects through airways, wounds,  
111 urinary tract, ear canal, via ocular and implanted medical devices (e.g., catheters or  
112 ventilators). Thereby, it is the main cause of eschars, conjunctivitis, keratitis, corneal  
113 ulcer, osteomyelitis, otitis, urinary infections, surgical site infections, bloodstream  
114 infections in ICUs and hospital-acquired pneumonia in immunocompromised indi-  
115 viduals, mainly in patients with severe burn wounds, AIDS, lung cancer, chronic  
116 obstructive pulmonary disease, bronchiectasis, and cystic fibrosis [16–18].

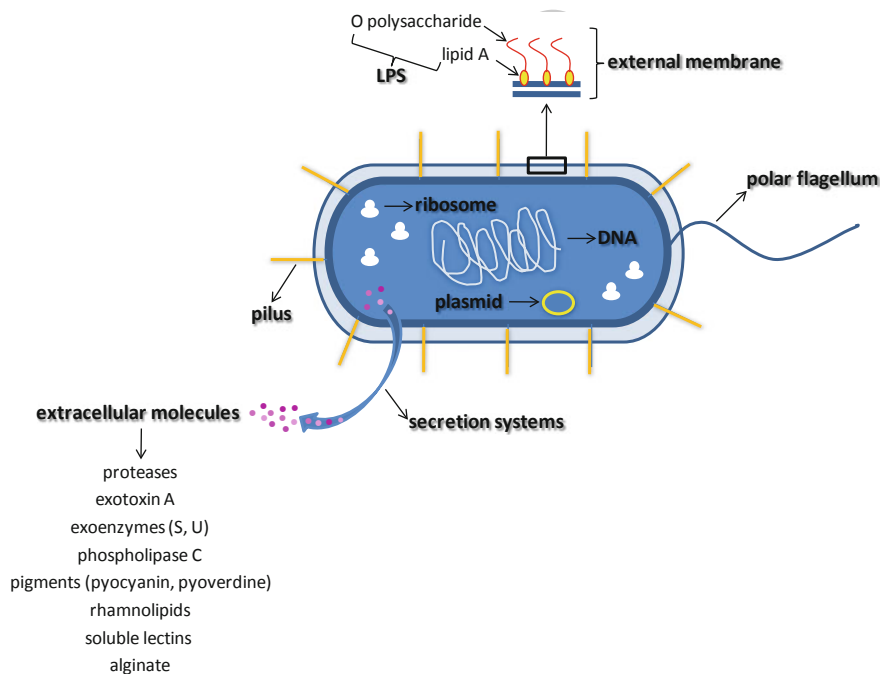
117 It is known that Gram-negative bacteria are common causes of a huge diversity  
118 of infections including, intra-abdominal infections (IAIs), urinary tract infections  
119 (UTIs), ventilator-associated pneumonia (VAP), and bacteremia [19]. In particular,  
120 *P. aeruginosa* is one of the most important pathogens in the hospital setting, being  
121 responsible for 27% of all pathogens and 70% of all Gram-negative bacteria  
122 causing health care-associated infections in the USA, and it is the most common  
123 Gram-negative organism causing VAP and the second most common organism  
124 causing catheter-associated UTIs [7, 19]. The Centers for Disease Control and  
125 Prevention found that *P. aeruginosa* totalized 7.1% of health care-associated  
126 infection in the USA in 2011, being the second most common cause of pneumonia  
127 in hospital settings and the third most common Gram-negative bacterium to cause  
128 bloodstream infections [20]. *P. aeruginosa* is also a major cause of concern in the  
129 cystic fibrosis setting, being the most common pathogen isolated from cystic  
130 fibrosis sputum, and approximately 70% of adult cystic fibrosis patients are  
131 chronically colonized by this microorganism [21, 22].

132 The pathogenic potential of *P. aeruginosa* is not only due to its metabolic/genetic  
133 versatility and both intrinsic and acquired antibiotic resistance. Its ability to form  
134 biofilm and to produce an arsenal of virulence attributes, including cell-associated  
135 determinants (e.g., lipopolysaccharide, pili, and flagellum) and soluble secreted  
136 factors (e.g., extracellular polysaccharides, exotoxins, pigments, and proteases), is  
137 very important for the survival and adaptation of this pathogen in distinct envi-  
138 ronments [17, 22, 23].

