Contribution of the Clp Protease to Bacterial Survival and Mitochondrial Homoeostasis

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Keywords

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Abstract

Fast adaptation to environmental changes ensures bacterial survival, and proteolysis represents a key cellular process in adaptation. The Clp protease system is a multi-component machinery responsible for protein homoeostasis, protein quality control, and targeted proteolysis of transcriptional regulators in prokaryotic cells and prokaryote-derived organelles of eukaryotic cells. A functional Clp protease complex consists of the tetradecameric proteolytic core ClpP and a hexameric ATP-consuming Clp-ATPase, several of which can associate with the same proteolytic core. Clp-ATPases confer substrate specificity by recognising specific degradation tags, and further selectivity is conferred by adaptor proteins, together allowing for a fine-tuned degradation process embedded in elaborate regulatory networks. This review focuses on the contribution of the Clp protease system to prokaryotic survival and summarises the current state of knowledge for exemplary bacteria in an increasing degree of

interaction with eukaryotic cells. Starting from free-living bacteria as exemplified by a non-pathogenic and a pathogenic member of the Firmicutes, i.e., *Bacillus subtilis* and *Staphylococcus aureus*, respectively, we turn our attention to facultative and obligate intracellular bacterial pathogens, i.e., *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Chlamydia trachomatis*, and conclude with mitochondria. Under stress conditions, the Clp protease system exerts its pivotal role in the degradation of damaged proteins and controls the timing and extent of the heat-shock response by regulatory proteolysis. Key regulators of developmental programmes like natural competence, motility, and sporulation are also under Clp proteolytic control. In many pathogenic species, the Clp system is required for the expression of virulence factors and essential for colonising the host. In accordance with its evolutionary origin, the human mitochondrial Clp protease strongly resembles its bacterial counterparts, taking a central role in protein quality control and homoeostasis, energy metabolism, and apoptosis in eukaryotic cells, and several cancer cell types depend on it for proliferation.

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Introduction to the Clp Machinery

The caseinolytic protease Clp, an energy-dependent, multimeric degradation machinery, plays a crucial role in protein homeostasis and quality control in prokaryotic cells and prokaryote-derived organelles of eukaryotic cells. The finely tuned Clp system is pivotal for bacterial survival under stress conditions, as damaged proteins are recognised as substrates [Meyer and Baker, 2011] and a multitude of major stress regulators are controlled by Clp-mediated regulatory proteolysis in various organisms [Frees et al., 2007]. Heat-shock chaperone induction, natural competence, motility, and sporulation are escape strategies regulated by the Clp machinery [Frees et al., 2007; Elsholz et al., 2017], and many pathogenic species were attenuated in the host environment when components of the Clp protease system were deleted [Brötz-Oesterhelt and Sass, 2014].

A functional Clp protease complex is composed of an ATP-consuming hexameric Clp-ATPase, which belongs to the Clp/Hsp100 chaperones of the AAA+ (ATPases associated with cellular activities) superfamily and the barrel-shaped proteolytic core ClpP (Fig. 1a, b). The central component ClpP is a self-compartmentalised serine peptidase with catalytic triads secluded within an internal degradation chamber formed by two stacked heptameric rings. The ClpP peptidase itself exhibits solely peptidolytic activity, whereas for protein hydrolysis it associates with Clp-ATPases, which unfold and translocate protein substrates in an ATP-consuming process into the secluded degradation chamber of ClpP [Olivares et al., 2016]. The catalytic triads of ClpP have very low cutting preference, and substrate specificity is conferred by the cognate Clp-ATPases. Usually, bacteria possess different Clp-ATPases, such as ClpX, ClpA, ClpC, or ClpE, which interact with the same proteolytic core [Frees et al., 2007; Olivares et al., 2016]. In this review, we use the term “Clp protease” to include all Clp-ATPase/ClpP combinations of a given organism to signify that they share the same proteolytic core.

Clp-ATPases recognise their substrates via certain degradation tags, which are either intrinsic to a particular protein sequence and only exposed under certain conditions [Maglica et al., 2008], or attached to proteins on demand, such as the *ssrA*-tag translated from a transfer-messenger (tm)RNA to rescue stalled ribosomes [Flynn et al., 2001]. Substrate specificity is further regulated by the interaction of the Clp-ATPases with specific adaptor proteins, like SspB and ClpS in *Escherichia coli* or McsB in *Bacillus subtilis* [Kirstein et al., 2009b]. Adaptor pro-

teins can, for example, enhance the affinity of a given Clp-ATPase to a particular protein substrate or stabilise the functional oligomeric state of a Clp-ATPase [Kirstein et al., 2009b]. Thus, the adaptor proteins offer an additional level of fine regulation to modulate Clp protease activity. For instance, in *E. coli*, *ssrA*-tagged proteins can be degraded by ClpXP as well as ClpAP, and the two adaptors SspB and ClpS help direct the substrate flow between the two protease complexes [Kirstein et al., 2009b]. Phosphorylation is a further means of regulating proteolysis. In *B. subtilis*, the arginine kinase McsB not only acts as an adaptor protein but distinguishes proteins via arginine phosphorylation for better recognition by ClpC and subsequent degradation by ClpCP [Trentini et al., 2016].

Most bacterial species encode only a single *clpP* gene, resulting in a homo-tetradecameric proteolytic core with 14 identical subunits. However, the increasing number of studies on ClpP proteins from pathogenic bacteria such as *Chlamydia trachomatis*, *Clostridium difficile*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, or *Pseudomonas aeruginosa* revealed the existence of distinct ClpP isoforms within the same organism, forming distinct homomeric tetradecamers or mixed heteromeric tetradecamers [Schmitz et al., 2014; Hall et al., 2017; Gatsogiannis et al., 2019; Lavey et al., 2019; Pan et al., 2019]. For instance, the intracellular pathogens *M. tuberculosis*, *L. monocytogenes*, and *C. trachomatis* contain a mixed ClpP1P2 hetero-tetradecamer, one heptameric ring comprising only ClpP1 and the opposite ring only ClpP2 [Schmitz et al., 2014; Gatsogiannis et al., 2019; Pan et al., 2019], as shown in Figure 1c.

This review starts with a free-living non-pathogenic soil organism to continue with pathogens in increasing dependence on host cells and concludes with mitochondria as an example for a bacteria-derived organelle. In following this structure, it also starts with Clp systems studied for many years, where a substantial level of information exists, regarding structure, regulatory networks, and physiological targets, towards systems studied only more recently and where few, if any, natural substrates are known.

***B. subtilis*: Role of Clp in Protein Homeostasis and Environmental Stress Management**

The Clp machinery of *B. subtilis* was among the first studied, with a particular interest in regulatory proteolysis networks. Here, *B. subtilis* is discussed as an example of the contribution of the Clp protease to a versatile bac-

Fig. 1. Operation mode and composition of the exemplary Clp protease complexes discussed in this review. **a** Model for the general operation mode of a Clp protease complex. Clp-ATPase-mediated substrate degradation by the proteolytic core ClpP is fuelled by ATP hydrolysis to unfold and translocate the polypeptide chain into the degradation chamber of ClpP. Further substrate specificity is achieved by the interaction of the Clp-ATPases with specific adaptors. **b** Cryo-EM structure of the *L. monocytogenes* ClpX hexamer (PDB: 6SFW) in interaction with the heptameric binding surface of the exclusive interaction partner ClpP2 within the hetero-tetradecameric *Listeria* ClpP1P2 (PDB: 6SFX) proteolytic core [Gatsogiannis et al., 2019]. The *Listeria* ClpXP1P2 cryo-EM structure is depicted in a surface fill model representation with ClpX coloured in raspberry red and ClpP1 and ClpP2 in dark and light grey, respectively. A 90° rotation of the cryo-EM structure, now exposing the apical surface of ClpP1, illustrates the symmetry mis-

match between the hexameric ClpX unfoldase and the heptameric ClpP1P2 core. The structures were visualised using PYMOL (<https://pymol.org/2/>). **c** Schematic overview of the composition of selected Clp protease complexes. In principle, due to the homomeric nature of the proteolytic ClpP core in *B. subtilis* and *S. aureus*, the Clp-ATPases ClpX, ClpC, and in *B. subtilis* also ClpE can bind to both sides of the tetradecameric barrel, which also refers to mitochondrial ClpX and ClpP [Kang et al., 2002]. Contrariwise, in *L. monocytogenes*, *M. tuberculosis*, and *C. trachomatis*, comprising a heteromeric ClpP1P2 core, ClpP2 acts as the exclusive interaction partner for the Clp-ATPases ClpX and ClpC1 in *M. tuberculosis* [Leodolter et al., 2015], ClpX in *L. monocytogenes* [Gatsogiannis et al., 2019], and ClpX in *C. trachomatis* [Pan et al., 2019]. The composition of ClpCP in *L. monocytogenes* and *C. trachomatis* has not been determined (n.d.) so far.

terial lifestyle and efficient stress compensation. *B. subtilis* masters survival in diverse environments, such as soil, marine ecosystems, the gut of invertebrates and vertebrates, and in association with plant roots [Brito et al., 2018]. Adaptation to different environments and changes within a given environment requires flexible adjustment of the bacterial proteome. Besides differential protein expression and activation, targeted proteolysis of regulatory factors plays a crucial role in managing the required alterations, as does the degradation of misfolded or aggregated proteins accumulating under stress. AAA+ proteases carry out this task, and the Clp protease is a prominent member of those [Kirstein et al., 2009b].

