Identification of substrate proteins of FtsH during sporulation of *Bacillus subtilis*

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Zusammenfassung

FtsH ist eine ATP- und Zn^{2+}-abhängige Metalloprotease, welche mittels zweier Transmembran-Segmente in der cytoplasmatischen Membran verankert ist. Sie ist die einzige beschriebene Membran-verankerte AAA-Protease bei Bakterien mit verschiedenen regulatorische Funktionen. Eine *ftsH*-Knockout Mutante zeigt einen pleiotropen Phänotyp. Dazu gehören filamentöses Wachstum der Zellen, Sensitivität gegenüber einem Hitzeschock und osmotischen Schock, und die Zellen können nicht mehr sporulieren. Kürzlich konnten wir zeigen, dass *ftsH*-Knockout Zellen nicht das Sporulations-Stadium II erreichen aufgrund einer zu geringen Menge an Spo0A~P. Außerdem haben wir Spo0E, eine Spo0A~P-spezifische Phosphatase, als erstes Substrat von FtsH identifiziert. Da die Sporulationsfrequenz in einer *spo0E ftsH* Doppelmutante nur teilweise wiederhergestellt wird, vermuten wir, dass FtsH weitere Substratproteine abbaut, die die Sporulation negativ beeinflussen. Um weitere Proteine zu identifizieren, wurden zwei verschiedene Strategien angewendet. Mittels der 2D-Gel Technik wurden die Proteome einer *ftsH* Wildtyp- und einer *ftsH*-Knockout-Mutante miteinander verglichen. Es konnten eine Reihe von Proteinen identifiziert werden, die in Abwesenheit von FtsH entweder vermehrt oder reduziert produziert wurden. Eines der mengenmäßig etwa 4-fach erhöhten Proteine wurde als Spo0M identifiziert. Da *ftsH* nicht mit der Transkription von *spo0M* interferiert, wurde ein *in-vitro*-Proteolysetest mit gereinigten Komponenten etabliert. Es konnte gezeigt werden, dass Spo0M ATP- und Zeit-abhängig von FtsH abgebaut wird. In der zweiten Strategie wurde zunächst eine $ftsH^{trap}$ Mutante konstruiert und auf Verlust der Proteolyse-Aktivität getestet. Protease Trap-Mutanten sind noch in der Lage ihre Substrate zu binden, können diese aber nicht mehr abbauen. Mit Hilfe einer GST- $ftsH^{trap}$ Mutante konnte das Membran-Protein YwnF gebunden und dann mittels Massen-Spektrometrie identifiziert werden. Weitere Experimente sind notwendig, um YwnF als Substrat-Protein zu verifizieren. Der letzte Teil der Dissertation galt dem *eag*-Gen, welches mit *spo0E* ein bicistronisches Operon bildet. Die Konstruktion und Analyse einer *eag* Insertions-Mutante ergab einen leichten Anstieg in der Sporulationsfrequenz und in der Menge an Spo0A. Eine Transkriptionsfusion zwischen dem Promotor des *spo0E-eag* Operons und dem *lacZ* Reportergen zeigte einen Anstieg in der β-Galactosidase Aktivität ab t_0 bei Wachstum der Zellen in Sporulationsmedium. Da es sich bei Eag vermutlich um ein integrales Membranprotein handelt, kann es

überschüssige Mengen an Spo0E binden und dadurch eine Dephosphorylierung von Spo0A~P verhindern. Alternativ oder zusätzlich kann Eag Spo0E binden und es FtsH als Modulatorprotein zum Abbau präsentieren.

Summary

FtsH is an ATP- and Zn^{2+}-dependent metalloprotease anchored in the cytoplasmic membrane by two transmembrane segments. It is the unique membrane-bound AAA-protease in bacteria that performs a variety of regulatory functions. In *B. subtilis,* an *ftsH* knockout exhibits a pleiotropic phenotype such as filamentous growth, sensitivity towards heat, osmotic shock and cells are unable to sporulate. Recently, it has been shown that *ftsH* knockout cells fail to entry sporulation stage II due to lack of a sufficient amount of Spo0A~P and the first substrate of FtsH identified in *B. subtilis* is the Spo0E phosphatase, a negative regulator that dephosphorylates Spo0A~P. However, the sporulation frequency in a *spo0E ftsH* double mutant strain was only partly restored, we hypothesized that FtsH might degrade additional substrate proteins involved in sporulation. To identify these proteins, two different strategies were applied. By using the 2D gel technique, the proteomes of an *ftsH* wild-type strain was compared with an *ftsH* null mutant. Several proteins were identified to be either up- or down-regulated in the absence of FtsH. One of them up-regulated about 4-fold was identified as Spo0M. Since *ftsH* did not interfere with transcription of *spo0M*, an *in vitro* proteolysis assay was established using purified components. It was shown that Spo0M was degraded by FtsH in an ATP- and time-dependent way. In the second strategy, an $ftsH^{trap}$ mutant was constructed and tested for loss of its proteolytic activity. Protease trap mutants are still able to bind substrate proteins, but are unable to degrade them. By using $FtsH^{trap}$ fused to a GST-tag, YwnF, a membrane protein, was trapped and identified as a substrate of FtsH by mass spectrometry. However, further experiments will be required to confirm YwnF as a target of FtsH. The last part of this thesis was focused on the *eag* gene, which forms a bicistronic operon with Spo0E. Construction and analysis of an *eag* insertion mutant exhibited a slight increase in the sporulation frequency and in the amount of Spo0A. A transcriptional fusion between the promoter of the *spo0E-eag* operon and the *lacZ* reporter gene revealed an increase in the β-galactosidase activity from t_0 when the cells were grown in sporulation medium. Since the Eag protein may be an integral membrane protein, it may bind excess Spo0E thereby preventing it from dephosphorylating Spo0A~P. Alternatively, Eag may bind Spo0E and present it as a modulator to FtsH for degradation.

1. INTRODUCTION

1.1. *Bacillus subtilis* - the most important genetic model organism of the Gram-positive bacteria

B. subtilis is one of the best model systems of the Gram-positive bacteria to study cell structure, chromosome dynamics, cell division, regulation of metabolism and gene expression (Kroos, 2007). For many decades, a significant research in the mechanism of endospore formation and the regulation of the different stages during the process has improved our understanding of various basic processes in bacteria (de Hoon et al., 2010). In addition, interactions between protein-protein and protein - DNA consisting in regulatory mechanisms, signaling pathways, feed-forward network motifs, and posttranslational regulation have been characterized in details to provide information of many cellular differential and developmental processes (de Hoon et al., 2010; Kroos, 2007). With the wealth of fundamental knowledge and numerous potential applications contributed by studies in *B. subtilis*, this bacterium deserves to be the most important generic model organism of the Gram-positive bacteria.

1.1.1. Overview of regulatory network in sporulation of *B. subtilis*

1.1.1.1. Morphology of *B. subtilis* sporulation and formation of protective structures

Under optimal conditions for vegetative growth, *B. subtilis* cells divide by binary fission to produce two identical daughter cells. By contrast, depletion of carbon, nitrogen or phosphate can initiate sporulation (Suel et al., 2006).

The sporulation process only begins when the starving cell has completed DNA replication. The two chromosomes are then segregated with their replication origins anchored at each cell pole and the origin-distal region at mid-cell (Teleman et al., 1998). Sporulation begins with the formation of a polar septum, creating two membrane-bound compartments of different sizes: the smaller forespore (prespore) and the much larger mother cell (Fig. 1.1) (Errington, 2003). Initially, only about one third of the chromosome is trapped in the forespore compartment, the remaining portion of the chromosome is then translocated into the forespore whereas the other chromosome is localized in the mother cell (Ptacin et al., 2008). Shortly after asymmetric division, under the control of the compartment-specific transcription factors, two parallel programs of gene expression are initiated in each compartment (de Hoon et al., 2010). Although the septum prevents

diffusion of regulatory proteins between the two compartments, it is not closed completely. The precise inter-compartmental signaling between two compartments is still connected to direct the spatial and temporal progression of the developmental process (de Hoon et al., 2010).

Following asymmetric division, the next morphological event of sporulation is the forespore engulfment. This process is directed by mother cell-specific proteins that facilitate the mother cell membrane migrating around the forespore and release the forespore as a free protoplast completely enclosed in the mother cell (Morlot et al., 2010; Pogliano et al., 1999). The forespore is now entirely surrounded by two membranes, its inner and outer membranes. Next, both the inner and outer forespore membranes secrete material into the space between the two membranes to synthesize the cortex, a modified peptidoglycan that is less tightly crosslinked than the cell wall (Popham, 2002). Simultaneously, the forespore chromosome is condensed into a toroidal nucleoprotein structure by binding of small, acid soluble spore proteins (Kroos, 2007). At least 70 individual coat proteins are generated in the mother cell to assemble a multi-layered structure on the forespore surface, building a spore coat outside the cortex to protect it from unfavorable environmental conditions (Kroos, 2007). In addition to the spore coat synthesis, the forespore begins to be dehydrated to prepare for dormancy. Finally, the mature spore is released due to lysis of the mother cell (de Hoon et al., 2010). The mature spores are admitted as the most resistant form of life on our planet (Nicholson et al., 2000) to preserve the bacterial genome from heat, desiccation, radiation, oxidation and considered as an efficient way to escape from predation mechanisms in higher organism (Klobutcher et al., 2006; Laaberki and Dworkin, 2008). However, the spore is constructed for responses to specific germinants. As soon as environmental conditions become favorable for vegetative growth, *B. subtilis* quickly abandons the dormant state to germinate (Setlow, 2003).

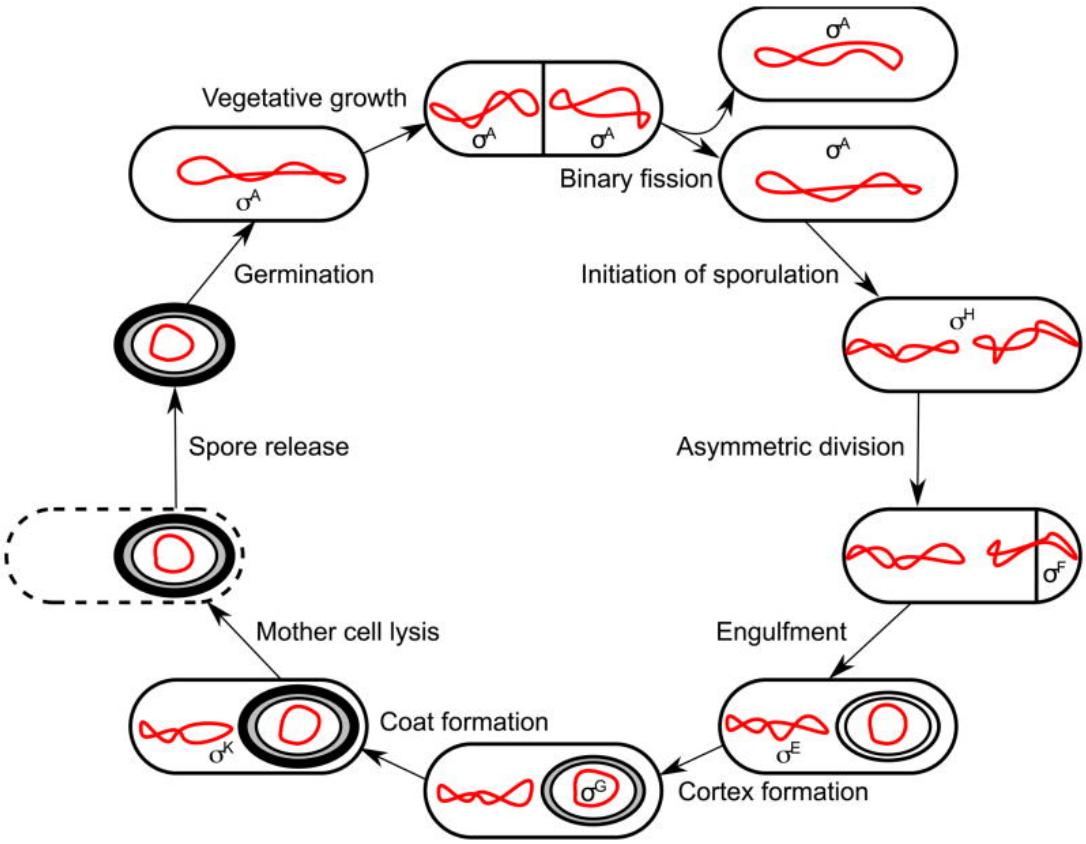

Fig. 1.1. Morphology of the B. subtilis life cycle. *The temporal and compartment-specific sigma* (σ) *factors required for each stage of sporulation are indicated. Cells divide by binary fission to generate two identical daughter cells under conditions of vegetative growth. During initiation of sporulation, the DNA chromosome completes replication and duplication. Each chromosome (red) is oriented with its origin-proximal region anchored at the opposite cell poles. During asymmetric division, the polar septum generates two unequal membrane-bound compartments: a large mother cell and a small forespore containing about one-third of the chromosome in its compartment. The remaining portion of the forespore chromosome is translocated into the forespore after asymmetric division. Engulfment begins with the migration of the mother cell membrane around the forespore membrane to release the forespore as a free protoplast in the mother cell. Next, the cortex is synthesized between the inner and outer forespore membranes. The mature spore is released into the environment due to the lysis of the mother cell. The B. subtilis spore can exit in a dormant spore state for long periods of time, but quickly resumes vegetative growth in the presence of favorite conditions. This figure was taken from de Hoon et al., 2010.*

1.1.1.2. Key transcriptional regulators during *B. subtilis* sporulation

The sporulation in bacteria is controlled by a complex cascade of regulatory interactions in which sigma factors serve as dominant regulators of this process (Losick and Stragier, 1992). There are two sigma factor cascades with compartment-specific activities organized spatially and temporally to direct gene expression during the different stages of endospore formation. Sigma factors σ^F and σ^G regulate gene expression in the early and late stages of forespore whereas σ^E and σ^K control gene expression in the early and late state in the mother cell, respectively (Losick and Stragier, 1992). The master regulator of sporulation, Spo0A~P, and σ^H play a key role during initiation of this process by activating these two σ factor cascades and by regulating transcription of genes in the predivisional cell to prepare for the endospore formation (Eichenberger et al., 2004). A brief functional description of key proteins in the network is summarized by Kroos, 2007 and shown in Table 1.1.

Table 1.1. Key transcriptional regulators during B. subtilis sporulation. This table was taken from Kroos, 2007

Protein	Aliases	Function
σ^A	RpoD, SigA	Major σ factor in growing cells; entry into sporulation
σ^H	Spo0H	Entry into sporulation
Spo0A		Entry into sporulation; activity persists in the mother cell
σ^F	SpoIIAC, SigF	Early forespore gene expression
RsfA	YwfN	Regulator of σ^F-dependent gene expression
σ^E	SpoIIGB, SigE	Early mother cell gene expression
SpoIIID		Regulator of σ^E-dependent gene expression, primarily[a]
GerR[b]	YlbO	Regulator of σ^E-dependent gene expression
σ^G	SpoIIIG, SigG	Late forespore gene expression
SpoVT	YabL	Regulator of σ^G-dependent gene expression
σ^K	SpoIVCB/SpoIIIC[c], SigK	Late mother cell gene expression
GerE		Regulator of σ^K-dependent gene expression

(a): SpoIIID also represses some σ^K-dependent genes (Halberg and Kroos, 1994; Ichikawa and Kroos, 2000).

(b): Ger, germination; a mutation in a ger gene interferes with this process, which involves rehydration of the spore and outgrowth of a rod-shaped cell in response to nutrients.

(c): σ^K is encoded in two genes, spoIVCB and spoIIIC, which are separated by 48 kb until joined by site-specific recombination in the mother cell to form the sigK gene (Stragier et al., 1989).

1.1.2. Genetic networks and key regulators controlling initiation of sporulation

Initiation of sporulation in *B. subtilis* is induced by nutritional, cell density, and cell cycle signals that result in an elevated concentration of Spo0A~P (Kroos, 2007). Due to nutrient deprivation, *B. subtilis* cells leave vegetative growth and enter the stationary

phase (termed transition state). In order to survive, the cells redirect their metabolism and physiology in different ways to deal with starvation (Phillips and Strauch, 2002). The cell's first priorities are to regulate the alterations in gene expression to utilize alternative nutrients and to successfully compete with other species for scarce resources. Various extracellular proteases and other degradative enzymes are produced and the alternate pathways are applied to maximize the utilization of nutrient resources (Strauch, 1993). A variety of antibiotics and antimicrobial compounds are secreted during this stage to out-compete with other microbial species. Cells also establish a genetically competent state to uptake exogenous DNA and sporulating cells are able to cannibalize non-sporulating cells (Gonzalez-Pastor et al., 2003). Sporulation is only committed as a last resort when all other attempts to grow, to compete and to survive have been exhausted. Once initiation of sporulation has occurred, there is no turning back (Phillips and Strauch, 2002). Two key regulatory proteins involved in the inititation of sporulation are σ^H and Spo0A and another important factor is AbrB, a negative regulator that regulates various stationary phase responses during initiation (Errington, 1993).

1.1.2.1. Activation of Spo0A, the master regulator of phase 0, occurs through a phosphorelay

Spo0A, the master regulator of stage 0, is activated by phosphorylation via a phosphorelay, an expanded version of a two-component system including protein kinases and phosphatases (Molle et al., 2003; Muchova et al., 2004). When cells enter the transition phase, unknown starvation signals trigger autophosphorylation at an invariant histidine residue of one of five sensor kinases (KinA through KinE) (Ireton et al., 1993; Jiang et al., 2000). The phosphoryl group is then transferred sequentially from the kinase(s) to Spo0F, then to Spo0B and finally to the response regulator Spo0A (Burbulys et al., 1991). Dephosphorylation of Spo0F~P may be caused by at least four Rap proteins, RapA, RapB, RapE and RapH (Perego and Hoch, 1996; Baker and Neiditch, 2011). It was thought that these Rap proteins function directly as phosphatases. Indeed, dephosphorylation of Spo0F~P is caused by the binding of Rap phosphatases to Spo0F~P stimulating its autophosphatase activity (Piggot and Hilbert, 2004; Core and Perego, 2003). The Rap proteins are inhibited specifically by their corresponding pentapeptides PhrA, PhrB, PhrE and PhrH. Their specific activity on the target Rap phosphatase is determined by the amino acid sequence of each pentapeptide (Core et al., 2001). Spo0A~P

itself is also dephosphorylated by the action of three phosphatases: Spo0E is produced during the transition state and two homologues, YisI and YnzD, are present during the vegetative phase of growth (Perego and Hoch, 1987). Spo0E acts as a negative regulator of sporulation by specifically dephosphorylating Spo0A~P and converting it into an inactive form. Overproduction of Spo0E represses sporulation and deletion of *spo0E* results in inappropriate timing of sporulation (Fig. 1.2) (Perego and Hoch, 1991).

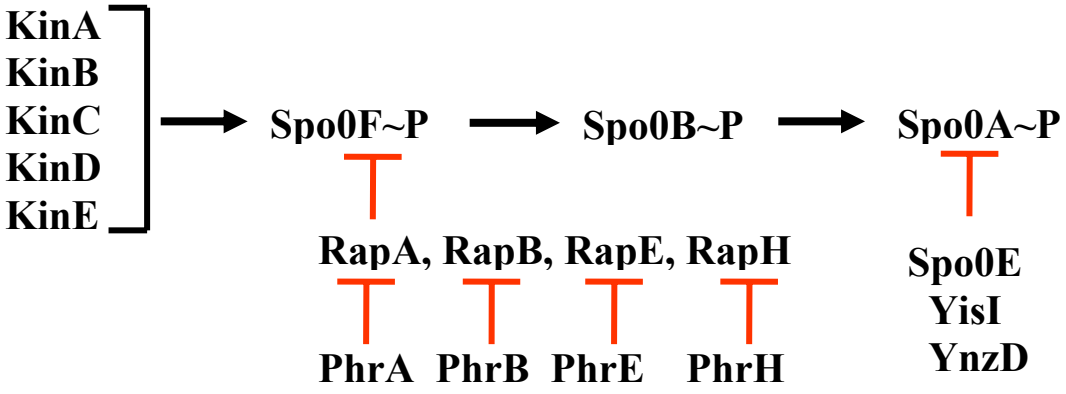

Fig. 1. 2: Schematic representation of the phosphorylation of Spo0A. *Spo0A is indirectly phosphorylated by a multicomponent phosphorelay involving the kinases KinA, KinB, KinC, KinD, KinE and two intermediate proteins. The kinases phosphorylate Spo0F resulting in Spo0F~P. Then, the phosphoryl group will be transferred to Spo0B and finally to Spo0A to activate it. Phr peptides sense cell density and inhibit several Rap phosphatases that can dephosphorylate Spo0F~P; Spo0E, YisI and YnzD can dephosphorylate Spo0A~P.*

1.1.2.2. Positive and negative autoregulatory loops control production of Spo0A~P

Production of Spo0A~P is controlled by both positive and negative regulatory loops during initiation of sporulation (Grossman, 1995). Spo0A~P can directly stimulate its own expression and contribute to the transcription of genes that regulate further accumulation of Spo0A~P via a positive feedback loop involving in AbrB and σ^H (Fig. 1.3). Transcription of the *spo0A* gene is controlled by a mechanism called "promoter switching mechanism", involving two Spo0A~P - dependent promoters: a vegetative σ^A-recognized promoter, P_v, and a sporulation σ^H-recognized promoter, P_s, controlled by the amount of phosphorylated Spo0A (Chastanet and Losick, 2011). It has been proposed that

a low level of *spo0A* is transcribed from P_v during the exponential phase of growth while P_s is silent because of the absence of Spo0A~P and σ^H. When Spo0A~P is formed via the phosphorelay, transcription of the *spo0A* gene is switched from P_v to P_s (Chastanet et al., 2010). Once activated, Spo0A~P represses transcription of *abrB* causing derepression of transcription of the *spo0H* gene coding for the sigma-H protein, thereby stimulating transcription of *spo0A* from a sigma-H-recognized promoter. σ^H also directs transcription of two response regulators, *kinA* and *spo0F*. As a result, a positive feedback loop is set up to control production of Spo0A~P (Fig. 1.3) (Britton et al., 2002).

