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ClpB chaperone as a promising target for antimicrobial therapy: A narrative review

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ABSTRACT

The Clp/Hsp100 family, part of the ATPase associated with various cellular activities (AAA+) superfamily, includes caseinolytic peptidase B (ClpB), a highly conserved protein found in bacteria, fungi, protozoa, and plants. Notably, ClpB is present in all ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. ClpB plays a crucial role in reactivating and disaggregating proteins, enabling pathogens to survive under host-induced stress and conferring thermotolerance to bacterial cells. Infections caused by ESKAPE pathogens are particularly challenging due to their resistance to broad-spectrum antibiotics and biofilm formation, posing a significant global health threat as they are often multidrug-resistant, extensively drug-resistant, and pan-drug-resistant. Given its absence in human cells and its essential role in bacterial survival under stress, ClpB is a promising target for antimicrobial therapy. Targeting Hsp100 family proteins could lead to the development of novel antifungal and antiprotozoal treatments. This review explores the function of ClpB in the survival of ESKAPE pathogens and the protozoan *Plasmodium falciparum*. Relevant research findings were compiled using academic databases, and data analysis was performed using Clustal Omega Multiple Sequence Alignment and Boxshade tools.

KEYWORDS: Molecular chaperone; ClpB; ESKAPE pathogens; Thermotolerance; Heat shock proteins; *Plasmodium falciparum*

1. Introduction

Proteins are the most adaptive and complex macromolecules in all biological systems. They are synthesized on ribosomes as linear

chains composed of up to thousands of amino acids. These chains must fold into their native state, forming a few closely related three-dimensional structures. Proteins participate in most biological events and carry out numerous crucial biological reactions. Therefore, maintaining proteome homeostasis, or proteostasis, is essential for organisms. Proteostasis encompasses protein synthesis, folding, refolding, and degradation. Preventing or reducing protein aggregation and maintaining proteins in a soluble, active state are critical outcomes of protein quality control and homeostasis[1,2].

Summary

Question: What is the role of ClpB in microorganisms, including ESKAPE pathogens and malarial parasites?

Findings: This literature review demonstrates key insights into the structure and function of ClpB. ClpB aids in the disaggregation of protein aggregates, a process crucial for pathogen survival under stress after host invasion, and shows high sequence conservation in ESKAPE pathogens and malarial parasites.

Meaning: ClpB enhances the virulence and survival of several significant pathogens, including ESKAPE pathogens and malarial parasites. Due to its essential role in bacterial survival under stress and its absence in metazoans, ClpB presents a promising target for developing novel antimicrobials.

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Molecular chaperones are proteins that assist other proteins in achieving their active, native structures without being part of the final structure themselves[1]. In the cellular environment, unstructured polypeptides emerging from ribosomes can sometimes misfold, although most fold correctly into their active states. Under stress conditions, some proteins may also misfold and lose their native structures. However, cells have evolved molecular chaperones to counteract protein aggregation by disassembling protein aggregates into unstructured polypeptides. These released polypeptides can either refold into their active native structures with the help of other molecular chaperones or be degraded by the cell's protease machinery. Examples of these molecular chaperones include caseinolytic peptidase B (ClpB) in bacteria, heat shock protein 101 (Hsp101) in plants, and Hsp104 in yeast. These proteins belong to the Hsp100 family, characterized by monomers with a molecular weight of approximately 100 kDa[2,3]. Notably, Hsp100 family proteins are absent in animals and humans but are present in plants, yeast, and bacteria[4,5].

ClpB is highly conserved throughout bacteria, and ClpB orthologs are found in fungi, protozoans and plants. These organisms also include *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Enterococcus faecium* (*E. faecium*), *Acinetobacter baumannii* (*A. baumannii*), and *Enterobacter* spp., which are also known as ESKAPE pathogens (Supplementary Figure 1) and malarial protozoan *Plasmodium falciparum* (*P. falciparum*) (Supplementary Figure 2)[2]. Many researchers have examined how ClpB responds to various stressful stimuli. As a molecular chaperon, ClpB offers defense against heat, low pH, oxidative stress osmolarity changes, nutrient deficiency, and ethanol. As a result, ClpB-deficient mutants show drastically reduced survival when subjected to these stimuli. Additionally, ClpB has been linked to the regulation of virulence factor expression in several harmful bacteria. Therefore, ClpB renders thermotolerance of bacterial cells, aids in their survival in stressful situations and the virulence of pathogenic bacteria, facilitates the dissolution of protein aggregates, and keeps cellular proteins soluble where they can be refolded with the assistance of other co-chaperones present in the cell[6].

For this review, literature analysis was conducted using the UniProt, ScienceDirect, and PubMed to gather research findings and relevant materials. These platforms provided access to crucial papers and information that support the topic. Clustal Omega, a multiple sequence alignment program, was employed to compare sequences and identify conserved regions, facilitating further evaluation of the sequence data collected from UniProt. For sequence visualization, the Boxshade tool was used to highlight significant patterns and variations.

2. AAA+ Chaperone ClpB: Its structure and function

The Casein lytic proteinase/heat shock protein 100 (Clp/Hsp100)

proteins are chaperones that act to remodel/disassemble protein complexes and/or aggregates using the energy of adenosine-5'-triphosphate (ATP). Clp/Hsp100 family is a member of the ATPases associated with diverse cellular activities (AAA+) superfamily proteins. This superfamily of proteins consists of highly conserved regions containing approximately 220 amino acids, frequently referred to as AAA domain or nucleotide-binding domain (NBD), and requires ATP energy to perform their functions. The AAA+ superfamily can be classified into two classes based on the quantity of AAA domains. Two highly conserved NBDs are found in Class I proteins, such as ClpA, ClpB, ClpC, and ClpE, and are connected by a linker region. ClpX and ClpY are examples of Class II proteins with just one NBD. These proteins can mediate the ATP-dependent unfolding or disassembly of protein-DNA or protein-protein complexes[7].

Figure 1 shows how the AAA+ superfamily and their domain organization are divided into Class I and Class II. NBD-1 is shown in yellow, and NBD-2 is shown in light green in this diagram. The middle domain is shown in red color. ClpB middle domain is comparatively prominent among other AAA+ proteins. The N-terminal domain is shown in blue color. ClpY, also known as HslU, lacks the N-terminal domain but has another domain called the I domain, which is displayed in brown color. Domain I is embedded in its NBD[8–10].

Class I proteins ClpA, ClpC, and ClpE, and Class II proteins ClpX and ClpY/HslU are chaperones that are associated with Clp proteases (ClpP and ClpQ) form complexes (ClpAP, ClpEP, ClpCP, ClpXP, and ClpYQ/HslUV) to function together in protein quality control. These chaperones form a hexameric ring structure with a pore and connect to the Clp protease core. Upon strenuous conditions, proteins unfold and lose their native structure and tend to form aggregates. These chaperones recognize these substrates before turning them into aggregates, unfold them, and spool them into the protease core, which hydrolyzes long polypeptide chains into 10-15 amino acid length peptide fragments[11]. Unlike these, ClpB lacks essential structural features to associate with Clp proteases; hence, it is involved only in protein disaggregation and not proteolysis. There is a synthetically engineered protein called BAP (ClpB variant that associates with ClpP), which can associate with ClpP and degrade proteins by hydrolysis in the presence of ClpP[9].

ClpB consists of six identical monomers (Figure 2), each comprising four main domains. The N-terminal domain is linked to the rest of the protein *via* a conserved linker. This linker connects to the first nucleotide-binding domain (NBD-1), followed by a distinctive, flexible, coiled middle (M) domain. The middle domain then links to the second nucleotide-binding domain (NBD-2), positioning itself between the two NBDs[3,6,12]. ClpB has also been observed in a heptameric assembly under non-physiological conditions[13].