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## 2 *Pseudomonas aeruginosa*: Establishing and Maintaining an Infection

In order to establish an infection, *P. aeruginosa* [17, 24]. These factors act together not only causing injuries on the host epithelial cell lining but also induce dysfunctions in bacterial physiology, such as shape, membrane permeability, and protein synthesis, as well as overcome host defenses, down-modulating the immune responses and preventing *P. aeruginosa* endocytosis and obstructing clearance mechanisms, thereby allowing this microbe to persist in cells/tissues and to establish an infection in the host [25, 26]. The virulence of *P. aeruginosa* is mediated by multiple mechanisms, but the major contributor is the production of extracellular proteases. In general, these enzymes regulate multiple cellular and physiological processes and are essential to



**Fig. 2** Virulence factors expressed/produced by *P. aeruginosa* cells: (i) lipopolysaccharide (LPS) that induces cytokine production, (ii) pili that help bacterial adherence to the respiratory epithelial cells, (iii) flagellum that participates in mobility, adherence, and internalization events, (iv) extracellularly released molecules like proteases (responsible for the cleavage of key host proteins), exotoxin A (inhibition of host protein synthesis), exoenzyme S (induces cytotoxic effect), exoenzyme U (antiphagocytic effect), phospholipase C (cleavage of membrane phospholipids), pigments (many biological effects, like pyocyanin that induces free radicals in host cells), rhamnolipids (detergent action), soluble lectins (inhibition of beating of lung cells), and alginate (phagocytosis inhibition, antifungal action, and host immune responses)

151 the success of the infection. They degrade a wide array of host proteins, impairing  
152 host defenses and destroying physical barriers that normally prevent attachment and  
153 penetration of the bacteria [26–28].

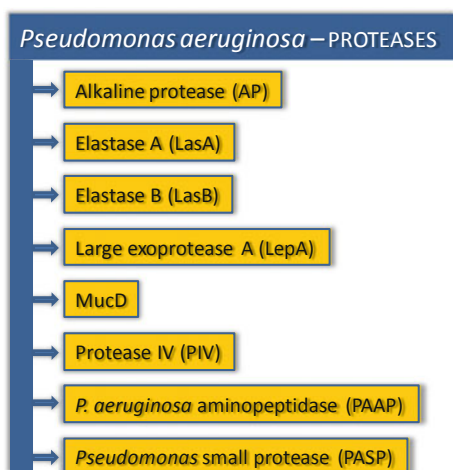
### 154 3 Proteolytic Enzymes Produced by *Pseudomonas* 155 *aeruginosa* 156

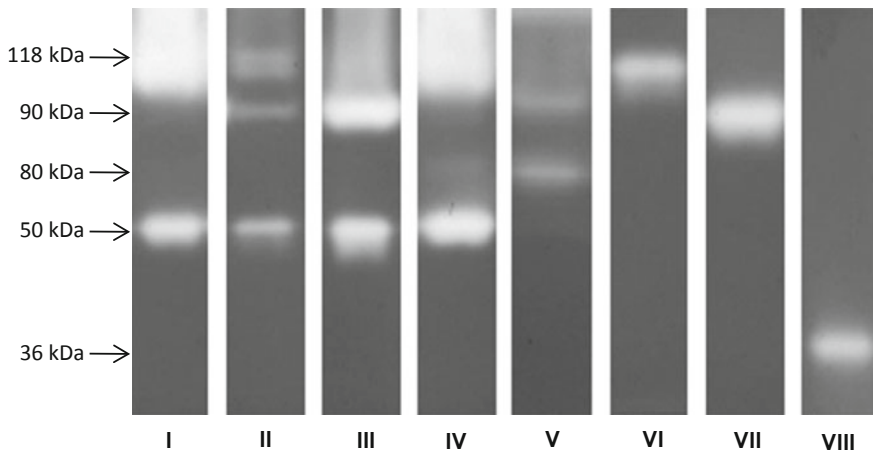
157 *P. aeruginosa* is able to extracellularly release different kinds of proteases (Fig. 3),  
158 which together are responsible for invasion and destruction of host tissues. Because  
159 of the relevant roles played by proteases on the physiopathology of *P. aeruginosa*,  
160 it has been shown that the majority of environmental and clinical strains of  
161 *P. aeruginosa* exhibited proteolytic activity, particularly elastase activity [29–31].  
162 According to Stover and co-workers [32], approximately 3% of the whole  
163 *P. aeruginosa* genome is composed by open reading frames that encode proteases  
164 [32]. Thus, the high genomic variability allows the bacterium to adapt its virulence  
165 arsenal machinery to support the variations of environment conditions, and for that,  
166 protease production in *P. aeruginosa* can vary greatly (Fig. 4) [32].

