Chapter 4
General and Regulatory Proteolysis in *Bacillus subtilis*

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Abstract The soil-dwelling bacterium *Bacillus subtilis* is widely used as a model organism to study the Gram-positive branch of Bacteria. A variety of different developmental pathways, such as endospore formation, genetic competence, motility, swarming and biofilm formation, have been studied in this organism. These processes are intricately connected and regulated by networks containing e.g. alternative sigma factors, two-component systems and other regulators. Importantly, in some of these regulatory networks the activity of important regulatory factors is controlled by proteases. Furthermore, together with chaperones, the same proteases constitute the cellular protein quality control (PQC) network, which plays a crucial role in protein homeostasis and stress tolerance of this organism. In this review, we will present the current knowledge on regulatory and general proteolysis in *B. subtilis* and discuss its involvement in developmental pathways and cellular stress management.

Introduction

The soil bacterium *Bacillus subtilis* encounters quickly changing and often unfavorable conditions in its natural habitat. During evolution these environmental cues might have been important for the establishment of a wide variety of elaborate stress response and developmental pathways, which *B. subtilis* cells exploit in order to adapt to their environment. For example, *B. subtilis* cells can grow normally even above 50 °C and they are quite resistant to osmotic stress. Furthermore, when the cells enter stationary phase the bacterium can differentiate into many different cell types, such as endospores, cells competent for DNA uptake and biofilm forming cells. At the heart of these pathways are complex signal transduction systems, which

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integrate different environmental cues to modify gene expression in order to respond to the external conditions [1].

Interestingly, controlled protein degradation is intricately involved in these regulatory pathways. Here the stability of transcription factors or other cellular regulators, which e.g. directly affect signal transduction pathways, are specifically modulated by proteolysis. At first glance, the control of cellular regulatory proteins by proteolysis might be considered rather inefficient and wasteful, given the amount of energy required for the synthesis of such a protein. However, using proteolysis in regulatory modules has certain advantages and can thus be beneficial to the survival of cells. The first advantage of protein degradation is its irreversibility. By irreversibly removing one component of a pathway, equilibriums can be shifted and bistable states can be stabilized. Secondly, proteolytic systems can exhibit very fast response times. For example, if a protein is constitutively degraded and then stabilized in response to a signal, this results in a fast, switch-like behavior [2–4].

At the same time protein degradation is also utilized as part of the cellular protein quality control (PQC) system. Proteases, in conjunction with chaperone systems, degrade a large variety of unfolded, misfolded or damaged proteins, which would otherwise be detrimental to cellular function. The cellular PQC system becomes especially important during heat, oxidative or salt stress, which are potentially damaging conditions for proteins but also other cellular components [5–7].

Protease Systems

Hsp100/Clp and AAA+(ATPases Associated with Various Cellular Activities) Proteases

Here, we will focus on the role of Hsp100/Clp and related AAA+protease complexes in regulatory and general proteolysis. These bipartite ATP fueled protease complexes consist of a hexameric ring of Hsp100/Clp proteins, which are ATP driven unfoldases of the AAA+family associated with a barrel-like structure formed by the higher oligomeric peptidase complex. The active peptidase sites are not accessible to substrates outside of the compartmentalized protease, unless the substrate proteins are recognized, unfolded and transferred by the associated hexameric ATPases into the proteolytic chamber [8–10].

In *B. subtilis* the ATPase components are the Hsp100/Clp proteins ClpC, ClpE, or ClpX, which associate with the double-heptameric ClpP peptidase complex, ClpY which associates with the ClpQ peptidase, or the closely related AAA+protease complexes with a similar architecture; LonA, LonB and the membrane associated FtsH [4]. Of these seven different ATP driven proteases, ClpCP and ClpXP are intricately involved in cellular signaling processes and developmental programs such as sporulation, competence development, motility, biofilm formation and in stress response pathways. Interestingly, the same protease complexes serve as an

integral part of the PQC network through general proteolysis of unfolded or misfolded proteins. The important role of these proteases in *B. subtilis* is supported by the relatively severe and pleiotropic phenotypes of *clp* mutants [4].

The synthesis of ClpC, ClpP and ClpE is controlled by the repressor CtsR (see section "Heat Shock Response") and strongly induced by heat stress [11–14]. ClpE synthesis is very tightly regulated and it is present in only very low amounts in non-stressed cells [15]. To date, the functions assigned to ClpE suggest a role in protein disaggregation and possible subsequent degradation of aggregated proteins as well as modulation of the heat shock response [16].

ClpC is present in non-stressed cells and plays an important regulatory role during normal logarithmic growth. During heat shock ClpCP is produced in high amounts and can degrade or disaggregate protein aggregates [17–20]. An interesting and unusual feature of ClpC is its absolute requirement of adaptor proteins for its activity. Adaptor proteins modulate the substrate specificity of the Clp/Hsp100 ATPases, usually by tethering the substrate to the ATPase [4]. However, for ClpC it could be demonstrated that in the absence of an adaptor protein, ClpC is monomeric and the ATPase is inactive. The adaptors have been shown to bind to the N-terminal domain (NTD) and linker domain of ClpC and trigger its oligomerization into a hexameric ring-shaped complex with ATPase activity [20–23].

Currently, three adaptor proteins for ClpC have been identified: MecA targets the competence master regulator ComK for degradation by ClpCP [24], its paralog YpbH permits degradation of unfolded and aggregated model proteins *in vitro* [20, 25]. MscB has recently been identified as an adaptor protein, which targets the class III heat shock repressor CtsR to ClpCP for degradation [21]. The identification of different adaptor proteins, which can target various substrates for degradation by ClpCP might explain the concurrent involvement of ClpC in many distinct regulatory and general processes [4]. There is some evidence that more unknown ClpC adaptors might exist. For example, *in vitro* degradation experiments with the known *in vivo* ClpCP substrates MurAA [26] and SpoIIAB [27] were unsuccessful, possibly because the correct adaptor was missing from the reaction.

ClpX is quite abundant under non-stress conditions and its stress-induced expression is regulated by an unknown mechanism. Mutants in *clpX* have a pleiotropic phenotype of slow growth on solid media, an apparent long lag phase during growth in liquid medium, and a defect in competence, which is mostly due to over-production of the ClpXP substrate Spx [28, 29]. Spx is an important transcriptional regulator of oxidative stress in *B. subtilis* (see section "Oxidative Stress Response and Spx"). The degradation of Spx is modulated by the adaptor protein YjbH and its recently identified anti-adaptor protein YirB [30–32]. ClpX is also involved in the degradation of SsrA-tagged proteins and in the degradation of unfolded and aggregated proteins [33].

LonA and LonB are hexameric proteases, in which the ATPase domain and the protease domain are present on a single polypeptide chain [34]. The ClpYQ (CodWX) complex is homologous to the HslUV complex in *Escherichia coli* [35, 36] and structurally resembles the Clp protease complexes, although the protease component ClpQ exhibits sixfold and not sevenfold symmetry [37, 38]. FtsH is a

zinc metalloprotease, which is embedded in the cytoplasmic face of the membrane [39]. The deletion of *ftsH* displays a pleiotropic phenotype in *B. subtilis* [39, 40].

Regulated Intramembrane Proteolysis

We will also briefly discuss regulated intramembrane proteolysis (RIP), which is a process that both prokaryotes and eukaryotes use for signaling across cell membranes. Here a signaling module consists of a cytosolic transcription factor that is kept inactive either as a preprotein with a trans-membrane domain or by a membrane-localized inhibiting factor. The trans-membrane activation of the regulatory protein is initiated and completed by the consecutive action of two membrane-localized proteases on this preprotein (or inhibiting factor). Upon an external signal the site-1 protease initiates the signaling cascade by processing of the preprotein (or inhibitor). This first proteolytic event makes its trans-membrane portion accessible to the site-2 protease (also named intramembrane cleaving protease (I-CLiP), resulting in cleavage of the intramembrane domain thereby causing release and activation of the cytosolic transcription factor [41–43]. In *B. subtilis* RIP is involved, not only in the envelope stress response, but also during sporulation in signal transduction between the mother cell and forespore [43–45] (see section "Membrane Stress and Regulated Intramembrane Proteolysis (RIP) of the Extracytoplasmic Function (ECF) Sigma Factors").

