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REVIEW

Armand-Frappier Outstanding Student Award — Role of ATP-dependent proteases in antibiotic resistance and virulence[1]

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Abstract: ATP-dependent proteases are found in nearly all living organisms and are known to play important roles in protein quality control, including protein degradation and protein refolding. ATP-dependent proteases have been well characterized in *Escherichia coli*. However, in the opportunistic human pathogen *Pseudomonas aeruginosa*, the role of these proteases is only starting to be understood. This review will discuss the most recent research regarding the role of ATP-dependent proteases, particularly Lon and ClpP, in *P. aeruginosa*. These studies have revealed that despite the fact that they are not traditional regulators, these proteases are involved in regulating a multitude of processes, including antibiotic resistance and virulence, implicating a broad array of functions that these intracellular proteases have in *Pseudomonas*. These results are also relevant in the context of drug therapy, since ClpP and Lon are good candidates to become novel therapeutic targets to combat *Pseudomonas* infections.

Key words: ATP-dependent proteases, Lon protease, antibiotic resistance, virulence, *Pseudomonas aeruginosa*.

Résumé : Les protéases dépendantes de l'ATP se trouvent dans presque tous les organismes vivants et elles sont reconnues pour jouer des rôles importants dans le contrôle de la qualité des protéines, notamment dans la dégradation des protéines et le repliement des protéines. Les protéases dépendantes de l'ATP ont été bien caractérisées chez *Escherichia coli*. Toutefois, le rôle de ces protéases commence seulement à être compris chez le pathogène opportuniste de l'humain *Pseudomonas aeruginosa*. Cet article de synthèse discutera de la recherche la plus récente en ce qui concerne le rôle des protéases dépendantes de l'ATP, particulièrement Lon et ClpP, chez *P. aeruginosa*. Ces études ont révélé que malgré le fait qu'elles ne soient pas des régulateurs traditionnels, ces protéases participent à la régulation d'une multitude de processus incluant la résistance aux antibiotiques et la virulence, ce qui veut dire que ces protéases intracellulaires exercent un large spectre de fonctions chez *Pseudomonas*. Ces résultats sont aussi importants dans le contexte de la thérapie médicamenteuse puisque ClpP et Lon sont de bons candidats pour devenir de nouvelles cibles thérapeutiques afin de combattre les infections à *Pseudomonas*. [Traduit par la Rédaction]

Mots-clés : protéases dépendantes de l'ATP, protéase Lon, résistance aux antibiotiques, virulence, *Pseudomonas aeruginosa*.

Introduction

ATP-dependent proteases belonging to the AAA+ (*A*TPases *a*ssociated with a variety of cellular *a*ctivities) family, for example, Lon, Clp, HslU, and FtsH, were first described in *Escherichia coli* (Swamy and Goldberg 1981). However, ATP-dependent proteases are widespread in nature and can be found in almost all living organisms, including eubacteria, archaebacteria, and eukaryotes (Snider et al. 2008). This review will focus mainly on the Lon and ClpP proteases from *Pseudomonas aeruginosa* and will highlight their importance in antibiotic resistance, the cellular stress response, virulence-related phenotypes, and virulence in this microorganism.

Pseudomonas aeruginosa is a major opportunistic human pathogen. Indeed, this Gram-negative bacterium is the third leading cause of nosocomial infections in North America, causing, for example, ventilator-associated pneumonia and urinary tract infections. Furthermore, *P. aeruginosa* chronic infections in cystic fibrosis patients eventually lead to lung damage and respiratory failure and have been associated with increased morbidity and mortality (Hutchison and Govan 1999; Gibson et al. 2003). *Pseudomonas* infections are generally difficult to eradicate owing to the high intrinsic resistance of this microorganism, together with its ability to develop resistance to common antibiotics through adaptation and mutation. For this reason, *P. aeruginosa* is referred to as a "Superbug", i.e., a microorganism resistant to most antibiotics available in the market. For a more detailed description of antibiotic resistance mechanisms in *P. aeruginosa* please refer to our recent review article (Breidenstein et al. 2011). Since the resistance rates and the number of *Pseudomonas* infections have been increasing over the last few years, according to reports from the National Nosocomial Infections Surveillance system, it is becoming clear that *P. aeruginosa* represents a major problem in Western society (Gaynes and Edward 2005). In this context, the search for new antibiotics and novel drug targets for antipseudomonal therapy is an important endeavour but poses a big challenge. ATP-dependent proteases have been demonstrated to fulfill the criteria for good drug targets in other pathogens, and here we will discuss the case for *P. aeruginosa*.

Structural characteristics of ATP-dependent proteases

As mentioned above, ATP-dependent proteases like Lon, ClpXP, and ClpAP belong to the family of AAA+ proteases. Lon is an 87 kDa cytoplasmic serine protease that associates into hexameric rings in Gram-negative bacteria and exhibits a Ser^{679}-Lys^{722} dyad,

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which is responsible for its catalytic activity (Botos et al. 2004). Lon is present in a wide range of microorganisms, but some bacterial species, such as *Listeria monocytogenes* and *Staphylococcus aureus*, lack the Lon protease. Lon proteases are divided into 2 subfamilies: LonA (*E. coli* and *P. aeruginosa*) and LonB (*Archaeglobus fulgidus*). LonA subfamily members contain an N-terminal domain for substrate recognition, an ATP-binding domain, and a proteolytically active C-terminal domain for cleavage, whereas members of the LonB subfamily lack the N-terminal domain and have instead a membrane domain acting as an anchor (Rotanova et al. 2006). Although most bacteria only have 1 type of Lon, some species, like *Bacillus subtilis*, possess both types of Lon proteases (Rotanova et al. 2004). Furthermore, Lon and other ATP-dependent proteases bear the group name "self-compartmentalized" or "chambered" proteases, since the substrate cleavage site is sequestered within a digestion chamber. There is only restricted access to the active site, and only unfolded substrates can enter the chamber for cleavage. The Lon protease consists of a homosubunit complex, indicating that the ATPase and the proteolytic active sites are formed by a single polypeptide chain (Butler et al. 2006). In contrast, the ATP-dependent serine protease complexes ClpXP and ClpAP (clp: *c*ase*i*no*l*ytic *p*rotease), for example, are organized in a heterosubunit complex, wherein the N-terminal ATP-binding domain and the proteolytic domain are found in different polypeptide chains. The ATP-binding proteins ClpX and ClpA, also known as translocases, recognize specific peptide sequences (tags) of unfolded proteins (Siddiqui et al. 2004; Hinnerwisch et al. 2005; Baker and Sauer 2006). Upon recognition, these subunits bind to the tags and translocate the unfolded protein into the proteolytic chamber of the self-compartmentalized ClpP protease. ClpP then proteolytically degrades the unfolded protein chain into small peptides (Thompson et al. 1994). In the cytoplasm, bacterial cells also have adaptor proteins responsible for binding the target proteins and subsequently transporting them to the protease complexes for degradation (Sauer et al. 2004). One such adaptor protein is ClpS, which is known to interact with ClpA (Dougan et al. 2002). ClpS recognizes and binds to specific substrates, so-called N-degrons (containing leucine, tyrosine, phenylalanine, or tryptophan residues), from the N-end rule pathway (Erbse et al. 2006; Roman-Hernandez et al. 2011) and delivers them to the ClpAP complex for degradation. In doing so, it inhibits the degradation of other substrates that are usually recognized by ClpA, as ClpS accesses the same N-terminal binding domain as other ClpA substrates. Thus, in the presence of ClpS, the ClpAP complex degrades N-degrons, whereas in the absence of ClpS, this complex degrades other unfolded proteins (Roman-Hernandez et al. 2011).