167 The expression of extracellular proteolytic enzymes in *P. aeruginosa* is directly  
168 influenced by environmental factors and changes in the physicochemical properties  
169 of culture medium (e.g., nutrients, temperature, pH, and aeration), which signifi-  
170 cantly modulate the production of these crucial virulence factors [26, 33]. In  
171 addition, the amount of protease produced depends on the cell cycle moment (e.g.,  
172 lag, exponential, or stationary growth phase) and on the growing lifestyle (e.g.,  
173 planktonic or biofilm). For instance, the total protease production (Fig. 5a) as well  
174 as the specific elastase secretion increases along the first 48 h of **in vitro** cultivation

AQ3

**Fig. 3** Proteases secreted by  
*P. aeruginosa* cells





**Fig. 4** Production of extracellular proteases in clinical isolates of *P. aeruginosa* recovered from different anatomical sites. The proteolytic profiles were characterized by sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin as the protein substrate. Profile I—118 + 50 kDa; Profile II—118 + 90 + 50 kDa; Profile III—90 + 50 kDa; Profile IV—118 + 80 + 50 kDa; Profile V—90 + 80 kDa; Profile VI—118 kDa; Profile VII—90 kDa, and Profile VIII—36 kDa

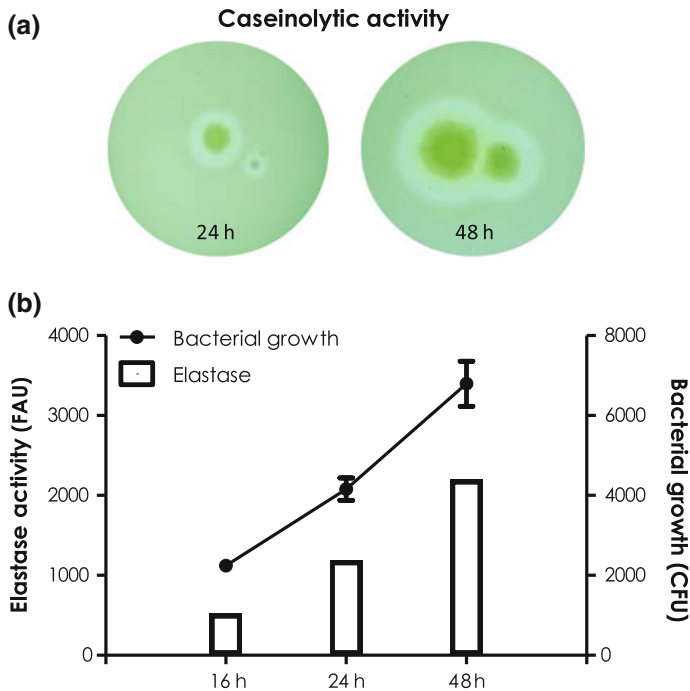
175 of *P. aeruginosa* planktonic cells (Fig. 5b). Further, according to Hastie and  
176 co-workers [34], after 85 h of bacterial growth, the elastase production dropped off.

### 177 3.1 Elastase B

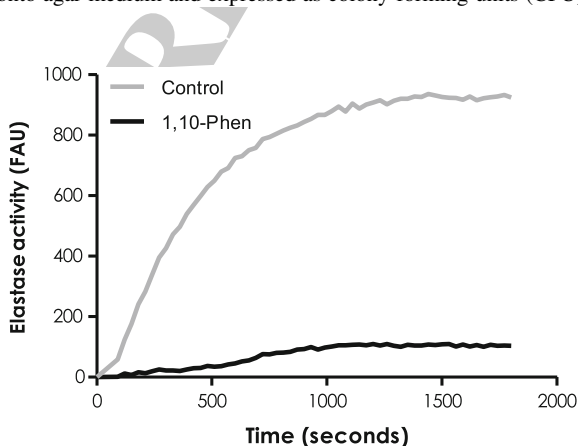
178 One of the best proteases characterized in *Pseudomonas* is elastase B (LasB), also  
179 known as pseudolysin. This 33-kDa enzyme belongs to the M4 thermolysin-like  
180 family of neutral, Zn-dependent metallo-endopeptidases (Fig. 6). This enzyme is  
181 encoded by *lasB* gene as a pre-pro-protein, containing at the N-terminal region a  
182 signal peptide of 23 amino acids that transport the enzyme through the inner  
183 membrane to periplasmic place by bacterial secretory system [35].