Regulatory Proteolysis

Competence Development

Competence is the ability of a subpopulation of stationary phase *B. subtilis* cells to take up DNA from the environment and incorporate it into their genome by homologous recombination. This process is controlled by the master regulator ComK, a transcriptional activator, which controls genes required for DNA binding, processing and transport into the cell. During logarithmic growth, ComK is inhibited [46, 47] and targeted by MecA for degradation by ClpCP [24]. This process results in a very low steady state concentration of ComK and ensures a very tight control of competence during exponential growth. At high cell density, however, a quorum sensing pathway activates the expression of the anti adaptor ComS, which binds to MecA with high affinity and thus competes with the binding of ComK to MecA [24, 48] (Fig. 4.1a). Subsequently, the released ComK activates its transcription from the *comK* promotor, leading to positive auto-regulation and bi-stable competence gene expression in the subpopulation of cells that reach a threshold level of ComK by stabilization due to the proteolytic ComS-anti adaptor switch (Fig. 4.1a) [49–51].

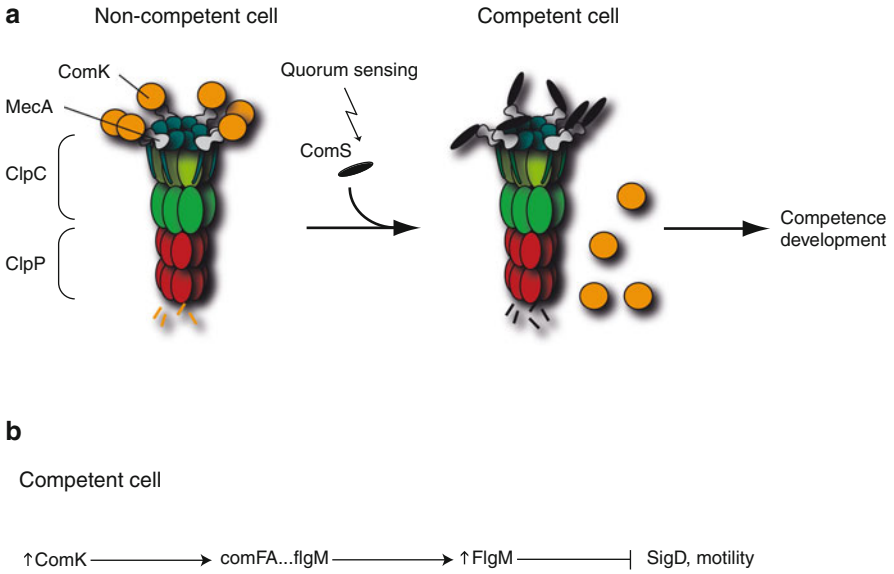

Fig. 4.1 (**a**) **Competence regulation by ClpCP/MecA**. In non-competent cells, ComK, the master regulator of competence development, is inhibited by MecA and targeted for degradation by ClpCP/MecA. This results in a low concentration of ComK. At high cell densities, a quorum sensing signal activates a cascade culminating in the expression of the peptide ComS. ComS is also a target of the ClpCP/MecA complex and competes with ComK for binding to MecA. Due to the high concentration of ComS, this peptide is preferentially degraded. In turn, ComK accumulates and activates the *comK* promotor. This positive autoregulatory loop results in high levels of ComK and competence development. (**b**) **Motility regulation by ClpCP/MecA**. The gene encoding FlgM (the flagellar anti-sigma factor) is located downstream of the competence genes *comFA*, separated by a weak terminator. Under conditions of high ComK levels, transcriptional read-through from *comFA* into *flgM* results in accumulation of FlgM and inhibition of σ^D-dependent motility gene expression

Swimming Motility

Swimming motility is a post-exponential process in *B. subtilis*, during which single flagellated cells swim through liquid medium by a stochastic run-and-tumble path that is guided by chemotaxis. In contrast, swarming motility describes a coordinated movement of tight bundles of cells over semi-solid surfaces, which requires flagellar rotation and the presence of surfactants [52]. Both processes require the expression of motility genes, which are organized in a hierarchy of two classes of genes: the first class of early flagellar genes is encoded in the 27 kb *fla/che* operon, which is transcribed by RNA polymerase (RNAP) in complex with the housekeeping sigma factor (σ^A). Examples of early flagellar genes are those encoding the ring complexes and the hook basal body complex. σ^D is an alternative sigma factor, which is encoded close to the 3′-end of the *fla/che* operon. The second class of late flagellar genes consist of separate transcriptional units, which all exhibit a σ^D-dependent promoter.

These genes include those encoding Hag, the structural subunit of the flagellum, and FliD, the cap of the flagellum, as well as their cognate export chaperones. The promoter binding activity of σ^D is inhibited by the anti-sigma factor FlgM [53, 54] possibly by direct protein-protein interaction as was demonstrated for FlgM and σ^{28} [55]. In *Salmonella*, FlgM is exported by the flagellar type III secretion system after completion of the hook basal body, releasing the flagellar sigma factor and enabling efficient production of flagellin [56, 57]. In *B. subtilis* it is not known, whether FlgM is exported or inactivated by a different mechanism.

Already shortly after the discovery of the *clp* genes in *B. subtilis*, a motility defect of the *clpP*, *clpX* and *clpC* mutants was described [58]. In the case of *clpC*, this phenotype was examined in detail, and was shown to be dependent on *comK*. A ComK-controlled competence gene *comFA* happens to be located directly in front of the operon containing *flgM*, separated by a weak terminator. Consequently, when high levels of ComK are present in the cell (i.e. in a *clpC* mutant or in competent cells), *comFA* is activated, resulting in read-through transcription of *flgM* (Fig. 4.1b). The excess production of FlgM anti-sigma factor inhibits σ^D and leads to a block of σ^D-dependent gene expression and motility (Fig. 4.1b) [59].

The biological implication of this mechanism may be to ensure mutually exclusive development of motility and competence. Competent cells are physiologically very different from exponentially growing cells and presumably it would be a waste of energy for them to express flagella. According to this model, the protease ClpCP together with the adaptor protein MecA would then promote motility gene expression during exponential phase by maintaining low ComK levels through proteolysis.

Interestingly, one study reported a *comK*-independent effect of *clpC* on swimming motility [60]. This suggests that other ClpCP substrates may also be involved in the regulation of motility. One candidate is the response regulator DegU, which is degraded by ClpCP/MecA [61] and represses the promotor of the *fla/che* operon in its phosphorylated form [62]. The specific recognition and degradation of only phosphorylated DegU [61] by ClpCP could be a very interesting regulatory mechanism and should be analysed in more detail.

However, the effect of the DegS-DegU two component system appears to be complex and is controversially discussed, because DegU has also been described as an activator of the *fla/che* promotor both in its phosphorylated and unphosphorylated forms [63–65]. The activating effect of DegU also seems to be stronger in strains, in which the flagellar activator SwrA is expressed. The presence of SwrA leads to hyper-flagellation required for swarming motility and SwrA is not expressed in laboratory strains due to a frameshift mutation in the *swrA* gene [65–67]. Whether the *comK*-independent effect of *clpC* on motility is due to *degU* awaits further characterization.

Recently, an interesting connection between FlgM and the ClpCP substrate DegU was discovered. Kearns and co-workers found that phosphorylated DegU (DegU ~ P) acts as a positive transcriptional regulator of *flgM* [68]. By degrading DegU ~ P, ClpCP would then facilitate the expression of the σ^D-regulon by yet another mechanism that acts on *flgM* transcription.