Prokaryotic organisms are directly affected by changes in their environment, and adverse conditions, such as nutrient shortage, unfavourable temperature, or exposure to toxic agents, require an immediate protective response and sometimes the induction of a long-term survival programme. In *B. subtilis*, regulatory proteolysis by the Clp machinery helps to: (1) better cope with external stress such as elevated temperatures, (2) actively move to a more favourable environment, (3) take up external DNA from the environment to enrich the gene pool, and, should everything else fail, to (4) secure long-term survival by starting the sporulation programme (Fig. 2a).

In *B. subtilis*, ClpP, as well as the ATPases ClpC and ClpE, belong to the heat-shock regulon. *ClpP* is transcribed as a monocistronic gene, *clpC* forms an operon with the adaptors *mcsA* and *mcsB*, while *clpX* and *clpE* are encoded in distinct operons [Derre et al., 1999; Krüger et al., 2001; Frees et al., 2007]. To allow for well-restrained proteolysis in general and efficient but transient induction under stress, the Clp system is controlled by several regulatory circles. Interestingly, the Clp machinery autoregulates its expression by recognising its own repressor CtsR as a proteolytic substrate. CtsR is a dimeric repressor, highly conserved in low-GC Gram-positive bacteria and positioned as the first gene in the *clpC* operon. The repressor negatively regulates the expression of *clpP*, *clpE*, and the *clpC* operon (including its own gene) by binding to a hepta-nucleotide direct repeat within the respective promoter regions and interfering with the RNA polymerase E6A holoenzyme [Derre et al., 1999; Krüger et al., 2001; Elsholz et al., 2010b]. Under non-stressing conditions, the active conformation of CtsR binds to DNA, repressing the transcription of *clpP*, *clpE*, and *clpC* and its own expression. Upon heat exposure, CtsR acts as a thermosensor and undergoes a conformational change that reduces its binding affinity for DNA [Derre et al., 2000; Elsholz et al., 2010b]. CtsR-controlled genes are tran-

scribed, i.e., the respective *clp* genes and many other heat-shock genes. *McsA* and *McsB* play a central role in CtsR degradation during heat stress, and auto-phosphorylation of *McsB* is essential for CtsR degradation by ClpCP [Kirstein et al., 2005; Kirstein et al., 2007; Elsholz et al., 2010b]. After initial strong heat-shock protein expression, the heat-shock response declines. CtsR is re-activated after 30 min of heat exposure, and phosphorylated *McsB* is either de-phosphorylated by YwIE or rapidly degraded by ClpCP to prevent the degradation of activated CtsR [Elsholz et al., 2010b]. For an extensive overview outlining the regulation of CtsR we refer readers to Frees et al. [2007] and Elsholz et al. [2010a]. Besides ClpCP, ClpEP and ClpXP were also shown to degrade CtsR, albeit under different conditions. While ClpEP degrades CtsR upon severe heat-shock, ClpXP degrades CtsR at 37°C [Derre et al., 2000; Krüger et al., 2001; Miethke et al., 2006]. ClpC, independently of ClpP, is presumed to play a positive role by binding and protecting CtsR against degradation by ClpXP at 37°C [Derre et al., 2000]. ClpX does not belong to the CtsR regulon, and its accumulation during heat stress is prevented due to instability of the *clpX* mRNA at 50°C [Gerth et al., 1996; Gerth et al., 2004].

A further level of regulation is added to the heat-shock response by a regulatory feedback loop between the transcription factor Spx and the CtsR regulon. Spx is well known as an oxidative stress regulator, and in addition Spx plays an important role in the heat-shock response and thermotolerance development [Runde et al., 2014]. Spx was shown to induce the CtsR regulon and directly mediate *yjbH* and *clpX* expression [Rochat et al., 2012]. ClpXP, together with the adaptor YjbH, degrades Spx under non-stressed conditions [Garg et al., 2009; Nakano et al., 2002b; Larsson et al., 2007; Runde et al., 2014], and the induction of *clpX* and *yjbH* expression by Spx was discussed to represent a negative regulatory loop, promoting the reduction of Spx levels [Rochat et al., 2012]. However, upon oxidative stress, ClpXP activity is impaired [Zhang and Zuber, 2007], and when ClpXP is incapable of degrading Spx, ClpCP controls the cellular Spx levels [Rojas-Tapias and Helmann, 2019]. Moreover, *McsB* modulates Spx activity through arginine phosphorylation and promotes Spx degradation by ClpCP as an adaptor protein [Rojas-Tapias and Helmann, 2019]. In short, the transcriptional regulator Spx positively contributes to the activity of the CtsR regulon, and induces also the expression of *clpX* and *yjbH*, whereas the CtsR regulon and especially the Clp proteases together with their adaptors mediate the inactivation of Spx, preventing negative effects of Spx overaccumulation, i.e., reduced growth, spor-

<div><div>a</div><div><i>B. subtilis</i></div><div><div> <div>heat shock: CtsR, 37°C</div><div>competence: Spx*</div><div>motility: indirectly via Spx*</div><div>sporulation: SpoIVA CmpA required</div></div></div></div> <div><div> <div>heat shock: CtsR, elevated °C McsA and McsB required</div><div>competence: ComK*, ComS*, Spx* MecA* required</div><div>motility: ComK*, DegU-P</div><div>sporulation: SpoIIAB</div></div></div> <div><div> <div>heat shock: CtsR, elevated °C immediately after CtsR- dissociation from the DNA</div></div><div>ClpC-MecA without ClpP bind SpoA-P</div></div>	<div><div>b</div><div><i>S. aureus</i></div><div><div> <div>virulence factor expression: Rot, positive regulation of RNAIII</div><div>β-lactam resistance: Sle1</div><div>SOS-response: LexA</div><div>stress response: Spx</div></div></div><div><div> <div>toxin-antitoxin systems: MazE, Axe1, Axe2</div><div>SOS-response: RecA contribution: LexA</div><div>cell division: FtsZ</div><div>UDP-GlcNAc synthesis: GlmS</div><div>heat shock: CtsR</div></div><div>ClpX without ClpP stimulates <i>rot</i> and <i>spa</i> expression, controls <i>SaPI5-</i> and <i>ess</i>-locus-encoded genes ClpP affects <i>WalRK</i> regulated genes ClpXP affects expression of <i>IsdB</i>, <i>tst</i>, <i>sed</i> and <i>sec</i></div></div></div>	<div><div>c</div><div><i>M. tuberculosis</i></div><div><div> <div>trans-translation: ssrA-tagged proteins</div></div><div><div> <div>trans-translation: ssrA-tagged proteins</div></div><div>ClgR, WhiB1 and CarD: substrate-candidates of ClpP1P2, no ClpATPase assigned yet ClpC1: implied in toxin-antitoxin regulation</div></div></div></div>
<div><div>f</div><div>Mitochondria</div><div><div> <div>mitochondrial respiration: NOA1*, SDHB, SDHA</div><div>ribosome biogenesis/ maturation: NOA1*, ERAL1</div><div>autophagy: PINK1 turnover</div><div>translation: EFG1</div></div></div></div>	<div><div>d</div><div><i>L. monocytogenes</i></div><div><div> <div>ssrA-tagged proteins* <i>in vitro</i></div></div><div>ClpP2 involved in listeriolysin O expression</div></div></div>	<div><div>e</div><div><i>C. trachomatis</i></div><div><div> <div>trans-translation: ssrA-tagged proteins*</div></div></div></div>

Fig. 2. Natural substrates of Clp proteases and physiological roles discussed in this review. Clp proteases of *B. subtilis* (a), *S. aureus* (b), *M. tuberculosis* (c), *L. monocytogenes* (d), *C. trachomatis* (e), and mitochondria (f), and the functions reported so far. In all the heteromeric ClpP1P2 complexes discussed in this review, Clp-ATPase interaction was only observed with ClpP2. Exemplary natural protein targets discussed in this review and shown to accumulate in *clp* deletion mutants and/or to interact in Clp pull-down

assays and/or supported by physiological evidence in whole-cell studies are marked in bold. Substrates for which we are aware of *in vitro* digestion are marked by an asterisk [Turgay, 1998; Nakano et al., 2002b; Nakano et al., 2003; Kirstein et al., 2009a; Al-Furoukh et al., 2014; Balogh et al., 2017; Pan et al., 2019]. In addition, most Clp proteases are also involved in general protein quality control. For an extensive overview of Clp protease substrates, we refer readers to Feng et al. [2013] and Bhandari et al. [2018].

ulation, and competence as observed in $\Delta yjbH$, $\Delta clpX$, and $\Delta clpC$ mutants [Nakano et al., 2002b; Larsson et al., 2007]. This well-studied example of the heat-shock response illustrates how multilayered circles of regulatory proteolysis can rapidly elicit and later quench a stress response.