184 The first and the most studied substrate of elastase B is bovine and human elastin  
185 [36–38]. Some reports correlate the elastinolytic activity of elastase B to *Pseu-*  
186 *domonas* infections in cystic fibrosis patients [39–43]. Histological studies have  
187 detected altered elastin fibers in lung alveoli of cystic fibrosis patients on autopsy,  
188 indicating a probable elastase activity on cystic fibrosis lung [39]. In addition, the  
189 elastase activity is associated with vascular inflammation during *P. aeruginosa*  
190 infection, since the disorganization of elastin fiber in vascular tissue caused by  
191 protease degradation was observed [44]. Previously, our group analyzed the pro-  
192 duction of virulence attributes in 96 clinical strains of *P. aeruginosa* recovered from  
193 patients attended at hospitals located in three states of Brazil (Espírito Santo, Minas  
194 Gerais, and Rio de Janeiro), and it was shown that all bacterial strains exhibited a





**Fig. 5** Protease detection in *P. aeruginosa*. **a** Total extracellular protease production was analyzed by the degradation of casein (1%) incorporated into Luria Bertani agar medium up to 48 h at 37 °C. **b** The elastase activity was measured in the cell-free culture supernatant obtained from *P. aeruginosa* cells grown in tryptic soy broth up to 48 h at 37 °C, using the fluorogenic peptide substrate Abz-Ala-Gly-Leu-Ala-*p*-Nitro-Benzyl-Amide. Results were expressed as fluorescence arbitrary units (FAU). In parallel, the number of bacterial cells along each time point was evaluated by plating cells onto agar medium and expressed as colony-forming units (CFU)



**Fig. 6** Elastase of *P. aeruginosa* is a typical zinc-metalloprotease. The purified elastase B is able to cleave the fluorogenic peptide substrate Abz-Ala-Gly-Leu-Ala-*p*-Nitro-Benzyl-Amide along the time. Conversely, 1,10-phenanthroline (1,10-Phen), a metalloprotease inhibitor, at 10  $\mu$ M was able to block the substrate cleavage. FAU, fluorescence arbitrary units

195 homogeneous elastase activity, with an average of  $1069.28 \pm 213.95$  fluorogenic  
196 arbitrary units (FAU) with no correlation with the original anatomical site of iso-  
197 lation [16]. On the other hand, *P. aeruginosa* strains recovered from trachea, uri-  
198 nary tract, and wounds of patients attended at University Medical Center/Texas  
199 Tech Health Sciences Center were able to produce different amounts of elastase  
200 [45]. Woods and co-workers [46] showed that Canadian *P. aeruginosa* strains  
201 isolated from acute lung infections showed the highest production of elastase  
202 ( $0.053 \pm 0.021$  mg/ml) compared with elastase activity of strains isolated from  
203 burns, wounds, cystic fibrosis lung, and blood.