Notably, under normal growth conditions the Lon and ClpP proteases are responsible for 70%–80% of energy-dependent protein degradation in *E. coli* (Maurizi 1992). Thus, these ATP-dependent proteases can degrade unstable and misfolded proteins by translocating them into the proteolytic chamber, where peptide bond cleavage occurs. The *P. aeruginosa* Lon and ClpP proteases have, respectively, 84% and 86% sequence similarity to their *E. coli* counterparts (www.pseudomonas.com), and this high level of identity among evolutionarily divergent proteins implies critical functional attributes. Although intriguingly unlike other highly conserved proteins, these ATP-dependent protease subunits are dispensable under normal broth growth conditions, and mutants lacking them remain viable. It appears likely that, as for other bacteria, these intracellular proteases play a significant role in the degradation of proteins in *P. aeruginosa*. This role might be partly redundant, but evidence suggests that the ATP-dependent proteases also have specific downstream effects on aspects of virulence, antibiotic resistance, and metabolism.

Effect of protease disruption on antibiotic susceptibility

Outer membrane permeability in *P. aeruginosa* is remarkably low, approximately 12–100 times less than that of *E. coli* (Hancock 1998), constituting 1 of the main mechanisms underlying intrinsic antibiotic resistance in this bacterium. Nevertheless, while low outer membrane permeability leads to a reduced rate of drug uptake, equilibration of hydrophilic molecules across the outer membrane is still managed within seconds. This clearly indicates that the high intrinsic resistance of *Pseudomonas* also relies on other secondary mechanisms, such as inducible AmpC β-lactamase production (Masuda et al. 1999) and constitutive and inducible expression of efflux pumps (RND and MexAB-OprM/MexXY-OprM) (Li et al. 1995, 2000). Additionally, *P. aeruginosa* exhibits acquired and adaptive resistance (for review, Breidenstein et al. 2011). However, it is increasingly evident that other, yet unknown, mechanisms contribute to antibiotic resistance in this pathogen. Recent studies have identified new candidate genes involved in altered antibiotic resistance to several antibiotic classes (fluoroquinolones, aminoglycosides, and β-lactams) by screening mutant libraries of *P. aeruginosa* (Brazas et al. 2007; Breidenstein et al. 2008; Schurek et al. 2008; Dotsch et al. 2009; Alvarez-Ortega et al. 2010). The genes identified in these screenings belong to a wide range of functional classes, including the ATP-dependent proteases. For instance, the disruption of the genes encoding the Lon (PA1803) and ClpP (PA1801) proteases independently leads to supersusceptibility to the fluoroquinolone ciprofloxacin (Brazas et al. 2007; Breidenstein et al. 2008), while mutations affecting the adaptor protein ClpS confer resistance to β-lactam antibiotics, such as piperacillin, imipenem, aztreonam, and ceftazidime (Alvarez-Ortega et al. 2010; Fernandez et al. 2012). Overall, the involvement of intracellular proteases in the antibiotic resistance of *P. aeruginosa* has not been fully determined. However, recently Breidenstein et al. (2012*a*) determined that the Lon protease participates in ciprofloxacin supersusceptibility by modulating the SOS DNA-damage response.

Role of the Lon protease in the SOS response triggered by ciprofloxacin and involvement in cell division

The SOS stress response is known to be an important transcriptional response to DNA damage that has been best characterized in *E. coli*. The *E. coli* DNA-damage repair system comprises 43 genes (Courcelle et al. 2001), whereas, to date, only 15 genes in the *P. aeruginosa* genome have been identified to be directly involved in the SOS response of this bacterium (Cirz et al. 2006). The SOS regulon is controlled by the repressor LexA and includes the genes *recA*, *sulA*, *lexA*, *recN*, *recX*, and *dinG*, among others. The promoter regions for each of these genes contain a consensus binding sequence for LexA (SOS box) that comprises 16 nucleotides: CTGTATAAATAACAG (the underlined nucleotides are 100% conserved), with the *P. aeruginosa* site having high homology to that found in *E. coli* (Cirz et al. 2006). During normal growth, the SOS response genes are negatively regulated by the autorepressor LexA, which binds to the SOS box located upstream of each gene. Therefore, depending on how strong this binding is, no or only slight transcription of the SOS response genes occurs. Upon DNA damage (from treatment with ciprofloxacin or mitomycin C, UV light exposure, etc.), RecA forms filaments with the single-stranded DNA while stimulating autoproteolysis of the transcriptional repressor LexA (Butala et al. 2009). As a result, all SOS repair genes, including *recA*, are transcribed to assist in overcoming DNA damage. Once the damage has been repaired, LexA binds again to the SOS box, thereby repressing the expression of the genes involved in the SOS response.

Studies in *E. coli* have demonstrated that the Lon protease is involved in degrading the cell division inhibitor SulA once the DNA damage has been repaired (Mizusawa and Gottesman 1983). However, the mechanism by which Lon affects the supersusceptibility of *P. aeruginosa* to ciprofloxacin, an antibiotic commonly used for treatment in the clinic, was only recently elucidated (Breidenstein et al. 2012*a*). Thus, microarray and follow-up RT-qPCR analysis revealed that the Lon protease is important for full induction of the SOS response upon exposure to ciprofloxacin, a DNA damaging agent. Generally, LexA–RecA and SulA are highly upregulated in response to environmental stress to overcome DNA damage and to inhibit cell division of cells containing damaged DNA, respectively. In a *lon*-deficient mutant, it is likely that the DNA damage is not repaired as effectively as in the wild type, since the SOS response was observed to be considerably weaker.

One prominent *lon* mutant phenotype (from which the gene gets its name) is filamentation due to defective cell division leading to abnormal cell elongation. The Lon protease is likely to work through SulA to regulate cell division, leading to this phenotype (Brazas et al. 2007). Thus in wild-type cells, the Lon protease is involved in degrading the cell division inhibitor SulA. However, in *lon* mutants this degradation does not occur and thus cell division is blocked.

Nevertheless, Lon does not appear to act through SulA to regulate the transcriptional levels of SOS response genes. Indeed, a strain overexpressing *sulA* did not show the increased susceptibility to ciprofloxacin nor dysregulation of the SOS regulon, as observed in a *lon*-deficient strain (Breidenstein et al. 2012*a*). Therefore, it seems that the Lon protease might be acting at the protein level by cleaving other proteins involved in the SOS response. By using SDS–PAGE and Western immunoblots with specific antibodies (Breidenstein et al. 2012*a*), it was demonstrated that RecA, a key player in the SOS response, does not accumulate in the *lon*-deficient strain upon subinhibitory ciprofloxacin exposure. This suggests that the cellular levels of RecA present in the mutant are significantly lower than in the wild type and would fail to induce the autocleavage of the repressor LexA, which is necessary for the activation of the SOS system. Thus, it seems clear that Lon is important for RecA function. A model has been proposed based on the known repressors of RecA, such as RecX, RecR, and RdgC (Umezu et al. 1994; Stohl et al. 2003; Drees et al. 2004, 2006), by which it is suggested that these are inhibited by the Lon protease. This inhibition would enable autoamplification of RecA and subsequent DNA damage repair. In the case where the Lon protein is inactive owing to mutation, the repressor proteins would stay intact, limited RecA amplification would occur, and damage would not be adequately repaired. This would ultimately lead to supersusceptibility to DNA damaging agents (fluoroquinolones, UV light, etc.), a phenotype also shared by mutants in genes involved in the SOS response and DNA repair (*recA*, *recN*, and *recG*) (Kidambi et al. 1996; Brazas et al. 2007; Breidenstein et al. 2008).