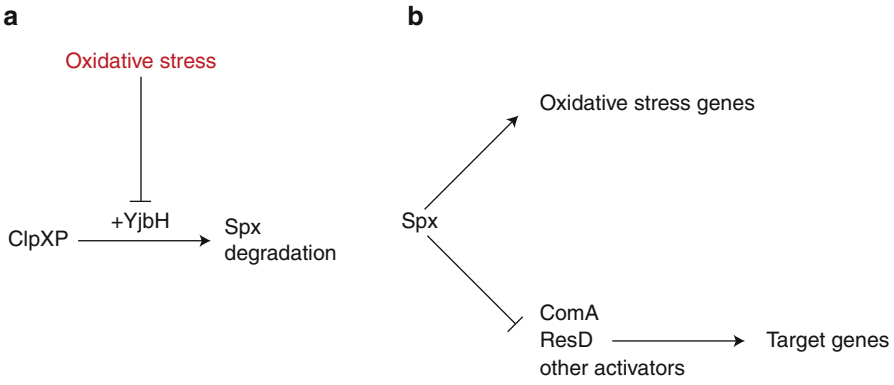

Fig. 4.2 Oxidative stress response mediated by Spx. (**a**) During normal growth, Spx is rapidly degraded by ClpXP, assisted by the adaptor protein YjbH. In response to oxidative stress, Spx is stabilized. (**b**) Spx acts as a transcriptional regulator, which positively regulates the expression of oxidative stress response genes and negatively regulates gene expression governed by transcriptional activators such as ComA and ResD

Stress Responses

Oxidative Stress Response and Spx

The oxidative stress response in *B. subtilis* and other Gram-positive bacteria is governed by the transcriptional regulator Spx. The *spx* gene (*yjbD*) was originally identified as a suppressor of *clpP* and *clpX*. Subsequently, Spx was shown to be a substrate of the ClpXP protease that is constantly degraded during normal growth (Fig. 4.2). Importantly, upon oxidative stress, introduced by diamide (an agent that introduces the formation of cytosolic disulfide bonds), paraquat or H_2O_2 treatment, the degradation of Spx is inhibited (Fig. 4.2). In *clpX* or *clpP* mutants, Spx accumulates to high levels under non-stress conditions, which has a detrimental effect on growth and leads to frequent suppressor mutations [28, 29]. Spx proteolysis is enhanced by the ClpX adaptor protein YjbH [30, 32], which itself might be regulated by oxidative stress [30]. Interestingly, it was proposed that ClpX can be inactivated by oxidation of a conserved zinc cluster, which is part of its N-terminal domain [69]. Taken together, the inactivation of ClpX and YjbH would result in rapid stabilization of Spx after redox stress. Recently, a novel anti-adaptor YirB was discovered [31]. The expression of this protein stabilizes Spx by inhibition of YjbH-mediated degradation of Spx by ClpXP, both *in vivo* and *in vitro* [31]. The expression of Spx is also transcriptionally induced during stress by the inactivation of the repressors PerR and YodB, which normally bind and repress one of the five known *spx* promoters [70, 71]. Furthermore, *spx* transcription is activated by heat shock and other stresses [72].

Spx acts as a negative regulator of a set of genes, including competence genes. According to the interference model, which has been directly demonstrated for the

response regulators ResD and ComA [73], Spx inhibits the transcriptional activation of genes by binding to the C-terminal domain (CTD) of the alpha subunit of the RNA polymerase (RNAP). Thereby the transcription of genes, which require transcriptional activators that bind to the CTD of the alpha subunit are inhibited (Fig. 4.2). The formation of this inhibitory Spx-alpha CTD complex has also been investigated at the molecular level by X-ray crystallography [74].

Interestingly, analysis of the Spx dependent transcriptome, suggested that Spx also positively influences the transcription of a large number of genes [75]. Recent experiments suggest that this positive influence could be achieved directly by Spx in complex with the CTD of the RNAP alpha subunit by enhancing the binding of RNAP to certain promoters [76, 77]. The genes within the Spx regulon encode enzymes with a role in processing or detoxification of reactive oxygen and nitrogen species, i.e. thioredoxin, thioredoxin reductase and superoxide dismutase (Fig. 4.2) [28, 29, 75].

Similar to the proteolytic switch involving ClpCP/MecA and ComK, ClpXP-mediated Spx proteolysis may serve as a very tight control mechanism of Spx under non-stress conditions, combined with a rapid response time following oxidative stress.

Heat Shock Response

In contrast to *E. coli*, where the heat shock response is controlled by the alternative sigma factor RpoH (σ^{32}), four different mechanisms of the heat shock response have been described for *B. subtilis* [78]. Class I heat shock genes, including the *dnaK* and *groEL/groES* operons, contain operator sites known as CIRCE-elements in their 5′-untranslated regions, which are bound and repressed by HrcA, which is activated by the chaperonin GroEL [79]. Promoters of class II heat shock genes are bound by the alternative sigma factor σ^{B}, and are induced not only by heat shock, but also by other kinds of stress [80]. The class III heat shock genes are controlled by the transcriptional repressor CtsR and include the *clpC* operon (containing *ctsR*, *mcsA*, *mcsB* and *clpC*) and the *clpE* and *clpP* genes [12–14, 81]. Heat shock genes, which are controlled by an unknown mechanism, have been grouped together in class IV. These genes include *htpG* (encoding Hsp90), *clpX* and the *lon* genes [78]. The class V heat shock genes are activated by extracellular protein folding stress as well as heat shock and are controlled by the two-component system CssS/CssR [82, 83].

CtsR

In this section, we will focus on the class III heat shock genes and the regulation of its repressor CtsR by proteolysis. CtsR is degraded by ClpCP, mediated by its adaptor protein McsB during heat stress, but not at normal growth temperatures [14, 21]. McsB is not only an adaptor protein, but also a protein kinase [84]. McsB was first characterized as a tyrosine kinase [84] and later shown to be the first protein arginine kinase [85]. McsB autophosphorylates arginine residues in the presence of McsA [86] and can phosphorylate substrate proteins, among them CtsR [85]. The kinase activity of McsB is inhibited by ClpC and counter-acted by the arginine

phosphatase YwlE [84, 87]. The general impact and physiological significance of arginine phosphorylation awaits further characterization. Importantly, as an adaptor protein McsB only binds and activates ClpC and targets CtsR for degradation in the presence of McsA when it is activated and autophosphorylated [21, 88]. Interestingly, phosphorylated McsB displays a high affinity for ClpC *in vitro* and thus can compete for binding to ClpC with the other adaptor protein MecA [21]. In addition, phosphorylated McsB can interfere with the DNA binding of CtsR [84, 85], leading to de-repression of CtsR-controlled genes. Based on these results a titration model for the activation of the class III heat shock genes was suggested, in which heat is sensed by an increase of unfolded proteins targeted by MecA to ClpCP [21, 84]. However, recently it was demonstrated that CtsR directly senses a temperature shift, resulting in its inactivation and subsequent release from DNA [88]. Neither MscB arginine phosphorylation nor CtsR degradation were required for this process, suggesting that proteolysis serves primarily to remove inactive CtsR molecules from the cell. This finding is also corroborated by the observation that CtsR controls heat shock genes in organisms that lack a McsB homologue. The current model for the activation of class III heat shock genes is as follows: CtsR changes its conformation at high temperatures, causing it to dissociate from its target class III heat shock genes resulting in their transcription. At the same time, ClpC is removed from the inhibitory McsA-McsB-ClpC complex, possibly involving MecA targeting unfolded proteins to ClpC, which results in the activation of the MscB arginine kinase activity. McsB phosphorylates itself and CtsR in the presence of McsA. Phosphorylated, heat-inactivated CtsR cannot rebind its operator sites and is also targeted for degradation by the proteolytic McsA-McsB-ClpCP complex, thus stabilizing the class III heat shock "ON" state (Fig. 4.3a).

Effect of Oxidative Stress on McsA

The CtsR regulon is not only de-repressed by heat shock, but also by oxidative stress [89, 90]. Recently, the mechanism of CtsR inactivation was shown to be very different from heat inactivation [87]. First of all, the activation of the class III heat shock genes by oxidizing agents is markedly slower than activation by heat stress, suggesting an indirect mechanism. Furthermore, McsB, but not its kinase activity, is required for this process in contrast to heat activation, which depends only on CtsR [88] and CtsR is not degraded during oxidative stress. McsA, the co-adaptor and modulator of CtsR, contains two clusters of conserved cysteine residues, which could constitute sensor domains to oxidative stress. Indeed, both clusters (one of which contains a zinc ion in its reduced state) were oxidized by disulfide stress *in vivo* [87]. Furthermore, McsA is also irreversibly modified by oxidative stress, resulting in a faster migrating form on SDS-PAGE gels. On the basis of N-terminal and C-terminal tagging experiments, this result was interpreted as proteolytic cleavage of McsA. Interestingly, the presence of the smaller band depended on the presence of ClpC and ClpP, suggesting a direct or indirect involvement of ClpCP in the putative cleavage reaction. Importantly, modification of McsA by oxidative stress led to dissociation of the McsA-McsB complex. McsB is able to release CtsR from DNA also in the absence

