# Regulated proteolysis in Gram-negative bacteria — how and when?

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Abstract | Most bacteria live in a dynamic environment where temperature, availability of nutrients and the presence of various chemicals vary, which requires rapid adaptation. Many of the adaptive changes are determined by changes in the transcription of global regulatory networks, but this response is slow because most bacterial proteins are stable and their concentration remains high even after transcription slows down. To respond rapidly, an additional level of regulation has evolved: the degradation of key proteins. However, as proteolysis is an irreversible process, it is subject to tight regulation of substrate binding and degradation. Here we review the roles of the proteolytic enzymes in Gram-negative bacteria and how these enzymes can be regulated to target only a subset of proteins.

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Intracellular proteolysis is a fundamental process that regulates gene expression and removes misfolded proteins in response to external stimuli and normal homeostasis. The importance of proteolysis is underscored by the existence of at least one protease in all cells and by the regulatory defects of mutants that lack one or more proteases[1–6]. Because proteolysis irreversibly destroys proteins, intracellular proteases evolved to avoid random, uncontrolled degradation. As a result, proteolysis is a carefully regulated process.

Regulated proteolysis in the cytoplasm of Gram-negative bacteria is carried out by five ATP-dependent proteases: ClpAP, ClpXP, Lon, HslUV and FtsH. Three additional proteases — ClpCP, ClpEP and the bacterial proteasome — are found in several Gram-positive bacteria. The active sites of ATP-dependent proteases are hidden inside oligomeric structures, which form a compartmentalized proteolytic chamber[7,8] (FIG. 1a). To enter this proteolytic chamber, substrates must pass through a regulatory substrate-binding domain that acts as a 'gatekeeper', which is an oligomeric structure that forms an opening (referred to as the pore) that is too narrow to allow random diffusion of most folded proteins into the proteolytic chamber. Protease substrates are first bound by the substrate-binding domain of the protease, which contains a molecular motor that couples ATP binding and hydrolysis with substrate unfolding and translocation (FIG. 1b). This molecular motor belongs to the AAA+ family of proteins (ATPases associated with diverse cellular activities) and, therefore, ATP-dependent proteases are often referred to as AAA+ proteases. The structure–function relationship and the mechanism of protein degradation by AAA+ proteases are discussed broadly in a recent review[9].

The spatial oligomeric organization of AAA+ proteases is highly conserved, although some variation exists (FIG. 1a). Fourteen subunits of ClpP form a proteolytic chamber that can interact with either ClpA or ClpX hexamers. Similarly, the HslUV protease is formed by the interaction of the dodecameric HslV proteolytic domain with the hexameric AAA+ HslU subunit. By contrast, in Lon and FtsH, both the AAA+ and protease domains are part of a single polypeptide chain that oligomerizes into a barrel-shaped homohexamer. Unlike the other proteases, FtsH is anchored to the inner membrane by an amino-terminal transmembrane helical segment.

ATP-dependent proteases combine strict selectivity and unusually broad substrate specificity. Indeed, they can interact with many protein substrates (for example, Lon can degrade about half of all *Escherichia coli* proteins when they are misfolded[10,11]), but can successfully avoid random degradation of non-substrate proteins. In this Review, we describe the mechanisms by which these proteases achieve this specificity in Gram-negative bacteria and how this specificity can be regulated.

## Substrate specificity and recognition

Each ATP-dependent protease degrades many different substrates with a varying degree of specificity. The substrate specificity of bacterial proteases is determined by the recognition of short substrate sequences, known as degradation tags or degrons[12], by the substrate-binding domain of the protease. Degrons are often present at the amino terminus or the carboxy terminus of substrates,



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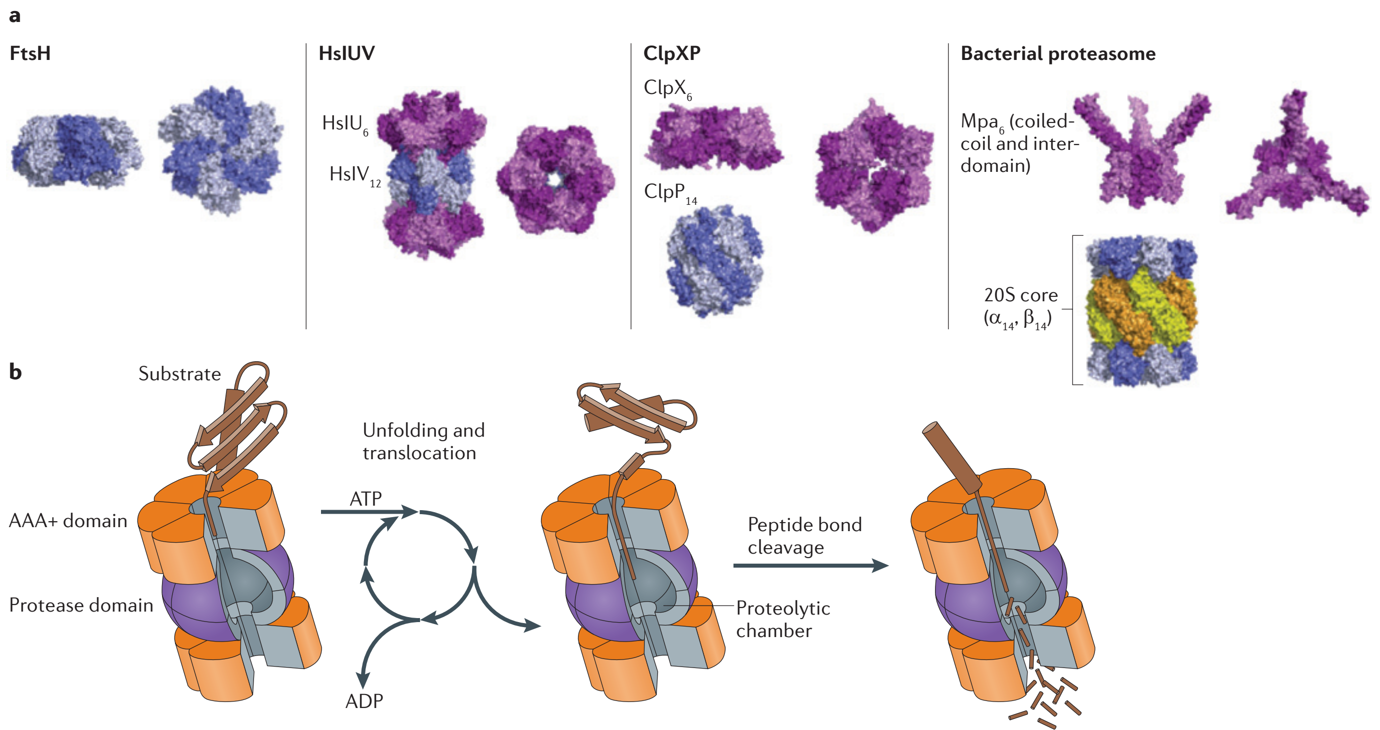