In conclusion, the Lon protease appears to modulate the levels of specific proteins involved in the SOS response, thereby promoting resistance to DNA-damaging agents like fluoroquinolone antibiotics, but this appears to be independent of its effects on SulA and cell division.

Proteases play crucial roles in adaptation to physiological and environmental changes

Bacteria are exposed throughout their life cycles to changing environmental conditions. For example, they may encounter physiological stress conditions, such as changing pH, the presence or absence of oxygen and different nutrients, osmotic shock and temperature shifts, among others. Therefore, bacteria exhibit regulatory mechanisms that have specifically evolved to adapt to the changing environment, and certain regulatory proteins are known to play crucial roles in this adaptation process. Traditionally these regulators bind to DNA and influence gene expression directly, and the list of genes that they regulate through binding to their promoter regions or through the dysregulation of other regulators is termed a regulon. ATP-dependent proteases have also been demonstrated to contribute to adaptation in diverse bacterial species, including *P. aeruginosa*, but they do not bind to DNA and instead are unconventional regulators acting presumably by modulating the stability of more conventional transcriptional regulators. An example of this was provided above, whereby Lon regulates cell division and the DNA damage response by influencing the stability of the SulA and (through RecA) LexA transcriptional regulators, respectively.

In many bacteria, the Lon protease also belongs to the heat-shock regulon and is the main protease involved in the degradation of misfolded proteins (Gottesman 1996). Misfolding occurs frequently during heat shock, and therefore, it is necessary that the Lon protease is present in high quantities under these conditions to prevent protein aggregate accumulation inside the cell. Indeed, *lon* expression is induced during heat shock at 42 °C, which is consistent with the fact that the Lon protease is required to unfold misfolded proteins and for their subsequent degradation (E.B.M. Breidenstein and R.E.W. Hancock, unpublished data). Examples of known Lon target proteins in *E. coli* include SulA (cell division inhibitor) (Mizusawa and Gottesman 1983), RcsA (transcriptional activator for capsule synthesis) (Torres-Cabassa and Gottesman 1987), the antitermination protein (N protein) of phage λ (Maurizi 1987), and certain short-lived regulatory proteins (Gottesman 1996). Furthermore, *lon* expression is induced under subinhibitory and lethal aminoglycoside exposure, which can impact on microbial persistence (Marr et al. 2007). Intriguingly, *P. aeruginosa* has another ATP-dependent protease, AsrA (*a*minoglycoside-induced *s*tress *r*esponse *A*TP-dependent protease), encoded by the gene PA0779, which has 60% similarity to the Lon protease. It was recently shown to be highly upregulated by bacteriostatic and lethal concentrations of tobramycin and to control the heat-shock stress response to tobramycin in *Pseudomonas* (Kindrachuk et al. 2011). Indeed, this protease has been proposed as a key mediator of the heat-shock response. Several ATP-dependent proteases are present in *P. aeruginosa*, and although they have overlapping functions, they also have quite distinct properties (Fernandez et al. 2012). This is consistent with our observations that while the Lon protease is involved in general stress responses, it also plays distinct and specialized roles in the bacterial cell.

Involvement of the Lon protease in virulence-related properties

It is well documented that the ability of *Pseudomonas* to form biofilms and to move in different environments (motility) contributes to the virulence of this microorganism. To date, 4 different types of motility have been described in *P. aeruginosa*, namely swarming, swimming, twitching, and surfing. Swimming motility occurs in aqueous environments and is mediated by the single polar flagellum normally present in *Pseudomonas* cells (Kohler et al. 2000; Dasgupta et al. 2003), whereas twitching motility relies on the presence of type IV pili. Twitching enables bacteria to pull themselves along surfaces through extension, surface adherence, and retraction of pili (Skerker and Berg 2001). Swarming motility is a social behaviour displayed by bacterial cells when growing on a surface of certain viscosity in the presence of poor nitrogen sources, such as amino acids, and is dependent on both type IV pili and flagella (Overhage et al. 2008). A fourth distinct type of motility, surfing, was only recently described as being dependent on the presence of mucin, a glycoprotein present at mucosal surfaces. Like swarming, surfing is a complex adaptation that involves the coordinated expression of hundreds of genes. However,

unlike swarming, surfing motility is flagella- but not pili-dependent (Yeung et al. 2012).

In addition to their involvement in antibiotic resistance, the *P. aeruginosa* ATP-dependent proteases Lon and ClpP and adaptor protein ClpS are important in motility, which is considered a virulence-related property (Marr et al. 2007; Fernandez et al. 2012). For example, *lon* mutants exhibit a drastic deficiency in swarming motility. Additionally, *lon* mutants have a moderate deficiency in swimming and also major twitching and surfing deficiencies (Marr et al. 2007; Breidenstein et al. 2012*b*). Thus, the Lon protease seems to play a major role in modulating motility, although the molecular mechanisms behind the involvement of the Lon protease in these processes are still largely unknown, since only a few flagella and pili genes are dysregulated in a *lon* mutant (E.B.M Breidenstein and R.E.W. Hancock, unpublished data). Interestingly, the ClpP and ClpS proteins also play a critical role in motility. Thus, the motility phenotypes displayed by *clpP* mutants are fairly similar to those of *lon* mutants, as they exhibit a swarming, swimming, and twitching deficiency (Fernandez et al. 2012), although the influence of ClpP on the newly described surfing motility has not yet been examined. ClpS-deficient mutants exhibit a swarming defect and a slightly reduced ability to swim, indicating that ClpS also impacts on motility. In contrast, 7 other probable ATP-dependent proteases as well as ATP-binding subunits of the ClpP protease do not seem to be involved in motility, as mutation of these genes does not alter the motility of *P. aeruginosa* (Fernandez et al. 2012). These results demonstrate that ATP-dependent proteases can play distinct roles in the cell depending presumably on their specific targets.

Biofilm formation is a "social" behaviour that involves the coordinated formation of structured microbial aggregates on surfaces. It requires the involvement of flagella, pili, and a large array of genes, including several traditional transcriptional regulators (Klausen et al. 2003). It is thought to be highly medically pertinent, since more than 65% of all infections involve biofilms. Biofilm formation is initiated by flagella motility that allows planktonic (free-swimming) cells to approach a surface, then attach more strongly, and finally grow into a heterogeneous mushroom-shaped structure held together by a matrix of extracellular polysaccharides, proteins, and DNA. Cells in biofilm growth mode are notably (10- to 1000-fold) resistant to the action of most antimicrobials (Hoyle and Costerton 1991; O'Toole and Kolter 1998; Costerton et al. 1999; Whiteley et al. 2001). This has been attributed to a variety of factors, including restricted penetration into the extracellular matrix, antibiotic-destroying enzymes trapped and concentrated in this matrix, the presence of highly resistant "persister" cells, stress responses within the biofilm, or the stationary growth phase within the biofilm that limits the action of those antibiotics that can only kill logarithmically growing cells (Lewis 2001; Mah and O'Toole 2001; Spoering and Lewis 2001; Pamp et al. 2008).