A negative feedback loop is also controlled by Spo0A~P via Spo0E and its repressor, AbrB. Transcription of *spo0E* is repressed by AbrB and is derepressed during early sporulation due to Spo0A~P repression of *abrB* (Perego and Hoch, 1991). An increase in the amount of the Spo0E phosphatase causes the removal of phosphate from Spo0A~P that converts it into the inactive form and prevents cells from entry into sporulation. This negative feedback loop presumably functions in the maintenance of a subpopulation of cells that do not sporulate under these conditions or delays fast Spo0A~P induction (Grossman, 1995; Chastanet et al., 2010).

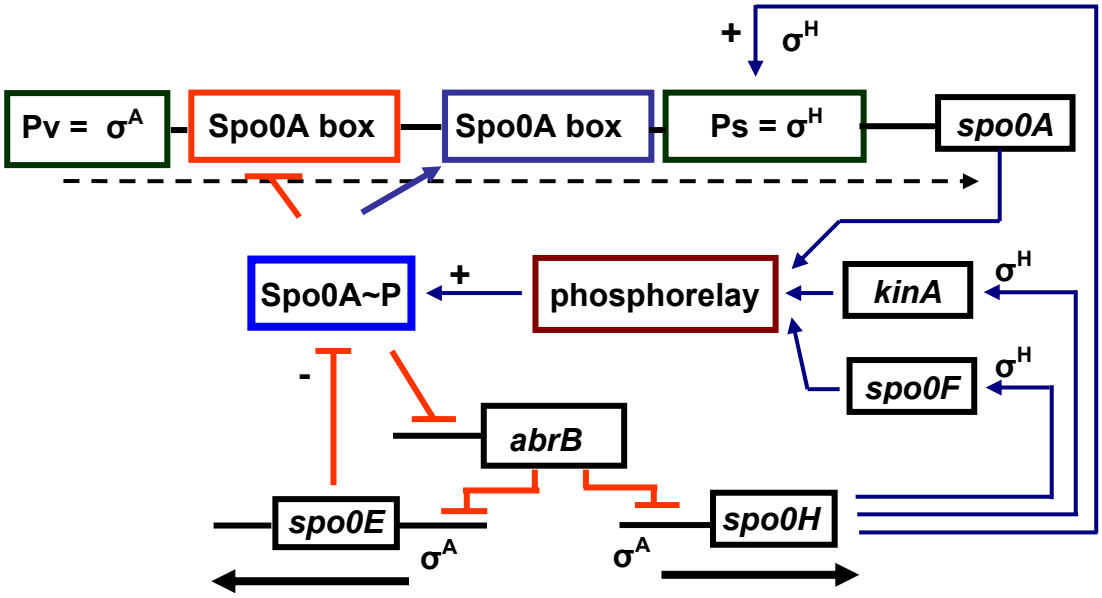

Fig. 1.3. Schematic representation of positive and negative regulatory loops controlling the production of Spo0A~P. *Spo0A is activated through the phosphorelay. Spo0A~P represses transcription of abrB. A decrease in AbrB protein causes derepression of transcription of spo0H, leading to increased transcription of spo0A and two response regulators of the phosphorelay, kinA and spo0F [a positive feedback loop (+)]. The decrease of AbrB level also causes derepression of spo0E, leading to increased accumulation of the phosphatase that removes phosphate from Spo0A~P thereby setting upon a negative feedback loop (-).*

1.1.2.3. Role of phosphorylated Spo0A during initiation of sporulation

The master regulator of sporulation, Spo0A~P, is a DNA-binding protein activated through a phosphorelay (Molle et al., 2003). It is a member of the response regulator family of two-component regulatory systems consisting of two distinct domains. The highly conserved N-terminal domain called phosphoacceptor (or receiver) domain containing an invariant aspartic acid residue (Asp-56) is the target of phosphorylation by the phosphorelay and mediates dimerization of Spo0A. The C-terminal DNA-binding (or effector) domain which is responsible for binding to specific DNA sequences, called 0A boxes, regulates transcription of target genes (Perego et al., 1991; Muchova et al., 2004).

Dimerization of Spo0A after phosphorylation is required to target 0A boxes (Asayama et al., 1995). Spo0A~P acts as a repressor and activator protein regulating a

total of 121 genes including genes with vegetative σ^A-recognized promoters as well as sporulation σ^H-recognized promoters (Seredick and Spiegelman, 2001; Molle et al., 2003). It plays two major roles in the cell's adaptive responses to starvation. First, a low amount of Spo0A~P initially represses transcription of *abrB* that causes an increase in AbrB-dependent gene expression during the transition state. Second, when the Spo0A~P concentration reaches a critical level, it will regulate expression of genes required for entry into sporulation (Phillips and Strauch, 2002). The basal level of Spo0A~P is not constant from cell to cell. These regulatory loops and the interconnectedness of the phosphorelay influences production of Spo0A~P and results in a bi-stable switch - a state where some cells in the population accumulate a higher level of Spo0A~P than others (Kroos, 2007; Dubnau and Losick, 2006). Cells with a high level of Spo0A~P produce killing factors to lyse those cells with a low Spo0A~P level to get more nutrients resulting in a delay in initiation of sporulation. They also resist their killing factor by synthesizing an export pump and an immunity protein to protect them from the toxin (Grossman, 1995; Dubnau and Losick, 2006). About 60% of the cells with a sufficient amount of Spo0A~P, called Spo0A-ON stage, are able to sporulate while the remaining 40% are in the Spo0A-OFF stage and fail to enter into sporulation. This mechanism is called "bistability" which means the simultaneous existence of two subpopulations in one population of genetically identical cells. The explanation for this mechanism is still unknown.

1.1.2.4. Sigma H, a positive regulator of sporulation

Sigma-H (σ^H) is an alternative RNA polymerase sigma factor activating the transcription of many genes required for formation of the polar septum, the initiation of cell-type-specific expression and activation of Spo0A (Burkholder and Grossman, 2000). Many sporulation genes are directly activated by sigma-H including *spo0A, spo0F, kinA, spo0M, spoVG,* and *spoVS* and the *spoIIA* operon (Bai et al., 1990; Johnson et al., 1983; Predich et al., 1992; Han et al., 1998; Resnekov et al., 1995; Wu et al., 1991).

Sigma-H also regulates transcription of some members of the *phr* family, coding for secreted peptide pheromones that inhibit specifically the corresponding Rap phosphatases, modulating cell entry into genetic competence, sporulation, and other processes (Perego and Brannigan, 2001; Lazazzera et al., 1999; McQuade et al., 2001). Several of the genes transcribed by sigma-H are also under control of σ^A-dependent promoters including *spo0A*, *ftsA* (cell division), *dnaG* (DNA replication), *sigA* (encoding sigma-A, the major sigma factor), and *citG* (tricarboxylic acid cycle) (Britton et al., 2002).

Sigma-H also contributes indirectly to the expression of Spo0A and KinB by activating expression of *sinI* and repressing *sinR* - a *spo0A* synthesis repressor, thereby activating indirectly the *spo0A* synthesis (Bai et al., 1993). In addition, sigma-H stimulates expression of CSF (competence stimulating factor), which inhibits the RapB phosphatase that dephosphorylates Spo0F~P and thereby contributes to the increase in *spo0A* transcription during the early stage of sporulation (Fig. 1.4). In turn, Spo0A~P contributes to the induction of sigma-H by repressing its transcriptional repressor, AbrB (Burkholder and Grossman, 2000).

Regulation of sigma-H itself is quite complicated. *spo0H,* coding for sigma-H, is transcribed from a σ^A-dependent promoter and directly under negative control of AbrB which is in turn repressed by Spo0A~P (Burkholder and Grossman, 2000; Strauch, 1995; Weir et al., 1991). Under appropriate conditions, increased levels of Spo0A~P result in repression of AbrB resulting in enhanced levels of *spo0H* transcription. Therefore, a high level of Spo0A~P is produced resulting in more repression of AbrB and increasing levels of *sigH* transcription, thereby establishing a self-reinforcing cycle to regulate both sigma-H and Spo0A.

Sigma-H plays important roles in response to a diversity of external conditions including pH, carbon source, and existence of amino acids. It controls many genes involved in several cellular processes including proteolysis, cell wall metabolism, transport, and cytochrome biogenesis that helps cells to adapt to conditions of starvation and impacts physiological decisions during entry into stationary phase (Britton et al., 2002).

Schematic representation of regulation of sigma-H transcription and its regulation of expression and activation of Spo0A is illustrated and shortly explained in Fig. 1.4.

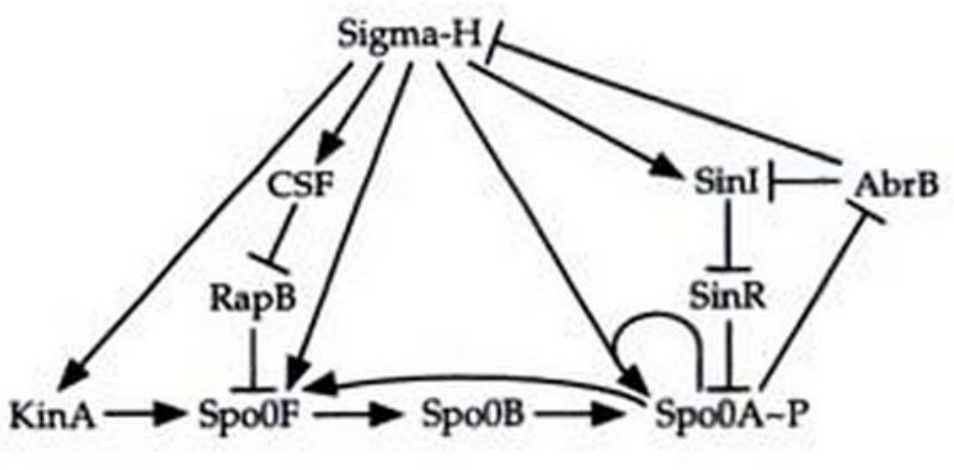

Fig. 1.4. Regulation of sigma-H transcription and its regulation of expression and activation of Spo0A. *Sigma-H regulates transcription of kinA, spo0F, and spo0A, contributing to the high level accumulation of Spo0A~P. Sigma-H also stimulates the activation of Spo0A by regulating expression of the secreted peptide pheromone, CSF, which inhibits the phosphatase, RapB, that dephosphorylates Spo0F~P. Similarly, sigma-H regulates expression of sinI, which inhibits sinR, resulting in further derepression of spo0A transcription. This figure was taken from Burkholder and Grossman, 2000.*

1.1.2.5. AbrB, an important transcription factor during initiation of sporulation

AbrB is a transcriptional repressor that plays an important role during initiation of sporulation (Kroos, 2007). This DNA-binding protein plays a role as a repressor of several competence genes as well as genes expressed during the transition state (Strauch and Hoch, 1993; Strauch et al., 1989b).

At least three sporulation genes controlled by AbrB are *spo0E* (Perego and Hoch, 1991), *spo0H* (Weir et al., 1991) and *spoVG* (Zuber and Losick, 1987). AbrB also regulates an antibiotic-synthetic gene, *tycA* (Fürbass et al., 1991; Robertson et al., 1989), and *abrB* itself (Strauch et al., 1989a). Over 40 different genes are directly regulated by AbrB and many other genes indirectly due to its influence on the transcription of other regulatory proteins. For example, the regulatory proteins ScoC, Abh, SinR and SigH are controlled by AbrB. These proteins also regulate numerous genes in different regulatory networks leading to a wide variety of genes controlled indirectly by AbrB (Phillips and Strauch, 2002).

AbrB acts as a DNA-binding factor and controls gene expression in at least three different ways (Dixon et al., 2001; Errington, 1996; Johnson et al., 1983). First, AbrB acts

as a unique repressor of some genes that are constitutively expressed during all phases of growth in an *abrB* mutant strain. In most of the cases, AbrB plays a role as a preventer to become a factor in series of redundant regulatory networks for ensuring that no regulator has complete control over genes that must remain silent during active growth. AbrB also plays a role as an activator of some genes when it represses activation of other repressors (two negatives = a positive) (Phillips and Strauch, 2002).

The transcription of the *abrB* gene is autoregulated. In an active growth state, the AbrB concentration is maintained at a threshold that is sufficient for its regulatory activity (Strauch et al., 1989a). During starvation, Spo0A, a repressor of *abrB* transcription, is activated by phosphorylation through the phosphorelay, resulting in a decrease of the AbrB level below the threshold for its negative regulatory activity thereby increasing the expression of AbrB-repressed genes. The genes under control of AbrB may function in many metabolic and physiological processes, including production of extracellular degradative enzymes, antibiotics, motility, development of competence, transport systems, oxidative stress response, phosphate, nitrogen and amino acid metabolism, cell surface components and sporulation (Phillips and Strauch, 2002).

1.2. The metalloprotease FtsH

1.2.1. Introduction of FtsH

FtsH is member of the AAA family (ATPases associated with a variety of cellular activities) inserted into the cytoplasmic membrane by two transmembrane segments (Schumann, 1999). It is comprised of an N-terminal region with two transmembrane segments and a C-terminal cytoplasmic region consisting of AAA-ATPase and Zn^{2+}-metalloprotease domains. While other AAA proteases are located in the cytoplasm, FtsH is a unique membrane-bound AAA protease able to degrade integral membrane proteins. It plays crucial roles in controlling the quality of membrane proteins by rapidly degrading abnormal membrane proteins and some short-lived proteins present in the cytosol (Ito and Akiyama, 2005).

Bacterial cells with FtsH malfunction in bacteria result in cell division defects and growth arrest (Bieniossek et al., 2006). In *E. coli*, the FtsH protease is essential for growth whereas it is dispensable in *B. subtilis*. However, *ftsH* mutant cells in *B. subtilis* appear more sensitive to heat, salt, and defective for cell division and sporulation (Kiran et al.,

2009). Orthologs of FtsH also exist in chloroplasts and mitochondria of eukaryotes (Bieniossek et al., 2006). The FtsH protease of *Arabidopsis thaliana* contributes to the tolerance of the plant to uplifted temperatures. It may alleviate light stress by degrading photodamaged photosystem II D1 protein and unassembled thylakoid membrane proteins (Chen et al., 2006). The loss of a close FtsH-orthologs in humans results in hereditary spastic paraplegia (Bieniossek et al., 2006).

1.2.2. Discovery of FtsH

The *E. coli ftsH* gene was discovered and described independently by four groups through detection of different phenotypes, thereby received four different designations: *ftsH*, stands for filamentous temperature-sensitive; *tolZ*, exhibits tolerance against colicins and *hflB*, causes high frequency of lysogenization by phage lambda and *mrsC,* stands for mRNA stability (Schumann, 1999).

In *B. subtilis*, the *ftsH* gene has been discovered separately by three different groups. First, the group of Schumann detected FtsH as an insertion mutant causing a growth defect under hyperosmotic conditions (Geisler and Schumann, 1993). Later, *ftsH* was detected by the group of S. Cutting as a regulatory factor of SpoVM, a protein requiring for spore cortex and coat formation (Cutting et al., 1997) and the group of P. Zuber identified *ftsH* as an essential gene for fermentation and nitrate respiration (Nakano et al., 1997).

In general, the *ftsH* gene is present in one single copy in the examined prokaryotic genomes except for cyanobacteria such as *Synechocystis* (J05708), which has four *ftsH* genes in its genome (Nixon et al., 2005). Yeast genomes contain three copies of the gene (Schnall et al., 1994), whereas plant genomes possess a larger *ftsH* gene family. For example, the *Arabidopsis* genome has 12 *ftsH* genes and mutations in these genes result in leaf color variegation (Chen et al., 2006).

1.2.3. The structure of FtsH

The membrane-bound metalloprotease FtsH is a ring-like homo-hexamer complex that carries the AAA and proteolytic domain on the same polypeptide chain (Ito and Akiyama, 2005). The FtsH monomer of *E. coli* consists of 647 amino acid residues with a calculated molecular mass of 71.0 kDa. FtsH is an integral cytoplasmic membrane protein

and both the amino- and carboxy-termini are exposed into the cytoplasm (Narberhaus et al., 2009). The N-terminus with only seven amino acids extending into the cytoplasm is followed by the two transmembrane segments and a large cytoplasmic region of approximately 520 residues consists of the ATPase and the Zn^{2+}-dependent protease domain (Ito and Akiyama, 2005).

The ATPase domain consists of the conserved Walker A and B motifs arranged to coordinate ATP in combination with Mg^{2+} and water molecules to support for nucleotide binding and hydrolysis, and the second region of homology (SRH) carrying conserved arginine residues ‘‘arginine fingers’’ for oligomerization and nucleotide hydrolysis (Bieniossek et al., 2009). They form the substrate entrance gate with a diameter of about 15 Å containing a conserved phenylalanine at position 228 required for substrate recognition and translocation. Substrates are pulled through a narrow gate using the energy of ATP hydrolysis for unfolding and translocation into the protease domain. The C-terminal region of FtsH is the protease domain containing the zinc-binding HEXXH motif, a conserved sequence feature for Zn-dependent metalloproteases with two histidines coordinating a zinc atom and the glutamate plays a crucial role in catalytic function (Narberhaus et al., 2009). Three conserved leucine residues at positions 567, 574 and 584 of this region form a leucine zipper which plays a key role for degradation of RpoH and λ *c*II (Shotland et al., 2000b).

In *E.coli*, FtsH interacts with the HflKC membrane protein complex to form a large membrane-spanning holoenzyme (Saikawa et al., 2004). HflK and HflC are cytoplasmic membrane proteins that form a hetero-multimeric complex (HflKC) and further interact with the FtsH hexamers within the membrane to form a large complex of about 1 MDa. It has been suggested that HflKC exerts proteolytic modulation of FtsH depending on the class of substrates, membrane-integrated or soluble. The membrane-integrated and soluble substrates are presented to FtsH via different pathways so that HflKC might be a regulatory factor of substrate selection (Akiyama, 2009).

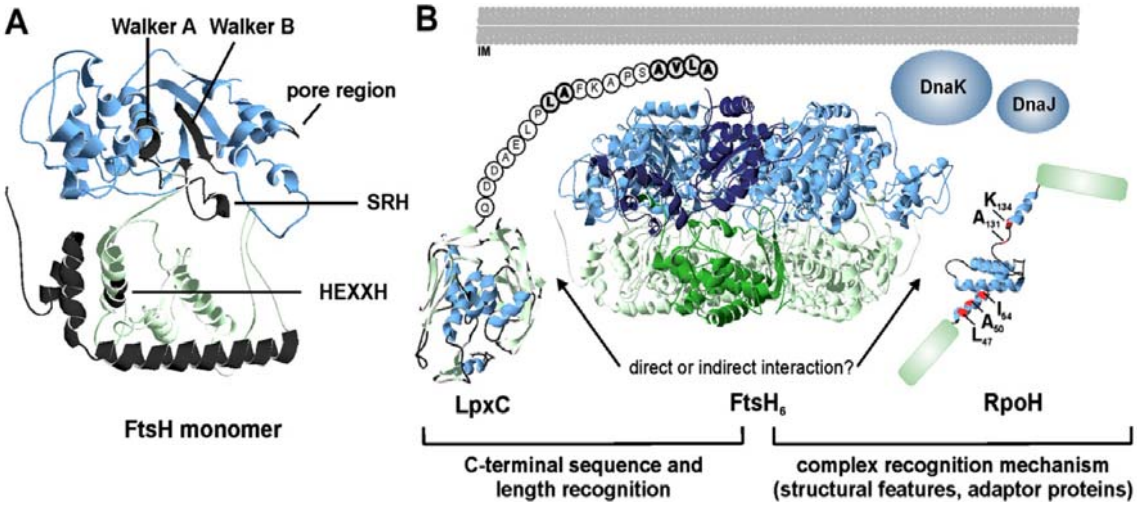

Fig. 1.5. Cartoon representation of FtsH structural features and degradation mechanisms by FtsH. *(A) Cartoon representation of one soluble subunit of FtsH from Thermus aquaticus. The ATPase domain and the protease domain are displayed in blue and green, respectively. The Walker A and B motifs, the pore region, the second region of homology (SRH), the zinc binding motif HEXXH and the helices at the C-terminus are shown in black. (B) The hexameric structure of FtsH is displayed using the same colors as in (A). LpxC structure, an example of a degradation mechanism starting at a free terminus, is shown on the left. The specific sequence and length for the E. coli C-terminal degradation signal on LpxC is indicated in the single letter amino acid code. On the right, regions 2.1-C of RpoH is shown as a model for a complex degradation mechanism with important residues colored in red. This figure was taken from Narberhaus et al., 2009.*

1.2.4. Substrate binding

FtsH interacts with cytoplasmic and membrane-bound substrates in different ways. It was proposed that recognition of protein substrates by FtsH is presumably initiated by binding of FtsH with its substrate occuring on the outer surface of the α-helical subdomain (Niwa et al., 2002). The conserved region in the C-terminus of FtsH may also contribute to the substrate binding (Shotland et al., 2000b). A degradable substrate recognized by FtsH is then translocated into the FtsH proteolytic chamber.

The proposed model for FtsH substrate binding is illustrated and described in Fig. 1.6 and Fig. 1.7 (Ito and Akiyama, 2005). For soluble protein substrate binding, the

substrate initiation region for degradation by FtsH may be first recognized by the outer surface of the helical subdomain of the ATPase domain (*purple*). The substrate is then scanned by FtsH for the ability to interact with the α-helical subdomain of the ATPase domain. After being recognized, the substrate is delivered into the ATPase chamber through the gate formed by the pore residues on the membrane side (*orange*) and then translocated further into the proteolytic chamber for proteolysis (Fig. 1.6). While the first step does not require ATP, the subsequent steps need to be coupled to ATP hydrolysis for their activity (Fig. 1.6).