204 LasB is also able to cleave other host extracellular matrix proteins, such as  
205 collagen type III and IV. Interestingly, after subcutaneous injection of purified  
206 elastase B into mice, an intense degradation of basement membranes was observed,  
207 and elastase B was responsible for severe hemorrhage and tissue damage [47].  
208 Several studies have demonstrated that LasB-associated epithelial disruption is  
209 mediated by the attack to intracellular tight junctions and cytoskeleton reorgani-  
210 zation via inhibition of protein kinase C and activation of EGFR, ERK1/2 and  
211 NF $\kappa$ B, urokinase, and protease-activated receptor 2 (PAR-2) [48–53]. Elastase B  
212 can also interfere with the host bacterial clearance by degrading several components  
213 of innate and adaptive immune defense, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ),  
214 interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-2 (IL-2), monocyte chemotactic protein-1  
215 (MCP-1), and epithelial neutrophil activating protein-78 (ENA-78) [52–57]. In  
216 addition, it was shown that elastase B was efficient in the inactivation of key  
217 components of the complement system such as fluid-phase and cell-bound C1 and  
218 C3 and fluid-phase C5, C8, and C9 [44]. This multifunctional enzyme is also able to  
219 cleave surfactant protein A and D (SP-A and SP-D), also known as collectin. SP-A  
220 and SP-D are synthesized by alveolar type II epithelial cells and are responsible for  
221 the recognition and binding to oligosaccharides present on the cell surface of many  
222 bacteria to be phagocytized by host macrophages [58]. Previously, Meyer and  
223 co-workers [59] have reported that a decrease on the SP-A and SP-D levels in  
224 bronchoalveolar lavage (BAL) was observed in the lung of cystic fibrosis indi-  
225 viduals. Also, SP-D knockout mice were more sensible to *P. aeruginosa* corneal  
226 infections when compared to wild-type animals, and only the wild-type mice  
227 recovered completely of the infection [60]. Based on this, elastase B was suggested  
228 to be responsible for the SP-D degradation in the eye [25, 26]. Furthermore,  
229 pseudomonal elastase can interact with host adaptive immune system by degrading  
230 immunoglobulins [61–63]. Bainbrigde and Flick [61] showed that elastase B was  
231 able to cleave IgG molecules recovered from cystic fibrosis patients and the  
232 degradation products bound to IgG-receptors of human neutrophils, thereby  
233 inhibiting the opsonization of bacterial invaders. Lomholt and Kilian [63] reported  
234 the IgA degradation in tears from patients infected with *P. aeruginosa*. They also  
235 observed that isogenic mutants of *P. aeruginosa* knockout to either elastase or  
236 alkaline protease were not able to completely inhibit the IgA degradation, indicating  
237 that several proteases were working in concert to cleave IgA.

238 Furthermore, elastase B plays a key role in the differentiation of pseudomonal  
239 biofilms. Tielen et al. [64] showed that strains that overexpress *lasB* gene were not

240 able to form robust biofilms, and they observed the formation of few microcolonies  
241 after 72 h of contact with glass surface. Those authors also assigned that *lasB*-  
242 overexpressed strain shifted the composition of its extracellular polymeric sub-  
243 stances, reducing the alginate content as well as enhancing the rhamnolipids con-  
244 centration [64]. However, Yu et al. [65] demonstrated that elastase B is crucial for  
245 biofilm formation. They observed that  $\Delta lasB$  mutant decreased the biofilm for-  
246 mation through down-regulation of rhamnolipids synthesis.

### 247 3.2 Elastase A

248 Another extracellular protease produced by *P. aeruginosa* is elastase A (LasA),  
249 a metalloprotease that belongs to the subgroup A of M23 family of staphylolytic  
250 or  $\beta$ -lytic zinc metallo-endopeptidases. LasA is codified as an elastase A  
251 pre-pro-protein with molecular mass of 40 kDa [66, 67]. After its synthesis in  
252 intracellular bacterial environment, LasA is secreted via type II secretion machinery  
253 and when it is secreted to the extracellular space, LasA is immediately converted to  
254 its mature and active form of 27 kDa due to the cleavage by other pseudomonal-  
255 secreted endopeptidases, such as LasB, LysC, and protease IV [68, 69].

256 Elastase A is also called as staphylolysin, because it is able to cleave the pen-  
257 taglycine bonds in the peptidoglycan of *Staphylococcus aureus* [70]. As well, LasA  
258 degrades several glycine-rich synthetic peptides [71]. However, LasA exhibited a  
259 limited elastinolytic activity [72]. Kessler and co-workers [71] showed that LasA  
260 prefers cleaving Gly-Ala peptide bonds within the Gly-Gly-Ala sequences sur-  
261 rounded by apolar sequences. Such sequences are uncommon in elastin, resulting in  
262 low elastinolytic activity [26, 73]. Besides its own intrinsic elastinolytic activity,  
263 LasA enhances significantly the elastinolytic activity of other proteases, including  
264 LasB in *P. aeruginosa*, but also human leukocyte elastase and human neutrophil  
265 elastase [74, 75]. Moreover, LasA is responsible for inducing shedding of the host  
266 cell surface proteoglycan syndecan-1 (co-receptor proteins), which has been shown  
267 to be important for *P. aeruginosa* survival [25, 26].