A disbalance in protein homoeostasis can result from external hazards such as heat stress or exposure to antibacterial agents, and ClpP, ClpC, ClpX, and ClpE help *B. subtilis* maintain a balanced, functional proteome in a changing environment. Their adhesion to aggregated heat-damaged proteins, as well as their degradation of defective proteins, clearly demonstrate their role in protein quality control, especially under stress conditions [Krüger et al., 2000; Miethke et al., 2006]. Additionally, ClpP and ClpC participate in general protein turnover under non-stressed conditions, again efficiently preventing the accumulation of protein aggregates [Krüger et al., 2000; Kock et al., 2004]. Proteome rearrangements are also important during shifts of growth phases and nutrient shortage. Although *clpP* is dispensable for *B. subtilis* during exponential growth in rich media at moderate temperatures, the Clp protease becomes essential when cells enter stationary phase or are grown in minimal media [Msadek et al., 1998]. Typical stationary phase adaptive responses, such as degradative enzyme synthesis, motility, natural genetic competence, and sporulation, are regulated by ClpP [Msadek et al., 1998]. In the light of survival strategies, motile cells have the advantage that they can actively change their environment by moving from unfavourable to more favourable conditions. In that sense, flagella represent an efficient coping strategy. Flagella are elaborate proton motive force-fuelled, rotating machines, and their assembly depends on the coordinated expression of various genes and transcription factors, among them DegU and 6^D [Tsukahara and Ogura, 2008]. The alternative sigma factor 6^D is encoded on the *fla/che* operon besides other flagella machinery genes. Once 6^D is activated, genes required for filament synthesis are expressed, including *hag* [Mukherjee and Kearns, 2014]. The Hag protein represents the flagellin monomer, and about 12,000 subunits form one flagellum [Oshiro et al., 2019]. In *B. subtilis*, deletion of *clpP*, *clpC*, *clpX*, or the adaptor *mecA* results in the loss of motility [Liu and Zuber, 1998; Msadek et al., 1998; Molière et al., 2016]. One way for ClpCP to regulate the swimming motility in *B. subtilis* is via ComK. The ComK transcription factor, which also plays a major role in competence regulation (see below), indirectly promotes the expression of *flgM*, which encodes the anti-sigma factor of 6^D . ComK is a ClpCP substrate, and by de-

grading ComK, ClpCP positively affects 6^D -dependent *hag* transcription, followed by flagellar assembly [Liu and Zuber, 1998]. Moreover, ClpCP targets DegU in its phosphorylated form [Molière et al., 2016]. DegU is the response regulator of the DegS-DegU two-component system that controls several post-exponential responses, among them motility. High DegU-P levels prevent expression of the *fla/che* operon, while DegU activates the same operon in its unphosphorylated or low-phosphorylated state. In contrast to the direct effects of ClpCP, ClpXP stimulates motility indirectly through degradation of the oxidative stress regulator Spx, a repressor of the *fla/che* operon and *hag* expression [Molière et al., 2016].

Developmental processes during stationary phase also include the physiological state of natural competence. Natural competence allows a subpopulation of *B. subtilis* cells to import foreign DNA, thereby enriching the gene pool [Turgay et al., 1997]. The autoregulatory transcription factor ComK is also central to competence development [Liu and Zuber, 1998]. During exponential growth, the adaptor MecA targets ComK for degradation by ClpCP, preventing its accumulation [Turgay et al., 1997; Turgay, 1998]. However, dependent on quorum-sensing mediated signals, the response regulator ComA is phosphorylated. ComA-P interacts with the RNA polymerase (RNAP), activating the transcription of the *srf* operon, resulting in the synthesis of the anti-adaptor protein ComS [D'Souza et al., 1994; Hamoen et al., 1995]. ComS binds to MecA, thus releasing ComK to initiate competence gene transcription. Thereby, ComS and MecA become substrates for ClpCP-mediated degradation themselves [Turgay et al., 1997, 1998]. In the context of natural competence, the transcription factor Spx prevents the development of this physiological state. Spx forms a quaternary complex with ComK-MecA-ClpC, resulting in enhanced binding of ComK to MecA-ClpC and inhibition of competence development [Nakano et al., 2002a]. Notably, Spx also represents a ClpCP-MecA substrate, clearly shown to be degraded *in vitro* [Nakano et al., 2002b] as well as *in vivo* [Rojas-Tapias and Helmann, 2019]. The fact that Spx and ComK are both substrates of the same Clp protease complex involving the same adaptor, and even being bound simultaneously, suggests competition of both substrates during the degradation process by ClpCP-MecA. Furthermore, Spx binds to the α -subunit of RNAP, preventing competence development by disrupting ComA-P-RNAP mediated transcription initiation [Nakano et al., 2003; Zhang et al., 2006]. Eventually, ComS is not synthesised, leading to the degradation of ComK and inhibition of competence. ClpXP contributes

to *srf* transcription activation, which results in ComS synthesis, by degradation of Spx [Nakano et al., 2000; Nakano et al., 2003]. Hence, the lack of competence in *clpP* and *clpX* deletion mutants results from an accumulation of Spx [Nakano et al., 2002b].

Genes controlling competence and sporulation are organised in tightly interconnected networks [Grossman, 1995]. Therefore, it is reasonable that the Clp protease is involved in both physiological processes. For instance, mutants deficient in ClpP show a defect in both competence development and sporulation, and mutants lacking ClpC are deficient in sporulation, as well [Msadek et al., 1998]. A rationale why ClpC and ClpP positively regulate sporulation was provided by the observation that SpoI^{IAB} is specifically degraded by ClpCP [Pan et al., 2001]. SpoI^{IAB} is an anti-sigma factor, sequestering σ^F , a forespore-specific sigma factor, in an inactive state. However, expression of σ^F and other sigma factors essential for sporulation depends also on the accumulation of Spo0A, a transcription factor and the master regulator of sporulation, in its phosphorylated form [Grossman, 1995]. In this regard, it was recently found that Spo0A-P binds to ClpC-MecA, independently of ClpP. Binding of Spo0A-P by ClpC-MecA might result in a bulky complex and prevent recruitment of the RNA polymerase. This way, ClpC-MecA directly repress the transcription of Spo0A-dependent genes without degrading the actual target [Tanner et al., 2018]. These examples show how a Clp-ATPase can have opposing roles in the regulation of the same developmental process, thereby allowing for fine-tuning. While ClpCP and ClpC-MecA affect sporulation at an early stage, ClpXP plays an important role at a later stage during sporulation, i.e., quality control of the spore envelope. SpoIVA is essential for the formation of the spore basement layer, on top of which coat proteins assemble [Roels et al., 1992; Mckenney et al., 2013]. In cells defective in spore envelope assembly, CmpA mediates degradation of SpoIVA by ClpXP, eventually leading to the destabilisation of the forespore and resulting in cell lysis. By participating in a critical checkpoint of the sporulation program, ClpXP and CmpA preserve the integrity of the bacterial population [Tan et al., 2015].

In summary, the Clp system in *B. subtilis* coordinates a variety of tasks, ranging from fundamental stress responses to regulatory proteolysis of specific transcription factors, several of which govern survival mechanisms. Therefore, the Clp protease machinery substantially contributes to the generalist lifestyle of *B. subtilis*.