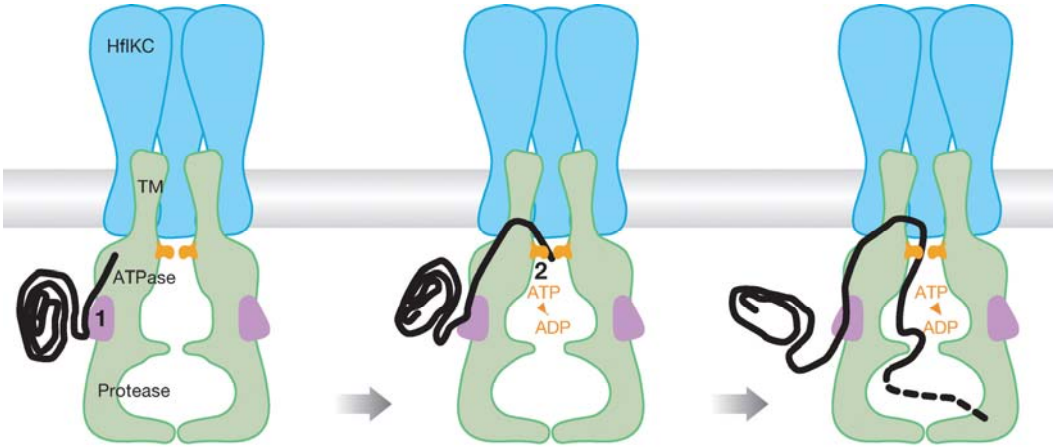

Fig. 1.6. Schematic representation of a possible entry route for soluble protein substrates*. FtsH first recognizes substrates at the outer surface of the ATPase domain (purple), then delivered into the ATPase chamber through the pore residue-formed gate (orange) and then translocated into the proteolytic chamber for proteolysis. This figure was taken from Ito and Akiyama, 2005*.

In case of the membrane protein substrates, recognition of a membrane protein substrate may first occur within the membrane by the interaction of the FtsH and substrate transmembrane regions in association with HflKC. HflKC may control access of membrane protein substrates to FtsH within the membrane. FtsH then recognizes a cytoplasmic tail of the substrate membrane protein that protrudes sufficiently into the cytoplasm and test for its interaction with the α-helical subdomain of the ATPase. The recognized substrate is then dislocated out of the membrane, and the rest of the steps may occur in a similar way as described for soluble substrates (Fig. 1.7).

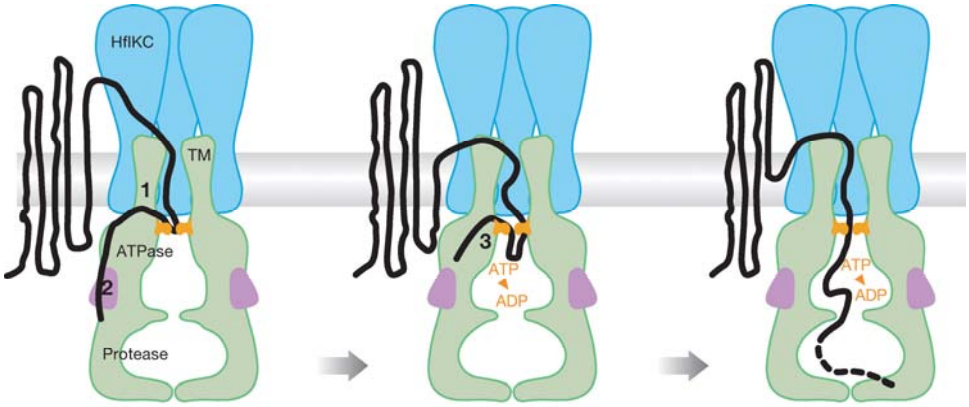

Fig. 1.7. Schematic representation of a possible entry route for membrane protein substrates *HflKC may initially control the interaction of membrane protein substrates with FtsH within the membrane allowing FtsH to scan for substrate membrane protein recognition. The remaining steps may occur similarly to degradation of soluble substrates. This figure was taken from Ito and Akiyama, 2005.*

1.2.5. Mechanism of substrate recognition and degradation by FtsH

The mechanism of FtsH protein substrate degradation depends on Zn^{2+} and ATP in which ATP-hydrolysis causes conformational changes and creates a mechanical force to unfold the substrate and translocate it into the proteolytic chamber. The proteolytic reaction of FtsH may occur in an initiation-signal-dependent manner in which substrate unfolding depends on an initiation signal and then extends along the polypeptide chain.

An unstructured region at either end or internal of the polypeptide is required for degradation initiation and this part of the substrate should not be folded or associated tightly with other proteins. So far, there are two principal pathways to explain the FtsH degradation mechanism. The first pathway is based on the recognition of motifs located at the N- or C-terminal ends of the substrates and the second is more complicated depending on structural internal features of protein substrates. In both cases, specialized adaptors and targeting proteins are involved (Narberhaus et al., 2009).

1.2.5.1. Recognition of N- or C-terminal motifs for FtsH degradation

FtsH may degrade membrane and soluble substrate proteins via different pathways (Akiyama, 2009). In case of degradation of membrane-anchored proteins, an unstructured and flexible N- or C-terminal tail is exposed into the cytoplasm serves as a

signature to cause initiation of proteolysis by FtsH. The minimal length of an exposed peptide is approximate 20 amino acids at the N-teminus and only ten for a C-terminally exposed peptide. For example, degradation of the membrane substrate YccA depends on a stretch of approximate 20 amino acid residues at the N-terminal cytosolic tail (Chiba et al., 2000). Shortening of the tail was reported to cause stabilization of this protein (Ito and Akiyama, 2005). No specific sequence is recognized for degradation initiation because some non-substrate membrane proteins can be converted into FtsH substrates by adding a cytosolic tail of sufficient length (about 20 residues) on either the N- or C-terminal side (Chiba et al., 2000; Chiba et al., 2002; Nishiyama et al., 2000).

Although recognition of terminal degradation signals is a common way for many FtsH substrate proteins, the process still depends on the mechanism of target interaction and membrane dislocation that might be different from a variety of FtsH substrates (Kihara and Ito, 1998; Kihara et al., 1999; Chiba et al., 2002).

Recognition of a free-end is also typical for the degradation of some soluble FtsH substrates such as SsrA-tagged polypeptides, phage protein λ CII and LpxC. In the case of LpxC degradation, an unstructured C-terminus is responsible for proteolysis. The largely non-polar motif consisting of the final 11 residues (-LAFKAPSAVLA) with the first two (LA) and last four (AVLA) amino acids play a key role for proteolysis. The critical length of 20 amino acids at the C-terminus is required for recognition of the non-polar residues at the C-terminus of LpxC and conserved amino acids at the entrance channel of FtsH (Yamada-Inagawa et al., 2003). Stabilization of the protein occurs when removing the tail or replacing at least two residues by the polar aspartic acid and has no effect on its activity (Führer et al., 2006; Führer et al., 2007). This indicates that the C-terminus is the unique feature required for targeting LpxC to FtsH.

1.2.5.2. Complex substrate recognition mechanisms

Several protein substrates of FtsH are not recognized by free terminal signals. Instead, an internal degradation motif is required for proteolysis (Okuno et al., 2006b). The best-studied example is the alternative heat shock sigma factor RpoH (σ^{32}). Neither end of RpoH is essential for proteolysis, but an internal region within the N-terminus can initiate degradation of this protein (Bertani et al., 2001; Tomoyasu et al., 2001).

Amino acids L47, A50 or I54 in region 2.1 of RpoH are important for FtsH-dependent proteolysis (Horikoshi et al., 2004; Obrist et al., 2009; Obrist and Narberhaus, 2005; Yura et al., 2007). The substitutions at these positions have revealed to protect RpoH from degradation and all three amino acids are assumed to line up on one face of an α-helix (Fig. 1.5B) (Narberhaus et al., 2009).

Another important region for RpoH degradation has determined to map in region C containing the two residues A131 and K134 that are essential for degradation by FtsH. The substitution of these two residues causes stabilization of RpoH. Therefore, a minimal RpoH fragment consisting only of regions 2.1 and C has been shown to be degraded by the FtsH protease (Obrist et al., 2009).

1.2.6. Biological functions of the FtsH protease

The protease FtsH and its orthologs exit in eubacteria, chloroplasts, and mitochondria and play a key role in quality and regulatory control within the cell (Bieniossek et al., 2009). FtsH is essential in *E. coli* and most of the Gram-negative bacteria but not all of them. In the alpha-proteobacterium *Caulobacter crescentus*, FtsH is not essential for viability. *ftsH* mutant cells are viable when growing at normal conditions, but are highly sensitive to antibiotics, high salt concentrations and elevated temperature. Cells are unable to carry out morphological and physiological adaptations during stationary phase and become more susceptible to death than the other wild-type cells under nutrient-limited conditions (Fischer et al., 2002).

In Gram-positive bacteria, the *ftsH* gene is not essential but an *ftsH* knock-out can cause severe pleiotropic effects (Deuerling et al., 1997). FtsH degrades both soluble and membrane-bound proteins with more than a dozen already described in various bacteria (Table 1.2). An overview of FtsH substrates might underline its important function in different species (Narberhaus et al., 2009).

1.2.6.1. Membrane proteins as substrates of FtsH

The FtsH protease regulates a variety of cellular processes in *E. coli* (Fig. 1.8). It controls the quality of misfolded and incorrectly inserted membrane proteins and functions as a chaperone to refold abnormal proteins or a protease able to degrade them.

One example is YccA, a short-lived membrane protein of unknown function. It has been suggested to be naturally degraded by FtsH, and its function seems to be linked to biofilm formation (Beloin et al., 2004).

FtsH also degrades unassembled membrane proteins such as the subunit SecY of the SecYEG translocase and $F_0\alpha$ of the H^+-ATPase. Degradation of these proteins only occurs when they fail to assemble with their partner proteins (Akiyama et al., 1996a; Akiyama et al., 1996b). SecY forms a stable translocon complex with SecE and SecG allowing translocation of presecretory proteins through the cytoplasmic membrane or integration into the lipid bilayer of newly synthesized membrane proteins. Therefore, incomplete assemblies of the translocon could be harmful to the cell (Akiyama et al., 1996b). The $F_0\alpha$ is a subunit of a proton channel across the membrane and its redundance might be also harmful to the cells (Akiyama et al., 1996a). Therefore, these examples show that FtsH protects cells from the harmful conditions by degrading abundant membrane protein subunits when they failed to form functional complexes (Ito and Akiyama, 2005).

1.2.6.2. Cytoplasmic substrates of FtsH

FtsH degrades a majority of cytoplasmic substrates of FtsH (Fig. 1.8 and Table 1.2) and many of them are short-lived soluble substrates. At least three substrates of FtsH are bacteriophage encoded proteins and they belong to the group of short-live proteins. The *cII* gene product is a transcription factor required for setting up the lysogenic cycle (Kihara et al., 1997; Shotland et al., 1997; Shotland et al., 2000a). The Xis protein is responsible for excision of prophage DNA from the bacterial genome (Leffers and Gottesman, 1998). The *cIII* gene product is a competitive inhibitor of FtsH (Herman et al., 1997). By degrading these substrates, FtsH exhibits its regulatory impact on the development and life cycle of infecting by degrading their key regulatory molecules (Ito and Akiyama, 2005).

FtsH also degrades SsrA-tagged proteins where the SsrA-tag consists of 11 residues added to stalled nascent chains during translation to enable ribosome recycling and remove of abnormal proteins from the cell (Lies and Maurizi, 2008; Herman et al., 1998). In another case, FtsH can degrade *E. coli* apo-flavodoxin in *in vitro* proteolytic

tests but the effect of FtsH on flavodoxin levels *in vivo* is still unknown (Okuno et al., 2006a; Okuno et al., 2006b).

FtsH is considered as the only essential AAA protein in *E. coli* due to its regulation on the level of LpxC, the key enzyme in lipid A biosynthesis. Both too much and too little lipid A is lethal for *E. coli*. Thus, FtsH maintains a sufficient amount of lipid A within the cells. FtsH also plays a dual role in LPS biosynthesis by degrading KdtA, a KDO transferase, catalyzes the KDO attachment to lipid A (Katz and Ron, 2008). Therefore, FtsH acts as the crucial protease required for protein and membrane lipid homeostasis (Narberhaus et al., 2009).

Another important function of FtsH is to regulate expression of σ^{32}, the heat shock sigma factor required for heat shock or other stress responses in *E. coli*. Regulation of σ^{32} by FtsH is assumed to involve its association with the DnaKJ chaperone system in which the DnaK chaperone is assumed to have a positive role in the degradation by presenting σ^{32} to FtsH (Tatsuta et al., 2000; Tatsuta et al., 1998; Tomoyasu et al., 1998).

FtsH also affects the proteolytic degradation of the alternative sigma factors SigF (σ^{F}) in *C. crescentus* that indirectly regulates the oxidative stress response in stationary phase (Varez-Martinez et al., 2006). The σ^{W} of *B. subtilis* might be another substrate of FtsH (Zellmeier et al., 2003). The Spo0E phosphatase involved in dephosphorylation of Spo0A~P has been shown to be a substrate of FtsH, and the recognition sequence is located in the C-terminal end (Le and Schumann, 2009). SpoVM has been shown to be a target and an inhibitor of the FtsH protease (Cutting et al., 1997). It shares structural similarities with λ CIII, another target and inhibitor of FtsH in *E. coli*, implying that both proteins share comparable inhibition and degradation mechanisms toward to FtsH (Kobiler et al., 2007).

FtsH is involved in nitrogen metabolism in *Corynebacterium glutamicum* due to its degradation of the GlnK protein, a response protein for nitrogen starvation. Under nitrogen starvation conditions, GlnK interacts with AmtR to induce expression of nitrogen starvation genes. In the medium with high nitrogen concentrations, GlnK is sequestered to the cytosolic membrane to interact with the transporter AmtB, which results in blocking ammonium uptake (Strosser et al., 2004).

In *Synechocystis sp.* PCC 6803, a phototropic model organism that possesses four copies of the *ftsH* gene in its genome, FtsH2 is thought to be involved in osmoregulation

by degradation of the cytoplasmic glycosyl glycerol (GG) synthase GgpS (Stirnberg et al., 2007). This uncomplexed GgpS is degraded by FtsH2 when it fails to form a complex with the GG phosphate phosphatase GgpP to catalyze GG synthesis.

In summary, FtsH is a protease with many talents that degrades a wide variety of structurally and functionally diverse substrates present either in the cytoplasm or in the cytoplasmic membrane. Numerous FtsH substrates have been identified in various bacteria and shown in Table 1.2 (Narberhaus et al., 2009). However, a great deal of FtsH substrates remain to be discovered to clarify the physiological importance of FtsH in prokaryotic organisms as well as in eukaryotic cells (Narberhaus et al., 2009).

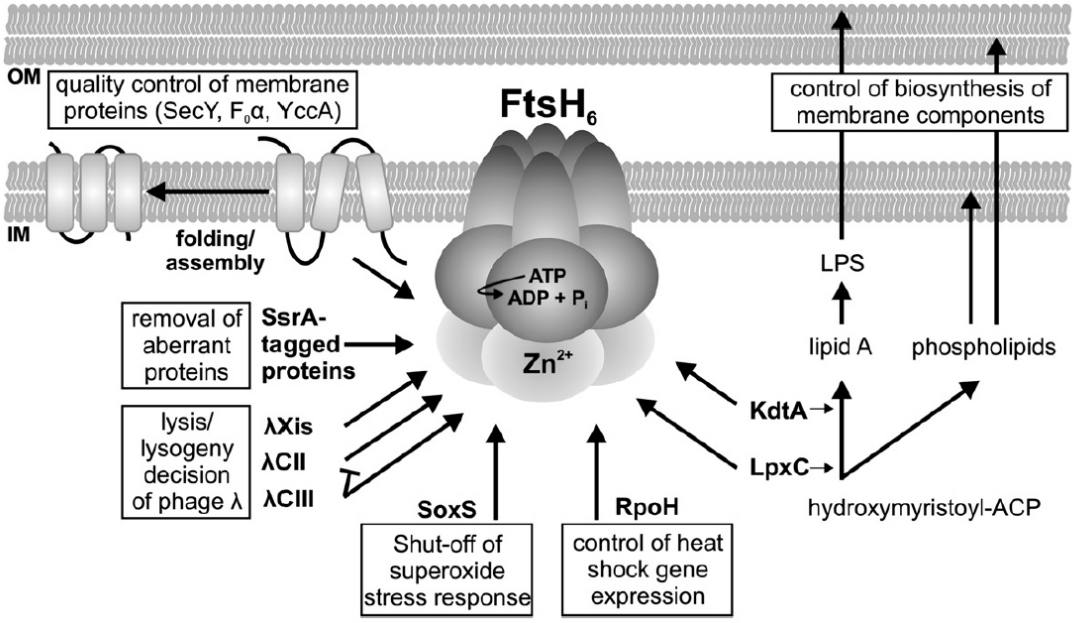

Fig. 1.8. Schematic view of FtsH functions in E. coli. *The hexameric FtsH protease controls quality of membrane proteins by either refolding misfolded proteins or degrading unassembled membrane proteins. FtsH degrades λ-encoded substrates, and is involved in the superoxide stress response, heat shock gene expression and controls the synthesis of membrane components. IM: inner membrane; OM: outer membrane; LPS: lipopolysaccharides. This figure was taken from Narberhaus et al., 2009.*

Table 1.2. Identified cytoplasmic substrates of the FtsH protease in bacteria.

Adaptor or modulator proteins and localization of degradation signal are given if analyzed; ND: not determined. This table was taken from the Narberhaus et al., 2009.

Protein	Organism	Adaptor/modulator proteins; Localization of degradation signal
SsrA-tag	*E. coli*	The tag itself
λ CII	Phage λ/*E. coli*	HflD, HflK/C; C-terminus
Λ CIII	Phage λ/*E. coli*	Internal
Λ Xis	Phage λ/*E. coli*	ND
SoxS	*E. coli*	N-terminus (Lon)
Flavodoxin	*E. coli*	Internal
LpxC	*E. coli*	C-terminus
KdtA	*E. coli*	ND
RpoH (σ^{32})	*E. coli*	DnaK/J, GroEL/ES; internal
RpoH (σ^{32})	*C. crescentus*	ND
σ^{F}	*C. crescentus*	ND
σ^{W}	*B. subtilis*	ND
SpoVM	*B. subtilis*	Internal
Spo0E	*B. subtilis*	C-terminus
GgpS	*Synechocystis sp. PCC 6803*	ND
GlnK	*C. glutamicum*	ND

1.3. The objective of the thesis

As already mentioned, a *B. subtilis ftsH* null mutant is viable, but exhibits a pleiotropic phenotype including a drastically reduced sporulation efficiency (Deuerling et al., 1997; Le and Schumann, 2009). Further analysis has shown that the amount of Spo0A is significantly reduced in such a knockout mutant (Le and Schumann, 2009). I hypothesized that FtsH may degrade one or more proteins involved in reducing the level of phosphorylated Spo0A. One sporulation-specific protein has been recently identified, the phosphatase Spo0E, which specifically dephosphorylates Spo0A~P (Le and Schumann, 2009). Since a *spo0E ftsH* double knockout restored the sporulation frequency to only 0.85% (wild type: ~ 60%), additional protein(s) have to be identified as substrate(s) of FtsH. Therefore, the objective of this doctoral thesis was first to identify additional substrate proteins by using two different techniques and second, to understand their function. Two experimental approaches were applied. The first is 2D-gel proteomics.

The hypothesis for this approach is the substrates might be overproduced in an *ftsH* null mutant when compared with an *ftsH* wild-type strain. By the 2D gel electrophoresis technique, these proteins can be detected and identified by mass spectrometry. Another approach is called "FtsH trap-mutant". The aim of this approach was to construct an FtsH mutant which binds substrates without cleaving them. Therefore, the substrates can be trapped in the proteolytic chamber of FtsH *in vivo* and co-purified with FtsH by a pull-down assay. Finally, the role of the *eag* gene located downstream of *spo0E* was analyzed.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strains

The bacterial strains used in this study are listed in Table 2.1

Table 2.1. Bacterial strains used in this study

Strains	Description	Source
	Escherichia coli	
DH10B	*mcrA* Δ(*mrr hsdRMS mcrBC*) φ80*d lacZ*M15 Δ*lacX74 deoR recA1 araD139* Δ(*ara leu*)*7697*	Bethesda Research Laboratories (BRL)
BL21	*E. coli* B F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal*	BRL
A8926	*sfhC zad-220::Tn10 ΔftsH3::kan*	Tatsuta et al., 1998
BHEQ	A8926 P_{IPTG} *–ftsHE424Q* (Amp^R)	This study
AL60	A8926 P_{IPTG} *–GST-FtsH* (Amp^R)	Le and Schumann, 2009
	Bacillus subtilis	
1012	*leuA8 metB5 trpC2 hsrM1*	Saito et al., 1979
WW01	1012 *ftsH::erm* (Erm^R)	Wehrl et al., 2000
BH1	1012 P_{spo0M} - *bgaB* (Neo^R)	This study
BH2	1012 P_{spo0M} - *bgaB ftsH::erm* (Neo^R) (Erm^R)	This study
BH3	1012, *pbgaB* (Neo^R)	This study
BH4	1012 P_{IPTG}-*GST-fstH*^{trap} (Cm^R)	This study
BH5	1012 P_{IPTG}-*GST-ftsH*$^+$ (Cm^R)	This study
BH6	1012 P_{IPTG}-*GST* (Cm^R)	This study
BH7	1012 P_{IPTG}-GST-*ftsH*^{trap} Δ*ftsH::erm* (Cm^R) (Erm^R)	This study
BH8	1012 P_{IPTG}-*GST-ftsH*$^+$ Δ*ftsH::erm* (Cm^R) (Erm^R)	This study

BH9	1012 P_{IPTG} *-GST* Δ*ftsH::erm* (Cm^R) (Erm^R)	This study
AB07	1012 Δ*spo0E::bleo* ($Bleo^R$)	Le and Schumann, 2009
AM01	1012 Δ*eag::*pMUTIN4 (Erm^R)	A. Maier
AM02	1012, $P_{skf\text{-}lacZ}$, *eag::*Pmutin4 (Spc^R) (Erm^R)	A. Maier
AM03	1012, *amyE::*$P_{skf\text{-}lacZ,}$ (Spc^R)	A. Maier

2.1.2. Plasmids

The plasmids used in this study are listed in Table 2.2

Table 2.2. Plasmids used in this study

Name	Description	Reference
pBgaB	Integration plasmid carrying the promoter-less *bgaB* gene, Neo^R	Mogk et al., 1996
pBH1	*spo0M* promoter inserted into pBgaB, Neo^R	This study
pGEX-2T	Expression vector with GST-tag, Amp^R	Amersham
pBH2	pGEX-2T with GST – *spo0M* fusion, Amp^R	Le and Schumann, 2009
pGST-*ftsH*	$ftsH^+$ gene from *B. subtilis* inserted into pGEX-2T, Amp^R	This study
pBH3	pGEX2T carrying *ftsHE424Q* mutant, Amp^R	This study
pHT08	A plasmid-based expression vector for *B. subtilis* with the IPTG-inducible P_{grac} promoter, Cm^R	Nguyen et al., 2007
pBH4	Fusion GST - *ftsHE424Q* mutant inserted into pHT08, Cm^R	This study
pBH5	Fusion GST- $ftsH^+$ inserted into pHT08, Cm^R	This study
pBH6	GST inserted into pHT08, Cm^R	This study
pMUTIN4	Integration plasmid to create a gene fusion with the *lacZ* reporter gene, Erm^R	Vagner et al., 1998
pMUTIN4-*eag*	*eag* gene inserted into pMUTIN4, Erm^R	A. Maier

2.1.3. Oligonucleotides

The oligonucleotides used in this study are listed in Table 2.3

Table 2.3. Oligonucleotides used in this study

Name	Sequence (5' to 3')	Description
ON01	CACCAGGAATTCATCGGTCTAAACTGAAATCG	5' end of *spo0M* promoter
ON02	CACCAGGAATTCTCCGGCACTTGCCGCAAGCTT	3' end of *spo0M* promoter
ON03	TCTGTTGGATCCATGTCATTTTTTAAGAAGCTTGCGGCA	5' end of *spo0M* gene
ON04	TCCCGGGGATCCCTATTACTCAACGTATTGGTCTAGGATCT	3' end of *spo0M* gene
ON05	CTTATCACCAAGGCGGACACACCGT	mutagenic primer with substitution of *FtsHE424Q*
ON06	CAATTCAAGCTTGTCACGATTTTCAGTCAGGA	flanking at 3' end of *ftsH* gene
ON07	CACCATGGATCCATGAATCGGGTCTTCCGTAATACCA	5' end of *ftsH* gene
ON08	CACCATGACGTCATGTCCCCTATACTAGGTTATTGGA	5' end of *ftsH* gene
ON09	CACCATGACGTCATTACTCTTTCGTATCGTCTTTCT	3' end of *ftsH* gene
ON10	CACCATGGATCCATGTCCCCTATACTAGGTTATTGGA	5' end of *GST* gene
ON11	CTGGTGGGATCCTTATCAAACAGATGCACGACGAGATCCA	3' end of *GST* gene

2.1.4. Media

Luria-Bertani broth (LB medium): 1 % ($^{w}/_{v}$) tryptone, 0.5 % ($^{w}/_{v}$) yeast extract, 1 % ($^{w}/_{v}$) NaCl.