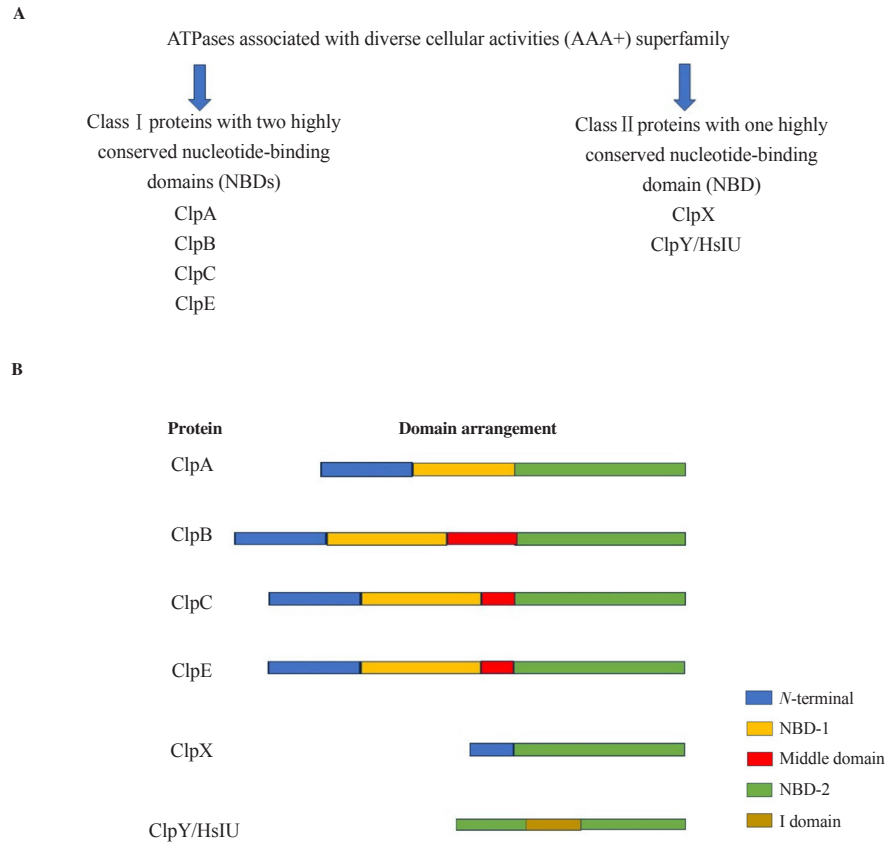


Figure 1. (A) Superfamily classification of Clp proteins and (B) domain organization of ClpA, ClpB, ClpC, ClpE, ClpX, and ClpY/HsIU.

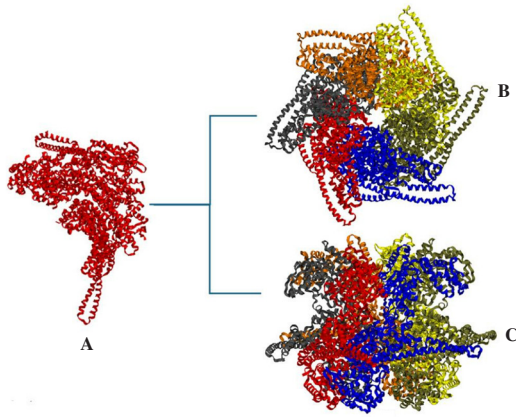


Figure 2. (A) Structure of the ClpB monomer (PDB ID: 1QVR). Structural model of hexameric ClpB (B: top view; C: side view). In panels (B) and (C), each monomer is colored differently for clarity.

The *Escherichia coli* ClpB monomer (Figure 3) consists of 857 amino acids. The first 146 amino acids form the *N*-terminal domain which connects to NBD-1 by a conserved linker spanning amino acids 147 to 163. NBD-1 extends from amino acid 164 to 413, followed by the middle domain, which spans amino acids 414 to 525 and resides between NBD-1 and NBD-2. NBD-2 starts at amino acid 526 and ends at 769, leading to the final 87 amino acids that constitute the *C*-terminal domain[2].

The alpha helices in the mobile, globular-shaped *N*-terminal domain facilitate the recognition and binding of aggregates. It has been shown that specific substrates can disaggregate without the *N*-terminal domain. However, in the absence of a functional DnaK, the *N*-terminal domain becomes crucial for optimal bacterial growth[2,14].

A unique feature of bacterial ClpB, absent in other bacterial chaperones, is its middle domain. This middle domain is wrapped around the exterior edge of the hexameric ClpB in a coiled-coil structural configuration[2,15].

The two nucleotide-binding domains (NBD1 & NBD2) seen in bacterial ClpB are thought to have evolved separately. Walker A, Walker B, Sensor 1, Sensor 2, and arginine finger are a few of the conserved sequence motifs found in these NBDs, which all generally share a similar structure[5]. Walker A and Walker B motifs serve as crucial binding and hydrolysis sites for ATP. Walker A directly interacts with the phosphate groups of ATP, and its sequence contains the conserved lysine, which is required to facilitate ATP binding. Walker B is required to coordinate the interactions of the Mg^{2+} ion and water, which are crucial for ATP hydrolysis. Walker B motifs have two critical conserved residues, aspartate and glutamate, and they also interact with bound nucleotides. The aspartate residue ensures that the Mg^{2+} ion is appropriately coordinated. The glutamate residue activates water molecules, which is necessary for

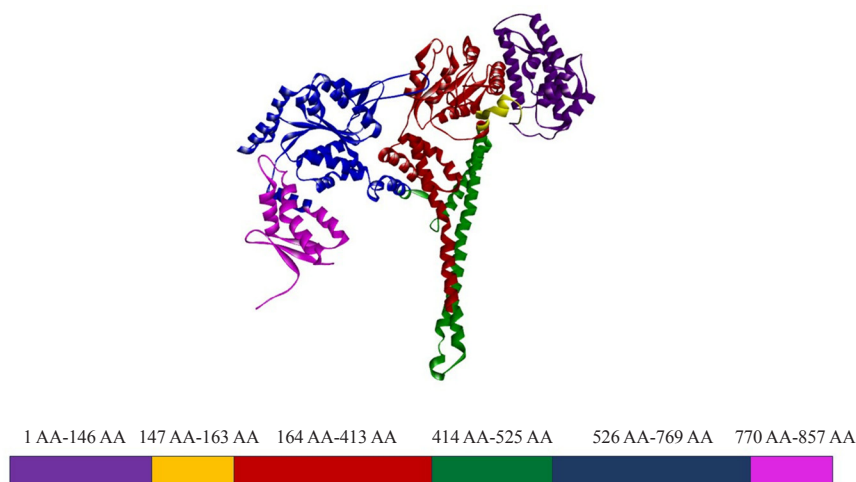


Figure 3. The crystal structure of ClpB (PDB ID:1QVR). *N*-terminal domain is shown in purple color, linker in yellow color, the first nucleotide binding domain in red color, middle domain in green color, the second nucleotide binding domain in blue color, and *C*-terminal domain in pink color.

the catalysis of the ATP hydrolysis step[16].

Near the *C*-terminus of Walker B motifs, there is a consensus area on AAA+ proteins known as the second region of homology (SRH). There is a little motif called Sensor 1 at the *N*-terminus of SRH and another motif called the arginine finger at the *C*-terminus of SRH. Since they are situated between Walker A and Walker B regions, the polar residues in the Sensor 1 motif are crucial for ATP hydrolysis (Figure 4). The binding and hydrolysis of ATP and the ATP-dependent oligomerization of AAA+ proteins depend on the arginine residues present in arginine finger motifs. The *C*-terminal terminus of NBD also contains the Sensor 2 motif, which has a vital conserved arginine. According to structural analyses, the arginine in Sensor 2 is in close proximity to the bound ATP's gamma phosphate. As a result, the ATP binding and hydrolysis pathway linked to AAA+ proteins requires Sensor 2[2,16].

ClpB needs an additional ATP-dependent chaperone, DnaK, to function in protein disaggregation and reactivation in the cell. DnaK is an omnipresent molecular chaperone that participates in protein refolding along with DnaJ and GrpE nucleotide exchange factors[3,17].

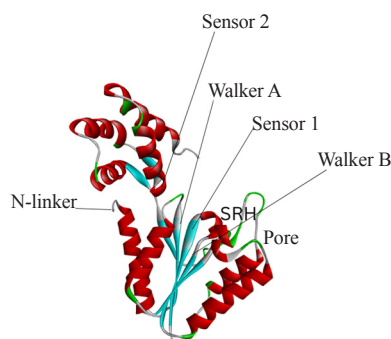


Figure 4. The crystal structure of *N*-ethylmaleimide-sensitive fusion protein (PDB ID: 1D2N) is used here as a model AAA+ domain to show the positions of structural elements N-linker, Walker A, Walker B, Sensor 1, Sensor 2, SRH, and the pore region. SRH: second region of homology.