Figure 1 | **Architecture and mechanism of ATP-dependent proteases. a** | Surface representations of bacterial ATP-dependent proteases for which hexameric three-dimensional structural data are available. Top view (right) and side view (left) representations are shown for FtsH (bound to ADP; protein databank identifier (PDB id): 2DHR )[114], HslUV (bound to ATP; PDB id: 1KYI)[7], ClpX (bound to ADP; PDB id: 3HWS)[115], and the coiled-coil and inter-domains of Mpa (PDB id: 3M9B)[116], the proteasomal regulatory protein of *Mycobacterium tuberculosis*. Side views alone are shown for ClpP (PDB id: 3MT6)[117] and the proteasomal 20S core particle (PDB id: 2FHH)[118]. The number of monomers in the complex is indicated in subscript. **b** | ATP-dependent proteases degrade substrates by a complex mechanism. After substrate binding, repeated cycles of ATP hydrolysis fuel substrate unfolding and translocation. In the proteolytic chamber of the protease domain, peptide bonds of the substrate are cleaved in an ATP-independent manner.

where they can be easily accessed and bound. Each protease recognizes specific degrons, and this determines its specificity. For example, Lon selectively recognizes misfolded proteins by interacting with degrons that are inaccessible when the substrate is properly folded[13], whereas FtsH recognizes primarily membrane-bound substrates[14,15]. A substrate can be recognized by more than one protease if it carries multiple degrons.

The best-studied example of specific substrate binding is the interaction of ClpXP with the SsrA degron. SsrA is a short amino acid sequence (AANDENYALAA in *E. coli*) that is appended to the C terminus of proteins in a process called *trans*-translation. During a shortage of charged tRNAs, or when a translated mRNA lacks a stop codon, the ribosomal A-site becomes unoccupied, resulting in translation stalling. When this occurs (once in every 200 translation events in *E. coli*[16,17]), a transfer-messenger RNA (tmRNA) enters the empty A-site of the ribosome. This tmRNA carries a coding sequence that extends translation by 11 amino acids, followed by a stop codon that allows translation to terminate properly. Consequently, the polypeptide is released from the ribosome with the SsrA degron as the C terminus. As this tag is efficiently recognized by ClpX, and to a lesser extent by other proteases, *trans*-translation products are rapidly degraded[16,18–20]. Recognition of the SsrA degron by ClpX is mediated by three sets of flexible loops, which are located at different positions along the axial pore: the RKH, pore 2 and GYVG loops[21–23]. The GYVG loops are conserved among AAA+ proteases and have a central role in substrate translocation[24]; the two other types are poorly conserved in AAA+ proteins other than ClpX and determine much of the specificity of ClpX for SsrA. Mutations in the RKH loops alter the specificity of ClpX in general and reduce specificity for SsrA, indicating that these loops act as selectors for substrate entrance to the pore. Once in the pore, the two C-terminal Ala residues in SsrA interact with the pore 2 loops, which are positioned deep in the pore and can readily deliver the substrate to the degradation chamber. After initial binding, substrate translocation can occur with negligible sequence specificity, as evident by efficient translocation of a range of polymeric chains by ClpX[25].

The different substrate specificities of bacterial proteases, together with their expression patterns and cellular localization, allow each protease to degrade specific classes of substrates and therefore to regulate certain physiological processes. It should be noted, however, that there is also a high degree of overlap. In addition, the mechanistic and kinetic properties of the proteases



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Box 1 | **Formation of aggregates in the absence of regulated proteolysis**

Failure to degrade or refold misfolded proteins often results in the formation of insoluble protein aggregates. Although it was shown that proteases can degrade aggregated proteins *in vitro*[31,105], there is no convincing evidence for such a process *in vivo*. It seems that aggregated proteins have to be disaggregated by chaperones before they can be processed by proteases (FIG. 2). It is possible that not degrading protein aggregates *in vivo* offers bacteria an advantage. Protein aggregates are spontaneous by-products of protein unfolding and are regarded as a dead-end in the 'life cycle' of a protein[106]. They are insoluble and inactive (with the exception of natively aggregated structures such as curly fimbriae and sex pili) and cannot be further processed before they are refolded by chaperones. Protein aggregates are often regarded as toxic to cells, as in the case of amyloid diseases such as neurodegenerative disorders and type II diabetes.