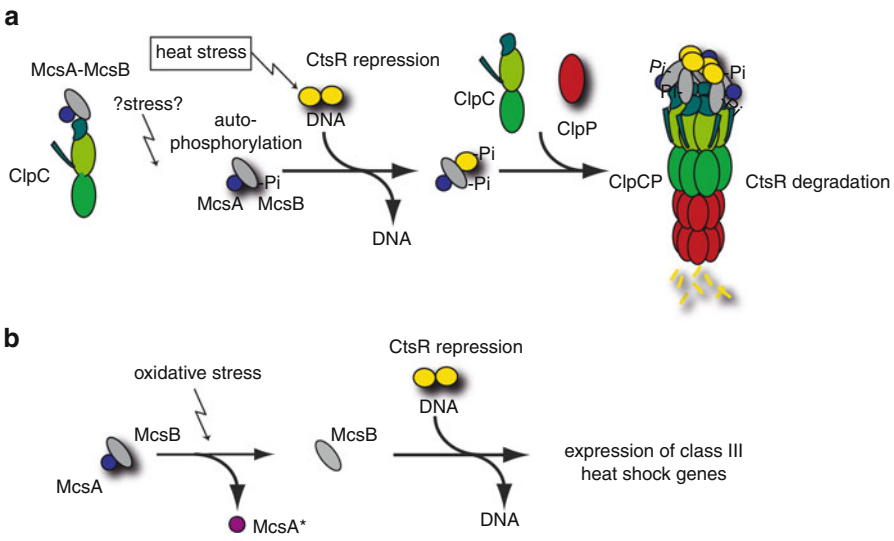

Fig. 4.3 Regulation of class III heat shock genes. (**a**) **CtsR regulation by heat**. In non-stressed cells, CtsR represses transcription of class III heat shock genes by binding to CIRCE operator sites. The kinase activity of MscB is inhibited by ClpC. Heat stress is directly sensed by CtsR, which is released from the DNA. This leads to derepression of class III heat shock genes. At the same time, the interaction of ClpC with McsB is disrupted by stress (possibly by unfolded proteins targeted for degradation by ClpCP/MecA) and McsB auto-phosphorylates in the presence of McsA. Phosphorylated McsB further contributes to release of CtsR from DNA and targets CtsR for degradation by ClpCP. (**b**) **CtsR regulation by oxidative stress**. McsA is oxidized and presumably processed by redox stress and dissociates from McsB. Monomeric McsB, which is not phosphorylated under these conditions, releases CtsR from DNA. Class III heat shock genes are derepressed

of McsA [84] and McsA acts as an inhibitor of McsB with regards to the ability to remove CtsR from DNA [14]. Thus, the results presented by Gerth and colleagues led to the following model [87]. In response to oxidative stress, McsA is proteolytically cleaved following modification of its cysteine residues, resulting in its dissociation from McsB (Fig. 4.3b). In turn, McsB is able to bind to CtsR and remove the protein from its operator DNA, causing derepression of the CtsR regulon [87] (Fig. 4.3b). Consequently, the heat shock "sensing" repressor, CtsR is also involved in responding to oxidative stress, via the McsA-McsB system.

Membrane Stress and Regulated Intramembrane Proteolysis (RIP) of the Extracytoplasmic Function (ECF) Sigma Factors

Regulated intramembrane proteolysis (RIP) is used by both prokaryotes and eukaryotes for signaling across cell membranes. RIP has been studied in great detail in *E. coli* using the activation of the extracytoplasmic function (ECF) sigma factor σ^E as a paradigm. ECF sigma factors constitute a special group of sigma factors (Group

IV of the σ^{70} subfamily) that are involved in the signaling of extracytoplasmic conditions and are characterized by a two-domain architecture [44]. There are seven ECF sigma factors in *B. subtilis* (σ^M, σ^V, σ^W, σ^X, σ^Y, σ^Z, and YlaC), whereas *Streptomyces coelicolor* contains about 50. The best characterized ECF sigma factor in *B. subtilis* is σ^W. This sigma factor is activated by alkaline stress, phage infection, salt stress, antimicrobial peptides and antibiotics such as vancomycin resulting in the expression of genes involved in the response to envelope stress and antibiotics [44]. In analogy to the *E. coli* σ^E/RseA system, *sigW*, the gene encoding σ^W is cotranscribed with *rsiW*, encoding a membrane bound, σ^W anti-sigma factor [91] (see also [92]). The cytoplasmic N-terminal domain of RsiW binds to, and inhibits the activity of, σ^W [91] (Fig. 4.4a). However, in contrast to the cascade in *E. coli*, site-1 cleavage is not executed by any of the DegS homologs HtrA, HtrB or HtrC [93]. Instead, PrsW (YpdC) is the site-1 protease of RsiW [94, 95] (Fig. 4.4a). PrsW is homologous to the eukaryotic type II CAAX prenyl endopeptidase family [94] and removes 40 amino acids from the extracellular domain of RsiW, cleaving between alanine 168 and serine 169 [96]. Additional processing by an unknown protease is required to facilitate cleavage by the site-2 protease and in a reconstituted *E. coli* system the tail-specific protease (Tsp) is able to fulfill this task [96] (Fig. 4.4a). The site-2 protease of RsiW was identified as RasP (YluC) [91] a zinc metalloprotease with an extracytoplasmic PDZ-domain and four transmembrane helices (Fig. 4.4a). This protease belongs to the site-2 protease (S2P) family of I-CLiPs [96] and processes RsiW after site-1 cleavage close to two highly conserved alanine residues [97]. This process requires the presence of the ABC transporter EcsAB, but the mechanism of this regulation remains unknown [98]. After site-2 cleavage and release from the cytoplasmic face of the membrane, the RsiW fragment remains bound to σ^W in the cytosol, until it is degraded by ClpXP [97] (Fig. 4.4a). The degradation of this fragment, by ClpXP or ClpEP but not cleavage by site-2 proteolysis, requires a stretch of three alanine residues at the new C-terminus of RsiW [97].

Two publications suggest that the substrate specificity of RasP is much broader than previously anticipated. Surprisingly, the cell-division protein FtsL is cleaved by RasP. Importantly, this cleavage is required for normal cell division, demonstrated by a short cell phenotype of a strain, in which FtsL could not be processed [99]. Furthermore, the cell division protein DivIC was shown to protect FtsL from proteolysis [100].

Recently, Akiyama and colleagues have demonstrated that RasP can also act as a signal peptide peptidase (SPP) that cleaves signal peptides (SP) in the membrane, after they have been removed from preproteins by the action of signal peptidase [101]. Likewise, the *E. coli* I-CLiP protease RseP, was also shown to act as the site-2 protease of pro-σ^E. Consistent with these findings, deletion of *rseP* inhibited SP cleavage *in vivo* while in contrast disruption of *sppA* (previously proposed to be the *E. coli* SPP) had no effect on SP cleavage [101].

It is fascinating to see how a protease, which was originally thought to have only one or two regulatory substrates, is suddenly found to be involved in such a broad fundamental process as signal peptide cleavage during protein secretion. These