3. Proposed mechanisms of ClpB-mediated protein aggregate reactivation

The mechanism of ClpB-mediated protein aggregate reactivation and disintegration is still unknown. It has been proposed that six ClpB monomers of each 95 kDa assemble into a 575 kDa hexamer of biologically active bacterial ClpB unit in the presence of ATP or adenosine-5'-diphosphate (ADP)-like nucleotides. The self-associating monomers in the hexamer's core create a small channel. Upon detecting aggregates within the cell, DnaJ will initially attach to the aggregates and then recruit DnaK. The aggregates may become somewhat looser and remodelled as a result of DnaJ and DnaK binding. ClpB needs co-chaperone DnaK to identify and target protein aggregates inside the cell. Through its middle domain, ClpB interacts with DnaK, activating ClpB and delivering substrate to it. ClpB hydrolyzes ATP to separate polypeptides from aggregates. By passing polypeptide ends (*N* or *C* terminus) or exposed loops of polypeptides along the central channel *via* ATP hydrolysis, ClpB dissociates protein clumps (Figure 5)[5].

According to the loop extrusion mechanism, ClpB can pull the peptide from both arms of the loop or shift to a single-arm translocation when trying to extract a polypeptide loop. The substrate can either refold as the extracted polypeptide leaves ClpB's central channel spontaneously or with the aid of co-chaperones. If it is unfeasible to extract peptides from aggregates, a ClpB hexamer splits into its monomers and then reassembles to interact with a different new loop or terminal. The aggregate breakdown is quick and effective because of the ability of many ClpB machines to work concurrently on the same aggregate (Figure 5)[18].

4. Role of ClpB in ESKAPE pathogens

ESKAPE pathogens are a group of opportunistic bacteria that cause

infections that are extremely difficult to treat due to their resistance to a broad spectrum of antibiotics and biofilm formation. When biofilms are formed, bacteria, including all ESKAPE pathogens inside biofilms, express around a thousandfold more resistance to antimicrobial agents in contrast to planktonic cells. Therefore, from a therapeutic perspective, these bacteria pose a global hazard because they are frequently multidrug-resistant, extensively drug-resistant, and pan-drug-resistant[19,20]. The impact of ClpB in ESKAPE pathogens is summarized in Table 1.

4.1. *E. faecium*

Enterococcus species are Gram-positive facultative anaerobes commonly observed in chains or pairs. They naturally inhabit the guts of humans and animals. Among the more than 20 species, *E. faecium* and *Enterococcus faecalis* are the most clinically significant. While cross-infection can occur in hospitalized patients, most *Enterococcus* infections result from endogenous acquisition[21].

Enterococcus species employ several mechanisms to adapt to

stress. Proteins such as DnaK, DnaJ, GrpE, and ClpB, recently characterized in *E. faecium* as part of a stress-induced multi-chaperone system, assist the cell in recovering from heat stress and ensure proper folding of newly synthesized proteins[6,22]. However, the exact mechanism of ClpB function in *E. faecium* remains undetermined[23].

4.2. *S. aureus*

S. aureus is a commensal organism found on human skin and can spread within and across healthcare institutions, including hospitals and communities. Individuals are more likely to become infected with *S. aureus* through close contact with carriers, prolonged hospital stays, surgical procedures, or medical devices such as implants or injections[24,25]. This bacterium can cause a wide range of infections, from mild superficial skin infections to severe conditions like sepsis and pneumonia, which can be fatal. Antibacterial resistance complicates the treatment of *S. aureus* infections, and an effective vaccine is not yet available. Methicillin resistant *S. aureus* strains

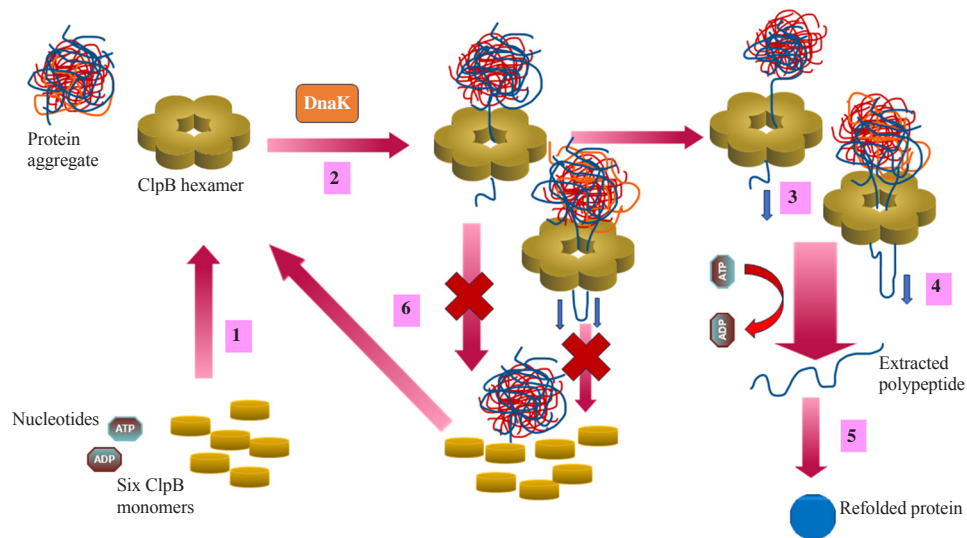


Figure 5. Two proposed mechanisms of ClpB-mediated protein disaggregation are shown in this picture. ClpB hexamer will assemble in the presence of adenosine 5'-triphosphate (ATP) or adenosine 5'-diphosphate (ADP) into an active hexamer with a central channel (1). With the aid of DnaK, ClpB detects protein aggregates and directs them towards aggregates (2). ClpB pulls an exposed end of the polypeptide (3) or loops (4) from the aggregate through its central channel with the use of energy from adenosine triphosphate hydrolysis. Extracted polypeptide exits the ClpB central channel and starts refolding on its own or with the aid of other co-chaperones (5). If a complete polypeptide fails to be extracted from an aggregate, ClpB hexamer dissociates into its monomeric units and will be assembled again at the same or a different location on the aggregate to restart the process (6).

Table 1. The impact of ClpB in ESKAPE pathogens.

Species	Role of ClpB in virulence	Reference
<i>Enterococcus faecium</i>	Helps the cell recover from heat stress	[6,22]
<i>Staphylococcus aureus</i>	Helps the growth in higher temperatures as well as for intracellular replication in invasive staphylococcal infection	[28]
<i>Klebsiella pneumoniae</i>	Withstands extreme environmental conditions and as a component of T6SS	[31,32]
<i>Acinetobacter baumannii</i>	Survives the oxidative stress of macrophages	[35]
<i>Pseudomonas aeruginosa</i>	Helps the growth and survival in higher temperatures	[38]
<i>Enterobacter spp.</i>	Acts as a component of T6SS	[40]

appeared soon after methicillin was introduced into clinical use and are the most infective staphylococci, becoming a global public health problem and a significant contributor to antibiotic resistance[26]. Consequently, the extensive array of toxins and other virulence factors produced by *S. aureus* and their roles in disease pathogenesis have garnered significant attention[27].

According to the findings of Frees and colleagues in 2004, ClpB is essential for *S. aureus* in establishing induced thermotolerance and growth at high temperatures. Their study demonstrated that *clpB* mutants were completely eliminated at 45.5 °C as the incubation temperature increased. In contrast, the wild-type strains formed colonies of the same size and frequency at 37 °C. Although *S. aureus* is primarily recognized as an extracellular pathogen, intracellular replication is now believed to play a significant role in the persistence and frequency of invasive staphylococcal infections by protecting against host defenses and antibiotics. The experiment also revealed that the *clpB* mutant could not replicate in MAC-T cells, a bovine mammary epithelial cell line. These findings suggest that intracellular replication depends on stress tolerance. Based on the discovery that ClpB is a cellular protein quality control system component, it is hypothesized that the accumulation of denatured polypeptides and their aggregates prevents the mutants from replicating inside the cells[28].

4.3. *K. pneumoniae*

Klebsiella species typically reside in the human intestines without causing illness and are also found in human excrement. While infections caused by *Klebsiella* are rare in healthy individuals, they are more common in hospitalized patients, particularly those using ventilators or intravenous catheters and those on prolonged courses of certain antibiotics. Recently, *Klebsiella* spp. has developed notable resistance to the carbapenem class of antibiotics[29]. The Gram-negative, aerobic, and multidrug-resistant *K. pneumoniae* can cause respiratory and urinary tract infections and has been associated with bloodstream infections in immunocompromised individuals[30,31].

A study conducted by Motiwala *et al.* using protein genomes of 100 *Klebsiella* strains (including 57 *K. pneumoniae* strains) showed that 98% of the examined strains, except for *Klebsiella variicola* DX120-E and *Klebsiella variicola* KP5-1, expressed ClpB. *K. pneumoniae* has developed several defense mechanisms to withstand extreme environmental conditions, including caseinolytic proteins for maintaining protein homeostasis in response to internal and external environmental changes[31]. Additionally, ClpB shares high sequence similarity with TssH, a protein component of the Type VI secretion system (T6SS). TssH is involved in the disassembly and recycling of the T6SS protein complex after it delivers effector proteins essential for virulence. Due to this similarity, it is suggested that ClpB also

aids in the disassembly and recycling of T6SS protein complexes, thereby maintaining the efficacy of T6SS and contributing to *K. pneumoniae* virulence[32].