As a social phenomenon, biofilm formation is controlled in part by quorum sensing. Quorum sensing is an intercellular communication system where a certain threshold of bacteria is required to produce a strong signal. Once this threshold is reached, autoinducer signal molecules achieve sufficiently high concentrations to enter neighbouring cells and trigger changes in their gene expression patterns (Schuster and Greenberg 2006). *Pseudomonas aeruginosa* produces 2 homoserine lactone autoinducer molecules: 3-oxododecanoyl homoserine lactone (product of the Las system) and *N*-butyryl homoserine lactone (product of the Rhl system) as well as the *Pseudomonas* quinolone signal 3,4-dihydroxy-2-heptylquinoline (Smith et al. 2002; Diggle et al. 2006). The homoserine lactone regulatory systems LasRI and RhlRI are regulated at the transcriptional level by LasR and RhlR, respectively. The LasRI system is able to regulate the RhlRI system, implicating a regulatory cascade or hierarchy (Brint and Ohman 1995). The Lon protease is a negative regulator of quorum sensing in *P. aeruginosa*. In particular, Takaya et al. (2008) showed that the Lon protease represses LasRI expression by degrading the autoinducer synthase LasI. The degradation of LasI, in turn, leads to the negative regulation of the RhlRI system, and as a result, *lon* mutants have increased levels of RhlR. With this in mind, it is not surprising that these mutants overproduce the quorum-sensing-regulated pyocyanin. Additionally, the connection between quorum-sensing signalling and biofilm formation also explains to a certain extent the altered biofilm formation observed in *lon*-deficient mutants. Interestingly, the biofilm-forming phenotype of *lon* mutants is strain dependent. Thus, a PA14-based *lon* mutant showed a strong increase in biofilm formation compared with the wild type (E.B.M. Breidenstein and R.E.W. Hancock, unpublished data), whereas the mutation of *lon* in a PAO1 strain background leads to decreased biofilm formation (Marr et al. 2007). This discrepancy in biofilm formation between the 2 strain backgrounds is in accordance with previously published data on the relationship between the 2 surface-associated behaviours — swarming and biofilms — in strains PA14 and PAO1. Thus, these 2 processes are often, but not always, coregulated in PAO1 mutant strains (Overhage et al. 2008), whereas there is often an inverse regulation in strain PA14 (Caiazza et al. 2007; Yeung et al. 2010). In addition to Lon, mutants in the ClpP and ClpS proteases (which are motility deficient) also exhibited a deficiency in biofilm formation. Swarming motility and biofilm formation have been implicated as important for in vivo growth, and therefore, the altered phenotypes displayed by strains carrying mutations in ATP-dependent proteases hint that these protease mutants might to some extent control virulence.

While the Lon and ClpP proteases have been previously shown to be involved in motility and biofilm formation in other bacteria, the involvement of ClpS in these processes is novel and was only recently demonstrated (Fernandez et al. 2012). Overall, intracellular proteases exert regulatory functions affecting a multitude of processes that go far beyond their expected role in stress responses.

Pathogenicity is influenced by intracellular proteases

Apart from the virulence-related defects observed in *lon* and *clpP* mutants in various microorganisms, several studies have demonstrated a role for these proteases in virulence in vivo. For instance, a *clpP* mutant in *S. aureus* has been shown to exhibit strongly decreased virulence. Likewise, ClpP has been demonstrated to be required for virulence in a skin abscess mouse model (Frees et al. 2003). Thus, fewer or no lesions were observed when a *clpP* mutant was injected into mice, when compared with the wild type, and fewer mutant strain bacteria were recovered from lesions compared with the wild type. Similarly, *lon* mutants also have reduced pathogenicity in Gram-negative bacteria, such as *Salmonella enterica* (Takaya et al. 2003), *Campylobacter jejuni* (Cohn et al. 2007), and *P. aeruginosa* (Breidenstein et al. 2012*b*). In *S. enterica*, the Lon protease has been shown to be important for systemic infection of mice and regulates bacterial survival within macrophages (Takaya et al. 2003). Indeed, *lon* mutants cannot survive and proliferate in murine macrophages. Recently, the Lon protease has been shown to contribute to pathogenesis in *P. aeruginosa* infections (Breidenstein et al. 2012*b*), including both acute and chronic infection models. The acute infection mouse model indicated that mice infected with the *lon* mutant exhibited only minor signs of stress 4 h postinfection, whereas mice infected with the same numbers of the wild-type strain appeared very sick and stressed. This was consistent with the observation that a lower bacterial load from the bronchoalveolar lavage was recovered from the mutant strain compared with the wild type. Furthermore, a chronic infection model in rats, established by encasing *Pseudomonas* in agar beads that were infiltrated into the lungs, showed that the *lon* mutant had a 500-fold reduced ability to compete with the wild-type strain

in vivo, whereas no difference in maintenance could be observed during in vitro growth. The ability to withstand killing by amoebae has been related to virulence (Alibaud et al. 2008). It was observed that compared with the wild-type strain, *P. aeruginosa lon* mutants were more susceptible to phagocytosis by amoebae and required a smaller number of amoebae for plaque formation. Overall, the Lon protease seems to contribute to establishment of infection and bacterial colonization. In contrast, Lon exerted the opposite effects in the plant pathogen *Pseudomonas syringae*, which exhibited an increase in virulence for plants upon mutation of the Lon protease (Bretz et al. 2002). In particular, it was shown that tissue necrosis occurred 3 h earlier with the *lon* mutant than with the wild type. This discrepancy might relate to the different hosts or pathogens and highlights that the regulation mediated by the Lon protease may have variable impacts.

To understand the participation of such proteases in pathogenicity, it is important to analyze whether intracellular proteases like Lon can affect the expression of virulence factors. In *P. aeruginosa*, the secretion of virulence proteins occurs mainly through 4 types of secretion systems (Type I, II, III, VI) that lead to secretion of virulence factors into the extracellular milieu or directly into the cytosol of the host cell. In particular, the Type III needle-like contact-dependent secretion apparatus, involving around 40 genes, plays an important role in virulence in *P. aeruginosa*. The exoproteins (e.g., ExoU, ExoT, ExoY, and ExoS), which are known to contribute to lung tissue damage, are transported through an assembled needle. For a detailed review on Type III secretion please refer to Hauser (2009). Interestingly, we demonstrated that several selected genes involved in Type III secretion were downregulated in a *lon* mutant (Breidenstein et al. 2012*b*). This is consistent with the possibility that Lon participates in virulence, to some extent, by enabling the full expression of Type III effector proteins. Similar results were observed in *Yersinia*, in which the lack of Lon was associated with a lower expression of Type III secretion proteins and reduced virulence (Jackson et al. 2004). In contrast, a *P. syringae lon* mutant exhibited an increase in Type III secretion system expression, which is consistent with an increase in virulence as mentioned previously (Bretz et al. 2002).