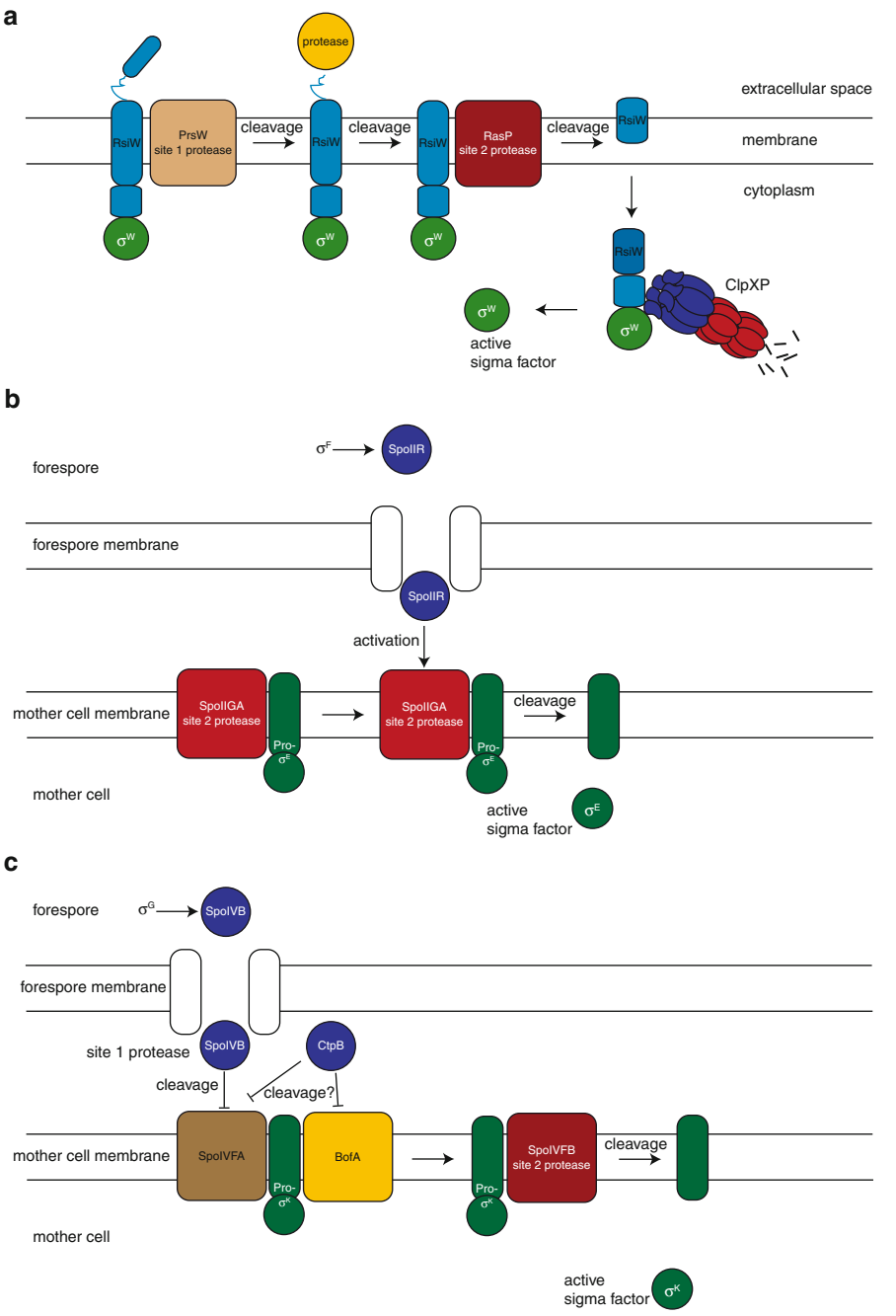

Fig. 4.4 Regulated intramembrane proteolysis (RIP) in *B. subtilis*. (**a**) RIP in the extracytoplasmic stress response. The extracytoplasmic stress response is controlled by the alternative sigma factor σ^W. In non-stressed cells, σ^W is bound and inhibited by the cytoplasmic domain of the membrane-embedded anti-sigma factor RsiW. Upon exposure to stress, the extracytoplasmic domain of

experiments suggest that many membrane localized proteases including FtsH and HtrA (DegP) may be involved in both regulation and PQC, just like the cytosolic protease systems.

Sporulation

Sporulation is a developmental pathway that is initiated in stationary phase under nutrient-limiting conditions and by which cells form durable and highly resistant endospores. These spores are able to preserve their genetic information during adverse environmental conditions and can later germinate, when nutrients are more abundant. During the early stage of sporulation, a *B. subtilis* cell divides asymmetrically into a smaller forespore and a larger mother cell. Subsequently, the forespore is engulfed by the mother cell through membrane fusion and eventually the mother cell lyses, releasing the developed spore. The importance of proteolysis in the sporulation of *B. subtilis* cells, is supported by the observation that FtsH, ClpP, ClpC and ClpX are all required for sporulation [27, 40, 58, 102, 103].

The early steps of sporulation (i.e. the formation of the asymmetric septum) are regulated by phosphorylation of the response regulator Spo0A, which is controlled by a phosphorelay system involving five different histidine kinases and several phosphatases. A self-reinforcing cycle involving Spo0A, the alternative sigma factor σ^H and the repressor AbrB, contributes to the production of high levels of phosphorylated Spo0A and entry into the sporulation pathway. Briefly, in its phosphorylated form, Spo0A represses the *abrB* gene, leading to derepression of the *spo0H* gene, encoding the alternative sigma factor σ^H, which directs transcription of *spo0A*. This leads to higher levels of Spo0A and activation of a positive autoregulatory loop triggering the differentiation into a spore-forming cell. Spo0A and σ^H activate several down-stream genes, i.e. the *spoIIA* locus, encoding σ^F (see below) and initiates the formation of the asymmetric division septum. After the

RsiW is processed by the site-1 protease PrsW and by a second unknown protease. This cleavage makes the transmembrane domain of RsiW susceptible to cleavage by the site-2 protease RasP. Cleavage by RasP releases σ^W from the membrane. The RsiW fragment still bound to σ^W is degraded by ClpXP in the cytoplasm. Subsequently, σ^W binds to RNAP and activates the transcription of its target genes. (**b**) RIP in σ^E regulation. σ^E is produced in the cytoplasm of the mother cell during sporulation as a proprotein (pro-σ^E) with a transmembrane domain and inserted into the mother cell membrane. SpoIIR is expressed in the forespore under the control of σ^F and secreted into the intermembrane space between forespore and mother cell. SpoIIR activates the site-2 protease SpoIIGA. In turn, SpoIIGA cleaves pro-σ^E, resulting in the release of σ^E from the membrane, which then mediates the expression of early mother cell specific genes. (**c**) RIP in σ^K regulation. Pro-σ^K is produced in the mother cell cytoplasm and inserted into the membrane, where it forms a complex with BofA and SpoIVFA, which protect pro-σ^K from proteolysis by the site-2 protease SpoIVFB. The late forespore-specific sigma factor σ^G directs expression of the site-1 protease SpoIVB, which is secreted into the intermembrane space and proteolytically processes SpoIFA, resulting in the destabilization of the pro-σ^K-BofA-SpoIVFA complex. In turn, SpoIVFB cleaves pro-σ^K and σ^K is released from the membrane to activate the expression of late mother cell specific genes

formation of the division septum, four compartment-specific alternative sigma factors (responsible for the expression of different classes of sporulation genes) are activated in a controlled temporal and spatial order. First, σ^F is activated in the forespore, next σ^E is activated in the mother cell. Following spore engulfment, σ^G replaces σ^F in the spore and finally, σ^K directs gene expression in the late mother cell [45, 104].

FtsH

Interestingly, one of the Spo0A phosphatases, Spo0E, was identified as an FtsH substrate [105], explaining the earlier observation that Spo0A activity is reduced in *ftsH* mutant cells [40]. FtsH localization to the asymmetric division septum is also consistent with its role in sporulation [106]. Furthermore, the FtsH protease is regulated by the small peptide SpoVM, which is essential for sporulation. SpoVM, not only binds to and inhibits FtsH but also serves as a substrate of the protease [107, 108].

LonA and LonB

The LonA protease was first identified in a screen for factors that down-regulate σ^G activity. Although the mechanism of σ^G inhibition by LonA was not investigated in detail, it was suggested that LonA is directly responsible for the degradation of σ^G [109]. LonA and LonB were also reported to down-regulate σ^H levels posttranslationally at low pH during sporulation, although this had no effect on σ^H directed gene expression [103]. These data are consistent with the model that σ^H is targeted to LonA and LonB at low pH, and that LonA and LonB also influence a downstream process, which is required for σ^H–mediated gene expression. LonB seems to have a special role in sporulation, since the *lonB* gene features a σ^F–dependent promotor and is specifically expressed under sporulation conditions. Neither a *lonB* mutant nor a strain over-expressing LonB affected sporulation, but interestingly expressing *lonA* from the sporulation dependent *lonB*-promoter lead to a sporulation defect and the down-regulation of σ^G–dependent gene expression [110]. LonA localizes to the nucleoid of cells during normal growth and to the forespore compartment during sporulation. However, LonB localized to the forespore membrane early in sporulation and to the whole forespore during later stages of the process [111].