***S. aureus*: Role of Clp in Enabling Survival in the Host and Host Infection**

S. aureus is an important opportunistic human pathogen and can cause severe illnesses like pneumonia, endocarditis, sepsis, or toxic shock syndrome [Balasubramanian et al., 2017; Oliveira et al., 2018]. The excellent adaptive potential of *S. aureus* during colonisation of the human host is based on an arsenal of cell surface-associated and secreted virulence factors. Functions of the Clp system in *S. aureus* range from the general stress response to the regulation of virulence factors (Fig. 2b). Similar to *B. subtilis*, *clpP* and *clpC* in *S. aureus* are controlled by the heat-inducible CtsR regulon, and both are required for growth at high temperature [Frees et al., 2004]. *ClpX* expression is not induced upon heat stress and mutants lacking *clpX* can grow at high temperature and in the presence of puromycin-induced protein accumulation [Frees et al., 2003; Frees et al., 2004]. The absence of ClpX even results in an improved growth at high temperatures, possibly because increased binding of ClpC to ClpP leads to improved protein quality control [Frees et al., 2003]. The role of ClpXP for protein quality control at elevated temperatures was analysed comparing the growth of $\Delta clpP$, $\Delta clpX$, and *clpX*_{I256E} mutants. The $\Delta clpX$ _{I256E} mutant cannot form a complex with ClpP due to an amino acid substitution in the IGF loop of ClpX [Stahlhut et al., 2017]. At 45°C, the $\Delta clpX$ and *clpX*_{I256E} mutants grew as well as the wild-type, while the $\Delta clpP$ mutant did not grow [Stahlhut et al., 2017]. These results preclude a major role in the heat-shock response for ClpX. However, ClpX seems to be important for growth at decreased temperatures, since deletion of *clpX* substantially slowed growth at 30°C [Jensen et al., 2019a]. Deletion of *clpP* also affected growth at decreased temperatures, but the phenotype was less severe than for $\Delta clpX$, implying a ClpP-independent function of ClpX in coping with cold-shock [Stahlhut et al., 2017].

There also exists a connection between ClpX and the cell envelope, affecting cell wall hydrolysis and cell division, and the molecular interactions are beginning to emerge. Recently, ClpX was shown to localise close to the division septum, control the cell wall synthesis operon *mraZ-mraW-ftsI-pbpA*, and to serve an important function in septum formation and cell division independently of ClpP [Jensen et al., 2019a, b]. Cells lacking *clpX* were characterised by septum synthesis arrest and premature splitting of daughter cells at 30°C, eventually leading to cell lysis [Jensen et al., 2019a]. *ClpX* mutants showed increased levels of autolysins involved in daughter cell split-

ting (Sle1 and Atl) and, accordingly, deletion of the autolysins *sle1* and partly also of *atl* rescued the low-temperature growth defect [Jensen et al., 2019a]. Lipoteichoic acids, which are essential for viability under standard laboratory conditions, became non-essential in cells lacking the ClpX chaperone. Spontaneous loss of function mutations in the *ltaS* gene (lipoteichoic acid synthase) emerged in the ClpX mutants at 30°C and rescued the growth defect [Bæk et al., 2016]. In a *ltaS clpX* double mutant, autolysin levels as well as growth were restored to wild-type levels [Bæk et al., 2016]. Interestingly, β -lactam antibiotics rescued the $\Delta clpX$ growth defect at low temperature. Oxacillin, for example, allowed septum progression in cells lacking ClpX, thus preventing premature splitting. Also, inhibitors of the first step of wall teichoic acid biosynthesis, i.e., tunicamycin and tarocin A, stimulated the growth of *clpX* mutants [Jensen et al., 2019a].

Growth of *S. aureus* at low temperatures is not only supported by ClpX acting as an independent chaperone, but also by the ClpXP protease. The autolysin Sle1 was characterised as a substrate of ClpXP [Feng et al., 2013], and zymograms of cells lacking ClpX and ClpP showed increased Sle1 activity [Bæk et al., 2014]. Furthermore, Atl, which is sequentially cleaved to generate a mature, fully processed version of 62 kDa, accumulated in its intermediate form of 85 kDa in cells lacking ClpX and ClpP. Thus, ClpXP was speculated to indirectly affect Atl processing by controlling extracellular proteases [Bæk et al., 2014]. In summary, ClpX chaperone activity is critical for successful septum formation at decreased temperature, and the protease complex ClpXP regulates the stability of cell wall hydrolases, thus controlling the cell wall metabolism.

The overall importance of ClpP in *S. aureus* is highlighted by the reduced growth rate of $\Delta clpP$ mutants [Frees et al., 2003; Kirsch et al., 2020], potentially due to reduced levels of proteins involved in purine and pyrimidine biosynthesis [Frees et al., 2012]. A global proteome analysis by Kirsch et al. [2020], aimed to provide a global catalogue of ClpP and ClpX regulated proteins, outlining the effects of a *clpP* and a *clpX* knockout on the proteome composition. Both mutants were characterised by a strong downregulation of the *pur/pyr* operon, and metabolomic analyses confirm decreased nucleobase (adenine, cytidine, guanine) and nucleoside (adenosine and guanosine) levels [Kirsch et al., 2020]. Moreover, ClpP affects WalRK-regulated genes [Kirsch et al., 2020], and WalRK is an essential two-component system in *S. aureus*, controlling autolysin activity and cell wall degradation [Dubrac et al., 2007].

Regarding *S. aureus* virulence, mutants lacking *clpP* or *clpX* were incapable of establishing local skin abscesses in mice [Frees et al., 2003]. In a murine model of acute pneumonia, *clpP* or *clpX* deletion mutants showed a substantially lower bacterial load in the lung, less lung injury, and a reduced inflammatory response [Kim et al., 2020]. As *S. aureus* is the major cause of wound infections in diabetic patients, a global transcription analysis was conducted in diabetic mice to investigate how diabetes influences the infection process [Jacquet et al., 2019]. *S. aureus* adapted to the hyperglycaemic environment by altering the expression of various genes, among them *clpP*, *clpX*, and *clpC*, which were all upregulated [Jacquet et al., 2019]. Cells lacking *clpP* or *clpX* showed decreased α -haemolysin production *in vitro* and reduced dermonecrosis in mice [Jacquet et al., 2019].

Another mechanism by which *S. aureus* adapts to the antagonistic host environment is iron-scavenging from host haemoglobin. ClpXP is important for the expression of the haemoglobin receptor IsdB [Farrand et al., 2013], an essential component of the iron-uptake machinery in *S. aureus* necessary for iron acquisition in the host [Skaar and Schneewind, 2004]. Cells lacking *clpX* or *clpP* are characterised by reduced *IsdB* expression under iron-poor conditions [Farrand et al., 2013]. Using immunofluorescence, $\Delta clpP$ mutants were shown to present less IsdB on the cell surface. Decreased IsdB protein levels could also be detected in immunoblots of $\Delta clpX$ mutants. Both results correlate with decreased haemoglobin binding of $\Delta clpP$ and $\Delta clpX$ mutants, their inability to acquire iron from the host, and reduced bacterial burden in kidneys, livers, and hearts upon systemic infection of mice [Farrand et al., 2013].

Pathogenicity of *S. aureus* depends on the ability to produce a wide range of cell-wall associated and secreted virulence factors, such as protein A, α -haemolysin, and extracellular proteases [Lowy, 1998; Frees et al., 2003]. Mutants lacking ClpP or ClpX are characterised by reduced exoproteolytic activity, as well as strongly reduced α -haemolysin secretion [Frees et al., 2003]. The quorum sensing-dependent *agr* regulatory network is, among others, responsible for virulence factor expression, and induction of the *agr* operon results in the production of the main effector molecule RNAPIII. Cells lacking *clpP* or *clpX* contained decreased RNAPIII levels, implying both as positive regulators of RNAPIII [Frees et al., 2003; Michel et al., 2006]. Under physiological conditions, RNAPIII accumulates in the post-exponential growth phase and was proposed to interact with Rot (repressor of toxins), resulting in the degradation of Rot by ClpXP and thereby promot-

