The Role of ClpB in Bacterial Stress Responses and Virulence

Athar Alam, Jeanette E. Bröms, Rajender Kumar and Anders Sjöstedt*

Laboratory for Molecular Infection Medicine Sweden (MIMS), Department of Clinical Microbiology, Umeå University, Umeå, Sweden

Bacterial survival within a mammalian host is contingent upon sensing environmental perturbations and initiating an appropriate counter-response. To achieve this, sophisticated molecular machineries are used, where bacterial chaperone systems play key roles. The chaperones are a prerequisite for bacterial survival during normal physiological conditions as well as under stressful situations, e.g., infection or inflammation. Specific stress factors include, but are not limited to, high temperature, osmolarity, pH, reactive oxidative species, or bactericidal molecules. ClpB, a member of class 1 AAA+ proteins, is a key chaperone that via its disaggregase activity plays a crucial role for bacterial survival under various forms of stress, in particular heat shock. Recently, it has been reported that ClpB also regulates secretion of bacterial effector molecules related to type VI secretion systems. In this review, the roles of ClpB in stress responses and the mechanisms by which it promotes survival of pathogenic bacteria are discussed.

Keywords: ClpB chaperone, stress response, heat shock, type VI secretion, ClpB inhibitor

INTRODUCTION AND OVERVIEW

Upon infection of a host, most bacterial pathogens experience drastic changes in their environment, e.g., with regard to pH, temperature and osmolarity. In addition, host inflammatory responses recruit phagocytic cells, subjecting pathogens to additional adverse conditions, such as oxidative and nitrosative stresses. Bacterial survival then depends on molecular adaptations, so called stress responses, to handle the adverse conditions. Essential to these responses are the heat shock proteins (Hsps) which act as molecular chaperones to stabilize proteins and assist protein refolding under stressful conditions (Neckers and Tatu, 2008). DnaJ (Hsp40), GroEL (Hsp60), DnaK (Hsp70), HtpG (Hsp90), and ClpB (Hsp100) are some of the major bacterial molecular chaperones that function in cooperation by forming complex molecular networks, thereby maintaining the overall cellular protein homeostasis (Henderson et al., 2006).

ClpB is a member of the AAA+ family (ATPases associated with diverse cellular activities) that together with the DnaK system have the ability to disaggregate stress-denatured proteins. Like other members of the Hsp100 family, ClpB comprises four domains: an N-terminal domain connected with the remainder of the protein by a conserved linker, the first nucleotide binding domain (NBD-1) in which the unique flexible middle (M) domain is located, and a second NBD (NBD-2) (Lee et al., 2003). Translocation of unfolded protein substrates through the axial protein channel requires that NBD-1 and −2 must couple their ATPase activity (Deville et al., 2017). The M-domain is involved in the direct interaction of ClpB with DnaK (Haslberger et al., 2007), in the interaction of the monomer with...
neighboring ClpB monomers via their NBD-1 domains (Oguchi et al., 2012), and in the stabilization of the hexamer (del Castillo et al., 2011).

ClpB is highly conserved amongst bacteria, fungi, protozoa, and plants and its role under different stressful conditions has been much studied. It provides protection against, e.g., heat, low pH, osmotic- and oxidative stress, ethanol, and nutrient starvation (Meibom et al., 2008; Krajewska et al., 2017; Glaza et al., 2020; Tripathi et al., 2020). Thus, clpB-deficient mutants demonstrate tremendously decreased survival upon exposure to these stresses. Furthermore, ClpB has also been implicated to regulate the expression of virulence factors in several pathogenic bacteria (Frees et al., 2004; Yuan et al., 2007; Capestany et al., 2008; de Oliveira et al., 2011; Lourdault et al., 2011; Alam et al., 2018; Sangpuii et al., 2018). Therefore, ClpB is critical for survival and infectivity of a broad range of clinically relevant microorganisms.

In addition to its role in solubilizing stress-induced protein aggregates, a role of ClpB in type VI secretion (T6S) has recently been reported in the highly pathogenic bacterium Francisella tularensis (Brodmann et al., 2017; Alam et al., 2018, 2020). Here, ClpB apparently serves as a functional homolog of ClpV, harnessing energy through the hydrolysis of ATPs required for depolymerization of the IgA-IgB (homologs of Vibrio cholerae VipA-VipB) sheath for recycling and reassembly. Consequently, deletion of clpB leads to significantly reduced level of T6S and complete attenuation of F. tularensis in mice (Alam et al., 2018, 2020).