ClpCP

In the pre-divisional cell, σ^F is already transcribed (activated by Spo0A), but is kept inactive by its anti-sigma factor SpoIIAB. SpoIIAB is antagonized by its anti-anti-sigma factor SpoIIAA. According to the partner-switch model, SpoIIAB

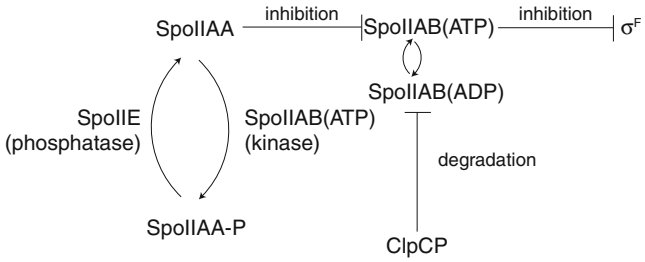

Fig. 4.5 Role of ClpC in σ^F regulation. The anti-sigma factor of σ^F, SpoIIAB can exist in an ATP-bound and an ADP-bound form. In the ATP-bound form, SpoIIAB binds to and inhibits σ^F. In the ADP-bound form, SpoIIAB is bound and inhibited by the anti-anti sigma factor SpoIIAA. SpoIIAB also acts as a kinase, which phosphorylates and inactivates SpoIIAA. SpoIIAA is dephosphorylated by the phosphatase SpoIIE. ClpCP degrades SpoIIAB, thus stabilizing the free active form of σ^F

can exist either in a complex with σ^F in its ATP-bound form, with SpoIIAA in the ADP-bound form or as a monomer bound to ADP. In the ATP-SpoIIAB-σ^F complex, SpoIIAB can act as a protein serine kinase that phosphorylates and inactivates SpoIIAA. The kinase activity of SpoIIAB is counteracted by the phosphatase SpoIIE. In the forespore after formation of the division septum, SpoIIE is able to dephosphorylate SpoIIAA, causing the formation of the inhibitory SpoIIAA-SpoIIAB-ADP complex and the release of σ^F, which can then interact with RNAP and activate its cognate promoters [45, 104] (Fig. 4.5). Interestingly, SpoIIAB is degraded by ClpCP but only in its monomeric form [27] (Fig. 4.5). This degradation is assumed to stabilize the free form of σ^F by shifting the equilibrium between the complexes described above. Targeting of SpoIIAB to ClpCP depends on a tripeptide motif (LCN) located at the C-terminus of SpoIIAB [27, 112]. Importantly, the proteolytic tag (LCN) on SpoIIAB is required for both normal σ^F-dependent gene expression and sporulation [27]. Given the degradation of SpoIIAB cannot be reconstituted *in vitro* it is plausible that an unknown adaptor protein or targeting mechanism may be responsible for the recognition of the LCN tag and targeting of SpoIIAB to ClpCP for degradation. However, SpoIIAB is also unstable *in vivo* when overexpressed during exponential growth, suggesting that the putative adaptor protein is unlikely to be expressed only during sporulation.

Interestingly the genes for SpoIIE, ClpC and ClpP are located close to the origin of replication of the *B. subtilis* chromosome and therefore are expressed together early in forespore development supporting the proteolytic part of the molecular mechanism resulting in forespore specific activation of σ^F [113]. This model is also supported by the localization of ClpC during sporulation, as determined from GFP-fusion experiments [114]. While ClpX-GFP and ClpP-GFP form foci close to the polar septum of the mother cell, ClpC-GFP exhibits a dynamic localization. The polar foci that are normally observed for ClpC-GFP during growth, delocalize early during sporulation. During engulfment of the

prespore, ClpC-GFP forms a helical pattern in the mother cell with a number of ClpC foci co-localizing with the engulfing membrane. Later, ClpC forms foci at the distal prespore membrane, which subsequently delocalize. Using GFP fusions to truncated ClpC and ClpX, a quantitative measure of ClpC and ClpX in the forespore and mother cell was achieved, which displayed a diffuse localization pattern avoiding the complication of foci formation. Interestingly, the truncated GFP-ClpC protein localized preferentially to the forespore, whereas GFP-ClpX localized to the mother cell. This localization could be explained by the presence of a putative σ^F–dependent promoter in the *clpC* operon between *ctsR* and *mcsA*. Finally, the GFP-LCN fusion protein is degraded exclusively in the forespore, while the GFP-AAV fusion protein, which is targeted to ClpXP, is only degraded in the mother cell. This distribution of the various components could contribute to preferential degradation of SpoIIAB in the forespore and to σ^F-activity. The biological implication of the preferred location of ClpX and the complex dynamic distribution of ClpC during sporulation remains to be elucidated [114].

ClpXP

ClpX mutants have a sporulation defect and are compromised in σ^H-dependent gene expression [103, 115]. Later, it was reported that ClpX influences RNAP holoenzyme composition, triggering preferred incorporation of σ^H into RNAP during stationary phase [115]. The sporulation defect of the *clpP* and *clpX* mutants is also partially suppressed by mutating *spx* [116]. Spx protein accumulates in *clpP* and *clpX* mutant cells. By binding to the RNAP alpha CTD, Spx interferes with the binding of other activators, such as ComA to RNAP [73]. The ComP-ComA two-component system is required for the expression of the *srf* operon [117], which is required for sporulation [73] and for production of the RapC and RapF phosphatases involved in dephosphorylation of Spo0A. Taken together, these data suggest that *clpX* positively regulates sporulation by at least two different mechanisms, one of which is dependent on *spx*. These mechanisms are still not very well understood and await further investigation.

RIP in Sporulation

Communication between the forespore and mother cell compartments during two different stages of sporulation is mediated by regulated intramembrane proteolysis. Both the early mother cell-specific sigma factor σ^E and the late mother cell specific sigma factor σ^K are synthesized as preproteins with an N-terminal transmembrane domain that is inserted into the membrane adjacent to the forespore [118–120]. In both cases, a signal to activate the sigma factors is initiated by the respective early or late forespore specific sigma factors, ensuring a strict temporal order of gene expression by communication across compartments [45, 104].

SigmaE (σ^E) SigmaE is synthesized as an inactive precursor protein with an N-terminal transmembrane domain [118]. Interestingly, the proprotein (pro-σ^E) is produced in both the mother cell and the forespore membrane, but is selectively removed from the forespore membrane [121, 122]. Conversion of pro-σ^E to the active form only occurs after formation of the polar septum [123]. Intramembrane proteolytic cleavage of the prosequence requires the membrane protein SpoIIGA [124–126] (Fig. 4.4b). Only relatively recently, it was biochemically demonstrated that SpoIIGA is a novel type of aspartic protease that cleaves pro-σ^E [127, 128]. The activation of SpoIIGA requires SpoIIR, which is produced in the forespore under σ^F control [129–131]. SpoIIR was shown to interact with SpoIIGA, but the mechanism by which SpoIIR activates SpoIIGA is still not understood. Processing of SpoIIGA by SpoIIR or another site-1-protease has not been observed. Instead Kroos and colleagues have proposed the formation of inactive oligomers by SpoIIGA, which are dissociated by binding of SpoIIR [128]. In summary, SpoIIR is synthesized in the forespore under the control of σ^F and activates the membrane localized SpoIIGA, which in turn proteolytically activates the membrane associated pro-σ^E to form the transcription factor σ^E.

SigmaK σ^K is proteolytically processed by the intramembrane zinc metalloprotease SpoIVFB, which resembles eukaryotic site-2 proteases [132, 133]. SpoIVFB forms a complex with the proteins SpoIVFA and BofA [120, 134] (Fig. 4.4c). BofA inhibits the proteolytic activity of SpoIVFB toward pro-σ^K and SpoIVFA indirectly inhibits SpoIVFB by recruiting BofA to the complex [134]. Activated by σ^G, the serine protease SpoIVB is produced in the forespore and presumably secreted into the intermembrane space between forespore and mother cell, where it cleaves SpoIVFA [135, 136] (Fig. 4.4c).

According to one model [136], based on the heterologous expression of the signaling complex in *E. coli,* SpoIVB induced cleavage of SpoIVFA destabilizes the heterotrimeric complex, which leads to BofA degradation by another secreted serine protease, CtpB [137] and activation of SpoIVFB. Based on *in vitro* experiments in detergent micelles and membrane vesicles, Campo and Rudner suggest another model, in which the SpoIVFB-SpoIVFA-BofA complex remains intact even after cleavage of SpoIVFA by SpoIVB and argue that this cleavage leads to a conformational change promoting activation of SpoIVFB [138]. Furthermore, these authors demonstrated that CtpB processes SpoIVFA, but not BofA. CtpB is also cleaved by SpoIVB, but this processing appeared to have no effect on the activation of CtpB *in vivo* [139]. Contrary to initial assumptions, CtpB is mainly produced in the forespore under the control of σ^G, just like SpoIVB [139]. In summary, both models propose that SpoIVB and CtpB jointly contribute to the proteolytic processing and activation of the SpoIVFA, which leads to the activation of the site-2 protease SpoIVFB and the processing of pro-σ^K into its active form. Subsequently, σ^K is released from the membrane to recruit RNAP to its cognate promoters (Fig. 4.4c).