ATP-dependent proteases as potential targets for antimicrobial therapy

As discussed above, ATP-dependent proteases influence virulence in several microorganisms, including *P. aeruginosa*. For this reason, it seems possible that ATP-dependent proteases could be used as targets for antimicrobial therapy. Indeed, a recent study showed that the *S. enterica* Lon protease can be inhibited by the peptidyl boronate compound MG262, which is a proteasome inhibitor and is commonly used for inhibiting the activation of a key transcription factor in host innate immunity, NFκB (Frase et al. 2006). Both the peptide and the boronic acid moieties appear to be important for the inhibition of Lon. In screening a series of commercially available peptide-based proteasome inhibitors, MG262 was demonstrated to be the most potent inhibitor of *S. enterica* Lon activity. However, this study did not involve specific design but rather the screening of a series of molecules designed for other targets, and further research is necessary to design compounds that specifically target bacterial Lon, without affecting the mammalian proteasome, which would cause potential toxicity for man. Other potent inhibitors, the β-lactones, target the ClpP ATP-dependent protease of *S. aureus*, which is also involved in virulence. By inhibiting ClpP, these inhibitors lead to a drastic decrease in the expression of virulence factors (Bottcher and Sieber 2008).

The participation of Lon proteases in virulence also makes them interesting targets for live vaccine development. Interestingly, immunization with a *Salmonella lon*-deficient strain has been shown to protect mice against a subsequent oral challenge with a virulent *Salmonella* strain and even a challenge with virulent *Listeria monocytogenes* (Matsui et al. 2003).

To date, there are no known inhibitors for the *P. aeruginosa* Lon protease; however, the involvement of the ATP-dependent Lon protease in the virulence of this pathogen indicates that it could be a good antimicrobial target. Indeed, mutation of the *lon*-encoding gene in *P. aeruginosa* leads to a significant reduction in virulence, as well as lower resistance to fluoroquinolones (Brazas et al. 2007; Breidenstein et al. 2008, 2012*b*). The very high homology between *S. enterica* and *P. aeruginosa* Lon proteases indicates the possibility that inhibitors like MG262 could serve as templates. Nonetheless, further research is still required to test the efficacy of MG262 to inhibit *P. aeruginosa* Lon and to identify new potential inhibitors based on this template. In addition, further studies should be undertaken to investigate whether a *P. aeruginosa lon* mutant would be a good candidate for the development of a live vaccine against *Pseudomonas* infections.

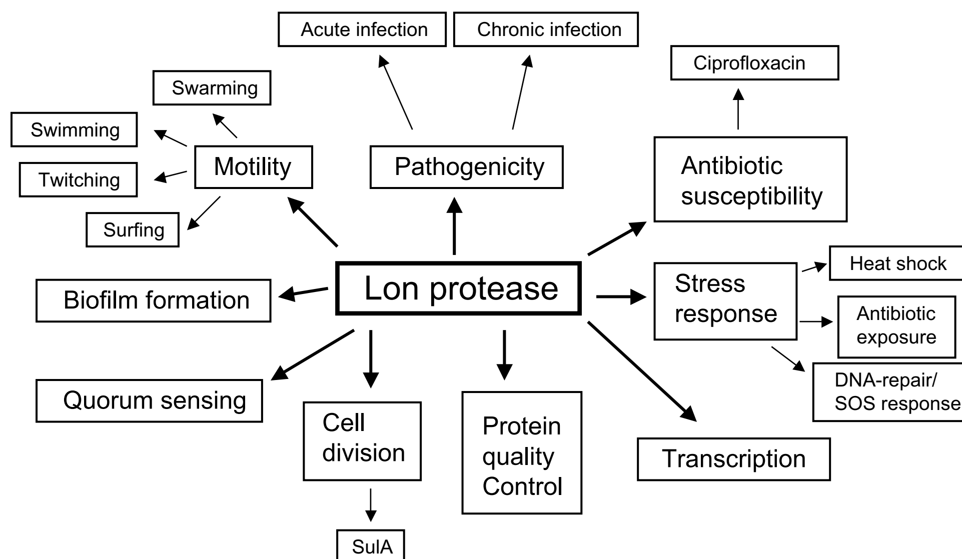
Conclusions

In this review, we have reported the most recent findings regarding the importance of intracellular ATP-dependent proteases in *P. aeruginosa*, in particular Lon and ClpP, in cellular functions, other than their known role in protein quality control (Sauer and Baker 2011). Indeed, there is an increasing body of evidence that these intracellular proteases play crucial roles in the coordination of antibiotic resistance, motility, biofilm formation, virulence, and stress adaptation in this microorganism (Fig. 1). For example, mutations in the genes encoding the Lon and ClpP proteases have been identified to exhibit increased susceptibility to ciprofloxacin (Brazas et al. 2007; Breidenstein et al. 2008; Fernandez et al. 2012). Furthermore, these mutants show defects in motility (swarming, swimming, twitching) and biofilm formation (Marr et al. 2007; Fernandez et al. 2012). These observations in *P. aeruginosa* are consistent with studies in other microorganisms but extend the impressive range of Lon-mediated cellular functions (Msadek et al. 1998; Cohn et al. 2007; de Bruijn and Raaijmakers 2009).

Based on these observations, we (Breidenstein et al. 2012*a*) have hypothesized that the Lon protease regulates and coordinates resistance to ciprofloxacin and key virulence determinants of *P. aeruginosa* by modulating the stability of key transcriptional regulators. The mechanistic basis was defined for the involvement of the Lon protease in protecting *P. aeruginosa* from DNA damage by exposure to ciprofloxacin or UV light by modulating the SOS response. Furthermore, we have found (E.B.M. Breidenstein and R.E.W. Hancock, unpublished data) that the Lon protease impacts on global regulation at the transcriptional level, since a *lon* mutation causes dysregulation of hundreds of genes. Therefore, the Lon protease seems to be a key modulator in *P. aeruginosa* cells. In addition, we recently determined that the Lon protease is involved not only in determining ciprofloxacin resistance, motility, biofilm formation, correct cell division, and the DNA damage (SOS) and other stress responses, but also in virulence, since *lon* mutants were consistently less virulent in several in vivo models (Breidenstein et al. 2012*b*). These observations of decreased virulence for the *Pseudomonas lon* mutant correlate well with studies performed in *Salmonella* and *Campylobacter* (Takaya et al. 2003; Boddicker and Jones 2004; Cohn et al. 2007). This suggests that the Lon protease might have a common role in virulence throughout the bacterial kingdom.

The participation of the ATP-dependent Lon protease in virulence and antibiotic resistance suggests that the design of a specific inhibitor for this protease would be an attractive new therapeutic option to treat *P. aeruginosa* infections. Thus, the inhibition of Lon would attenuate the virulence of the bacterium and, at the same time, increase susceptibility to ciprofloxacin, all of which would be beneficial in clinical settings.

Fig. 1. Biological functions of the *Pseudomonas aeruginosa* Lon protease. As indicated in the figure, the Lon protease plays a diverse regulatory role in the bacterial cell, as it impacts on a wide variety of different processes.



Another protease, ClpP, which is known to work in concert with Lon, is also involved in antibiotic resistance and virulence-related properties (Fernandez et al. 2012). In contrast to the Lon protease, its importance in the virulence of *P. aeruginosa* has not been reported to date. Cohn et al. (2007), however, showed that a *clpP*-deficient strain in *C. jejuni* exhibited a lower level of invasion of epithelial cells compared with the wild-type strain. To our knowledge, nobody has addressed this question yet for the *clpP* mutant of *Pseudomonas*.

Overall, further research is required to fully understand how ATP-dependent proteases can act as global regulators inside the bacterial cell, as well as their potential as novel antimicrobial targets. This is ultimately relevant to the clinic, particularly for the treatment of cystic fibrosis patients, where *Pseudomonas* causes chronic lung infections that can lead to mortality. In these patients, *Pseudomonas* is very difficult to eradicate with the currently available antibiotics, and therefore, it is paramount to find new therapy alternatives. Within this context, ATP-dependent proteases would be good candidates for the development of new therapeutics against *P. aeruginosa*, as their inhibition has been shown to have potent effects in other microorganisms.