Molecular chaperones have the potential to serve as critical targets for the development of novel antimicrobials. For example, the Hsp70 and Hsp90 ATPases have been identified as drug targets for protozoan-derived infectious diseases in humans (Zininga and Shonhai, 2014, 2019). However, due to the high degree of sequence conservation among the Hsps across different domains of life, it is a challenging task (Glaza et al., 2020). ClpB is of special relevance as a drug target, since the homolog of ClpB, Skd3, also known as human ClpB, is conserved in many metazoan lineages, but differs significantly from bacterial and yeast proteins in domain structures. Skd3 lacks the characteristic microbial ClpB coiled-coil domain and contains a unique ankyrin-repeat domain (Erives and Fassler, 2015; Cupo and Shorter, 2020). In contrast, eu- and non-metazoan eukaryotes harbor Hsp104, which is more closely related to microbial ClpB (Oguchi et al., 2012).

This review aims to elucidate our current understanding of the ClpB chaperones of pathogenic bacteria and their potential contribution to virulence. Since ClpB affects infectivity and survival of a broad range of clinically relevant pathogenic microorganisms, the possibility of exploiting ClpB as a therapeutic target is also discussed.

**THE ROLE OF ClpB IN STRESS-TOLERANCE AND VIRULENCE**

One of the fundamental roles of ClpB is to mediate tolerance to stressful conditions, in particular heat, for a wide range of bacterial species (Figure 1 and Table 1), but if and how ClpB contributes to bacterial survival during infection has been less studied. The Escherichia coli ClpB has served as the prototype for studies of the essential mechanisms of Hsp100 disaggregases during heat shock and for the structural identification of the various domains (Squires et al., 1991; Mogk et al., 1999, 2015; Barnett et al., 2000; Rosenzweig et al., 2013). Due to the high degree of conservation among bacterial ClpB, the E. coli ClpB data is often being used to infer the structures and roles of ClpB proteins of other bacterial species.

*E. coli*, *F. tularensis*, Helicobacter pylori, Pseudomonas putida, Campylobacter coli, and Campylobacter lari are some of the pathogenic bacteria for which the role of ClpB in thermotolerance has been studied (Squires et al., 1991; Allan et al., 1998; Meibom et al., 2008; Ito et al., 2014; Alam et al., 2018, 2020; Riedel et al., 2020). *F. tularensis*, a highly infectious pathogen and a category A bioterrorism agent, is the etiological agent of the zoonotic disease tularemia. Deletion of the clpB gene causes a severe defect in survival at elevated temperature (Meibom et al., 2008; Alam et al., 2018, 2020). A similar effect was observed for a clpB mutant of *H. pylori*, the causative agent of gastric ulcers (Allan et al., 1998). In addition to thermosensitivity, an inability to disaggregate aggregated proteins was demonstrated for a clpB mutant of the opportunistic human pathogen *P. putida* (Ito et al., 2014; Table 1). Moreover, enhanced levels of clpB gene expression were observed at elevated temperature in Campylobacter, a genus containing one of the most important food-borne pathogen globally. Transcriptomic profiles of *C. coli* and *C. lari* at elevated temperatures showed enhanced gene expression of clpB and other genes encoding chaperones such as dnaK, groES, and groEL, indicating that multiple chaperones, including ClpB, play a vital role in the thermotolerance of *Campylobacter* spp. (Riedel et al., 2020).

In addition to its importance for thermotolerance, ClpB also plays a role in the general stress-tolerance of bacteria (Figure 1 and Table 1). A clpB null mutant of *Brucella suis*, the etiological agent of swine brucellosis, showed increased sensitivity not only to high temperature, but also to ethanol and acid pH (Ekaza et al., 2001). A specific role of ClpB during antibiotic-induced stress has also been reported in *Acinetobacter baumannii*, a multi-resistant, opportunistic human pathogen. Levels of clpB were dramatically increased in the presence of the carbapenem meropenem, or trimethoprim/sulfamethoxazole, indicating that the chaperone may play a key role for antibiotic resistance (Lazaretti et al., 2020). Similarly, inactivation of *ibpA/clpB* increased the susceptibility to the aminoglycoside tobramycin in the opportunistic human pathogen *P. aeruginosa* (Wu et al., 2015; Table 1).