4.4. *A. baumannii*

A. baumannii is responsible for several nosocomial infections associated with ventilator use, catheterization and surgical wounds. Thereby, they can cause infections in wounds, the bloodstream, the urinary tract and the lungs[33]. Due to its rapid acquisition of antibacterial resistance and ability to persist in adverse conditions, *A. baumannii*, a Gram-negative, motile coccobacillus, has become a significant global health burden over the past two decades, even in countries with advanced healthcare systems[34,35].

Macrophages play a crucial role in the host defense against *A. baumannii* by effectively phagocytosing and destroying the pathogen, thereby preventing the spread of infection[36]. The proteins ClpA and ClpB may protect *A. baumannii* from oxidative stress and the acidic environment of the phagolysosome, which are critical for its survival within macrophages. According to a study by Belisario *et al.*, the deletion of the *clpB* gene renders *A. baumannii* more susceptible to macrophage-mediated destruction compared to the deletion of *clpA*. This indicates that ClpB significantly enhances the survival of *A. baumannii* within macrophages, thereby increasing its virulence[35].

4.5. *P. aeruginosa*

Pseudomonas spp. are commonly found in the environment, with *P. aeruginosa* being the most prevalent pathogen causing infections in humans. *P. aeruginosa* can lead to bloodstream infections, pneumonia, and infections in other body parts following surgical or burn wounds. Hospitalized patients, particularly those on ventilators, with indwelling catheters, or with surgical or burn wounds, are at high risk of infection by *P. aeruginosa*[37]. Over 80% of individuals with cystic fibrosis suffer from chronic lung infections caused by this opportunistic bacterium, which is a major cause of morbidity and mortality in these patients. The high versatility of *P. aeruginosa* grants it the phenotypic flexibility to cause both acute and chronic infections in humans[38].

In their study, Mougous and colleagues investigated the roles of ClpV1 and ClpB in *P. aeruginosa*. They found that deletion of *clpV1* did not affect thermotolerance, whereas deletion of *clpB* resulted in thermos-sensitivity, indicating that ClpB is essential for thermotolerance. Additionally, they reported that a mutation in the ClpB analogous residue (E279A) significantly reduced the *in vivo* and *in vitro* activity of *P. aeruginosa*[38].

4.6. *Enterobacter* spp.

Enterobacter, a member of the Enterobacteriaceae family, is primarily associated with nosocomial infections. Currently, there are 22 recognized *Enterobacter* species, though not all are known to cause infections in humans. *Enterobacter* species are responsible for numerous hospital-associated infections and, less frequently, community-acquired diseases such as endocarditis, osteomyelitis, urinary tract infections, soft tissue infections, and lung infections. Additionally, various *Enterobacter* species can be found on human skin, in certain foods, water, sewage, and soil, and some species are part of the mammalian gastrointestinal microflora[39].

According to a genome analysis study conducted in 2020 by Mustafa and colleagues, all five strains of the *Enterobacter cloacae* complex — *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, *Enterobacter nimipressuralis*, and *Enterobacter asburiae* — express IcmF and ClpB, two T6SS signature proteins that play significant roles in virulence and host-microbe interactions. High expression of the *icmF* gene in all *Enterobacter cloacae* complex strains indicates that IcmF drives the secretion of effector proteins via the T6SS. Real-time qPCR results under *in vivo* mimicking conditions show that in the absence of *icmF* in *Enterobacter kobei* and *Enterobacter ludwigii*, *clpB* expression is upregulated. This suggests that ClpB may be crucial for the secretion of effector proteins by the T6SS in the absence of IcmF[40].

5. Role of ClpB in *P. falciparum*

Malaria is a mosquito-borne disease caused by the protozoan *Plasmodium*, significantly affecting populations worldwide, particularly in underdeveloped regions of Africa and Asia. *P. falciparum* is the primary cause of severe and fatal malaria cases[41]. Globally, malaria was treated with chloroquine, the first synthetic antimalarial drug. The parasite *P. falciparum*, known to cause the most deadly form of malaria in humans, has become resistant to several antimalarial medications including chloroquine[42]. The *P. falciparum* genome encodes six Clp proteins: two ClpB isoforms (PfClpB1 and PfClpB2/Hsp101), ClpC, and ClpM, which are ATPases, and ClpP and ClpR12, which are proteases[43].

Plasmodium belongs to the phylum Apicomplexa, characterized by the presence of the apicoplast, a unique organelle essential for the parasite's survival. The apicoplast is a promising target for new antimalarial drugs, as it involves critical biochemical processes necessary for the parasite's survival. During the blood stage of development, the apicoplast's primary function is the production of isoprenoid precursors[43].

All Clp proteins in *Plasmodium* are located in the apicoplast,

except for PfClpB2/Hsp101, which is found exclusively in the parasitophorous vacuole. PfClpB2/Hsp101 is a key component of the *Plasmodium* translocon of exported proteins (PTEX), a protein secretion complex (Supplementary Figure 3)[43].

The ability of *Plasmodium* to export hundreds of proteins that modify and control infected host cells is crucial for its intracellular survival within red blood cells. Exported proteins must first cross the plasma membrane of *Plasmodium* and then the parasitophorous vacuole membrane to reach the host cell cytosol. PfClpB2/Hsp101 is one of the five subunits of the PTEX complex, along with EXP2, PTEX150, TRX2, and PTEX88. Proteins destined for export through the parasitophorous vacuole membrane via the translocation pore EXP2 must be unfolded. The hexameric AAA+ protein PfClpB2 uses ATP hydrolysis to unfold and thread these proteins through the transmembrane conduit EXP2, actively driving the export process[43]. The hexameric structure of PfClpB2 forms a continuous channel that aligns with the heptameric EXP2 transmembrane pore, facilitating the passage of unfolded proteins into the host cell cytosol[44]. Inactivation of PfClpB2 in parasites results in a near-total blockade of protein export, causing substrates to accumulate in the vacuole and affecting all types of exported proteins[43].

PfClpB1 contains a 52-residue non-conserved insert in its middle domain (Supplementary Figure 2), in addition to its AAA+ sequence motifs. Like most AAA+ ATPases, ATP induces PfClpB1 to self-assemble into hexamers. PfClpB1 reactivates aggregated proteins similarly to bacterial ClpB, but this reactivation fails in the presence of *Escherichia coli* DnaK, DnaJ, and GrpE. The species-specific sequence of the ClpB middle domain likely causes poor interaction between PfClpB1 and the bacterial DnaK system. PfClpB1's chaperone activity maintains apicoplast protein folding, contributing to the survival of *P. falciparum*[45]. *In vitro* experiments have shown that apicoplast PfClpB1 exists in a hexameric state and exhibits ATPase-dependent chaperone function[43].

Both PfClpB2 and PfClpB1 are modular proteins composed of a short C-terminal domain, two NBDs that catalyze ATP-driven protein unfolding, an N-terminal domain for substrate binding, and a coiled-coil middle domain, characteristic of Class 1 Clp AAA+ proteins. Sequence alignment of PfClpB1 and PfClpB2 reveals both proteins' substrate-binding pore motifs and nucleotide-binding Walker A/B and Sensor-1/2 motifs. Both *Plasmodium* ClpBs have N-terminal leader sequences indicating their cellular localization (Supplementary Figure 2)[45].

6. Limited availability of antimicrobials

The antimicrobial resistance dilemma arises when bacteria, viruses, fungi, and parasites gradually adapt and become unresponsive

to antimicrobials. This leads to difficulties in curing infections, increased risk of disease transmission, severe illness, and even death[46,47]. This crisis is primarily driven by three key factors: the vast and interconnected global population facilitates the spread of pathogens; the widespread and often unnecessary use of antibiotics exerts strong evolutionary pressure, promoting antimicrobial resistance among microbes; antimicrobial-resistant phenotypes are becoming more prevalent as an evolutionary response to this extensive antibiotic use. Consequently, there is an urgent need for new antibiotics[48,49].

In a laboratory setting, a target must meet specific criteria to be identified as an antimicrobial target: it must be essential for the survival of the microorganism, widely distributed among the target organisms of concern, ideally have no human or other eukaryotic homologs, and be druggable or amenable to interaction *in vivo*, as well as susceptible to inhibition by small molecules or biotherapeutics[50].