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References

Alibaud, L., Kohler, T., Coudray, A., Prigent-Combaret, C., Bergeret, E., Perrin, J., et al. 2008. *Pseudomonas aeruginosa* virulence genes identified in a *Dictyostelium* host model. Cell Microbiol. **10**(3): 729–740. doi:10.1111/j.1462-5822.2007.01080.x. PMID:18042255.

Alvarez-Ortega, C., Wiegand, I., Olivares, J., Hancock, R.E.W., and Martinez, J.L. 2010. Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to β-lactam antibiotics. Antimicrob. Agents Chemother. **54**(1): 4159–4167. doi:10.1128/AAC.00257-10. PMID:20679510.

Baker, T.A., and Sauer, R.T. 2006. ATP-dependent proteases of bacteria: recognition logic and operating principles. Trends Biochem. Sci. **31**(12): 647–653. doi:10.1016/j.tibs.2006.10.006. PMID:17074491.

Boddicker, J.D., and Jones, B.D. 2004. Lon protease activity causes down-regulation of *Salmonella* pathogenicity island 1 invasion gene expression after infection of epithelial cells. Infect. Immun. **72**(4): 2002–2013. doi:10.1128/IAI.72.4.2002-2013.2004. PMID:15039320.

Botos, I., Menikov, E.E., Cherry, S., Tropea, J.E., Khalatova, A.G., Rasulova, F., et al. 2004. The catalytic domain of *Escherichia coli* Lon protease has a unique fold and a Ser-Lys dyad in the active site. J. Biol. Chem. **279**(9): 8140–8148. doi:10.1074/jbc.M312243200. PMID:14665623.

Bottcher, T., and Sieber, S.A. 2008. β-Lactones as specific inhibitors of ClpP attenuate the production of extracellular virulence factors of *Staphylococcus aureus*. J. Am. Chem. Soc. **130**(44): 14 400–14 401. doi:10.1021/ja8051365. PMID:18847196.

Brazas, M.D., Breidenstein, E.B.M., Overhage, J., and Hancock, R.E.W. 2007. Role of lon, an ATP-dependent protease homolog, in resistance of *Pseudomonas aeruginosa* to ciprofloxacin. Antimicrob. Agents Chemother. **51**(12): 4276–4283. doi:10.1128/AAC.00830-07. PMID:17893152.

Breidenstein, E.B.M., Khaira, B.K., Wiegand, I., Overhage, J., and Hancock, R.E.W. 2008. Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. Antimicrob. Agents Chemother. **52**(12): 4486–4491. doi:10.1128/AAC.00222-08. PMID:18824609.

Breidenstein, E.B.M., de la Fuente-Núñez, C., and Hancock, R.E.W. 2011. *Pseudomonas aeruginosa*: all roads lead to resistance. Trends Microbiol. **19**(8): 419–426. doi:10.1016/j.tim.2011.04.005. PMID:21664819.

Breidenstein, E.B.M., Bains, M., and Hancock, R.E.W. 2012*a*. Involvement of the lon protease in the SOS response triggered by ciprofloxacin in *Pseudomonas aeruginosa* PAO1. Antimicrob. Agents Chemother. **56**(6): 2879–2887. doi:10.1128/AAC.06014-11. PMID:22450976.

Breidenstein, E.B.M., Janot, L., Strehmel, J., Fernandez, L., Taylor, P. K. Kukavica-Ibrulj, I., et al. 2012*b*. The Lon protease is essential for full virulence in *Pseudomonas aeruginosa*. PLOS One. **7**(11): e49123. doi:10.1371/journal.pone.0049123.

Bretz, J., Losada, L., Lisboa, K., and Hutcheson, S.W. 2002. Lon protease functions as a negative regulator of type III protein secretion in *Pseudomonas syringae*. Mol. Microbiol. **45**(2): 397–409. doi:10.1046/j.1365-2958.2002.03008.x. PMID:12123452.

Brint, J.M., and Ohman, D.E. 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. J. Bacteriol. **177**(24): 7155–7163. PMID:8522523.

Butala, M., Zgur-Bertok, D., Busby, S.J. 2009. The bacterial LexA transcriptional repressor. Cell. Mol. Life Sci. **66**(1): 82–93. doi:10.1007/s00018-008-8378-6. PMID:18726173.

Butler, S.M., Festa, R.A., Pearce, M.J., and Darwin, K.H. 2006. Self-compartmentalized bacterial proteases and pathogenesis. Mol. Microbiol. **60**(3): 553–562. doi:10.1111/j.1365-2958.2006.05128.x. PMID:16629660.

Caiazza, N.C., Merritt, J.H., Brothers, K.M., and O'Toole, G.A. 2007. Inverse regulation of biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **189**(9): 3603–3612. doi:10.1128/JB.01685-06. PMID:17337585.

Cirz, R.T., O'Neill, B.M., Hammond, J.A., Head, S.R., and Romesberg, F.E. 2006. Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. J. Bacteriol. **188**(20): 7101–7110. doi:10.1128/JB.00807-06. PMID:17015649.

Cohn, M.T., Ingmer, H., Mulholland, F., Jorgensen, K., Wells, J.M., and Brondsted, L. 2007. Contribution of conserved ATP-dependent proteases of *Campylobacter jejuni* to stress tolerance and virulence. Appl. Environ. Microbiol. **73**(24): 7803–7813. doi:10.1128/AEM.00698-07. PMID:17933920.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. 1999. Bacterial biofilms: a

common cause of persistent infections. Science, **284**(5418): 1318–1322. doi:10.1126/science.284.5418.1318. PMID:10334980.

Courcelle, J., Khodursky, A., Peter, B., Brown, P.O., and Hanawalt, P.C. 2001. Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*. Genetics, **158**(1): 41–64. PMID:11333217.

Dasgupta, N., Wolfgang, M.C., Goodman, A.L., Arora, S.K., Jyot, J., Lory, S., et al. 2003. A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. Mol. Microbiol. **50**(3): 809–824. doi:10.1046/j.1365-2958.2003.03740.x. PMID:14617143.

de Bruijn, I., and Raaijmakers, J.M. 2009. Regulation of cyclic lipopeptide biosynthesis in *Pseudomonas fluorescens* by the ClpP protease. J. Bacteriol. **191**(6): 1910–1923. doi:10.1128/JB.01558-08. PMID:19114474.

Diggle, S.P., Cornelis, P., Williams, P., and Camara, M. 2006. 4-Quinolone signalling in *Pseudomonas aeruginosa*: old molecules, new perspectives. Int. J. Med. Microbiol. **296**(2–3): 83–91. doi:10.1016/j.ijmm.2006.01.038. PMID:16483840.

Dotsch, A., Becker, T., Pommerenke, C., Magnowska, Z., Jansch, L., and Haussler, S. 2009. Genomewide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **53**(6): 2522–2531. doi:10.1128/AAC.00035-09. PMID:19332674.

Dougan, D.A., Reid, B.G., Horwich, A.L., and Bukau, B. 2002. ClpS, a substrate modulator of the ClpAP machine. Mol. Cell, **9**(3): 673–683. doi:10.1016/S1097-2765(02)00485-9. PMID:11931773.