ing the expression of the mentioned secreted virulence factors [Frees et al., 2005]. Besides acting as an ATPase partner for regulatory proteolysis of Rot by ClpXP, ClpX on its own was shown to promote the expression of *spa*, encoding the surface-bound, Fc-receptor scavenger, protein A [Frees et al., 2003, 2005]. ClpX stimulates *rot* expression, and *spa* expression is induced upon a certain Rot threshold in the cell. In addition, ClpX stimulates translation of the *spa* mRNA [Jelsbak et al., 2010]. Besides the widely expressed α -toxin and extracellular proteases, a smaller percentage of *S. aureus* strains produces harmful staphylococcal superantigens (SAGs), i.e., the toxic shock syndrome toxin TSST-1, or staphylococcal enterotoxins (SEs) like SEC and SED, causing severe illness [Spaulding et al., 2013]. The gene encoding TSST-1 is *tst*, which is located on pathogenicity islands (SaPI) [Lindsay et al., 1998]. SEC and SED are mostly associated with food intoxications. SEC is encoded on a SaPI and a plasmid, while SED is solely encoded on a plasmid [Etter et al., 2020]. A study analysing toxin transcript and protein levels in the $\Delta clpP$, $\Delta clpX$, and *clpX*_{I256E} mutant set concluded that the functional ClpXP protease is required for the induction of *sec*, *sed*, and *tst* in *S. aureus* clinical isolates, although with strain-specific differences [Schelin et al., 2020]. Moreover, it was shown that ClpXP controls *tst* expression via a variety of transcriptional regulators, i.e., SarU, SarX, SarT, and MgrA [Schelin et al., 2020]. ClpX was also shown to regulate the expression of further pathogenicity island-encoded genes via ClpP-dependent and ClpP-independent mechanisms [Stahlhut et al., 2017; Jensen et al., 2019b]. For instance, proteins involved in excision and replication of SaPI5 are controlled independently of ClpP [Jensen et al., 2019b]. Furthermore, ClpX controls the expression of the *ess* locus. The *ess* locus encodes genes for the type VII secretion machinery as well as secretory proteins, i.e., toxins. The type VII secretion system impacts virulence and is linked to persistent infections caused by *S. aureus* [Burts et al., 2008; Wang et al., 2016; Jensen et al., 2019b].

Treatment of infections caused by *S. aureus* strains using β -lactams are complicated by the continuous spread of methicillin-resistant *S. aureus* (MRSA) strains [Vestergaard et al., 2019], and the ClpXP protease influences the level of β -lactam resistance in *S. aureus*. The MRSA strain JE2, like many other community-acquired MRSA strains, displays heterogeneous β -lactam resistance, with only a small percentage of highly resistant and a larger fraction of low-level resistant cells. Deletion of *clpP* or *clpX* resulted in a homogenous high-level β -lactam resistance phenotype in JE2, although the PBP2a level remained

constant [Bæk et al., 2014]. A key ClpP substrate in that context is the autolysin Sle1, and cells lacking ClpXP protease activity are characterised by high Sle1 levels and β -lactam hyper-resistance [Bæk et al., 2014]. In turn, deletion of *sle1* in JE2 wild-type cells or in a *clpX*_{I265E} mutant incapable of interacting with ClpP led to hyper-susceptibility towards β -lactams and MIC levels comparable to methicillin-sensitive *S. aureus* strains [Thalsø-Madsen et al., 2019]. In summary, ClpXP controls β -lactam resistance via degradation of Sle1, which itself strongly contributes to resistance.

Comparing the two protease subtypes ClpXP and ClpCP, it becomes clear that they perform distinct roles in *S. aureus*. ClpXP is the major protease involved in the regulation of the pathogenic lifestyle of *S. aureus*, while ClpCP plays a major role in protein quality control upon heat stress and under non-stress conditions. In particular, ClpXP is dispensable for degradation of damaged proteins at high temperatures, as cells expressing ClpX_{I256E} did not accumulate aggregated proteins upon heat stress [Stahlhut et al., 2017]. In contrast, cells lacking ClpP as well as cells lacking ClpC severely suffer from protein aggregation [Frees et al., 2012; Stahlhut et al., 2017]. Even at 37°C, protein aggregates appear in $\Delta clpP$ cells, and the CtsR regulon and the *ahpC-ahpF* operon, which reacts to oxidative stress, are highly induced in $\Delta clpP$ mutants. In contrast, there were fewer protein aggregates in cells expressing ClpX_{I256E}, abolishing ClpXP but retaining ClpCP function [Stahlhut et al., 2017]. This observation points towards a major function of ClpCP in protein quality control, also under non-stress conditions [Stahlhut et al., 2017]. Although all currently available data distinguish ClpXP as the major virulence-regulating Clp protease complex in *S. aureus*, there might also be some involvement of ClpCP. This notion is implicated by the observation that *clpP* deletion (effecting both ClpCP and ClpXP) resulted in a stronger decrease of *agr* transcription than the lack of ClpXP protease activity in the ClpX_{I256E} mutant [Stahlhut et al., 2017]. Distinct protease roles also correlate with distinct substrate specificities conferred by ClpX or ClpC. Feng et al. [2013] expressed a ClpP^{TRAP} mutant, which allows trapping of proteins inside the proteolytic chamber without degrading them due to a defective active site. This approach enabled the confirmation of already known substrates as well as the identification of various new ClpP substrates with regulatory or physiological functions. By expressing the ClpP^{TRAP} protein in cells lacking either ClpX or ClpC, different substrate specificities for ClpXP versus ClpCP were determined [Feng et al., 2013]. A variety of substrates were

identified. Prominent examples of ClpCP substrates were RecA, an important member of the SOS-regulon, the cell division protein FtsZ and GlmS, which catalyses the first step of UDP-N-acetyl-D-glucosamine synthesis. Regarding ClpXP substrates, especially Sle1 and Spx were trapped in high amounts [Feng et al., 2013]. Another ClpXP substrate is the SOS-response repressor LexA, which undergoes autocleavage upon DNA stress. The resulting N-terminal domain is mainly degraded by ClpXP, but ClpCP appears to contribute to the degradation. In contrast, the C-terminal domain was not degraded by Clp proteases [Cohn et al., 2011].

The Clp protease also affects biofilm formation by *S. aureus*, with *clpP* deletion increasing and *clpX* and *clpC* deletions decreasing the amount of biofilm formed. However, the molecular explanation is still elusive [Frees et al., 2004]. Last but not least, ClpCP degrades antitoxins in *S. aureus*. Toxin-antitoxin systems are small genetic elements encoding a toxin and its corresponding antitoxin, and upon environmental stress toxin production is enhanced. In *S. aureus*, MazF, for example, encodes an RNase toxin responsible for decreased growth rates as well as decreased *hla* and *spa* amounts [Schuster and Bertram, 2016]. MazE represents the corresponding antitoxin. Besides MazEF, *S. aureus* also produces the *axe1-txe1* and *axe2-txe2* toxin-antitoxin system. ClpCP was shown to degrade all three antitoxins [Donegan et al., 2010].

Taken together, the Clp system exerts a strong impact on the physiology of *S. aureus*, promoting colonisation and infection in the host environment in a manifold manner. ClpXP and ClpCP have a distinct focus, with multiple functions in virulence regulation for the former, and a prominent role in protein quality control for the latter. For clarification, two additional Clp-ATPases, ClpB and ClpL, are present in *S. aureus*. However, they lack the consensus tripeptide motif essential for the interaction with ClpP [Kim et al., 2001; Frees et al., 2004]. Therefore, they act as independent chaperones but are not further discussed in this review, as they do not provide substrates to the conserved proteolytic core. *S. aureus* is featured in this review as a particularly well-studied example of the impact of the Clp protease system on bacterial virulence. Notably, *clpP* deletion was also shown to attenuate the virulence of other clinically relevant species like *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, or *L. monocytogenes* [Gaillot et al., 2000, 2001; Kwon et al., 2004; Wang et al., 2007].

***M. tuberculosis*, *L. monocytogenes*, *C. trachomatis*: Importance of Clp for a Facultative or Obligate Intracellular Lifestyle**

M. tuberculosis is a rod-shaped, acid-fast, and facultative intracellular human pathogen. The lifecycle is characterised by two different metabolic states, one of active replication and a non-proliferative, persistent state [Glickman and Jacobs, 2001]. Switching between these lifestyles and ensuring extracellular as well as intracellular survival, demands high proteome plasticity. *M. tuberculosis* contains two ClpP homologs encoded in a bicistronic operon, with both *clpP1* and *clpP2* positioned downstream of *clgR*, the conserved activator of *clpP* expression in Actinobacteria [Personne et al., 2013]. Biochemical and structural studies demonstrated that the functional ClpP complex of *M. tuberculosis* is a hetero-tetradecamer composed of a ClpP1 and a ClpP2 homo-heptameric ring, designated ClpP1P2 and meaning that one ring is made of seven ClpP1 subunits, while the heptamer stacked to it exclusively contains ClpP2 [Ingvarsson et al., 2007; Akopian et al., 2012; Ollinger et al., 2012]. Although originating from co-transcribed genes, ClpP1 and ClpP2 are surprisingly dissimilar in their amino acid sequence, and both mycobacterial ClpP homologs share relatively low sequence identities with ClpP proteins from other bacteria (Fig. 3).