### 268 3.3 Alkaline Protease

269 Another pseudomonal protein shown to be important for phagocytic evasion is  
270 alkaline protease (AprA), which is also known as aeruginolysin. Alkaline protease  
271 is a 50-kDa zinc-metalloprotease, member of subfamily B of the M10 peptidase  
272 family and metzincin superfamily. AprA, encoded by *aprA* gene, has a C-terminal  
273 secretion signal located within the last 50 amino acids residues necessary to be  
274 translocated and secreted by AprD, AprE, and AprM membrane proteins, which  
275 form the bacterial type I secretory machinery [35].

276 It was reported that alkaline protease is able to degrade a large number of host  
277 proteins, including fibronectin and laminin, important components of basal lamina  
278 and endothelium. Therefore, alkaline protease develops an important function in

279 invasion and hemorrhagic tissue necrosis in infections caused by *P. aeruginosa*  
280 [76]. Furthermore, this protease was found in many isolates of *P. aeruginosa*  
281 recovered from different human anatomical sites with especial elevated expression  
282 in clinical isolates from eyes, gastrointestinal tract, and mucoid wounds exacerbated  
283 in cystic fibrosis patients [25, 61]. AprA is important to bacterial escape from the  
284 host immunological defenses, degrading complement proteins (C1q, C2, and C3)  
285 and cytokines (IFN- $\gamma$ , TNF- $\alpha$  and IL-6) [76]. Also, alkaline protease and elastase B  
286 are able to inhibit chemotaxis of neutrophils and block efficiently the phagocytosis,  
287 which gives the pathogen an advantage in escaping from phagocyte cells that are  
288 one of the first lines of host defense mechanisms [25, 31, 77, 78]. Moreover,  
289 alkaline protease is able to inhibit flagellin recognition by TLR5 due to the  
290 degradation of free flagellin monomers, helping *P. aeruginosa* cells to avoid the  
291 immune detection [79]. This enzyme has also been shown to aid *P. aeruginosa*  
292 survival in iron limitations conditions during human infections by cleaving trans-  
293 ferrin that increase the siderophore-mediated iron uptake [80]. Gupta and  
294 co-workers [81] also reported that treatment of mouse corneal tissue with alkaline  
295 protease (50 ng) increases the binding of *P. aeruginosa* to the epithelial surface.

### 296 3.4 Protease IV

297 *P. aeruginosa* secretes a serine-type protease designated as protease IV (PIV) or  
298 lysyl endopeptidase (PrpL), a 26-kDa protease belonging to the chymotrypsin  
299 family S1 that has been demonstrated to be an important virulence factor in the  
300 rabbit cornea, but is found in clinical isolates recovered from all the anatomical sites  
301 analyzed [35, 82]. Its catalytic domain is formed by the triad His<sub>72</sub>, Asp<sub>122</sub>, and  
302 Ser<sub>198</sub>. Moreover, it was demonstrated that the residue Ser<sub>197</sub> adjacent to Ser<sub>198</sub> is  
303 critical to the catalytic activity [83]. Protease IV is encoded by *piv* gene (PA4175),  
304 with a full length of 48 kDa, which is initially expressed in the cytoplasm in a  
305 pre-pro-enzyme form and then processed to the 26-kDa mature protease after its  
306 secretion into the extracellular milieu [83].

307 PIV participates in the tissue invasion/damage processes and hemorrhagic events  
308 due to the cleavage of fibrinogen. It is well known that fibrinogen is required after  
309 vascular damage, but the degradation of fibrinogen by PIV leads to hemorrhage  
310 during *P. aeruginosa* infection [84]. PIV is also important to evade host immune  
311 defenses because it is able to degrade plasminogen, immunoglobulin, C1q and C3,  
312 and host antimicrobial peptide LL-37 [25, 68]. Furthermore, Malloy and co-workers  
313 [82] observed that PIV degrades the surfactant proteins, SP-A, SP-D, and SP-B, by  
314 a time- and dose-dependent way in cell-free bronchoalveolar lavage fluid. Those  
315 authors reported that degradation of SPs by protease IV reduced the association  
316 among bacteria and alveolar macrophage. Interestingly, the incubation of pul-  
317 monary surfactant with pseudomonal protease IV reduced the ability of the sur-  
318 factant to diminish the superficial tension within the lung [82]. Protease IV has been  
319 shown to be an iron-regulated protein, suggesting that its expression is regulated  
320 irrespective of *quorum sensing* system, which is distinct from other pseudomonal