7. ClpB as a druggable target

The bacterial heat shock response has emerged as a highly specific and distinctive target, opening a new field of research for developing novel antimicrobial agents. When a pathogen invades a host, it encounters abrupt pH fluctuations, heat shock, and oxidative stress. The pathogen's molecular response to these stresses is crucial for its survival and replication[51]. Hsps are present across all domains of life, and their amino acid sequences are highly conserved. This conservation may partly explain why no successful inhibitors have been discovered to target the stress response mechanism despite evidence supporting the bacterial heat shock response as a promising target for antimicrobial drug development[52,53].

The Hsp100 family member ClpB, exhibit a high degree of sequence conservation including in ESKAPE pathogens and malaria parasites. Due to its absence in metazoans and its essential role in bacterial survival under stress, ClpB presents a novel opportunity for developing innovative antifungal and antiprotozoal medications. ClpB orthologs are also present in lower eukaryotic fungi and protozoans, further supporting its potential as a therapeutic target[52,53].

8. Conclusion

This review summarizes the current knowledge on the role of the Hsp100 molecular chaperone ClpB in enhancing the virulence and survival of several significant pathogens, including ESKAPE pathogens and malarial parasites, which cause life-threatening

infections in humans. ClpB reactivates and disaggregates protein aggregates induced by various stresses within the host cell, a process crucial for the pathogen's survival under stress. ClpB is essential for all the pathogenic microbes discussed in the review, linking its function to the pathogen's adaptation to unfavorable conditions. The antibiotic resistance of ESKAPE pathogens and malarial parasites is a rising global issue that needs urgent attention. Notably, ClpB is absent in human and animal cells, making it an attractive target for drug discovery and development. Controlling protein aggregation mediated by ClpB could help prevent severe human infections. However, further *in vivo* and *in vitro* studies are required to explore the potential of ClpB as a drug target.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

CBR conceptualized the framework of the review and supervised the project design along with RMN and UR. SJU and SS conducted the literature search, compiled studies for data acquisition, and prepared the initial draft. SJU and RMN created and refined the visual materials.

RMN, UR, NNS, and CBR handled the definition of intellectual content, provided insights, and verified references. NNS assessed data accuracy, provided feedback, and suggested additional literature. RMN, UR, and NNS guided the manuscript's direction, ensuring scientific rigor and addressing key theoretical aspects.

CBR conducted the final critical revision of manuscript editing and review and approved the submission as the guarantor. All contributors aided in writing the manuscript, participated in discussions, and addressed reviewer comments.

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ClpB chaperone as a promising target for antimicrobial therapy: A narrative review

Running title : ClpB as a novel druggable target

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<i>E. faecium</i>	1	MNTEKMTTITQEAIAFAQAVTRKHQDTIAHWKIFLC-PNHFGNFYTDAGLDVESF
<i>S. aureus</i>	1	MDUNKMTYAVQSALQCAVELISQKFKLQNIETEAISALNESESLYKSTITERANIEVDQI
<i>K. pneumoniae</i>	1	MRIDRITNKFQALADAAQSLAIGHDNOETIEPIHLMSALLNQEGGSVPPLITSAGINAGKI
<i>A. baumannii</i>	1	MRFEFETNRITQALSDAQSLAMGRDHTATAGTHLSTLLEEPS--NISLLQQAGARIPEL
<i>P. aeruginosa</i>	1	MRIDRITSKIQLALSDAQSLAVGHDHPATIEPVHLISALLEQQGGSIKPLIMQVGFDAAL
<i>E. cloacae</i>	1	MRIDRITNKFQALADAAQSLAIGHDNOETIEPIHLMSALLNQEGGSVPPLITSAGINAGQI
<i>T. thermophilus</i>	1	MNERWTOAAREALACACVLAQRMKHQATLPHLWAVLLKDERSLAWRLLEKAGAPKAI

<i>E. faecium</i>	60	EHEIDRLDEYEVVSGGNVQYGOILSQNFFRLNEADQRESFGDEFISTEVVILALVKL
<i>S. aureus</i>	61	NKAYEDKINTYASVEGDNIQYQYISQANQLITKAEASYMKEYEDEISMVHIIRSAMDI
<i>K. pneumoniae</i>	61	ETDIEQALSRLPQVEGTG--GDVQPSQILVRLNLCDNLACKKKDNFISSEIFVLAALS
<i>A. baumannii</i>	59	KQKIEQALKDAPTIANPT--GDVININPFAVKALNLADRYAQKAGDEFISTIWVILG-LAE
<i>P. aeruginosa</i>	61	ESGINKELDALPKIQSPT--GDVILSQILARILNQADNLAAQKGDQFISSEIFVILAAME
<i>E. cloacae</i>	61	ETAITQALSRLPQVEGTG--GDVQPSQILVRLNLCDNLACKKRGDNFISSEIFVLAALS
<i>T. thermophilus</i>	61	KELQERELARLPKVEGAE--VGQYLISRLSGALNRALMEELKDRVAVTLVLALALAA

N-terminal and linker

<i>E. faecium</i>	120	KNYPLTVYLKNGLEKELEKNTIEVRGGERVTSQNOEEQYKALEKYGVLDIVQVKSGKM
<i>S. aureus</i>	121	DQTTKH-YLNK---VEVTHEIILKKVRGGNHVTSONPEVNYBALAKYGRDLVEVRQGM
<i>K. pneumoniae</i>	119	RGTLTD-LIKSAGATANVTQATIQVRGGESVNDQGAEDQRAALKKTVDLTERAEQGKL
<i>A. baumannii</i>	116	TGETKN-ILSAVGVPDSTLKVIENTRGSEKVMNNHEDQDSDLNKYTDLTERALSGLK
<i>P. aeruginosa</i>	119	NTRLGK-LLLGQGVSRKALENANIRGGEAVNDPNVEESRQALDKYTVDLTKRAEEGKL
<i>E. cloacae</i>	119	RGTLTD-LIKSAGATANVTQATIEKVRGGESVNDQGAEDQRAALKKTVDLTERAEQGKL
<i>T. thermophilus</i>	119	TPGLEPG-----LEALNGAKELRGGRVTQTEHAESTYNALEQYGLDLTRDAEGKL

<i>E. faecium</i>	180	DPVIGRDEEIRDVIRLSRRTKKNPVLIGEPGVGKTAIVEGLAQIRIVKVPENLKDRTI
<i>S. aureus</i>	177	DPVIGRDEEIRNTIRLSRRTKKNPVLIGEPGVGKTAIVEGLAQIRIVKVPESILDKTV
<i>K. pneumoniae</i>	178	DPVIGRDEEIRRTIQVLQRRTKKNPVLIGEPGVGKTAIVEGLAQIRIVNGEVEPLGKGRV
<i>A. baumannii</i>	175	DPVIGRDDIIRRTIQVLSRRTKKNPVLIGEPGVGKTAIVEGLAQIRIVNGEVEPLGKNKRV
<i>P. aeruginosa</i>	178	DPVIGRDDIIRRTIQVLQRRTKKNPVLIGEPGVGKTAIVEGLAQIRIVNGEVEPLGKDKRL
<i>E. cloacae</i>	178	DPVIGRDEEIRRTIQVLQRRTKKNPVLIGEPGVGKTAIVEGLAQIRIVNGEVEPLGKGRV
<i>T. thermophilus</i>	170	DPVIGRDEEIRRVIQILLRRTKKNPVLIGEPGVGKTAIVEGLAQIRIVKVPPEGLGKGRV

Walker A

<i>E. faecium</i>	240	FSLDMGALVAGAKFRGEFEERLKAVLKEVKKSGRITLFFIDELHTNVGAGKTEGSM DAGN
<i>S. aureus</i>	237	FELDLSALVAGAKYRGEFEERLKAVLKEVKESIGRITLFFIDELHTNVGAGKTGDGAM DAGN
<i>K. pneumoniae</i>	238	LALDMGALVAGAKYRGEFEERLKAVLTLSKQEGNVILFIDELHTNVGAGKADGAM DAGN
<i>A. baumannii</i>	235	LSLDIGSLVAGAKYRGEFEERLKAVLKULAKHEGESITLFFIDELHTNVGAGKGDGAM DAGN
<i>P. aeruginosa</i>	238	LALDMGALVAGAKFRGEFEERLKAVLNELGKQEGRVILFIDELHTNVGAGKAGAM DAGN
<i>E. cloacae</i>	238	LALDMGALVAGAKYRGEFEERLKAVLNELGKQEGNVILFIDELHTNVGAGKADGAM DAGN
<i>T. thermophilus</i>	230	WSLQMGSLVAGAKYRGEFEERLKAVIQEVQSQGESVILFIDELHTNVGAGKAGAM DAGN