It is important to note that, unlike most bacteria, both mycobacterial ClpP homologs are essential for *M. tuberculosis*, even under standard growth conditions in culture broth *in vitro* [Ollinger et al., 2012]. Reduction of functional mycobacterial ClpP1P2 via *clpP* knock-down or active site mutagenesis resulted in severe attenuation in a mouse lung infection and deficiency to replicate within macrophages [Carroll et al., 2011; Raju et al., 2012]. Although it remains to be elucidated which Clp-ATPase is involved and how the regulatory network is arranged, mycobacterial ClpP1P2 was reported to degrade its regulator ClgR (Fig. 2c). Other strong candidates for Clp-mediated degradation include essential transcription factors such as WhiB1 and CarD, accumulation of which proved to be detrimental or even fatal for *M. tuberculosis*, offering a rationale for the essentiality of the Clp protease in this species [Raju et al., 2014; Yamada and Dick, 2017].

In vitro studies on the degradation of substrates by purified *M. tuberculosis* ClpP1P2 are only just emerging. One group of substrates demonstrated to be digested by ClpP1P2 are *ssrA*-tagged proteins. Such proteins are released from the ribosome in the process of trans-translation. For *ssrA*-protein degradation, mycobacterial ClpX

Fig. 3. Phylogenetic clustering of the ClpP proteins discussed in this review. **a** ClpP protein homology analysis based on CLANS (cluster analysis of sequences). A total of 597 unique sequences were extracted from the Swiss-Prot database, and lmClpP1 (Q8Y7Y1) was extracted from the TrEMBL database. The p value threshold was set to $1e-81$. Atypical ClpP species such as ct-ClpP1 (P38002), lmClpP1 (Q8Y7Y1), mt-ClpP1 (P9WPC5), and mtClpP2 (P9WPC3) are highlighted. **b** Zoomed view of the large cluster comprising over 70% (437/598) of total input sequences. Most ClpP species are highly conserved, including saClpP (P63786), bsClpP (P80244), and lmClpP2 (Q9RQI6). Human mitochondrial hsClpP (Q16740) and ctClpP2 (O84712) are located at the margin of the highly conserved ClpP cluster, outlining distinct sequence variation. bs, *B. subtilis*; ct, *C. trachomatis*; hs, *Homo sapiens*; lm, *L. monocytogenes*; mt, *M. tuberculosis*; sa, *S. aureus*.

and ClpC1 both interact exclusively with ClpP2 and not with ClpP1 [Leodolter et al., 2015; Nagpal et al., 2019]. Recently, ClpC1 has been reported to be heavily involved in the regulation of a myriad of toxin-antitoxin systems [Ziems et al., 2021]. When the isolated *M. tuberculosis* ClpP1P2 core was studied, it emerged that catalytic activity was strongly stimulated by the addition of certain activator peptides, which did not act as substrates but stabilised the catalytically competent conformation of the active sites [Akopian et al., 2012; Liu and Chien, 2014; Schmitz et al., 2014; Famulla et al., 2016]. A cryo-EM study recently revealed that this characteristic feature of *M. tuberculosis* ClpP is caused by the absence of a typical β -sheet within the handle region of ClpP and the resulting flexibility in this region that shifts the tetradecamer into an inactive compact conformation [Vahidi et al., 2020]. The peptide agonists increase order in this region of ClpP and restore the active extended conformation of the barrel. It is assumed that within the cell peptide products serve this agonistic function, generated by a Clp-ATPase feeding an unfolded polypeptide through the entrance

pores of ClpP [Schmitz and Sauer, 2014; Schmitz et al., 2014]. As the interaction of separate ClpP proteins, i.e., ClpP1 plus ClpP2, plus product activation seems to be required for an active proteolytic core, the mycobacterial Clp protease appears particularly tightly regulated [Raju et al., 2012; Schmitz et al., 2014; Alhuwaider and Dougan, 2017].

L. monocytogenes is a facultative intracellular pathogen with two distinct *clpP* genes, which, unlike *M. tuberculosis*, are not co-transcribed. Among the ClpPs of *Listeria*, ClpP2 is very similar to the ClpP proteins found in single-ClpP organisms and possesses the canonical Ser-His-Asp catalytic triad, whereas ClpP1 shares only 41% identity with ClpP2 (Fig. 3), and in the catalytic triad of ClpP1 the conserved Asp is exchanged for Asn [Zeiler et al., 2011, 2013]. Active-site directed labelling with various β -lactone suicide inhibitors, size exclusion chromatography, and peptidase activity assays found ClpP2 of *L. monocytogenes* to form an active tetradecameric peptidase, while ClpP1 alone adopted only an inactive heptamer that resisted β -lactone labelling [Böttcher and

Sieber, 2008; Zeiler et al., 2011]. However, upon co-expression or mixing of both homologs, ClpP2 activated ClpP1 by hetero-oligomerisation [Zeiler et al., 2011; Dahmen et al., 2015]. In the resulting ClpP1P2 hetero-complex, two homo-heptamers of exclusively ClpP1 and ClpP2 are stacked onto each other, and all 14 catalytic residues within both ClpP subunits are correctly aligned for catalysis, greatly enhancing its proteolytic capabilities [Dahmen et al., 2015]. In corroboration of this finding, in the presence of cognate ClpX, the proteolytic activity of the ClpP1P2 hetero-complex was 10-fold higher than that of the ClpP2 homo-tetradecamer [Dahmen et al., 2015].

The importance of Clp proteins in *Listeria* pathogenesis is conspicuous. While the physiological function of ClpP1 remains largely elusive so far, the deletion of *clpP2* is fatal for *L. monocytogenes* during intracellular growth in macrophages and rendered the mutant avirulent in mice [Gaillot et al., 2000; Gaillot et al., 2001]. This lack of intracellular survival has been linked to the depletion of the *Listeria*-specific haemolysin listeriolysin O (Fig. 2d), a major virulence factor responsible for phagosome escape during *Listeria* infection [Gaillot et al., 2000]. As for its corresponding Clp-ATPases, ClpX has been shown to mediate the degradation of *ssrA*-tagged proteins by ClpXP1P2 *in vitro*, and knock-out mutagenesis of *clpC* and *clpE* revealed that the lack of either or both chaperones caused pleiotropic physiological impairments, including deficiencies in cell division, general stress tolerance, and virulence [Nair et al., 1999, 2000; Balogh et al., 2017].

Chlamydia trachomatis is a Gram-negative obligate intracellular bacterium with an obligatory biphasic, parasitic lifestyle that preferably infects human epithelial cells. *C. trachomatis* encodes two ClpP homologs, which are not co-transcribed and only ClpP2 shares a relatively high sequence identity with most other ClpP homologs (Fig. 3). In terms of the ClpP oligomer organisation, the chlamydial ClpP proteins are different insofar as ClpP1 as well as ClpP2 formed only heptamers *in vitro*. Consequently, both subunits are required for the assembly of the functional hetero-tetradecamer. The fact that the two *clpP* genes of chlamydia are encoded in separate chromosomal locations, although their protein products strictly depend on each other for catalytic function, suggests a safeguard mechanism on the genomic level, only granting protease action when correct transcription is ensured [Pan et al., 2019]. In biochemical studies, the active sites of ClpP1 and ClpP2 contributed about equally to the total catalytic rate of the proteolytic core, while ClpX interacted only with ClpP2 [Pan et al., 2019].

C. trachomatis is an obligate parasite and possesses a very reduced genome size of only about 1 Mb. Genome comparison reveals that many intracellular proteolytic systems known from free-living bacteria, such as DegS(HtrH), HslUV(Cl pQY), Thr/Cys proteases and the prokaryotic proteasome, are not available, which emphasises the contribution of the remaining ones. In the unique chlamydial lifecycle, alternating between infectious elementary bodies (EB) and replicative reticulate bodies (RB), proteome plasticity is essential and unimpeded Clp function is expected to play a critical role in ensuring protein turnover during cell development and pathogenesis [Rodgers et al., 2010; Pan et al., 2019; Wood et al., 2020]. Degradation of *ssrA*-tagged proteins by chlamydial ClpXP1P2 has been demonstrated in recent studies, both *in vitro* and in intracellular chlamydia (Fig. 2e) [Pan et al., 2019; Wood et al., 2020]. Genetic manipulation of the obligate intracellular pathogen is challenging and has been managed only as of late. In a recent study by Wood et al. [2020], the authors attempted to disrupt the functionality of the protease system by overexpressing mutated, inactive ClpX and ClpP2 variants within chlamydial cells. Titrating the defective proteins against the remaining wild-type forms is expected to result in non-functional mosaic complexes and yield a knock-down phenotype. Overexpression of inactive ClpX or ClpP2 led to a sharp drop in recoverable inclusion-forming units (i.e., released infectious EB particles), although likely due to different physiological reasons. The authors propose a model in which ClpP2 is required for the transition of the EB to RB state in the developmental cycle and ClpX for reproduction and infectivity of the EBs [Wood et al., 2020]. Natural substrates and regulatory proteolysis of the *C. trachomatis* Clp protease are the subject of current research, including the role of ClpP1. The observation that *M. tuberculosis*, *L. monocytogenes*, and *C. trachomatis* contain hetero-tetradecameric ClpP cores, engaging only ClpP2 in asymmetric interactions with the Clp-ATPases investigated so far, implies an expanded regulatory capacity, which might be crucial for survival or intracellular persistence [Leodolter et al., 2015; Gatsogiannis et al., 2019].