However most aggregates that result from protein unfolding stresses are not amyloid but are disordered aggregates; although there is a correlation between the occurrence of disordered aggregates and damage to the cell[107], the fact that they are toxic is not strongly supported by evidence. Instead, the damage to *Escherichia coli* cells during a protein unfolding stress was shown to result from the loss of activity of cellular proteins and not necessarily from aggregate formation[108]. If disordered protein aggregates are not toxic, it makes sense to avoid their degradation, as they serve as a pool of potentially active proteins that require disaggregation and refolding by chaperones. Following disaggregation and solubilization, should refolding fail, the misfolded — now soluble — proteins can be degraded by proteases.

Nevertheless, this rationale cannot overrule degradation of protein aggregates under certain conditions. In fact, a recent report provides a clue for a possible crosstalk between proteases and the disaggregation chaperone machinery[109]. IbpA and IbpB, two proteins associated with inclusion bodies, were shown to form specific interactions with the protease Lon[109]. Although the physiological relevance of these interactions is currently unclear, it is tempting to speculate that a mechanism exists which enables the attack of protein aggregates by proteases under certain conditions.

N-end rule

The regulation of protein stability by the first amino acid of a protein. For example, the presence of a Lys or Arg severely decreases the half-life of the proteins through regulated proteolysis.

are also important determinants of substrate selection. For example, FtsH has a weak unfoldase activity[26] and therefore can degrade only unfolded proteins, whereas ClpA, which has two AAA+ domains, is a powerful unfoldase and can efficiently degrade stably folded substrates[27]. Furthermore, the ability of a protease to unfold substrates also depends on the substrates themselves; Lon can allosterically adjust its activity according to the properties of the substrate and can operate in various activity modes depending on the degron[28]. Some degrons induce Lon to operate in a rapid degradation mode, whereas others induce high ATP hydrolysis rates and shut off degradation, allowing Lon to interact with certain substrates without degrading them[28].

Adaptor proteins enable fine-tuning of proteolysis, by biasing the specificity of a protease towards certain substrates or a class of substrates according to physiological requirements. Adaptor proteins increase the effective concentration of the substrate near the protease pore by tethering substrates to the protease, thus promoting efficient binding without inhibition of substrate processing. Three bacterial adaptors have been identified in Gram-negative bacteria: SspB, which increases the affinity of ClpX for SsrA-tagged proteins[29]; RssB, which is responsible for recognition of $\sigma^S$ by ClpX[30] (see below); and ClpS, which facilitates binding of N-end rule substrates to ClpAP. ClpS interacts with substrates that carry a large N-terminal hydrophobic residue (Tyr, Phe, Trp or Leu) in close proximity to a positively charged residue[31–34]; these residues are buried deep in a hydrophobic pocket of ClpS that determines substrate specificity[34,35]. Recently, 23 ClpS substrates were identified in *E. coli*, including PATase (putrecine N-transferase) and Dps (DNA protection during starvation)[36]. Strikingly, the original N terminus of these proteins was post-transcriptionally modified by truncation of four N-terminal amino acids. In the case of Dps, this occurs through an unknown mechanism, and for PATase this occurs by the addition of a primary destabilizing amino acid to its N terminus by LFTR (Leu/Phe tRNA-protein transferase)[36]. Currently, PATase is the only known LFTR substrate in *E. coli*, and it remains to be determined whether this is a general mechanism to regulate degradation.

The bacterial adaptor SspB dimerizes to form an SsrA-binding domain[37] that is leashed to ClpX by the two long unstructured C-terminal segments of the dimer. Using this mechanism, SspB increases the affinity of ClpX for SsrA about 10-fold, from ~2 μM in the absence of SspB to 200 nM in its presence[29,38]. Although the importance of SsrA tagging *in vivo* is clear, the conditions in which SspB-mediated degradation becomes an advantage have yet to be discovered. By contrast, the physiological importance of RssB-mediated $\sigma^S$ degradation is well understood and is discussed below.

## Proteolysis and protein quality control

Under stress conditions, such as heat shock and exposure to oxidative reagents, protein unfolding greatly increases and becomes a major problem for the cell, as unfolded proteins are often inactive and tend to accumulate as insoluble aggregates (BOX 1). To minimize protein unfolding and to cope with aggregates, all cells possess a network of proteases, chaperones and accessory factors, which are referred to as the protein quality control system (FIG. 2). However, under stress conditions chaperones are flooded with substrates, so coping efficiently with the mass of misfolded species becomes a serious problem. Furthermore, although it is energetically more cost-effective to refold a protein rather than to degrade and re-synthesize it, not all misfolded proteins can be refolded by chaperones; instead, such proteins can be degraded by ATP-dependent proteases, generating building blocks for *de novo* protein synthesis[39].