This is the second example where a developmental signal from the forespore is conveyed via different membrane localized proteases over two membranes, resulting in the transmembrane proteolytical activation of an alternative sigma factor.

Biofilm Formation

Biofilms are complex communities of bacteria growing on solid surfaces and air liquid interfaces held together by an extracellular matrix. The bacteria in this multicellular heterogenous biofilm population undertake a drastic lifestyle change from the better studied often motile and planktonic state, to this special sessile and stationary-phase developmental state [140, 141].

Laboratory strains of *B. subtilis* are unable to form robust biofilms due to mutations acquired during cultivation under laboratory conditions. However, biofilm formation has been investigated in this model organism, using "wild" *B. subtilis* isolates [142, 143]. It could be demonstrated that *B. subtilis* biofilms consist of long chains of individual cells that are held together laterally by an extracellular matrix composed of exopolysaccharides (EPS) and fibers of the amyloid-like protein TasA [144, 145]. The regulation of biofilm development is intricately connected with several other developmental processes e.g. sporulation, motility and competence development [146–149].

The operons encoding the EPS (*epsA-O*) and TasA (*tapA-sipW-tasA*) are controlled by the transcriptional repressor SinR, which is antagonized by the anti-repressor SinI. SinI in turn is activated by Spo0A, a global regulator of sporulation and other stationary phase processes, leading to expression of EPS and TasA and the formation of biofilm [149, 150]. By investigation of cell chaining during biofilm formation it could be demonstrated that SinR is involved in a bistable regulatory feedback loop with another transcriptional regulator, SlrR [151, 152] (Fig. 4.6). In this double negative feedback loop, SinR represses the *slrR* gene and SlrR protein forms a heterodimeric complex with SinR that switches the DNA binding specificity of SinR. Whereas SinR on its own represses *slrR*, *epsA-0* and *tapA-sipW-tasA*, these genes are derepressed in the presence of the SinR-SlrR complex. Interestingly, the SinR-SlrR dimer represses a completely different set of genes, among them the ORFs encoding autolysins (enzymes, which degrade peptidoglycan during cell separation) and flagellin [152]. This results in the production of more SlrR, titration of more SinR and progression into a biofilm-promoting SlrR "HIGH" state, in which biofilm genes are expressed while motility and cell separation genes are repressed (Fig. 4.6). During exponential growth, this pathway is activated stochastically, resulting in a small fraction of cells forming chains. However, when the culture is committed to biofilm formation, the entire population is forced into the SlrR "HIGH" state by the expression of SinI through Spo0A [152]. Biofilms are not infinitely stable, but exhibit a tendency to disassemble after several days of growth [153], which is accompanied by a decrease in cellular SlrR levels [154]. This observation prompted an investigation into how the switch from the SlrR "HIGH" state back to the SlrR "LOW" state is achieved [154]. Interestingly, SlrR bears a LexA-like autocleavage sequence in the linker between its N- and C-terminal domains. Notably, cleavage into a smaller SlrR species was observed *in vivo*. SlrR was also found to be unstable *in vivo* and this degradation depended on the presence of the autocleavage region. Furthermore, mutation of *clpC* partially stabilized SlrR in a late biofilm-forming culture and a *clpC* mutant exhibited severe cell chaining

during exponential growth, which was suppressed by an *slrR* mutant. These data imply that SlrR is removed by proteolysis during the late stages of biofilm formation to return the cell to the SlrR "LOW" state [154].

General Proteolysis

Degradation of Unfolded and Aggregated Proteins

B. subtilis must adapt to extreme and quickly changing temperatures in its natural environment. Consequently, this organism is very well suited to grow at temperatures well over 50 °C [155]. Growth at such high temperatures requires an intricate protein quality control (PQC) system to cope with an increased amount of unfolded and misfolded proteins, which tend to form toxic aggregates. In all organisms, PQC systems consist of chaperones and proteases [5, 156]. Chaperones such as DnaK and GroEL either bind to unfolded substrate proteins to prevent their aggregation, actively refold the substrates or provide a sequestered environment for protein folding [157]. In cooperation with the chaperones, proteases recognize unfolded, misfolded and aggregated proteins to degrade them in a controlled manner [6]. Finally, unfoldases, such as *E. coli* ClpB or the yeast homolog Hsp104, together with their cognate Hsp70 chaperone systems, actively solubilize and refold protein aggregates [158–160]. ClpB is a Clp/Hsp100 ATPase, which lacks the ability to form a complex with ClpP and acts as a protein disaggregation enzyme together with DnaK in conjunction with small heat shock proteins in *E. coli* [161–163].

Although many of the chaperone systems are shared between *B. subtilis* and *E. coli* (DnaK/DnaJ/GrpE, GroEL/GroES, trigger factor, HtpG), *B. subtilis* lacks homologues of the proteins involved in protein disaggregation in *E. coli* (i.e. ClpB or the small heat shock proteins IbpA and IbpB). This raised the question, whether aggregates can be dissolved and refolded by other chaperones in *B. subtilis* or whether aggregates are irreversibly removed by proteases. The relatively severe heat shock phenotypes of *clpC*, *clpX* and *clpP* mutants in contrast to the comparably mild effect of a *dnaK* mutant in *B. subtilis* seems to suggest that general proteolysis is very important for PQC in this organism. However, the direct contribution of ClpC and ClpP to PQC is difficult to assess, because these proteins are also involved in regulation of the stress response. Likewise, in *E. coli* DnaK plays an important function in the heat shock regulation of σ^{32} [164–169], which could partly explain the severe heat shock phenotype of the *E. coli dnaK* mutant. In *B. subtilis*, GroEL/GroES regulates the class I heat shock repressor HrcA [79], but DnaK is not known to be involved in heat shock regulation.

However, there is substantial evidence that Clp proteases are directly involved in the degradation of unfolded or aggregated proteins [17]. ClpC, ClpE, ClpX, and ClpP localize to inclusion bodies formed by expression of puromycyl peptides or the aggregating protein PorA [16, 19, 170, 171]. Also, the turnover of puromycyl peptides decreased in all four mutant strains [16, 19]. Finally, ClpC in the presence of the adaptor protein MecA was shown to degrade aggregated model proteins

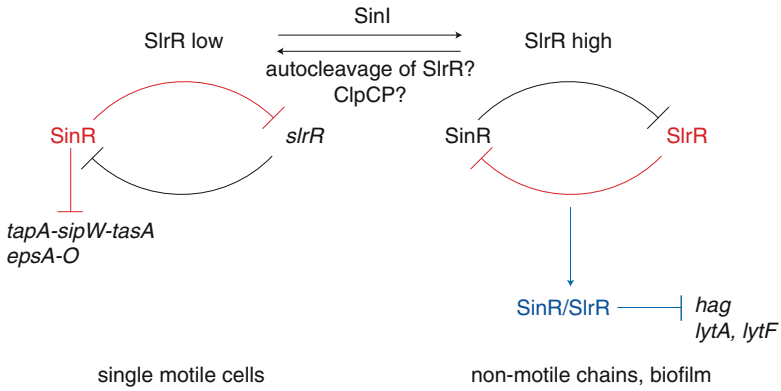

Fig. 4.6 The SlrR biofilm/motility switch. During exponential growth, SinR represses the biofilm operons *tapA-sipW-tasA* and *epsA-O* in most cells and motility genes and autolysins are expressed, resulting in single motile cells. The *slrR* gene is also repressed by SinR, which leads to a "low" steady state level of SlrR. The anti-repressor SinI is expressed in response to Spo0A activation in biofilm-forming cells or stochastically in exponential phase in a small number of cells. SinI inhibits SinR, which causes derepression of the *slrR* gene. SlrR forms a heterodimer with SinR. This heterodimer acts as a transcriptional repressor with different target specificity compared to SinR alone. The autolysin genes and *hag* encoding flagellin are directly repressed by SinR/SlrR, while the biofilm operons *tapA-sipW-tasA* and *epsA-O* and *slrR* are de-repressed. This double negative feedback loop results in a state, in which a high level of SlrR is expressed and cells are present as non-motile chains expressing biofilm genes. To return from the SlrR high state to the SlrR low state, LexA-like auto-cleavage of SlrR and ClpC are required, but the mechanism of this process is currently unknown

in vitro. Interestingly, the ClpC-MecA complex also displayed disaggregation activity in the absence of ClpP *in vitro* similar to *E. coli* ClpB [20]. This result suggests that ClpC could substitute for the ClpB-mediated disaggregation activity *in vivo*, although the ClpC-mediated disaggregation was slower than the ClpCP-mediated degradation *in vitro*. Whether ClpC function is always associated with ClpP *in vivo* remains to be elucidated.