Port

Walker B

NBD-1

<i>E. faecium</i>	300	MLKPM LARGELHLIGATTLD EYROYMEKDKALERRFQKVLVKEPTVEDTISILRGLKERY
<i>S. aureus</i>	297	MLKPM LARGELHLCIGATTLD EYRIEYIEKDSALERRFQKVAVSEPTVEDTISILRGLKERY
<i>K. pneumoniae</i>	298	MLKPALARGELHCVGATTLD EYROYIEKDAALERRFQKVFAEPSVEDTIAILRGLKERY
<i>A. baumannii</i>	295	MLKPALARGELRCVGATTLD EYROYIEKDAALERRFQKVLVDEPSVEDTIAILRGLKERY
<i>P. aeruginosa</i>	298	MLKPALARGELHCVGATTLD EYROYIEKDAALERRFQKVLVDEPSVEDTIAILRGLKERY
<i>E. cloacae</i>	298	MLKPALARGELHCVGATTLD EYROYIEKDAALERRFQKVFAEPSVEDTIAILRGLKERY
<i>T. thermophilus</i>	290	MLKPALARGELRLIGATTLD EYRIEYIEKDPALERRFQVYVDEPTVEDTISILRGLKERY

Sensor-1

<i>E. faecium</i>	360	EHHGVNIHONALVAAATLS DRYITDRQLPDKAIDLIDEAASATIRMEINSMPTELDQVTR
<i>S. aureus</i>	357	EYHHGVRIQDRALVAAAE L DRYITDRQLPDKAIDLIDEACATIRTEGNSMPTELDQVNR
<i>K. pneumoniae</i>	358	EHHHHVNIIDPAIVAAATLS HRYIADROLPDKAIDLIDEAASSIRMQIDSKPEELDRDLR
<i>A. baumannii</i>	355	ATHHGVNIIDSAITAAAK LSHRYITDRQLPDKAIDLIDEAASRIIMEIDSKPEALDKLDR
<i>P. aeruginosa</i>	358	EHHHGVSIIDCAITAAAK LSHRYITDRQLPDKAIDLIDEAASRIIMEIDSKPEELDRDLR
<i>E. cloacae</i>	358	EHHHHVNIIDPAIVAAATLS HRYIADROLPDKAIDLIDEAASSIRMQIDSKPEELDRDLR
<i>T. thermophilus</i>	349	EHHHGVRIISDAITAAATLS HRYITDRQLPDKAIDLIDEAAARIRMALESAPETIDALIR

<i>E. faecium</i>	420	RLMQLEETEEAALKKESDASKKRRLKNLDEELALRPEANAKMOMETEKEEVNSVSAKIA
<i>S. aureus</i>	417	RYMQLEETEEAALKNESDNASKQRLOELDEELANEKEKQAAQSRVSESEKKEIANLQEKHA
<i>K. pneumoniae</i>	418	RLIQLKLEEQQALKKESDEASLKRIDMLDEELAKERQMSVLEEEWKAEKASLSGQTITIA
<i>A. baumannii</i>	415	RLIQLKVQLEAAKKLEDAASKAEVTHLEKQIAEVEKEYNDEEVWKAECTIVEGKQQAQV
<i>P. aeruginosa</i>	418	RLIQLKIEREALKKEIDDAIRKRRAKLEEDIVKIEREYADLEEIWKSEKAEVQGSQAIIQQ
<i>E. cloacae</i>	418	RLIQLKLEEQQALKKESDASKKRIDMLDEELDKERQYSELEEEWKAEKASLSGQTITIA
<i>T. thermophilus</i>	409	KKIQLEETIEREALKKEKDPDSQERIKAEAEAKTETETIAKRAEWEREFEILRLKREAQH

<i>E. faecium</i>	480	EIDKAKHELEDAENNYDLERAAVTRHGTTIPOLEKELKELEAKAKD----SEIKMVQESVT
<i>S. aureus</i>	477	QLDESRQALEDAQTNNNLEAAELOYGTTIPOLEKELRELEDNFQDEQGEDTDRMITREVVT
<i>K. pneumoniae</i>	478	ELIQAKTATEQARRVGDIAFMSELQYQKIPLEKQLAAATQS-----EGKTMRLLRNKVT
<i>A. baumannii</i>	475	ELDKAIAFEKAQREGDLABAARLQYGVIPBLQKQLEQDVAE-----ENEFPILIRTKVT
<i>P. aeruginosa</i>	478	KLEQAKQEMEAARRRGDLESMAIRQYQTTIPDLERSLQMVQIH-----GKTENQLLRNKVT
<i>E. cloacae</i>	478	ELIQAKTATEQARRVGDIAFMSELQYQKIPLEKQLEIATQS-----EGKTMRLLRNKVT
<i>T. thermophilus</i>	469	RLDEVIRELEDAERQYDLNRAAEIRYGEIPKLEAEAEALSE-----KLRGARFVRLEVT

Middle domain

<i>E. faecium</i>	536	ENEIAQVVGRLTGIPVTKLVEGEREKLKINETLHHRVIGQDEAVVAVSDAVIRSRAGIQ
<i>S. aureus</i>	537	DEEIGDIVSQWTGIPVSKLVETEREKLIHSLILHHRVVGQDKAVILVSDAVVIRARAGIK
<i>K. pneumoniae</i>	533	DAEIAEVLARWTGIPVSKMSESERIKLLRMEQLHHRVIGQDEAVVAVSNATRRSRAGIS
<i>A. baumannii</i>	531	ENEIAEVVSAATGIPVAKMVGGEREKLHMEQLHHRVIGQDEAVVAVSNATRRSRAGIS
<i>P. aeruginosa</i>	533	DEEIAEVVSNWTGIPVSKMVEGEREKLRLMEQLHHRVIGQDEAVVAVSNATRRSRAGIA
<i>E. cloacae</i>	533	DAEIAEVLARWTGIPVARMVEGEREKLRLMEQLHHRVIGQDEAVVAVSNATRRSRAGIS
<i>T. thermophilus</i>	523	DEEIAEIVSRWTGIPVSKLVEGEREKLRLVEELHHRVVGQDEAVVAVADAIRRARAGIK

<i>E. faecium</i>	596	DPNRPVGSFSLFLGPTGVGKTELAKALAEFLDSEDHMVRIDMSEFMKHAVSRLVGAPPG
<i>S. aureus</i>	597	DPNRPVGSFSLFLGPTGVGKTELAKSLAASLFDSEKHMVRIDMSEFMKHAVSRLVGAPPG
<i>K. pneumoniae</i>	593	DPNRPVGSFSLFLGPTGVGKTELCITLANFLFDSDDAMVRIDMSEFMKHSVSRVLGAPPG
<i>A. baumannii</i>	591	DPNRPVGSFSLFLGPTGVGKTELCITLANFLFDSDDAMVRIDMSEFMKHSVSRVLGAPPG
<i>P. aeruginosa</i>	593	DPNRPVGSFSLFLGPTGVGKTELCALAEFLFDEEALVRIDMSEFMKHSVSRVLGAPPG
<i>E. cloacae</i>	593	DPNRPVGSFSLFLGPTGVGKTELCALAEFLFDSDDAMVRIDMSEFMKHSVSRVLGAPPG
<i>T. thermophilus</i>	583	DPNRPVGSFSLFLGPTGVGKTELAITLAATLFDDEEAMVRIDMSEFMKHAVSRLVGAPPG

Walker A

Pore

<i>E. faecium</i>	656	YVGVEEGGQLTEAVRRNPYSVILLDEVEKAHPDVFNILQVLDGRLTDSKGRVDFKNT
<i>S. aureus</i>	657	YIGHDEGGQLTEAVRRNPYSVILLDEVEKAHTDVFNILLQLDEGRLTDSKGRVDFKNT
<i>K. pneumoniae</i>	653	YVGVEEGGYLTEAVRRNPYSVILLDEVEKAHPDVFNILQVLDGRLTDGQGRVDFFRNT
<i>A. baumannii</i>	651	YVGVEEGGYLTEAVRRNPYSVILLDEVEKAHPDVFNILQVLDGRLTDSKGRVDFKNT
<i>P. aeruginosa</i>	653	YVGVEEGGYLTEARRKPYSVILLDEVEKAHPDVFNILQVLDGRLTDSHGRVDFFRNT
<i>E. cloacae</i>	653	YVGVEEGGYLTEAVRRNPYSVILLDEVEKAHPDVFNILQVLDGRLTDGQGRVDFFRNT
<i>T. thermophilus</i>	643	YVGVEEGGQLTEAVRRNPYSVILLDEVEKAHPDVFNILQLDDGRLTDSHGRVDFFRNT