But how does the protein quality control system decide which proteins should be refolded and which should be degraded? In eukaryotic cells, chaperone-mediated proteolysis can occur[40], but in bacteria the key principle in the balance between refolding and degradation seems to be competition between chaperones and proteases for substrate binding[41]. This competition is balanced towards refolding, as chaperones are far more abundant than proteases — GroE and DnaK, the two main chaperones in *E. coli*, are present at roughly 1,600 and 10,000 copies, respectively[42,43], whereas ClpXP, ClpAP and Lon are present at roughly 50–150 hexamers per cell[21] (E.G., unpublished observations). Furthermore, chaperone recognition sequences are more common in proteins than proteolysis recognition sequences. For example, DnaK and its co-chaperone, DnaJ, interact with hydrophobic sequences that are rich in aliphatic residues, whereas Lon recognizes aromatic residues[13].



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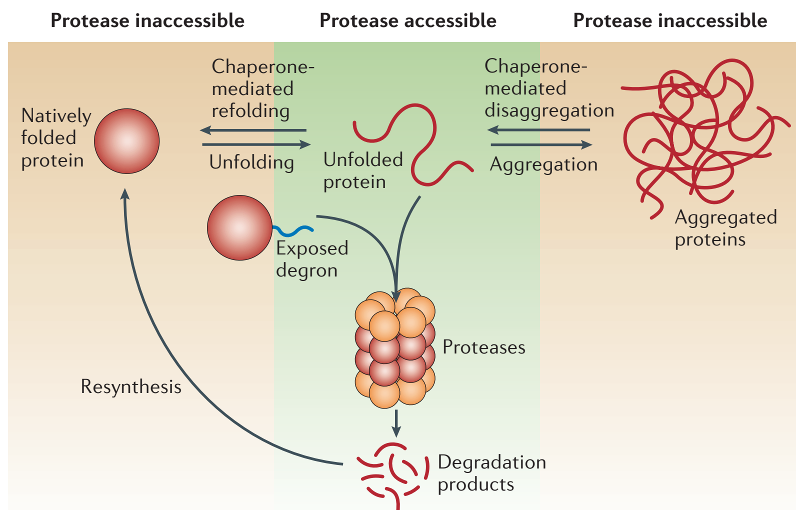


Figure 2 | **Proteases and protein quality control.** ATP-dependent proteases can access either misfolded proteins or the degrons of folded proteins. Unfolded proteins can either be degraded by proteases or refolded by chaperones. Otherwise, they tend to form insoluble aggregates. The aggregated proteins can be made accessible to proteases if they are re-solubilized by chaperones. The products of protein degradation can be used for *de novo* protein synthesis.

As aromatic residues are less abundant than aliphatic residues in proteins, misfolded proteins are more likely to be refolded than to be degraded. However, when a protein fails to be refolded by chaperones, or when multiple refolding cycles are required, interaction with a protease becomes more likely. Moreover, as interaction of Lon with multiple degrons on the same substrate can contribute synergistically to binding, a higher degree of substrate unfolding (which exposes several degrons) can facilitate substrate interaction with this protease[13].

Notably, SsrA-tagged proteins are a special case. As they are translated from an aberrant mRNA, SsrA-tagged proteins are usually misfolded and unlikely to fold properly, making any interaction with chaperones useless. The SsrA degron facilitates direct interaction and degradation by ClpXP, especially in the presence of SspB, and thereby allows the protein to bypass the competition between chaperones and quality control proteases such as Lon.

## Regulation of cellular processes

Protein degradation is widely used in bacteria to regulate physiological processes. For example, Lon regulates capsule synthesis in *E. coli* by degrading RcsA, a positive transcriptional regulator of capsule synthesis. Indeed, *lon* mutants are known to be mucoid as a result of overproduction of polysaccharide capsule. These mutants are also sensitive to ionizing irradiation, such as ultraviolet light and X-rays, owing to the increased half-life of the cell division inhibitor SulA in the absence of Lon (see below).

Since the initial description of the phenotypes of the *lon* mutant, many regulatory proteins have been found to be degraded by different proteases, and a range of physiological processes have been shown to be controlled by proteolysis[44,45] (FIG. 3). Although protein degradation seems wasteful as a way to control protein levels compared with preventing the synthesis of these proteins, regulation by proteolysis allows rapid and immediate control, and in systems of constant flow it is more efficient to act downstream when rapid response is required. Indeed, in stress responses, which require immediate action, regulation by proteolysis is the most obvious response. Below, we discuss several well-studied examples of cellular processes that are regulated by proteolysis, including the degradation of key metabolic enzymes, regulatory proteins and central regulatory circuits. We focus primarily on *E. coli*, although examples in other bacteria are also provided (see also BOX 2 for a description of the regulation of *Caulobacter crescentus* differentiation by localized proteolysis).

C1 compounds
Compounds that contain one C atom and are more reduced than $CO_2$. Such compounds include methane, methanol and formaldehyde.

Stringent response
The physiological changes that are elicited by amino acid starvation.

***Maintaining balanced lipopolysaccharide biosynthesis.*** Lipopolysaccharide (LPS) is an essential component of the outer membrane of Gram-negative bacteria and constitutes about 10% of the total cell lipids. LPS is vital for bacterial pathogenesis, and alterations in LPS lead to substantially decreased virulence[46–49].

LPS is composed of lipid A and core oligosaccharides, which start with two units of KDO (3-deoxy-D-manno-octulosonate). Two essential enzymes in LPS synthesis are regulated by proteolysis: FtsH regulates the levels of LpxC (UDP-3-O-acyl-*N*-acetylglucosamine deacetylase), which catalyses the first committed step in the biosynthesis of lipid A[50,51], and of KDO transferase (KdtA), which attaches the two KDO residues to the lipid A precursor (lipid IVA) to form the minimal essential structure of LPS (KDO2–lipid A)[52]. The effect of FtsH on these LPS enzymes is constitutive, so FtsH regulates the concentration of both the lipid moiety (lipid A) and the sugar moiety (KDO-based core oligosaccharides) of LPS, ensuring a balanced synthesis of LPS, which is essential for viability[53].