Mitochondrial Clp: Bacteria-Derived Protease Mediating Protein Homeostasis in Eukaryotic Cells

The importance of the Clp machinery manifests in the high diversity of bacteria relying on this system, and the Clp protease was probably one of the benefits, the α -proteobacterium that later evolved to mitochondria,

brought into the proto-eukaryotic cell [Valera-Alberni and Canto, 2018]. In recent eukaryotic cells, the mitochondrial Clp protease makes an important contribution to protein quality control and homeostasis as well as the regulation of oxidative phosphorylation (OXPHOS), mitochondrial ribosome biogenesis, various stress response pathways and signalling cascades (Fig. 2f) [Voos, 2013; Valera-Alberni and Canto, 2018]. Although only the mitochondrial Clp protease will be discussed further in this review, it should be mentioned that also two further organelles contain a Clp protease, i.e., chloroplasts and the apicoplast of the parasitic phylum Apicomplexa [Yu and Houry, 2007; Bhandari et al., 2018].

The metabolism of the eukaryotic cell depends on an immaculate mitochondrial function at all times. Besides their prominent role in bioenergetics by performing the tricarboxylic acid cycle and OXPHOS, mitochondria are essential for amino acid and fatty acid metabolism, steroid hormone production, and cofactor synthesis [Voos, 2013]. They serve as a signalling hub for various stress responses, innate immunity and apoptotic pathways, perform central functions in cell cycle regulation and cell differentiation, and mitochondrial dysfunction is a central factor in cellular senescence [Antico Arciuch et al., 2012; Voos, 2013; Sun et al., 2016]. To ensure functional integrity, mitochondrial protein homeostasis is mandatory and maintained by a set of chaperones and proteases interconnected in a functional network. The Clp protease is one of three major proteases responsible for protein turnover in the mitochondrial matrix [Calvo and Mootha, 2010; Voos, 2013].

The genes of human mitochondrial *clpP* and *clpX* are encoded in the nucleus on chromosome 19 and 15, respectively, and translated as precursor proteins with an N-terminal mitochondrial targeting sequence to be removed upon uptake into the organelle [Kang et al., 2002]. Human mitochondrial ClpP is highly homologous to its bacterial equivalents, in sequence as well as in structure [Kang et al., 2002, 2004]. The proteolytic core is composed of a single ClpP homolog, and ClpX is the only Clp-ATPase present [Kang et al., 2002, 2004]. Structural and functional conservation allowed an interaction of mitochondrial ClpP with *E. coli* ClpX, resulting in a functional complex, capable of recognising *E. coli*-specific substrates and their degradation tags, like the *ssrA*-tag [Kang et al., 2004]. In contrast, human mitochondrial ClpXP could not process *ssrA*-tagged substrates *in vitro*, while the model substrate casein was efficiently degraded [Kang et al., 2004].

Efforts to elucidate natural substrates and regulation networks of mitochondrial ClpXP have only just begun

[Fischer et al., 2015; Wong and Houry, 2019]. Several potential substrates and interaction partners were recently proposed, based on proteins accumulating in *clpP* and/or *clpX* deletion mutants or ClpP/ClpX pull-down experiments and further candidates are discussed based on human homologs of bacterial proteins [Cole et al., 2015; Fischer et al., 2015; Seo et al., 2016]. To our knowledge, the only substrate verified *in vitro* is NOA1, an essential mitochondrial GTPase involved in the regulation of mitochondrial respiration and mitochondrial ribosome biogenesis [Heidler et al., 2011; Kolanczyk et al., 2011; Al-Furoukh et al., 2014]. Further ClpXP substrates deduced from proteomics and physiological investigations in whole-cell studies include SDHB and ERAL1 [Seo et al., 2016; Szczepanowska et al., 2016]. SDHB is the iron sulphur subunit B of the succinate dehydrogenase complex required for a functional respiratory complex II, and its misfolding and accumulation in the absence of ClpXP disrupts the electron transport chain in mitochondria [Seo et al., 2016]. ERAL1 is a putative 12S rRNA chaperon, the timely removal of which from the small ribosomal subunit is essential for the maturation of mitochondrial ribosomes [Szczepanowska et al., 2016]. SDHA, subunit A of the succinate dehydrogenase, and elongation factor EFG1 also accumulated in *clp* deletion mutants, further substantiating the role of ClpXP in mitochondrial bioenergetics and translation [Szczepanowska et al., 2016; Mirali and Schimmer, 2020]. Additional discussed substrates include proteins involved in amino acid and fatty acid metabolism, the pyruvate dehydrogenase complex, protein import as well as chaperones [Cole et al., 2015; Fischer et al., 2015; Deepa et al., 2016; Seo et al., 2016; Szczepanowska et al., 2016].

Most networks of regulatory proteolysis involving ClpXP in mitochondria are still elusive and an active field of research. The best-understood participation of the Clp protease in a pathway is the mitochondrial unfolded protein response (UPR^{mt}) in the model organism *Caenorhabditis elegans*. UPR^{mt} represents the primary signalling pathway for restoration of protein balance in mitochondria and occurs when defective proteins accumulate in the matrix, import of proteins into mitochondria is hampered, or if the established stoichiometry between proteins encoded in the nucleus and proteins encoded in the mitochondria is disturbed [Pellegrino et al., 2013]. Several factors signal protein stress to the nucleus, among them proteins, the uptake of which into the matrix fails, and ClpXP, which generates peptide fragments that are exported into the cytoplasm. In response, nucleus-encoded chaperones and proteases are upregulated and import-

ed into the mitochondria to re-establish protein homeostasis. In mammalian cells, ClpXP also plays an important role in reducing protein stress in the mitochondrial matrix by degrading defective proteins and its level is elevated during the UPR^{mt} [Al-Furoukh et al., 2015; Deepa et al., 2016; Seiferling et al., 2016]. Moreover, ClpXP was shown to be involved in the turnover of the mitophagy kinase PINK1 [Greene et al., 2012; Pellegrino et al., 2013]. PINK1 accumulates on the outer membrane of defective mitochondria, recruiting the ubiquitin ligase Parkin and causing the activation of the downstream autophagy machinery to direct the functionally dead organelle to lysosomes. Furthermore, there are hints suggesting a contribution of the Clp protease in mitochondrial fusion and fission dynamics, as mitochondria of *clpP* knock-down cells were smaller and more scattered throughout the cell and the dynamin-related protein Drp1 accumulated, which is known to induce fission [Deepa et al., 2016].

The ClpXP protease is almost omnipresent in eukaryotic cells, but different cell types seem to depend on it to a different extent. A strong expression of human mitochondrial ClpP was observed in skeletal muscle followed by heart, liver, and pancreas, while significantly lower expression levels were determined in the brain, placenta, lung, and kidney [Bross et al., 1995; Wong and Houry, 2019]. In a myoblast cell line, knock-down of mitochondrial ClpP resulted in impaired proliferation and triggered a failure to differentiate into myotubes, while the mitochondria showed morphological defects, a reduced respiration rate, decreased membrane potential, and increased ROS production [Deepa et al., 2016]. Dysfunction of human mitochondrial ClpP can also lead to human disease. A direct link is established for the Perrault syndrome, a rare human recessive genetic disorder, causing severe sensorineural hearing loss, neurological defects, and developmental delay as well as ovarian dysfunction in females [Gispert et al., 2013; Bhandari et al., 2018]. Different gene defects in six distinct genes were identified to cause the Perrault syndrome (types 1–6 respectively), among them *clpP* mutations (Perrault syndrome type 3). Seven different point mutations in the *clpP* gene were identified in patients, impairing the docking site of the interacting Clp ATPase or the catalytic triad and one mutation even both in parallel [Brodie et al., 2018]. The defect results in an unstable ClpP protein with an impaired structure and shorter half-life, leading to overall mitochondrial dysfunction [Ahmed et al., 2015; Dursun et al., 2016; Bhandari et al., 2018]. In the neurodegenerative disease Friedrich ataxia (FRDA), ClpP upregulation was attributed to disease progression, although indirectly, as the

genetic defect resides in the gene encoding the mitochondrial protein frataxin, an iron chaperone contributing to the biosynthesis of Fe-S clusters in mitochondria [Guillon et al., 2009]. Loss of frataxin function results in low amounts of Fe-S proteins in mitochondria and other compartments, and consequently reduced OXPHOS performance and elevated ROS levels [Guillon et al., 2009; Fischer et al., 2015]. In a study using a mouse model for FRDA, ClpP and the protease Lon were both upregulated during the middle stage of the disease, in parallel with a further decrease of Fe-S proteins, and Fe-S protein proteolysis was hypothesised [Guillon et al., 2009].