Difco Sporulation Medium (DSM): 0.8 % ($^{w}/_{v}$) Nutrient Broth, 0.1 % ($^{w}/_{v}$) KCl and 1 mM $MgSO_4.7H_2O$. Adjust the pH to 7.4 with KOH. After autoclaving, the medium was supplied with 0.5 mM $CaCl_2$, 0.01 mM $MnCl_2$ and 0.001 mM $FeCl_2$

Agar was added to 1.5 % ($^{w}/_{v}$) to prepare plates.

2.1.5. Antibiotics

Concentrations of the antibiotics used in this study are given in Table 2.4.

Table 2.4. Antibiotic solutions used in this study

Antibiotic	Concentration of stock solution (mg/ml)	Dissolved in	Final concentration (µg/ml)
Ampicillin	50 - 100	70% ethanol	100
Chloramphenicol	20	Ethanol	10
Erythromycin	1 or 100	Ethanol	1 or 100
Neomycin	10	Water	10
Spectinomycin	100	Water	100
Bleomycin	20	Water	1 or 5

2.1.6. Chemicals and enzymes

All standard enzymes and chemicals used for common buffers and solutions were purchased from Sigma-Aldrich, Merck or Roth, Karlsruhe, Germany. Other chemicals, solutions, buffers and kits were purchased from the suppliers listed below:

- New England Biolabs: Taq DNA polymerase, T4 DNA-Ligase
- Fermentas: Restriction enzymes, DNA ladder and Protein Molecular Weight Marker
- Amersham: ECL™ Reagent, antibody
- Pierce: *Dithiobis[succinimidyl propionate]* (DSP) cross linker
- Qiagen: PCR purification kit, gel-extraction kit, midi purification kit
- Roche: Complete Protease inhibitor cocktail, Alkaline phosphatase, Lysozyme, Proteinase K, RNase A

2.1.7. Antibodies

Table 2.5. Antibodies used in this study

Name	Dilution for Immunoblotting	Reference
Spo0A	1 : 5000	Fujita et al., 2005
GST	1 : 5000	Amersham™
Anti-Rabbit IgG	1: 10000	Amersham™

2.2. Methods

2.2.1. Identification of FtsH substrates by proteomics

2.2.1.1. Growth conditions

B. subtilis strains 1012 and its isogenic *ftsH* mutant (WW01) were cultivated at 37°C under vigorous agitation in Difco Sporulation Medium (DSM), erythromycin was added to a final concentration of 50 μg/ml during cultivation of WW01 strain (Δ*ftsH::erm*). Samples were taken at stage 0 (t_o) and experiments were repeated twice.

2.2.1.2. Sample preparation

Cells were harvested at stage 0 (t_o) by centrifugation (6.000 x g, 4°C, 10 min), washed in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA), resuspended in TE urea buffer (8 M urea, 2 M thio-urea) and disrupted by ultrasonication. After centrifugation (20,000 x g, 4°C, 30 min), the protein concentration of the extract was determined with the RotiNanoquant Kit (Roth, Karlsruhe, Germany).

2.2.1.3. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

To separate by 2D-PAGE, protein extracts (500 mg protein/sample) were loaded onto duplicate immobilized pH gradient (IPG) strips, pH 4-7 by rehydration for 18-24 h in

a solution containing 8 M urea, 2 M thiourea, 20 mM DTT, 1% w/v CHAPS and 0.5% v/v Pharmalyte 3-10.

The isoelectric focusing (IEF) step was carried out by using the Multiphor ll unit (Amersham Pharmacia Biotech) with voltage profile applied: linear increase from 0 to 500 V for 500 V/h, 500 V for 2500 V/h, linear increase from 500 to 3500 V for 10 000 V/h, and a final phase of 3500 V for 35 000 V/h (Büttner et al., 2001).

The IPG strips were sequentially equilibrated 2 x 15 min in SDS - equilibration buffer (50 mM Tris-HC1, pH 8.5, 2% w/v SDS, 6 M urea, 30% w/v glycerol, 1% bromophenol blue) first containing DTT (1% w/v) and followed by equilibration buffer containing iodoacetamide (5% w/v), then washed with SDS - PAGE running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) (Görg et al., 1995).

The second dimension separation was done in polyacrylamide gels of 12.5% acrylamide and 2.6% bisacrylamide using Investigator 2-D electrophoresis system (Genomic Solutions, Chelmsford, MA, USA). Gels were routinely run overnight at 2 W/gel with cool temperature (15^{o}C) (Büttner et al., 2001). After fixing in a solution containing 40% v/v ethanol, 10% acetic acid for 1 h, gels were stained for 20 h in the Coomassie Blue staining solution (10% v/v phosphoric acid, 10% w/v ammonium sulfate, 0.12% w/v Coomassie G-250) diluted with methanol (20% v/v, final concentration). Removal of excess Coomassie stain was done by washing gels two times with distilled water (Voigt et al., 2004).

2.2.1.4. Proteome analysis and mass spectrometry for protein identification

After staining, the gels were scanned in transmission mode at a resolution 300 dpi with a color depth of 8 bit - 256 gray levels. For comparing the gels, the quantitative analysis of the 2-D PAGE images were analyzed by the Delta 2D software using dual channel imaging (Bernhardt et al., 1999). The gel images were organized into two groups: wild-type and knock-out *ftsH* and defined with separate colors: green and red, respectively. The corresponding spots between the two groups were matched to create a combined image and spot density values of each pair of protein spots were calculated based on their colors (http://www.decodon.com/Support/Documentation/4.1/getting_started/de/Quickguide.pdf) Protein spots showing an induction of at least two-fold at stationary phase were excised, digested with trypsin and analyzed by MS/MS (Voigt *et al.*, 2004).

2.2.1.5. Construction of plasmids and recombinant strains

2.2.1.5.1. Construction of pBH1 for the spo0M transcription analysis

Plasmid pBH1 carrying the transcriptional fusion between the *spo0M* promoter region and the *bgaB* reporter gene (P_{spo0M}-*bgaB*) was constructed by PCR amplification of the *spo0M* promoter region using primers ON1 and ON2 with chromosomal DNA of strain 1012 as template, inserted at the unique *Eco*RI site of pBgaB (an integrative pBgaB vector allowing insertion of the transcriptional fusion at the *amyE* locus (Mogk et al., 1996) (Fig. 2.1), transformed into *B. subtilis* 1012 and selected on LB plates containing neomycin to result in strain BH1. The *ftsH::erm* mutation was subsequently introduced into the strain BH1 by transformation yielding strain BH2.

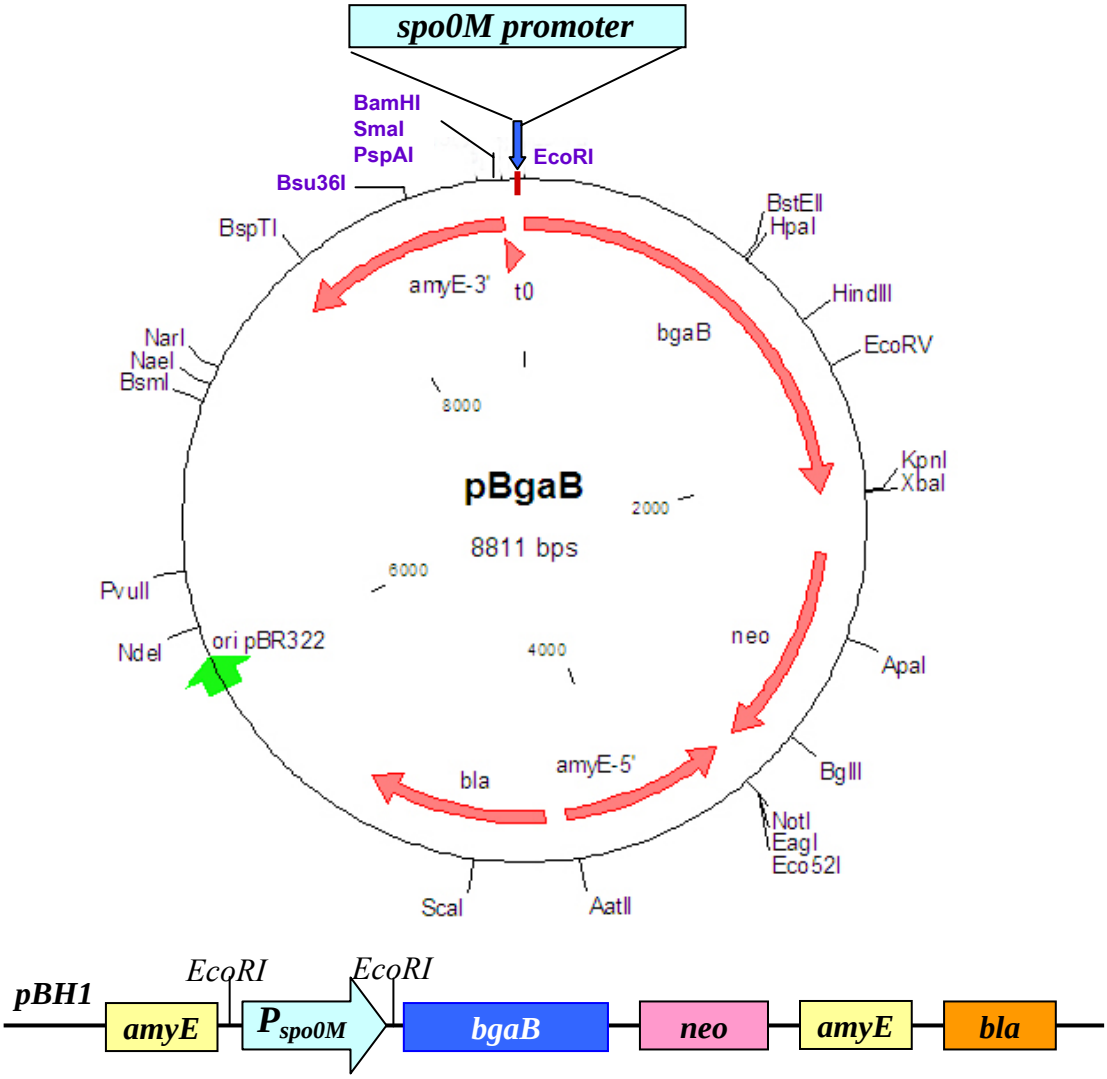

Fig 2.1: Construction of pBH1 for the spo0M transcription analysis

2.2.1.5.2. Construction of pBH2 for spo0M expression

spo0M gene was amplified from *B. subtilis* 1012 strain as a template using the primers ON3 and ON4, inserted downstream of the glutathione S-transferase (*gst*) gene at the unique site *Bam*HI in pGEX-2T resulting in plasmid pBH2 (Fig. 2.2). The plasmid was transformed into strain BL21 resulting in strain BH2 that allows expression of the fusion protein GST-Spo0M induced by IPTG and its purification by a GSTrap-HP-column (Amersham).

Plasmid pGST-*ftsH* for production of GST-FtsH carrying the *ftsH* gene from *B. subtilis* was constructed by Ai Le (Le and Schumann, 2009) and transformed into the *E. coli* strain A8926, an *E. coli* strain carrying an *ftsH* knockout to avoid producing two types of *ftsH* genes, one amplified from *E. coli* and the other from the *B. subtilis* DNA template (Tatsuta et al., 1998).

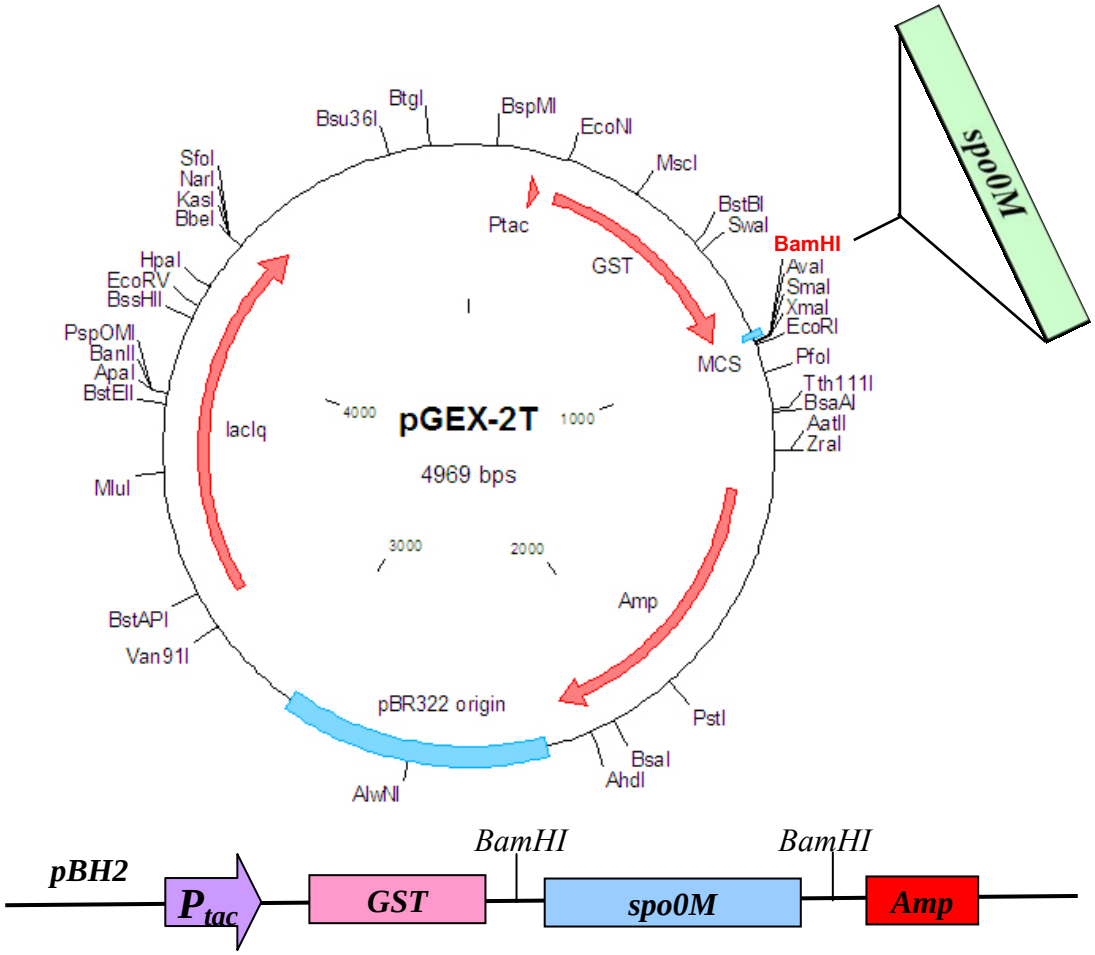

Fig 2.2: Construction of pBH2 for spo0M expression

2.2.1.6. Expression and purification of GST-tagged proteins

Expression of the GST-tagged Spo0M was induced by addition of 1 mM IPTG (final concentration) to the cell cultures in LB/Amp medium, at an OD_{600} of 0.8, 37°C. Then, the cultures were transferred to 25°C and further grown overnight. Cells were harvested by centrifugation (10 min, 8.000 x g, 4°C), resuspended in lysis buffer (140 mM NaCl, 10 mM Na_2HPO_4, 27 mM KCl, 1.8 mM KH_2PO_4, pH 7.3) containing 20 mM DTT, 10 µl Complete Protease Inhibitor Cocktail (Roche Diagnostics), and lysed by ultrasonication.

Samples were then centrifuged and the supernatants were used for purification using a 1-mL GSTrap HP column coupled to the Äkta-Purifier FPLC system (GE Healthcare, Munich, Germany).

2.2.1.7. β-Galactosidase assays

Cells were grown in DSM at 37°C and samples were harvested at the time points indicated by centrifugation, frozen immediately and stored at -80°C. The experiments were repeated three times. Before analysis, the frozen cells were resuspended in LacZ buffer (Miller, 1972) containing 0.1 mM PMSF and 100 µg of lysozyme per ml and disrupted by ultrasonication. Samples were assayed for BgaB activity as described (Mogk *et al.*, 1996).

2.2.1.8. Proteolysis experiments

Degradation reactions were carried out as described (Tomoyasu et al., 1995). The complete reaction mixture (30 µl) comprised of following components: 50 mM Tris/acetate (pH 8.0), 12.5 mM zinc acetate, 5 mM magnesium acetate, 50 mg/ml of BSA, 80 mM NaCl, 1.4 mM β-mercaptoethanol, 5 mM ATP, 100 mg/ml of the target GST-tagged protein (or 100 mg/ml of β-casein, used as a positive control for the proteolytic reaction by purified GST-FtsH), 50 mg/ml of purified *B. subtilis* GST-FtsH. Reactions were carried out at 37°C for the time points indicated, and samples were analyzed by 15% SDS-PAGE and Coomassie blue staining.

2.2.2. Identification of FtsH substrates by the trap-mutant approach

2.2.2.1. Construction of FtsH^{trap}

To construct an FtsH^{trap}, a conserved glutamate residue in the Zn-binding motif (HEEXH) of FtsH was replaced with glutamine (E424Q) to inactivate the protease activity of FtsH. A two-step PCR using an internal primer ON5 carrying a designed E424Q mutation and two different flanking primers, ON6 and ON7, were applied.

In the first step, the mutagenic primer ON5 and a reverse flanking primer ON6 with p*GST-ftsH* as a template was used to generate the "megaprimer" carrying the E424Q mutant. In the second step, the megaprimer and the forward flanking primer ON7 were used to amplify an *ftsH* mutant flanking by *Bam*HI and *Hin*dIII sites. The plasmid p*GST-ftsH* was cleaved with *Bam*HI and *Hin*dIII to remove the wild-type *ftsH* allele and replaced by *ftsHE424Q* mutant allele to obtain plasmid pBH3 followed by transformation into *E. coli* A8926 resulting in strain BHEQ allowing expression of the *ftsH* mutant (FtsH^{trap}) under control of the IPTG-inducible promoter.

2.2.2.2. Construction of plasmids and strains in *B. subtilis* for protein trapping by FtsH^{trap} *in vivo*

Plasmids expressing *GST-ftsH^{trap}* and *GST-ftsH^{+}* in *B. subtilis* were constructed by PCR using primers ON8 and ON9 to amplify *GST-ftsH^{trap}* and *GST-ftsH^{+}* with p*GST-ftsHE424Q* and p*GST-ftsH* as template, respectively. These amplification products were cleaved with *Aat*II, cloned into the *Aat*II fragment of pHT08 (a plasmid-based IPTG-inducible expression vectors for *B. subtilis*) to obtain plasmids pBH4 and pBH5 (Fig. 2.3). These plasmids were transformed into *B. subtilis* 1012 resulting in strains BH4 and BH5, allowing GST expression under control of the IPTG-inducible *Pgrac* promoter. An *ftsH::erm* knockout allele was introduced into these strains to obtain strain BH7 and BH8, respectively.

As a negative control for *in vivo* protein trapping, a plasmid expressing GST was constructed by PCR amplification of the GST gene using primers ON10 and ON11 with pGEX-2T (Fig. 2.3). This plasmid was transformed into *B. subtilis* 1012 resulting BH6 strain. Next, an *ftsH::erm* knockout was introduced into strain BH6 to obtain strain BH9.