The ability to survive an otherwise lethal heat shock after preconditioning at a sublethal temperature (a process known as thermotolerance) is directly connected to the presence of ClpB (in *E. coli*), or Hsp104 (in yeast) [158–163]. Interestingly, although *B. subtilis* lack homologs of both ClpB and the small heat shock proteins, *B. subtilis* cells exhibit thermotolerance. Future research will be aimed at elucidating the involvement and interplay of chaperones and proteases in PQC, stress response and thermotolerance.

Trans-translation and SsrA-Tagging in B. subtilis

Trans-translation is a process by which bacteria rescue stalled ribosomes. Ribosome stalling can occur for a number of reasons, i.e. as a consequence of damaged mRNAs lacking a stop codon, during the translation of proteins containing

numerous rare codons or as a regulatory process. This translational block is relieved by an unusual small RNA, *ssrA* (also known as tmRNA), which structurally resembles both a tRNA and a mRNA [172]. The *ssrA* RNA binds to stalled ribosomes with the help of the protein SmpB and triggers translation to resume. Alanine, which is conjugated to the tRNA acceptor stem of *ssrA* is first incorporated into the polypeptide chain. Subsequently, the ribosome deciphers the sequence encoded in the mRNA-like domain of *ssrA* and adds a short peptide tag of 15 amino acids to the C-terminus of the polypeptide. This peptide sequence, known as the SsrA-tag, is recognized by proteases and targets the tagged protein for rapid degradation [173]. Proteolysis of SsrA-tagged proteins is believed to be important for survival of the bacterial cell, because truncated proteins arising from translational stalling are often misfolded and tend to aggregate [172]. In contrast to *E. coli*, where different proteases can degrade SsrA-tagged proteins, ClpXP is the only protease identified with this activity in *B. subtilis* [33]. Although LonA, LonB, and ClpYQ have yet to be tested [33], ClpCP, ClpEP and FtsH all lack the ability to degrade SsrA-tagged proteins. Although the sequence of the *B. subtilis* SsrA tag (AGKTNSFNQNVALAA) and the *E. coli* SsrA tag (ANDENYALAA) are quite divergent, the four C-terminal residues are identical. These C-terminal alanine residues are critical for degradation by ClpXP while the upstream sequence of the *E. coli* SsrA tag is required for ClpA and SspB binding [33] and consistently, *B. subtilis* lacks the adaptor protein SspB, which enhances the targeting of SsrA-tagged proteins to ClpXP in *E. coli* (see also [174]). In *B. subtilis* the abundance of SsrA-tagged proteins increased at 50 °C and the nature of the tagged proteins varied considerably between 37 and 50 °C [175]. Only eight SsrA-tagged substrates have been identified to date [175]. At 37 °C, three tagged proteins were identified, two of unknown function, YqaP and YtoQ, along with TreP (trehalose permease, a component of the phosphotransfer system). At 50 °C, five tagged proteins were identified, YloN (unknown function), PerR (repressor of oxidative stress genes), TufA (elongation factor Tu), FolA (dehydroneopterin aldolase) and GsiB (a σ^B-dependent general stress protein of unknown function) [175].

The *B. subtilis ssrA* gene is dispensable under non-stress conditions, but becomes essential for growth at high temperature, high concentration of ethanol or cadmium chloride [176], and low temperature [177]. The *ssrA* gene is induced by heat by an unknown mechanism [176] and resides in an operon with *secG* (component of the SecYEG translocon channel), *yvaK* (carboxyl esterase), *rnr* (RNase R) and *smpB*, which is subject to complex regulation and features five promotors, including a weak σ^B-dependent and a heat-inducible σ^A-dependent promoter [177]. These data indicate that trans-translation and/or SsrA-tagging is important during stress, when translational stalling may occur more often or may be more damaging to the cell. One result reported by Muto and colleagues, suggests that trans-translation and not the degradation of the SsrA-tagged proteins is important for growth at high temperature [176]. In this experiment, the two C-terminal alanine residues of the SsrA tag were replaced by aspartic acid residues and the strain ($ssrA^{DD}$) was grown at high temperature. In contrast to cells, in which *ssrA* was completely removed or the alanylation site was mutated, the $ssrA^{DD}$ strain grew as well as the wild type at high temperature [176]. These data suggest that potentially

aggregated SsrA-tagged substrates can be dealt with by other chaperones and proteases, which are highly abundant during heat stress, and that the principle task of the trans-translation system is the reconstitution of functional ribosomes.

Localization of Clp Proteases During Regulatory and General Proteolysis

Recently, the cellular localization of Clp proteases fused to GFP was investigated by three independent studies [111, 114, 171]. Interestingly, ClpP and its Clp ATPases were not localized uniformly throughout the cells, but clustered in 1–2 foci close to the cell poles. Furthermore, ClpP colocalized with its ATPases in CFP/YFP double labeling experiments [111, 114, 171]. The foci were still present in long filamentous cells induced by depletion of FtsZ, implying that localization depends on occlusion from the nucleoid. During cell division, ClpP foci localized to the division site, suggesting an unknown activity of ClpP in this process. ClpP clusters were also monitored within a growing microcolony. Under these conditions, the foci were highly dynamic. Clusters appeared and disappeared quickly at different locations in the cell, whereas individual clusters did not move very far. After heat shock, more foci of ClpP, ClpX and ClpC appeared, and ClpE formed visible clusters that were not observed during non-stress conditions. Also, the intensity of the clusters increased after heat stress. Strikingly, the adaptor protein McsB and the ClpCP substrate CtsR localized to the same locations as ClpCP after heat shock. This pattern already occurred at 30 °C in an *ywlE* mutant, in which McsB is hyperphosphorylated, and was unchanged in a trapped complex with ClpC. Although colocalization was not directly demonstrated, these results suggest that regulatory proteolysis of CtsR during heat shock is confined to specific locations in the cell. Furthermore, ClpP and all three ATPases colocalized with PorA-aggregates at the cell poles, demonstrating that general proteolysis is also localized close to the poles [171].

Antibiotics and Proteolysis

Recently, ClpP was identified as a target for acyldepsipeptide (ADEP) antibiotics, in both Gram-negative and Gram-positive bacteria. ADEP has an unusual mode of action, because it activates and dysregulates the protease systems, which results in subsequent cell death [178]. It could be demonstrated that ADEP binds specifically to a pocket on ClpP, which is necessary for the interaction with the associated Hsp100/Clp ATPase [179]. The binding of ADEP thereby interferes with the protease complex formation and the recognition of cellular substrates is prevented [180]. In addition, ADEP binding induces a conformational change, which opens the ClpP complex resulting in the recognition of larger unfolded proteins for proteolysis [179, 180]. Thereby substrate recognition, normally controlled by the ATPase is prevented, and

unfolded proteins i.e. nascent polypeptide chains emanating from the ribosome are recognised and degraded by ClpP [180]. Recently the cell division protein FtsZ was identified as a new substrate for the activated ADEP-ClpP complex in *B. subtilis*, explaining the observed inhibition of cell division mediated by ADEP [181].

Another compound specifically designed to inhibit ClpP [182] was shown to be effective e.g. against ClpP of apicoplasts in *Plasmodium falciparum* [183].

A third compound with antibiotic activity against *Mycobacterium tuberculosis*, cyclomarin, was identified and it could be demonstrated that cyclomarin targets ClpC in this organism [184].

The recent discovery of antibiotics, which target regulatory and general proteolysis in bacteria, highlights the importance of the described protease systems for bacterial physiology.

Concluding Remarks

Although tremendous progress has been made in the last couple of years in the field of proteolysis in *B. subtilis*, many processes involving proteases are still poorly understood. Novel substrates involved in regulatory pathways continue to be discovered and the exact role of proteolysis in protein quality control remains to be elucidated. These are only a few examples of the exciting questions in the field of proteolysis in *B. subtilis* to be addressed in the future.

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