***Regulation of growth at increased temperatures.*** An increase in temperature is a major challenge for unicellular organisms, as enzymes — and thus metabolic pathways — respond differently to temperature increases, making it difficult to maintain an overall metabolic balance. *E. coli* and various other microorganisms maintain balanced growth at increased temperatures by slowing down metabolism through limiting the availability of Met[54–56]. In addition to being used for protein synthesis, Met is required for the synthesis of polyamines (spermidine and spermine) and the transfer of C1 compounds, which are required in a large number of pathways, including the biosynthesis of purines and pyrimidines (FIG. 4). In addition, inhibition of protein synthesis blocks RNA synthesis through the stringent response and DNA replication at initiation (as initiation requires protein synthesis).

When *E. coli* cells are exposed to temperatures above 30°C, the growth rate changes depending on the availability of endogenous Met. The limitation of Met as a function of temperature is mediated by the first enzyme in Met biosynthesis, homoserine O-succinyltransferase



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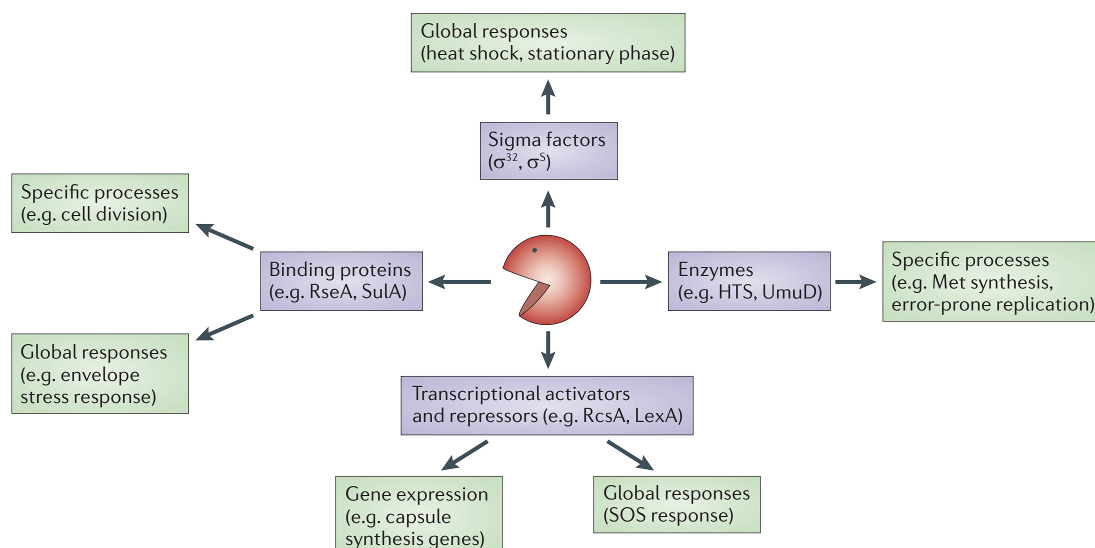


Figure 3 | **Regulation of cellular processes by proteolysis.** Shown are the possible regulatory targets of bacterial proteases and the cellular processes that are affected by their degradation.

(HTS)[55]. This enzyme is extremely thermolabile and unfolds at temperatures above 25°C. The activity of HTS decreases with increasing temperature, and at 46°C the enzyme is inactive and forms aggregates, resulting in an absolute Met requirement[55,57–59]. The temperature-dependent unfolding of HTS results in its proteolysis at a rate that increases with temperature[56]. The degradation of HTS is carried out by ATP-dependent proteolysis[56] and provides an additional level of control over the availability of Met and the growth rate at increased temperatures (FIG. 5).

***Regulation of the heat shock response through the levels of $\sigma^{32}$.*** One of the best-studied regulatory networks is the heat shock response, which involves the induction of expression of a large number of proteins upon increases in temperature. For example, in *E. coli* the heat shock response is induced upon a shift from about 18°C to any temperature up to 45°C[60]. This heat shock regulon contains genes coding for components of the protein quality control system as well as factors required for the quality control of translation[61]. In Gram-negative bacteria, the expression of the heat shock regulon is controlled by the transcriptional activator $\sigma^{32}$, which recognizes different promoters than the housekeeping sigma factor $\sigma^{70}$.

In *E. coli*, $\sigma^{32}$ is synthesized at a basal level at all temperatures, but its cellular concentration remains low as a result of rapid proteolysis by FtsH, assisted by the DnaK and GroESL chaperone systems[62–65], leading to a $\sigma^{32}$ half-life of ~ 1 min[62]. Upon a shift to higher temperatures, more $\sigma^{32}$ is produced; this, together with a decrease in the levels of free chaperones (which are engaged in rescuing denatured proteins) leads to a stabilization and accumulation of $\sigma^{32}$ and the concomitant induction of heat shock genes (FIG. 5).

However, regulated proteolysis of $\sigma^{32}$ as a function of temperature is not observed in all Gram-negative bacteria. For example, in the alphaproteobacterium *Agrobacterium tumefaciens*, $\sigma^{32}$ is a stable protein at all temperatures, and it is not clear whether proteolysis has a role in the heat shock response in this species[66].