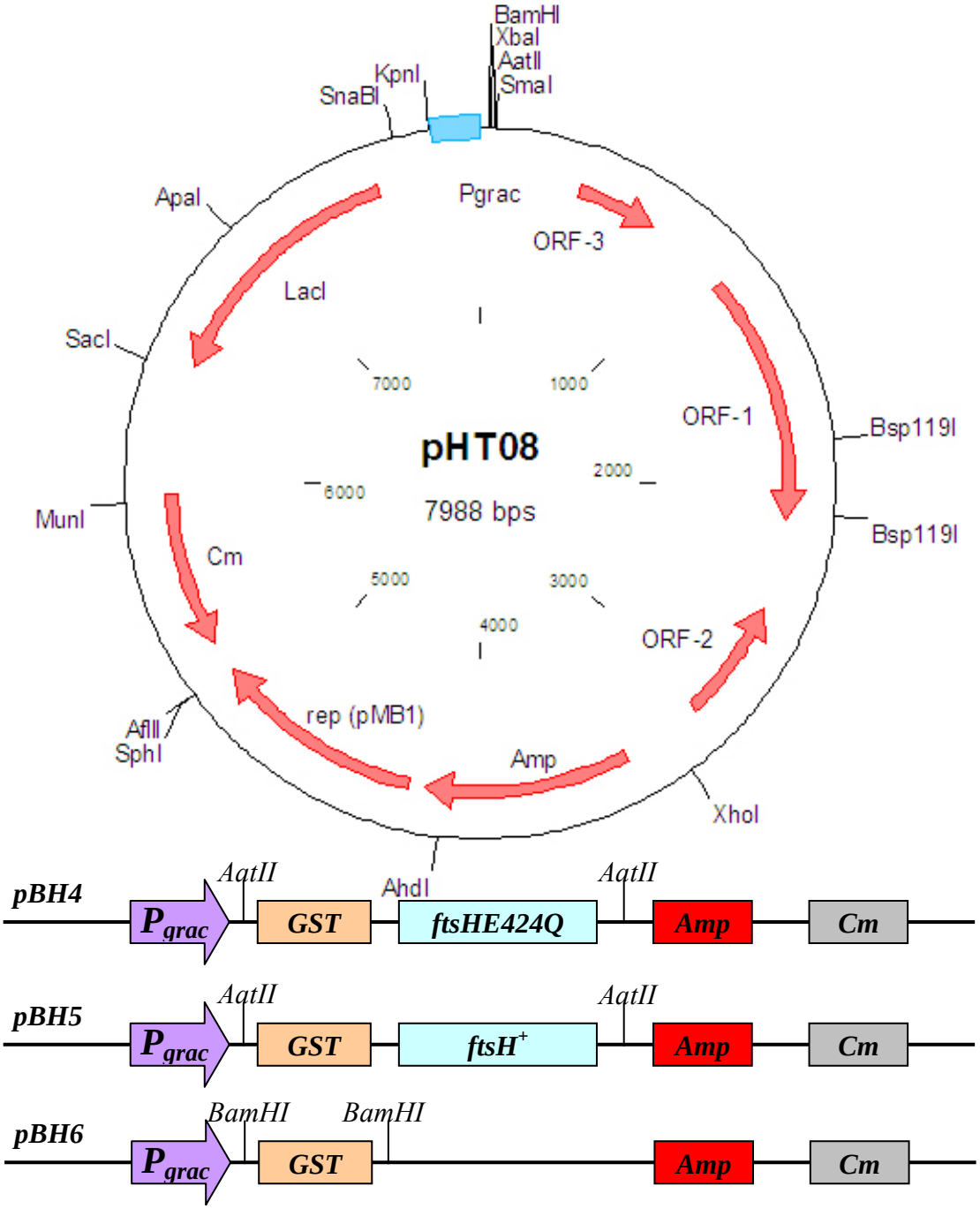

Fig 2.3: Construction of plasmids pBH4, pBH5 and pBH6 for in vivo protein trapping using the FtsH^{trap} mutant. *The amplicons of GST-ftsH^{trap}, GST-ftsH^{+} and the GST gene were inserted into pHT08 resulting in pBH4, pBH5, pBH6 allowing expression of GST-ftsH^{trap}, GST-ftsH^{+} and GST protein in B. subtilis under control of the IPTG-inducible P_{grac} promoter.*

2.2.2.3. Complementation of the *ftsH* alleles in an *ftsH* knockout strain

2.2.2.3.1. Morphology complementation in the wild-type and ftsH^{trap}

Cells of *ftsH* knockout strains BH7 and BH8 allowing *ftsH^{trap}* and *ftsH^{+}* expression under control of the IPTG-inducible P_{grac} promoter were grown in DSM medium, induced with IPTG at an OD_{578} of 0.5, collected samples at stationary phase, washed and resuspended in ddH_2O. Mixtures of 5 µl of suspensions with 10 µl of 1% agarose were spread onto a glass slide and the cell's morphology was observed under the microscope.

2.2.2.3.2. Sporulation complementation

B. subtilis strains were inoculated into DS medium and incubated with shaking for 24 h at 37°C. The experiments were repeated twice. Cells were harvested from 10 ml cultures, resuspended in 1 ml potassium phosphate buffer (10 mM K_2HPO_4, 50 mM KCl, 1 mM $MgSO_4$) and heated for 30 min at 80°C. Samples of the heated cultures as well as of the untreated parental culture were diluted and plated on DS medium for viable cell counting.

2.2.2.4. Identification of FtsH substrates by the pull-down assay

2.2.2.4.1. Sample preparation for protein trapping in vivo

Strains BH7, BH8, BH9 for expression of GST-FtsH^{trap}, GST-FtsH^{+} and GST, respectively, were routinely grown in 1 liter of DSM with chloramphenicol 5 µg/ml and erythromycin 1 µg/ml at 37°C to an OD578 of 0.5, induced with 0.1 mM IPTG and further grown until cells reached the transition state (stage 0 of sporulation). The experiments were repeated three times.

Then, the cultures were taken and chilled. Cells were harvested by centrifugation at 4°C, 8.000 x g, washed and resuspended in 7 ml of lysis buffer (140 mM NaCl, 1,8 mM KH_2PO_4, 27 mM KCl, 10 mM Na_2HPO_4, 5% glycerol, pH 7.3) containing 20 mM DTT, 10 µl Complete Protease Inhibitor Cocktail (Roche Diagnostics). Cells were disrupted by using a Mixer Mill at 15 Hz, 5 x 30s.

2.2.2.4.2. Ex vivo cross - linking with DSP

After disruption of the cells by the Mixer Mill, freshly DSP *(Dithiobis-[succinimidylpropionate])* stock at 100 mg/ml was prepared in DMSO, 150 µl DSP stock was added into 7 ml cell lysate and allowed cross-linking reaction proceeding for 5 min at room temperature. Then, the unreacted DSP was quenched by adding 100 mM Tris pH 8.5, and stirred for 5-10 min at room temperature.

After crossed linking with DSP, the lysates were centrifuged for 30 min at 21.000 x g to separate the cytoplasmic fraction (supernatant) from the membrane fraction (pellet). The membrane proteins in the pellet were solubilized with the non-ionic detergent NP-40 (final concentration 0.5%), the cell debris were removed by centrifugation (25.000 x g, 4^{o}C, 30 min) and collected as the supernatant containing membrane fraction.

2.2.2.4.3. Pull-down assay for FtsH substrate trapping in vivo

The membrane fraction and the supernatant were added to 1 ml glutathione agarose beads, gently agitated at 4^{o}C for 4 h, washed with 5 volumes of PBS buffer (140 mM NaCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.3), eluted in 0.5 ml elution buffer (10 mM GSH reduced glutathione in 50 mM Tris-HCl, pH 8.0), and the elution fraction was taken for analyses by SDS-PAGE. The protein bands were detected by silver staining and individual proteins were identified by Mass spectrometry.

2.2.2.5. SDS-PAGE and Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was peformed according to the method of Laemmli (1970) using 10, 12 or 15% SDS-polyacrylamide gels.

Western blotting was performed as described by Towbin et al., (1979) with the detection step using an ECL Western blotting detection kit (Amersham). The signals were recorded by the LAS4000 machine (Fujifilm) and analyzed by Multi Gauge Ver 3.1 Software (Fujifilm).

2.2.2.6. Silver Staining

Gels were fixed by Fixing Solution (50% Ethanol, 10% glacial acetic acid, 0.05% formalin [35% formaldehyde]) and then shaken for 1 h. Gels were rinsed with Rinse Solution (50% Ethanol) and shaken for 5 min (2X). The Rinse Solution was replaced with Sensitizer (0.02% sodium thiosulphate), shaken for 2 min and then washed with milli-Q water and shaken for another 2 min. Gels were stained with Staining solution (0.2% silver nitrate, 0.075% formaldehyde 37% v/v) and shaken for 20 min. Then, the gel was washed with water for 1 min (5 - 6X) and developed with Developer Solution (5% sodium carbonate, 0.05% formaldehyde 37% v/v, 0.0004% sodium thiosulphate), shaken for 5 - 10 min as required. A Stop Solution was added to stop the reaction. Gels were washed with milli-Q water (3X) and kept at 4ºC in 1% glacial acetic acid for analyses and FtsH substrate identification by mass spectrometry.

3. RESULTS

3.1. Identification of FtsH substrate proteins by proteomics

3.1.1. Identification of the Spo0M protein as a putative substrate of FtsH by 2D-gel electrophoresis

To identify putative substrates of FtsH, the proteomes of a wild type *ftsH* and its isogenic *ftsH::erm* knockout strain taken at stage 0 were analyzed by 2D-gel electrophoresis and individual protein spots were identified by mass spectrometry. The protein quantity of each spot in both strains was compared and analyzed by the Delta 2D software. Approximately 50 proteins were strongly increased or decreased in the *ftsH* knockout strain as compared to the wild type strain (Table 3.1 and Table 3.2). According to the SubtiList functional categories, the identified proteins were classified into functional groups and most of them perform basic metabolic functions in the cell such as translation, amino acid metabolism, glycolysis and being part of the tricarboxylic acid (TCA) cycle (Table 3.3 and Table 3.4).

Table 3.1. List of proteins increasing in an ftsH knockout strain

No.	Ratio	Accession number	Protein name
1	4.54962	Spo0M	Sporulation-control gene
2	3.73728	YjoA	Unknown similar to unknown proteins
3	3.13626	Tkt	Transketolase
4	3.04021	TufA	Elongation factor Tu
5	2.84877	GlnA	Glutamine synthetase
6	2.59722	YoxD	Unknown similar to 3-oxoacyl- acyl-carrier protein reductase
7	2.38021	SodA	Superoxide dismutase
8	2.36637	YjbG	Unknown similar to oligoendopeptidase
9	2.3513	Icd	Isocitrate dehydrogenase
10	2.34449	Dat	Probable D-alanine aminotransferase
11	2.31573	FabI	Enoyl-acyl carrier protein reductase
12	2.31573	YvqH	Unknown similar to unknown proteins from *B. subtilis*
13	2.31118	Ddl	D-Alanyl-D-alanine ligase A
14	2.31118	Pgk	Phosphoglycerate kinase
15	2.28535	YvgN	Unknown similar to dehydrogenase
16	2.27029	BkdB	Lipoamide acyltransferase
17	2.19324	YceC	Unknown similar to tellurium resistance protein
18	2.19095	Hag	Flagellin protein
19	2.15821	Pyk	Pyruvate kinase
20	2.12796	Tpx	Probable thiol peroxidase
21	2.0957	Eno	Enolase
22	2.0957	SucC	Succinyl-CoA synthetase (beta subunit)
23	2.08144	YvaB	Unknown similar to NAD(P)H dehydrogenase (quinine)
24	2.05179	GcvPB	Probable glycine decarboxylase (subunit 2)
25	2.05179	YumC	Unknown similar to thioredoxin reductase
26	2.00393	AsnS	Asparaginyl-tRNA synthetase
27	2.00393	CysS	Cysteinyl-tRNA synthetase
28	2.00393	DhaS	Aldehyde dehydrogenase

Table 3.2. List of proteins decreasing in an ftsH knockout strain

No.	Ratio	Accession number	Protein name
1	0.49203	Drm	Phosphopentomutase
2	0.49203	RocD	Ornithine aminotransferase
3	0.48638	GapB	Glyceraldehyde-3-phosphate dehydrogenase
4	0.48287	PyrAB	Carbamoyl-phosphate synthetase (catalytic subunit)
5	0.48002	CysC	Putative adenylylsulfate kinase
6	0.48002	PyrH	Uridylate kinase
7	0.47701	NusG	Transcription antitermination factor
8	0.46497	PnpA	Polynucleotide phosphorylase (PNPase)
9	0.46428	Fmt	Methionyl-tRNA formyltransferase
10	0.44283	ResD	Two-component response regulator involved in aerobic and anaerobic respiration
11	0.44283	SucD	Succinyl-CoA synthetase (alpha subunit)
12	0.44091	PdhC	Pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit)
13	0.43359	Upp	Uracil phosphoribosyltransferase
14	0.42343	OppD	Oligopeptide ABC transporter (ATP-binding protein) (initiation of sporulation, competence developme
15	0.41514	RocG	Glutamate dehydrogenase (major)
16	0.39438	OdhA	2-Oxoglutarate dehydrogenase (E1 subunit)
17	0.38879	IolD	Myo-inositol catabolism

Table 3.3. Functional groups of proteins increasing in the absence of FtsH according to the SubtiList database

COGs	Protein Name	Functional groups
		Information storage and processing
COG0050J	TufA	Elongation factor Tu
COG0017J	AsnS	Asparaginyl-tRNA synthetase
COG0215J	CysS	Cysteinyl-tRNA synthetase
		Cellular processes and signaling
COG2310T	YceC	Unknown similar to tellurium resistance protein
COG1181M	Ddl	D-alanyl-D-alanine ligase A
COG1344N	Hag	Flagellin protein
COG2077O	Tpx	Probable thiol peroxidase
COG0492O	YumC	Unknown similar to thioredoxin reductase
		Metabolism
COG0538C	Icd	Isocitrate dehydrogenase
COG0508C	BkdB	Lipoamide acyltransferase
COG0045C	SucC	Succinyl-CoA synthetase (beta subunit)
COG1012C	DhaS	Aldehyde dehydrogenase
COG0021G	Tkt	Transketolase
COG0126G	Pgk	Phosphoglycerate kinase
COG0469G	Pyk	Pyruvate kinase
COG0148G	Eno	Enolase
COG0174E	GlnA	Glutamine synthetase
COG1003E	GcvPB	Probable glycine decarboxylase (subunit 2)
COG0115EH	Dat	Probable D-alanine aminotransferase
COG0623I	FabI	Enoyl-acyl carrier protein reductase
COG1182I	YvaB	Unknown similar to NAD(P)H dehydrogenase (quinone)
COG0605P	SodA	Superoxide dismutase
		General function prediction only - function unknown
COG4326R	Spo0M	Sporulation-control gene
COG0300R	YoxD	Unknown similar to 3-oxoacyl- acyl-carrier protein reductase
- NA -	YjbG	Unknown similar to oligoendopeptidase
- NA -	YvqH	Unknown similar to unknown proteins from *B. subtilis*
COG0656R	YvgN	Unknown similar to dehydrogenase
COG2318S	YjoA	Unknown similar to unknown proteins

COG: Clusters of Orthologous Groups; NA: Not Available

Table 3.4. Functional groups of proteins decreasing in the absence of FtsH according to SubtiList database

COGs	Protein Name	Functional groups
		Information storage and processing
COG1185J	PnpA	Polynucleotide phosphorylase (PNPase)
COG0223J	Fmt	Methionyl-tRNA formyltransferase
COG0250K	NusG	Transcription antitermination factor
		Cellular processes and signaling
COG0745TK	ResD	Two-component response regulator involved in aerobic and anaerobic respiration
		Metabolism
COG0074C	SucD	Succinyl-CoA synthetase (alpha subunit)
COG0508C	PdhC	Pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit)
COG0567C	OdhA	2-Oxoglutarate dehydrogenase (E1 subunit)
COG1015G	Drm	Phosphopentomutase
COG0057G	GapB	Glyceraldehyde-3-phosphate dehydrogenase
COG4992E	RocD	Ornithine aminotransferase
COG0444EP	OppD	Oligopeptide ABC transporter (ATP-binding protein) (initiation of sporulation, competence developme
COG0334E	RocG	Glutamate dehydrogenase (major)
COG3962E	IolD	Myo-inositol catabolism
COG0458EF	PyrAB	Carbamoyl-phosphate synthetase (catalytic subunit)
COG0528F	PyrH	Uridylate kinase
COG0035F	Upp	Uracil phosphoribosyltransferase
COG0529P	CysC	Probable adenylylsulfate kinase

The proteomic approach was used to identify FtsH substrates to understand the function of FtsH during sporulation. I hypothesize that these protein substrates are supposed to be overproduced in an *ftsH* knockout and function during sporulation. As a result, among 28 proteins significantly increased in the absence of FtsH, the most abundant protein was identified as Spo0M with its predicted function as a sporulation control gene. The Spo0M level increased about 4.5-fold in the *ftsH* null mutant (Fig. 3.1).

The *spo0M* gene has been shown to control sporulation during the process from stage 0 to stage II (Han et al., 1998). An σ^H-like promoter has been detected in the upstream region of *spo0M*, and it has also been shown to be down-regulated by benzoate at pH 7.0 or by a low external pH (Kitko et al., 2009). A *spo0M* null mutant is viable,

blocked at stage 0, and its sporulation frequency is reduced by 20- to 100-fold. If the *spo0M* gene is inserted into a high-copy number plasmid, the sporulation frequency is reduced, indicating that overproduction of the Spo0M protein results in a negative effect on sporulation (Han et al., 1998).

Since Spo0M is overproduced at the beginning of stage 0 in the absence of FtsH, we first asked whether FtsH regulates expression of Spo0M directly or indirectly.

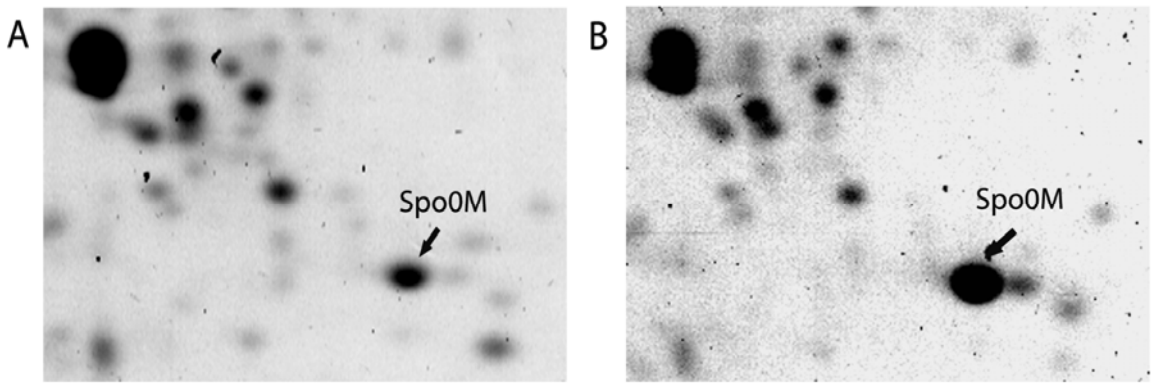

Figure 3.1. Comparative proteomics of a wild type ftsH and null mutant strain. *(A) Strains 1012 (ftsH$^+$) and (B) WW01 (ftsH::erm) were grown in DSM to stage 0 at 37°C. Then, intracellular proteins were separated by 2D electrophoresis. Proteins were separated by a pH gradient of 4 to 7 in the first dimension followed by the second dimension separation of SDS-PAGE. Gels were stained by Coomassie brilliant blue, and the protein spot of Spo0M is indicated.*

3.1.2. FtsH does not influence expression of *spo0M*

In principle, FtsH could regulate the amount of Spo0M indirectly through modulation of a negative regulator or directly through its degradation. To analyze for an indirect influence, the promoter region of *spo0M* was transcriptionally fused to the *bgaB* reporter gene and cells carrying the *bgaB* reporter gene were allowed to sporulate in DS medium (DSM). At the beginning of the stationary phase, samples were removed at intervals and assayed for β-galactosidase activity in the wild type *ftsH* and the isogenic knockout strain. The results are shown in Fig. 3.2.

During transition from the exponential growth phase to the stationary phase, expression of *bgaB* fused to the *spo0M* promoter clearly increased, while the *bgaB*

activity was not expressed from the vector control (*bgaB* gene without *spo0M* promoter fusion) (Fig. 3.2, strain BH3). No difference in the BgaB activity was found between the wild-type *ftsH* and its isogenic insertion mutant (Fig. 3.2, strain BH1 and BH2). This result clearly demonstrates that FtsH is not involved in regulation of transcription of *spo0M*.

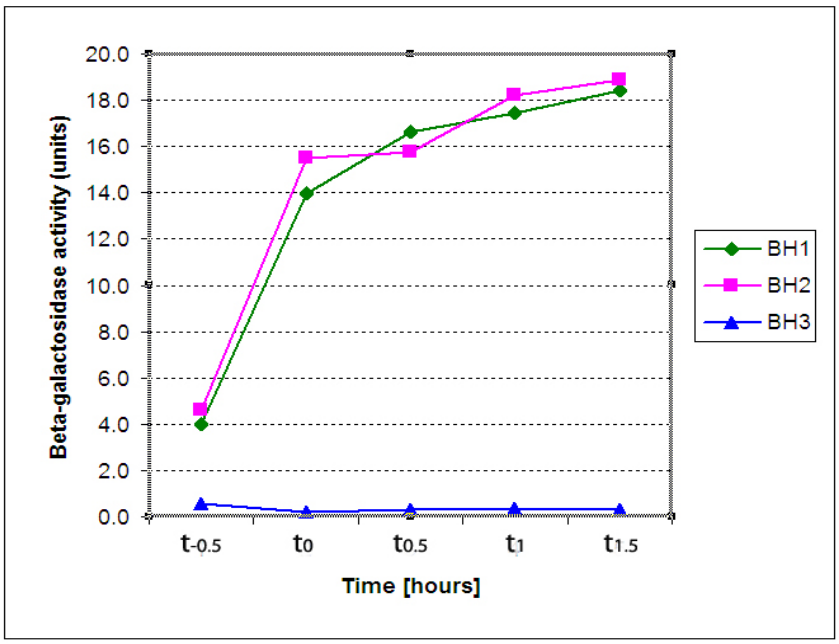

Figure 3.2. FtsH does not influence transcription of spo0M. *B. subtilis strains BH1, BH2 and BH3 containing plasmid pBH1 (P_{spo0M}-bgaB), pBH1 with an ftsH::erm knockout and a promoter-test vector pBgaB, respectively, were grown in DSM at 37°C, and aliquots were withdrawn at the indicated time points for measurement of β-galactosidase activities where t_0 indicates entry into the transition phase.*

3.1.3. Spo0M is confirmed as a substrate protein of FtsH by an *in vitro* degradation experiment

Since FtsH is not involved in the regulation of transcription of *spo0M*, I assumed that it directly modulates Spo0M activity by degradation. Therefore, the *in vitro* degradation of Spo0M by FtsH was tested. First, the Spo0M fused translationally to a GST-tag, was overproduced and purified. The purified GST-Spo0M fusion was incubated with purified GST-FtsH in the presence and absence of ATP. Reactions were carried out under standard conditions as described (Tomoyasu et al., 1995).