Besides promoting stress tolerance, ClpB plays an important role in invasiveness and/or host survival of multiple important bacterial pathogens (Table 1), such as *Leptospira interrogans*, *Yersinia enterocolitica*, *Francisella noatunensis*, *F. tularensis*, *Piscirickettsia salmonis*, *Mycoplasma pneumoniae*, *Salmonella typhimurium*, *Mycobacterium tuberculosis*, *Porphyromonas gingivalis*, *Enterococcus faecalis*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Badger et al., 2000; Chastenet et al., 2004; Frees et al., 2004; Yuan et al., 2007; Capestany et al., 2008; Kannan et al., 2008; Conlan, 2011; de Oliveira et al., 2011;
FIGURE 1 | A summary of ClpB’s so-far established roles in pathogenic bacteria, including T6S (Francisella only). Schematic figure illustrating the importance of ClpB in various stress responses, T6S and virulence. Model of the T6S in extended (left), contracted (central), and disassembled (right) forms of canonical and Francisella T6S is shown, where ClpB acts as an energizer. Canonical T6S subunits from Escherichia coli are labeled in black and Francisella T6S subunits, which are encoded within the Francisella Pathogenicity Island (FPI), are labeled in blue.

Lourdault et al., 2011; Alam et al., 2018, 2020; Sangpuii et al., 2018; Harnagel et al., 2020; Kêdzierska-Mieszkowska and Arent, 2020; Tripathi et al., 2020). In the case of *L. interrogans*, the causative agent of the emerging zoonotic disease leptospirosis, a *clpB* mutant not only showed enhanced susceptibility to high temperature, nutrient-depletion, and oxidative stress, but was also attenuated in a gerbil animal model of acute leptospirosis (Lourdault et al., 2011; Kêdzierska-Mieszkowska and Arent, 2020). Similarly, a *Y. enterocolitica clpB* mutant demonstrated defective invasion of human laryngeal epithelial cells, Hep-2, and reduced expression of important virulence factors, including invasin and flagellin (Badger et al., 2000). *F. noatunensis* ssp. *noatunensis* is the etiological agent of francisellosis in Atlantic cod. In the absence of ClpB, the resulting mutant showed attenuation in a zebrafish model and also provided efficient protection in zebrafish challenged with wild-type bacteria (Lampe et al., 2017). Moreover, *clpB* mutants of *F. tularensis* subspecies *holartica* and *tularensis* were found to be defective for T6S, susceptible to elevated temperature, and completely attenuated in mice (Alam et al., 2018, 2020). Such mutants also serve as highly efficacious vaccines in animal models of tularemia (Conlan, 2011; Alam et al., 2018). *P. salmonis*, the etiological agent of salmonid rickettsial septicemia (SRS), a disease that affects a wide variety of cultivated fish species, demonstrated significantly higher levels of ClpB during intramacrophage growth in a salmon cell line; indicating that this permits the pathogen to adapt to the hostile intracellular conditions and facilitates replication (Isla et al., 2014). A growth-promoting status of ClpB was also observed in *M. pneumoniae*, an important cause of community-acquired pneumonia, since loss of ClpB resulted in impaired replication under permissive growth conditions (Kannan et al., 2008). ClpB also plays a vital role in the survival in chicken of *S. typhimurium*, a major cause of gastroenteritis globally, since a *clpB* mutant was found to display reduced survival at 42°C in poultry macrophages and during exposure to hypochloric acid and paraquat (Sangpuii et al., 2018). Moreover, the mutant showed decreased dissemination *in vivo* (Sangpuii et al., 2018). *M. tuberculosis*, the causative agent of tuberculosis, is one of the most important pathogens globally. It was demonstrated that a *clpB* mutant of *M. tuberculosis* has aberrant cellular morphology, impaired biofilm formation and reduced cellular infectivity (Tripathi et al., 2020). In addition, the mutant was sensitive to oxidative stress and defective for the maintenance of dormant bacteria (Harnagel et al., 2020; Tripathi et al., 2020). Furthermore, the purified ClpB protein from *M. tuberculosis* showed potent biological activity and induced release of pro-inflammatory cytokines from a human macrophage cell line (Tripathi et al., 2020). A vital role of ClpB was also observed in *P. gingivalis*, an important cause of chronic periodontal disease, where a *clpB* mutant showed...
defective thermotolerance and also decreased cellular invasion and marked attenuation in a mouse model (Yuan et al., 2007; Capestany et al., 2008). ClpB of the Gram-positive bacterium L. monocytogenes, an etiological agent of human meningitis, was not involved in tolerance to heat, high salt, or cold; but played a role for virulence in mice (Chastanet et al., 2004). S. aureus, a major cause of skin infections and several systemic infections, was susceptible to elevated heat stress and a clpB mutant demonstrated diminished intracellular multiplication within bovine mammary epithelial cells (Frees et al., 2004). Similarly, E. faecalis lacking ClpB demonstrated defective thermotolerance, as well as attenuation in a Galleria mellonella model (de Oliveira et al., 2011). Altogether, the published data unequivocally demonstrate that ClpB of many bacterial species play a key role for their survival during numerous forms of stress conditions and for their virulence in experimental models.