***Modulating transcript stability through proteolysis of the RNA chaperone CspC.*** One unique feature of the heat shock response is its transient nature; after the rapid increases caused by the shift to higher temperatures, the expression of heat shock genes returns to a steady-state level[67]. This decline in expression occurs while the stress factor (high temperature) is still present and is essential to maintain balanced growth at the new increased temperature.

Two factors in *E. coli* control the reduced expression of heat shock factors during this process. First, DnaK and GroE are themselves heat shock proteins, and their levels increase with higher activity of $\sigma^{32}$, allowing

Box 2 | **Bacterial differentiation and localized proteolysis**

*Caulobacter crescentus* is one of the few bacteria with a defined life cycle, and proteolysis plays an important part in this. Each division produces two distinct cell types with specialized developmental programmes: a sessile cell and a swarmer cell. The sessile cell has a long stalk with an adhesive tip, which anchors the cell to surfaces. It goes through a division cycle to form two distinct daughter cells, another stalked sessile cell and a swarmer cell. By contrast, the swarmer cell is motile, with a polar flagellum, polar pili and a polar chemotaxis apparatus. This cell eventually differentiates into a sessile cell by losing its flagellar motor, pili and chemotaxis apparatus, and replacing them with a stalk.

Proteolysis has two essential roles in this complex life cycle: it removes the structural parts of the flagellum and components of the chemotaxis machinery during the swarmer-to-sessile cell transition[110–112] and controls the levels of the regulatory proteins, including cell cycle proteins. The ClpXP protease catalyses the degradation of the master regulator CtrA at specific time points in the cell cycle and thereby allows the initiation of DNA replication and cell division. ClpXP is part of a proteolytic complex that is localized to the pole of the *C. crescentus* sessile cell. This localization of the proteolytic complex ensures that degradation of CtrA and initiation of cell division occur only in the sessile cell[113].



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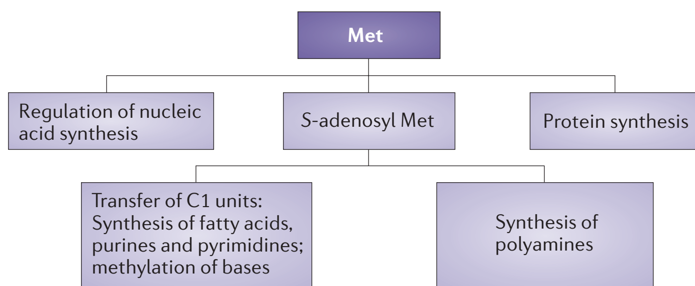


Figure 4 | **Multifaceted functions of Met.** The sulphur-containing amino acid Met is the amino-terminal amino acid in most proteins and is also incorporated into proteins as encoded by the sequence of their genes. In addition, Met contributes C1 units for a large number of pathways, including the biosynthesis of purines and pyrimidines, through *S*-adenosyl Met, and is a building block for the synthesis of polyamines (spermidine and spermine). Met deficiency inhibits all these reactions and also blocks RNA synthesis through the stringent response and initiation of DNA replication (which requires protein synthesis).

them to recapture $\sigma^{32}$ and thereby limit the induction of heat shock genes ('titration model' (REFS 68–71)). Second, there is a considerable decrease in the half-life of the transcripts of heat shock genes at increased temperatures[61]. For example, the transcripts of many $\sigma^{32}$-dependent genes are stabilized by the RNA chaperone cold shock protein C (CspC)[61] (FIG. 5). However, the levels of CspC decrease upon shifts to high temperatures owing to proteolysis and aggregation, thereby lowering the stability, and therefore half-life, of mRNAs of heat shock genes[61].

It should be noted that the $\sigma^{32}$ mRNA contains an RNA thermometer element[72] that has essential secondary structures for the characteristic thermoregulation, and can induce an increase in the total transcript levels upon an increase in temperature. However, there is no evidence that this regulatory element involves CspC or proteolysis.

The CspC-mediated reduction in the stability of heat shock gene transcripts is essential for the proper shutdown of the heat shock response. This is physiologically important because high levels of heat shock proteins (for example, caused by overexpressing $\sigma^{32}$ (REF. 61)) is harmful and results in slower growth at high temperatures. Similarly, growth slows down after a shift to 42°C when CspC is overexpressed, indicating that temperature-dependent proteolysis of CspC is an essential part of the heat shock response and is required for balanced growth at high temperatures[61]. As the concentration of heat shock proteins is important for optimal growth at high temperatures, modulation of stability of heat shock gene transcripts probably occurs in other bacteria as well.

***$\sigma^E$ and regulation of the envelope stress response.*** As the periplasmic space of bacteria is not accessible to the cytoplasmic protein quality control system and lacks ATP, which is required for the activity of key components of the cytoplasmic protein quality control system, bacteria contain a dedicated periplasmic protein quality control system. This system includes biosynthetic enzymes, proteases and chaperones that do not rely on ATP hydrolysis. The genes encoding these enzymes are transcriptionally regulated by a dedicated sigma factor, $\sigma^E$. A large subset of the 89 $\sigma^E$-regulated genes identified in *E. coli* K12 are conserved across genomes; these encode proteins that participate in biosynthesis and maintenance of LPS and porins[73,74]. At steady-state conditions, $\sigma^E$ remains associated with the membrane, as it is bound by the cytoplasmic domain of the transmembrane regulator protein RseA. However, under conditions that stimulate protein unfolding in the periplasmic space, a cascade of proteolytic events leads to cleavage of RseA and the concomitant release of $\sigma^E$ (REF. 75,76); this initiates what is known as the envelope stress response.