As shown in Fig. 3.3, GST-Spo0M is clearly degraded by FtsH in the presence of ATP, but remained stable in its absence. We could already show that the GST-tag itself does not serve as a substrate for GST-FtsH (Le and Schumann, 2009). From this result, we conclude that Spo0M is a substrate protein of the FtsH-metalloprotease.

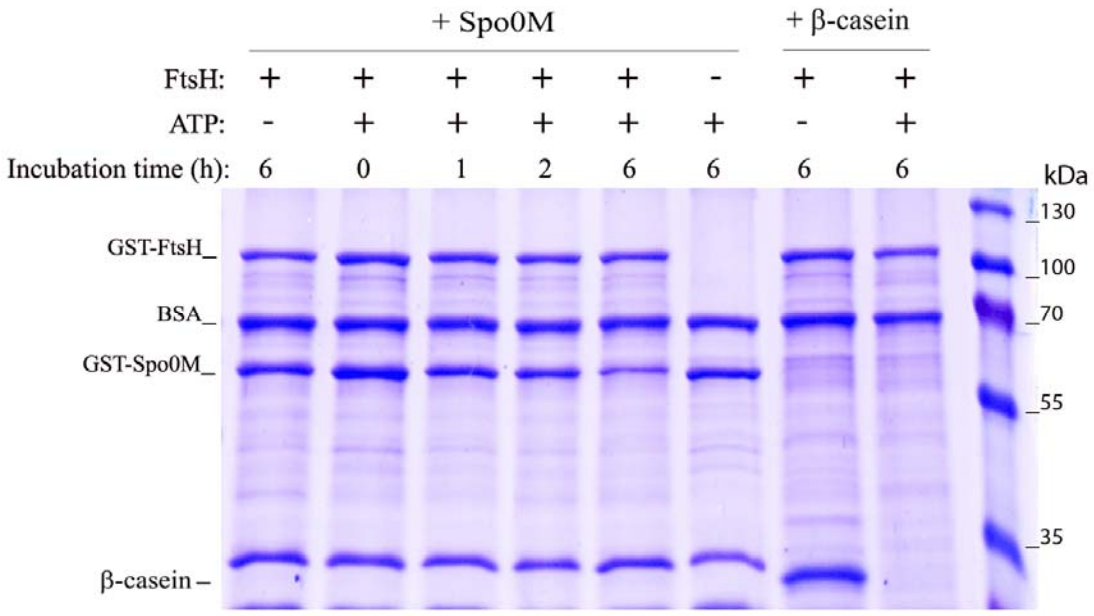

Figure 3.3. Spo0M acts as a substrate protein for FtsH degradation. *The mixture of purified GST-FtsH and GST-Spo0M was incubated by conditions described under Materials and Methods. Aliquots of the reaction mixtures were analyzed by SDS-PAGE and Coomassie blue staining.*

3.2. Identification of FtsH substrate proteins by the *ftsH*^{trap} mutant

This approach relies on the fact that ATP-dependent proteases bind to substrate proteins, unfold them and translocate the unfolded polypeptides into the proteolytic chamber where they are degraded into peptides (Sauer and Baker, 2011). It has been demonstrated that inactivation of the proteolytic activity by exchange of the appropriate amino acid allows binding of the substrate protein but not its degradation. These mutants have been termed trap (Flynn et al., 2003). First, an *ftsH*^{trap} was constructed which was fused cotranslationally to a GST-tag. Next, the mutant protein was overproduced in *E. coli*, purified and inoculated with β-casein. After proving loss of its proteolytic activity, the mutant protein was tested *in vivo* to identify natural substrate proteins of *B. subtilis*.

Using an FtsH^{trap} fused with the GST-tag, substrates of *B. subtilis* FtsH should be trapped in the proteolytic chamber *in vivo*. Using cross-linking experiments, FtsH^{trap} substrates could be co-purified with the mutant protease by using a pull-down assay with the GST-tag, resolved by SDS-PAGE and identified by mass spectrometry.

3.2.1. Construction and characterization of FtsH^{trap}

To selectively inactivate the protease activity of FtsH, the glutamate of the zinc-binding motif HEGGH was replaced by glutamine (E424Q). This mutation was expected to abolish proteolytic activity of FtsH with a minimum of structural perturbations in the matrix of the metalloprotease and retains the ATPase activity (Jayasekera et al., 2000). Therefore, the FtsH^{trap} mutant *(ftsHE424Q)* was cotranslationally fused to the GST-tag at its N-terminus, and the GST-*ftsH^{trap}* fusion was expressed under control of an IPTG-inducible P_{tac}-promoter and purified via glutathione-GST affinity chromatography.

3.2.1.1. Determination of expression of FtsH^{trap} and its controls by IPTG induction

To identify substrate proteins of FtsH by using FtsH^{trap} *in vivo*, the experiments were performed in the *ftsH* mutant strains BH7, BH8, BH9. These strains carry plasmids pBH4 (*GST-ftsH^{trap}*), pBH5 (*GST-ftsH^{+}*), pBH6 (*GST*) that allow expression under control of the IPTG-inducible P_{grac}-promoter. A Western blot analysis using anti-GST antibodies was carried out to test for expression in *B. subtilis* after IPTG-induction. The results are shown in Fig. 3.4. After IPTG-induction, strains BH7 and BH8 expressed a fusion protein with a molecular weight about 100 kDa as expected corresponding to GST-FtsH^{+} or GST-FtsH^{trap}, respectively, and strain BH9 expressed a protein of about 26 kDa representing the GST-tag protein.

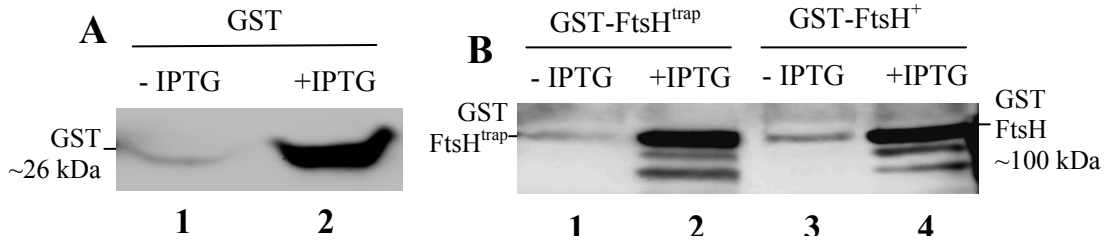

Figure 3.4. Western blot analysis to detect expression of FtsH^{trap}, FtsH^{+} and GST in strains BH7, BH8, BH9 under control of the IPTG-inducible P$_{grac}$ promoter. *Expression was induced by addition of 1 mM IPTG (final concentration) to the cell cultures at an OD_{578} of 0.5; samples were collected at the stationary phase and analyzed by Western blot using GST antibodies. (A): Expression of GST in strain BH9 under control of IPTG. (B): Expression of FtsH^{trap} and FtsH^{+} in strains BH7 and BH8, respectively, without and with IPTG induction.*

3.2.1.2. Physiological characterization of the *ftsH^{trap}* mutant *in vivo*

In *B. subtilis*, an *ftsH* null mutant results in a pleiotropic phenotype, where most of the cells grow as large filaments and are deficient in sporulation. We asked whether these phenotypes can be observed also in the presence of the *GST-ftsH^{trap}* mutant allele. Wild type GST-FtsH was analyzed as positive control in the absence of the chromosomal copy of *ftsH*.

3.2.1.2.1. Expression of FtsH^{+} restores the wild type phenotype, while FtsH^{trap} is defective in phenotypic complementation

To analyze for phenotypic complementation, morphologies of strains BH7 and BH8 complemented with IPTG-inducible expression of *ftsH^{trap}* and *ftsH^{+}*, respectively, were analyzed by light microscopy. In the absence of IPTG induction, cells of strains BH7 and BH8 exhibit a filamentous phenotype as observed in strain WW01 (Δ*ftsH:erm*) (Fig. 3.5). Expression of *ftsH^{+}* after IPTG-induction in strain BH8 exhibited the normal rod-shaped morphology similar to the morphology in strain 1012 (wild type strain), while expression of *ftsH^{trap}* in strain BH7 exhibited a filamentous phenotype (Fig. 3.5). It can be concluded that the *ftsH^{trap}* mutant is unable to complement an *ftsH* knockout.

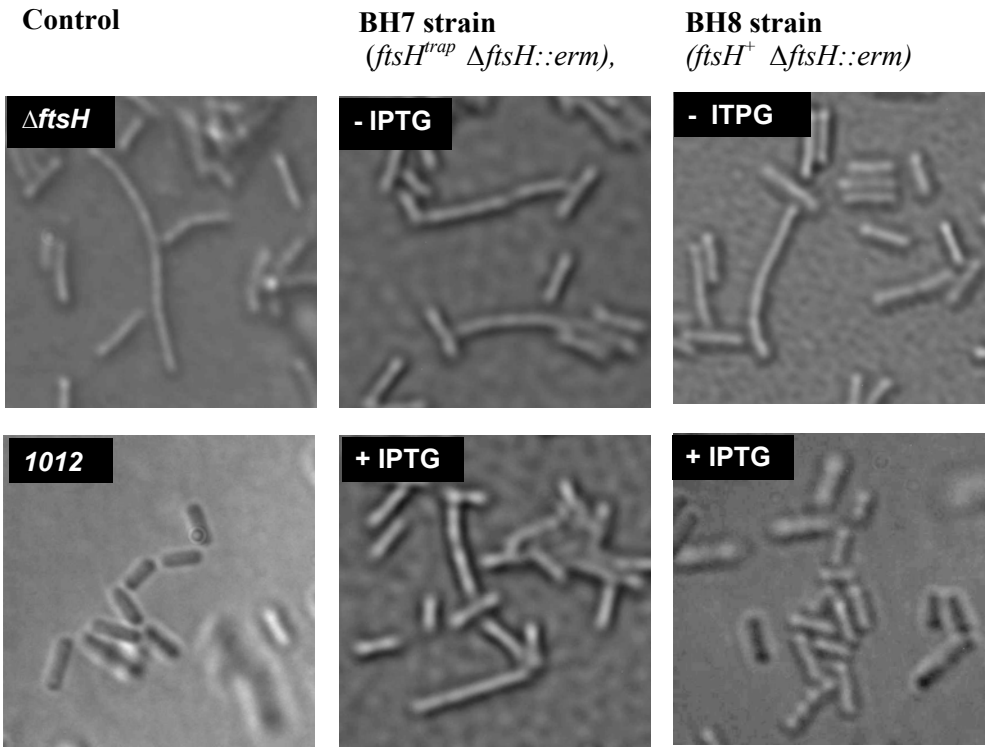

Figure 3.5. Cell morphology of the ftsH knockout strain complemented with FtsH$^+$ and FtsH^{trap}. *BH7: an ftsH null mutant complemented with FtsH^{trap}. BH8: an ftsH null mutant complemented with wild type FtsH. (- IPTG): no added IPTG; (+ IPTG): induced by IPTG.*

3.2.1.2.2. Expression of ftsH$^+$ in a depletion strain shows recovery of the sporulation frequency while ftsH^{trap} does not

To further test for functional complementation, the sporulation frequencies of strains BH7 and BH8 carrying the *ftsH^{trap}* and *ftsH$^+$* alleles, respectively, were determined and compared to those of wild type and *ftsH* knockout strains. The results are shown in Table 3.5.

As usual, the sporulation efficiency of strain 1012 was 48%, while an *ftsH* null mutant was almost deficient in sporulation (0.003%). A considerable restoration in sporulation was observed in strain BH8 (28%) indicating that expression of *ftsH$^+$* is able to restore the sporulation defect of the *ftsH* null mutant. In contrast to *ftsH$^+$*, expression of *ftsH^{trap}* in strain BH7 was unable to complement the sporulation frequency. In the presence or absence of IPTG induction, the sporulation frequency of this strain was comparable to that obtained with strain WW01 (*ftsH::erm*).

Table 3.5. Sporulation frequencies of complemented strains BH7 and BH8 induced by IPTG or not in comparison with wild type and ftsH knockout strains.

Strains	**Cells/ml**	**Spores/ml**	**Sporulation frequency (%)**
B. subtilis 1012	4.8×10^8	2.3×10^8	48
WW01 (Δ*ftsH::erm*)	3.9×10^6	1.1×10^2	0.003
BH7, Δ*ftsH::erm, ftsH*^{trap}, no IPTG	1.9×10^4	3.0×10^0	0.02
BH7, Δ*ftsH::erm, ftsH*^{trap}, (+) IPTG	8.4×10^3	5.6×10^0	0.07
BH8, Δ*ftsH::erm, ftsH*$^{+}$, no IPTG	8.0×10^5	3.0×10^1	0.3
BH8, Δ*ftsH::erm, ftsH*$^{+}$, (+) IPTG	1.3×10^7	3.6×10^6	28

3.2.1.3. Construction and characterization of FtsH^{trap} *in vitro*

To confirm that the *ftsH*^{trap} mutant (E424Q) is defective in proteolysis, an *in vitro* degradation assay was performed to test the ability of FtsH^{trap} to degrade β-casein. A mixture of purified FtsH^{trap} was incubated with β-casein in the presence of ATP as described in 2.2.1.8. As shown in Fig. 3.6, within 4 h of incubation, no degradation of β-casein in the presence of ATP by FtsH^{trap} was observed, while β-casein was clearly degraded by the wild type FtsH within 4 h under the same reaction conditions. From this result, it can be concluded that mutagenesis of residue E424 to glutamine Q424 abolished the proteolytic activity of FtsH *in vitro*. Therefore, an FtsH^{trap} containing this mutation (E424Q) is defective in degradation of β-casein due to lack of its proteolytic activity.

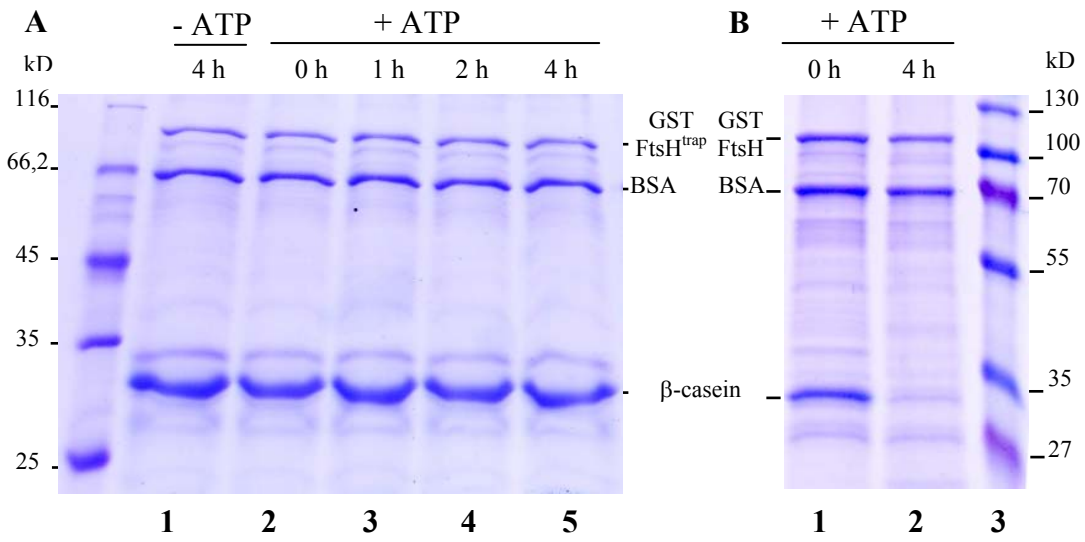

Figure 3.6. In vitro degradation of β-casein with FtsH^{trap} in comparison to wild type FtsH. *Reactions were carried out as described under Materials and Methods. Samples (10 µl) were taken and analyzed by 12% SDS-PAGE. (A) Incubation of FtsH^{trap} with β-casein for up to 4 h without IPTG (lane A1) or with ATP. (B) Incubation of FtsH^{+} with β-casein for 4 h in the presence of ATP.*

3.2.2. Identification of FtsH substrates *in vivo* using the GST-FtsH^{trap} variant

To identify FtsH substrates by using GST-FtsH^{trap} *in vivo*, I hypothesized that FtsH substrates will be degraded by FtsH^{+} while they will be trapped in the proteolytic chamber of FtsH^{trap} due to the proteolytic activity in FtsH^{+} and FtsH^{trap} strains.

By SDS-PAGE gel comparison, protein bands present after purification of GST-FtsH^{trap} and not present in the GST-FtsH^{+} strain are supposed to be substrates of FtsH. To stabilize substrate(s) in the proteolytic chamber, a cross-linking step was added to prevent dissociation of substrate(s) during the purification process.

3.2.3. Most FtsH^{trap} and its co-purified proteins were detected in the membrane fraction and DSP cross-linking caused protein aggregation

FtsH is anchored into the cytoplasmic membrane by two trans-membrane domains located near the N-terminus of the protein and both of the N- and C-terminus are exposed into the cytoplasm (Bieniossek et al., 2009). Therefore, we expected that FtsH and its co-purified substrate proteins can be isolated together with the membrane fraction. To

confirm this assumption, isolation of GST-FtsH^{trap} was carried out using both the cytoplasmic and the membrane fraction. The effect of DSP cross-linking on trapping substrate proteins *in vivo* was also examined by comparison of the amount of purified proteins in both the presence and absence of DSP. The purified and co-purified proteins were resolved by SDS-PAGE and visualized by silver-staining. The results are shown in Fig. 3.7.

As expected, most of the GST-FtsH^{trap} protein was detected in the membrane fraction, while the major of soluble GST expressed in strain BH9 was present in the cytoplasmic fraction. Unexpectedly, the cross-linking experiment was not effective enough for trapping of substrate proteins. As shown in Fig 3.7, the amount of purified and co-purified proteins detected after cross-linking is very low in comparison with those prepared in the absence of cross-linker. It is known that cross-linking can create protein aggregates, which can not be resolved by SDS-PAGE (Thermo Scientific, Pierce Crosslinking Technical Handbook). Indeed, cross-linking experiments are difficult to be carried out because they require an optimal amount of cross-linker for each specific experiment. Another disadvantage of cross-linking is that they make data analysis become difficult and unreliable due to non-specific interactions (Thermo Scientific, Pierce Crosslinking Technical Handbook). Therefore, we decided to continue the FtsH^{trap} experiment *in vivo* without cross-linking.

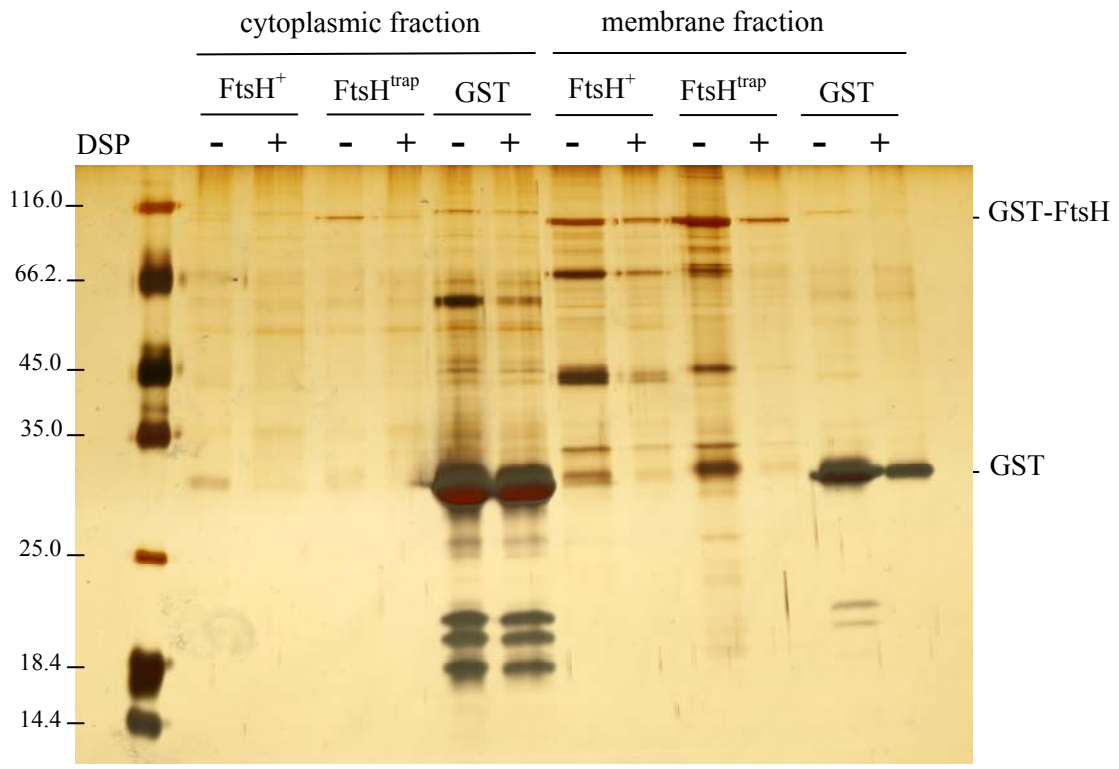

Figure 3.7. Analysis of purified FtsH and co-purified proteins in the absence or presence of the DSP cross-linker in the cytoplasmic and the membrane fraction. *DSP cross-linker was either added (+) or omitted (-), as indicated, cytoplasmic and membrane fractions of strains BH7 (GST-FtsH^{trap}), BH8 (GST-FtsH^{+}), BH9 (GST) were purified, separated by 15% SDS-PAGE and visualized by silver staining.*

3.2.4. Identification of potential FtsH substrate by SDS-PAGE and silver staining

The experiments for protein trapping *in vivo* in strains BH7, BH8 and BH9 were repeated at least three times, and samples were taken for purification and SDS - PAGE analysis. Proteins were resolved in a 10% SDS-PAGE to identify the potential FtsH substrates with a molecular weight higher than 25 kDa and in a 15% SDS-PAGE to detect the FtsH substrates with a molecular weight lower than 25 kDa. There were seven protein bands trapped in the FtsH^{trap} strain and not present in the FtsH^{+} strain numbering from 1 to 7 in the gels (Fig. 3.8 & 3.9). All these bands and the regions (sites) corresponding to these bands in the GST-FtsH^{+} strain were excised and sent for mass spectrometry analysis.