Walker B

NBD-2

<i>E. faecium</i>	716	VLIMTSNIGSQLLLEGVTAEGTIPEAAVQVNTLRGNFRPEFINRIDEVVFHPLSLDN
<i>S. aureus</i>	717	IIIMTSNIGSQVLLNVKGTGITESTEKAVITNNAYFKPEILNRDDIVLFKPLSLID
<i>K. pneumoniae</i>	713	VVIMTSNLGSDLIQEFRGELD-YGHMKDLVGVVSNFRPEFINRIDEVVFHPLGEKH
<i>A. baumannii</i>	711	VIVMTSNLGSDVREITGEGAT-DEVEFTIVNAVSHFRPEFINRIDEVVFHSLKKA
<i>P. aeruginosa</i>	713	VVIMTSNLGSAQIQEFAGERE-A--QAAVDAVNAHFRPEFINRIDEVVFHPLAREQ
<i>E. cloacae</i>	713	VVIMTSNLGSDLIQEFRGELD-YSHMKDLVGVVSNFRPEFINRIDEVVFHPLGEKH
<i>T. thermophilus</i>	703	VILTSNLGSEPLILEGQKGP-YERIRDEVFKVIOQHFPEFINRIDEVVFHPLTKEQ

Sensor-1

<i>E. faecium</i>	776	KGIVKMWQAQARLEHQEILTISDEAKTWAEINYEPAVGARPLKREITRENETPLA
<i>S. aureus</i>	777	SMIVKILQNLIRLLQRSIEVSDIAKAWLGQEAYPEVGARPLKREVOQIETPLA
<i>K. pneumoniae</i>	771	IASIAQIQLORLYKRLEERGYESQMSDEALKLISANGYDPVYGARPLKRAIQOOIENPLA
<i>A. baumannii</i>	769	IRGIAIQLODRLRSLVRDMSLTVDSDAFDLIIAGHDPVYGARPLKRAIQOOIENPLA
<i>P. aeruginosa</i>	769	IAGIAIQLGRLRKRLAEREISLESQALDKLIAGHDPVYGARPLKRAIQOOIENPLA
<i>E. cloacae</i>	771	IASIAQIQLORLYKRLEERGYESHISDEALKLISANGYDPVYGARPLKRAIQOOIENPLA
<i>T. thermophilus</i>	762	IRQIVTIQLSYLRLARLAERISLEITFAKDFLAERGYPVYGARPLRRVIQRENETPLA

Sensor-2

<i>E. faecium</i>	836	KEI V ACH V M P K S K T T T T - - L D G Q L H F K T E E L E E I V
<i>S. aureus</i>	837	R M M I K E G F P E G T T I K V N N S D N N L T F N V E K I H E - -
<i>K. pneumoniae</i>	831	Q Q I L S G E L V P G K T I E L V K D D - R I V A V Q - - - - -
<i>A. baumannii</i>	829	Q K I L S G D F V A G D T I L V K G E N G - H L V F D K L K L S - - -
<i>P. aeruginosa</i>	829	Q L I L A G K F A F G A S I S A K T E G D - E I V F A - - - - -
<i>E. cloacae</i>	831	Q Q I L S G E L I P G K V I R I E A N D D - R I V A V Q - - - - -
<i>T. thermophilus</i>	822	Q K I L A G E V K E G D R T Q V D T G P A - G L V F A V P A R V E - -

C-terminal

Supplementary Figure 1. Sequence alignment of ClpB proteins of ESKAPE pathogens.

Using the Clustal Omega Multiple Sequence Alignment tool provided by Bioinformatics Institute EMBL-EBI available at <https://www.ebi.ac.uk/jdispatcher/msa/clustalo> , the ClpB sequences of common ESKAPE pathogens were compared. Boxshade version 3.3, available at <https://junli.netlify.app/apps/boxshade/>, was then used to shade the alignments. Conserved residues are highlighted in black, similar amino acids are highlighted in grey, and distinct amino acids are not highlighted. Gaps in the sequence alignment are denoted by the symbol (). Structural regions are underlined as **purple**-N-terminal and linker, nucleotide binding domains (NBDs), **red**-NBD-1 and **blue**-NBD-2, **green**-middle domain and **magenta**-end C terminal. Residues of NBD-1, Walker A and Walker B are presented in blue and orange lettering, while those of NBD-2, Walker A and Walker B residues are presented in green and yellow lettering, respectively. Pore loop residues of NBD-1 and NBD-2 are highlighted in purple and light green while sensor 1 residues of NBD-1 and NBD-2 are highlighted with teal and magenta. Sensor 2 residues of NBD-2 are highlighted in green. All these regions were identified by referring to the crystal structure of ClpB derived from *Thermus thermophilus* (PDB ID: 1QVR)[12].

The following strains were used in the alignment:

E. faecium- tr|A0A132PA02|A0A132PA02_ENTFC, *Enterococcus faecium*

S. aureus- sp|Q7A6G6|CLPB_STAAN, *Staphylococcus aureus* (strain N315)

K. pneumoniae- tr|W9B407|W9B407_KLEPN, *Klebsiella pneumoniae*

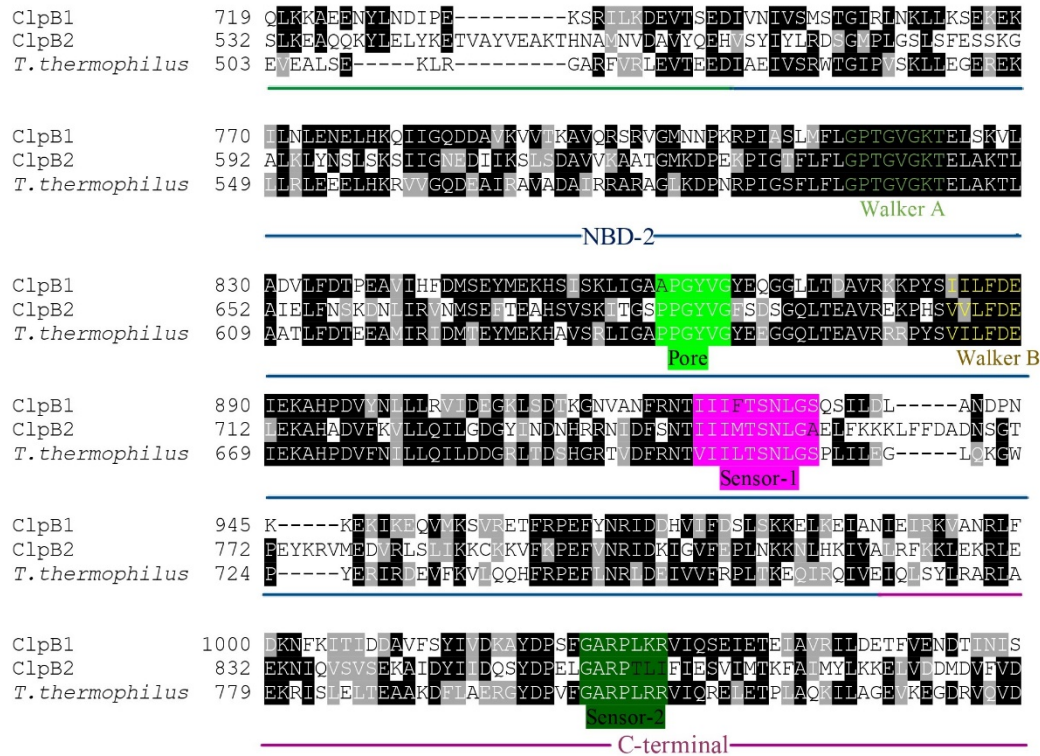
A. baumannii- tr|V5VGK1|V5VGK1_ACIBA, *Acinetobacter baumannii*

P. aeruginosa- sp|Q9HVN5|CLPB_PSEAE, *Pseudomonas aeruginosa* (strain ATCC 15692 / DSM 22644 / CIP 104116 / JCM 14847 / LMG 12228 / 1C / PRS 101 / PAO1)