The envelope stress response starts with the activation of the trimeric periplasmic protease DegS, which becomes activated after binding the C-terminal β-strand of unfolded or nascent outer membrane proteins[77–79]. Activated DegS cleaves the periplasmic domain of RseA[80], leading to activation of the membrane-embedded protease RseP (also known as YaeL). Activated RseP then cleaves the transmembrane domain of RseA, allowing the RseA cytoplasmic domain (still bound to $\sigma^E$) to diffuse away from the membrane[81]. Finally, degradation of the RseA cytoplasmic domain by ClpXP releases $\sigma^E$, which becomes free to initiate gene expression[82] (FIG. 5).

Control of RseA cleavage is mediated not only through allosteric activation of DegS, but also by sequestration of RseA. The periplasmic protein RseB binds RseA and prevents its cleavage by DegS, regardless of the activation level of the protease[83], thereby serving as a control against random cleavage[84]. Although it is not clear which physiological trigger disrupts the interaction between RseA and RseB, it has been suggested that lipoproteins may interact with RseB and titrate it away from RseA[85]. For example, Lpp (also known as Braun's lipoprotein) is essential for anchoring peptidoglycan to the outer membrane. Lpp may fail to be inserted into the outer membrane under stress conditions and consequently accumulate in the cytoplasm; under such conditions, Lpp could interact with RseB[85]. If this model is indeed correct, a picture arises in which both RseB and DegS sense periplasmic stress by binding unfolded polypeptides. RseB responds by releasing RseA, whereas DegS responds by cleaving it, eventually resulting in activation of the envelope stress response.

***Stationary phase and signal transduction tagging.*** Stationary phase is controlled by the master regulator of the general stress response, $\sigma^S$ (also known as RpoS). This sigma factor is a transcriptional activator for many genes that are essential for coping with numerous environmental stresses, carbon starvation and growth during stationary phase[86]. The levels of $\sigma^S$ are low during exponential growth, but increases significantly upon entering stationary phase[87]. Similarly to $\sigma^{32}$, expression of $\sigma^S$ is regulated at several levels, including protein stability.

Proteolytic degradation of $\sigma^S$ is carried out by the protease ClpXP and requires the adaptor activity of RssB, a two-component response regulator that binds $\sigma^S$. Optimal interaction of RssB with $\sigma^S$ *in vitro* requires



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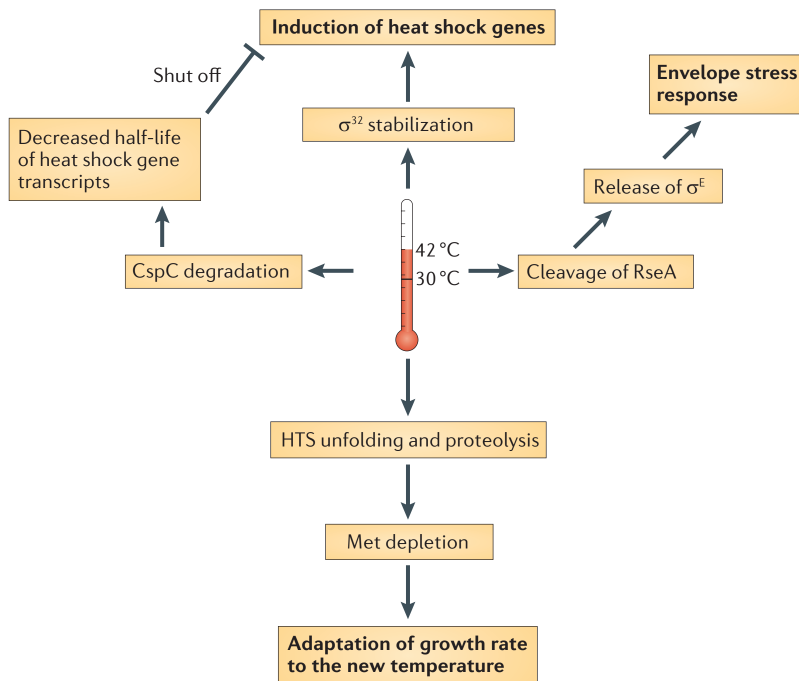


Figure 5 | **Proteolysis in the adaptation to temperature increases.** Schematic representing the various events of regulated proteolysis that take place upon a shift to higher temperatures (most *Escherichia coli* experiments involve a temperature increase from 30°C to 42°C). These include induction of heat shock genes through the expression of $\sigma^{32}$, activation of the envelope stress response through the release of $\sigma^E$ and adaptation to the new temperature through Met depletion and the decreased half-life of the heat shock gene transcripts.

phosphorylation of Asp58 in RssB, as mutation of this phosphorylation site results in increased $\sigma^S$ stability and activity *in vivo*[86]; however, the kinase involved has yet to be identified. RssB is a typical 'orphan' response regulator, as no cognate His sensor kinase is encoded in an operon with *rssB* or close to it, although there is some evidence for a connection of RssB to the Arc two-component system, which can regulate $\sigma^S$ activity at a transcriptional level[88].