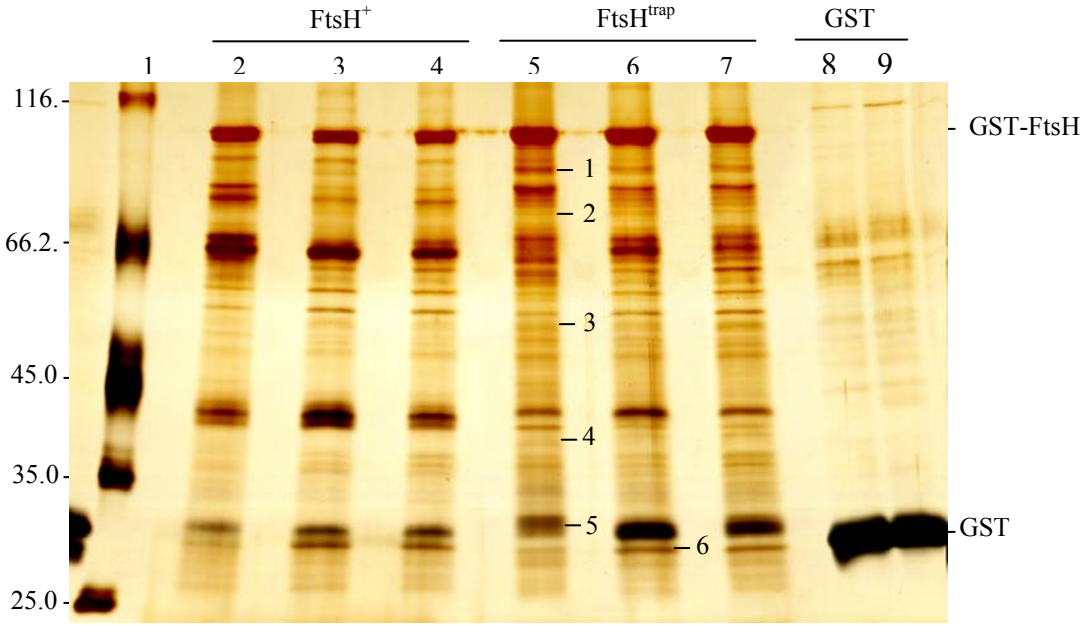

Figure 3.8. Analysis of proteins by 10% SDS-PAGE after copurification with GST-FtsH^{+} and GST-FtsH^{trap}. *Protein bands copurifying with GST-FtsH^{trap} but not with FtsH^{+} and GST were numbered from 1 to 6 and excised for mass spectrometry identification.*

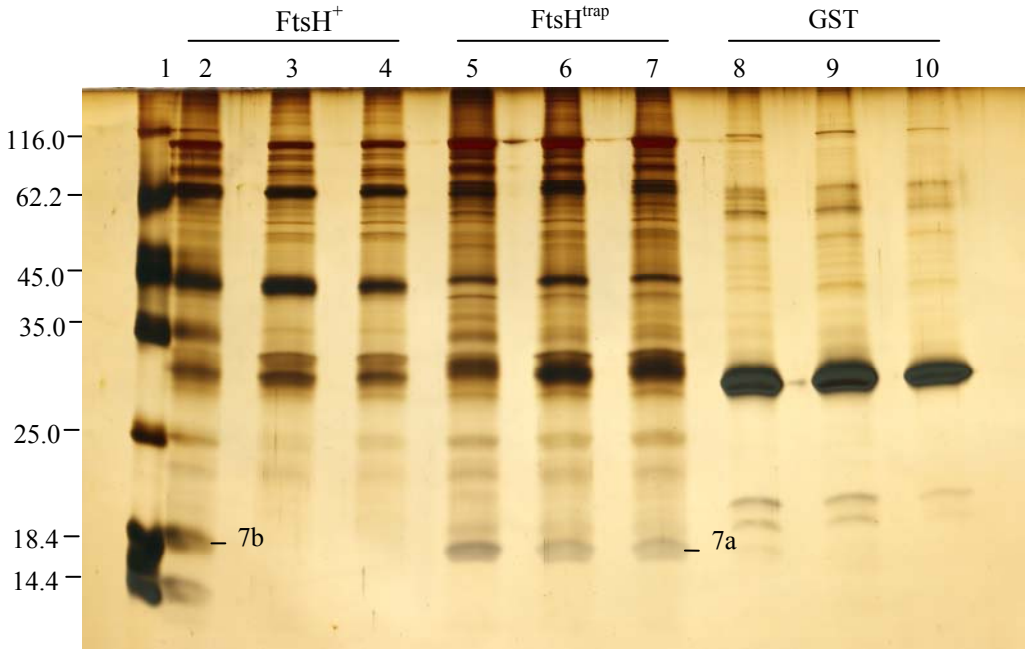

Figure 3.9. Analysis of proteins by 15% SDS-PAGE after copurification with GST-FtsH^{+} and GST-FtsH^{trap}. *Only one band numbered "7a" was detected in GST-FtsH^{trap} but not in GST-FtsH^{+} and GST samples. A band numbered "7b" in lane 2 supposed to be a contaminated protein from lane 1 and band "7a" were excised and identified by mass spectrometry.*

3.2.5. YwnF was identified as a potential substrate of FtsH

The names of the trapped protein bands identified by mass spectrometry are presented in Table 3.6.

Table 3.6: Identification of protein bands trapped by GST-FtsH^{trap}

Number of the protein band	Protein name
1	FtsH
2	FtsH
3	FtsH
4	FtsH
5	GST
6	GST
7a	YwnF
7b	Unknown

Most of the protein bands identified corresponded to FtsH (bands 1 to 4) suggesting that GST-FtsH^{trap} was partially degraded *in vivo*. Protein bands 5 and 6 revealed as GST although their size as determined by SDS-PAGE turned out to be higher than the calculated molecular mass. Remarkably, a strong protein band of about 17 kDa pulled-down clearly by GST-FtsH^{trap}, but not by GST-FtsH^{+} was identified as YwnF protein. It is a small protein with 144 amino acids and predicted as a membrane protein with two transmembrane domains involving amino acids 31-53 and 63-80, the function of which is still unknown (www.Uniprot.org).

The band "7b" at lane 2 (Fig 3.9) with the same size of YwnF is of unknown origin and could be a contaminating protein present in *B. subtilis*. Possibly, it represents the β-lactoglobulin of milk from sheep because the 18.4 kDa band of molecular weight the ladder in lane 1 consist of the β-lactoglobulin presence sheep's milk so that the mass spectrometry could not identify its name from the protein databank of *B. subtilis*.

In summary, by using the GST-FtsH^{trap} approach, one trapped protein, YwnF, was identified as a putative protein substrate of FtsH. However, further experiments *in vitro* and *in vivo* showing degradation of this trapped protein by FtsH are required to clarify and confirm this conclusion.

3.3. Is the Eag protein involved in the regulation of the activity of Spo0E?

The *eag* gene has been identified as an open reading frame downstream of the *spo0E* gene coding for a phosphatase (Perego and Hoch, 1987), where *eag* stands for *spo0E*-associated gene. It codes for a protein of 143 amino acids with a molecular weight of 16.4 kDa. Eag protein is predicted to be an integral inner membrane protein with two potential transmembrane segments. Since no promoter has been identified upstream of *eag*, it is assumed that it forms a potential bicistronic operon with *spo0E* (Perego and Hoch, 1987). Is *eag* involved in sporulation as described for the upstream gene, *spo0E*? Since Spo0E has been identified as a substrate of FtsH (Le and Schumann, 2009), we speculate that the Eag protein of still unknown function might be involved in regulation of the synthesis or activity of the Spo0E phosphatase. To test this hypothesis, we first isolated an *eag* disruptant mutation by integration of a complete plasmid thereby destroying the reading frame of *eag*.

3.3.1. Construction of an *eag* null mutant by insertion of the pMUTIN4 integration vector

The integration vector pMUTIN4 has been widely used to construct insertion mutants in chromosomal genes of *B. subtilis* (Vagner et al., 1998). This vector plasmid is unable to replicate in *B. subtilis* and allows fusion of the promoter-less *lacZ*-gene to the promoter of the gene to become inactivated (Vagner et al., 1998). In a first step, about 300 bp of the gene to be inactivated are amplified by PCR and inserted in front of the *lacZ* reporter gene. Next, this recombinant plasmid is transformed into the appropriate *B. subtilis* strain, and insertion mutants are selected on erythromycin-containing plates. Insertion at the correct site is confirmed by Southern-blotting. By using this technology, strain AM01 was obtained.

Is the *eag* gene expressed during sporulation? To answer this question, strain AM01 was grown in DSM and aliquots were withdrawn before and at different time points after t_0. Strain *yrdB::*pMUTIN4 was analysed as a control. The gene *yrdB* does not play a role during sporulation. Measurement of the β-galactosidase activities of both strains are presented in Fig. 3.11. While the β-galactosidase activities of the *yrdB* insertion mutant dropped from 8 to about 2 units when cells entered the transition phase, that of the

*eag::*pMUTIN4 strain increased from 1 to 5.5 units (Fig. 3.11). This result indicates that *eag* is induced during phase 0 of sporulation.

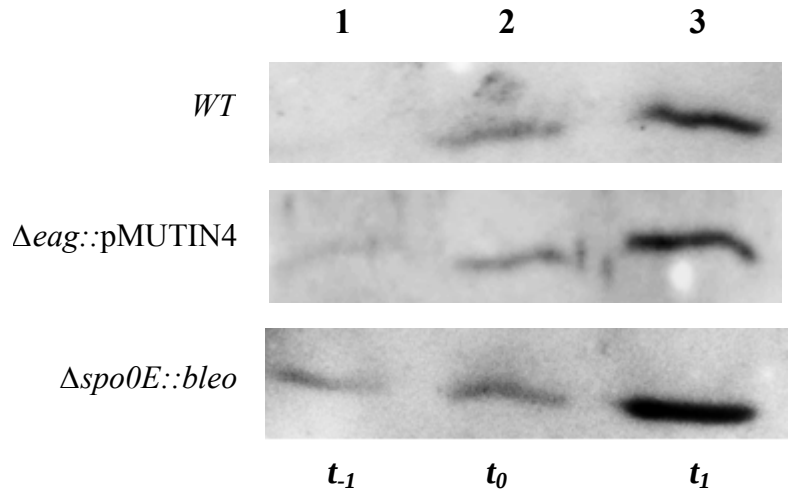

Fig. 3.10. Western blot analysis to detect the amount of Spo0A in wild type B. subtilis and two knockout strains. *Cells were grown in DSM at 37°C and aliquots were taken at the time point indicated (t_{-1}, t_0, t) and analysed by Western blot using Spo0A antibodies.*

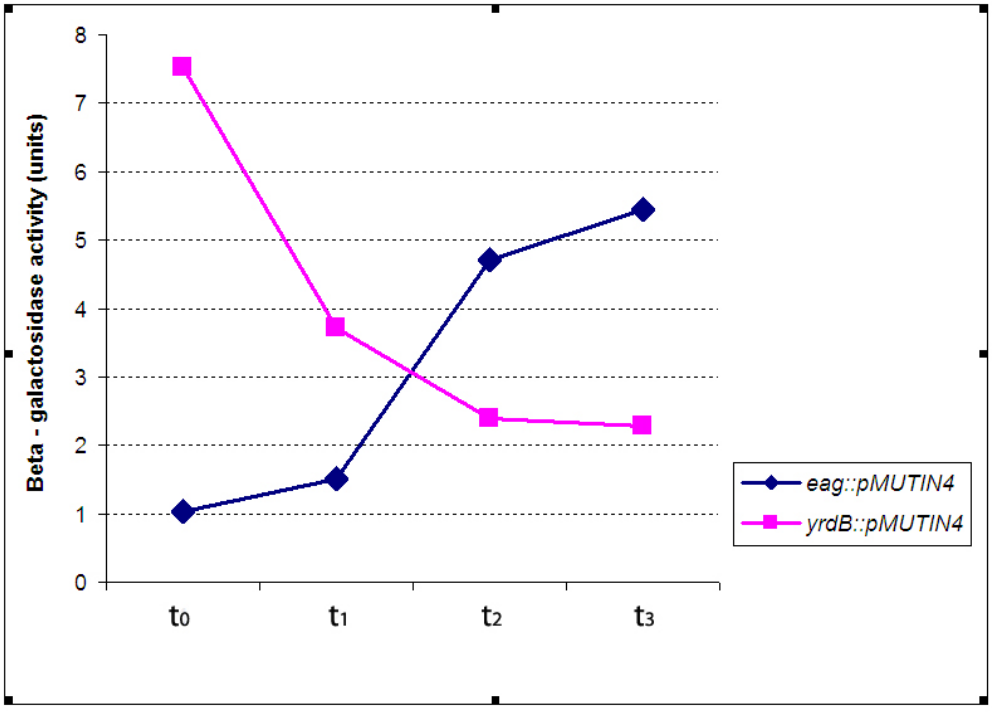

Fig. 3.11. β-Galactosidase activities of strains yrdB::pMUTIN4 and eag::pMUTIN4. *Cells were grown in DSM at 37°C and aliquots were taken at the time point indicated (t_0, t_1, t_2, t_3) and analysed for β-galactosidase activities.*

3.3.2. Does the *eag* gene affect the sporulation frequency?

Strain AM01 (*eag::*pMUTIN4), 1012 (positive control) and AB07 (*spo0E::bleo*) were grown in DSM, and spores were prepared and analyzed as described under Materials and Methods. The results in Table 3.7 show that the wild type strain 1012 exhibited a sporulation frequency of about 52%, while that of its isogenic *spo0E::bleo* derivative was 82% that the sporulation frequency is increased in the absence of the Spo0E phosphatase has already published (Perego and Hoch, 1991) and confirmed by our group (Le and Schumann, 2009). Inactivation of the *eag* gene resulted in a slightly increased sporulation frequency as compared to the wild type strain (58 versus 52%, see Table 3.7). This result suggests a negative influence of *eag* on the sporulation process.

Table 3.7. The sporulation frequencies of the B. subtilis strains 1012, eag::*pMUTIN4 *and spo0E::bleo.

Strains	Viable cells/ml	Spores/ml	Sporulation frequency
1012	4.8×10^8	2.5×10^8	0.52
*1012, eag::*pMUTIN4	6.8×10^8	3.9×10^8	0.58
1012, spo0E::bleo	6.2×10^8	5.1×10^8	0.82

3.3.3. Does the *eag* gene influence the amount of Spo0A protein?

Spo0A is the master regulator of the sporulation phase 0. This protein becomes phosphorylated through the phosphorelay (Burbulys et al., 1991), and Spo0A~P is a DNA-binding protein which acts either as an activator or a repressor depending on the location of the binding site termed OA-box (Perego et al., 1988). Spo0A~P regulates a total of 121 genes directly (Fujita et al., 2005).

Spo0A~P is subject to direct regulation of its activity of the phosphatase Spo0E (Stephenson and Perego, 2002). This phosphatase specifically dephosphorylates Spo0A~P. Next, we asked whether *eag* can influence the amount of Spo0A produced. Two different experiments were carried out to answer this question. First, we directly visualized to Spo0A by immunoblotting and second, we measured the enzymatic activity of a transcriptional fusion dependent on the amount of active Spo0A. Three different strains, wild type 1012 and its isogenic insertion mutants *spo0E::bleo* and *eag::*pMUTIN4

were grown in DSM and aliquots were withdrawn at different time points before and after entry into the transition phase. The Spo0A protein present in these aliquots was visualized by an immunoblot using antibodies raised against Spo0A. As already published, the amount of Spo0A in wild type cells was below the detection level at t_{-1}, started to appear at t_0 and further increased at t_1 (Fig. 3.10). When the *spo0E::bleo* strain was analysed, in contrast to the wild type extracts, Spo0A was already present at t_{-1} and accumulated to a higher amount at t_1 as compared to the wild type strain (Fig. 3.10). In the absence of a functional *eag* gene, small amounts of Spo0A can be detected at t_{-1} and the amount present at t_1 is comparable to the amount present at the same time point in the wild type strain. Therefore, we conclude that the *eag* gene does not influence the amount of Spo0A significantly. As already mentioned, Spo0A~P acts either as a transcriptional activator or repressor. As to its role as an activator, it has been shown that there are two classes of promoters. While some need only a small amount of Spo0A~P to become activated, others need higher amounts (Fujita et al., 2005). The promoter preceeding the *skf* operon needs only a small amount of active Spo0A. The transcriptional P_{skf}- *lacZ* fusion was introduced by transformation into two different strains, the wild type 1012 used as a control and the *eag::*pMUTIN4 strain AM01. Both strains were grown in DSM, and aliquots were taken at t_{-1}, t_0, t_1 and t_2. The β-Galactosidase activities increased from about 2 units to 80 units in the wild type strain, while it increased up to 120 units at t_2 in the *eag* disruption mutant (Fig. 3.12). This result indicates that *eag* exerts a minor effect on the amount of active Spo0A, either by decreasing its amount or favouring its dephosphorylation or both.

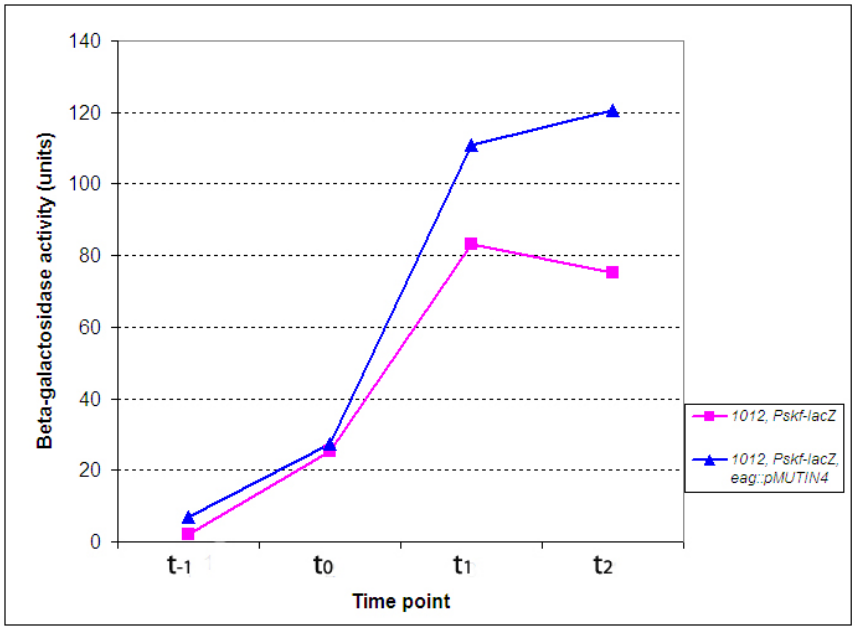

Fig. 3.12. β-Galactosidase activity of an eag knockout and wild type strain. *Cells containing the P_{skf}-lacZ fusion integrated at the amyE locus were grown in DSM at 37°C. Samples were taken at the indicated time points for measurement of β-galactosidase activity.*

4. DISCUSSION

The present doctoral thesis deals with different aspects which will be discussed separately: (1) Identification of the Spo0M protein as a novel substrate, (2) construction of an *ftsH*^{trap} mutant allowing identification of novel substrate protein, and (3) putative role of the Eag protein in modulating the activity of the Spo0E phosphatase.

4.1. Identification of the Spo0M protein as a novel substrate

FtsH is the unique ATP-dependent and membrane-bound protease universally conserved in both prokaryotes and eukaryotes (Okuno et al., 2006b). In *B. subtilis*, cells of an *ftsH* knockout strain fail to sporulate presumably due to the absence of a sufficient amount of Spo0A or/and phosphorylated Spo0A (Spo0A~P) for entry into the sporulation programme (Deuerling et al., 1997; Le and Schumann, 2009).

The first target of FtsH in *B. subtilis* identified by our group was the Spo0E phosphatase, involved in dephosphorylation of Spo0A. Since a *spo0E ftsH* double knockout restored the sporulation frequency to only 0.85% while the sporulation in wild type strains is approximate 60%, we reasoned that additional stage 0-dependent protein(s) are substrates of FtsH (Le and Schumann, 2009). By using the proteomic approach and further analysis in terms of transcription and post-translational modifications, Spo0M was confirmed as a target of FtsH, the second substrate of FtsH which was identified in *B. subtilis*.

4.1.1. Spo0M, a target of FtsH and its function in sporulation

In an attempt to identify protein substrates of the FtsH metalloprotease involved in stage 0 of sporulation in *B. subtilis*, the proteomics approach using the 2D gel techniquewas applied to compare the proteome of an *ftsH* wild-type strain to an *ftsH* null mutant. One of the most abundant proteins identified in the *ftsH* knockout strain was Spo0M, a sporulation control protein of stage 0. Using a *bgaB* reporter system, the *spo0M* promoter was fused transcriptionally with the *bgaB* reporter gene (P_{spo0M}-*bgaB*) and expression analysis did not show any influence of FtsH on transcription of *spo0M* gene. It implied that FtsH might have a negative regulation on the stability of Spo0M through its

proteolytic activity. An *in vitro* proteolytic assay using purified components revealed that Spo0M was degraded by FtsH, indicating that it is a substrate of the FtsH protease.