321 proteases [69]. Protease IV has also been correlated to ring abscess lesions present  
322 in pseudomonal keratitis [68]. Corroborating this finding, Engel et al. [85] showed  
323 that protease IV-deficient mutants exhibited lower ocular virulence in rabbits when  
324 intrastromally infected.

### 325 **3.5 Pseudomonas Small Protease**

326 *P. aeruginosa* small protease (PASP) is described as a 18.5-kDa secreted  
327 zinc-dependent leucine aminopeptidase. *PASP* gene has been found in a large  
328 number of *P. aeruginosa* clinical strains, but its higher expression is found during  
329 the ocular infection [86]. Previous reports showed that *PASP* is found only in the  
330 bacterial supernatant culture. According to Tang and co-workers [86], the sequence  
331 of *PASP* gene appears to have a signal peptide consistent with that needed for type  
332 II secretion system.

333 Direct inoculation of purified *PASP* into the rabbit cornea causes severe ocular  
334 pathology, including epithelial erosion and ulcer in stroma, edema, and neutrophil  
335 infiltration into the corneal stroma [87]. *PASP* has also been demonstrated to  
336 cleavage host proteins required for maintaining structure of cornea, such as colla-  
337 gens, fibrinogen (but not fibrin), complement C3, and antimicrobial peptide LL-37.  
338 Studies of *PASP*, coupled with those of *PIV*, strongly support the hypothesis that  
339 *Pseudomonas* proteases play a major role in keratitis [87].

### 340 **3.6 Large Exoprotease A**

341 Large exoprotease A (*LepA*) is an exoprotease with molecular mass of ~100 kDa  
342 produced by *P. aeruginosa*. *LepA*, as well as thrombin and trypsin, cleaves human  
343 protease-activated receptors (PARs) 1, 2, and 4 in order to activate the critical  
344 transcription factor NF- $\kappa$ B, which is associated with host inflammatory and  
345 immune responses [49, 88].

### 346 **3.7 MucD**

347 *MucD* was reported to be a serine endoprotease that is localized within the  
348 periplasmic space. Data suggest that *MucD* induced a significant reduction on the  
349 levels of IL-1 $\beta$ , neutrophil-chemoattractant chemokines KC, and macrophage-  
350 inflammatory protein-2 (MIP-2) in the early stages of bacterial infection as well as it  
351 inhibited the recruitment of polymorphonuclear (PMN) cells into the cornea. Fur-  
352 thermore, a decrease in PMN cells recruited to infection site favored the estab-  
353 lishment of infection by *P. aeruginosa*. *MucD* may be secreted to the extracellular  
354 space, interfering with the biological functions of cytokines and chemokines, but  
355 further investigation is needed to understand the mechanisms underlying the role of  
356 *MucD* in keratitis [89, 90].

### 3.8 Aminopeptidase

The *P. aeruginosa* aminopeptidase (PAAP) or leucine aminopeptidase has been speculated as complementary enzyme to the activity of other endopeptidases. PAAP has an important function in bacterial physiology; it acts releasing free amino acids/small peptides from protein fragments produced by the others *P. aeruginosa* endopeptidases, thereby providing low molecular mass nutrients that can be taken up by the bacterium, which in turn may promote bacterial growth and proliferation [26].

## 4 Conclusions

*P. aeruginosa* is a metabolically versatile bacterium that can cause a wide range of severe opportunistic infections in hospitalized patients. To cause this huge variety of infections, *P. aeruginosa* has an arsenal of proteases that are involved in critical events of bacterial pathogenicity and virulence, which are important for survival in the host, tissue invasion, and evasion of host immune defenses. Therefore, this review has highlighted the importance of each pseudomonal protease in bacterial physiology and/or in infectious events. In this context, inhibitors able to block the proteases produced by *P. aeruginosa* cells would represent a new drug class quite promising to combat this widespread bacterial pathogen.

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