Enterobacter- tr|A0A3S0FSR9|A0A3S0FSR9_9ENTR, *Enterobacter* sp. WCHEn090040

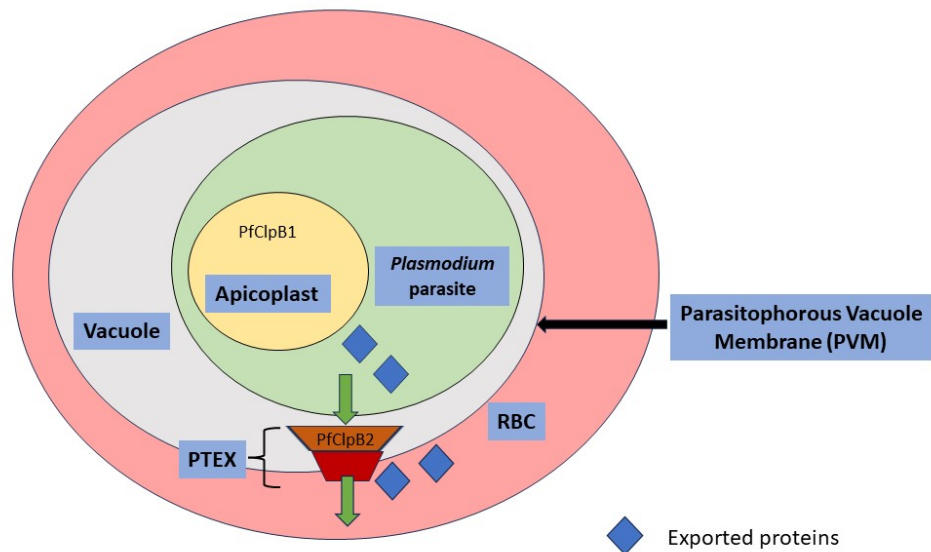
Thermus thermophilus (strain ATCC 27634 / DSM 579 / HB8)

ClpB1	1	MVNSFFCFVITGLIYVWDITYSKKAKIFFNKNDFISIKNTHWDIYDKKKYFFIGNNHLK
ClpB2	1	MTRRYLKYYIFVTLLEFVQ-----IN----
<i>T. thermophilus</i>	1	-----
ClpB1	61	NEESFLPEVRKDYKSKIKEYKNSTNGIYHNNKNRLSYTINDQVNYDNNMTSGINKRRKV
ClpB2	23	-NVLCAPD---NKQE-----
<i>T. thermophilus</i>	1	-----
Leader sequence		
ClpB1	121	KDSSIHMNNSYEKNRNKNKALFMSDEEYTNISDDYTEKAWEAISLNKITEKYDSAYVE
ClpB2	34	-----OGKYLNRTINILNACKNIKSYCHNKIK
<i>T. thermophilus</i>	1	-----NNLERNTAAREALAQGVTAQEMKHCALT
ClpB1	181	AEMLLALINDSPDGLAERLLKESGIDTQLLVQETDDYLKQPKPSCFGEQKII-GRIT
ClpB2	62	PIHILSALAKSD---YGSTLFKENNVNAAANKHYIDIALEQTRAGAPLDNKSKIINSAEV
<i>T. thermophilus</i>	31	LPHIW-AVILKDERSLAWRLLEKAGADPKALKELORELAQLPKVEGA-EVGQYI-TSRI
ClpB1	240	QTVLSTSKRLKKEINDEYISTEHLISISESKFTRPWLKYNINYEKIKKAEKIRCK
ClpB2	119	KETLALAEAAANKKSPKVDVEHLISGLSNDEL-VNEIEN-EVYTDEAKKAILKR-KFE
<i>T. thermophilus</i>	88	SGALNRAEALMEELKDRYVAVITLILAEATPGI-----PGLEAKGALKELRGG
N-terminal and linker		
ClpB1	300	KKVTSKTPFTYCALEKYSRDLTALARAGKLDPVIGRDNEIRRAIQILSRRTKNNPILQG
ClpB2	176	KTKKDKDCKGTLYTEQGSNNNEKVRNGKLQGYGRDEEIRATIESLLRYNKNSPVLVG
<i>T. thermophilus</i>	139	RTVQTEHAESTYNALQYGTDLTRLAAEGKLDPVIGRDDEEIRRIQILLRRTKNNPVLQG
ClpB1	360	DPGVGKTAIVEGLAKIVCGDVPISLKGRKTVSLDMSSLAGAKYRGDFEERLKSILKEV
ClpB2	236	NPGITGKTIIVEGLIVYRIEKGDVPELQGYTVISLNRKFTSGISYRGEFETRMNIIKEI
<i>T. thermophilus</i>	199	DPGVGKTAIVEGLAQRIVKGDVPISGLKGRITVSLMCSLAGAKYRGFEERLKAIVQEV
Walker A		
ClpB1	420	QDAEGOVVFIDEIHTVVGAGAVAEGAIDAGNLLKPLARGELRCIGATTVSEYRFIEK
ClpB2	296	KIKKKNKILFDEIHLIGAGK-AEGGTDAANLLKPLSGEIKLIGATTIAEYRKFIES
<i>T. thermophilus</i>	259	VDSQGEVILFIDEIHTVVGAGK-AEGAIDAGNLLKPLARGELRLIGATTIDEYREIEK
Walker B		
Sensor-1		
NBD-1		
ClpB1	480	DKALERRFQQLIVEQPSVDETISILRGLKEFYEVHHGVRIIDSAIVQAAVLSDRYISYRE
ClpB2	355	CSAFERRFEKILVEPPSVDMTKILRLSKSYENFYGINIDKALVAAAKISDRRIKDRM
<i>T. thermophilus</i>	317	DPALERRFQPIYVIEETVDETISILRGLKEKYEVHHGVRIIDSAITAAATLSHRVITERR
ClpB1	540	LPDKAIDLIDEAASNKIQLSKKEIQLENTEKQIQLEMEKISILGDKQKNLF-NYSSVA
ClpB2	415	LPDKAIDLINKACSEIQVLSGKERIIDVTERDIERLSYEISTLEKDVDRVSKKKYNKI-
<i>T. thermophilus</i>	377	LPDKAIDLIDEAAARLRVALESAPFEEIDALERKKIQLETEREALKKKKD-----
Non-conserved insert		
ClpB1	599	NTHNNNNSSISSNSSSYGNAEETEATVDYTKSPNFLKKRINEKEIDRLKMIORIMSEL
ClpB2	474	-----IEFEFEK
<i>T. thermophilus</i>	426	-----PDSQERLKATAEIAKL
Middle domain		
ClpB1	659	KEQKILDSSTESYVDNIRAKERIDVVKTEIEKAERYDLNRAAEIRFETLPDLK
ClpB2	481	KEQLKKYYEENVITGERLKKKEIEKKINDIK---ELTONYVYSNKEPPI-----ELQN
<i>T. thermophilus</i>	443	TEETAKRAEWEREIEILKLREAQHRLEVRREIELAEROVDLNRAAEIRYGELEPKLEA



Supplementary Figure 2. Sequence alignment of ClpB1 and ClpB2 of *Plasmodium falciparum*.

Using Clustal Omega Multiple Sequence Alignment tool provided by Bioinformatics Institute EMBL-EBI available at, <https://www.ebi.ac.uk/jdispatcher/msa/clustalo>, ClpB1 and ClpB2 sequences with gene IDs PF08_0063 and PF11_0175 of *Plasmodium falciparum* were compared. Boxshade version 3.3, available at <https://junli.netlify.app/apps/boxshade/>, was then used to shade the alignments. Conserved residues are highlighted in black, similar amino acids are highlighted in grey, and distinct amino acids are not highlighted. Gaps in the sequence alignment are denoted by the symbol (-). Structural regions are underlined as **Orange**- Leader sequence, **purple**- N-terminal and linker, nucleotide binding domains (NBDs), **red**- NBD-1 and **blue** - NBD-2, **green** - middle domain and **magenta** end C-terminal. Non-conserved inserts with 52 residues in ClpB2 are shown in brown lettering. Residues of NBD-1, Walker A and Walker B are presented in blue and orange lettering, while those of NBD-2, Walker A and Walker B residues are presented in green and yellow lettering, respectively. Pore loop residues of NBD-1 and NBD-2 are highlighted in purple and light green, while sensor 1 residues of NBD-1 and NBD-2 are highlighted with teal and magenta. Sensor 2 residues of NBD-2 are highlighted in green[45].



Supplementary Figure 3. Schematic presentation of the *Plasmodium vivax* parasite within a parasitophorous vacuole in an infected red blood cell (RBC). The ClpB proteins, PfClpB1 (located in the apicoplast) and PfClpB2 (localized in *Plasmodium vivax*), play crucial roles in the parasite's biology. The Plasmodium translocon of exported proteins (PTEX), a vacuole membrane protein complex, is responsible for secreting malaria effector proteins into the RBC. Upon invasion of a human red blood cell by the *Plasmodium* parasite, PTEX facilitates the translocation of effector malarial proteins across the parasitophorous vacuole membrane. Details for the diagram were adapted[43].