Spo0M was identified as a novel sporulation-control protein by the Ochi group (Han et al., 1998). The *spo0M* gene codes for a 257-amino-acid protein with a molecular weight of 29.6 kDa and has a strong negative charge (pI = 4.3). This protein has no significant homology to any known protein sequence and transcription is under control of sigma H, a transcriptional regulator that plays key roles during initiation of sporulation, and to be down-regulated by benzoate at pH 7.0 or by low external pH (Kitko et al., 2009). *spo0M* gene is not essential for cell viability but a *spo0M* gene disruption mutant is sensitive to lysis during growth and the sporulation process is blocked from stage 0 to stage I (Han et al., 1998).

If the first target of FtsH - the Spo0E phosphatase - is involved in dephosphorylation of Spo0A, the second FtsH target is supposed to be involved in regulation the amount of Spo0A. By using a transcriptional fusion, expression of Spo0A was shown to be significantly impaired at both the expression level and the timing in the *spo0M* disruption mutant. Thus, the sporulation frequency is decreased by 20- to 100-fold in the absence of Spo0M. Surprisingly, overproduction of Spo0M also results in negative effects on the sporulation frequency (Han et al., 1998). In this study, our results could show that Spo0M is a target of the FtsH protease, which may play a role in maintaining a low but sufficient amount of Spo0M to allow sporulation (Fig. 3.1). We asked the question: What is the "right concentration" of Spo0M for sporulation? How is the function of FtsH in modulating the amount of Spo0M during initiation of sporulation?

4.1.2. The function of FtsH during in the regulation of Spo0M

As shown by the *in vitro* FtsH degradation assay, FtsH fails to completely degrade Spo0M within 6 h (Fig. 3.3). We asked why a small amount of Spo0M still remains intact for such a long time? There are at least two explanations. First, a small amount of GST-Spo0M may stay refractory to degradation, or/and Spo0M may be present naturally in two conformations and only one of them is recognized by FtsH while the other not, in accordance with three other substrate proteins LpxC and KdtA of *E. coli* (Führer et al., 2006; Katz and Ron, 2008) and Spo0E of *B. subtilis* (Le and Schumann, 2009). In the case of proteolysis of LpxC by FtsH, LpxC is an enzyme of the LPS biosynthesis pathway that

catalyzes the biosynthesis of lipid A. Both accumulation and depletion of LpxC are lethal to *E. coli*. Therefore, regulation of FtsH on LpxC levels plays a pivotal role for cell viability (Langklotz et al., 2011). By continuously degrading LpxC, a low amount of LpxC is maintained and thus, assures the proper equilibrium between LPS and phospholipids (Langklotz et al., 2011).

It is likely that the function of FtsH on regulation of Spo0M is similar to that on LpxC. Both overexpression and disruption of the *spo0M* gene have negative effects on sporulation. Therefore, the FtsH protease is probably required to degrade Spo0M and keep it at a low level sufficient for sporulation. The next question is how FtsH modulates the level of Spo0M?

4.1.3. The mechanism of substrate recognition by the FtsH protease

FtsH is a protease with many talents that degrades a variety of substrates located in the cytoplasm and cytoplasmic membrane (Narberhaus et al., 2009). It might use different mechanisms for recognizing substrate proteins, depending on their location or function. Several years ago, it was shown that FtsH recognizes C-terminal non-polar tails of some cytoplasmic proteins and degrades them (Ito and Akiyama, 2005). The thermostability of protein substrates also influences their degradation by FtsH. If the protein is more thermostable, it will be less degradable by FtsH, even when carrying a good recognition tag for FtsH degradation. For example, FtsH is unable to degrade some tightly folded substrate proteins like GFP or DHFR even if a SsrA-tag has been added. Therefore, both a specific sequence recognition and thermostability (or folding state) of proteins might be used as criteria of the degradation decision (Herman et al., 2003).

So far, two principally different mechanisms have been described how FtsH recognizes their substrates. The first depends on free N- or C-terminal ends and the second relies on structural features of the substrate protein (Narberhaus et al., 2009). For example, FtsH degrades σ^{32} at multiple sites due to its endoprotease activity, while the C-terminus of the Spo0E phosphatase is responsible to proteolysis by FtsH (Tomoyasu et al., 1995; Le and Schumann, 2009).

Since Spo0M has already been confirmed to be a substrate of FtsH, we can ask which free-end or internal structural feature of Spo0M is responsible for its recognition by

FtsH. Is a free N- or C-terminal end or the endopeptidase activity of FtsH responsible for its degradation? This has to be clarified by additional experiments.

4.2. Construction of an FtsH^{trap} mutant allowing identification of novel substrate proteins

Until now, more than a dozen protein substrates of FtsH have been identified in different bacteria. Due to the physiological importance of FtsH in cell, it can imply that many substrates of FtsH may stay to be discovered. FtsH is a member of the AAA protease family consisting of two major domains, an ATPase and a proteolytic domain. The ATPase domain is responsible for binding and unfolding of substrate proteins then to translocate the unfolded substrates into the protease domain for degradation (Herman et al., 2003). Recently, to identify substrates of the AAA ClpXP protease, a proteolysis-inactive ClpXP mutant was constructed able to bind the substrates without degrading them. This mutant has been called a trap mutant (Flynn et al., 2003). By fusion to a His-tag allowing for simple purification, followed by separation of the substrates by 2D gel technique and identification by mass spectrometry, a set of substrates of ClpXP have been identified to clarify the role of ClpXP during the cellular processes. Therefore, this trapping method has been shown as an effective way for discovery of new protein substrates (Flynn et al., 2003).

In this study, with the purpose of identification of new FtsH substrates, an approach similar to that of ClpXP^{trap} was applied. An FtsH^{trap} mutant was constructed by substituting the glutamate residue with glutamine in the zinc-binding motif HEGGH to inactivate the proteolytic activity of FtsH. The fusion of FtsH^{trap} with the GST-tag allows for the rapid purification of the FtsH^{trap} and its co-purified substrates present in the proteolytic chamber. Using wild type *ftsH* as a control, it was revealed that in an *ftsH* knockout strain, expression of wild type *ftsH* under control of IPTG can recover the proteolytic activity of FtsH while expression of the *ftsH*-allele cannot. It can be implied that *in vivo*, artificial expression of the wild type *ftsH* gene can degrade its substrates, while these substrates will be kept in the proteolytic chamber of FtsH^{trap}. A quick comparison shows that some protein bands with the purified FtsH^{trap} are not present in the wild-type FtsH on a SDS-PAGE (Fig. 3.8 and Fig. 3.9). As a result, YwnF, a membrane protein, was identified as a potential substrate protein of FtsH. However, it is expected

that more FtsH substrates can be identified with the $FtsH^{trap}$ mutant protein. Theoretically, the $FtsH^{trap}$ protein is not only able to trap full-length FtsH substrates but also fragments consisting of N- or C-terminal residues that play an important role during FtsH degradation. Unfortunately, due to their low molecular weight, these small peptides could not be detected by 1-D SDS-PAGE. Therefore, to discover new substrates of FtsH, the 2D-gel analysis should be applied to separate and identify fragments captured by $FtsH^{trap}$ and full-length proteins as well. Remarkably, *B. subtilis ftsH* itself is a heat-shock gene belonging to class VI (Schumann, 2003). It is involved not only in sporulation but also in stress adaption and protein secretion (Deuerling et al., 1995). Using the $FtsH^{trap}$ approach, the set of FtsH substrates identified will be representative only under particular experimental conditions. For example, in this study, $FtsH^{trap}$ was used to identify the FtsH substrates during initiation of sporulation. To fully understand the function of FtsH during cellular processes in *B. subtilis,* the identification of additional FtsH-substrates under different physiological conditions will be required, e.g, secretion or different physical and chemical stress conditions to discover a set of new substrates and to clarify regulatory pathways controlled by FtsH.

Cytoplasmic membrane proteins play important roles in transport of molecules or exchange of environmental signals across the membrane. So far, our understanding of these processes and their mechanisms are still limited (Akiyama, 2009).

In cells, FtsH acts as a chaperon to control quality of membrane protein by refolding misfolded proteins or degrading proteins that fail to insert correctly into the membrane. There are three native membrane substrates of FtsH identified in *E. coli*. SecY, the central membrane subunit of the Sec-dependent protein translocation machinery and $F_0\alpha$, a subunit of the F_0 sector of the proton ATPase are degraded by FtsH when they failed to assemble into the respective complex (Kihara et al., 1995; Akiyama et al., 1996a) while YccA, a stress-inducible membrane protein, is slowly degraded by FtsH when overexpressed (Kihara et al., 1998).

Because the proteolytic active site of FtsH is located within the cytoplasm, FtsH presumably recognizes cytoplasmic tails of substrate membrane proteins to initiate its dislocation into the proteolytic chamber for subsequent proteolysis. Some additional interactions with other membrane proteins may involve the proteolytic functions of FtsH (Kihara and Ito, 1998; Akiyama et al., 1998). FtsH is known to form a larger complex with the membrane proteins HflK and HflC in *E. coli* and work together to regulate the

degradation of membrane protein substrates (Kihara et al., 1996). As suggested by Akiyama (Akiyama, 2009), the *HflKC* complex may control membrane substrates accessing to FtsH within the membrane for recognition by FtsH. It requires a proper positioning of FtsH to interact with the substrate and to create the force for dislolcation from conformational changes and ATP-hydrolysis. Therefore, the substrate is then delivered into the protease active site for subsequent proteolysis (see Fig. 4.1; Akiyama, 2009).

In this study, using an *ftsH*^{trap} mutant, the membrane protein YwnF was identified as a substrate of FtsH. To confirm that YwnF trapped by FtsH^{trap} is really a substrate of FtsH, an *in vitro* reaction system is required. Both purified FtsH and YwnF will be performed in an *in vitro* degradation assay in the presence of ATP. However, the assay for analyzing activities of FtsH against membrane protein substrates has only used detergent-solubilized components, which is not appropriated to characterize the membrane-integrated substrate degradation (Akiyama and Ito, 2003). Recently, an *in vitro* system for characterization of dislocation and degradation of membrane proteins by FtsH has been established using reconstituted proteoliposomes carrying purified FtsH and a substrate membrane protein. This system consists of two inverted membrane vesicles or two proteoliposomes, one carrying the enzyme and the other a substrate, and it requires ATP for degradation (Akiyama and Ito, 2003). In future, we will apply this *in vitro* system to test the degradation of the membrane protein YwnF by FtsH. Besides, by using the YwnF fusion to the GST tag, it is expected to increase the solubility of YwnF so that the degradation experiment assay of purified component of GST-FtsH and GST-YwnF can be performed to confirm YwnF as target of FtsH.

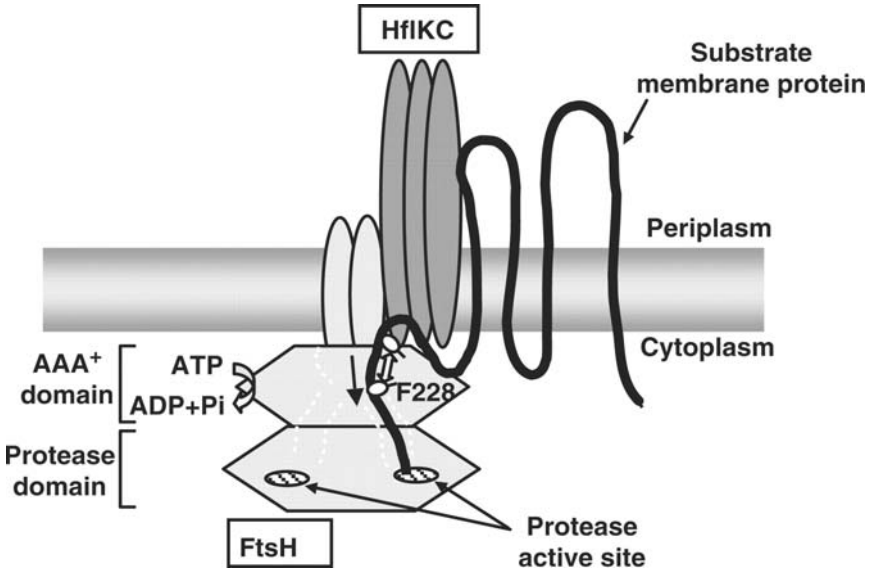

Fig. 4.1. A model of membrane protein degradation by FtsH. *HflKC may control substrate interaction with FtsH within the membrane allowing recognition of a cytoplasmic tail of a membrane substrate by FtsH. Due to ATP-hydrolysis-dependent conformational changes of the ATPase domain, the substrate is then delivered into the proteolytic chamber for proteolysis. This figure was taken from Akiyama, 2009.*

4.3. Putative role of the Eag protein in modulating the activity of the Spo0E phosphatase

The *eag* gene, first described by Perego and Hoch (1987), codes for a protein of 143 amino acids with a molecular weight of 16.4 kDa. The *eag* gene is located downstream of *spo0E* and both genes form a bicistronic operon. The *spo0E* gene codes for a small protein (9.8 kDa) consisting of 85 amino acids that functions in dephosphorylation of Spo0A~P. Upstream of the start codon of *spo0E* is a ribosome binding site, and further upstream are sequences corresponding to a *sigma-A* dependent promoter (Fig. 4.2). A potential stem-loop structure similar to Rho-independent terminators locates downstream of this gene and is followed by a second ribosome binding site with the open reading frame of the *eag* gene and another putative terminator located at the end of this gene (Fig. 4.2) (Perego and Hoch, 1987).

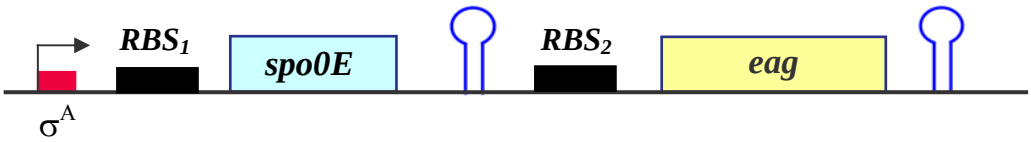

Fig. 4.2. Genomic organisation of the spo0E-eag region in B. subtilis. *spo0E gene forms a bicistronic operon with eag. Both of genes have their own ribosome binding site (RBS) and are separated by a terminator sequence.*

Due to the genomic organization of the *eag* gene, we asked whether it is involved in regulation of *spo0E*. By using a transcriptional *lacZ* reporter gene system, it could be shown that the *eag* gene is induced during phase 0 of sporulation (Fig. 3.11). The analysis of sporulation also revealed that *eag* has a negative influence on the sporulation frequency. From these results, it can be hypothesized that *eag* exerts a minor effect on the amount of active Spo0A, either by reducing its amount or favouring its dephosphorylation or both.

By which mechanism, transcription of the *eag* gene occurs when preceded by a transcriptional terminator and no obvious promoter? The *recA-recX* operon of *E. coli* may serve as an example (Pages et al., 2003). This operon exhibits an organization comparable to that of *spo0E-eag*. It contains just one promoter upstream of *recA* and a putative terminator between *recA* and *recX*. Two different transcripts have described, one corresponding to *recA* and the other to both *recA-recX* genes in which the full-length transcript represents only about 5-10% of the total amount of transcripts. The *recX* expression is shown to be down-regulated at the translational level about 500-fold as compared to *recA* (Pages et al., 2003). Similarly, the *eag* gene of *B. subtilis* may influence the activity of the Spo0E phosphatase in the same way with *recA* and *recX* transcription.

We assume that only small amounts of Eag are produced which modulate either the synthesis or the activity of Spo0E. Eag may interfere with either transcription or translation of *spo0E* or it may directly interact with the Spo0E protein as described for *recX*, a new SOS gene located 220 bp downstream of *recA*, and two genes are co-transcribed in *E. coli*. RecX protein acts as a negative regulator of RecA activities by inhibiting the RecA-dependent strand exchange reaction and co-protease activity by slow depolymerization of RecA-DNA filaments (Galkin et al., 2011). We prefer the second possibility and suggest the following model shown in Fig. 4.3.

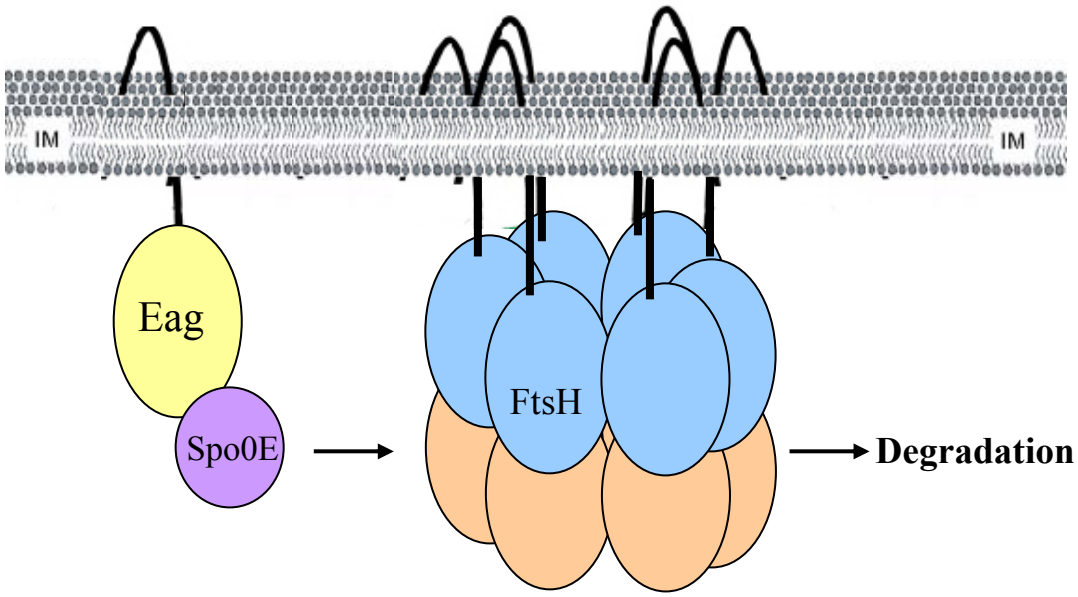

Fig. 4.3. Hypothetical model how Eag may modulate the activity of the Spo0E phosphatase. *Eag may bind Spo0E to prevent it from dephosphorylating Spo0A~P and even transfer it to FtsH for degradation.*

The Eag protein has been assumed to be integrated into the cytoplasmic membrane. It may bind Spo0E, thereby preventing Spo0E from interacting with Spo0A~P followed by dephosphorylation. This model could be tested by artificial overproduction of the Eag protein. If the model is correct, this should result in an increase in the sporulation frequency and also in the amount of Spo0A. In addition, Eag may transfer Spo0E to the FtsH protease followed by degradation. This hypothesis is suggested since both Eag and FtsH are intergral membrane proteins and may stay close together in the membrane. If Eag really transfers Spo0E to FtsH, it may act as an adapter protein - a protein that recognizes substrate proteins of ATP-dependent proteases and transfers them to the appropriate protease. Examples are ClpS which cooperates with ClpAP of *E. coli* (Schmidt et al., 2009) and MecA of *B. subtilis* transferring substrate proteins to ClpCP protease (Kirstein et al., 2006; Mei et al., 2009).

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List of abbreviations and symbols

Abbreviation	**Denotation**
σ	Sigma Factor
2D-Gel	Two-Dimensional Gel
2D-PAGE	Two-Dimensional Polyacrylamide Gel Electrophoresis
AAA	ATPases Associated with a Variety of Cellular Activities
ATP	Adenosine-5'-Triphosphate
Amp^R	Resistant to Ampicillin
BSA	Bovine Serum Albumin
Cat	Gene Coding for Chloramphenicol-Acetytransferase
CHAPS	3-[(3-Cholamidopropyl)Dimethylammonio]-1-Propanesulfonate
Cm^R	Resistant to Chloramphenicol
COG	Clusters of Orthologous Groups
CSF	Competence Stimulating Factor
ddH_2O	Double Distilled Water
DHFR	Dihydrofolate Reductase
DMSO	Dimethyl Sulfoxide
DSM	Difco Sporulation Medium
DSP	Dithiobis[Succinimidyl Propionate]
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
Erm	Gene Coding for Erythromycin Resistance
Erm^R	Resistant to Erythromycin
GFP	Green Fluorescent Protein
GSH	Reduced Glutathione
GST	Glutathione-S-Transferase
IEF	Isoelectric Focusing
IPG	Immobilized pH Gradient
IPTG	Isopropyl-ß-D-Thiogalactoside

KDO	3-Deoxy-D-Manno-Oct-2-Ulosonic Acid
LB	Luria-Bertani
LPS	Lipopolysaccharide
MS	Mass Spectrometry
Neo	Gene Coding for Neomycin Resistance
Neo^R	Resistant to Neomycin
OD	Optical Density
$OD_{578\ (600)}$	Optical Density at a Wavelength of 578 (or 600) nm
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride
P_{grac}	An IPTG inducible promoter, which consists promoter of P_{groES} and *lac* operator
RBS	Ribosome Binding Site
Rpm	Revolution or Round per Minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Spc	Gene Coding for Spectimomycin Resistance
Spc^R	Resistant to Spectinomycin
SRH	The Second Region of Homology
TE	Tris – EDTA
TCA	Tricarboxylic Acid
Tris	Tris-(Hydroxymethyl)-Aminomethane
t_x	Stage x of Sporulation Program
WT	Wild-Type

Publication

Research in Microbiology

The sporulation control gene *spo0M* of *Bacillus subtilis* is a target of the FtsH metalloprotease

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The *eag* gene of *Bacillus subtilis* influences the activity of the Spo0E phosphatase

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Construction of *ftsH*^{trap} mutant to isolate FtsH protein substrate in *Bacillus subtilis*,

Hue Bach Thi Nguyen and Wolfgang Schumann.

in preparation.

Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbstständig verfasst zu haben und keine anderen als die von mir angegebenen Quellen oder Hilfsmittel verwendet zu haben.

Ferner habe ich weder an der Universität Bayreuth, noch an einer anderen Hochschule versucht eine Dissertation einzureichen, oder mich einer Promotionsprüfung zu unterziehen.

Hue Bach Thi Nguyen

Bayreuth, Januar 2012