THE ROLE OF ClpB FOR T6S

The type VI secretion systems comprise the most common secretion machinery among Gram-negative bacteria, present in more than 25% of all proteobacteria. T6S is used to translocate effector molecules directly into neighboring cells, commonly a bacterial competitor (Coulthurst, 2019). The machinery is composed of 13 to 14 core components, with a set of regulatory effector molecules directly into neighboring cells, commonly a bacterial competitor (Coulthurst, 2019). The AAA+ ATPase ClpV has been shown to act as an energizer for T6S. Its action promotes sheath disassembly and the dynamic recycling for repeated rounds of firing, disassembly and reassembly (Figure 1; Bönemann et al., 2009; Pietrosiuk et al., 2011; Kube et al., 2014). For some bacteria, the energy may be provided through the activity of ATPases distinct to ClpV, which are encoded outside of the T6S cluster. In support, only a partial loss of the activity of ATPases distinct from ClpB cannot be excluded. Notably, in the malaria parasite, a ClpB-like protein of the Hsp101 family is essential for export across the parasitophorous vacuolar membrane into the erythrocyte and it was demonstrated that the protein functions in a complex that serves as a convergent step in a multi-pathway export process (Beck et al., 2014).

ClpB AS A THERAPEUTIC TARGET

The global threat of antibiotic-resistant bacteria shows no sign of being resolved and the arsenal of clinically useful antibiotics becomes more and more limited. Bacterial chaperones remain one set of underexploited targets for antibiotic development. In particular, ClpB belongs to the group of potential drug targets, since mammals do not have Hsp100 homologs, other than human ClpB/Skd3 which is significantly different from the microbial ClpB in domain structures (Erives and Fassler, 2015; Cupo and Shorter, 2020). The development of specific inhibitors of ClpB might not only be useful as a novel antibiotic for otherwise antibiotic-resistant bacterial strains, but also as a means to understand the molecular mechanism of this chaperone.

Currently, only a few ClpB inhibitors have been identified (Grimminger et al., 2004; Martin et al., 2013; Kuczyńska-Wisnisk et al., 2017; Glaža et al., 2020; Singh et al., 2020). Guanidinium chloride specifically inhibits the ATP hydrolysis by Hsp104 of Saccharomyces cerevisiae and also the ClpB function of Ehrlichia chaffeensis (Grimminger et al., 2004). Thus, it may serve as a general inhibitor of members of the AAA+ protein family, but this remains to be proven. Two other ClpB inhibitors, called compounds 3 and 6, inhibit the functional properties and the growth of E. coli, thus displaying antimicrobial activity under thermal or oxidative stress conditions (Martin et al., 2013). Compound 3 competes with substrate binding and modifies the ATPase activity of ClpB, while compound 6 hampers the substrate-induced improvement of its ATPase activity (Martin et al., 2013). Further, the specific interaction of the compounds with the chaperone is essential for their antimicrobial action. This, in combination with only moderate cytotoxicity, suggests that they could be used as leads for development of new antimicrobials (Martin et al., 2013).
# TABLE 1 | The impact of the chaperone ClpB on bacterial growth, survival, and virulence in various bacterial species.