Drees, J.C., Lusetti, S.L., and Cox, M.M. 2004. Inhibition of RecA protein by the *Escherichia coli* RecX protein: modulation by the RecA C terminus and filament functional state. J. Biol. Chem. **279**(51): 52 991–52 997. doi:10.1074/jbc.M409050200. PMID:15466870.

Drees, J.C., Chitteni-Pattu, S., McCaslin, D.R., Inman, R.B., and Cox, M.M. 2006. Inhibition of RecA protein function by the RdgC protein from *Escherichia coli*. J. Biol. Chem. **281**(8): 4708–4717. doi:10.1074/jbc.M513592200. PMID:16377615.

Erbse, A., Schmidt, R., Bornemann, T., Schneider-Mergener, J., Mogk, A., Zahn, R., et al. 2006. ClpS is an essential component of the N-end rule pathway in *Escherichia coli*. Nature, **439**(7077): 753–756. doi:10.1038/nature04412. PMID:16467841.

Fernandez, L., Breidenstein, E.B.M., Song, D., and Hancock, R.E.W. 2012. Role of intracellular proteases in the antibiotic resistance, motility, and biofilm formation of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **56**(2): 1128–1132. doi:10.1128/AAC.05336-11. PMID:22123702.

Frase, H., Hudak, J., and Lee, I. 2006. Identification of the proteasome inhibitor MG262 as a potent ATP-dependent inhibitor of the *Salmonella enterica* serovar Typhimurium Lon protease. Biochemistry, **45**(27): 8264–8274. PMID:16819825.

Frees, D., Qazi, S.N., Hill, P.J., and Ingmer, H. 2003. Alternative roles of ClpX and ClpP in *Staphylococcus aureus* stress tolerance and virulence. Mol. Microbiol. **48**(6): 1565–1578. doi:10.1046/j.1365-2958.2003.03524.x. PMID:12791139.

Gaynes, R., Edwards, J.R., and the National Nosocomial Infections Surveillance System. 2005. Overview of nosocomial infections caused by gram-negative bacilli. Clin. Infect. Dis. **41**(6): 848–854. doi:10.1086/432803. PMID:16107985.

Gibson, R.L., Burns, J.L., and Ramsey, B.W. 2003. Pathophysiology and management of pulmonary infections in cystic fibrosis. Am. J. Respir. Crit. Care Med. **168**(8): 918–951. doi:10.1164/rccm.200304-505SO. PMID:14555458.

Gottesman, S. 1996. Proteases and their targets in *Escherichia coli*. Annu. Rev. Genet. **30**: 465–506. doi:10.1146/annurev.genet.30.1.465. PMID:8982462.

Hancock, R.E.W. 1998. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. Clin. Infect. Dis. **27**(Suppl. 1): S93–S99. PMID:9710677.

Hauser, A.R. 2009. The type III secretion system of *Pseudomonas aeruginosa:* infection by injection. Nat. Rev. Microbiol. **7**(9): 654–665. doi:10.1038/nrmicro2199. PMID:19680249.

Hinnerwisch, J., Fenton, W.A., Furtak, K.J., Farr, G.W., and Horwich, A.L. 2005. Loops in the central channel of ClpA chaperone mediate protein binding, unfolding, and translocation. Cell, **121**(7): 1029–1041. doi:10.1016/j.cell.2005.04.12. PMID:15989953.

Hoyle, B.D., and Costerton, J.W. 1991. Bacterial resistance to antibiotics: the role of biofilms. Prog. Drug Res. **37**: 91–105. PMID:1763187.

Hutchison, M.L., and Govan, J.R. 1999. Pathogenicity of microbes associated with cystic fibrosis. Microbes Infect. **1**(12): 1005–1014. doi:10.1016/S1286-4579(99)80518-8. PMID:10617932.

Jackson, M.W., Silva-Herzog, E., and Plano, G.V. 2004. The ATP-dependent ClpXP and Lon proteases regulate expression of the *Yersinia pestis* type III secretion system via regulated proteolysis of YmoA, a small histone-like protein. Mol. Microbiol. **54**(5): 1364–1378. doi:10.1111/j.1365-2958.2004.04353.x. PMID:15554975.

Kidambi, S.P., Booth, M.G., Kokjohn, T.A., and Miller, R.V. 1996. *recA*-dependence of the response of *Pseudomonas aeruginosa* to UVA and UVB irradiation. Microbiology, **142**(4): 1033–1040. doi:10.1099/00221287-142-4-1033. PMID:8936329.

Kindrachuk, K.N., Fernandez, L., Bains, M., and Hancock, R.E.W. 2011. Involvement of an ATP-dependent protease, PA0779/AsrA, in inducing heat shock in response to tobramycin in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. **55**(5): 1874–1882. doi:10.1128/AAC.00935-10. PMID:21357290.

Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jorgensen, A., Molin, S., et al. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Mol. Microbiol. **48**(6): 1511–1524. doi:10.1046/j.1365-2958.2003.03525.x. PMID:12791135.

Kohler, T., Curty, L.K., Barja, F., van, Delden, C., and Pechere, J.C. 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182**(21): 5990–5996. doi:10.1128/JB.182.21.5990-5996.2000. PMID:11029417.

Lewis, K. 2001. Riddle of biofilm resistance. Antimicrob. Agents Chemother. **45**(4): 999–1007. doi:10.1128/AAC.45.4.999-1007.2001. PMID:11257008.

Li, X.Z., Nikaido, H., and Poole, K. 1995. Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**(9): 1948–1953. doi:10.1128/AAC.39.9.1948. PMID:8540696.

Li, X.Z., Zhang, L., and Poole, K. 2000. Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **45**(4): 433–436. doi:10.1093/jac/45.4.433. PMID:10747818.

Mah, T.-F.C., and O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. **9**(1): 34–39. doi:10.1016/S0966-842X(00)01913-2. PMID:11166241.

Marr, A.K., Overhage, J., Bains, M., and Hancock, R.E.W. 2007. The Lon protease of *Pseudomonas aeruginosa* is induced by aminoglycosides and is involved in biofilm formation and motility. Microbiology, **153**(2): 474–482. doi:10.1099/mic.0.2006/002519-0. PMID:17259618.

Masuda, N., Gotoh, N., Ishii, C., Sakagawa, E., Ohya, S., and Nishino, T. 1999. Interplay between chromosomal β-lactamase and the MexAB-OprM efflux system in intrinsic resistance to β-lactams in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43**(2): 400–402. PMID:9925544.

Matsui, H., Suzuki, M., Isshiki, Y., Kodama, C., Eguchi, M., Kikuchi, Y., et al. 2003. Oral immunization with ATP-dependent protease-deficient mutants protects mice against subsequent oral challenge with virulent *Salmonella enterica* serovar Typhimurium. Infect. Immun. **71**(1): 30–39. doi:10.1128/IAI.71.1.30-39.2003. PMID:12496146.

Maurizi, M.R. 1987. Degradation in vitro of bacteriophage λ N protein by Lon protease from *Escherichia coli*. J. Biol. Chem. **262**(6): 2696–2703. PMID:2950089.

Maurizi, M.R. 1992. Proteases and protein degradation in *Escherichia coli*. Experientia, **48**(2): 178–201. PMID:1740190.

Mizusawa, S., and Gottesman, S. 1983. Protein degradation in *Escherichia coli*: the *lon* gene controls the stability of sulA protein. Proc. Natl. Acad. Sci. U.S.A. **80**(2): 358–362. doi:10.1073/pnas.80.2.358. PMID:6300834.