Binding of RssB to $\sigma^S$ is regulated in response to various starvation conditions through the activity of Ira (inhibitors of RssB activity) proteins, regardless of the phosphorylation state of RssB. These anti-adaptor proteins bind RssB to prevent its interaction with $\sigma^S$, thereby promoting stabilization of the sigma factor. IraP, the first anti-adaptor to be discovered[89] in *E. coli*, is expressed during phosphate starvation[90] (and magnesium starvation in *Salmonella enterica* subspecies *enterica* serovar Typhimurium[91]). The two other anti-adaptors, IraM and IraD, are expressed in response to magnesium starvation and DNA damage, respectively[92].

Overall, $\sigma^S$ degradation by ClpXP can be controlled by both RssB phosphorylation and anti-adaptor-mediated sequestration. The existence of at least three Ira proteins that all respond to distinct stress conditions suggests that each anti-adaptor modulates $\sigma^S$ stability to an extent that allows optimal induction of the general stress response under specific conditions.

***The SOS response as an example for a regulatory circuit.*** One of the first indications for the control of the expression of a large network by regulated proteolysis involves the SOS response, the bacterial response to DNA damage. Regulated proteolysis affects the SOS response through two mechanisms, proteolysis of regulatory elements and proteolysis of several SOS-induced proteins, which together fine-tune their cellular concentrations to minimize inhibition of growth and error-prone repair processes.

The key regulator of the SOS response is the LexA repressor, which inhibits the expression of ~ 40 genes[93–95] that contain a 20-nucleotide-long SOS box (also known as a LexA box) in their promoter[96]. Single-stranded DNA resulting from DNA damage activates RecA, which then induces self-cleavage of LexA[97–99]. Cleaved LexA does not repress transcription of the SOS regulon and is rapidly degraded by ClpXP, as the new C-terminal sequence of LexA is very similar to the C-terminal sequence of the SsrA degron. This C-terminal LexA fragment is presumably a substrate for Lon as well as ClpXP[98].

Many of the proteins in the SOS regulon are toxic if expressed for too long, so their removal by proteolysis following the repair of DNA damage is essential[94,95]. The cell division inhibitor SulA was one of the first proteins to be discovered as a substrate for intracellular proteolysis. It is one of the most short-lived *E. coli* proteins, as it is degraded by Lon and HslUV to allow septation following recovery from DNA damage[1,100,101]. Other proteins of the SOS regulon that are regulated by proteolysis include UvrA (which is part of the nucleotide excision repair DNA repair pathway and the cellular levels of which are tuned to the level of DNA damage[94,102]) and the Umu proteins (which are involved in error-prone DNA replication[103,104]). The extensive involvement of proteolysis in the regulation of the SOS response allows rapid shut-off of this response after the DNA damage has been repaired. Thus, the deleterious effects of SOS proteins can be arrested as soon as their activity is no longer required.

## Conclusions and future directions

Although transcriptional control has been considered the major regulatory element in bacterial physiology, regulated proteolysis offers a robust control mechanism that is subject to fine-tuning. This powerful mechanism complements transcriptional control and provides the required means for cell cycle changes and for adaptation to stress conditions. Although, the importance of proteolysis as a key factor in regulating a broad range of bacterial networks has only recently become fully apparent, it is now clear that proteolysis is involved in many studied bacterial networks.

Even though these regulatory bacterial systems have been studied extensively, key facts are still missing, and for some systems it is not yet clear how proteolysis is triggered and how the substrates are recognized. Furthermore, most of the available data are from *E. coli*, and many physiological processes can differ in other Gram-negative bacteria. Regulatory proteolysis in pathogens is especially important, as they have to cope with various stresses and



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environments and had to develop novel control systems. Thus, it is likely that many proteolysis-regulated processes remain to be discovered.

Moreover, only a few proteolysis substrates have been recognized so far, as regulated proteolysis is sometimes difficult to detect for various reasons. First, regulatory proteolysis substrates are usually present at very low cellular concentrations, making them difficult to identify by proteomic studies. Second, the relevant protease that cleaves a substrate *in vivo* can be difficult to identify, as the substrate can be degraded by several proteases. Third, the isolation of mutants is often complicated, as some of the genes coding for the regulators are essential. Last, the accumulation of proteolysis substrates can be lethal, as is the case for SulA[1,100].

We expect that the advances in genomics and functional genomics, including transcriptomics and proteomics, will help to identify additional systems that are regulated by proteolysis. For example, it should be possible to detect unstable proteins by searching proteomes for proteins which decrease in levels in pulse-chase experiments. In addition, it should be possible to search for genes with high transcript levels but low protein levels, which would suggest that the proteins are degraded through proteolysis. It will be especially interesting to determine whether systems involved in environmental survival and in the ability to carry out processes that are vital for the environment, such as the nitrogen cycle, carbon cycle and even bioremediation of pollutants, are also regulated by proteolysis.

Last, but not least, very little is known about the roles of regulated proteolysis in pathogenesis. Interactions with the host involve a series of stresses — for example, changes in pH and temperature, oxidative stress and iron starvation — during which proteolysis is crucial. The use of genomics and functional genomics will provide data on the role of proteolysis in the control of gene expression during the pathogenic process. For example, it will be informative to examine the proteomic changes after infection, or upon exposure to host cells in the pathogen as well as in the host. These data should make it possible to identify potential host-related bacterial regulatory circuits in which proteolysis plays a crucial part, and will pave the way for a better understanding of the pathogenic process and of bacterial–host interactions.


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**Competing interests statement**

The authors declare no competing financial interests


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