## Role of ClpB in bacterial stress responses

| Species                          | Heat shock | Other stresses                                      | Additional observations for clpB mutants | Attenuation in indicated host model or cell infection                                                | References                                      |
|----------------------------------|------------|-----------------------------------------------------|----------------------------------------|----------------------------------------------------------------------------------------------------|------------------------------------------------|
| Acinetobacter baumannii          | NT         | S (beta-lactams)                                    |                                        | DHR82 canine macrophage cell line                                                                | Lazaretti et al., 2020                          |
| Brucella suis                    | S          | S (ethanol, pH)                                     |                                        |                                                                                  | Ekaza et al., 2001                               |
| Campylobacter coli, lari         | S          | NT                                                  |                                        |                                                                                  | Riedel et al., 2020                              |
| Ehrlichia chaffeensis             | T          | NT                                                  |                                        |                                                                                  | Grimminger et al., 2004; Zhang et al., 2013      |
| Enterococcus faecalis            | S          | T (oxidative stress)                                |                                        |                                                                                  |                                             |
| Escherichia coli                 | S          | S (oxidative stress)                                |                                        |                                                                                  |                                             |
| Francisella novicida             | S          | NT                                                  | ↓ T6S                                  | J774 cell line, Mice                                                                        | Alam et al., 2017                               |
| Francisella tularensis subsp. tularensis | S   | S (pH)                                              | ↓ T6S                                  |                                                                                  | Alam et al., 2018                               |
| Francisella tularensis subsp. holarctica | S  | S (oxidative stress, pH, ethanol)                    | ↓ T6S                                  |                                                                                  | Meibom et al., 2008; Alam et al., 2018           |
| Helicobacter pylori              | S          | NT                                                  |                                        |                                                                                  | Allan et al., 1998                              |
| Leptospira interrogans           | S          | S (oxidative stress) S (nutrient-limitation)        |                                        |                                                                                  | Lourdault et al., 2011; Kędzierska-Mieszkowska and Arent, 2020 |
| Listeria monocytogenes           | T          | T (osmosis/salt) T (cold)                           |                                        |                                                                                  | Chastanet et al., 2004                           |
| Mycobacterium tuberculosis       | NT         | S (oxidative stress)                                | Aberrant cellular morphology, impaired biofilm formation and defective maintenance of dormant bacteria | THP-1 cell line                                                                          | Harnagel et al., 2020; Singh et al., 2020; Tripathi et al., 2020 |
| Mycoplasma pneumoniae            | NT         | NT                                                  |                                        |                                                                                  | Kannan et al., 2008                             |
| Piscirickettsia salmonis         | NT         | NT                                                  |                                        | SHK-1 salmon cell line                                                                    | Isla et al., 2014                               |
| Porphyromonas gingivalis         | S          | T (pH) T (oxidative stress)                         |                                        | Mice; Gingival epithelial cells and Human coronary artery endothelial cells                 | Yuan et al., 2007; Capestany et al., 2008        |
| Pseudomonas putida               | S          | NT                                                  |                                        |                                                                                  | Ito et al., 2014                                 |
| Pseudomonas aeruginosa           | NT         | S (tobramycin)                                      |                                        |                                                                                  | Wu et al., 2015                                 |
| Salmonella typhimurium            | S          | S (oxidative stress)                                |                                        |                                                                                  | Sangpui et al., 2018                            |
| Staphylococcus aureus            | S          | T (oxidative stress)                                |                                        |                                                                                  | Frees et al., 2004                              |
| Vibrio cholerae                  | S          | S (pH) S (oxidative stress)                         | ↓ Invasin expression ↓ Motility        |                                                                                  | Nag et al., 2005                                |
| Yersinia enterocolitica          | NT         | NT                                                  |                                        |                                                                                  | Badger et al., 2000                             |

1Abbreviations used are as follows: S, sensitive; T, tolerant; NT, not tested. ↓Decreased.
Three inhibitors of *M. tuberculosis* ClpB have been identified and they also inhibit the ATPase activity of *E. coli* ClpB and yeast Hsp104 (Singh et al., 2020). In addition, DBeQ, which is derived from an inhibitor of the human AAA+ ATPase P97, an anti-tumor target, inhibited *E. coli* proliferation and appeared to selectively target ClpB (Glaza et al., 2020).

Collectively, the identification of these ClpB inhibitors demonstrates the potential of the protein as a therapeutic target.

**CONCLUSION**

The ATP-dependent ClpB protein is a disaggregase and a key member of a multi-chaperone system that efficiently inhibits and reverses protein aggregation. As such, ClpB is critical for the survival of various microorganisms exposed to stress, but it also confers vital functions during normal physiological conditions. In bacteria, loss of ClpB is commonly associated with fatal thermosensitivity, but it may also lead to susceptibility to other forms of stress, such as reactive oxidative species, antibiotics and bactericidal molecules as well as changes in osmolarity and pH. More recent work has identified a critical role of ClpB related to T6S. Thus, in *F. tularensis*, the absence of ClpB leads to T6S dysfunction and impaired bacterial virulence. This also suggests that the ATPase activity of ClpB may provide the energy required for functional T6S, thereby substituting for ClpV proteins in bacteria where these are absent. In view of the many central roles of ClpB, it is a logical therapeutic target and recent work serves as proof of concept for this hypothesis.

**AUTHOR CONTRIBUTIONS**

AA, JEB, and AS conceptualized the manuscript, involved in the generation of the figure, and critically revised the manuscript. AA, JEB, RK, and AS wrote the manuscript. All authors have read the article and approved it for publication.

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**Conflict of Interest**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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