Msadek, T., Dartois, V., Kunst, F., Herbaud, M.L., Denizot, F., and Rapoport, G. 1998. ClpP of *Bacillus subtilis* is required for competence development, motility, degradative enzyme synthesis, growth at high temperature and sporulation. Mol. Microbiol. **27**(5): 899–914. doi:10.1046/j.1365-2958.1998.00735.x. PMID:9535081.

O'Toole, G.A., and Kolter, R. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28**(3): 449–461. doi:10.1046/j.1365-2958.1998.00797.x. PMID:9632250.

Overhage, J., Bains, M., Brazas, M.D., and Hancock, R.E.W. 2008. Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. J. Bacteriol. **190**(8): 2671–2679. doi:10.1128/JB.01659-07. PMID:18245294.

Pamp, S.J., Gjermansen, M., Johansen, H.K., and Tolker-Nielsen, T. 2008. Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. Mol. Microbiol. 68(1): 223–240. doi:10.1111/j.1365-2958.2008.06152.x. PMID:18312276.

Roman-Hernandez, G., Hou, L.Y., Grant, R.A., Sauer, R.T., and Baker, T.A. 2011. The ClpS adaptor mediates staged delivery of N-end rule substrates to the AAA+ ClpAP protease. Mol. Cell, **43**(2): 217–228. doi:10.1016/j.molcel.2011.06.009. PMID:21777811.

Rotanova, T.V., Melnikov, E.E., Khalatova, A.G., Makhovskaya, O.V., Botos, I., Wlodawer, A., et al. 2004. Classification of ATP-dependent proteases Lon and comparison of the active sites of their proteolytic domains. Eur. J. Biochem. **271**(23–24): 4865–4871. doi:10.1111/j.1432-1033.2004.04452.x. PMID:15606774.

Rotanova, T.V., Botos, I., Melnikov, E.E., Rasulova, F., Gustchina, A., Maurizi, M.R., et al. 2006. Slicing a protease: structural features of the ATP-dependent Lon proteases gleaned from investigations of isolated domains. Protein Sci. **15**(8): 1815–1828. doi:10.1110/ps.052069306. PMID:16877706.

Sauer, R.T., and Baker, T.A. 2011. AAA+ proteases: ATP-fueled machines of protein destruction. Annu. Rev. Biochem. **80**: 587–612. doi:10.1146/annurev-biochem-060408-172623. PMID:21469952.

Sauer, R.T., Bolon, D.N., Burton, B.M., Burton, R.E., Flynn, J.M., Grant, R.A., et al. 2004. Sculpting the proteome with AAA(+) proteases and disassembly machines. Cell, **119**(1): 9–18. doi:10.1016/j.cell.2004.09.020. PMID:15454077.

Schurek, K.N., Marr, A.K., Taylor, P.K., Wiegand, I., Semenec, L., Khaira, B.K., et al. 2008. Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **52**(12): 4213–4219. doi:10.1128/AAC.00507-08. PMID:18824604.

Schuster, M., and Greenberg, E.P. 2006. A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. Int. J. Med. Microbiol. **296**(2–3): 73–81. doi:10.1016/j.ijmm.2006.01.036. PMID:16476569.

Siddiqui, S.M., Sauer, R.T., and Baker, T.A. 2004. Role of the processing pore of the ClpX AAA+ ATPase in the recognition and engagement of specific protein

substrates. Genes Dev. **18**(4): 369–374. doi:10.1101/gad.1170304. PMID: 15004005.

Skerker, J.M., and Berg, H.C. 2001. Direct observation of extension and retraction of type IV pili. Proc. Natl. Acad. Sci. U.S.A. **98**(12): 6901–6904. doi:10.1073/pnas.121171698. PMID:11381130.

Smith, R.S., Harris, S.G., Phipps, R., and Iglewski, B. 2002. The *Pseudomonas aeruginosa* quorum-sensing molecule *N*-(3-oxododecanoyl)homoserine lactone contributes to virulence and induces inflammation in vivo. J. Bacteriol. **184**(4): 1132–1139. doi:10.1128/jb.184.4.1132-1139.2002. PMID:11807074.

Snider, J., Thibault, G., and Houry, W.A. 2008. The AAA+ superfamily of functionally diverse proteins. Genome Biol. **9**(4): 216. doi:10.1186/gb-2008-9-4-216. PMID:18466635.

Spoering, A.L., and Lewis, K. 2001. Biofilms and planctonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J. Bacteriol. **183**(23): 6746–6751. doi:10.1128/JB.183.23.6746-6751.2001. PMID:11698361.

Stohl, E.A., Brockman, J.P., Burkle, K.L., Morimatsu, K., Kowalczykowski, S.C., and Seifert, H.S. 2003. *Escherichia coli* RecX inhibits RecA recombinase and coprotease activities in vitro and in vivo. J. Biol. Chem. **278**(4): 2278–2285. doi:10.1074/jbc.M210496200. PMID:12427742.

Swamy, K.H., and Goldberg, A.L. 1981. *E. coli* contains eight soluble proteolytic activities, one being ATP dependent. Nature, **292**(5824): 652–654. doi:10.1038/292652a0. PMID:7019728.

Takaya, A., Suzuki, M., Matsui, H., Tomoyasu, T., Sashinami, H., Nakane, A., et al. 2003. Lon, a stress-induced ATP-dependent protease, is critically important for systemic *Salmonella enterica* serovar Typhimurium infection of mice. Infect. Immun. **71**(2): 690–696. doi:10.1128/IAI.71.2.690-696.2003. PMID: 12540547.

Takaya, A., Tabuchi, F., Tsuchiya, H., Isogai, E., and Yamamoto, T. 2008. Negative regulation of quorum-sensing systems in *Pseudomonas aeruginosa* by ATP-dependent Lon protease. J. Bacteriol. **190**(12): 4181–4188. doi:10.1128/JB.01873-07. PMID:18408026.

Thompson, M.W., Singh, S.K., and Maurizi, M.R. 1994. Processive degradation of proteins by the ATP-dependent Clp protease from *Escherichia coli*. Requirement for the multiple array of active sites in ClpP but not ATP hydrolysis. J. Biol. Chem. **269**(27): 18 209–18 215. PMID:8027082.

Torres-Cabassa, A.S., and Gottesman, S. 1987. Capsule synthesis in *Escherichia coli* K-12 is regulated by proteolysis. J. Bacteriol. **169**(3): 981–989. PMID:3029041.

Umezu, K., and Kolodner, R.D. 1994. Protein interactions in genetic recombination in *Escherichia coli*. Interactions involving RecO and RecR overcome the inhibition of RecA by single-stranded DNA-binding protein. J. Biol. Chem. **269**(47): 30 005–30 013. PMID:7962001.

Whiteley, M., Bangera, M.G., Bumgarner, R.E., Parsek, M.R., Teitzel, G.M., Lory, S., et al. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature, **413**(6858): 860–864. PMID:11677611.

Yeung, A.T., Bains, M., and Hancock, R.E.W. 2010. The sensor kinase CbrA is a global regulator that modulates metabolism, virulence, and antibiotic resistance in *Pseudomonas aeruginosa*. J. Bacteriol. **193**(4): 918–931. doi:10.1128/JB.00911-10. PMID:21169488.

Yeung, A.T., Parayno, A., and Hancock, R.E.W. 2012. Mucin promotes rapid surface motility in *Pseudomonas aeruginosa*. MBio, **3**(3): e00073-012. doi:10.1128/mBio.00073-12. PMID:22550036.

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