Mitochondrial Clp in Cancer Cells: Capitalising on a Bacterial System for Survival under Stress

The characteristic unrestrained growth and proliferation of cancer cells make cell homeostasis a particular challenge. Extensive reprogramming of cellular metabolism, as a direct or indirect consequence of tumorigenic mutations, is a hallmark of cancer cells. It involves major modifications in energy metabolism (e.g., substantially increased glycolysis and lactic-acid fermentation), alternative precursor flows in anabolism (e.g., more biosynthetic precursors derived from glycolysis), and versatile, opportunistic routes of nutrient acquisition, to name just a few [Pavlova and Thompson, 2016]. Contrary to a previous notion that cancer cells would generate ATP predominantly by glycolysis, recent studies indicate that, despite their heavy fermentation, most tumours still rely on mitochondrial OXPHOS for ATP generation and that mitochondria serve multiple central functions in tumour cell progression, including protein homeostasis and quality control [DeBerardinis and Chandel, 2016; Zong et al., 2016]. The requirement for constant nutrient and ATP replenishment due to unchecked growth, elevated ROS levels due to increased metabolic rates, acidosis due to fermentation, and hypoxia due to increased oxygen demand or during metastatic dissemination, all represent factors that impose stress on tumour cells. Cancer cells have developed multifarious strategies to alleviate the stress, and with regard to preserving mitochondrial function, multiple chaperones and proteases are upregulated, including ClpXP.

In about half of all patients with primary acute myeloid leukaemia (AML), ClpP was overexpressed, suggesting a role in pathology [Cole et al., 2015]. A protein-protein interaction study in AML cells yielded SDHA as a primary interaction partner of ClpP, in agreement with reduced

respiratory complex II activity, oxygen consumption, and viability of the cells upon *clpP* knock-down [Cole et al., 2015]. Notably, these inhibitory effects were only observed in AML subtypes distinguished by ClpP overexpression, whereas AML cells with a normal ClpP amount were not affected, raising hopes for ClpP as a target in subtypes of cancer with an elevated ClpP amount. Apart from AML, ClpP levels were also found increased in other hematologic malignancies as well as solid prostate tumours and sarcomas [Cole et al., 2015]. In a study with mitochondria from prostate carcinoma cells, immunoprecipitation experiments suggested the formation of a complex between ClpXP, the Hsp90-like chaperone TRAP-1, and the oncoprotein survivin [Seo et al., 2016]. Survivin and ClpXP also interacted with the respiratory II complex subunit SDHB. The authors propose that a ClpXP-survivin-TRAP-1 complex acts as a protein quality control system, combining the refolding capability of the chaperone(s) and proteolytic activity of the protease, and that the complex supports SDHB function, thereby contributing to the regulation of respiration. Knock-down of ClpXP resulted in an accumulation of misfolded SDHB, impaired OXPHOS, oxidative stress, reduced tumour cell proliferation, and inhibition of metastasis [Seo et al., 2016]. These examples show that certain tumours can exploit ClpXP-mediated protein homeostasis to maintain mitochondrial function and tumour cell survival under stress.

Conclusion

Bacterial survival strongly depends on proteome flexibility to enable adaptation and on proteome homeostasis to safeguard essential functions. Protein quality control helps to cope with protein damage under stress, and targeted proteolysis of central regulatory factors (often transcription factors) enables a response to changing environmental conditions. In that general regard, there are strong parallels between all bacterial species featured in this review, and also between bacteria and mitochondria, and in all of them the Clp protease machinery makes essential contributions to both the maintenance of protein homeostasis and targeted regulatory proteolysis. Even in bacteria, like Firmicutes, where Clp protease function is dispensable under non-stressing growth conditions *in vitro* (i.e., in the presence of sufficient nutrients, at moderate temperatures, and in the absence of inhibiting agents), the Clp protease becomes essential, when stress increases (e.g., when bacteria enter the stationary phase or the host environment). By pairing the central ClpP

core with different cognate Clp-ATPase partners, not only flexibility in the selection of proteolytic substrates is generated, but it is also guaranteed that the options of protein refolding and protein turnover are intricately linked.

Important specific contributions of the Clp protease system to bacterial survival range from the degradation of defective, misfolded, or aggregated proteins and the general rescue of stalled ribosomes to precise regulatory control of the heat-shock response, natural competence, sporulation, motility, virulence, and lifecycle management. In the well-studied system of *B. subtilis*, it emerged that in addition to the proteolytic core ClpP and the different cognate Clp-ATPases, a variety of adaptor proteins fine tune the proteolytic response. It also became clear that by interacting with one versus the other of its Clp-ATPase partners, ClpP can either promote or inhibit a given development process. Rather as a rule than an exception, the Clp protease also recognises its proteolytic activators or repressors as substrates generating an elaborate and entwined network of regulatory control. It is to be expected that in species, where investigations on the Clp machinery have commenced more recently and where only the rudiments of substrates and interaction partners are known so far, similar regulatory layers await discovery. The asymmetric ClpP1P2 cores discovered in several bacterial species so far, including the intracellular species discussed here, offer further regulatory potential, as the assembly of two different ClpP homologs, sometimes even expressed from distinct chromosomal locations, are required for catalytic function. Also, eukaryotic cells capitalise from the capacity of the Clp protease to alleviate protein stress and perform regulatory proteolysis to ensure proper mitochondrial function and cellular homeostasis.

Due to its prominent role in regulating bacterial survival and virulence as well as proliferation and metastasis of cancer cells, the Clp protease holds promise as an antibiotic and anti-tumour drug target. Adding to this is the fact that the protease proved to be druggable by more than one compound class and that its inhibition as well as activation showed therapeutic potential. Acyldepsipeptides of the ADEP class dysregulate ClpP by inhibiting its natural functions, yet at the same time stimulating unregulated proteolysis of non-native substrates [Kirstein et al., 2009a; Famulla et al., 2016]. ADEP has broad-spectrum anti-Gram-positive antibiotic activity, displaying low minimal inhibitory concentrations and potent *in vivo* efficacy [Brötz-Oesterhelt et al., 2005; Brötz-Oesterhelt and Vorbach, 2021]. The specific inhibition of the

Clp protease is likewise promising and pursued as an anti-virulence strategy. β -Lactones and the dihydrothiazepine-derivative 334 represent ClpP and ClpX inhibitors, respectively. β -Lactones were shown to attenuate *S. aureus* in mouse infections, and 334 was reported to attenuate global virulence factor synthesis *in vivo* [Weinand et al., 2014; Fetzter et al., 2017]. Cyclomarin, lassomycin, and ecumicin are antibacterial agents that deregulate the Clp-ATPase component of the Clp protease complex [Malik and Brötz-Oesterhelt, 2017]. Regarding anti-tumorigenic potential, ADEP derivatives showed some potential, and for the imipridone ONC 201, a first-in-class anticancer compound in clinical trials, ClpP has recently been established as the target [Wong et al., 2018; Graves et al., 2019; Ishizawa et al., 2019; Wong and Houry, 2019].

The ubiquitous Clp protease system represents an especially versatile machinery, involved in various cellular homeostasis processes and coping strategies. It will be fascinating to unravel additional functions in bacteria and eukaryotic cells and further explore its therapeutic potential.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.I. drafted the passages on *B. subtilis* and *S. aureus*, prepared Figure 2, and assembled the final document. Y.T. drafted the passages on mitochondrial ClpP. S.P. drafted the passages on *L. monocytogenes*, *M. tuberculosis*, and *C. trachomatis*, and prepared Figure 3. L.R. drafted the passage on the introduction to the Clp system and prepared Figure 1. H.B.-O. devised the concept and structure of this review and finalised the text.

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